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Alcohol and Gut-Derived Inflammation

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In large amounts, alcohol and its metabolites can overwhelm the gastrointestinal tract (GI) and liver and lead to damage both within the GI and in other organs. Specifically, alcohol and its metabolites promote intestinal inflammation through multiple pathways. That inflammatory response, in turn, exacerbates alcohol-induced organ damage, creating a vicious cycle and leading to additional deleterious effects of alcohol both locally and systemically. This review summarizes the mechanisms by which chronic alcohol intake leads to intestinal inflammation, including altering intestinal microbiota composition and function, increasing the permeability of the intestinal lining, and affecting the intestinal immune homeostasis. Understanding the mechanisms of alcohol-induced intestinal inflammation can aid in the discovery of therapeutic approaches to mitigate alcohol-induced organ dysfunctions.

Key words: Alcohol consumption; alcohol use, abuse, and dependence; chronic alcohol use; organ damage; gastrointestinal (GI) tract; liver; metabolites; gut-derived inflammation; intestinal inflammation; intestinal microbiota

The gastrointestinal (GI) tract, as the first line of contact with anything ingested into the body, is at particular risk for damage by toxins. And mounting research suggests that poor gastrointestinal health plays a significant role in the body's overall health. Connecting the dots, anything that may cause GI damage, may have consequences far beyond the intestines. In fact, researchers have begun to discover that alcohol, particularly if consumed chronically and in larger amounts, induces a process initiated in the gut that promotes inflammation throughout the body (Patel et al. 2015). This alcohol-induced intestinal inflammation may be at the root of multiple organ dysfunctions and chronic disorders associated with alcohol consumption, including chronic liver disease, neurological disease, GI cancers, and inflammatory bowel syndrome. This review summarizes the mechanisms by which chronic alcohol intake leads to intestinal inflammation. These mechanisms include alcohol's influences on intestinal microbiota, on the integrity of the barrier between the intestine and the rest of the body, and on immune function within and outside the GI tract. The factors that can modify alcohol-induced gut inflammation and organ damage and the resulting pathologies that are a consequence of gut-derived inflammation are described. Although there may be large gender, racial, and interindividual variations in alcohol's effect on the GI tract, depending on differences in alcohol absorption, distribution, and elimination, they are not the focus of the current review.

Alcohol Metabolism and the Gut

Once consumed, alcohol is absorbed mainly in the upper intestinal tract by diffusion and then enters the liver via the portal vein. Therefore, the effect of alcohol on the distal small intestine and colon should largely come from its circulatory levels. That said, the luminal concentration of alcohol in the latter parts of the small intestine, close to the colon, reaches up to 200 mg/100 ml within an hour of drinking 2 to 2 ½ standard alcoholic drinks (0.8 g/kg) (Halsted et al. 1973).

The majority of alcohol metabolism in humans occurs in the liver, in cells called hepatocytes. During social drinking, defined here as an average of two standard drinks, the body typically processes the ingested alcohol with no

deleterious effects through a process called oxidative conversion, during which the enzyme alcohol dehydrogenase (ADH) converts alcohol into the toxin acetaldehyde. Acetaldehyde dehydrogenase (ALDH) then converts acetaldehyde into acetate. Another alcohol metabolism pathway, the microsomal ethanol–oxidizing system (MEOS), handles a small portion of alcohol metabolism in social drinkers but a significant portion of alcohol metabolism when the body needs to process larger amounts of alcohol. MEOS leads to the production of oxygen free radicals, which can cause cellular damage. Although the majority of alcohol metabolism occurs in hepatocytes, the enzymes involved in the oxidative metabolism of alcohol also are present in the intestinal mucosa (Cederbaum 2012) and intestinal bacteria also produce acetaldehyde in the GI tract. In addition, less commonly, nonoxidative alcohol metabolism occurs in the intestines via reactions with membrane phospholipids and/or free fatty acids. This alternative pathway may become particularly relevant when intestinal injuries occur after chronic alcohol consumption (Elamin et al. 2013*a*).

Therefore, both the small and large intestine can be affected by alcohol and its metabolites as the result of its oxidative and nonoxidative metabolism. Metabolism of alcohol in the GI tract can then lead to disruption of tissue homeostasis toward a chronic state of intestinal inflammation (Patel et al. 2015; Rao et al. 2004). As will be discussed in this review, mounting evidence shows that alcohol induces intestinal inflammation through various pathways, including changes in intestinal microbiota composition (Engen et al. 2015; Mutlu et al. 2012) and function (Couch et al. 2015), increased permeability of the intestinal mucosa (Tang et al. 2015), and disruptions of the immune system of the intestinal mucosa (Cook 1998).

Underlying Mechanisms for Alcohol and Gut-Derived Inflammation

Alcohol and Intestinal Microbiota

The intestine houses more than 500 bacterial species and achieves bacterial homeostasis when the ratio between "good" bacteria and pathogenic bacteria is appropriately balanced. "Dysbiosis" occurs when disease or environmental factors disrupt the bacterial balance (Belizário and Napolitano 2015). Disruption to the normal gut flora also occurs when there is an overall overgrowth of bacteria. Studies show that alcohol promotes both dysbiosis and bacterial overgrowth (Mutlu et al. 2012; Schnabl and Brenner 2014), which in turn leads to an increase in the release of endotoxins, produced by gram-negative bacteria (Rao et al. 2004). Endotoxins activate proteins and immune cells that promote inflammation (Elamin et al. 2013*a*; Keshavarzian et al. 2009). This section discusses evidence supporting alcohol's role in altering intestinal microbiota.

Bacterial Overgrowth

Studies in animals and humans confirm that alcohol increases intestinal bacteria (Canesso et al. 2014). This overgrowth may be stimulated directly by alcohol, but some studies suggest that it also could be an indirect byproduct of poor digestive and intestinal function caused by alcohol consumption. For example, studies of patients with liver cirrhosis (both caused by alcohol and not) found an association between patients with abnormal intestinal motility—the intestine's ability to move food along—and bacterial overgrowth (Chang et al. 1998). Other studies found a connection between alcohol, bile acid, and bacterial overgrowth. Specifically, alcohol can alter bile-acid metabolism and, in turn, bile acids can affect intestinal bacteria (Schnabl and Brenner 2014). Studies in rats show that alcohol decreases certain bile acids (Xie et al. 2013) and treating rats with bile acids reversed bacterial overgrowth (Lorenzo-Zúñiga et al. 2003).

Bacterial Dysbiosis

More recent studies use DNA sequencing technology to assess intestinal microbiota populations and indicate a correlation between alcohol and changes in the ratio between beneficial or "good" bacteria, such as strains of *Lactobacillus* and *Bifidobacterium*, and pathogenic bacteria, such as proteobacteria and bacilli (Canesso et al. 2014). For example, mice chronically fed alcohol display a decrease in good bacteria and an increase in bacteria that boost endotoxin production (Bull-Otterson et al. 2013). In another study, researchers found a significant shift in intestinal microbiota composition in rats chronically fed alcohol, but they could prevent the shift by giving the rats *Lactobacillus GG* bacteria and a diet that included probiotic oats (Mutlu et al. 2009). Connecting dysbiosis to alcohol-induced health problems, several studies find that probiotic and synbiotic interventions, which stimulate the growth of beneficial bacteria, attenuate liver injury in rats (Forsyth et al. 2009) and liver dysfunction in cirrhotic patients (Liu et al. 2004). Alcohol-induced bacterial overgrowth also may increase the risk of inflammation because intestinal bacteria can independently metabolize alcohol, producing excess acetaldehyde in the colon, which increases production of proinflammatory alcohol metabolites (Zhong and Zhou 2014).

Alcohol-Induced Intestinal Hyperpermeability

The intestinal barrier regulates the passage of materials between the GI tract and the bloodstream, allowing for the absorption by the blood of key nutrients and preventing the absorption of noxious substances. It is made up of a layer of water, mucous gel, and epithelial and connective tissue. The epithelial

layer can become leaky or "permeable," allowing pathogens and other deleterious substances into the bloodstream.

Studies in humans demonstrate that a subset of people with alcohol use disorder (AUD) in fact have increased intestinal permeability, measured using a method called Cr-EDTA, which examines excretion of orally administered chromium (Leclercq et al. 2014). In addition, those people with AUD and with increased permeability are more likely to have liver disease (Keshavarzian et al. 1999), indicating that intestinal permeability may be a mediator of organ damage in some people with AUD. Another study showed that not only is gut permeability increased in people with AUD, it is increased enough to allow large macromolecules through the intestinal barrier (Parlesak et al. 2000). Endotoxins—also known as lipopolysaccharides (LPS)—are large macromolecules and, as expected, the same study found that plasma endotoxin levels increased in parallel with increases in gut permeability (Parlesak et al. 2000).

But exactly how does alcohol induce intestinal permeability? The short answer is by disrupting the epithelial cells themselves (transepithelial permeability) and by disrupting the spaces between the epithelial cells (paracellular permeability), which consist of tight junctions, the cytoskeleton, and several associated proteins (see figure below). Trans-epithelial permeability is caused by direct cellular damage. For example:

- Alcohol causes cell death (Pijls et al. 2013), which leads to changes in the intestine that include mucosal ulcerations, erosions, and loss of

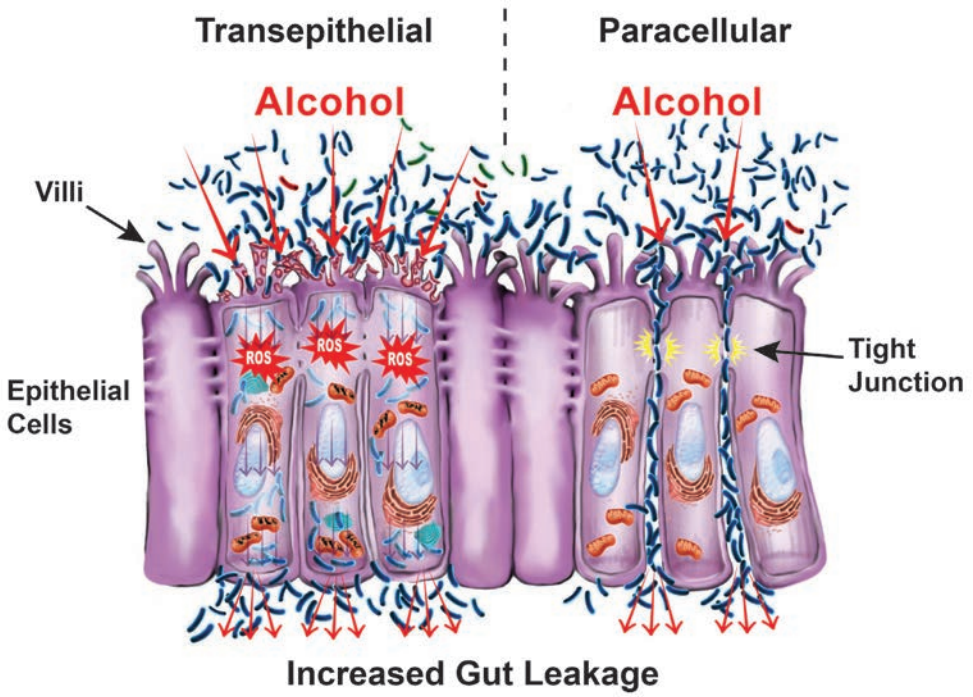


Figure The intestinal barrier regulates the passage of materials, including microbial products, between the inside of the intestine (where food and drink go) and the cells and blood vessels on the other side of the epithelial cell layer lining the inside of the intestine. Alcohol disrupts the intestinal barrier, increasing its permeability, in two ways: via transepithelial mechanisms (cells on the left), which allow material to pass directly through the epithelial cells, and paracellular mechanisms (cells on the right), which allow material to pass through the junctions between the epithelial cells. Alcohol and its metabolites trigger transepithelial mechanisms by damaging the cells directly and weakening cell membranes via several mechanisms including oxidative stress caused by reactive oxygen species (ROS). Alcohol's metabolites trigger paracellular mechanisms by disrupting the proteins that create the tight junctions linking cells and proteins that stabilize cells' cytoskeletons. Increased permeability of the intestinal barrier allows bacteria and the toxins they create to leave the gut and infiltrate other organs through the bloodstream.

epithelium mainly at the villi tips (Rocco et al. 2014);

- Acetaldehyde forms DNA adducts that cause direct cellular damage (Malaguarnera et al. 2014); and
- Reactive oxygen species (ROS) released during alcohol metabolism cause direct cellular damage via oxidative stress (Forsyth et al. 2014).

Alcohol and its metabolites cause paracellular permeability by acting on the tight-junction complex, which melds two adjoining cells together. For example, acetaldehyde destabilizes tight junctions by redistributing proteins (Dunagan et al. 2012); alcohol and its metabolites alter the expression of tight-junction proteins (Wang et al. 2014); and alcohol nonoxidative metabolites cause tight-junction redistribution, disrupting its barrier function (Elamin et al. 2013*b*). In addition, studies show that alcohol destabilizes cells' cytoskeletons, the cell borders that give them their structure (Elamin et al. 2014). There also is growing evidence that alcohol causes the overexpression of microRNAs (miRNAs), which are small stretches of noncoding RNA that silence gene expression (Tang et al. 2014). Specifically, alcohol can lead to overexpression of miRNAs that influence genes associated with gut-barrier integrity (Lippai et al. 2014).

Alcohol Modulation of Mucosal Immunity

Gut inflammation results from an inflammatory response mounted by the immune system against alcohol and its metabolites. Alcohol affects intestinal mucosal immunity via several mechanisms (see sidebar). In particular, it may first decrease the innate immune response in the mucosa, resulting in increased susceptibility to intestinal pathogens (Zhou et al. 2013). Subsequently, as found in studies in cell cultures, alcohol may trigger an immune system response and upregulation of molecules that promote the inflammatory response, including a release of inflammatory immune cells, such as leukocytes and mast cells (Fleming et al. 2001).

As mentioned earlier, alcohol-related bacterial overgrowth and dysbiosis may lead to increased endotoxin production in the gut, which can bind to cells on the intestinal mucosa, causing local inflammation, and translocate to extraintestinal sites, causing systemic inflammation (Leclercq et al. 2014). Studies also show that alcohol can directly modulate both innate and adaptive immunity, further contributing to gut and gut-derived inflammation. For example, a study in mice found that alcohol inhibits the intestine's immune response for clearing hazardous bacteria (Sibley and Jerrells 2000), and other studies find that alcohol suppresses intestinal mucosal immune cell activity (Cook 1998). Additional studies find myriad ways that alcohol affects mucosal immunity, including the following:

- By reducing the amount of antimicrobial molecules intestinal cells secrete, which leads to bacterial overgrowth (Schnabl and Brenner 2014);
- By suppressing the signaling molecule, interleukin-22, which negatively affects antimicrobial peptides (e.g., Reg3β and Reg3γ) and intestinal mucosal integrity (Rendon et al. 2013); and
- By suppressing signal molecules and immune T cells and thereby suppressing the intestinal mucosal immune response and bacterial clearance (Trevejo-Nunez et al. 2015).

Modifying Factors for Alcohol-Induced Gut-Derived Inflammation

As described above, alcohol causes gut-derived inflammation, which is related to other alcohol-associated pathologies. However, not all people with AUD develop disease, and those who do have varying degrees of disease severity. Although the extent of disease depends in large part on the extent of alcohol use and likely involves inherent individual characteristics, including genetics, race, and age, there are some adjustable factors that affect alcohol-induced intestinal inflammation and, therefore, may prevent or slow the progression of alcohol-related disease. Here, we discuss the roles of two adjustable environmental factors: circadian rhythm and diet.

Circadian Disruption

Circadian rhythm, also known as the biological clock, refers to an internal cycling of various biological processes. Chronic alcohol use can lead to a disrupted biological clock, which in turn can have a wide range of health-related consequences.

In terms of gut-related inflammation, studies in cell cultures, mice, and humans suggest that a disrupted circadian rhythm exacerbates alcohol-related gut leakiness. For example, one study (Summa et al. 2013) found that alcohol-fed CLOCK mutant mice—who have a disrupted circadian cycle—showed more evidence of gut leakiness than alcohol-fed wild-type mice. A study in humans (Swanson et al. 2015), including a group of shift workers who often have disrupted circadian rhythm, came to a similar conclusion. The researchers assessed circadian rhythm by measuring participants' blood melatonin levels, using low melatonin as a marker for disrupted circadian rhythm. They found that low melatonin correlated with gut leakiness in people with AUD.

Although it is unclear how circadian disruption amplifies alcohol-induced gut permeability, there are some hints from recent studies. For example, gut microbes have circadian oscillations, and circadian disruption can lead to dysbiosis in mice fed a high-fat diet (Voigt et al. 2014), which in turn can induce intestinal inflammation and hyperpermeability. In addition, timing

of lipid metabolism and bile-acid synthesis are regulated by the local hepatic circadian rhythm (Bailey et al. 2014). Together, the evidence on circadian rhythm suggests a looping cycle where circadian disruption promotes alcohol-induced intestinal inflammation and alcohol disturbs circadian rhythm, which may further propagate intestinal hyperpermeability and inflammation.

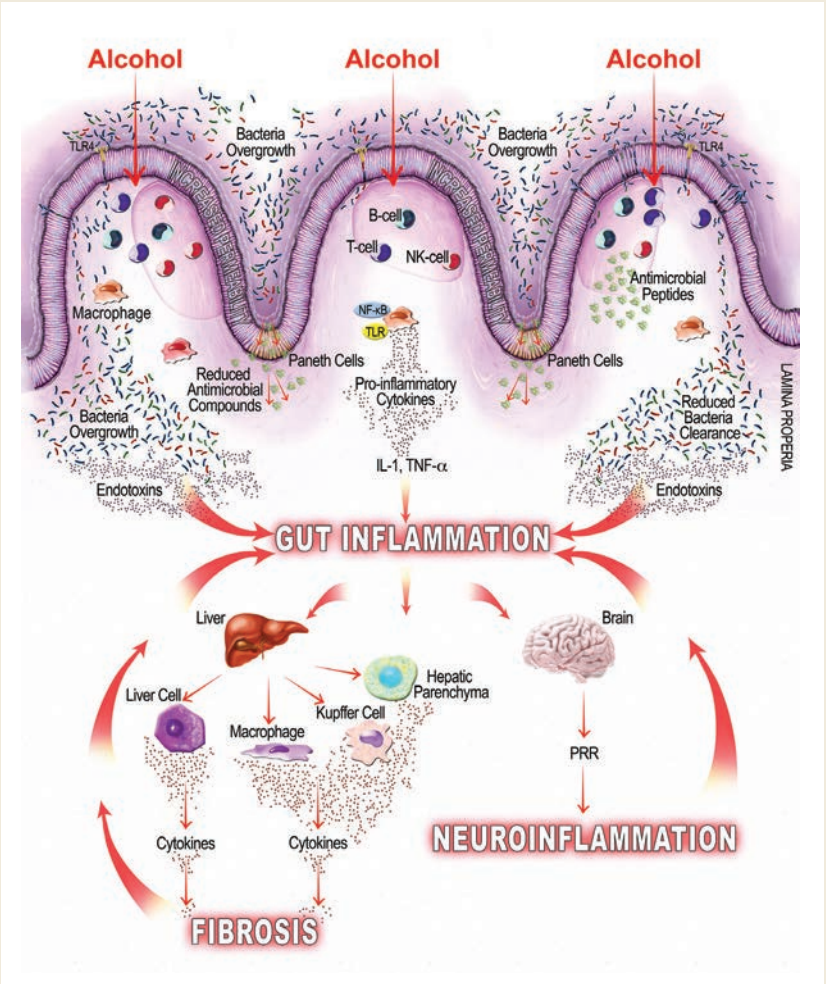
Diet

Various studies show that nutrition can modify alcohol-induced gut inflammation and, subsequently, extraintestinal organ damage. Because people with AUD typically have altered diet composition, a focus on changing dietary habits might attenuate alcohol-related diseases. The following section reviews a sampling of studies on different diets and alcohol use.

Fat. Studies examining high-fat diets find conflicting results. Some find fats propagate alcohol's effects on the intestine, and some find they attenuate alcohol's harmful effects. The contrast likely reflects the variety

Alcohol's Effect on Immunity and Inflammation

Alcohol can induce intestinal inflammation through a cascade of mechanisms that subsequently lead to inflammation and organ dysfunction throughout the body, in particular in the liver and brain. One mechanism is by increasing bacterial loads and the permeability of the intestinal wall (see figure) allowing bacteria to leak through, leading to local and systemic effects by affecting mucosal immunity and via endotoxin release, respectively. Alcohol also affects mucosal immunity by suppressing one of the intestine's main lines of defense against bacteria, Paneth cells that secrete antibacterial compounds. Suppressed Paneth cells secrete fewer antibacterial compounds, which can allow additional intestinal bacteria overgrowth and allow their byproducts (i.e., endotoxins) entrance through the intestinal barrier. The bacteria, via endotoxins, trigger an inflammatory response by the intestine's immune system, causing a release of proinflammatory cytokines. The endotoxins and cytokines can then enter the liver, directly interacting with hepatocytes and with liver immune cells, causing local cytokine release that leads to fibrosis and causes additional inflammation. The gut inflammation can also spread endotoxins and cytokines into the bloodstream where they can enter the central nervous system (CNS), causing neuroinflammation.



of fats found in high-fat foods. Generally, studies seem to support the idea that unsaturated fats increase gut permeability and some kinds of saturated fats are protective.

Studies have examined the effects of several types of saturated fats given as supplements to alcohol-exposed mice. One (Cresci et al. 2014) found that tributyrin, a triglyceride fat found in butter and margarine, prevented alcohol-induced tight-junction disruption, which in turn protects against intestinal hyperpermeability. Another (Chen et al. 2015*a*) examined saturated long-chain fatty acids (SLCFAs), which are found in coconut oil, peanut oil, and dairy products. The researchers observed that the intestinal bacteria in mice chronically fed ethanol produced far less SLCFAs than mice not fed ethanol, and they also had lower levels of tight-junction proteins. That changed after the researchers gave the ethanol-fed mice SLCFA supplementation. Indeed, the mice given supplementation had higher levels of tight-junction proteins than ethanol-fed mice without supplementation. SLCFA supplements also prevented dysbiosis (Chen et al. 2015*a*).

Unsaturated fats had less favorable effects. In one study (Kirpich et al. 2012), mice fed alcohol and unsaturated fats had increased fatty liver changes and suppressed mRNA expression of tight-junction proteins compared with mice fed alcohol and saturated fat. These findings suggest that an unsaturated-fat diet in conjunction with chronic alcohol use increases intestinal permeability.

Oats. Oats, which are rich in fat, fiber, protein, vitamins, and minerals, have long been associated with cardiovascular health and, more recently, examined for a possible role in gastrointestinal health. Several preclinical studies suggest that oats may attenuate alcohol's deleterious effects on the digestive system. In one study (Keshavarzian et al. 2001), two groups of rats received increasing doses of alcohol and either oats or regular rat chow for a period of 10 weeks. The oats-fed rats had significantly lower endotoxin levels than the chow-fed animals. Another study (Tang et al. 2009) found that alcohol-fed rats given oat supplementation showed fewer signs of gut inflammation and alcohol-induced hyperpermeability than rats fed alcohol and regular rat chow. More recently, researchers examined supplementation with glutamine, an amino acid found in oats. The study in mice found that glutamine supplements ameliorated alcohol-induced intestinal leakiness and improved alcohol-induced liver injury (Chaudhry et al. 2016).

Vitamins and Minerals. People with AUD often are deficient in certain vitamins and minerals, including zinc and vitamin D, either from direct effects of alcohol consumption or poor diet. Those deficiencies, in turn, may have deleterious effects on the digestive system. A study in mice (Zhong et al. 2013) found a relationship between zinc deficiency and gut leakiness. The study compared mice fed alcohol and a zinc-deficient diet with mice fed alcohol and a zinc-adequate diet. The zinc-deficient mice showed increased intestinal permeability and higher plasma endotoxin levels (for more on zinc, see the article by McClain).

Another study, conducted in intestinal cell culture and mice, examined whether vitamin D might protect gut health from alcohol exposure. In the cells, treating with vitamin D protected the cells from ethanol damage. In the mice, higher vitamin D levels measured in blood correlated with increased resistance to changes that lead to intestinal injury (Chen et al. 2015*b*). These findings suggest that vitamin D deficiency may promote the deleterious effects of alcohol on the gut barrier and, perhaps, that vitamin D supplementation may attenuate those effects.

The Clinical Relevance of the Alcohol-Induced Gut-Derived Inflammation

Alcohol-induced gut inflammation is believed to promote several disease states both within the GI tract, in the form of gastrointestinal cancers and inflammatory bowel disease, and outside the GI tract, in the form of, for example, liver disease and neuroinflammation (Rao et al. 2004). The following section briefly reviews a sample of the conditions associated with alcohol-related gut inflammation.

Alcohol and GI Cancers

Chronic alcohol consumption is associated with increased risk of major gastrointestinal cancers including cancer of the esophagus, stomach, and colon (colorectal cancer). The risk generally increases as alcohol consumption increases and in combination with other lifestyle-related factors, such as smoking tobacco or metabolic syndrome. And although alcohol was initially thought to act as a direct carcinogen, research instead suggests that alcohol-induced gut inflammation may be at fault (Thrift et al. 2011).

Systemic inflammation seen in metabolic syndrome and obesity increases risk of several types of epithelial cancers, including those in the gastrointestinal tract (Feakins 2016), suggesting that the systemic inflammatory state created by alcohol-induced gut inflammation also may contribute to alcohol-induced carcinogenesis in the GI tract and other organs. This process can snowball because, as cells transition to a cancerous state, ADH activity increases while ALDH activity may decrease (Testino et al. 2011). This leads to an increased oxidation rate and a decreased ability to clear alcohol metabolites (Testino et al. 2011), which in turn can further promote carcinogenesis through direct effects on DNA, oxidative stress, and gut inflammation (Jelski and Szmitkowski 2008).

Alcohol and Inflammatory Bowel Disease (IBD)

Several lifestyle factors such as smoking and diet affect the incidence and severity of IBD, most likely by modulating gut inflammation (Swanson et al. 2010). Alcohol consumption also may influence the course of IBD through associated gut inflammation (McGuckin et al. 2009); however, its effect in patients with IBD only has been studied in a few small studies. One study (Swanson et al. 2011), for example, examined the impact of 1 week of moderate (24 g to 36 g ethanol daily) red wine consumption on clinical disease activity and other noninvasive markers associated with increased risk of future disease flare. The study found no significant changes in indices of clinical disease but did find subclinical increases in markers for disease activity, including intestinal permeability. Such findings suggest that chronic alcohol consumption could increase the long-term risk for disease flare in IBD and supports the need for additional study.

Gut–Liver Axis

Approximately 20 to 30 percent of heavy drinkers (people who drink more than 30 grams/day for at least 10 years) develop clinically significant alcoholic liver disease, including alcoholic steatohepatitis and cirrhosis (Gramenzi et al. 2006). Several factors, such as the amount and duration of alcohol consumption, obesity, and gender, seem to moderate a person's risk and progression of alcoholic liver disease. In addition, studies find that alcohol-induced gut inflammation can contribute to liver injury by increasing intestinal permeability and the likelihood that gut-derived endotoxins enter the liver. One study (Keshavarzian et al. 1999) found that people with AUD who also have liver disease are much more likely to have intestinal permeability: more than 40 times more likely than people without AUD and more than 20 times more likely than people with AUD who do not have liver disease. In alcohol-fed rats, gut leakiness is evident 2 weeks after alcohol initiation; after another 2 weeks, endotoxemia develops and then liver injury, suggesting an intermediary role for endotoxemia on liver injury (Keshavarzian et al. 2009).

Once gut leakiness begins, endotoxins can enter the liver via the portal vein that drains from the gut. In the liver, gut-derived substances interact with the liver's hepatocytes, parenchymal cells, and immune cells. Alcohol exposure increases LPS levels in portal and systemic circulation (Wheeler et al. 2001), and that can have a host of deleterious effects:

- Initiating endotoxin-mediated hepatocellular damage by activating the innate immune system and leading to an increase in ROS and inflammatory cytokines, leukotrienes, and chemokines (Purohit et al. 2008);
- Activating signaling pathways that lead to proinflammatory cytokine release associated with liver fibrosis (Seki and Schnabl 2012); and
- Activating immune cells that can lead to liver inflammation and eventual fibrosis (Szabo et al. 2012).

Gut–Brain Axis

It is well established that the brain helps control the gut, and recently research suggests the opposite also is true: the gut can influence brain function (Hsiao et al. 2013). In fact, some evidence suggests that alcohol-induced intestinal permeability and LPS can influence psychological and cognitive function. For example, among a group of alcohol-dependent, noncirrhotic patients hospitalized for detoxification, the subset that showed signs of intestinal permeability and LPS also had higher scores on measures of depression, anxiety, and alcohol cravings and scored worse on measures of selective attention (Leclercq et al. 2012). These findings suggest that some of the biological and behavioral changes seen in people with AUD may extend from the systemic inflammatory response triggered by changes in the gut.

Although the mechanisms by which the gut–brain axis conveys the effect(s) of alcohol on the central nervous system (CNS) are not well established, several studies suggest that systemic inflammation, like that caused by alcohol-provoked leaky gut, can influence the nervous system in several ways. For example, alcohol-induced gut inflammation can result in a systemic inflammation that subsequently affects neuronal function and may drive some symptoms of alcohol withdrawal, including autonomic disturbances and anxiety (Retson et al. 2015). In addition, elevated cytokines caused by the inflammatory response may be able to enter the brain and disrupt the blood–brain barrier, starting a vicious cycle that perpetuates alcohol's effects on the CNS (Banks et al. 2015). Alcohol-induced dysbiosis may have its own effect on the CNS via vagal afferent nerve fibers, which influence areas of the brain implicated in AUD, including the thalamus, hippocampus, amygdala, and prefrontal cortex. Specifically, accumulating evidence suggests that alcohol-induced dysbiosis and gut microbiome may contribute to modifications in the vagal response and neuroinflammation in the CNS linked with alcohol-associated behaviors (Gorky et al. 2016). Other studies link microbiota alterations and endotoxins with neuroinflammation (Szabo and Lippai 2014) and anxiety-like behavior (Bercik et al. 2011; Hsiao et al. 2013). Studies in mice and humans suggest that antimicrobials and probiotics can positively influence brain function in healthy people, holding out promise that targeting gut microbiota in people with AUD might help defray alcohol's influence on brain function (Bercik et al. 2011; Tillisch et al. 2013).

Conclusions

Through multiple pathways, alcohol induces gut inflammation, which in turn promotes broad-spectrum pathologies both inside and outside the GI tract. In fact, many alcohol-related disorders, including cancers, liver disease, and neurological pathologies, may be exacerbated or directly affected by this alcohol-induced gut inflammation. The inflammation itself results from oxidative and nonoxidative pathways of alcohol metabolism that lead to a leaky gut, bacterial overgrowth, dysbiosis, and alterations in the mucosal immune system. As research uncovers the mechanisms by which alcohol affects gut inflammation and how that inflammation influences disease, researchers may be able to develop better strategies to prevent, or treat, conditions associated with chronic alcoholism. Already, studies are suggesting ways to modify diet and intestinal flora that may help alleviate some of the risks associated with chronic heavy drinking. Controlled trials are needed to assess the use of dietary supplementation with micronutrients in preventing or reversing alcohol effects.

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The authors declare that they have no competing financial interests.

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Neuroimmune Function and the Consequences of Alcohol Exposure

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Induction of neuroimmune genes by binge drinking increases neuronal excitability and oxidative stress, contributing to the neurobiology of alcohol dependence and causing neurodegeneration. Ethanol exposure activates signaling pathways featuring high-mobility group box 1 and Toll-like receptor 4 (TLR4), resulting in induction of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells, which regulates expression of several cytokine genes involved in innate immunity, and its target genes. This leads to persistent neuroimmune responses to ethanol that stimulate TLRs and/or certain glutamate receptors (i.e., N-methyl-D-aspartate receptors). Alcohol also alters stress responses, causing elevation of peripheral cytokines, which further sensitize neuroimmune responses to ethanol. Neuroimmune signaling and glutamate excitotoxicity are linked to alcoholic neurodegeneration. Models of alcohol abuse have identified significant frontal cortical degeneration and loss of hippocampal neurogenesis, consistent with neuroimmune activation pathology contributing to these alcohol-induced, long-lasting changes in the brain. These alcohol-induced long-lasting increases in brain neuroimmune-gene expression also may contribute to the neurobiology of alcohol use disorder.

Key words: Alcohol use, abuse, and dependence; alcohol effects and consequences; alcohol exposure; binge drinking; immunity; neuroimmune responses; neuroimmune genes; neurodegeneration; brain; microglia; stress axis; stress responses; oxidative stress; glutamate receptors; Toll-like receptors; cytokines; high-mobility group box 1; nuclear factor-kappa B

To a large extent, signaling processes between neurons in the brain are distinct from signaling mechanisms between cells in the immune system and use different signaling molecules. However, some proteins first discovered within the immune system act as both peripheral immune-signaling molecules and brain-signaling molecules. These neuroimmune factors include various cytokines, Toll-like receptors (TLRs), and high-mobility group protein box 1 (HMGB1). In the brain, both neurons and supporting glial cells (both astrocytes and microglia) contribute to the release of and responses to these neuroimmune factors. Neuroimmune signaling in the brain not only is a part of the innate immune response, but its effects also persist for long periods and could contribute to long-lasting changes in neurobiology.

Studies found that brain neuroimmune signaling is activated in models of binge drinking and neurodegeneration, suggesting another pathway through which alcohol may affect brain function. This review defines the roles of various cellular compartments and signaling molecules involved in neuroimmune activation, including the role of the stress axis in the communication between the central and peripheral immune systems and in sensitizing the neuroimmune response to alcohol. The article also will offer evidence from animal studies and postmortem human alcoholic brain studies that neuroimmune signaling may increase alcohol drinking and risky decision making and (in alcohol-treated animals) blunt the ability to change, decreasing behavioral flexibility.

Neuroimmune Signaling in the Alcoholic Brain

Monocytes and Innate Immune Genes

Innate immune genes are associated with rapid first-line responses to infections that involve primarily immune cells called monocytes (e.g., the acute-

phase response). These responses include increases in multiple cytokines as well as in their cellular receptors. Together, these changes amplify expression of a large number of genes through kinase signaling pathways that converge on two transcription factors called nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein-1 (AP-1). NF-κB and AP-1 promote expression of innate immune cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β), as well as of TLRs and cytokine receptors (see figure 1). In addition, innate immune responses include the activation of proteases and oxidases, particularly cyclooxygenase and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase,[1] as well as of major histocompatibility complex (MHC) signaling molecules, such as beta-2 microglobulin.

[1] NADPH oxidase is an enzyme that produces reactive oxygen species (ROS)—for example, during ethanol metabolism—thereby increasing oxidative stress and contributing to cell damage.

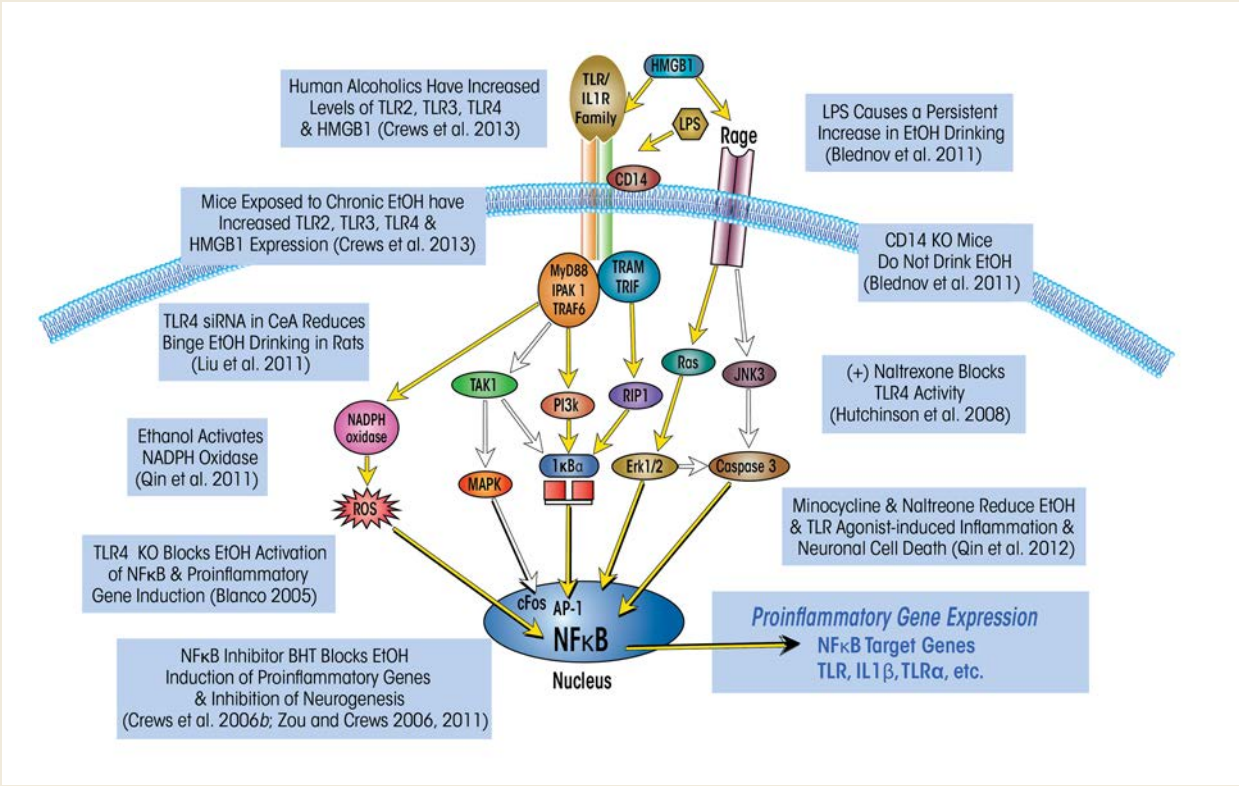

Figure 1 Simplified schematic of the Toll-like receptor (TLR) and the receptor for advanced glycation end products (RAGE) signaling cascades. Stimulation of TLRs with high-mobility group box 1 protein (HMGB1) and other inflammation-inducing agents leads to the generation of reactive oxygen species (ROS) and downstream activation of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF)-κB. Similarly, HMGB1 activation of the RAGE receptor results in downstream activation of NF-κB and induction of ROS. Transfer of NF-κB to the nucleus induces proinflammatory gene expression, neuroimmune induction, and cell death. Expression of several TLRs (i.e., TLR2, TLR3, and TLR4) and HMGB1 is upregulated in the postmortem human alcoholic brain and mouse brain following exposure to ethanol (Crews et al. 2013); this is accompanied by an upregulation of NADPH oxidase expression (Qin et al. 2011). Interestingly, blockade of neuroimmune signaling, either genetically (Blanco 2005) or pharmacologically (Crews et al. 2006*b*; Qin et al. 2012; Zou and Crews 2006, 2011), prevents ethanol-induced neuroimmune-gene induction and neurodegeneration. The neuroimmune system also contributes to alcohol-drinking behavior, because activation (Blednov et al. 2001) or blockade of this system (Blednov et al. 2011; Liu et al. 2011) increases and decreases self-administration, respectively.

NOTE: AP-1: activator protein-1; CD14: cluster of differentiation 14; ERK: extracellular signal–regulated kinase; IKK: inhibitor of NF-κB; IRAK 1: interleukin-1 receptor–associated kinase 1; JNK: c-jun N-terminal kinases; LPS: lipopolysaccharide; MAPK: mitogen-activated protein kinase; MyD88: myeloid differentiation primary response gene 88; NADPH oxidase: nicotinamide adenine dinucleotide phosphate-oxidase; PI3K: phosphatidylinositol-4,5-bisphosphate 3-kinase; RIP: receptor interacting protein; TAK1: transforming growth factor beta–activated kinase 1; TRAF: tumor necrosis factor receptor–associated factor; TRAM: TRIF-related adaptor molecule; TRIF: TIR-domain-containing adaptor–inducing interferon-beta.
SOURCE: Adapted from Crews et al. 2011, 2013.

The NF-κB–mediated transcription of proinflammatory genes, in turn, is amplified within and across cells by induction of TLRs and cytokine receptors (e.g., those that belong to the IL-1β receptor family), which induce innate immune gene expression. Amplification of innate immune gene induction across cells and tissues can cause pathology, such as sepsis. Sepsis and systemic inflammatory-response syndrome involve a "cytokine storm." This potentially fatal innate immune reaction consists of positive feedback loops between cytokines and immune and tissue cells, resulting in highly elevated levels of cytokines in the blood, multiorgan failure, and death (Osterholm 2005). Models of sepsis that involve activation of an acute phase-like response lead to increases in the levels of multiple cytokines in the blood that occur in two distinct phases. First, both TNF-α and IL-1β levels increase during the first several hours after infection but then subside. Subsequently, levels of HMGB1, a ubiquitously expressed, cytokine-like protein that can activate TLR4 and potentiate cytokine responses, increase about 16 hours after infection and remain elevated for several days (Wang et al. 2001). In mouse models, sepsis-induced death that occurs several days after infection is associated with HMGB1 and is prevented by treatment with antibodies blocking HMGB1. Survivors of sepsis show prolonged increases in serum HMGB1 and cognitive deficits that can be prevented with HMGB1-antibody treatment (Chavan et al. 2012). About half of the patients released from the hospital after surviving a cytokine storm–sepsis insult die within 5 years (Quartin et al. 1997). Thus, innate immune responses can be long lasting and can induce pathology long after they initially have been activated. However, although most studies support a central role for NF-κB–mediated transcription of proinflammatory cytokines, proteases, and oxidases in the innate immune response, both the precise mechanisms that regulate individual cell or cytokine activation and the contributions of tissues and cells in vivo to amplification of specific innate immune genes are poorly understood.

Monocytes are the primary cells mediating the innate immune response. They are found in all tissues, including the brain. Monocytes in the brain, which also are referred to as microglia, fall into two main categories: proinflammatory M1 monocytes/microglia and trophic M2 monocytes/microglia. M1 monocytes/microglia participate in the acute proinflammatory responses of the innate immune system; in addition, they also convey signals to the adaptive immune cells (i.e., T and B cells) through the MHC molecules they carry on their cell surface. These signals help create a persistent sensitization to pathogens (e.g., in the form of antibodies that mediate immunization). These proinflammatory effects occur in response to pathogens as well as tissue damage, cell death, and degeneration. Thus, M1 microglia and other monocyte-like cells consistently express multiple cytokine receptors and TLRs that, when activated, induce innate immune genes, such as proinflammatory cytokines, proteases, and oxidases, which help to break down, process, and remove damaged cells and tissue. In contrast, the M2 monocytes/microglia mediate a delayed response that initiates wound-healing trophic signaling and seem to be critical for healing. Both monocytes in general and brain microglia in particular can have both proinflammatory M1 and trophic M2 phenotypes (Colton 2009; Michelucci et al. 2009).

Although the M1 and M2 phenotypes are poorly understood, monocyte proinflammatory activation clearly is linked to NF-κB–mediated transcription of multiple innate immune genes. Activation of monocyte NF-κB by both pathogens and tissue damage involves TLR4 (see figure 1). This receptor responds to endotoxin released by certain bacteria (e.g., lipopolysaccharide [LPS]) as well as to HMGB1. Proinflammatory gene induction also is amplified by cytokine–receptor-activated release of HMGB1 that further contributes to innate immune gene induction.

The role of these innate immune-signaling molecules is well characterized within the immune system, but only recently these molecules have been discovered to also contribute to brain signaling. Thus, studies have indicated that MHC molecules contribute to brain development (Huh et al. 2000), to most neurodegenerative diseases (Gage 2002; Glass et al. 2010), and to alcohol and other drug dependence (Crews 2012). Neuroimmune signaling in the brain has not been extensively studied, and most knowledge on this subject is based on the assumption that monocyte responses elsewhere in the body reflect microglial and brain innate immune responses.

The Immune Response in the Brain

The immune system is not normally active in the healthy brain. Thus, the healthy normal brain does not contain antibodies and has only one type of immune cell, the microglia. During fetal development, neurons, astrocytes, and all other brain cells are formed from one embryonic structure (i.e., the ectoderm), whereas microglia migrate from another embryonic structure (i.e., the mesoderm) to the brain at a specific time (Ginhoux et al. 2010). In the healthy brain, the number of ramified or "resting" microglia equals that of neurons, and these cells contribute to the integration of sensory systems and overall survey of the brain milieu (Raivich 2005). Along with astrocytes, they modulate important metabolic, trophic, and synaptic functions in addition to responding to brain–damage-induced neuroimmune responses (Farina et al. 2007; Streit et al. 2004). Microglia respond to endogenous or exogenous insults with distinct morphological changes in shape (i.e., they develop "bushy" or "amoeboid-like" pheno-

types) as well as with marked alterations in gene expression, including proinflammatory innate immune-response genes (Graeber 2010). However, it sometimes is unclear whether microglia are responding to a brain insult or causing it through the release of proinflammatory cytokines. Microglia respond to and signal through both neuroimmune and neurotransmitter signals. For example, acetylcholine—an important neurotransmitter involved in multiple brain functions, including cognition—inhibits proinflammatory activation in both peripheral monocytes and brain microglia and has anti-inflammatory effects.

Some studies found an increase in the expression of the microglial marker Iba-1 in the brains of alcoholic individuals (see figure 2) (He and Crews 2008), suggesting that microglia contribute to the neurobiology of alcoholism. Microglia in postmortem human alcoholic brain and chronic alcohol-treated mouse and rat brain show increased MHC gene expression, but not the bushy or phagocytic activation profiles associated with marked brain damage. Chronic ethanol treatment also increases microglial TLR4 expression (Vetreno et al. 2013). Thus, microglia are the only immune cells in healthy brain and are integrated into the brain's responses to both neurotransmitters and neuroimmune signals. They also seem to contribute to chronic alcohol-induced responses.

Alcohol, Neuroimmune Signaling, and Neurodegeneration

Chronic binge-drinking models repeatedly found that ethanol exposure increases the expression of a variety of neuroimmune genes in the brain and that these alterations may persist over long periods (see figure 1). For example, one study found that chronic ethanol exposure induced the neuroimmune gene cyclooxygenase 2 (COX2) in multiple cortical and limbic brain regions long after physical signs of withdrawal had subsided (Knapp and Crews 1999). However, ethanol did not induce COX2 in transgenic mice lacking TLR4, suggesting that this process involves TLR4 (Alfonso-Loeches et al. 2010). Chronic alcohol exposure also altered the activity of NF-κB and another regulatory protein, cyclic AMP-responsive element binding protein (CREB). Specifically, ethanol treatment of HEC brain slice cultures increased NF-κB binding to DNA probes modeling gene promoter regions and decreased CREB binding to DNA probes modeling CREB-responsive gene promoter DNA (Zou and Crews 2006).

The CREB family of transcription factors is activated by phosphorylation; they promote neuronal survival, protecting neurons from excitotoxicity and apoptosis by regulating the transcription of pro-survival factors (Lonze and Ginty 2002; Mantamadiotis et al. 2002). Conversely, NF-κB is known widely for its ubiquitous roles in inflammatory and immune responses (O'Neill and Kaltschmidt 1997). Accordingly, NF-κB and CREB have different target genes. For example, CREB targets the neuropeptide Y and brain-derived neurotrophic factor (BDNF) genes, both of which are involved in promoting neuronal growth and resilience to insults, including protection against excitotoxicity and neuronal death (Lonze and Ginty 2002). Regular excitation of neurons increases synaptic plasticity related to CREB and induces synaptic proteins and BDNF. In contrast, excessive excitation triggers activation of certain extrasynaptic receptors for the neurotransmitter glutamate (i.e., *N*-methyl-D-aspartate [NMDA] receptors) and excitotoxicity, resulting in either rapid or delayed neuronal death, which is associated with reduced CREB (Hardingham and Bading 2010). Chronic ethanol exposure interferes with the normal functions of CREB. Thus, the levels of CREB phosphorylation and CREB–DNA binding as well as of the target gene BDNF all were decreased in the rat

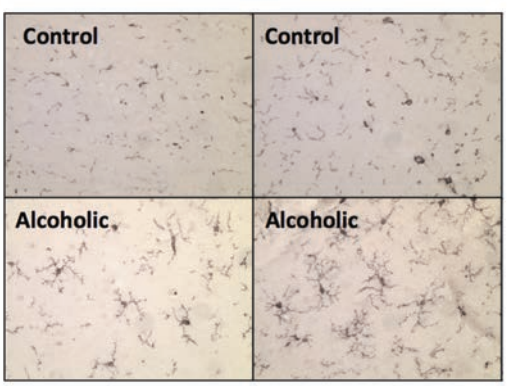

Figure 2 Microglial activation, as indicated by expression of the microglial marker Iba-1, is increased in postmortem alcoholic brain. The photomicrographs depict microglia from postmortem brain samples of alcoholics and control subjects. The number of Iba-1–positive microglia (dark stains) is higher in the alcoholic than in the control samples.

SOURCE: He and Crews 2008.

frontal cortex following a 24-hour withdrawal from chronic ethanol exposure (Pandey et al. 1999, 2001). In addition, neuropeptide Y levels were reduced in the cortex following ethanol treatment, an effect that was accompanied by reduced levels of phosphorylated CREB (Bison and Crews 2003).

The reciprocal relationship between NF-κB and CREB transcription sensitizes neurons to excitotoxicity (Zou and Crews 2006). This reciprocal relationship appears to result from the actions of kinases, such as protein kinase A, which activate CREB transcription but inhibit NF-κB activation. However, the reciprocal relationship also may represent differences between neuronal and glial signaling pathways, because regulation of CREB transcription principally occurs in neurons whereas NF-κB activation of proinflammatory genes primarily occurs in microglia.

In summary, ethanol can directly increase NF-κB–mediated transcription of proinflammatory genes in the brain as well as decrease trophic protective-factor transcription by reducing CREB transcription. Together, these effects decrease the brain's resilience to insults.

Roles of HMGB1 and TLR4

Ethanol induces neuroimmune genes through multiple mechanisms. One mechanism involves alcohol-induced release of HMGB1,[2] which increases NF-κB–mediated transcription of proinflammatory cytokines (Crews et al. 2013; Zou and Crews 2014). Several transmitters and neuroimmune-signaling receptors as well as neuronal excitability increase the release of HMGB1 (Maroso et al. 2010). This protein is a TLR4 agonist that acts through multiple signaling mechanisms in the brain, thereby influencing astrocytes and microglia, as well as neurogenesis, neurite growth, and excitability in adjacent neurons. HMGB1 released by neuronal activity stimulates TLR4 receptors, resulting in IL-1β release and increased phosphorylation of a subunit of the NMDA receptor (i.e., the NR2B subunit), which in turn increases susceptibility to seizures (Maroso et al. 2010; Vezzani et al. 2011). Actively released HMGB1 is acetylated, and ethanol increases HMGB1 acetylation in brain slice cultures. The acetyl-HMGB1 initially is found primarily in the cell's cytosol, likely in vesicles, before its concentration in the surrounding fluid increases progressively, consistent with neuronal release (Zou and Crews 2014). The importance of ethanol-induced release of HMGB1 and resulting TLR4 activation to ethanol-induced neurodegeneration and behavioral pathology was demonstrated in studies using cells and animals that no longer produced TLR4 (i.e., TLR4 knockout cells and mice). The experiments showed that knockout of TLR4 markedly blunted chronic–ethanol-induced neurodegeneration and induction of proinflammatory gene expression (Alfonso-Loeches et al. 2010; Blanco et al. 2005; Fernandez-Lizarbe et al. 2009; Pascual et al. 2011; Valles et al. 2004).

Additional studies found that ethanol treatment induced neuroimmune genes in microglia and astrocyte primary cultures as well as in mice and that this induction was dependent on the expression of TLR4. These receptors are always present on microglia, making microglia a key component of drug-induced neuroimmune activation (Alfonso-Loeches and Guerri 2011; Schwarz and Bilbo 2013). In addition, TLR4 is integral to ethanol-induced dopamine release (Alfonso-Loeches and Guerri 2011), damage to white matter (Alfonso-Loeches et al. 2012), and other pathologies associated with chronic–ethanol-induced changes in the brain (Pascual et al. 2011). In cultured cells, ethanol treatment increases innate immune gene expression in a time-dependent fashion, mimicking responses to LPS or IL-1β administration, although ethanol induces a much smaller response (Crews et al. 2013). In vivo, ethanol induces neuroimmune genes in the brains of wild-type mice, but not TLR4 knockout mice (Alfonso-Loeches et al. 2010). These studies support the hypothesis that TLR4 signaling is critical to many of the effects of alcohol on the brain.

It is not clear why signaling through TLR4 but not via other cytokine receptors seems to contribute significantly to ethanol responses, because all of these receptors generally belong to the same receptor superfamily (i.e., the TLR–IL1-R superfamily) (Wald et al. 2003) and share kinase cascades in monocytes and microglia that all converge upon NF-κB. The findings suggest that the TLR4s on neurons or other brain cells may have some unique properties that differ from NF-κB activation by receptors for TNF-α, IL-1β, and other cytokines (e.g., TNF receptor) which induce NF-κB transcription of proinflammatory cytokines. Further complicating the picture, the TLR4 signaling pathway is not the only one affected by ethanol exposure. Vetreno and colleagues (2013) found that chronic intermittent treatment of adolescent rats also led to persistent increases in the expression of another receptor stimulated by HMGB1, called receptor for advanced glycation end products (RAGE) (see figure 1). Although the mechanisms remain complicated, together these studies suggest that HMGB1–TLR4 and perhaps RAGE signaling (which are found on multiple brain cells types) as well as neuronal–glial neuroimmune signaling and microglial–astrocyte activation all contribute to alcohol-induced brain damage.

Effects of Acute vs. Chronic Ethanol Exposure

Although chronic alcohol treatment increases proinflammatory gene expression in the brain through activation of TLR4, this is confounded by acute alcohol inhibition of TLR4 signaling in monocytes and possibly

[2] HMGB1 also is known as amphoterin (Huttunen and Rauvala 2004).

other cells. Time-dependent acute and chronic opposing effects of ethanol confound many studies (Crews et al. 2006*a*, 2011; Szabo and Mandrekar 2009). Acute ethanol suppresses the innate immune response to LPS, a TLR4 agonist, in both in vivo and in vitro models. For example, LPS-induced TNF-α and IL-1β production is blunted in blood monocytes obtained from healthy human volunteers after acute alcohol exposure (Crews et al. 2006*a*; Szabo et al. 1993, 1995, 2001). In animal models, acute ethanol exposure attenuates the TNF-α, IL-1β, and IL-6 immune responses to LPS (Pruett et al. 2004). Similarly, in in vitro models, addition of ethanol (25 mM) just before LPS blunts induction of TNF-α (Szabo et al. 1993, 1995, 2001). In contrast, chronic in vitro ethanol exposure of astrocytes, microglia, and brain slices induces NF-κB transduction of proinflammatory genes through activation of TLR4 signaling (Crews et al. 2013; Fernandez-Lizarbe et al. 2009; Zou and Crews 2014).

While it is unclear if the presence of acute ethanol exposure antagonizes TLR4 on all cell types, other TLRs are not acutely blocked by ethanol (Crews et al. 2006*a*). Upregulation of TLRs by chronic alcohol treatment can lead to sensitization. In mice, binge treatment with ethanol for 10 days (5 g/kg/day), followed by LPS 24 hours later when alcohol had cleared, resulted in a marked increase in proinflammatory gene induction (Qin and Crews 2012*b*). Ethanol treatment increased the responses to LPS-induced proinflammatory cytokines in liver, blood, and brain. The responses were transient in blood and liver but were long lasting in brain. Similarly, chronic 10-day alcohol treatment sensitized mice to the proinflammatory response to Poly:IC, a compound that activates TLR3 (Qin and Crews 2012*a*). Thus, the effects of ethanol on brain neuroimmune signaling are in part related to increases in TLRs (see figure 1) that increase neuroimmune signaling and cytokines, such as IL-1β, during chronic ethanol treatment, although the presence of alcohol can blunt TLR4 responses during intoxication.

Ethanol Induction of HMGB1–TLR Signaling in the Brain

As mentioned previously, studies investigating the mechanisms of ethanol induction of proinflammatory genes in the brain have shown that chronic ethanol increases expression of TLRs as well as the TLR4 receptor agonist HMGB1. Studies of chronic 10-day ethanol treatment of mice (Crews et al. 2013), chronic in vitro treatment of rat brain-slice cultures (Zou and Crews 2014), and analyses of postmortem human alcoholic brain (Crews et al. 2013) all found increased expression of HMGB1, TLR4, TLR3, and TLR2 (see figure 3).[3] Increases in receptors and agonists are common in innate immune signaling, and these findings suggest that chronic alcohol, through induction of HMGB1 and TLR4 as well as the less well characterized RAGE receptor, may contribute to increases in neuroimmune-gene expression. Brain-slice culture experiments found that ethanol could induce HMGB1 release, which then increased proinflammatory gene expression. This process could be blocked by pharmacological antagonists or knockdown of TLR4 (Crews et al. 2013; Zou and Crews 2014). Studies in adolescent rats (Vetreno and Crews 2012), adolescent mice (Coleman et al. 2014), and adult mice (Qin et al. 2007, 2008, 2013) found long-lasting increases in neuroimmune-gene induction following alcohol treatment.

In humans, levels of HMGB1 and TLR expression in specific brain regions (e.g., the orbitofrontal cortex) have been shown to correlate with lifetime alcohol consumption (Crews et al. 2013) (see figure 4). Alcoholic subjects who vary greatly in the duration and amounts of active drinking bouts exhibited a large variation in lifetime alcohol consumption that correlated with increased HMGB1–TLR expression in the frontal cortex. In contrast, moderate-drinking humans consumed much less alcohol than alcoholics and exhibited much lower HMGB1–TLR expression. This interesting correlation only could occur if ethanol induction of HMGB1–TLR was persistent and cumulative with binge-drinking episodes (see figure 4). Together, these studies suggest that HMGB1–TLR4 signaling is increased by chronic binge drinking, contributing to the persistent and sustained induction of proinflammatory signaling in brain.

[3] Researchers have identified 13 TLRs (i.e., TLR 1-13) in mammals (Medzhitov 2001; Takeda et al. 2003); however, only TLR2, TLR3, and TLR4 have been assessed in alcoholic brain.

Mechanisms of Neurodegeneration Related to Alcohol's Effects on Neuroimmune Signaling in the Brain

Role of NADPH Oxidase and Oxidative Stress

One innate immune gene induced by ethanol and LPS is NADPH oxidase, a multi-subunit enzyme that catalyzes the formation of the reactive oxygen species (ROS), superoxide, and thereby increases oxidative stress. NADPH oxidase first was characterized as a phagocytic oxidase in monocytes, where it was hypothesized to contribute to the oxidation of infectious agents. The superoxide produced by NADPH oxidase can increase NF-κB transcription, thereby creating another amplifying loop of proinflammatory signaling (see figure 1). More recent studies have found that there are multiple genes and forms of NADPH oxidase.

Qin and Crews (2012*b*) discovered that LPS and ethanol can increase expression of NADPH oxidase subunits, particularly the superoxide-forming $gp91^{phox}$ subunit, in the brain and that ethanol treatment of mice increased superoxide formation in

the brain as well as neuronal death. Inhibition of oxidases both reduced superoxide formation and protected against alcohol-induced neuronal death. Other studies in mice demonstrated that LPS treatment induced neuroimmune-gene expression, NADPH-oxidase activity, and oxidative stress that persisted for at least 20 months and led to neurodegeneration (Qin et al. 2013). Prolonged induction of NADPH oxidase and oxidative stress in the brain could contribute to the persistent increase in NF-κB transcription observed after alcohol exposure, because ROS can activate NF-κB. These findings are consistent with the hypothesis that oxidative stress, by inducing innate immune genes, significantly contributes to alcoholic brain damage and alcoholic neurodegeneration.

In addition to enhancing ROS levels, alcohol exposure decreases endogenous antioxidant levels, thereby reducing the body's natural defense against ROS and again increasing oxidative stress (Henderson et al. 1995). Specifically, ethanol decreases the levels of the antioxidant glutathione and the cellular activity of antioxidative enzymes, such as glutathione peroxidase, catalase, and superoxide dismutase. Furthermore, a synthetic superoxide dismutase/catalase mimetic (EUK-134) and a water-soluble analog of vitamin E (Trolox), both of which are well-known antioxidants, protected developing hypothalamic neurons from oxidative stress and cellular apoptosis caused by ethanol-treated microglia medium (Boyadjieva and Sarkar 2013).

Role of Hyperexcitability and Excitotoxicty

Another mechanism contributing to alcoholic neurodegeneration and associated with HMGB1–TLR4 signaling is the excessive stimulation of receptors that results in neuron damage and cell death (i.e., excitotoxicity). Chronic ethanol treatment of neurons leads to increased sensitivity to excitotoxicity (Chandler et al. 1994). This effect primarily involves the neurotransmitter glutamate and its receptors.

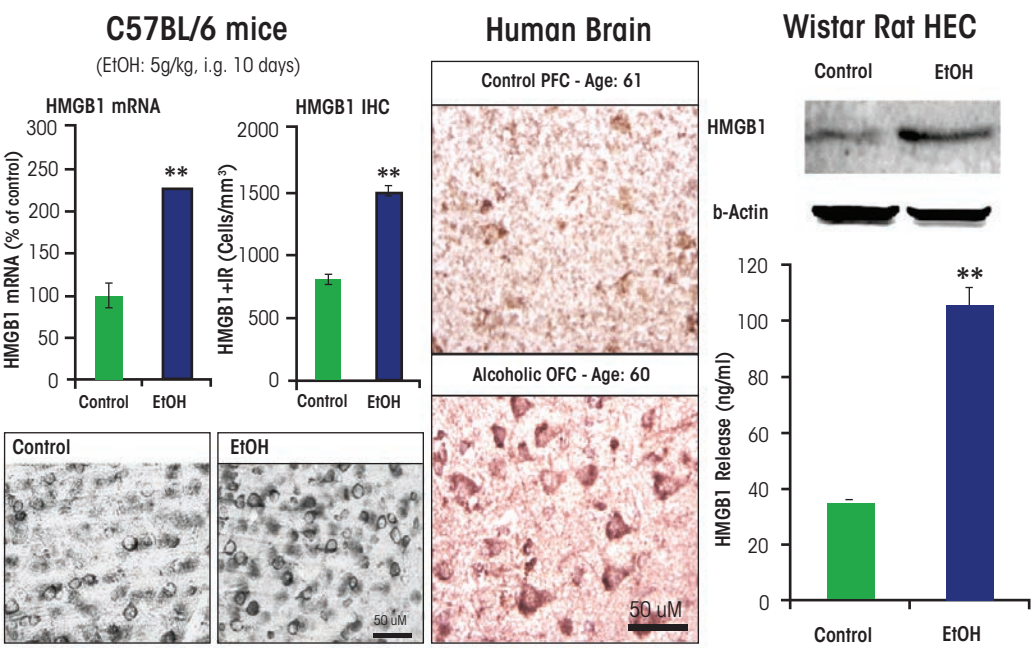

Figure 3 Alcohol increases high-mobility group box 1 (HMGB1) expression in mouse brain, and human brain and induces HMGB1 release from rat brain slices. **(Left)** Chronic ethanol treatment of mice for 10 days increases expression of HMGB1 mRNA and protein. **(Middle)** Postmortem human alcoholic orbitofrontal cortex (OFC) has significantly more HMGB1-immunoreactive cells than seen in age-matched moderately drinking control subjects. **(Right)** Ethanol causes the release of HMGB1 into the media from hippocampal-entorhinal cortex (HEC) slice culture.

NOTE: ** $P < 0.01$, relative to the corresponding control group.
SOURCE: Adapted from Crews et al. 2013.

However, the relationship between ethanol and glutamate receptors is complex. Thus, although ethanol enhances overall glutamate excitotoxicity, in neuronal primary cultures it blocks excitotoxicity associated with a specific type of glutamate receptor (i.e., the NMDA receptor). This is consistent with many studies finding that ethanol inhibits NMDA receptors (Chandler et al. 1998). Yet at the same time, HMGB1–TLR4 signaling (Balosso et al. 2014) and IL-1β receptor signaling (Viviani et al. 2003)—both of which, as described above, are induced by chronic ethanol—increase NMDA receptor-mediated calcium flux, neuronal excitability, and excitotoxicity through activation of kinase signaling cascades, including activation of Src kinase and tyrosine-kinase (see figure 5). Furthermore, Suvarna and colleagues (2005) found that ethanol increases NMDA excitability in the hippocampus through kinase activation that alters receptor trafficking, leading to increased numbers of NMDA receptors containing the NR2B subunit at the synapse.

Another mechanism through which chronic ethanol induces hyperexcitability involves neuroimmune inhibition of glial glutamate transporters (Zou and Crews 2005). Thus, in brain-slice cultures, ethanol potentiates excitotoxicity by causing blockade of the molecules that normally remove glutamate from the synapse into glial cells and may perhaps even induce glutamate release from those cells (Zou and Crews 2006, 2010).

As indicated above, ethanol causes HMGB1 release, creating hyperexcitability that disrupts synaptic plasticity and sensitizes to excitotoxicity. HMGB1 is massively released during brain damage, resulting in persistent neuroimmune-gene induction (Kim et al. 2006). Maroso and colleagues (2010) found that increased HMGB1 release was associated with hippocampal excitability that caused seizures, leading to persistent increases in HMGB1 and excitability. Ethanol has modest cumulative effects with repeated chronic exposure, further exacerbating excitability and excitotoxicity resulting from increased neuroimmune signaling. Thus, the global neurodegeneration associated with alcoholism, with the most severe losses observed in the frontal cortex, is secondary to the persistent and progressive neuroimmune activation.

Neuroimmune-Gene Expression in Postmortem Human Alcoholic Brain

In addition to the HMGB1–TLR4 signaling cascade, multiple other proinflammatory genes are increased and have been detected postmortem in the brains of alcoholics. Initial

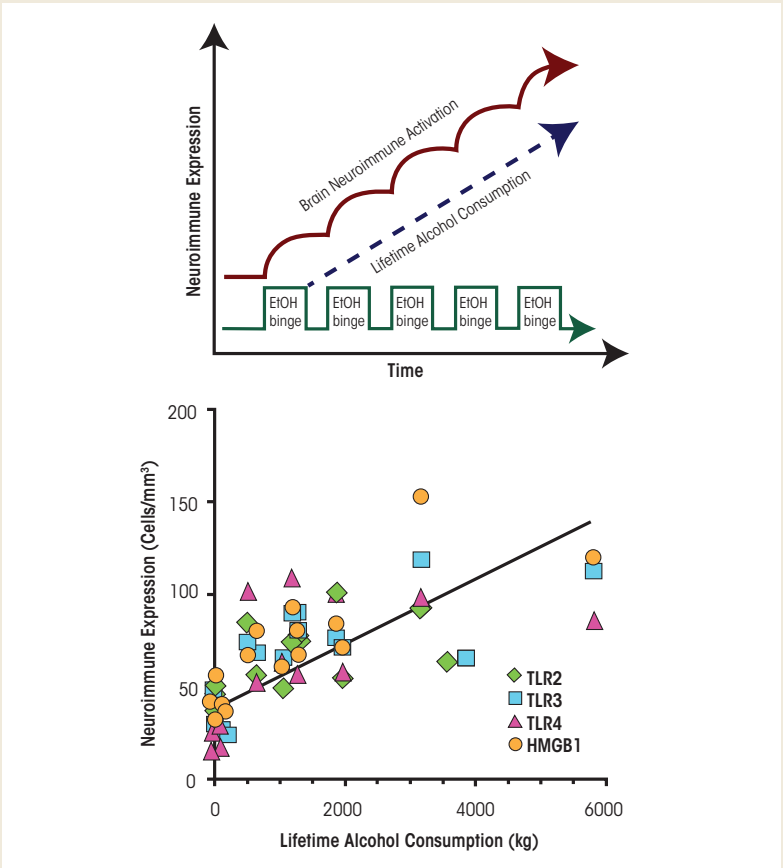

Figure 4 Cycles of chronic alcohol consumption lead to persistently increased neuroimmune-gene expression. **(Top)** Repeated ethanol (EtOH) binges result in increased brain neuroimmune activation (i.e., microglial and astrocytic activation as well as upregulated neuroimmune-gene expression). **(Bottom)** In humans, lifetime alcohol consumption is positively correlated with neuroimmune signal immunoreactivity. Symbols indicate levels of Toll-like receptor (TLR) 2, TLR3, TLR4, and high-mobility group box 1 (HMGB1) in individual moderate drinkers and alcoholics. Results for moderate drinkers are clustered along the Y-axis because of their low lifetime alcohol consumption and similar neuroimmune expression. Alcoholic subjects show a more than 10-fold variation in lifetime alcohol consumption as well as considerable variation in expression of all four neuroimmune genes.

NOTE: Correlations are as follows: TLR2: $r = 0.66$ ($p < 0.01$); TLR3: $r = 0.83$ ($P < 0.001$); TLR4: $r = 0.62$ ($P < 0.01$); HMGB1: $r = 0.83$ ($P < 0.001$).
SOURCE: Crews et al. 2013.

human brain studies focused on microglia and the proinflammatory cytokine monocyte chemotactic protein-1 (MCP-1, also known as CCL2), which among the cytokines tested was induced most robustly by ethanol in brain-slice cultures (Zou and Crews 2012). Additional studies also showed increased levels of MCP-1 protein in the ventral-tegmental area, amygdala, nucleus accumbens, and hippocampus (He and Crews 2008). In addition to MCP-1, expression of the microglial marker Iba-1 also was increased. These studies indicate that neuroimmune-gene expression is increased in the human alcoholic brain.

Subsequent studies focusing on the prefrontal cortex, specifically the orbital frontal cortex (OFC), found increased levels of HMGB1 as well as TLRs (specifically TLR2, TLR3, and TLR4) in postmortem alcoholic brain (Crews et al. 2013). Furthermore, NADPH oxidase was increased in alcoholic OFC, consistent with increased oxidative stress as found in mice. The HMGB1 receptor RAGE also was increased in postmortem human alcoholic brain (Vetreno et al. 2013). Finally, studies detected increased IL-1β inflammasome markers in the hippocampus of postmortem alcoholic brains that could contribute to loss of neurogenesis. These observations indicate that multiple neuroimmune genes are increased in alcoholic brain and likely contribute to neurodegeneration and the neurobiology of alcoholism in humans.

Researchers also investigated the relationship between alcohol drinking and neuroimmune-gene expression in alcoholics and control subjects. Interestingly, two forms of correlations were found linking neuroimmune-gene expression to alcohol consumption and alcoholism. The first correlation involved the age at drinking onset (Vetreno et al. 2013). Adolescent drinking is known to increase risk of developing alcohol dependence, with the risk decreasing with every year of delaying alcohol-use initiation across adolescence (for more information, see the sidebar). Studies found that in the OFC, a negative

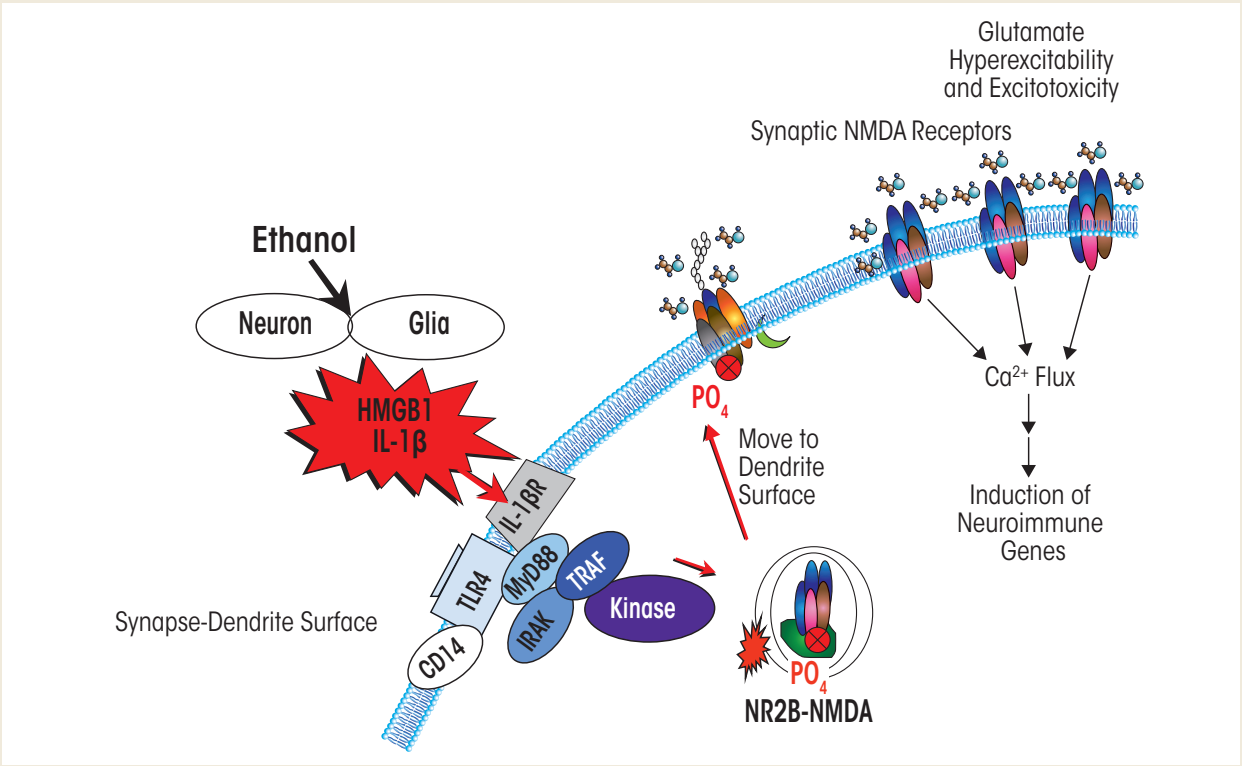

Figure 5 Simplified schematic depicting how neuroimmune signaling leads to neuronal hyperexcitability and the neurobiology of addiction. Alcohol and stress activate neurons and glia in the central nervous system, resulting in the release of various neuroimmune signals (e.g., high-mobility group box 1 [HMGB1] and interleukin-1beta [IL-1β]) that activate neuroimmune receptors (e.g., Toll-like receptors [TLRs]). Neuroimmune receptor stimulation leads to phosphorylation, and thus activation, of glutamatergic *N*-methyl-D-aspartate (NMDA) receptors that are transported to the cell surface (Iori et al. 2013; Maroso et al. 2010). The increased number of NMDA receptors increases Ca^{2+} flux, triggering further induction of neuroimmune genes, and also promotes glutamate hyperexcitability and excitotoxicity.

correlation existed between HMGB1–TLR4 expression and age at drinking onset, with lower HMGB1–TLR4 expression in individuals who initiated alcohol use later. The second correlation involved the amount of alcohol consumed, with total lifetime alcohol consumption positively correlated with OFC expression of HMGB1, TLR4, TLR3, TLR2, and RAGE (Crews et al. 2013). These findings further support the role of neuroimmune signaling in alcoholic brain and alcoholic neurodegeneration.

Role of Microglia in Mediating Alcohol Actions in the Brain

Given their role in facilitating inflammation, it is not surprising that alcohol-activated microglia have been implicated in alcohol-induced inflammatory pathways. In rats, intermittent and chronic alcohol exposure can activate microglia while concomitantly increasing expression of proinflammatory cytokines and neuronal cell death, providing indirect evidence for the role of microglia in alcohol-induced neuroinflammation and neurotoxicity (Alfonso-Loeches and Guerri 2011; Chastain and Sarkar 2014; Zhao et al. 2013). Alcohol can activate microglia directly, via stimulation of TLRs, or indirectly, via neuronal damage and subsequent release of damage-associated molecular patterns that include HMGB1, resulting in the accumulation of microglia in the brain (i.e., reactive microgliosis) (Alfonso-Loeches and Guerri 2011). Microglial TLR4 seems to be necessary in alcohol- induced activation of microglia and subsequent microglial production of inflammatory mediators and apoptosis of neighboring neurons (Fernandez-Lizarbe et al. 2009, 2013). In an in vitro study (Boyadjieva and Sarkar 2010), microglia-conditioned media enhanced ethanol-induced apoptosis of cultured hypothalamic neurons. Interestingly, the neuronal cell death induced by microglia-conditioned media could be abolished if TNF-α was inactivated in the cultured cells, suggesting that microglial TNF-α production plays a key role in ethanol-induced neurotoxicity in developing neurons. The mechanism by which alcohol induces neuronal cell death may involve upregulation of NF-κB expression, which then stimulates release of TNF-α, resulting in neuronal apoptosis (Crews and Nixon 2009; Guadagno et al. 2013). Stimulation of the transcription factor AP-1 and release of IL-1β, IL-6, and transforming growth factor β (TGF-β1) also may contribute to alcohol-induced neuronal apoptosis (Alfonso-Loeches and Guerri 2011; Chen et al. 2006).

In addition to releasing cytokines, stimulated microglia contribute to neurotoxicity by secreting ROS (Takeuchi 2010). ROS, such as superoxide, hydrogen peroxide, and nitric oxide, can break down cell membranes and induce cell death. After alcohol exposure, ROS levels increase both as a natural byproduct of alcohol metabolism and as a result of enhanced cellular respiration, thus creating oxidative stress and leading to neuronal cell death (Guerri et al. 1994; Montoliu et al. 1995). Several studies have implicated microglia in the alcohol-induced production of ROS and resulting neurotoxicity. Qin and Crews (2012*a*) demonstrated that mice exposed to chronic alcohol showed increased levels of NADPH oxidase, superoxide, microglial activation, and cell death in cortical and hippocampal brain regions. Inhibition of NADPH oxidase during alcohol administration decreased superoxide, microglial activation, and cell death, directly linking ROS production to alcohol-induced microglial activation and neurotoxicity. In accord with these in vivo findings, in vitro studies showed that microglia-conditioned media enhanced ethanol-induced ROS production and oxidative stress in cultured hypothalamic neuronal cells and increased apoptotic cell death (Boyadjieva and Sarkar 2013*a*). Through these mechanisms, as well as the ethanol-related decreases in antioxidants discussed earlier, ethanol-activated microglia can induce apoptotic cell death and cell death in cultured fetal hypothalamic neurons from rat, suggesting that microglia may help facilitate ethanol-induced neurotoxicity by ROS.

Another cellular signaling mechanism by which alcohol induces neuronal apoptosis involves increased neuronal release of TGF-β1. Alcohol-induced elevation of TGF-β1 levels in neuronal cells is accompanied by a host of molecular and chemical changes related to cell death, including the following (Chen et al. 2006; Kuhn and Sarkar 2008):

- Increased expression of a protein called E2F1, whose overexpression sensitizes cells to apoptosis;
- Reduced expression of two key regulators of cell-cycle progression (i.e., cyclin D1 and cyclin-dependent kinase-4);
- Elevated levels of mitochondrial proapoptotic proteins bak, bad, and bcl-xs;
- Lowered levels of the antiapoptotic protein bcl-2; and
- Increased production of the apoptotic enzyme caspase 3.

Interestingly, in transformed cells, inhibition of NF-κB or ROS abrogates TGF-β1 stimulation of cell functions (Tobar et al. 2010). Hence, the ROS–NF-κB–TGF-β1 signaling cascade is a possible mechanism by which alcohol induces the apoptotic process in neurons—a process that is modulated by microglia. Another mechanism might relate to the microglial ability to reduce production of BDNF and cyclic adenosine monophosphate (cAMP) in neurons following ethanol activation. Thus, hypothalamic neuronal cell cultures treated with ethanol-activated microglia-conditioned medium showed decreased levels of both of these compounds. Treatment with BDNF or dibutyryl

cAMP decreased the changes in the levels of intracellular free radicals, ROS, nitrite, glutathione, and catalase as well as neuronal apoptotic cell death that otherwise occurred when these cultures were treated with ethanol-activated microglia-conditioned medium. These findings suggest that ethanol increases the production of certain microglia-derived factors, thereby reducing cellular levels of cAMP and BDNF and increasing cellular oxidative stress and apoptosis in neuronal cells (Boyadjieva and Sarkar 2013*b*). However, further studies are needed to fully elucidate the mechanism(s) by which ethanol-activated signaling induces neuronal death.

Microglia also may mediate the effects of alcohol administration on the development of new neurons (i.e., neurogenesis). Alcohol exposure can result in decreased hippocampal neurogenesis, an effect that may underlie alcohol-related neurodegeneration (Crews et al. 2006; Morris et al. 2010). However, alcohol exposure followed by a period of abstinence results in increased hippocampal neurogenesis, which may serve a regenerative purpose. Interestingly, this process is preceded by microglial proliferation, raising the possibility that microglia may facilitate some regenerative mechanisms in recovery from alcohol exposure (McClain et al. 2011; Nixon et al. 2008).

In addition to being implicated in alcohol-induced neurotoxicity, microglia also might contribute to the processes that lead to the development of alcohol use disorder. Recent studies in rodents support a role for microglia in voluntary alcohol drinking and preference. In a quantitative-trait locus analysis of six strains of mice that differ in voluntary alcohol-drinking behavior, alcohol-preferring animals exhibited an increase in the expression of β-2-microglobulin, an NF-κB target gene involved in microglial MHC immune signaling (Mulligan et al. 2006). In addition, knockout of the β-2-microglobulin gene in mice decreased voluntary alcohol consumption and preference (Blednov et al. 2012). Finally, treatment with minocycline, an antibiotic and selective inhibitor of microglia, reduced voluntary alcohol consumption in adult mice (Agrawal et al. 2014). These studies suggest microglia might mediate alcohol preference and might contribute to the development of alcohol use disorder.

Neuroimmune Signaling Integrates CNS Responses to Alcohol and Stress

The Stress Axis and the Peripheral Immune System

Alcohol activation of immune signals and cytokine production in the brain affects not only cellular functions in the brain but also immune-system function in the periphery. The body's main stress response systems—the hypothalamic–pituitary–adrenal (HPA) axis and the sympathetic nervous system (SNS)—are major pathways by which the brain and the immune system communicate. When the HPA axis is activated by a stressful situation, the hypothalamus releases corticotropin-releasing hormone (CRH), which acts on the pituitary to induce the release of adrenocorticotropic hormone. This hormone in turn acts on the adrenal glands to stimulate the release of stress hormones (i.e., glucocorticoids), including cortisol in humans and corticosterone in rodents. These hormones then help coordinate the body's response to the stress. The SNS is part of the autonomic nervous system that regulates the body's unconscious activities to maintain its normal functions. One of the main processes coordinated by the SNS is the fight-or-flight response to stress.

Alcohol has a potent activating effect on the HPA axis as well as on neuroimmune signaling; therefore, these effects may integrate the responses of the central nervous system (CNS) to alcohol and stress (figure 6). For example, TNF-α, IL-1β, and IL-6 act upon the HPA axis and SNS, both directly via local effects and indirectly via the CNS (Besedovsky and del Rey 1996; Pickering et al. 2005; Wilder 1995). Furthermore, CRH has a variety of complex effects on immune cells (Elenkov et al. 1999) and modulates immune/inflammatory responses through receptor-mediated actions of glucocorticoids on anti-inflammatory target immune cells (Tsigos and Chrousos 2002). In contrast, elevated glucocorticoid levels in the prefrontal cortex are proinflammatory, potentiating LPS–TLR4 activation of NF-κB and other proinflammatory signals (Munhoz et al. 2010). The neurotransmitter norepinephrine that is released by SNS activation also disturbs inflammatory cytokine networks and innate immune-cell function. Similarly, the hypothalamic peptide β-endorphin (BEP), whose release is stimulated by CRH during HPA activation, can inhibit stress-hormone production and activate peripheral immune functions (Sarkar and Zhang 2013). All of these findings suggest that the stress–HPA axis, commonly thought to involve anti-inflammatory glucocorticoid actions, also contributes to stress–alcohol responses in the brain that can increase proinflammatory HMGB1–TLR–cytokine signaling.

HPA hormones influence the immune system in multiple ways (figure 6). Glucocorticoids prevent the migration of leukocytes from the circulation into extravascular regions, reduce accumulation of various immune cells (i.e., monocytes and granulocytes), and suppress the production and/or action of many cytokines and inflammatory mediators (Hermann et al. 1995; Sheridan et al. 1998; Zhang et al. 1998). They also inhibit a number of cytokines, including IL-1α, IL-1β, IL-6, IL-12, IFN-γ, TNF-α, granulocyte-macrophage colony-stimulating factor, and chemokine (C-C motif) ligand 5 (RANTES)

Adolescence and Persistent Neuroimmune Expression in the Brain

Adolescence is a developmental stage characterized by increased play behavior, thrill seeking, risk taking, puberty, and transition to independence. During this stage, the brain continues to develop; in particular, the frontal cortex continues to exhibit structural changes that coincide with maturation of adult behaviors and executive functions (Ernst et al. 2009). The developing brain is uniquely sensitive to alcohol, making adolescence a critical period of risk for developing alcohol use disorder (AUD) (Crews et al. 2007). Adolescence also is a period of experimentation, as exemplified by findings that alcohol-use initiation use typically begins during those years. The age of drinking onset is associated with various alcohol-related characteristics, including prevalence of lifetime AUD, as well as violence, fights, and injuries associated with alcohol use (Brown et al. 2008; Dawson et al. 2008; Sher and Gotham 1999). The younger the age of drinking onset, the more likely the person will develop AUD. In addition, binge drinking peaks during late adolescence.

The high prevalence of binge drinking among adolescents increases the importance of understanding how binge drinking might affect the adolescent brain. Studies found that a younger age of drinking onset is associated not only with an increased risk of lifetime AUD but also correlates with a smaller brain size and greater expression of high-mobility group box 1 (HMGB1) and Toll-like receptor 4 (TLR4), as well as other neuroimmune signaling receptors (Vetreno et al. 2013). These associations likely result both from pre-existing conditions that mature into dysfunctional behavior and from alcohol-induced factors than change over the life course and increase dysfunctional behavior, perhaps by altering brain maturation. The contributions of these two factors can only be determined by controlled experiments in which adolescent alcohol exposure is the only variable and genetic and other factors play no role. Such studies cannot be done in humans but are being done in animals (primarily rats) whose genetic background and environment can be controlled. The essential need to understand the neurobiology and impact of adolescent drinking on adulthood resulted in the formation of a consortium called NADIA, funded by the National Institute on Alcohol Abuse and Alcoholism, which addresses the contribution of adolescent alcohol abuse to adult psychopathology.

Adolescents have an immature response to alcohol, characterized by unique factors that differ from the adult response to alcohol. For example, adolescent rats show greater ethanol-induced memory impairment in certain tasks (e.g., the Morris water maze and discrimination tasks) than do adults (Land and Spear 2004; Markwiese et al. 1998). Similarly, humans who initiate alcohol use in their early 20s are more sensitive to the effects of alcohol on multiple memory tasks compared with those who start drinking in their late 20s (Acheson et al. 1998). Also, compared with adults, adolescents exhibit more potent inhibition of NMDA receptor-mediated synaptic activity in the hippocampus (Swartzwelder et al. 1995) as well as greater induction of long-term potentiation (LTP) (Martin et al. 1995). Adolescents, who already exhibit social behaviors, also are uniquely sensitive to the social facilitative effects of ethanol (Varlinskaya and Spear 2002). Consistent with findings in humans, adolescent rats are more sensitive to binge-drinking models of brain damage, particularly in the frontal cortex (Crews et al. 2006). Interestingly, adolescent rats are less sensitive than adults to certain effects of alcohol, such as the sedative (Little et al. 1996; Silveri and Spear 1998), motor impairing (Little et al. 1996, White et al. 2002*a,b*), social inhibitory (Varlinskaya and Spear 2002), and aversive (Anderson et al. 2010) effects. Adolescent rats also show electrophysiological differences from adults in the hippocampus, particularly a reduced sensitivity to γ-aminobutyric acid (GABA) type A (GABAA) receptor-mediated inhibition (Carr et al. 2003; Sullivan et al. 2006; Yan et al. 2009, 2010). The reduced sedative sensitivity to alcohol and increased alcohol-induced cognitive disruption observed in adolescent animals is consistent with findings in humans that adolescents have high rates of binge drinking and are at particularly high risk of alcohol-related traffic crashes. The continuous increase in high binge-drinking levels in human adolescents over the past decade justifies the need to study the long-term consequences of adolescent alcohol abuse in more detail.

Like adult alcohol exposure, adolescent exposure induces neuroimmune genes in the brain; furthermore, in humans, the effect on neuroimmune genes correlates with age of drinking onset. Indeed, Vetreno and Crews (2012) found that intermittent binge-ethanol treatment in adolescent rats increased expression of multiple innate immune genes in the frontal cortex during adulthood. Interestingly, whereas expression of the critical neuroimmune signaling receptor TLR4 decreased during adolescence in controls, expression of this receptor increased and remained elevated into adulthood in adolescents with binge ethanol exposure (Vetreno and Crews 2012). In contrast, the expression of HMGB1 in the frontal cortex

increased during adolescence in control subjects, and this increase was exacerbated by adolescent binge ethanol exposure. Moreover, adolescent alcohol exposure resulted in a persistent increase in adult HMGB1 and TLR4 levels that may represent adolescent-like HMGB1–TLR4 signaling in these adults.

As mentioned in the main article, HMGB1–TLR4 signaling induced by alcohol exposure can enhance sensitivity at the NMDA glutamate receptor, which can counteract ethanol's direct inhibitory effects on this receptor. Accordingly, adults with persistent increases in HMGB1–TLR4 signaling resulting from adolescent alcohol exposure might experience adolescent-like tolerance to alcohol's sedative effects, and perhaps increased adolescent-like cognitive disruption as well. Although adolescent alcohol exposure does not markedly disrupt adult learning tasks, adolescent intermittent binge exposure induces deficits in reversal learning in adult rats (Vetreno and Crews 2012) and mice (Coleman et al. 2011). These studies are consistent with the hypothesis that the adolescent brain is vulnerable to long-lasting changes that persist through maturation into adulthood. Persistent neuroimmune-gene induction likely contributes to continuous slow neurodegeneration as well as to more specific insults on key neurotransmitters that mature during adolescence (Crews et al. 2007; Vetreno and Crews 2012) and may also be related to a persistent loss of behavioral flexibility. Together, the persistent loss of ability to adapt to changes, low sedative response to alcohol, and increased sensitivity to cognitive disruption associated with adolescent alcohol exposure all are likely to promote and sustain high alcohol-drinking levels. These in turn will promote more alcohol consumption and the chances that AUD will develop in addition to alcoholic neurodegeneration.

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(Sapolsky et al. 2000; Wiegers et al. 2005). At the same time, glucocorticoids increase the activity of TGF-β by activating a latent form of the cytokine (Oursler et al. 1993), which may indirectly affect the immune response, because TGF-β inhibits activation of T cells and macrophages. Glucocorticoids also increase production of IL-10, an anti-inflammatory cytokine that blocks NF-κB transcription and inhibits antigen presentation and T-cell activation (de Waal Malefyt et al. 1991). Finally, glucocorticoids suppress maturation, differentiation, and proliferation of immune cells, including innate immune cells, T cells, and B cells.

Norepinephrine released after SNS activation also disturbs inflammatory cytokine networks by inhibiting production of immune-enhancing cytokines, such as IL-12 and TNF-α, and by upregulating production of inhibitory cytokines, such as IL-10 and TGF-β (Webster et al. 2002). Additionally, norepinephrine affects peripheral natural killer (NK) cells, a subset of lymphocytes that are a first-line defense against viral infections, tumor growth, and metastasis via their unique cytolytic action (Herberman and Ortaldo 1981). These cells carry receptors for norepinephrine (i.e., β-adrenergic receptors) on their surfaces (Madden et al. 1995). The cytolytic activity of NK cells involves the synergistic actions of the pore-forming protein perforin and the serine protease granzyme B to cause apoptosis of target cells (Graubert et al. 1996). Among the HPA hormones, glucocorticoids and CRH both are potent inhibitors of NK-cell activity in vitro and in vivo. Hypothalamic CRH inhibits NK activity and IFN-γ production through actiavation of the SNS, which causes release of catecholamines (e.g., norepinephrine) from the spleen and activation of β-adrenergic receptors on NK cells (Irwin et al. 1990). Thus, it appears that the hormones secreted during stress by the HPA axis and SNS have inhibitory effects on peripheral immune functions that contrast with their actions in the CNS.

During activation of the HPA axis, secretion of CRH and catecholamines also increases the secretion of BEP in the hypothalamus. In a feedback mechanism, BEP regulates the secretion of CRH in a hypothalamic region called the paraventricular nucleus (PVN) (Plotsky 1986). In the PVN, the BEP-releasing neurons act on CRH-releasing neurons and inhibit CRH release, thus regulating the activity of the stress system (Plotsky et al. 1993). BEP acts by binding to δ- and μ-opioid receptors; accordingly, treatment with a μ-opioid receptor antagonist results in increased CRH release (Boyadjieva et al. 2006). BEP affects immune-system function through a variety of mechanisms. By binding to δ- and μ-opioid receptors BEP modulates neurotransmission in sympathetic and parasympathetic neurons via neuronal circuitry within the PVN, ultimately resulting in activation of NK-cell cytolytic functions in the spleen (Boyadjieva et al. 2006, 2009; Sarkar et al. 2011). If incubated with human bone marrow mononuclear cells or NK-enriched cell populations, BEP enhances NK activity (Mathews et al. 1983). In animal models, chronic BEP infusion into the blood vessels in the brain enhances NK-cell activity in vivo, and this effect is eliminated by the opioid antagonist naloxone (Jonsdottir et al. 1996). BEP also can inhibit T-cell proliferation (van den Bergh et al. 1993) as well as antibody production (Morgan et al. 1990).

Abnormalities in BEP neuronal function are correlated with a higher incidence of cancers and infections in patients with schizophrenia, depression, and fetal alcohol syndrome and in obese patients (Bernstein et al. 2002; Lissoni et al. 1987; Polanco et al. 2010; Zangen et al. 2002). Interestingly, BEP-cell transplantation in the hypothalamus suppresses various cancers in rat models by activating innate immune-cell functions and altering inflammatory and anti-inflammatory cytokine milieus (Sarkar et al. 2008). In this setting, chronic alcohol use suppresses BEP neuronal activity and is connected with increased infection rates and higher incidence of cancer. Thus, alcohol and stress seem to paralyze adaptive innate immune functions by inducing complex changes in NK cells and other adaptive immune signaling that in the brain primarily involves microglial–astrocyte–neuronal HMGB1–TLR signaling.

Effects of Immune System Activation on Brain Function

The interaction between the brain and the immune system is not unidirectional—that is, immune-system responses also may influence responses in the brain. Recent studies indicate that ethanol causes HMGB1 release in the gut, which activates TLR4. As a result, the gut leaks LPS-like bacterial products, thereby stimulating proinflammatory cytokine induction in the liver, which in turn leads to increased levels of TNF-α and other cytokines in the blood. Qin and Crews (2007, 2012*b*) discovered that LPS-induced increases in serum TNF-α as well as proinflammatory cytokines led to gene induction in the brain. The proinflammatory cytokines in the blood can be transported across the blood–brain barrier (BBB) by their receptors (e.g., TNFR) (Banks and Erickson 2010; Qin et al. 2007). Using intraperitoneal injections of LPS to stimulate proinflammatory responses in the liver and other tissues and induce proinflammatory cytokines, researchers discovered parallel increases in TNF-α in the blood and brain (Qin et al. 2007). In transgenic mice lacking TNF receptors, however, LPS increased TNF-α only in the blood but not in the brain, suggesting that LPS–TLR4 induction of TNF-α in the blood leads to TNF transport by its receptors across the BBB and activation of proinflammatory responses in the brain. Transgenic

mice without the TNF-α receptor cannot transport the cytokine to the brain; consequently, the LPS–TLR4 proinflammatory response is amplified across peripheral tissues but does not spread to the brain.

Ethanol can increase proinflammatory cytokine levels in the blood by activating proinflammatory responses in the liver and other tissues. One mechanism seems to involve the ethanol-induced increase in gut permeability (or "leakiness") mentioned above (Ferrier et al. 2006). At high doses (at least 2 to 3 g/kg ethanol administered into the stomach), ethanol potentiates innate immune signaling in the gut (Ferrier et al. 2006). This disrupts the

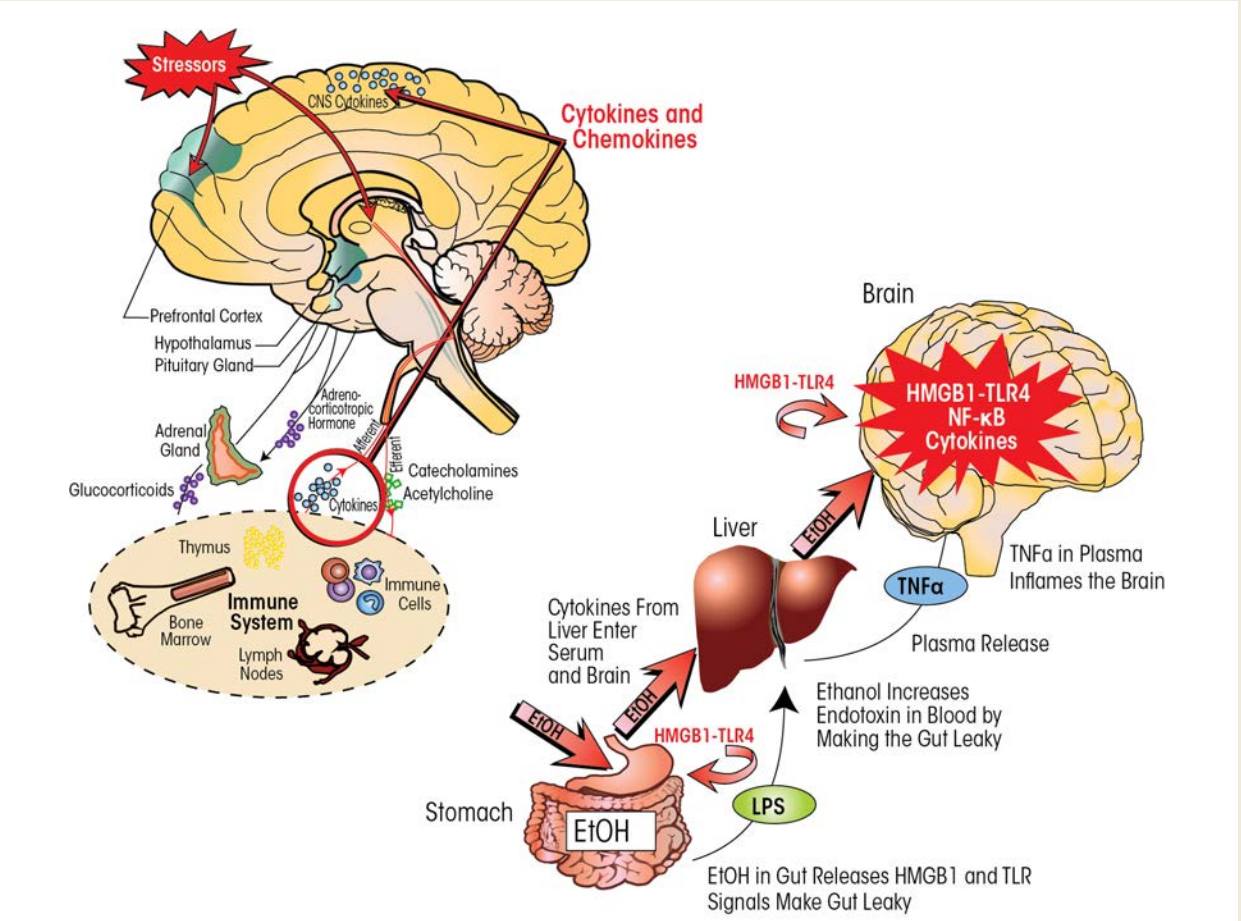

Figure 6 Neuroimmune signaling integrates central nervous system (CNS) responses to alcohol and stress. **(Left)** Stressors activate the body's stress response system, which is comprised of the hypothalamus, pituitary gland, and adrenal glands (i.e., HPA axis) as well as the stress hormones they produce (e.g., adrenocorticotropic hormone and glucocorticoids). Stress also activates the sympathetic nervous system, which secretes catecholamines. These hormones act on various organs and tissues that are part of the immune system. In response, immune cells secrete cytokines that via the blood are transported to the brain. There, these cytokines lead to brain neuroimmune-gene induction that sensitizes stress-response pathways. At the same time, the immune system communicates with the CNS through sensory (afferent) nerves that activate the brain in response to stressful stimuli. This communication pathway involves particularly the vagus nerve and the nucleus tractus solitarius in the brain stem. **(Right)** Alcohol influences neuroimmune signaling via its effects on the gastrointestinal tract. Consumed ethanol enters the stomach and gut and makes them "leaky" by inducing the release of high-mobility group box 1 (HMGB1), which in turn activates Toll-like receptor 4 (TLR4) in the gut. As a result, bacterial products such as lipopolysaccharide (LPS) can enter the blood and reach the liver. Both LPS and ethanol (which also reaches the liver via the circulation) contribute to inflammatory reactions in the liver, which lead to release of tumor necrosis factor-alpha (TNF-α) and other proinflammatory cytokines from the liver. These proinflammatory cytokines in the blood enter the brain and increase neuroimmune-gene expression. Chronic ethanol also increases expression of HMGB1–TLR4 signaling in the brain, leading to persistent and progressive increases in neuroimmune-gene expression in the brain.

connections between the cells lining the gut (i.e., gut tight junctions) and opens sites that allow gut bacteria and their endotoxins to enter the blood vessels leading to the liver, where they can initiate a proinflammatory response (Sims et al. 2010). Thus, high doses of ethanol increase systemic proinflammatory responses, which can then spread to the brain through TNF-α and likely other cytokines (see figure 6).

Although some in vitro studies have suggested that ethanol can interfere with the BBB, most in vivo studies do not show BBB damage following chronic ethanol treatment. Marshall and colleagues (2013) assessed BBB integrity by tracking a protein (i.e., albumin) that cannot cross an intact BBB in rats that were administered large amounts of alcohol for 4 days (a regimen that can induce alcoholic brain damage). The analyses found no evidence of albumin in the brain, indicating that the BBB had remained intact following the ethanol treatment. Using the same model, Crews and colleagues (2006) found that inhibition of NF-κB protected against the brain damage and inhibition of neurogenesis normally induced by this regimen. These findings are consistent with the assumption that proinflammatory responses in the brain mediate brain damage without causing BBB damage. Instead, the brain damage may be induced through direct activation of proinflammatory responses in the brain and/or systemic proinflammatory signals that are transported across the BBB and contribute to brain proinflammatory responses.

Although the levels of proinflammatory gene expression in the blood and brain parallel each other at early time points after initiation of an immune response, the brain's response to LPS is much smaller than that found in the liver and blood during the first few hours. Surprisingly, the blood and liver responses to LPS return to baseline over about 8 to 12 hours, whereas the increase in proinflammatory gene

Glossary

Antibody: Immune molecule (protein) produced by B cells that recognizes foreign molecules that have entered the body (i.e., antigens), binds to these molecules, and marks them for destruction by the body's immune system.

Astrocytes: Characteristic star-shaped non-neuronal cells in the brain and spinal cord that support the endothelial cells that form the blood–brain barrier and provide nutrients to the nervous tissue.

Cytokine: Any of a group of molecules, produced primarily by immune cells, that regulate cellular interactions and other functions; many cytokines play important roles in initiating and regulating inflammatory reactions.

Endotoxin: A highly toxic chemical component of the cell walls of certain bacteria that occur normally in the intestine. Endotoxin can be released into the bloodstream when bacteria die or there is an increase in gut permeability.

Excitotoxicity: Pathological process by which nerve cells are damaged or killed after being excessively stimulated by excitatory neurotransmitters (e.g., glutamate).

Kinase: An enzyme that transfers phosphate groups from one molecule (the donor) to a specific target molecule (the substrate).

Long-term potentiation (LTP): Process by which an episode of strong receptor activation at a synapse leads to a subsequent long-lasting strengthening of the signal transmission across that synapse (e.g., by inducing the accumulation of more receptor molecules at that synapse).

Macrophages: A type of immune cell that ingests foreign particles and microorganisms in a process called phagocytosis and which synthesizes *cytokines* and other molecules involved in inflammatory reactions.

Major histocompatibility complex (MHC): A highly diverse set of glycoproteins in the cell membranes of almost all cells that help to present foreign molecules (i.e., antigens) to other immune cells (i.e., T cells) to activate these cells and induce an immune response.

Microglia: Type of non-neuronal cell in the central nervous system (CNS) that acts as the first and main form of active immune defense in the CNS.

Monocytes: A type of white blood cell involved in the innate immune response; upon activation (e.g., in response to an infection) they move to the site of the infection, enter the tissues, and differentiate into *macrophages*, which then can engulf and destroy the pathogen.

Sepsis: The presence of pathogenic organisms or their toxic products in the blood or tissues.

Toll-like receptors (TLRs): A class of proteins that play a key role in innate immunity. They are located on *macrophages* as well as other brain cells (i.e., *astrocytes* and neurons) and are activated in response to various pathogens; this activation triggers additional innate immune responses and, eventually, adaptive immune responses.

expression in the brain persists for months. This leads to degeneration of dopamine neurons in the substantia nigra, a region in the midbrain involved in reward and addiction (Qin et al. 2007). Similarly, liver and blood responses to binge alcohol exposure appear to be small and transient, although they have not been extensively investigated. In contrast, brain expression of the proinflammatory cytokine MCP-1 persists for at least 1 week (Qin et al. 2008).

Exposure of C57BL/6 mice to 10 daily doses of ethanol followed by LPS results in increased LPS induction of proinflammatory cytokines in the liver, blood, and brain compared with control animals treated only with LPS (Qin et al. 2008). However, this ethanol-induced sensitization to the LPS response resulted in sustained increases in multiple proinflammatory cytokines, including TNF-α, IL-1β, and MCP-1 only in the brain, but not in the liver. The mechanism underlying the sustained brain response and transient liver response is not clear. The investigators noted that the anti-inflammatory cytokine IL-10, which inhibits NF-κB, was increased in the liver 1 week after alcohol treatment, but decreased in the brain (Qin et al. 2008). This suggests that anti-inflammatory mechanisms may contribute to the loss of the liver response. Further analyses found that mice pretreated with ethanol are sensitized not only to the TLR4 receptor agonist LPS but also to the TLR3 agonist Poly:IC (Qin and Crews 2012*a*). Similar to LPS, Poly:IC induces proinflammatory genes in the brain at 24 hours after 10 days of daily alcohol administration (5 g/kg/day). These findings suggest that chronic ethanol sensitizes proinflammatory TLR responses that are easily observed after the clearance of alcohol.

Taken together, the observations indicate that chronic ethanol sensitizes both systemic and brain responses to neuroimmune-gene activation through induction of HMGB1 and TLR proteins. Ethanol-induced leaky gut occurs after high binge-drinking doses, with gut ethanol exposure often being equivalent to the beverage content (i.e., 80 proof is 40 percent ethanol). As a result, bacterial products enter the circulation to the liver and activate liver monocytes (i.e., Kupffer cells), which then produce cytokines, including TNF-α. The TNF-α can be transported to the brain, activating brain neuroimmune signaling that persists for long periods (Qin et al. 2007). Thus, at least two mechanisms of ethanol activation of neuroimmune signaling exist—a direct activation within the brain and the spread of a systemic innate immune activation to the brain (figure 6).

Summary

Binge drinking stimulates neuroimmune-gene induction, which increases neurodegeneration through increased oxidative stress, particularly NADPH oxidase-induced oxidative stress. In addition, HMGB1–TLR4 and NF-κB signaling are increased, leading to enhanced expression of NF-κB target genes and, ultimately, to persistent and sensitized neuroimmune responses to ethanol and other agents that release HMGB1 or directly stimulate TLR receptors and/or NMDA receptors. Persistent neuroimmune-gene induction alters stress-coping mechanisms and the sympathetic nervous system, resulting in the HPA-mediated enhancement of peripheral cytokines, which further exacerbates the neuroimmune response. In addition to neuroimmune signaling, glutamate excitotoxicity also is linked to alcoholic neurodegeneration.

It has been proposed that, instead of simply being a side effect of excessive alcohol consumption, neuronal damage associated with drinking actually may underlie some of the mechanisms of developing alcohol use disorder (Crews and Boettiger 2009). The development of dependence is thought to result at least in part from a lack of inhibition of the subcortical mesolimbic reward system by the frontal cortex (Koob and Le Moal 1997). Alcohol-induced cell death in regions such as the prefrontal cortex may lead to lack of inhibition in subcortical reward areas such as the striatum, which in turn may reduce behavioral inhibition and increase motivation to drink. Repeated stimulation of the innate immune system during chronic or heavy alcohol consumption may facilitate this process, leading to decreased inhibition of the mesolimbic reward system and thus increased drinking (Crews et al. 2011). These processes may be particularly relevant in adolescence, when persistent and long-lasting increases in brain neuroimmune-gene expression and neurodegeneration may be associated with the development of alcohol use disorder.

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Effects of Alcohol on Tumor Growth, Metastasis, Immune Response, and Host Survival

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Most research involving alcohol and cancer concerns the relationship between alcohol consumption and cancer risk and the mechanisms of carcinogenesis. This review relates the amount and duration of alcohol intake in humans and in animal models of cancer to tumor growth, angiogenesis, invasion, metastasis, immune response, and host survival in specific types and subtypes of cancer. Research on the influence of alcohol drinking on human cancer patients is limited. Although there is more information in animal models of cancer, many aspects still are ill defined. More research is needed to define the mechanisms that underlie the role of alcohol on cancer progression in both animals and humans. Activation of the immune system can play a positive role in keeping cancer under control, but this also can facilitate cancer progression. Additionally, a functional immune system is required for cancer patients to achieve an optimal response to conventional chemotherapy. Insight into the underlying mechanisms of these interactions could lead to effective immunotherapeutic approaches to treat alcoholics with cancer. Defining the epigenetic mechanisms that modulate cancer progression also has great potential for the development of new treatment options not only for treating alcoholics with cancer but also for treating other alcohol-induced diseases.

Key words: Alcohol consumption; alcoholism; alcohol use duration; alcohol-induced disease; risk factors; cancer; cancer progression; tumor; metastasis; immune response; immune system; chemotherapy; host survival; angiogenesis; epigenetic mechanisms; treatment; animal models; human studies

Alcohol use and abuse have been implicated as etiological factors in the genesis of an increasing number of cancer types in both men and women. In 2012, the International Agency for Research on Cancer (IARC) listed both beverage alcohol (i.e., ethanol) and its major metabolite, acetaldehyde, as tumor-inducing substances (i.e., carcinogens) in humans. The most recent worldwide statistic from 2002 estimated that about 3.6 percent of all cancers, or 389,100 cases, are associated with alcohol consumption (Seitz and Stickel 2007). Cancers for which strong epidemiological evidence indicates that alcohol consumption is associated with an increased risk include, but are not limited to, esophageal, laryngeal, pharyngeal, stomach, colorectal, liver, pancreas, lung, prostate, breast, central nervous system, and skin cancers (Berstad et al. 2008; Boffetta and Hashibe 2006; Brooks and Zakhari 2013; de Menezes et al. 2013; Haas et al. 2012; Kumagai et al. 2013; Longnecker et al. 1995; Nelson et al. 2013; Rota et al. 2014*a*; Watters et al. 2010). The risk of developing a second aerodigestive-tract cancer also is higher in alcohol drinkers (Day et al. 1994; Lin et al. 2005; Saito et al. 2014).

Increased risk of cancer often is associated with high alcohol consumption; however, the specific dose–response relationship varies according to the site of cancer. A recent meta-analysis of 16 articles involving 19 cohorts of subjects with liver cancer (i.e., hepatocellular carcinoma) found a linear relationship between the amount of alcohol consumed and the risk of liver cancer compared with nondrinkers (Turati et al. 2014). Thus, consumption of three alcoholic drinks per day was associated with a moderate increase in risk, whereas consumption of about seven drinks per day was associated with an increase in risk of up to 66 percent. A similar linear relationship has been described for breast cancer risk (Scoccianti et al. 2014).

However, alcohol consumption does not increase the risk of all types

of cancer and may even be associated with a lower risk in some cases. For example, although alcohol consumption overall is associated with a higher risk of breast cancer in women, this association does not apply to all types of breast cancer. Thus, among women enrolled in the Women's Health Initiative the risk of estrogen-positive breast cancer was increased in those who drank alcohol, whereas the risk of triple-negative breast cancer[1] was reduced among drinkers compared with women who had never consumed alcohol (Kabat et al. 2011).

Interestingly, alcohol consumption also is associated with a lower incidence of several types of blood cancer, including non-Hodgkin's lymphoma (NHL) (Gapstur et al. 2012; Ji et al. 2014; Morton et al. 2005; Tramacere et al. 2012) and multiple myeloma (Andreotti et al. 2013). An analysis of 420,489 individuals diagnosed with alcohol use disorder (AUD) who were linked to the Swedish Cancer Registry also found a low risk of developing leukemia, multiple myeloma, and Hodgkin's disease (Ji et al. 2014). Another recent study also showed that alcohol drinking was not associated with increased risk of leukemia and that, in fact, light drinking (less than or equal to one drink per day) was associated with a modest 10 percent reduction in leukemia incidence (Rota et al. 2014*b*). In addition to blood cancers, alcohol consumption also is associated with a lower risk of thyroid cancer (de Menezes et al. 2013) and renal cell carcinoma (Song et al. 2012). In the case of renal cell carcinoma, a lower risk was noted even with consumption as low as one drink per day in both men and women, and higher alcohol intake conferred no further benefit. Finally, a retrospective, observational study of colon and rectum adenocarcinoma indicated that moderate alcohol consumption (less than 14 grams per day) was inversely associated with the incidence of rectal cancer. The investigators also found that moderate intake of beer and especially wine was inversely associated with distal colorectal cancer (Crockett et al. 2011).

In summary, it is well established that alcohol use and abuse is associated with a wide variety of cancers, and the number of these associations continues to grow. At the same time, it now is becoming clear that alcohol can have a preventative effect for certain cancers. Whereas the role of alcohol as a carcinogen is well established, the mechanism(s) by which it prevents cancer are largely unknown and an area for further research. Also, despite the potential beneficial effects of alcohol in the prevention of some cancers, it is important to remember that the detrimental effect of chronic alcohol abuse cannot be disregarded.

Although extensive epidemiologic evidence links the etiology of cancer to alcohol, very little information addresses the critical question of whether and how alcohol modulates tumor metastasis, survival, and the response to cancer therapy. One of the components in these processes is the immune system. Much research regarding the role of the immune response in oncogenesis has centered on hepatocellular cancer (for excellent recent reviews, see Aravalli 2013; Stauffer et al. 2012; Wang 2011). However, less is known regarding the role and interaction among alcohol consumption, immune modulation of tumor growth, blood vessel formation (i.e., angiogenesis), metastasis, and survival. These issues form the major emphasis of this review. It is well established that immunosurveillance by the innate and adaptive immune systems plays important roles in the prevention of cancer and in controlling cancer survival (Fridmann et al. 2012; Rocken 2010). However, direct or indirect interactions of the tumors with their microenvironment can facilitate immune evasion so that the tumor is not detected by the immune system and thus can spread uncontrolled. Tumors also release factors that can directly or indirectly suppress antitumor immune responses, thus facilitating angiogenesis, invasion of surrounding tissues, and metastasis to distant sites in the body (for a general review, see Jung 2011). (For more information on the processes involved in tumor metastasis, see the sidebar.) The following sections will review the role of alcohol in cancer growth and progression, both in humans and in animal models.

Alcohol, Tumor Growth, and Survival in Humans

Survival and Mortality

Statistics from 2002 indicate that approximately 3.5 percent of all cancer deaths are associated with alcohol (Seitz and Stickel 2007). A study of 167,343 adult subjects in rural southern India found that daily drinking for 30 or more years increased overall cancer-related mortality (Ramadas et al. 2010). Similarly, a study involving 380,395 men and women who were followed for 12.6 years as part of the European Prospective Investigation into Cancer and nutrition (EPIC) study indicated that compared with no or light-to-moderate consumption (i.e., 0.1 to 4.9 g alcohol/day), heavy (30 or more g/day) drinking in women and heavy to extreme (60 or more g/day) drinking in men was strongly associated with increased total mortality as well as deaths from alcohol-related cancers (Ferrari et al. 2014). However, the effect of alcohol on cancer-specific mortality is variable and depends on factors such as the amount of alcohol consumed, health status of the patient, and the type of cancer.

Survival of patients with oral cavity, pharyngeal, laryngeal, and esophageal cancer is generally reduced by drinking (Jerjes et al. 2012; Mayne et al. 2009; Thrift et al. 2012; Wang et al. 2012*a*; Wu et al. 2012; Zaridze et al. 2009). In Korean patients with head and neck and hepatocellular carcinoma the

[1] In estrogen-positive breast cancer, the cancer cells carry the estrogen receptor and depend on estrogen for growth. In contrast, in triple-negative breast cancer, the cancer cells carry neither estrogen nor progesterone or HER2 receptors.

death rate exhibited a dose-dependent relationship with consumption, with patients who drank between 124 and 289 g of alcohol per day showing the highest death rate (Park et al. 2006). Lower survival of patients with hepatocellular cancer also has been reported in Scotland (Dunbar et al. 2013), Russia (Zarizde et al. 2009), and Spain (Fenoglio et al. 2013). Shortened survival in drinkers as compared with nondrinkers with oral squamous cell carcinoma has been linked to the expression of hypoxia-inducible factor-1-alpha (HIF-1α), a biomarker associated with tumor invasion, metastasis, and progression of a variety of human cancers that also plays a central role in angiogenesis. Drinkers showed higher HIF-1α expression in the nucleus of their cancer cells than nondrinkers (Lin et al. 2008). Finally, although alcohol consumption lowers the incidence of NHL, it decreases patient survival of those with the disease (Battaglioli et al. 2006; Geyer et al. 2010; Talamini et al. 2008).

The effect of alcohol consumption on mortality of women with breast cancer is varied and difficult to interpret. In general, long-term low and moderate alcohol consumption does not seem to affect the survival of breast cancer patients (Flatt et al. 2010; Harris et al. 2012; Kwan et al. 2012; Newcomb et al. 2013). In fact, moderate drinking actually may benefit survival of young women with breast cancer (Barnett et al. 2008; Newcomb et al. 2013). On the other hand, several studies indicated that postmenopausal women with breast cancer who are high-intensity drinkers have lower survival than those with no or lower consumption (Holm et al. 2013; McDonald et al. 2002; Weaver et al. 2013).[2] In addition to patient age, the specific type of breast cancer may influence the effects of alcohol on survival. Thus, for women with estrogen receptor–positive breast cancer neither pre- nor postdiagnosis alcohol consumption was associated with breast cancer mortality (Ali et al. 2014). In women with estrogen receptor–negative disease, however, mortality was slightly reduced. Another study investigated the effect of pre- and postoperative alcohol consumption over a 3-year period in 934 Swedish primary breast cancer patients who had breast cancer surgery (Simonsson et al. 2014). The study found that both pre- and postoperative consumption of any amount of alcohol was weakly associated with a lower risk of early distant metastases and death. The associations were found in patients with axillary lymph node involvement but not in patients without lymph node involvement.

The effect of alcohol consumption on the incidence as well as the mortality of patients with prostate cancer was evaluated in a prospective cohort study of 194,797 men from the United States aged 50–71 years in 1995–1996 (Watters et al. 2010). The incidence of nonadvanced prostate cancer increased with increasing number of drinks per day, with a 25 percent increase in risk observed after high alcohol consumption (six or more drinks per day). However, an inverse correlation existed between alcohol consumption and deaths from prostate cancer, suggesting that alcohol consumption likely does not affect advanced or fatal prostate cancer.

In summary, several reports indicate that alcohol consumption decreases survival of patients with cancer, whereas other studies did not observe this association. The effect of alcohol consumption on mortality of women with breast cancer is particularly complex and seems to differ according to age, estrogen receptor status, and extent of alcohol drinking. Clearly, more breast cancer–specific studies are needed that correlate mortality with the properties of the cancer and the level of alcohol consumption.

Tumor Growth and Metastasis

The actual influence of alcohol consumption on tumor growth and metastasis is largely unknown in human cancer patients. Discriminant function analysis of 39 asymptomatic Italian patients with a total of 59 small hepatocellular carcinomas arising from cirrhosis revealed that, among other variables, alcohol intake was a good predictor of tumor doubling time and 2-year survival (Barbara et al. 1992). Another study of 35 Japanese patients with hepatocellular carcinoma and type C cirrhosis found that habitual drinkers consuming 80 g of ethanol per day for 5 years had a statistically significant ($P < 0.01$) shorter tumor-volume doubling time than did non-alcoholic patients (78 ± 47 days vs. 142 ± 60 days) (Matsuhashi et al. 1996).

Basal cell carcinoma—a type of skin cancer—is the most common cancer in humans and continues to increase in incidence. Although the cure rate is high and mortality and morbidity rates are low, aggressive basal cell carcinomas are not rare. In a Spanish study, a significant positive association existed between moderate (5 to 10 drinks per week) and high (more than 10 drinks per week) alcohol consumption and the presence of aggressive basal cell carcinomas (Husein-Elahmed et al. 2012).

Alcohol, Tumor Growth, Invasion, and Metastasis in Animal Models

Several studies using animal cancer models indicate tumor specific differences in the effect of alcohol on tumor growth and metastasis. These models included various types of breast cancer, melanoma, lung cancer, colon cancer, and hepatocellular carcinoma (For more information, see the sidebar "Effects of Alcohol on Tumor Growth, Invasion, Metastasis, and Survival in Animal Models"). Taken together, these studies and animal models did not allow for general conclusions regarding the impact of alcohol on tumor growth, metastasis formation, and disease progression, as findings differed significantly depending on

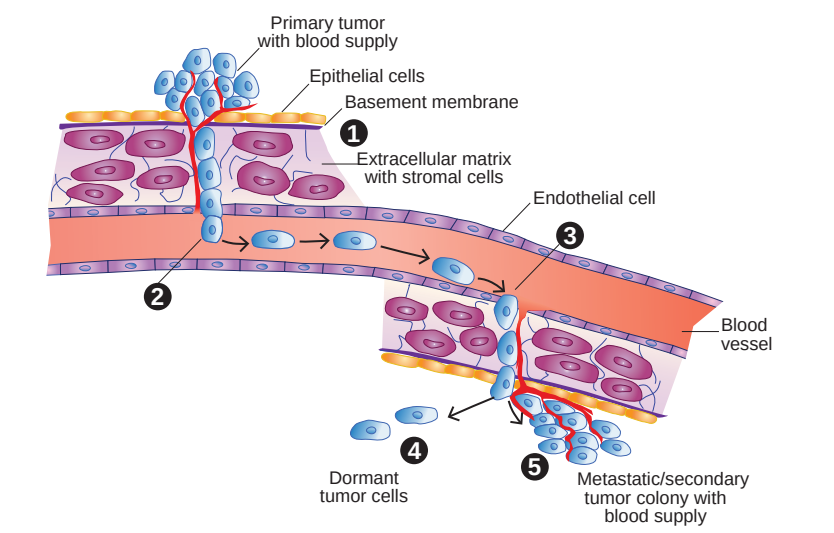
[2] The exception to this is a study in Russia indicating an inverse association between alcohol consumption and mortality (Zaridze et al. 2009).

tumor type. The alcohol model used as well as the duration of alcohol administration also are important variables and can affect the overall outcome (D'Souza El-Guindy et al. 2010), as is the amount of alcohol administered. For example, in studies assessing alcohol's effects on metastasis formation, acute administration of high doses of alcohol, which mimics binge drinking, generally increased metastasis, whereas longer-term alcohol administration either had no effect or decreased metastasis formation, depending on the amount of alcohol consumed by the animal. Several mechanisms have been suggested as to how acute alcohol may enhance metastasis formation, including alcohol-induced formation of as well as inhibition of various signaling molecules (i.e., cytokines and chemokines). However, although both of these mechanisms seem to contribute to the increase of metastases after acute administration, they do not account for the entirety of alcohol's effects. Another mechanism whereby alcohol could facilitate metastasis of certain cancers may involve disruption of the integrity of the cells lining the blood vessels (i.e., vascular endothelium). Thus, studies found that exposure to 0.2 percent (weight per volume [w/v]) ethanol in vitro, which promotes angiogenesis and invasion, interferes with the integrity of the vascular endothelium by inducing endocytosis of VE-cadherin (Xu et al. 2012). This molecule is an important component of certain junctions between cells (i.e., cellular adherens junctions). These changes in the vascular endothelium have been shown to allow for increased migration of human A549 lung adenocarcinoma cells, MDA-MB-231 breast cancer cells, and HCT116 colon cancer cells through single-cell layers of endothelial cells (Xu et al. 2012).

Researchers also examined the effects of alcohol administration on tumor growth. These studies found that high alcohol intake had no consistent effect on tumor growth across different tumors or within a specific tumor type. Low intake of alcohol generally has been associated with enhanced angiogenesis

Tumor Metastasis

Tumor metastasis is the ability of tumor cells to spread from their original site to other sites in the body and to re-establish growth, a new blood supply, and tumor colonies at the new location. **(1)** Cells that escape from a primary solid tumor invade into the surrounding normal tissue by passing through the basement membrane and extracellular matrix (ECM). Several factors are involved in the invasion process, including the ability to activate enzymes called matrix metalloproteinases (MMP), which are important for the tumor cells to degrade basement membranes and underlying stroma. **(2)** The escaped cells reach the blood either directly by actively passing through endothelial cells that line the blood vessels or passively through the lymphatic system, which ultimately carries the tumor cells to the blood. **(3)** Once in the blood, the tumor cells exit into tissues at the secondary site from small capillaries by passing through endothelial cells and then invading the basement membrane of the ECM. **(4)** Once at the secondary site, the tumor cells can lay dormant for extended periods of time, or **(5)** they re-establish growth to form metastatic tumor colonies (by proliferation of cells from a single tumor cell), and finally form a new blood supply (by stimulating the angiogenesis process) to nourish the metastatic tumor. Dormant cells also can proliferate at a future date and ultimately establish a new metastatic tumor. Factors that control the breaking of dormancy are largely unknown, and this is an active area of research.



(which promotes tumor growth), whereas high intake may have no effect.

As mentioned earlier, studies in humans found that alcohol's effects on breast cancer, its progression, and the associated mortality are influenced at least in part by the type of breast cancer involved, specifically its estrogen receptor status. However, animal models involving different breast cancer cell lines detected no consistent trend regarding the effect of alcohol consumption on tumor growth and progression associated with estrogen receptor expression. Estrogen generally suppresses breast cancer growth in vivo but increases in vitro migration of cells away from the original tumor. However, the relationship between estrogen supplementation, diet, caloric intake, and alcohol and their effects on subcutaneous breast cancer growth seem to be highly complex.

The effects of alcohol on in vitro invasion of surrounding tissue primarily have been studied in breast cancer and melanoma cells, with a variety of results. The evidence in melanoma suggests that ethanol can positively impact the extracellular membrane and augment expression of genes that suppress tumor metastasis, resulting in inhibition of metastasis. In addition, certain immune cells called natural killer (NK) cells seem to have some role in regulating the metastasis of breast cancers and melanomas. Clearly, more mechanistic research is needed in murine models to serve as a template for further examination of the complex interactions connecting alcohol to tumor growth, metastasis, and survival in humans.

Alcohol-Induced Immune Modulation and Tumor Progression

Although many factors influence tumor growth, metastasis, and survival in cancer patients, it is apparent that a functioning immune system plays an important role, not only because it helps control cancer progression but also because it is required for the effectiveness of common cytotoxic chemotherapeutic drugs (Bracci et al. 2014). Evidence that directly implicates immune cells from both the innate and adaptive immune systems in control of cancer growth and progression continues to accumulate. This has stimulated research directed toward developing effective immunotherapeutic approaches to treat cancer (for a review of the tumor immune response as well as approaches being taken to develop immunotherapeutics for cancer, see Harris and Drake 2013).

The innate immune response reacts rapidly to recognize and destroy cancer cells. This response is characterized by inflammatory reactions involving various mediators, including chemokines and cytokines that are produced by a variety of immune cells, such as macrophages, neutrophils, NK cells, and dendritic cells. Macrophages and neutrophils can exhibit antitumor activity as well as suppress immune response against tumor cells (i.e., have immunosuppressive activity). NK cells can destroy tumors on contact, and their antitumor function can be further stimulated by cytokines. Dendritic cells are important in presenting molecules that identify a cell as harmful or foreign (i.e., antigens) to other immune cells and are a bridge between the innate immune response and the B-cell and T-cell responses that characterize the adaptive immune system.

B cells can recognize tumor-cell antigens to ultimately produce antitumor antibodies. They also can have immunosuppressive activity. T cells can be classified according to certain molecules they exhibit on their surfaces, such as CD4, CD8, or CD25. They also can be classified according to their specific functions (e.g., as helper, cytotoxic, regulatory, or memory T cells). $CD4^+$ helper T cells can further be divided into Th1, Th2, and Th17 subpopulations based on the specific cytokines they produce and the reactions they induce in the body, which may either facilitate or suppress antitumor immune responses. Certain subsets of $CD4^+CD25^+$ T cells, known as regulatory T cells, generally are immunosuppressive. Cytokines released by Th1 helper T cells, in turn, can activate $CD8^+$ T cells, rendering them directly cytotoxic to tumor cells as well as enhance the activity of NK cells. Other populations of $CD8^+$ cells (i.e., tumor-specific and memory $CD8^+$ T cells) produce high levels of the cytokine interferon gamma (IFN-γ), which is important to the control of tumor metastasis and host survival. Finally, another population of T cells (i.e., NKT cells) that produce a wide variety of cytokines upon activation can function as immunoregulatory cells to either enhance or suppress antitumor immune responses, depending on the cytokine profile that they exhibit. Together, the cells of the immune response provide an intricate interactive control that governs tumor growth and progression. (For more information on the innate and adaptive immune systems and their responses, see the "Primer on the Immune Response," by Spiering.)

A Role for the Immune System in Control of Cancer Progression

Numerous findings with a variety of tumor types suggest that the numerous types of immune cells, particularly various T-cell subpopulations, are involved in controlling tumor progression, including the following:

- $CD8^+$ T cells, in particular a subtype expressing the memory phenotype ($CD8^+CD44^{hi}$) that produce high levels of IFN-γ, are key to controlling metastasis and host survival of different tumors (Erdag et al. 2012; Eyles et al. 2010; Fridman et al. 2012; Rosenberg and Dudley 2009).

- Increased tumor progression in patients with gastric cancer has been tied to increased peripheral blood levels of certain $CD4^+$ T-cell subpopulations, including Th22 ($CD4^+IL\text{-}22^+IL\text{-}17^-IFN\text{-}\gamma$) and

Th17 ($CD4^+IL\text{-}17^+IFN\text{-}\gamma$) cells (Liu et al. 2012).

- A multivariate analysis in metastatic breast cancer patients indicated that prolonged progression-free survival was correlated with increased $CD3^+CD4^+$ or $CD8^+CD28^+$ T cells. Conversely, elevated $CD8^+CD28^-$ T cells were associated with shortened progression-free survival (Song et al. 2013). These effects seem to be related to the cytokines produced by these cells, because patients with elevated $CD8^+CD28^-$ and $CD4^+CD25^+$ T cells had elevated levels of IL-6, and the patients that expressed elevated $CD8^+CD28^-$ T cells also exhibited decreased IFN-γ.

These data underscore the importance of immune cells in the progression of cancer.

Alcohol can modulate the body's immune responses, and it is possible that these alterations affect disease progression in cancer patients. For example, in a Chinese study of newly diagnosed NHL patients (Lin et al. 2009), alcohol addiction was associated with increased peripheral blood $CD4^+CD25^{hi}CD127(IL\text{-}7)^{lo}$ regulatory T cells, and these increases were higher in male than in female patients. However, the increased levels of these cells did not relate to the clinical features (e.g., age, tumor staging, cancer symptoms, pathological subtype, and short-term treatment efficacy). Therefore, the importance and significance of the elevated regulatory T cells is uncertain in NHL.

Another study of 25 patients with hepatocellular carcinoma in Japan (Yang et al. 2006) found an increase in $CD4^+CD25^+$ T cells in the tissue regions surrounding the tumor (i.e., the peritumoral region) compared with similar tissues in patients who had chronic hepatitis or liver cirrhosis but no hepatocellular carcinoma. The values were not correlated with the stage of the tumor.[3] These peritumoral $CD4^+CD25^+$ T cells had a regulatory phenotype, as indicated by an increased expression of several molecules (e.g., cytotoxic T lymphocyte antigen 4 [CTLA-4, CD152] and glucocorticoid-induced TNF receptor superfamily member 18 [GITR, CD357]), expression of a biomarker for regulatory T cells (i.e., FOXP3), and decreased expression of CD45RA. The numbers of these cells were inversely associated with the numbers of $CD8^+$ T cells. Additional observations suggest that these regulatory T cells may contribute to the progression of hepatocellular carcinoma by interfering with normal immune responses. Thus, isolated peritumor $CD4^+CD25^+$ T cells that were incubated with peripheral blood T cells from the same person and stimulated with certain antibodies, suppressed T-cell proliferation and activation of $CD8^+$ T cells (Yang et al. 2006).

The functionality of the innate immune system also can be correlated with tumor progression. A recent study compared innate immune-system functionality with the number of circulating tumor cells in patients with a variety of cancers. In patients with metastatic disease, these circulating tumor cells are promising as biomarkers for tumor progression and overall cancer survival, with relatively high circulating cell numbers correlated with a poor prognosis. The study, which included patients with metastatic breast, colorectal, and prostate cancer found decreased NK cell cytolytic activity and decreased expression of certain proteins (i.e., toll-like receptors 2 and 4) in patients with high circulating tumor cells compared with patients with relatively low numbers (Santos et al. 2014). Decreased NK cytolytic activity also has been linked with other types of cancer, including colorectal cancer (Kim et al. 2013), metastatic melanoma (Konjevic et al. 2007), and head and neck cancer (Baskic et al. 2013).

[3] Interestingly, the same cell type was decreased in the peripheral blood in the cancer patients compared with control patients.

In addition to the effects of specific types of lymphocytes on cancer growth and metastasis, chemokines also have important roles in cancer progression, terminal growth arrest of tumor cells (i.e., tumor growth senescence), angiogenesis, epithelial mesenchymal transition,[4] metastasis, and evasion of the immune system. Chemokines and their receptors often are altered in cancer patients, and their importance in cancer progression has been the subject of several recent reviews (Aldinucci and Colombatti 2014; de Oliveira et al. 2014; Sarvaiya et al. 2013).

Alcohol and Immune Effects in Patients with Cancer

A large body of literature indicates that alcohol consumption modulates many aspects of the innate and adaptive immune systems. Alcohol originally was described as immunosuppressive, and numerous studies support the immunosuppressive aspects of alcohol consumption on the innate and adaptive immune systems. However, it also is well documented that chronic alcohol administration can activate the immune system—especially dendritic cells, T cells, and NKT cells—in experimental animals as well as humans (Cook et al. 1991; Laso et al. 2007; Song et al. 2002; Zhang and Meadows 2005). This adds to the complexity of interpreting alcohol's effect on cancer progression and survival.

Few studies have specifically examined the interaction between alcohol and the immune response in cancer patients or in experimental animals implanted with cancer cells. Although human cancer patients often have immune deficits, few data are available that specifically address the effects of alcohol on immune parameters. The studies that are available examined the immune responses in patients with head and neck cancer. These patients often are immunodeficient because of

[4] Epithelial mesenchymal transition is a process whereby epithelial cells lose their innate cellular polarity and cell–cell adhesive properties to become mesenchymal cells, which lack polarity and have the ability to migrate and to invade through tissues.

their alcohol abuse and heavy tobacco use; however, the contribution of continued alcohol abuse to altered immune parameters in these patients has largely not been assessed.

An early study of patients with head and neck squamous cell carcinoma and a history of smoking and significant alcohol use found a deficiency in the percentage of certain T cells (i.e., $Th5.2^+$ IL-2–producing T cells) in peripheral blood compared with control patients who were hospitalized for elective surgical procedures (Dawson et al. 1985). The overall percentage of all T cells, as well as of $CD4^+$ T-, $CD8^+$ T-, B-, and NK cells, in contrast, did not differ between cancer and control patients. However, this effect cannot be clearly attributed to alcohol because the patients also were heavy tobacco users. Another study compared a different indicator of immune-system function (i.e., production of antigen-specific antibodies) using blood samples obtained from patients with squamous cell carcinoma of the oropharynx or larynx and healthy controls, some of whom had high alcohol consumption (i.e., 100 g/day) and/or excessive smoking (20 cigarettes per day for more than 5 years) (Wustrow 1991). The study found that among healthy participants, those with high alcohol consumption or smoking had a pronounced decrease of antigen-specific antibody production in vitro. The effect was more pronounced in heavy drinkers than in excessive cigarette smokers. Cancer patients who were heavy drinkers, in contrast, did not show any antigen-specific antibody production in vitro. However, after removal of a subset of white blood cells (i.e., mononuclear cells) from the peripheral blood, samples from two-thirds of the patients began to produce such antibodies, and antibody production reached the same level as that measured in the healthy subjects with high alcohol abuse and cigarette consumption. The author suggested that the decreased antigen-specific antibody production in the cancer patients could be related to upregulation of suppressive cells in these patients (Wustrow 1991).

More recent studies have evaluated the role of a protein called macrophage migration inhibitory factor (MIF), which is an important regulator of the innate immune response. This factor has been studied in patients with lip or intra-oral squamous carcinoma as well as in patients who consumed alcohol regularly (Franca et al. 2013). The analyses found a significant relationship between the incidence of intra-oral cancer, alcohol use, and the number of MIF-positive cells in the stroma. Thus, MIF in the stroma of intra-oral tumors (i.e., tongue, floor of mouth, and alveolar ridge) was decreased in patients who consumed alcohol. The importance of these findings is unknown, although patients with tumors that did not express MIF had a worse prognosis than patients that did.

Alcohol and Immune Interactions in Animal Models of Cancer

If human tumor cells are introduced (i.e., inoculated) into animals with functioning immune systems, they do not form tumors because they are recognized as foreign by the animal's immune system. However, human tumors often grow in animals with compromised immune systems, and such animals can be used as models for a variety of research questions, including studies regarding the roles of various immune cells in controlling cancer and the impact of alcohol on this process. One such study specifically examined the role of $CD4^+$ T cells in regulating tumor growth by implanting cells from a human lung cancer (i.e., the 201T human lung adenocarcinoma cell line) into the lungs of a strain of mice called BALB/c (Hunt et al. 2000). In this study, the mice were administered alcohol chronically for 8 weeks and then were injected with an anti-CD4 monoclonal antibody to deplete $CD4^+$ T cells. Initial experiments confirmed that normal, immunocompetent BALB/c mice did not form lung tumors. To examine the effect of alcohol, the mice were administered ethanol in their food[5] as well as 10 percent in their drinking water throughout the experimental period. After 8 weeks of ethanol administration or regular food, the mice were implanted with the tumor cells and also received one injection of the anti-CD4 antibody. Separate groups of mice were evaluated at 6 weeks and 13 weeks. Mice in the non–ethanol-fed control group injected with one dose of anti-CD4 antibody initially developed large tumors at 6 weeks, which significantly regressed thereafter. Compared with these control animals, the ethanol-fed mice exhibited significantly larger tumors at 6 weeks as well as a diminished ability to decrease their tumor size at 13 weeks. The findings suggest that this difference in the ability of the ethanol-fed mice to reduce their tumor burden results from an impaired immune system caused by chronic alcohol intake.

Another series of studies analyzed the interaction between chronic alcohol consumption and immune-system functioning in female C57BL/6 mice implanted with B16BL6 melanoma cells under the skin (i.e., subcutaneously). In these studies, the animals continuously received 20 percent w/v ethanol in the drinking water and generally were inoculated with B16BL6 melanoma after 12 weeks or longer of this treatment. The analyses found that in the alcohol-exposed, melanoma-bearing animals the overall numbers of peripheral blood lymphocytes (which include various types of immune cells) were lower than in water-drinking controls when determined 11, 14, and 17 days after tumor inoculation (Zhang et al. 2012). This was in contrast to normal mice not injected with melanoma cells, in which the number of lymphocytes was not altered by alcohol. The decrease in cells was not caused by cell death (i.e., apoptosis). Additional analyses demonstrated that the lowered lymphocyte numbers (i.e., lymphopenia)

[5] The food included blocks of a jelly-like material (i.e., agar-agar) containing 40 percent alcohol and 0.5 g/kg peanut butter.

were associated with a two- to fourfold decrease in mature B cells as well as in $CD4^+$ and $CD8^+$ T cells. Further examination demonstrated that the decrease in mature B cells in the blood was associated with impaired B-cell circulation resulting from a down regulation in the formation of compound called sphingosine-1-phosphate and its receptors. Formation of sphingosine-1-phosphate is mediated (i.e., catalyzed) by an enzyme called sphingosine kinase 1, which is an important regulator of tumor progression in melanoma and several other cancers (Meng et al. 2014). This enzyme and other components of the sphingosine-1-phosphate pathway currently are being examined as potential targets for cancer drug development (Pyne and Pyne 2013; Tabasinezhad et al. 2013). Zhang and colleagues (2012) concluded that the severe decrease in mature B cells in the blood of the alcohol-exposed and tumor-inoculated animals could result from inhibition of B-cell migration from the spleen to the blood resulting from impairment of the sphingosine-1-phosphate signaling pathway. The importance and role of mature B cells in antitumor immune responses is still unclear. They play a dual role by both inhibiting (Inoue et al. 2006) and facilitating antitumor immune response through production of cytokines and enhancement of T-cell activation (DiLillo et al. 2010). Thus, impaired circulation of B cells attributed to alcohol consumption (Zhang et al. 2012) could negatively affect T-cell function.

The investigators also analyzed the levels of the various types of blood cells in the spleen (Zhang et al. 2012). The spleen contains proportionally more B cells and fewer T cells than the peripheral blood; among the T cells, the spleen normally contains a higher proportion of $CD8^+$ T cells than the peripheral blood. The analyses found that alcohol consumption also led to a decrease in $CD8^+$ T cells in the spleen; however, this reduction was less remarkable than in peripheral blood. No changes in these cells were observed in the bone marrow. Furthermore, alcohol consumption reduced the overall numbers of B cells in the spleen, although it did not affect all types of B cells equally. Thus, there was no effect on splenic follicular B cells, whereas the number of immature T1 B ($CD19^+CD93^+CD23^-$) cells increased and the number of marginal zone B cells ($CD19^+CD1d^{hi}CD21^{hi}$) decreased.

Other analyses (Zhang and Meadows 2010) investigated the effects of chronic alcohol consumption on various types of $CD8^+$ T cells in mice with or without inoculation of B16BL6 melanoma

Glossary

Antibody: Immune molecule (protein) produced by *B cells* that recognizes foreign molecules that have entered the body (i.e., *antigens*), binds to these molecules, and marks them for destruction by the body's immune system.

Antigen: Any molecule that can bind specifically to an *antibody* and can induce an immune response.

B cells: One of the two main types of lymphocytes involved in the adaptive immune response; when activated by interacting with a specific *antigen*, they differentiate into specific subtypes and begin to produce *antibodies* that recognize the specific *antigen*.

Chemokines: Small proteins that serve as chemoattractants, stimulating the migration and activation of cells, particularly phagocytic cells and lymphocytes; they have a central role in inflammatory responses.

Cytokine: Any of a group of molecules, produced primarily by immune cells, that regulate cellular interactions and other functions; many cytokines play important roles in initiating and regulating inflammatory reactions.

Dendritic cell: A type of immune cell involved in the innate immune response that is characterized by a branched morphology; dendritic cells can bind to *antigens* and present these antigens to *T cells,* thereby initiating an adaptive immune response.

Macrophage: A type of immune cell that ingests foreign particles and micro-organisms in a process called phagocytosis and which synthesizes *cytokines* and other molecules involved in inflammatory reactions.

Natural killer (NK) cell: A type of immune cell involved in the innate immune response that can kill certain harmful cells, particularly tumor cells, and contributes to the innate immune response to cells infected with viruses or other intracellular pathogens.

Neutrophil: A type of immune cell involved in the innate immune response that engulfs and kills extracellular pathogens in a process called phagocytosis.

T cells: One of the two main types of lymphocytes involved in the adaptive immune response after activation through the interaction with a specific *antigen*. T cells can be divided into several subgroups that support other immune cells (helper T cells), kill invading pathogens or infected cells (cytotoxic T cells), or help turn off the adaptive immune response (regulatory T cells).

cells. These analyses yielded the following results:

- $CD8^+CD44^{hi}$ T memory cells produced high levels of IFN-γ and were important in the antitumor response to B16BL6 melanoma. Mice not inoculated with melanoma that chronically consumed alcohol had higher levels of these memory cells than mice that drank water.

- After melanoma inoculation, these $CD8^+CD44^{hi}$ T memory cells increased over a 2-week period in water-drinking animals. However, in mice that chronically consumed alcohol, these memory cells failed to expand in response to melanoma inoculation.

- The lack of expansion of the memory T cells in response to melanoma inoculation in the alcohol-consuming mice resulted from a reduced ability of these cells to proliferate in response to melanoma. Additional experiments examined the ability of $CD8^+$ T cells obtained from 2-week melanoma-bearing mice to proliferate in vitro in response to specific T cell stimulation (i.e., anti-CD3 and anti-CD28 antibodies). The analyses showed that proliferation of $CD8^+$ T cells was reduced by more than one-half in alcohol-consuming mice compared with cells from water-drinking mice.

- The number of $CD8^+$ T cells that specifically recognize a melanoma-specific antigen (i.e., gp100) was 2.5-fold lower in the spleen of the alcohol-consuming mice than in water-drinking control mice at three weeks after tumor inoculation, suggesting an impaired immune response.

- The percentage of IFN-γ–producing $CD8^+$ T cells, which have tumor-suppressive effects, initially displayed a robust increase until day 11 after melanoma inoculation, but exhibited an accelerated decay thereafter, suggesting enhanced inhibition of these cells related to an alcohol–melanoma interaction.

The investigators also analyzed the numbers of several types of cells whose production is induced by tumors and which produce factors that inhibit the antitumor functions of T cells, including myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages, T regulatory cells ($CD4^+CD25^+FOXP3^+$), regulatory B cells ($CD1d^{hi}CD5^+$), and NKT cells (Zhang and Meadows 2010; Zhang et al. 2012). Of these, the percentage of $CD11b^+Gr\text{-}1^{int}$ MDSCs, as well as the percentage of the $CD124^+$ subpopulation within the $CD11b^+Gr\text{-}1^{int}$ cells, was increased in the peripheral blood of alcohol-consuming mice as determined one week after tumor inoculation. These cells are known to suppress antitumor T-cell immune responses (Sinha et al. 2005; Terabe et al. 2005; Zhu et al. 2007). The percentages of T regulatory cells and tumor-associated macrophages did not differ between alcohol-consuming and water-drinking mice with melanoma tumors (Zhang and Meadows 2010).

The percentage and number of $CD3^+NK1.1^+$ invariant NKT cells was elevated in the blood of alcohol-consuming, B16BL6 melanoma-bearing mice especially at day 14 after tumor inoculation (Zhang et al. 2012). These cells have important regulatory functions and can either promote antitumor immune responses or inhibit them. Initially, these cells express a cytokine profile that favors antitumor immune responses (i.e., a high ratio of IFN-γ to IL-4). After repeated activation, however, these cells become anergic and switch to a cytokine profile that inhibits antitumor immune responses and favors tumor progression (i.e., a high ratio of IL-4 to IFN-γ) (Parekh et al. 2005). The invariant NKT cells from the alcohol-consuming, melanoma-bearing mice exhibit a high IL4/IFN-γ ratio, indicating that they express a cytokine profile favoring immune inhibition and tumor progression (Zhang et al. 2015).

Overall, very few studies have addressed the role of and interaction among alcohol, cancer, and the immune system once the cancer is established. It is important to understand these interactions, however, because many alcoholics have immune deficiencies and because a competent immune system is important to the success of many conventional drug therapies for cancer. In addition, new immune-enhancing approaches to cancer therapy are being developed. Finally, evidence from animal models and human studies suggests that appropriately combined chemotherapy and immunotherapy may be more beneficial than either therapeutic approach alone (Ardiani et al. 2013; Shi et al. 2014; van Meir et al. 2014; Wang et al. 2014).

Additional Avenues for Future Research

The interactions between alcohol use/abuse, the antitumor immune response, tumor growth, and spread of cancer are complex. A negative impact of alcohol on the immune system can lead to increased cancer mortality; however, studies also indicate that alcohol, generally in low doses, can have beneficial effects on mortality, depending on the cancer. Clearly, more mechanistic research is needed to define the complex interactions between cancer and alcohol. Additional research is likely to uncover targets to mitigate the detrimental effects of alcohol on mortality and to identify specific biochemical and molecular mechanisms involved in the beneficial effects of alcohol related to enhancing survival of cancer patients. This research could translate into the development of more effective and specific targeted approaches to treat cancer patients in general and especially those who abuse alcohol.

Because cancer is a collection of different diseases with diverse underlying causes, it is important that research

take into account the diversity in gene mutations and alterations involved in uncontrolled growth. In addition, future analyses must address the genetic instability that fosters metastasis, the major cause of death from cancer. It is becoming increasingly clear that genes which suppress metastasis (Meadows 2012) as well as signaling pathways that inhibit metastasis (Singh et al. 2014) can be regulated through epigenetic mechanisms[6] induced by the diet and dietary constituents, including alcohol. Alcohol-related epigenetic mechanisms include modulation of DNA methylation, histone acetylation/deacetylation, and expression of micro RNA (French 2013). These epigenetic mechanisms associated with alcohol also are known to affect the gastrointestinal-hepatic system (Shukla and Lim 2013) and may promote, for example, the progression of hepatic carcinoma. For the most part, alcohol-related epigenetic changes have not yet been associated with tumor growth, metastasis, and survival; however, alcohol-induced aberrant DNA methylation of certain genes plays a role in the control of breast cancer (Tao et al. 2011). Moreover, alcohol also can dysregulate the immune system through epigenetic mechanisms (Curtis et al. 2013), and this aspect of the association between alcohol, the immune system, and cancer progression needs to be explored further.

Another potential target for future research is a molecule called toll-like receptor 4, which is known to help regulate host innate immunity. This receptor recognizes the lipopolysaccharide (LPS) endotoxin, a molecule found on by certain bacteria that are part of the intestinal microflora. In the blood, LPS can induce strong immune reactions. Alcohol is known to facilitate the release of LPS from the gut into the systemic circulation, and this is a key factor in the pathogenesis of alcoholic liver disease (Petrasek et al. 2010). In addition to its response to LPS, toll-like receptor 4 can facilitate antitumor immune responses; however, emerging evidence also suggests that overactivation of this receptor is associated with tumor progression as well as tumor development (Mai et al. 2013). Although these observations need to be explored further, they suggest that this receptor could be a target for future agonist or antagonist targeted treatment for cancer, particularly for patients that abuse alcohol.

Continued research into the detrimental and beneficial effects of alcohol in human cancer patients and animal models of cancer is a key factor to understanding the complex interactions that affect tumor progression and survival, particularly in the context of alcohol use. This research has a strong potential to discover new immunotherapy and epigenetic approaches to cancer treatment as well as treatment of other alcohol-induced diseases.

[6] Epigenetic mechanisms are processes that alter the expression of certain genes without permanently altering the DNA building blocks (i.e., nucleotides) making up the genes; examples of epigenetic mechanisms include the temporary chemical modification (e.g., methylation or acetylation) of nucleotides or of the proteins (i.e., histones) around which the DNA is wrapped in the cell nucleus.

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Effects of Alcohol on Tumor Growth, Invasion, Metastasis, and Survival in Animal Models

Several studies using animal cancer models indicate tumor-specific differences in the effect of alcohol on tumor growth and metastasis. These models included various types of breast cancer, melanoma, lung cancer, colon cancer, and liver cancer (i.e., hepatocellular carcinoma).

Lung Cancer

One early study (Capel et al. 1978) investigated the effect of alcohol exposure on the growth and metastasis of Lewis lung carcinoma. Male animals from a type of mouse strain called C57BL/6 were exposed to 10 percent ethanol in their drinking water for 2, 4, 5, or 8 weeks before tumor cells were implanted into their thighs. Ethanol administration then was continued for 2 more weeks. The study found that ethanol exposure before tumor injection did not affect tumor growth. Furthermore, metastases were significantly reduced in the 2-week and 8-week ethanol groups but not in the 4-week and 5-week groups. Administration of ethanol for 2 weeks after tumor inoculation affected neither tumor growth nor metastasis. These investigators also evaluated the effect of 2-week pre- and postimplantation ethanol exposure (10 percent in drinking water) on the growth of another type of tumor called Ehrlich ascites carcinoma, which is a spontaneous murine mammary adenocarcinoma adapted to grow in fluid in the abdominal cavity (i.e., ascites). Exposure to ethanol before but not after tumor injection significantly decreased the tumor cell number.

Colon Cancer

Gu and colleagues (2005) assessed the effects of alcohol on human HT1080 colon cancer cells in a chick embryo model, focusing on variables related to the blood supply of the tumor. One of the variables analyzed was the expression of vascular endothelial growth factor (VEGF)—a growth factor that promotes blood vessel formation (i.e., is proangiogenic) and enhances tumor vascularization. The study found that exposure to 0.25 g/kg ethanol per day for 9 days resulted in a 2.2-fold increase in tumor volume as well as a 3.9-fold increase in the expression of VEGF mRNA in the tumor cells and a 2.1-fold increase in the amount of the blood vessels in the tumor (i.e., intratumoral vascular volume) compared with control embryos. Exposure of isolated tumor cells to 10 mM and 20 mM ethanol for 19 hours also increased VEGF mRNA and protein expression. The increased intratumoral vascular volume strongly correlated with the increase in tumor volume as well as with intratumoral connective tissue volume density. Finally, invasion of HT1080 cells from the tumor into blood vessels (i.e., intravasation), which occurs during metastasis, increased more than eightfold in response to ethanol. Ethanol did not have a direct effect on cell proliferation.

Hepatocellular Carcinoma

Because excessive alcohol consumption often is associated with liver disease, investigators also have examined the effects of alcohol on hepatocellular carcinoma. One study (Thompson et al. 2013) evaluated the effects of alcohol as well as a high-fat diet on hepatocellular carcinoma progression in male C57BL/6 mice with diet-induced obesity as well as in nonobese control mice. Both groups were injected with Hepa1-6 tumor cells. Ethanol was administered in drinking water at daily alternating concentrations of 10 percent and 20 percent for 6 weeks before the animals were injected with the tumor cells. The alcohol and dietary regimens then were continued for an additional 8 weeks. Gross numbers of tumors as well as tumor burden were lower in mice fed the high-fat diet with and without ethanol or the control diet with ethanol than in control animals receiving neither ethanol nor high-fat diet, with ethanol exerting a greater protective effect than the high-fat diet. However, the high-fat diet both alone and in conjunction with ethanol enhanced mortality in the tumor-bearing mice. Further analyses found that all tumor-bearing mice exhibited increased numbers of inflammatory white blood cells in their livers, regardless of whether they had received ethanol. The expression of some cytokines and their receptors also was altered in the tumor-bearing mice, and these changes in some cases depended on the animals' diet. Thus, tumor necrosis factor (TNF)-α and interleukin (IL)-6 receptor alpha mRNA expression were increased and transforming growth factor (TGF)-β mRNA expression was decreased in tumor-bearing mice receiving the high-fat diet compared with mice not injected with tumor. An analysis of seven cytokines (IL-1β, IL-6, IL-10, IL-12, IL-13, interferon gamma [IFN-γ], and TNF-α) in the pooled plasmas from each experimental group indicated no changes across the groups except

in the water-drinking, high-fat diet group, which exhibited depressed cytokine levels. There was no correlation with ethanol consumption compared with mice not injected with tumor cells.

Breast Cancer

The effect of ethanol on mammary cancer growth has been studied in a number of animal models, using both rodent and human tumor cell lines.

Studies Using Rodent Tumor Cell Lines

Wang and colleagues (2012) examined the effect of ethanol on the growth of the aggressive estrogen receptor–positive E0771 mouse mammary cancer in female C57BL/6 mice. The mice were given 2 percent ethanol in drinking water for half a day on each of 3 consecutive days before the E0771 tumor cells were inoculated into breast tissue (i.e., secondary mammary fat pad), and the ethanol feeding regimen then was continued for 24 days. The study found that the ethanol group exhibited higher primary tumor growth rates, increased final tumor weights, and a twofold increase in lung metastases compared with the water-drinking control group. Immunohistochemical analyses of the mammary tumor tissues also showed a higher density of tiny blood vessels in the ethanol group, indicating that ethanol promoted tumor angiogenesis. Finally, the investigators found increased expression of a chemokine called monocyte chemoattractant protein-1, or MCP-1 (also known as CCL2), which has been implicated in tumor development and angiogenesis, in tumor tissues from the ethanol group and in E0771 cells exposed to 0.2 percent ethanol. MCP-1 plays an important role in suppressing antitumor immune functions and facilitating tumor metastasis (Kudo-Saito et al. 2013), indicating another mechanism through which alcohol could promote breast cancer progression.

A related study using the same alcohol-feeding regimen confirmed alcohol's effects on growth and angiogenesis of E0771 inoculated into other female C57BL/6 mice (Lu et al. 2014). In that study, a molecule that can inhibit VEGF receptor 2 blocked alcohol's stimulatory effect on tumor growth, indicating that alcohol acts via a VEGF pathway.

Holcomb and colleagues (2012) examined the effects of various diets and supplements on the growth of estrogen receptor–positive mammary tumor cells (derived from mammary tumor virus-Wnt-1 transgenic mice) inoculated subcutaneously into female C57BL/6J mice that had their ovaries removed. Conditions tested included (1) 20 percent weight per volume (w/v) alcohol in drinking water; (2) a calorie-restricted diet with 30 percent fewer calories than normal; (3) a high-fat diet where 60 percent of calories were derived from fat; (4) a low-fat diet where 10 percent of calories were derived from fat; and (5) estrogen supplementation. The diets and alcohol were started when the animals were 8 weeks old and continued for 27 weeks. Estrogen pellets were implanted after 19 weeks of alcohol consumption, and tumors were implanted after 22 weeks. The results on tumor growth were similar to those obtained by Hong and colleagues (2011), with the high-fat diet and alcohol promoting tumor growth and estrogen suppressing it. Tumor growth was greatly inhibited in the mice receiving a high-fat diet as well as estrogen supplements. The calorie-restricted diet also inhibited tumor growth independent of the effects of alcohol and estrogen supplementation. Neither the diets nor alcohol affected VEGF.

Researchers also studied the effects of alcohol on estrogen receptor–negative mouse mammary tumors. One study involving estrogen receptor–negative Met-1 cancer cells used female FVB/N mice that consumed 20 percent w/v ethanol in drinking water for 18 weeks before they were injected subcutaneously with the cancer cells (Hong et al. 2010). Compared with water-drinking control mice, the ethanol-drinking animals developed palpable tumors earlier and also developed larger tumors. Several other parameters (i.e., insulin sensitivity, leptin levels in the blood, and estrogen levels) were elevated in the alcohol-consuming mice. These researchers also examined the effect of ethanol in vitro on the migration of the estrogen receptor–positive T47D breast cancer cell line. The results showed that cells exposed to different concentrations of ethanol from 0.1 percent to 0.5 percent exhibited increased migration, as did cells exposed to estrogen (20 nM). The combination of estrogen and 0.5 percent resulted in higher migration than either treatment alone.

In another study (Hong et al. 2011), the researchers examined the effects of alcohol on Met-1 tumor growth as a function of a diet (i.e., low fat vs. high fat) and estrogen supplementation (low dose vs. high dose). Mice consuming the high-fat diet developed larger tumors than did mice fed the low-fat diet; moreover, alcohol ingestion increased the

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final tumor volume in both dietary groups. Estrogen treatment suppressed tumor growth regardless of diet and alcohol consumption; however, mice treated with high-dose estrogen had slightly larger tumors than did mice treated with the low dose. Tumor tissues also were analyzed for the levels of various regulatory molecules hypothesized to potentially influence tumor progression, including phosphoinositide 3 kinase (PI3K/p85α); a protein kinase called Akt; the phosphorylated form of Akt (p-AktSer^{473}), a signaling molecule associated with cell proliferation, survival, and invasion; proliferating cell nuclear antigen (PCNA); and cleaved caspase-3, a molecule involved in programmed cell death (i.e., apoptosis), with the following results:

- PI3K (p85α) was not affected by alcohol, high-fat diet, or estrogen treatment.
- Alcohol and the high-fat diet increased expression of p-AktSer^{473}. In contrast, estrogen supplementation reduced phosphorylation of Akt in both the alcohol and high-fat diet groups.
- The high-fat diet and alcohol decreased the level of cleaved caspase-3, suggesting decreased apoptosis in the tumor tissue. Interestingly, estrogen reduced this signaling molecule in tumors from mice fed alcohol and high-fat diets.
- PCNA was not altered by any of the variables, indicating that cell proliferation was not affected.

Other researchers investigated the effects of alcohol on metastasis of the estrogen receptor–negative and natural killer (NK) cell-sensitive rat MADB106 mammary adenocarcinoma (Yirmiya et al. 1992). In this study, male Fischer 344 rats were administered only one alcohol dose (1.5 to 3.5 g ethanol/kg body weight into the peritoneal cavity) 1 hour before intravenous tumor inoculation. The higher ethanol doses (i.e., 2.5 g/kg and 3.5 g/kg) significantly increased the number of lung metastases, whereas the lowest dose (1.5 g/kg) did not. Administration of naltrexone, an opioid receptor antagonist used to treat alcohol dependence, did not modify the alcohol-related increase in metastasis. Ethanol's effects on lung metastasis seem to depend on the exact administration schedule. Thus, in a related study these researchers found that administration of 2.5 g/kg ethanol 24 hours before or after tumor inoculation did not affect lung metastasis (Ben-Eliyahu et al. 1996). Yirmiya and colleagues (1992) also administered ethanol in a liquid diet for 2 weeks before and 3 weeks after tumor inoculation and found that lung metastases were increased.

Alcohol's effects also seem to depend on the tumor type studied. When rats were injected intravenously with rat CRNK-16 leukemia cells instead of MADB106 cells, acute administration of 1.5 to 3.5 g of ethanol/kg/body weight reduced survival in a dose-related fashion, whereas maintenance on a liquid diet containing 5 percent w/v ethanol did not (Ben-Eliyahu et al. 1996; Yirmiya et al. 1992).

One of the ways in which the body defends itself against tumor cells involves their destruction by NK cells. The investigators also analyzed alcohol's effects on NK-cell activity, finding that neither acute injection nor dietary administration of ethanol in these experiments affected NK-cell activity against MADB106 cells when determined in an in vitro assay (Yirmiya et al. 1992). When MADB106 and CRNK-16 cells were incubated with ethanol in vitro, the numbers of these cells were reduced after 5 days. These effects were significant for MADB106 cells at ethanol concentrations of 0.2 percent, 0.5 percent, and 1.0 percent ethanol and for CRNK-16 cells at 0.5 percent and 1.0 percent ethanol. Ethanol had no effect in rats that were depleted of NK cells, and metastasis was not affected after injection of the NK-insensitive C4047 rat colon cancer cell line (Ben-Eliyahu et al. 1996).

Tumor growth and metastasis also were studied in the highly metastatic estrogen receptor–negative 4T1.2 cells implanted into the mammary fat pad of female Balb/c that had consumed alcohol in drinking water for 4 weeks (Vorderstrasse et al. 2012). The analyses indicated that continuous availability of 1 percent, 5 percent, and 18 percent ethanol w/v did not affect either the establishment of tumors or the final mammary tumor weight, indicating a lack of effect on tumor growth. However, the animals receiving 18 percent ethanol exhibited a marked reduction in metastasis to secondary mammary glands and to the lung. Tumor-associated increases in spleen size (i.e., splenomegaly) also were significantly reduced in the 18-percent ethanol group. Lung metastasis tended to be lower in mice consuming 5 percent ethanol, but there was no effect among those consuming 1 percent ethanol. Although the animals receiving 18 percent ethanol showed suppressed metastasis, they were less healthy compared with the

other treatment and control groups as indicated by appearance, body condition, natural behavior, and provoked behavior. In vitro studies indicated that exposure of cells to 0.3 percent w/v ethanol did not affect 4T1.2 cell proliferation, colony formation, or invasion but did reduce cell migration twofold. Reduced cell migration may be related to changes in expression of CXCR4, a chemokine receptor that is important to migration and metastasis of breast and other cancers (Sarvaiya et al. 2013). Vorderstrasse and colleagues (2012) showed that expression of CXCR4 was suppressed 60 to 80 percent in tumors of mice consuming 18 percent ethanol; however, a correlation analysis indicated a lack of relationship between CXCR4 expression and metastasis. When the 4T1.2 cells were grown in 0.3 percent ethanol, however, CXCR4 expression was not altered, suggesting that expression was indirectly modulated by alcohol consumption in vivo.

Studies Using Human Tumor Cell Lines

Several studies examined the specific effects of ethanol on various aspects of disease progression in human breast cancer cell lines, including proliferation of cells. Singletary and colleagues (2001) found that incubation in 0.4 percent w/v ethanol increased cell proliferation in the estrogen receptor–positive MCF-7 and ZR75.1 breast cancer cells but not in the estrogen receptor–negative BT-20 and MDA-MB-231 cells. The effect of ethanol on MCF-7 cells also was correlated with increases in estrogen receptor alpha content. However, alcohol's effects are complex. When the MCF-7 cells were cultured together with human skin fibroblasts in 0.4 percent ethanol for 72 hours, ethanol suppressed estrogen receptor alpha expression compared with untreated cells (Sanchez-Alvarez et al. 2013). Thus, the tumor micro-environment is important in determining estrogen-receptor status and the effects of alcohol on breast cancer. Whether this study is relevant to patients with breast cancer is not known. However, additional studies are warranted, because estrogen receptor–negative breast cancer generally is more aggressive, and patients have a worse prognosis than patients with estrogen receptor–positive breast cancer. Moreover, conversion from estrogen receptor alpha positive to estrogen receptor alpha negative can occur (Hoefnagel et al. 2010).

Other studies focused more on the invasion and migration in vitro of estrogen receptor–positive and estrogen receptor–negative human breast cancer cells. One study (Ma et al. 2003) compared the effects of incubation in 0.4 percent w/v ethanol for 48 hours on various breast cancer cell lines. This treatment increased invasion of the estrogen receptor–positive MCF-7 and T47D breast cancer cells as well as the estrogen receptor–negative HS578T, MDA-MB231, and MDA-MB435 cells. Similarly, incubation for 48 hours in 0.1 percent and 0.2 percent w/v ethanol stimulated invasion of estrogen receptor–negative SKBR3 and estrogen receptor–positive BT474 breast cancer cells. In contrast, ethanol exposure did not affect invasion of HB2, an immortalized normal human breast tissue cell line, or estrogen receptor–negative BT20 breast cancer cells. The effects of ethanol may depend not only on the specific cell line examined but also on the ethanol concentration used. Thus, studies from another laboratory demonstrated that exposure to 0.1 percent, 0.2 percent, and 0.5 percent w/v ethanol enhanced invasion of T47D, MCF-7, and MDA-MB231 cells in a dose-dependent manner (Wong et al. 2011). Similarly, Aye and colleagues (2004) examined the effects of exposure for 48 hours to different ethanol concentrations on estrogen receptor–negative SKBR3 and estrogen receptor–positive BT474 breast cancer cells. For both SKBR3 and BT474 cells exposure to 0.1 percent and 0.2 percent w/v ethanol stimulated invasion. A higher dose of 0.4 percent w/v ethanol, however, inhibited invasion of SKBR3 cells and created mixed results for BT474, with one study (Aye et al. 2004) detecting no effect on invasion and another study (Xu et al. 2010) detecting increased invasion.

Invasive ability generally was related to the expression of ErbB2/neu, an epidermal growth factor (EGF) receptor that is amplified in 20 to 30 percent of breast cancer patients, with higher ErbB2/neu levels indicating higher risk of lymph node metastasis and poor prognosis. More detailed studies of the relationship between alcohol, ErbB2/neu, and invasion in the human breast cancer cell line T47D found that activation of the EGF receptor by addition of EGF did not significantly affect ethanol's ability to enhance invasiveness (Luo and Miller 2000). Conversely, prevention of ErbB2/neu production inhibited the ability of ethanol to increase migration (Luo and Miller 2000).

Numerous studies have sought to identify the mechanisms through which ethanol affects growth and migration of breast cancer cells or

the molecules that mediate these effects. The results of such investigations include the following:

- Studies in MDA-MD231 and T47D cells determined that ethanol induced altered adhesion to fibronectin, increased development of lamellipodia,[1] increased phosphorylation of a protein called focal adhesion kinase, and increased production of several proteins (e.g., ribosomal protein L7a, smooth muscle myosin alkali light chain), all of which can promote cell migration (Xu et al. 2010; Zhu et al. 2001).

- Exposure of T47D cells to 0.5 percent w/v ethanol decreased mRNA expression of *NM23*, a known metastasis suppressor gene (Wong et al. 2011). However, gene expression levels of two other metastasis suppressors, *KISS1* and *MKK4*, were increased by ethanol. Ethanol treatment also modulated the expression of more than 80 other genes associated with regulation of the extracellular matrix and cell adhesion. For example, ethanol induced expression of the gene encoding fibronectin receptor subunit integrin alpha 5 (ITGA5). This receptor activates signaling pathways supporting invasion, and reduced expression of ITGA5 significantly inhibits invasion. The ethanol-induced expression of this gene was blocked by overexpression of the *NM23* gene, indicating an important relationship between these two genes in controlling invasion of T47D breast cancer cells.

[1] Lamellipodia are projections on the mobile edge of a cell that allow the cell to move across a substrate.

- Studies also assessed alcohol's interactions with enzymes called matrix metalloproteinases (MMPs) such as MMP-2 and MMP-9 that are involved in the breakdown of the extracellular matrix in normal physiological processes as well as in disease processes, such as metastasis. Studies to date indicate no interactions between alcohol and MMPs (Aye et al. 2004; Ranuncolo et al. 2002).

Melanoma

In one of the first experiments conducted in melanoma, 6- to 8-week-old female CDBA/2F1 mice consumed water or 20 percent alcohol for 52 weeks before being inoculated in a leg with the Cloudman 8-91 melanoma tumor (Ketcham et al. 1963). When the tumors reached a size of 1.5 to 2.0 cm (about 28 days after tumor inoculation), the groups were divided in half, and half of each group had the primary tumor-bearing leg amputated. At 56 days after tumor implantation, the number and size of pulmonary metastases were recorded for all animals. The study detected no substantial or consistent effect of alcohol on the size or incidence of pulmonary metastases. However, surgical removal of the tumor-bearing leg decreased pulmonary metastasis in both ethanol-drinking and water-drinking groups.

Other studies in mice assessed the effects of acute and chronic alcohol consumption on tumor growth and metastasis using B16 melanoma and its more metastatic variants, B16F10 and B16BL6. An early study (Capel et al. 1978) found that mice given 10 percent ethanol in drinking water for 2 weeks before inoculation with B16 melanoma into the thigh showed no altered tumor growth or metastasis compared with water-drinking controls. In another study (Tan et al. 2007), tumor growth and angiogenesis were examined in C57BL/6 mice implanted subcutaneously with B16F10 melanoma cells. The mice had access to regular drinking water and to 1 percent ethanol in their drinking water for 12 hours each per day for 4 weeks, with tumor cells being implanted during the second week of ethanol administration. Compared with animals who only drank water, those who had access to ethanol developed palpable tumors sooner and had 2.2 times greater tumor weights at the end of the study. Analysis of the tumors indicated an increase in VEGF mRNA and VEGF protein, as well as increased tumor angiogenesis. Moreover, another marker of angiogenesis, VEGF-R1 (Flt-1), also was found in a greater number of tumor cells and endothelial cells in the surrounding tissue from the ethanol group compared with the control group.

Wu and Pruett (1999) determined the effects of acute ethanol administration (5 or 6 g/kg body weight) given through a tube inserted into the mouth (i.e., by oral gavage) on melanoma metastasis. Ethanol administered to female B6C3F1 mice 1 hour before intravenous inoculation of B16F10 melanoma cells greatly increased lung metastasis. The number of metastases also was increased when administration of 6 g/kg ethanol occurred 4 or 10 hours after tumor inoculation but not when it occurred 6 hours before tumor inoculation. About half of the increase in lung tumor nodules seemed to be related to ethanol-induced corticosterone production in the adrenal glands. Experiments

analyzing whether ethanol-related inhibition of some aspect of NK-cell function also might contribute to the increased metastasis produced found no indication of significantly reduced NK-cell numbers or activity. However, further studies directed at determining the role of NK cells in the acute ethanol-induced increase in melanoma lung metastasis showed that ethanol inhibited experimental induction of NK-cell activity (in response to poly I:C), resulting in approximately a twofold increase in lung tumor nodules compared with control mice (Wu and Pruett 1999). Additional mechanistic studies indicated that a single dose of ethanol increased corticosterone levels (Collier et al. 2000) and also suppressed experimental induction of mRNA for a variety of cytokines (i.e., interferon alpha [IFN-α], interferon beta [IFN-β], IFN-γ, IL-6, IL-12, and IL-15) (Pruett et al. 2003). As a result, concentrations of IFN-α and IL-12 in the blood were decreased. Conversely, ethanol greatly enhanced the levels of IL-10 in the blood. However, these changes in serum cytokines were not mediated by corticosterone. Overall, these studies suggest that the increase in melanoma lung nodule formation after acute ethanol administration is complex and related to effects associated with some aspect of NK-cell inhibition, increased suppression of NK-cell activity by corticosterone, and corticosterone-independent effects on cytokines favoring tumor immune escape.

The effects of chronic alcohol consumption on tumor growth and metastasis of the highly invasive and spontaneously metastatic B16BL6 melanoma inoculated subcutaneously were studied in female C57BL/6 mice administered ethanol in drinking water. In an initial study, consumption of 2.5 percent, 10 percent, or 20 percent w/v ethanol in drinking water for 6 to 8 weeks before tumor inoculation and continuing thereafter did not affect primary tumor growth (Blank and Meadows 1996). However, the animals receiving 20 percent ethanol in their drinking water exhibited consistently reduced survival, lower tumor weight, and lower final body weight compared with the other groups. Alcohol exposure also affected metastasis. All three ethanol-exposed groups had reduced metastasis to the axillary lymph nodes, with the 10-percent and 20-percent ethanol groups showing reduced lung metastasis, and the 20-percent ethanol group showing reduced superficial metastasis to the kidneys. Metastasis did occur, however, in the draining inguinal lymph nodes in mice consuming 20 percent weight per volume ethanol for 12 weeks (Zhang et al. 2011*b*).

More specific experiments sought to determine the effect in mice on metastasis of B16BL6 melanoma of short-term and long-term exposure to 10 percent and 20 percent w/v ethanol before and after intravenous inoculation into a tail vein or inoculation into the skin of the pinna of the ear[2] (Meadows et al. 1993*a,b*; Spitzer et al. 2000; Zhang et al. 2011*a*). These experiments showed that 10 percent w/v ethanol did not affect metastasis after intravenous tumor inoculation in female C57BL/6 mice consuming alcohol for 2 weeks or spontaneous metastasis in mice injected 1 week after initiating ethanol feeding. However, lung metastasis was inhibited if intravenous injection of tumor cells occurred at 4, 6.5, 7, and 12 weeks after initiation of 20 percent w/v ethanol. Similarly, spontaneous metastasis to the lung was significantly inhibited in mice injected with melanoma at 1, 4, 6.5, and 10 weeks of consuming 20 percent ethanol. Ethanol did not prevent spontaneous metastasis to the draining cervical lymph nodes.

The antimetastatic effect of ethanol on the B16BL6 melanoma tumor was confirmed after injection of tumor cells in male C57BL/6J mice that carry a gene for obesity and which had consumed 20 percent ethanol in drinking water for 10 weeks (Kushiro and Nunez 2012). Different groups were analyzed for lung tumor metastasis over a period of 16 to 21 days. All of the water-drinking animals had developed visible lung metastases at 16 days after tumor injection, whereas some of the ethanol-drinking mice did not develop lung metastases until 21 days. More-over, the numbers of lung metastases in the ethanol-drinking mice were significantly lower at 21 days.

The contribution of NK cells to the inhibition of metastasis was evaluated in mice consuming 10 percent or 20 percent w/v ethanol for 4 weeks (Meadows et al. 1993*a*). Whereas consumption of 10 percent ethanol did not alter the NK cells' ability to destroy other cells (i.e., decrease cytolytic activity), animals consuming 20 percent ethanol showed decreased NK cytolytic activity. And although experimental stimulation of NK cells could enhance their cytolytic activity 4.3-fold in the ethanol-drinking animals, compared with 2.6-fold in the control animals, overall cytolytic activity still was lower in the ethanol group than in the control group. Treatment of mice with an antibody against NK cells (i.e., anti-NK1.1 antibody)

[2] This method of tumor placement facilitates spontaneous metastasis.

markedly decreased NK-cell cytolytic activity in both water- and ethanol-drinking animals. These same treatments were evaluated in mice injected with B16BL6 melanoma. Experimental stimulation of NK cells decreased the number of lung metastases in the water-drinking and 10-percent ethanol groups, but not in the 20-percent ethanol group. Inactivation of NK cells by administration of anti-NK1.1 antibody significantly increased lung metastases in the water-drinking and 10-percent ethanol groups, but not in the 20-percent ethanol group.

The data suggest that inhibition of NK-cell cytolytic activity in mice consuming 20 percent ethanol does not lead to enhanced metastasis following inoculation of B16BL6 melanoma. This lack of interaction between alcohol consumption and NK-cell cytolytic activity in B16BL6 melanoma lung metastasis was further confirmed in another strain of mice (i.e., beige mice) that naturally have low NK cytolytic activity (Spitzer et al. 2000). In other experiments to determine how ethanol decreases metastasis of B16BL6 melanoma, either isolated tumor cells grown in the presence of 0.3 percent ethanol or tumor cells from alcohol-consuming mice were inoculated into water-drinking mice (Blank and Meadows 1996). Mice that had received either tumor cells cultured in the presence of ethanol or derived from mice drinking 20 percent alcohol showed increased lung metastasis compared with control mice or those receiving tumor cells derived from mice drinking 10 percent ethanol. Thus, ethanol seems to increase the actual metastatic potential of melanoma cells. However, because alcohol drinking inhibits metastasis, there seem to be other factors induced by ethanol that counter this metastatic potential.

Further research was directed at identifying factors involved in expression of the antimetastatic effect associated with chronic (12 weeks) consumption of 20 percent w/v alcohol consumption. Zhang and colleagues (2011*a*) found that chronic alcohol consumption increased the numbers of immune cells (e.g., NK, NKT, $CD4^+$ T and $CD8^+$ T cells) that produce IFN-γ. IFN-γ is an essential factor in the control of melanoma metastasis (Blankenstein and Qin 2003; Ikeda et al. 2002). IFN-γ also mediates the antimetastatic effects of chronic alcohol consumption, because mice that could not produce IFN-γ did not show this effect (Zhang et al. 2011*a*). The investigators attempted to determine whether one immune cell population was more important than another in producing the antimetastatic IFN-γ. However, through a series of specific cell depletion experiments, they found that it did not matter which cell type produced the IFN-γ.

Other investigators (Kushiro and Nunez 2012) found that B16BL6 melanoma cells grown in 0.1 percent, 0.2 percent, or 0.5 percent ethanol showed considerably reduced cell invasion and, at the highest ethanol concentration, reduced cell motility and anchorage-dependent growth. Ethanol did not affect cell proliferation, apoptosis, or necrosis of the melanoma cells. In addition, the highest ethanol dose altered the expression of several genes that play prominent roles in regulating melanoma metastasis (i.e., the IL6, Nfkb, snail1, E-cadherin, Kiss1, Nm23-m1, and Nm23-m2 genes). These changes also could contribute to the antimetastatic effect of alcohol on melanoma.

Summary

Animal models have yielded some insights into the effects of alcohol on tumor growth, survival, and metastasis of different cancers, including breast cancer, lung cancer, liver cancer, and melanoma. However, because cancer is a collection of many different diseases and subtypes, each cancer or cancer subtype might not respond similarly to alcohol, as is evident from the research discussed here.

The relationship between alcohol and tumor progression is complex. Tumor metastasis involves many steps and also is controlled by many different signaling pathways. Because each cancer is unique, the specific connections between alcohol and the steps involved in cancer progression also by nature are complex. Thus, it is important to relate experimental results to specific types/subtypes of cancer as well as the amount of alcohol consumed, the route of alcohol administration, and the duration of alcohol administration. Clearly, more studies are needed to define the effect of alcohol on tumor progression and to determine the mechanisms associated with individual cancers and subtypes.

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Alcohol and Viral Hepatitis

Role of Lipid Rafts

Angela Dolganiuc, M.D., Ph.D.

Both alcohol abuse and infection with hepatitis viruses can lead to liver disease, including chronic hepatitis. Alcohol and hepatitis viruses have synergistic effects in the development of liver disease. Some of these involve the cellular membranes and particularly their functionally active domains, termed lipid rafts, which contain many proteins with essential roles in signaling and other processes. These lipid rafts play a central role in the lifecycles of hepatitis viruses. Alcohol's actions at the lipid rafts may contribute to the synergistic harmful effects of alcohol and hepatitis viruses on the liver and the pathogenesis of liver disease.

Key words: Alcohol abuse; alcohol use and misuse; alcohol disorder; liver; liver disease; hepatitis; hepatitis B virus; hepatitis C virus; lipid rafts

Alcohol is the most used and abused psychoactive drug worldwide. Alcohol use and misuse, including alcohol use disorder, can have devastating effects and account for 5.9 percent of deaths and 5.1 percent of the global burden of disease and injury, thereby also imposing a significant social and economic burden on society (World Health Organization 2015). Moreover, treatments for alcohol abuse have shown limited effectiveness (Grant et al. 1988; National Institute on Alcohol Abuse and Alcoholism 1998). Alcohol use disorder is a systemic disease that affects all organs and systems. Evidence suggests that risk of alcohol-related organ damage occurs with excessive alcohol intake, which is defined as binge drinking or heavy drinking. According to the National Institute on Alcohol Abuse and Alcoholism, binge drinking is defined as a pattern of alcohol consumption that brings the blood alcohol concentration (BAC) level to 0.08 percent or more. This pattern of drinking usually corresponds to consumption of 5 or more drinks on a single occasion for men and 4 or more drinks on a single occasion for women, generally within about 2 hours. Heavy drinking typically is defined as consuming 15 drinks or more per week for men and 8 drinks or more per week for women (Centers for Disease Control and Prevention [CDC] 2014). The liver is particularly susceptible to alcohol-induced damage. However, although many chronic heavy drinkers develop alcoholic liver disease (ALD), no consumption levels have been identified that predictably result in ALD. Factors that influence the susceptibility to ALD include gender, co-exposure to other drugs, genetic factors that either favor the development of addiction or affect alcohol-metabolizing enzymes, immunological factors, nutritional status, and infection with viruses targeting the liver (i.e., hepatotropic viruses).

Hepatitis viruses, and particularly hepatitis B virus (HBV) and hepatitis C virus (HCV), are responsible for most cases of chronic hepatitis in the United States. In 2013, almost 20,000 new cases of HBV infection and almost 30,000 new cases of HCV infection were estimated to occur in the United States (CDC 2015*a*). Worldwide, approximately 350 to 400 million people, or about 5 percent of the population, are chronically infected with HBV and about 180 million people, or 2 percent of the population, with HCV (El-Serag 2012). In chronic alcoholics, the prevalence of HCV infection as indicated by the presence of anti-HCV antibodies is higher than in the general population (Takase et al. 1993). Co-occurring viral hepatitis and alcohol use disorder adversely affect disease course and are associated with increased mortality and death at an earlier age (Kim et al. 2001; Sagnelli et al. 2012; Tsui et al. 2006; Wiley et al. 1998). The most serious complication of ALD is liver cirrhosis, which often progresses to hepatocellular carcinoma (HCC); indeed, about 20 percent of heavy drinkers develop cirrhosis during their lifetime, and this risk is much increased in the presence of co-occurring viral hepatitis (El-Serag 2012; Ishak et al. 1991). End-stage liver disease from viral hepatitis, together with ALD, is the main reason for liver transplantation in the United States (El-Serag 2012).

The mechanisms how alcohol and viral hepatitis together accelerate liver disease have been researched extensively over the last several decades. It is becoming clear that alcohol exposure, infection with hepatitis viruses, and the host's defense mechanisms against these offenders combine to contribute to the pathogenesis of liver disease and thus could be targets of therapeutic interventions. New antiviral drugs against HCV have been developed in recent years, and reasonably effective HBV treatments also are available (American Association for the Study of Liver Diseases 2015; Lok and McMahon 2009). Yet many patients either do not qualify for or cannot afford newer antiviral treatments. Further, exposure to alcohol, whether acute or chronic, light or heavy, may preclude eligibility for treatment of viral hepatitis. Additionally, many patients cannot achieve abstinence from alcohol or experience recurrent relapse (Becker 2008). Therefore, novel approaches are needed for the

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diagnosis and treatment of patients with coexisting alcohol use and viral hepatitis.

This article reviews some of the mechanisms underlying alcohol-induced liver injury and also explains the contributions of hepatitis viruses to liver disease, as well as the synergistic effects of alcohol and hepatitis virus infections on the liver. This discussion particularly focuses on the roles that the cellular membranes, and especially membrane domains called lipid rafts, play in these processes. Both alcohol and viral infections influence the functions of lipid rafts and the functional proteins they contain, which may exacerbate disease progression. The specific mechanisms underlying the effects of alcohol and hepatitis viruses on the cellular membranes and their contribution to liver disease pathogenesis, however, still remain to be fully elucidated.

Alcohol-Induced Liver Injury

Liver injury in ALD occurs as a result of multiple synergistic mechanisms, including impaired function of the main parenchymal liver cells (i.e., hepatocytes), imbalanced local (i.e., nonparenchymal) and systemic immune responses, and altered cross-talk between parenchymal and nonparenchymal cells in the liver.

Alcohol has diverse effects on the hepatocytes that result in significant disturbances of the cells' abilities to synthesize needed molecules and detoxify harmful products (Van Horn et al. 2001; Videla et al. 1973), pronounced deficits in antioxidant levels (Fernandez-Checa et al. 2002; Lauterburg and Velez 1988), and marked oxidative cellular stress (Tsukamoto 1993). These effects, together with additional changes in hepatocyte metabolism, lead to the accumulation of lipids in the alcohol-exposed hepatocytes (i.e., hepatic steatosis). The affected cells consume oxygen inefficiently, have reduced detoxifying ability, fail to synthesize needed compounds, and are more likely to undergo apoptosis (Nanji and Hiller-Sturmhoefel 1997). As a result of all of these changes, the cells also become more susceptible to other harmful influences, such as infections with hepatotropic viruses and dietary insufficiencies. Finally, alcohol exposure greatly enhances tumorigenesis in hepatocytes (Morgan et al. 2004).

Alcohol exposure also affects local immune responses by both hepatocytes and resident and nonresident immune cells. Hepatocytes are the first cells to encounter hepatotropic viruses, and activation of their cytokine signaling systems—including proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-1 and IL-6, and interferons (IFNs)—is key to the initiation of immune responses. Alcohol exposure has diverse effects on these immune responses. On the one hand, alcohol suppresses intracellular expression of type I IFNs (IFN-α/β) in human hepatocytes by reducing the expression of key positive regulators of type I IFN signaling pathways and inducing the expression of key negative regulators of IFN-α/β signaling (Plumlee et al. 2005; Ye et al. 2010). On the other hand, alcohol-exposed hepatocytes increase the expression of proinflammatory TNF-α (Mandrekar 2007; Plumlee et al. 2005). In addition, alcohol exposure results in differential activation of IL-1 pathways in hepatocytes versus other nonparenchymal cells (e.g., Kupffer cells). Thus, certain active molecules (i.e., the active fragment of caspase-1 and IL-1β) are elevated only in liver immune cells but not in alcohol-exposed hepatocytes. Innate immune pathways in hepatocytes also may regulate hepatocyte steatosis and hepatocellular injury. A signaling molecule called IRF3, which is an essential component of innate immunity and is required for hepatocyte apoptosis, may play a unique role in the processes leading to hepatocyte apoptosis in ALD and tying together alcohol-induced liver inflammation, metabolic disturbances, and cell death (Petrasek et al. 2013).

Alcohol-induced cross-talk between parenchymal and nonparenchymal liver cells (e.g., Kupffer cells) is another key component of liver disease (Cohen and Nagy 2011). The activation of Kupffer cell-specific signaling pathways involving innate immune molecules called toll-like receptors (TLRs), and in particular TLR4, is emerging as a required step in the progression of liver disease from steatosis to steatohepatitis in ALD. In addition, TLR4-mediated activation of Kupffer cells seems to be important for the formation of scar tissue (i.e., fibrogenesis) in the liver after chronic alcohol treatment (Adachi et al. 1994; Inokuchi et al. 2011). Other TLRs also influence the development of ALD. Thus, alcohol exposure augments signaling via TLR8 and TLR7, thereby inducing both IL-10 and TNF-α and downgrading IFN expression in myeloid cells (Pang et al. 2011). These effects may contribute to the persistent inflammation and impaired antiviral responses in ALD. Kupffer cells seem to govern the course of ALD, especially in the early stages of the disease, because deletion of these cells protects against alcohol-induced liver injury. The mechanisms underlying these effects are not fully understood but likely are multifactorial and include cell cross-talking between innate immune cells and other liver cells, such as stellate cells (Adachi et al. 1994). Stellate cells, in turn, can develop into myofibroblasts that play a central role in alcohol-induced fibrogenesis. Alcohol exposure triggers stress signals from hepatocytes that can activate myofibroblasts, which favor excess type 1 collagen synthesis and lead to progression of fibrosis (Siegmund and Brenner 2005). Additionally, TLR4 is a key molecule involved in signaling to, from, and inside of stellate cells, suggesting that innate immune pathways also contribute to this stage of ALD (Paik et al. 2003; Seki et al. 2007).

Hepatitis Viruses

Hepatitis viruses are a heterogeneous group of five unrelated hepatotropic viruses that cause inflammation of the liver. They include hepatitis viruses A, B, C, D, and E. Of

these, HBV and HCV are clinically most relevant in Western countries.[1]

HBV

HBV reproduces exclusively in hepatocytes. Each HBV particle contains a 3.2-kb open circular DNA encapsulated in a protein shell made of three envelope proteins and the enzymes HBV polymerase and cellular protein kinase C alpha (PKCα) (Wittkop et al. 2010). This complex is called the core particle or nucleocapsid. The nucleocapsid is further surrounded by a membrane derived from the previous host cell. When infecting cells, the viral envelope interacts with liver-specific receptors, leading to uptake into the cell (i.e., endocytosis) of the virus particle and release of the nucleocapsid (see figure 1). The nucleocapsid is transported to the nucleus, where the HBV genome is released and then transcribed into mRNAs that gives rise to three envelope proteins. In parallel, another viral mRNA is translated in the cytosol into the HBV core protein and viral polymerase. Then, the viral mRNA and the various viral proteins assemble to immature core particles in a membrane-enclosed cell structure called the Golgi apparatus. The HBV genomes mature within these core particles via reverse transcription of the pre-genomic mRNA to DNA. As soon as the mature virus is assembled, the viral particle release begins. Each virus particle is packaged into a cellular membrane coat from the Golgi apparatus and then released from the host cell, taking a bit of the cell membrane with it as an envelope.

Immune cells sense virus-infected cells, inducing a cytotoxic immune response. This response, combined with ongoing strong HBV DNA replication, often results in persistent, strong inflammatory disease (i.e., hepatitis), progressive fibrosis of the liver, and potentially in HCC (El-Serag 2012; Koziel 1998).

HCV

HCV is a positive-sense, single-stranded RNA virus that, like HBV, is thought to reproduce exclusively in hepatocytes (Paul and Bartenschlager 2014).[2] HCV replicates in humans and high-level primates; it causes acute infections and has very high propensity to progress to chronic infection. The HCV viral particle includes the HCV RNA genome, the core, and an envelope made up of two glycoproteins (i.e., E1 and E2), which are key to the initial viral attachment to its cellular receptor/co-receptors (Flint and McKeating 2000; Rosa et al. 1996). Numerous molecules can serve as HCV receptors, such as scavenger receptor class B type I, low-density lipoprotein receptors, CD81, claudin-1, occludin, epidermal growth factor receptor, and Niemann-Pick C1-like 1 cholesterol absorption receptor (for a review, see Lindenbach and Rice 2013). Following attachment to the entry receptors, HCV is internalized into the host hepatocyte via endocytosis (Bartosch et al. 2003; Blanchard et al. 2006) and the RNA genome is released into the cytoplasm (see figure 2). The HCV RNA serves as template for the translation of a single large precursor protein that is processed further into 10 individual viral proteins. The translation, folding, processing, and function of these viral proteins depend on a specific intracellular structure in the hepatocytes called a membranous web, which also hosts viral RNA replication to generate new HCV genomes and assists in the assembly of new infectious viral particles (Chao et al. 2012). The assembly and release of these virus particles is closely linked to lipid metabolism (Paul et al. 2014). Thus, the lipid composition of the viral envelope is dependent on cholesterol biosynthetic pathways and resembles several types of cholesterol (i.e., low-density lipoprotein and very-low-density lipoprotein, with associated apolipoprotein E and/or B). In fact, the virus particles share the outer lipid coat with certain structures (i.e., lipid rafts, which will be discussed below) in the cell membrane surrounding the host hepatocytes (Chang et al. 2007; Gastaminza et al. 2008; Merz et al. 2011; Miyanari et al. 2007).

HCV replication is kept in check by the combined efforts of innate and adaptive (i.e., cellular and humoral) immune responses. In some people, the acute infections are mild and with limited clinical manifestations. In about 70 percent of infected individuals, however, the HCV infection is not cleared and a chronic infection is established. Possible mechanisms contributing to chronic HCV infection include failure of several types of immune cells, including natural killer cells, dendritic cells, and CD4 T cells (Dolganiuc et al. 2012; Koziel 2005; Szabo and Dolganiuc 2008). Persistently high viral replication that leads to steatotic transformation of hepatocytes and the subsequent death of some of the infected cells as well as an exaggerated inflammatory response to the infection can promote the development of fibrosis and induce disease progression from chronic hepatitis to end-stage liver disease and HCC.

Synergistic Effects of HBV/HCV Infection and Alcohol Abuse on Liver Disease

HBV and Alcohol Abuse

The prevalence of drinking in the general population is high, with more than 70 percent of people over age 18 in the United States reporting that they drank alcohol in the past year (National Institute on Alcohol Abuse and Alcoholism 2015). Accordingly, a significant portion of patients with chronic HBV infection are believed to have concomitant ALD. Alcohol use disorder is one of several conditions that

[1] The hepatitis A virus usually only causes self-limiting infections. Hepatitis D virus requires the helper function of HBV to replicate and thus hepatitis D virus infections only occur in people infected with HBV. Hepatitis E virus primarily is found in Asia and Africa and is less common in Europe and the Americas. Chronic hepatitis E virus infection only has been observed in people receiving immunosuppressant treatment after an organ transplant. The effects of alcohol on hepatitis A, D, and E viruses are largely unknown.

[2] Some reports suggest that viral replication outside the liver may also occur (Revie and Salahuddin 2011).

may co-occur with chronic HBV infection and contribute to rapid progression of liver disease, increased likelihood of tumorigenesis, and accelerated progression of HCC (Ribes et al. 2006; Sagnelli et al. 2012). Thus, heavy alcohol intake in chronic HBV-infected patients is associated with a higher risk for developing liver cirrhosis; the prevalence of cirrhosis is about 2.5 times higher in patients with co-occurring HBV infection and alcohol abuse than in patients with only one of these conditions (Sagnelli et al. 2012). The prevalence of HCC and liver-related mortality also is higher in people with chronic HBV infection and concurrent heavy alcohol consumption (Hughes et al. 2011; Niro et al. 2010). Other co-occurring conditions that increase morbidity and mortality associated with chronic HBV infection and accelerate disease progression include infection with HCV, hepatitis D virus, and HIV (Ribes et al. 2006; Sagnelli et al. 2012).

Other studies found that alcohol promotes the presence of HBV particles in the blood (i.e., HBV viremia). For example, ethanol-fed mice showed up to sevenfold increases in the levels of HBV surface antigens (i.e., HBsAg) and viral DNA in the blood compared with mice fed a control diet. In addition, the ethanol-fed mice had elevated levels of

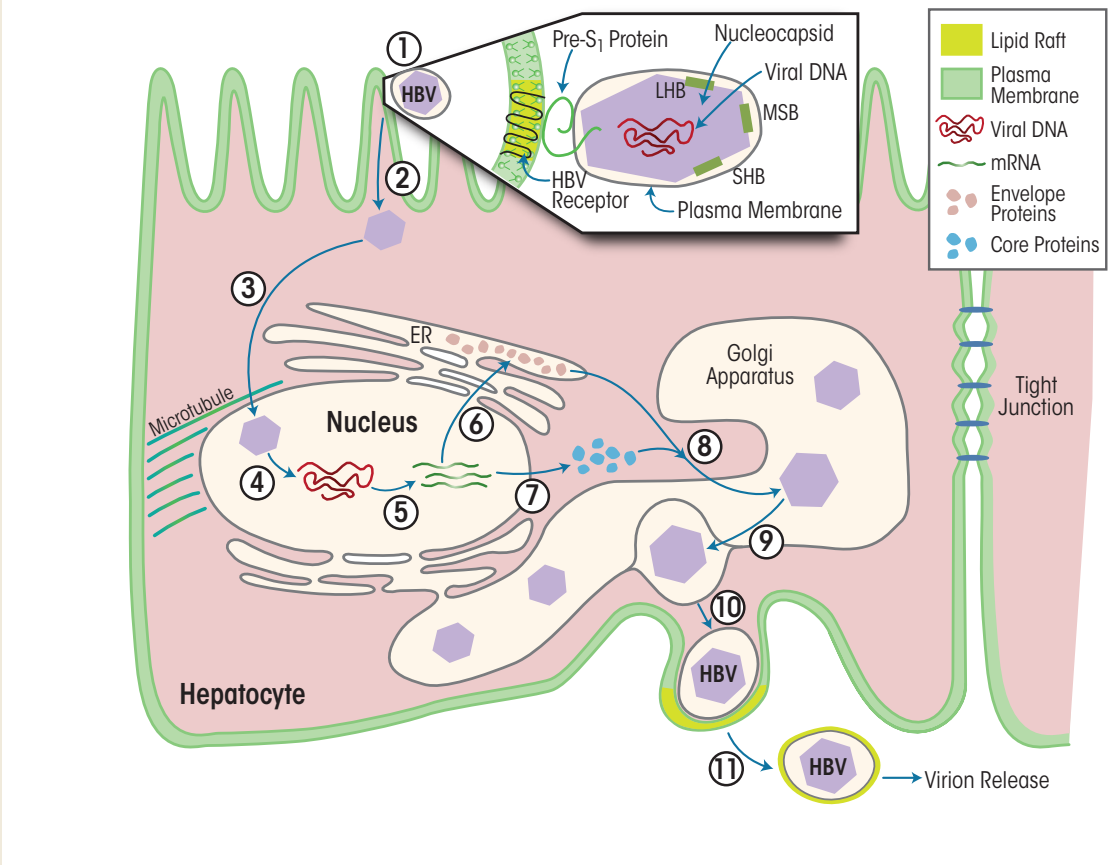

Figure 1 The life cycle of hepatitis B virus (HBV) and the role of lipid rafts. The HBV particles consist of an inner core particle (i.e., the nucleocapsid) that is made up of several envelope proteins, core proteins, and viral DNA. It is surrounded by a membrane derived from the previous host cell. **(1)** The virus particle attaches, presumably via the Pre-S1 protein, to unknown HBV receptors in the membrane of the cell. These receptors are located in membrane regions with characteristic lipid composition (i.e., lipid rafts). **(2)** The virus particle is taken up into the cell via a process called endocytosis and the nucleocapsid is released. **(3)** The nucleocapsid is transported into the nucleus, where **(4)** the DNA is released and **(5)** transcribed into mRNAs. **(6)** Some of the mRNAs are translated into the envelope proteins in the endoplasmic reticulum (ER). **(7)** Other mRNAs are translated into core proteins in the host cell's cytosol. **(8)** Envelope proteins, core proteins, and mRNA move to the Golgi apparatus and assemble into immature core particles. **(9)** The immature particles mature in the Golgi apparatus, including reverse transcription of viral mRNA into DNA. **(10)** The mature particles become surrounded by the Golgi apparatus membrane. **(11)** The mature particles are released from the host cell, taking a piece of cellular membrane with them as an envelope, including lipid raft pieces.

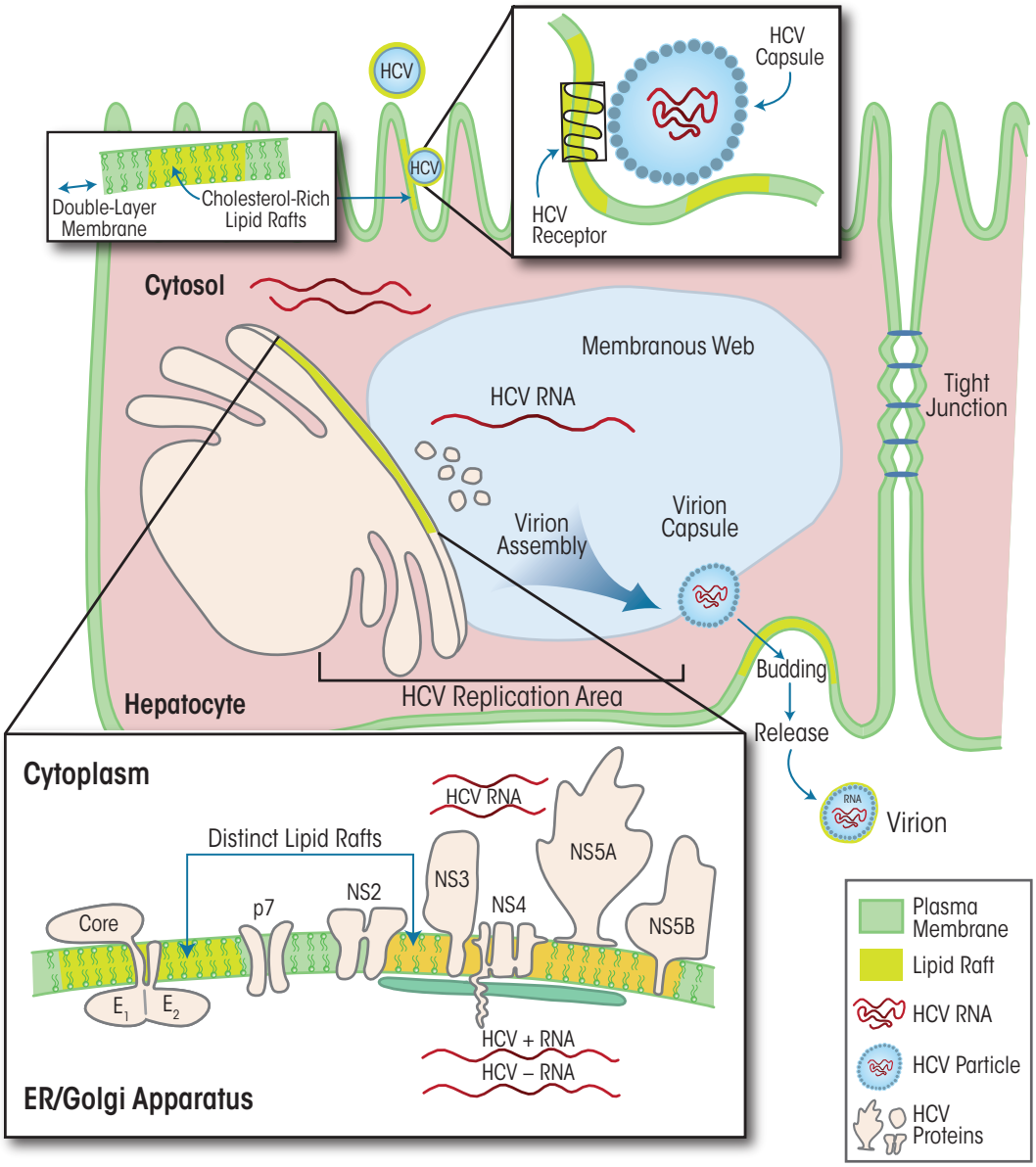

Figure 2 The life cycle of hepatitis C virus (HCV) and the role of lipid rafts. HCV particles attach to receptors in lipid-raft regions of the hepatocyte membrane, and the virus particles are taken up into the cell via endocytosis. The viral RNA is released and serves as template for the production of viral proteins at a structure called the membranous web, which also includes the membranes surrounding the endoplasmic reticulum (ER) and Golgi apparatus. The membranous web also is the site of assembly of new virus particles. During assembly and subsequent release of the viral particles, the particles obtain pieces of the cellular membrane as an outer envelope that shares the lipid composition of the membrane, particularly of the lipid rafts.

HBV RNA as well as increased expression of various viral proteins (i.e., surface and core proteins) and X antigens in the liver (Larkin et al. 2001).

HCV and Alcohol Abuse

The prevalence of chronic HCV infection is significantly elevated among people with alcohol use disorder (Fong et al 1994; Novo-Veleiro et al 2013) compared with the general population (prevalence of 1 to 2 percent) (CDC 2015*b*). Variables independently associated with HCV infection include female gender, injection drug use, and the presence of ALD (Novo-Voleiro et al. 2013). At the same time, patients with HCV infection have a higher prevalence of alcohol abuse and a longer duration of alcohol consumption compared with the general population (Degos 1999; Nevins et al. 1999; Pessione et al. 1998).

Chronic HCV infection results in the development of HCC in about 1 to 3 percent of patients after 30 years (Grebely and Dore 2011), contributing to the morbidity associated with HCV. The rate of HCC is substantially higher in people with HCV-related cirrhosis, reaching 2 to 4 percent per year in the United States, and even higher rates of up to 7 percent have been reported in Japan. Risk factors for the development of HCV-related HCC include male gender, age older than 55 years, and high levels of alcohol consumption (Grebely and Dore 2011; Hajarizadeh et al. 2013; Kim and Han 2012). Alcohol intake of 40 grams ethanol per day or more is associated with more rapid progression of liver disease associated with chronic HCV infection, including a more rapid increase in fibrosis and a doubled incidence of cirrhosis compared with patients with lower consumption levels (Wiley et al. 1998). Similarly, the risk of developing HCC is twice as high in patients with chronic HCV infection who drink heavily. Even small amounts of alcohol lead to an increased level of serum HCV RNA in patients with HCV infection (Cromie et al. 1996).

Alcohol, Cellular Membranes, and Lipid Rafts

Biological membranes surround the cells and create compartments within the cells, such as the endoplasmic reticulum and Golgi apparatus. Current models view cellular membranes as tri-dimensional lipid–protein complexes that are easily disturbed. Thus, even small stimuli, such as changes in pH, ion environment, or binding of a molecule to a protein receptor, can lead to profound changes in the composition, function, and integrity of the cellular membrane. Not surprisingly, therefore, alcohol also can alter the state of the cellular membranes and may thereby affect cellular function. At the same time, proteins embedded in the cellular membranes may serve as receptors and points of entry for viruses, such as HBV and HCV.

The specific structure and function of hepatocyte membranes contributes to the ability of hepatitis viruses to infect the cells. In contrast to nonparenchymal liver cells, hepatocytes are polarized cells—that is, they have two clearly defined ends (i.e., an apical and a basolateral side), which is reflected in the membrane structure. Thus, the apical and basolateral membranes each have characteristic components that cannot mix, partially because the two cellular domains are separated by structures called tight junctions that also ensure the connection between a hepatocyte and its neighboring cells. The composition of polarized membranes differs between both ends of the cell with respect to both protein composition and lipid repertoire. Additionally, the membranes of both polarized and nonpolarized cells can be divided into lipid rafts and non–lipid-raft domains. Lipid rafts are membrane sections ranging in size from 10 to 200 nm that are enriched in specific lipids (i.e., sterols, sphingolipids, or ceramide). The specific structure of these lipid rafts promotes protein–protein and protein–lipid interactions; in addition, many cellular processes occur in these membrane regions. In both hepatocytes and other cell types, the overall protein concentration in the lipid rafts is relatively low, although certain proteins are highly concentrated in these membrane sections (Prinetti et al. 2000). The association with a lipid raft can influence the function of a protein (Paik et al. 2003; Pike 2006; Sonnino and Prinetti 2013). For example, proteins within lipid rafts are less able to move to other membrane areas, which favors more stable interactions with other proteins in the same domain. Thus, the activation of a cellular protein that serves as a receptor in a lipid raft facilitates clustering of the receptor with its co-receptors. Because the outer envelope of animal viruses such as HBV and HCV is derived from the host membranes, the lipid composition of the viral envelope resembles that of the membrane from which the virus buds (Laine et al. 1972). The cellular lipids and lipid rafts obtained from the host often modulate the membrane fusion between virus and host cell that is mediated by viral proteins (Teissier and Pécheur 2007) and therefore could become important targets for efforts to disrupt the viral life cycle. In general, the viruses seem to attach primarily to membrane areas containing lipid rafts; it remains to be determined whether viruses gain infectivity advantage if they attach to lipid rafts located in the apical or basolateral domain of the cell (Lindenbach and Rice 2013).

Influence of Alcohol on Cellular Membranes and Lipid Rafts

The effect of alcohol exposure on cellular membranes, and lipid rafts in particular, depends on the cell type and its activity state as well as on the alcohol concentration and duration of exposure. It is important to remember, however, that alcohol's effects on the cellular membrane do not occur in isolation; rather, they are part of alcohol's global effects on the cell and on the tissue as a whole. In addition, liver-cell membranes may adapt to alcohol consumption (Rottenberg 1991), although it is difficult to determine which of those changes represent an adaptation and which represent pathological changes. Whether the adaptive changes of membrane

composition, structure, and function delay or accelerate the onset of the pathological changes in the liver of human alcoholics also still is unclear.

Alcohol's effects on cellular membranes can be indirect or direct (see figure 3). Indirect effects include, for example, the binding of acetaldehyde—which is a major metabolic product of ethanol and is found in high concentrations in the serum during alcohol abuse—to hepatocyte membranes. The acetaldehyde affects the structure of the cellular membrane, which leads to disruption of tight junctions, increased immune recognition of certain antigens, cell damage, DNA damage, and mutagenesis (Setshedi et al. 2010; Thiele et al. 2008). Alcohol's direct effects on the cellular membrane can be subdivided further into effects on the lipids and effects on the protein components. Of these, alcohol's effects on protein function probably have the greatest impact on both parenchymal and nonparenchymal liver cells. They occur during both the acute and the chronic phase of alcohol exposure and lead to significant functional impairment of the cells, which can cause cell death, tumorigenesis, altered intercellular communication, and increased susceptibility to additional insults, including viral infections. All of these can contribute to liver dysfunction. Studies have demonstrated that alcohol can impair the functions of proteins in cellular membranes and lipid rafts in liver cells in multiple ways, including actions on lipid-raft–associated signaling pathways (Dai and Pruett 2006; Dolganiuc et al. 2006). However, these studies have focused primarily on the outer cellular membrane and its lipid rafts; the effects of alcohol on intracellular lipid rafts (Chao 2012) remain to be characterized. Nevertheless, it is clear that as a result of the complex actions of alcohol on lipid-raft–associated signaling, the

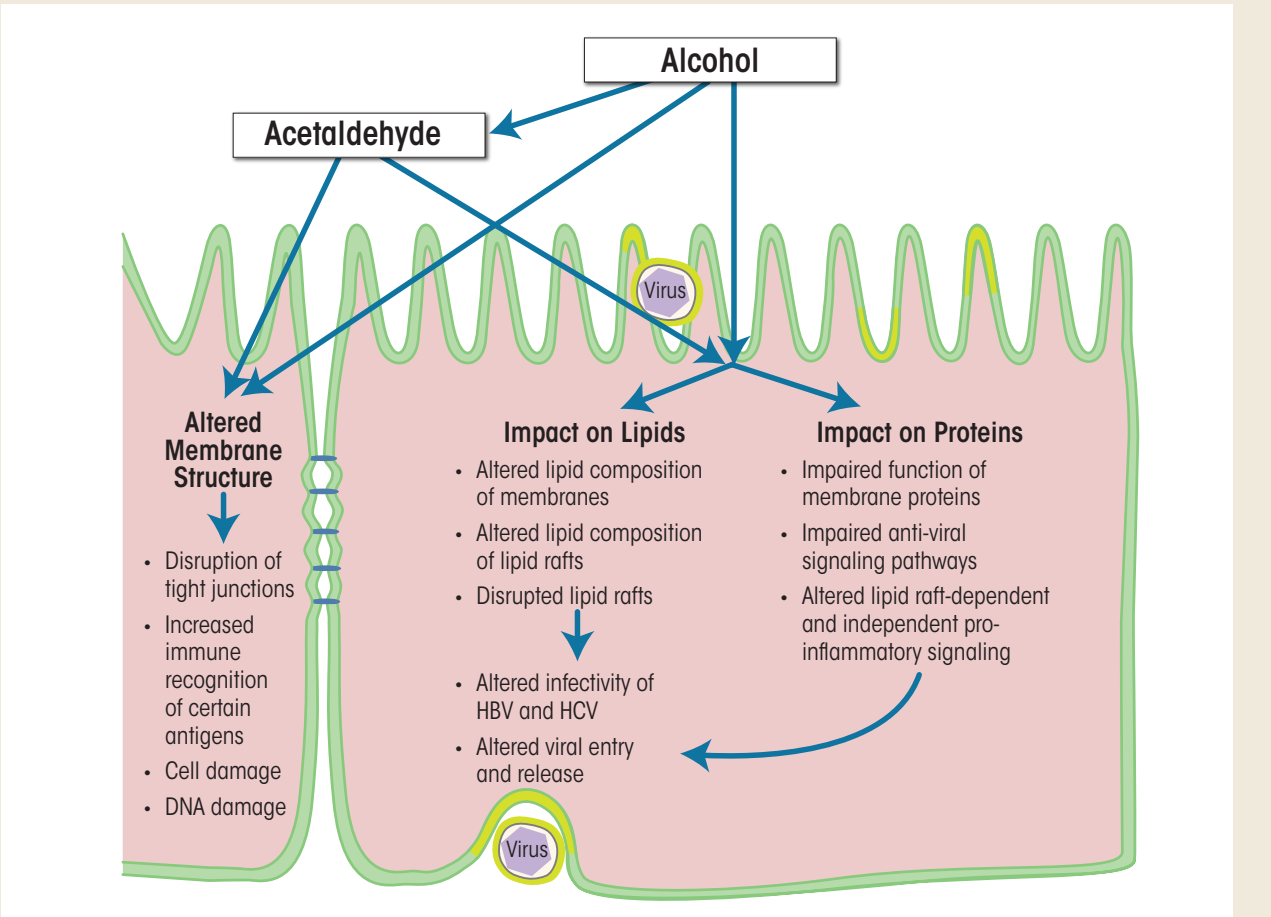

Figure 3 Alcohol's effects on virus-infected hepatocytes. Alcohol may exert its effects both directly and indirectly. Indirect effects are, for example, related to the actions of the alcohol metabolite, acetaldehyde. Alcohol can directly affect both lipids and proteins in the cell. Through a variety of mechanisms, these effects may alter the infectivity of and the cell's response to HBV and HCV, affecting both viral entry into the cells and release of viral particles from the cells.

liver cells are more likely to create a proinflammatory milieu and downregulate their antiviral defense mechanisms. For example, studies have detected interference with signal transduction systems (Aliche-Djoudi et al. 2011; Dolganiuc et al. 2006; Nourissat et al. 2008) as well as enhancement of oxidative stress (Nourissat et al. 2008). Additionally, the cells spend excessive resources on efforts aimed at maintaining cellular homeostasis (e.g., remodeling the cellular membrane or re-ordering metabolic priorities) and on mechanisms to counteract cell death (Dolganiuc et al. 2012; Donohue and Thomes 2014). More importantly, exposure to alcohol, especially prolonged exposure, increases the liver cells' vulnerability to second hits, including hepatitis viruses.

Effects of Alcohol Abuse and Hepatitis Virus Infection on Cellular Membranes

As described above, the cellular membranes and lipid rafts are important targets of alcohol's actions in the liver (Lieber 1980; Tsukamoto 1993) and are key in many aspects of alcohol-induced liver-cell dysfunction. Concurrent infection with HBV, HCV, and/or other viruses exacerbates alcohol's detrimental effects on liver function and leads to an accelerated course of liver disease (Ribes et al. 2006; Tsui et al. 2006). The mechanism underlying the synergistic effects of hepatitis viruses and alcohol, and particularly the role of cellular membranes and lipid rafts, are not yet fully understood.

For HBV, alcohol's effects on the membranes are relevant because the virus acquires its envelope from the membrane of the endoplasmic reticulum (Gerlich 2013). This envelope has a relatively high cholesterol content (Satoh et al. 2000), which is a key determinant of virus infectivity (Funk et al. 2007, 2008; Stoeckl et al. 2006). Thus, interference with the cellular membrane and lipid rafts during the viral life cycle, whether it is at the level of the host hepatocyte or cholesterol depletion from the virus membrane, has detrimental effects on the virus. Specifically, cholesterol-poor HBV virions take longer time to attach to, enter, and migrate inside the hepatocytes and are more likely to be cleared from the cells once they do enter (Funk et al. 2008). Alcohol exposure can distinctly alter the lipid composition of cellular membranes in general and lipid rafts in particular (Dolganiuc et al. 2006) and may thereby influence HBV infectivity. However, the precise effect of alcohol on the various steps of the HBV lifecycle remains largely unknown.

In addition to directly affecting both the virus and host parenchymal liver cells, alcohol influences anti-HBV immunity—an effect that also involves the cellular membrane as well as lipid rafts. HBV is known to interfere with normal T-cell function, and specifically with the T-cell receptor (TCR) that is responsible for recognizing and interacting with foreign antigens, thereby initiating an immune response. Thus, during HBV infection, the virus can impair the translocation of various components of the TCR (e.g., CD3f, ZAP-70, and Grb2) to lipid rafts; this is a hallmark of defective adaptive immune responses during chronic HBV infection (Barboza 2013; Larkin et al. 2001). Similarly, lipid-raft–dependent TCR localization and function are altered when adaptive immune cells are exposed to alcohol (Ishikawa et al. 2011). In particular, ethanol inhibits lipid-raft–mediated TCR signaling in CD4 T cells, resulting in suppression of IL-2 production (Ghare et al. 2011). Thus, alcohol acts synergistically with HBV to limit antiviral immunity. The consequences of alcohol's effects on the TCR of HBV- and HCV-infected individuals are largely unknown but remain of high interest because adaptive immunity plays an important role in viral clearance (Barve et al. 2002; Heim and Thimme 2014; Loggi et al. 2014).

Compared with HBV, the life cycle of HCV depends on cellular membranes and lipid rafts to an even greater extent. HCV attaches to the cellular membrane and binds to a variety of cellular receptors that also serve as signaling molecules or receptors for other host proteins; most of these receptors reside in lipid rafts or are recruited there upon viral sensing and signaling. For example, one study found that compared with control cells, lipid rafts of cells expressing an HCV-1b genome showed altered levels of 39 proteins, including new or increased expression of 30 proteins and decreased expression of 9 proteins (Mannova and Beretta 2005). These alterations also affect a signaling pathway called the N-ras–PI3K–Akt–mTOR pathway (Peres et al. 2003; Zhuang et al. 2002); modulation of this pathway is one of the strategies by which HCV inhibits apoptosis and prevents elimination of infected cells. Alcohol can target these signaling platforms and may exert detrimental effects on lipid rafts that contain several putative HCV receptors, which may affect HCV replication and survival of HCV-infected cells. Thus, alcohol has been shown to affect the PI3K–mTOR pathway in non-liver cells (Li and Ren 2007; Umoh et al. 2014). However, the effect of alcohol on the PI3K–mTOR pathway in parenchymal and nonparenchymal liver cells remains to be determined.

Alcohol also adversely affects many of the immune cells and pathways that are considered key to antiviral immunity to HCV. Thus, alcohol exposure enhances signaling via TLRs that mediate inflammation and impairs signaling via TLRs that mediate production of antiviral molecules, including interferons. Of note, some of the same pathways are targeted in similar ways by HCV, thus producing synergistic effects that promote inflammatory reactions and support the viral lifecycle in both parenchymal and nonparenchymal liver cells (John and Gaudieri 2014; Koziel 2005; Pang et al. 2011; Szabo et al. 2010).

Conclusions

Alcohol exposure and hepatitis viruses exploit common mechanisms to promote liver disease. Some of these mechanisms focus on the cellular membrane and its most active domains, the lipid rafts, which play critical roles in sustaining the lifecycle of both HBV and HCV. For HBV, the cellular

membranes and lipid rafts are particularly involved in viral entry; for HCV, lipid rafts additionally are required for formation and/or maintenance of HCV viral replication, virion assembly, and virion release from the host cell. Lipid rafts additionally influence viral survival indirectly because they serve as signaling platforms for a proinflammatory signaling cascade as well as for antiviral pathways, and they help regulate intracellular lipid storage within the parenchymal liver cells. Moreover, cellular membranes and lipid rafts play a crucial role in the immune-mediated cell defense in nonparenchymal liver cells. Alcohol affects membrane and lipid-raft function both directly and indirectly by modulating the proinflammatory cascade as well as antiviral pathways and intracellular lipid storage within the parenchymal liver cells and hampering the function of nonparenchymal liver cells through both lipid-raft–dependent and –independent mechanisms. The synergistic effects of hepatitis viruses and alcohol on the cellular membranes lead to impaired antiviral immunity and a proinflammatory milieu in the liver, thereby helping to sustain the viral lifecycle and promoting rapid progression and a more severe course of liver disease.

A better understanding of lipid-raft function may contribute to new approaches to treatment of viral and alcohol-related hepatitis, but knowledge of the structure and function of these cell structures is only beginning to emerge. For example, lipid-raft formation still is an enigma, and researchers are only now starting to investigate and understand the processes underlying lipid-raft activation, protein–lipid interactions, lipid-raft–dependent signaling, and other mechanisms through which lipid rafts direct the bioactivity of the various membrane constituents. Eventually, however, better understanding of cellular membranes and lipid rafts and their involvement in health and disease may lead to novel treatment approaches, including cellular- and subcellular-based personalized medicine approaches that also may lead to improved outcomes for patients with viral and/or alcohol-related hepatitis.

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Alcohol and HIV Effects on the Immune System

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HIV disease and alcohol independently influence the human immune system, so it stands to reason that, together, their influence may be additive. Here, we review the evidence that alcohol can exacerbate HIV's influence on the immune system, thereby affecting disease progression and transmission. We focus particularly on alcohol's effect on the mucosal immune system in the tissues of the gastrointestinal tract, the genital tract and the lungs, all of which play a role in transmission and progression of HIV disease.

Key words: Alcohol consumption; alcohol use disorder; immune system; immune function; HIV; HIV infection; acquired immunodeficiency syndrome (AIDS); immune response; mucosal immune system; gastrointestinal tract; genital tract; lungs; CD4+ T cells; antiretroviral therapy

Alcohol use disorder (AUD) and HIV infection both affect the immune system and frequently coexist in the same person, potentially multiplying the risk of infectious disease. Infectious disease, in turn, continues to be a major health concern and leading cause of morbidity and mortality worldwide, despite major advances in our understanding of the immune system, improvements in sanitation practices, and use of antibiotics, vaccines, and antiviral drugs.

Infection with HIV is particularly troublesome for the immune system because it infects and destroys immune system cells called T helper lymphocytes or $CD4^+$ T cells. Untreated, the disease progresses over a few years to AIDS, leading to eventual death for most people. The disease is transmitted from infected to uninfected people through biological fluids containing the virus, most commonly through sexual contact but also through contaminated needles and other means. Since its discovery in the early 1980s, HIV infection has become a pandemic, causing an estimated 36 million deaths. The World Health Organization estimates that in 2012, of the 35 million people living with HIV/AIDS (PLWHA), 2.3 million were newly infected and 1.6 million died of AIDS-related causes despite increased availability of effective antiretroviral therapy (ART) (Joint United Nations Programme on HIV/AIDS 2013).

AUD in the form of alcohol abuse and alcohol addiction are the most common and costly form of substance abuse in the United States and represent a global health problem. For PLWHA, rates of heavy drinking are even higher than those in the general population (Galvan et al. 2002). One study found that 82% of HIV-infected patients consumed alcohol, and half were classified as hazardous drinkers (Lefevre et al. 1995). Because AUD and HIV infection frequently coexist, studies have tried to understand the influence of alcohol consumption on the transmission and progression of HIV disease. For one, heavy alcohol consumption increases the risk of HIV transmission through its propensity to increase the likelihood of risky sexual behavior, including unprotected sex with multiple partners (Rehm et al. 2012; Shuper et al. 2009; Stall et al. 1986). However, as detailed at length in this issue, AUD also may affect innate immune defenses and adaptive immune responses and thereby potentially increase the likelihood of HIV transmission over and above alcohol's known behavioral associations with infection risk. Once infected, studies find that PLWHA with AUD perform poorly at multiple levels of the HIV treatment cascade, including adherence to ART, resulting in a higher likelihood of virologic nonsuppression (Azar et al. 2010; Chander et al. 2006; Palepu et al. 2003). Large observational studies show that hazardous alcohol consumption decreases overall survival in PLWHA in what seems to be a dose-dependent manner (Braithwaite et al. 2007). In 2010, this journal devoted an issue to the many consequences of alcohol consumption on HIV transmission with an emphasis on prevention, HIV disease pathogenesis, progression and treatment, and the impact of alcohol–HIV comorbidity on the lung, liver, heart, and brain (Bryant et al. 2010).

Here, we focus on the impact of alcohol on the host defense response to HIV infection. In particular, we review the evidence that alcohol exacerbates HIV's influence on

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the immune system and affects disease progression and transmission. In particular, we discuss alcohol's effect on the mucosal immune system in the tissues of the gastrointestinal tract, the genital tract, and the lungs, all of which play a role in transmission and progression of HIV disease.

Does Alcohol Interact With HIV?

Alcohol's effect on the immune system already is complex, but it is made even more complex in the context of HIV disease. Alcohol either can be immunosuppressive or immune activating for the cells of the innate and adaptive immune systems (Molina et al. 2010; Szabo et al. 2009). Intoxicating doses of alcohol generally are immunosuppressive, which can lead to an increase in the incidence and severity of infections, especially pneumonia (Szabo et al. 2009; Zhang et al. 2008), and could result in increased secondary infections in HIV-positive patients. In contrast, chronic alcohol consumption can be immune activating, causing chronic inflammation and oxidative stress resulting in conditions such as alcoholic liver disease, acute respiratory distress syndrome, and muscle wasting.

Studies that directly examine the interaction between alcohol and HIV disease related to the immune system have found mixed results (Bagby et al. 1998; Hahn et al. 2010). Early studies by Basgasra and colleagues (1993) administered alcohol to people uninfected with HIV then isolated peripheral blood mononuclear cells from their blood and infected the cells with HIV. The alcohol increased HIV replication. In similar experiments, Cook and colleagues (1997) also observed this response but only in lymphocytes from a subset of individuals. Other early studies suggested that AUD accelerated disease progression. For example, Fong and colleagues (1994) reported that a user who heavily abuses alcohol rapidly progressed to AIDS shortly after seroconversion. That said, evidence of alcohol's influence on blood $CD4^+$ T cell counts in HIV-positive patients is mixed. For example, Pol and colleagues (1996) observed improved $CD4^+$ cell counts after HIV-positive alcoholic patients who were not receiving ART stopped drinking. However, a recent study did not see an association between alcohol consumption and a change in $CD4^+$ cell counts over time in the absence or presence of ART (Conen et al. 2013). And in a cross-sectional study of 325 participants, daily alcohol use did not statistically correlate with lower $CD4^+$ cell counts among patients taking or not taking ART (Wu et al. 2011). In contrast, Baum and colleagues (2010) found that frequent alcohol users treated with ART were more likely to show a decline in $CD4^+$ cell counts independent of baseline $CD4^+$ cell count or HIV load. Likewise, this study revealed an increased likelihood of declining $CD4^+$ cell counts in patients not on ART. Samet and colleagues (2007) also observed lower $CD4^+$ cell counts in HIV patients not on ART, but heavy alcohol consumption by subjects on ART was not associated with lower $CD4^+$ cell counts. A French study (Carrieri et al. 2014) following HIV infected subjects with access to ART found that low alcohol consumption, defined as less than 10 grams per day, was associated with higher $CD4^+$ cell counts when compared with abstainers. In addition, participants in this study who were categorized as moderate alcohol consumers had $CD4^+$ cell counts similar to abstainers. These investigators suggested that low alcohol consumption might be a proxy to healthier behaviors encompassing things like diet and exercise.

Reports on the effect of alcohol on viral load in patients on antiretroviral therapy also vary among studies. One study (Wu et al. 2011) found higher viral loads in HIV patients on ART who consume alcohol, whereas another study (Samet et al. 2007) did not. Patients who consume alcohol may have lower adherence to ART, resulting in higher levels of viremia (Baum et al. 2010). These higher viral loads, in turn, make patients more infectious during unprotected sex with uninfected partners, which becomes more likely when patients drink (Kalichman et al. 2013).

Epidemiological studies conducted prior to the use of ART failed to identify an effect of alcohol abuse on HIV disease progression (Coates et al. 1990; Kaslow et al. 1989). However, these studies faced many methodological obstacles, including difficulties obtaining accurate measures of alcohol use patterns and problems controlling for confounding factors such as variations in the HIV strain, patient age, demographics, ethnicity, time since infection, comorbidities, medication side effects, and a host of environmental influences. Researchers have gotten around these methodological issues by creating an animal model of HIV disease using simian immunodeficiency virus (SIV) in rhesus macaque monkeys (see textbox, "An Animal Model for Conducting Controlled Studies").

Alcohol, HIV, and the Mucosal Immune System

Many of the SIV studies examining the connection between alcohol, HIV, and the immune system have focused on the mucosal immune system, consisting of cells that reside in the tissues lining various parts of the body, particularly the gastrointestinal tract, genital tissue, and the lung. These areas represent a critical if not primary site of HIV infection. Further, converging evidence suggests that AUD influences mucosal tissue in a way that potentially increases HIV disease transmission and adversely affects disease pathogenesis, resulting in accelerated disease progression.

The Gastrointestinal Mucosal Immune System

Pathogenesis of Alcohol and HIV Infection

Both alcohol and HIV infection have profound and often overlapping adverse effects on the integrity and immunology of the gastrointestinal tract. It is now known that the intestinal tract plays a major role in the pathogenesis of HIV

An Animal Model for Conducting Controlled Studies

To conduct more controlled studies of alcohol's biological effects on HIV disease, researchers have turned to studies in rhesus macaque monkeys infected with simian immunodeficiency virus (SIV), a credible animal model of HIV disease (Bagby et al. 1998). Discovered in the 1980s, SIV is structurally, biologically, and genetically related to HIV. Like HIV, it enters host cells through $CD4^+$ surface proteins and causes $CD4^+$ T cell depletion, resulting in immunodeficiency that progresses to an AIDS-like state with opportunistic infections, muscle wasting, and neurological problems. This model provides many experimental advantages to studies in humans. It allows researchers to:

- Control the timing of infection, the route of infection, the dose of SIV, and the strain of SIV;
- Control the dose and timing of alcohol delivery prior to and during infection;
- Control the experimental variables such as nutrition, drug delivery, and behavior;
- Perform longitudinal studies from the earliest events through end-stage disease as a result of shortened duration relative to the clinical course in people living with HIV/AIDS (PLWHA) (median survival is less than 1 year with pathogenic strains of SIV); and
- Perform studies in either the absence or presence of antiretroviral therapy (ART).

With regard to alcohol delivery, in our alcohol–SIV model, we provide rhesus macaques (Macaca mulatta) with a nutritionally balanced primate chow supplemented with fruit and ad libitum water. Starting 3 months prior to inoculation with SIV and continuing for the duration of the study, the macaques receive either ethanol or sucrose via a gastric catheter (Bagby et al. 2003). This alcohol delivery approach is chosen to ensure each animal receives similar alcohol doses and achieves similar circulating alcohol concentrations. We use two alcohol delivery protocols to simulate chronic binge alcohol (CBA) consumption: 5-hour infusion sessions 4 days per week, or half-hour infusion sessions 7 days per week. Individual doses are adjusted to achieve an intoxicating plasma alcohol concentration of 0.23 to 0.27 percent measured 2 hours after initiating alcohol delivery in the daily protocol and 5 hours after initiating alcohol delivery in the 4-day-per-week protocol. These protocols model heavy chronic binge alcohol consumption, which is the most common pattern of excessive alcohol use in the United States (Centers for Disease Control and Prevention 2012). With both protocols, animals received 13 to 14 g of alcohol per kilogram per week. Animals are inoculated with SIV either intravenously or intrarectally. As a rule, SIV infection leads to a peak viral load after about 2 weeks, which then decreases over the next few weeks to a steady-state termed viral "set point." A high viral set point indicates that an animal will progress more rapidly to end-stage disease compared with animals with low viral set points (Staprans et al. 1999).

Overall, studies of this kind find that CBA increases virus production in tissue and plasma of SIV-infected animals (Bagby et al. 2006; Kumar et al. 2005; Nelson et al. 2013; Poonia et al. 2005). In two separate studies (Bagby et al. 2006; Nelson et al. 2013), CBA/SIV infected rhesus macaques had higher viral set points than sucrose controls, and progressed faster to end-stage disease with a median survival time of 374 versus 900 days, respectively (Bagby et al. 2006). As expected, blood $CD4^+$ lymphocyte numbers decreased during SIV infection but did not differ between CBA and sucrose-treated animals. Another study, however, found a positive correlation between plasma viral load and blood $CD4^+$ lymphocytes (mostly naïve T cells) in CBA but not sucrose-treated animals (Pahar et al. 2013).

These studies allow us to ask specific questions about the connection between alcohol, HIV disease, and the immune system and how those connections may influence the transmission and progression of HIV disease.

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infection throughout all stages of infection, and increasing evidence suggests alcohol exacerbates many of these effects. Because examining intestinal immune responses in humans is difficult, determining the biologic effects of alcohol and HIV infection requires carefully controlled studies in relevant animal models.

Early studies in the SIV model demonstrated that, regardless of the route of infection, the intestinal tract is the primary target for SIV replication, amplification, and marked $CD4^+$ T cell depletion in the earliest stages of infection (Veazey et al. 1998). These studies have been confirmed in HIV-infected humans (Brenchley et al. 2004; Mehandru et al. 2004), and the role of the gastrointestinal tract has since been a major focus of HIV research (Sandler et al. 2012). Indeed, research shows that, within the first few days of HIV infection, the virus rapidly infects and eliminates most of the $CD4^+$ T cells in the intestinal tract (Sandler et al. 2012). Because the gastrointestinal tract contains most of the lymphocytes in the body, this represents a profound loss of total $CD4^+$ T cells in the body (Douek 2007; Picker 2006). To best understand the interaction of alcohol and HIV on the intestinal immune system, we must first briefly explain the structure and compartmentalization of immune tissues in the gut. A more in-depth description of the gastrointestinal tract and a review of its immune system, also known as the gut-associated lymphoid tissue (GALT), is described by Hammer and colleagues, in this issue.

GALT comprises both specialized regions of lymphoid tissues, called Peyer's patches, and more diffuse lymphoid tissues scattered throughout the layer of the intestinal wall called the lamina propria. The specialized regions provide immune surveillance for the intestines and tend to initiate the gastrointestinal immune response. There is an even larger pool of differentiated T cells, called $CD4^+CCR5^+$ T cells, and plasma cells diffusely scattered throughout the intestine's lamina propria that serve as the "effector" arm of the intestinal immune system, actively battling antigens first encountered by the Peyer's patches (Mowat et al. 1997).

HIV/SIV infection targets and eliminates these activated effector $CD4^+CCR5^+$ T cells, which are crucial for providing help for essentially all major innate and adaptive immune responses in the gut including cytotoxic functions, cytokine regulation, and controlling the production and secretion of immunoglobulins, particularly IgA (Xu et al. 2013). The viruses also selectively and rapidly infect and eliminate key subsets of $CD4^+$ T cells that recent research shows are critical for the gastrointestinal immune response. Specifically, HIV and SIV target $CD4^+$ T cells that produce interleukin (IL)-17 (Th17) or IL-22 (Th22) (Brenchley et al. 2008; Cecchinato et al. 2010), which seem to be crucial for regulating innate immune responses, especially the development and maintenance of the GALT (Ouyang et al. 2008; Paiardini 2010). These findings suggest that selective targeting and elimination of key subsets of $CD4^+$ T cells in the intestine by HIV/SIV can have a profound influence on intestinal barrier function and adverse effects on both T- and B-cell responses in the gut (Klatt et al. 2010). Because alcohol also disrupts intestinal barriers and reduces Th17 cells (Asquith et al. 2014), it is likely that alcohol use in HIV patients results in even greater levels of intestinal barrier damage, leading to the leakage of intestinal microbes outside of the intestines—what is called "microbial translocation"—which can lead to inflammation and infections.

Microbial Translocation in Alcohol and HIV

Both alcohol consumption and HIV/SIV infection seem to disrupt the intestinal lining, disrupt intestinal barrier function, and lead to microbial translocation. Indeed, both short- and long-term alcohol consumption can cause intestinal barrier permeability and movement of luminal bacterial antigens into systemic circulation through the blood, resulting in chronic inflammation and eventually liver damage (Elamin et al. 2013; Parlesak et al. 2000). Research also finds microbial translocation in the blood of HIV patients and SIV-infected macaques in the absence of alcohol as evidenced by the presence of lipopolysaccharide (LPS) endotoxins (Brenchley et al. 2006*b*; Klatt et al. 2010; Sandler et al. 2012). In fact, bacteria leaking through damaged intestinal barriers as a result of HIV-induced destruction of the key regulatory $CD4^+$ T cells seems to be responsible for the systemic immune activation that is a hallmark of HIV infection (Brenchley et al. 2006*b*; Klatt et al. 2010; Sandler et al. 2012) and is a better predictor of HIV disease than plasma viral loads (Deeks et al. 2004). Indeed, levels of systemic activation appear to play a major role in the susceptibility to infection (Giorgi et al. 2002; Klatt et al. 2010, 2013) and disease progression (Deeks et al. 2004; Klatt et al. 2010).

Together, these findings suggest that the adverse effects of alcohol and HIV infection on microbial translocation may be additive. In addition, the inflammation and epithelial cell barrier damage that alcohol and its byproducts inflict on the intestinal mucosa (Elamin et al. 2013) may result in greater levels of mucosal and systemic immune activation, rendering patients more vulnerable to HIV transmission.

Microbial Flora and Innate Mucosal Immunity in HIV and Alcohol Abuse

It is increasingly clear that the intestinal microflora play a role in the health of the gastrointestinal immune system. Research clearly shows that alcohol use alters the normal intestinal microflora, leading to intestinal damage and increased levels of LPS leaking into the blood where it circulates throughout the body (Elamin et al. 2013). Together, this suggests that alcohol use in HIV patients may influence the rate of intestinal T cell turnover and innate mucosal immune responses.

As with microbial translocation, the combination of HIV and alcohol may have an additive effect on the intestinal microbiome. HIV infection alone is associated with changes in the intestinal microbiome of infected patients, and those

changes may be linked to HIV-induced alterations in the mucosal immune system (McHardy et al. 2013; Saxena et al. 2012). Although less studied, the combined effects of alcohol use and HIV infection are likely to induce marked changes in the intestinal microflora, and the innate immune responses in the gut, which should be a focus of future studies.

Intestinal T Cell Turnover in HIV Infection

As suggested in the preceding sections, SIV and HIV infection lead to chronic immune activation, which creates a pro-inflammatory "cytokine storm." This storm recruits and activates additional $CD4^+$ T cells into mucosal tissues, providing the virus with a continuous supply of activated memory $CD4^+$ T cells to infect (Mogensen et al. 2010; Wang et al. 2013). Continued activation, recruitment, and turnover of these viral target cells in damaged mucosal tissues may provide the "fuel" for continued viral replication and persistence in the gut. Indeed, it is increasingly clear that the intestinal tract is one of the major reservoirs for viral persistence of SIV in macaques (Ling et al. 2010) and of HIV in humans, even those on antiretroviral therapy (Avettand-Fenoel et al. 2011; Poles et al. 2006). One study found that alcohol increases the percentages of memory $CD4^+$ T cells in the gut, suggesting that alcohol use may increase turnover of viral target cells in intestinal tissues (Poonia et al. 2006). Although the mechanisms behind this still are under investigation, it is possible that repetitive damage to the intestinal epithelium by alcohol may simply result in chronic inflammation, which recruits these T cells to the intestine through the above pathways. Thus, alcohol use in HIV infection may result in increased turnover of viral target cells in the intestine, which may partially explain why macaques infected with SIV have significantly higher levels of viremia in primary SIV infection (Bagby et al. 2006; Poonia et al. 2005).

Although more studies are needed to define the mechanisms involved, it is increasingly clear that HIV and alcohol use may have synergistic pathology, resulting in greater rates of disease progression in HIV patients, fueled by the loss of intestinal mucosal cells and chronic immune activation due to microbial translocation. Because many of these same processes may also occur in rectal and genital mucosal tissues, HIV and alcohol use may interact similarly to increase susceptibility to HIV infection and early replication following sexual transmission, a proposition we examine below.

Alcohol and HIV Transmission via the Genital Tract

While HIV can be transmitted several ways, the primary method of transmission is through sexual contact. As such, it is critical to understand how AUD affects virus levels in genital fluids and the mucosal environment of the genital tract and rectum and how that may alter innate and adaptive immune responses and facilitate HIV transmission as well as susceptibility to infection. HIV levels present in the inoculating fluid—typically seminal or vaginal fluids—is a key factor in transmission of the virus from an infected individual to a noninfected host and typically is associated with HIV levels in the blood (Cohen et al. 2011). Because chronic alcohol abuse has been associated with increased plasma viral loads and more rapid disease progression (Baum et al. 2010; Rompalo et al. 1999), this population presents an increased risk for transmission. In female subjects, studies also have linked recent alcohol use with higher levels of virus in genital fluids when controlling for plasma HIV loads (Homans et al. 2012; Theall et al. 2008). These observations suggest that higher levels of HIV replication may occur in genital tissues of women that use alcohol, further increasing the potential for transmission. Similar studies in men have not been done.

Along with possibly promoting viral transmission, studies indicate that alcohol use by people who are uninfected may make them more susceptible to infection. In sexual transmission, HIV is acquired across the penile, vaginal, cervical, or rectal mucosa, and the integrity of the epithelial barrier and the innate defenses within these microenvironments provide the critical first lines of defense against HIV. The risk of HIV infection increases with increased inflammation within these local mucosal environments, likely as a result of the influx of HIV-susceptible cells and a potential breach in the epithelial barrier (Cohen 2004). Although no studies have examined in humans whether alcohol abuse can increase biological susceptibility to HIV infection, we recently found that chronic binge alcohol exposure that included intoxication at the time of exposure increased susceptibility to SIV infection (Amedee et al. 2014).

Studies have not directly evaluated the effects of chronic alcohol abuse on the genital mucosal environments. However, as detailed above, alcohol increases inflammation and the density of target $CD4^+$ T cells in the upper gastrointestinal tract, making it feasible that it causes similar changes in barrier integrity and lymphoid cell levels in the lower areas of the gastrointestinal tract, which could facilitate HIV entry across the rectal mucosa. Chronic alcohol use may similarly affect the genital mucosa of the penis, vagina, and cervix through changes in target cell distribution and alterations of the innate defenses within the microenvironment. The early virus–host interactions following sexual exposure to HIV have been characterized most extensively in models of the female genital tract, and these studies describe rapid diffusion and penetration of the virus through the vaginal epithelium, followed by the formation of an initial focus of infected cells in submucosal tissues (Carias et al. 2013). These virus-infected cells move to draining lymph nodes, allowing the virus to spread systemically through the body (Li et al. 2009). Research has identified several innate factors in the vaginal compartment as critical first lines of defense in limiting HIV infection, including epithelial barrier integrity and antiviral proteins in vaginal fluids (Anderson et al. 2014; Cole 2006). In addition, the microbial flora of the vagina can directly influence these innate defenses by altering the function and integrity of the epithelial barrier

and by influencing the inflammatory state of the compartment (Mirmonsef et al. 2012; Rose et al. 2012). Bacterial vaginosis, an infection characterized by an imbalance in the normal bacterial balance in the vagina and the absence of lactobacillus species in vaginal flora, has previously been associated with increased risk of HIV acquisition (Mirmonsef et al. 2012). In one study, women who reported alcohol use were less likely to have lactobacillus species present in their vaginal flora, leading to a flora consistent with that of bacterial vaginosis (Baeten et al. 2009).

Clearly, researchers need to conduct more directed studies to further define the specific alcohol-induced changes that alter innate defenses of the mucosal environments and that lead to an increased risk for HIV infection. As the numbers of HIV-infected persons continues to rise, it will become increasingly important to understand how chronic alcohol abuse affects genital virus expression and thus the risk of transmission. Additionally, the effects of alcohol on the genital microenvironment are important considerations in the development of pre-exposure prophylactic approaches. Alcohol-induced changes in microbial flora of the gut and vaginal compartments as well as changes to the epithelial barrier and innate defenses may alter the efficacy of antiviral approaches.

Alcohol, HIV, and the Lung

The overlap between HIV and alcohol continues in another mucosal tissue—the lung. Both HIV and alcohol increase susceptibility to opportunistic infections, in particular, infection with bacteria that cause pneumonia. It is well established in humans and animal models that the immunosuppression caused by HIV infection frequently leads to pulmonary infections and that alcohol abuse impairs lung host defenses, resulting in a higher incidence and severity of

Alcohol's Influence on Wasting Disease

Loss of muscle mass and what is called "wasting disease" is one hallmark of HIV in humans and SIV in rhesus macaques. Several studies suggest that chronic binge alcohol consumption (CBA) exacerbates this connection between muscle mass and SIV in part by triggering immune-related molecules. Although CBA- and sucrose-treated animals continued to gain weight and had comparable body weights during the first 300 days after SIV infection (Bagby et al. 2006), a closer evaluation of body composition revealed subtle but significant differences (Molina et al. 2006). For one, weight loss was a more common reason for euthanasia in alcohol-treated compared with sucrose-administered animals (Bagby et al. 2006; Molina et al. 2008), and muscle wasting was more pronounced in alcohol-consuming animals (Molina et al. 2008).

Chronic excessive alcohol consumption, even in the absence of SIV or HIV infection, alters the nutritional state, micronutrient availability, and tissue growth factor expression and activity (Molina et al. 2014). Indeed, approximately 50 percent of alcoholics show signs of alcoholic myopathy resulting from decreased muscle protein synthesis and accelerated breakdown of muscle proteins (Lang et al. 1999; Pacy et al. 1991; Preedy et al. 1994; Reilly et al. 1997; Teschner et al. 1988). Studies in SIV-infected rhesus macaques exposed to alcohol show that alcohol-mediated effects on muscle wasting are multifactorial, including decreased total caloric intake, altered nutrient selection, decreased nitrogen intake, and localized skeletal muscle inflammation and oxidative stress, which lead to a greater decrease in lean body mass and increase the incidence of AIDS (Molina et al. 2006, 2008).

Research in CBA/SIV macaques has begun to tease apart what is happening at the molecular and genetic level to breakdown the proteins that make up muscles and thereby decrease body mass. Simply put, within the skeletal muscle of CBA/SIV macaques there exists a molecular milieu that promotes the breakdown and inhibits the construction of new muscle.

The promotion of muscle breakdown seems to be regulated by the "ubiquitin-proteasome system (UPS)," which turns on any time muscles begin breaking down proteins because of conditions such as infections, burn injuries, fasting, and cancer (Fang et al. 1995; Hasselgren 1999; Llovera et al. 1994). The UPS is controlled by various molecules of the immune system, including glucocorticoids, catecholamines, and proinflammatory cytokines (Costelli et al. 1995; Price et al. 1994; Tiao et al. 1996). The genes encoding this system are stimulated in the skeletal muscle of emaciated AIDS patients (Llovera et al. 1998). In addition, studies in terminal stage SIV-infected rhesus macaques have shown a number of molecular markers related to muscle degradation, including significant increases in mRNA levels related to UPS, suppression of molecules such as insulin that promote muscle growth and increases in immune system molecules that promote inflammation (Molina et al. 2006, 2008). Similar imbalances in growth factors and pro-inflammatory molecules have been associated with wasting in AIDS patients (Franch et al. 2005; Nguyen et al. 1998).

pneumonia (Szabo et al. 2009; Young et al. 1989; Zhang et al. 2008), as detailed more specifically by Simet and Sisson, in this issue.

There are no reported clinical studies on the effect of AUD on the incidence and severity of opportunistic infections in PLWHA. And our longitudinal study of SIV infection to end-stage disease did not find more frequent secondary infections in alcohol-treated animals, nor were these infections, including pneumonias, a more likely underlying cause of end-stage disease in alcohol-treated animals (Bagby et al. 2006). This lack of a connection between alcohol and increased rate of lung infection may be attributed to the highly controlled biosafety practices used for these studies. Therefore, we set out to more fully address the influence of lung infection on host defense and SIV infection by measuring plasma and lung viral load in animals infected with pneumococcal bacteria load (Nelson et al. 2013). We chose pneumococcal infection because bacterial pneumonias are the most prevalent pulmonary complication in PLWHA (Wallace et al. 1993), and alcohol consumption is a well-known risk factor for bacteremic pneumococcal pneumonia in non–HIV- and HIV-infected populations (Nuorti et al. 2000).

We injected a sublethal dose of S. pneumoniae into a segment of the right lung of SIV-infected macaques. Twenty-four hours later, both alcohol-treated and sucrose control animals showed higher viral loads in bronchoalveolar lavage (BAL) fluid, which remained increased for at least 14 days in chronic alcohol-treated macaques. That said, we did not see differences between the alcohol-treated and sucrose control animals in plasma viral load, disease progression, or cytokine and neutrophil recruitment response. However, the early increase in local (lung) viral replication coincided with the host proinflammatory response that included nuclear factor (NF)-kB activation

Alcohol's Influence on Wasting Disease (*continued*)

Studies in CBA/SIV macaques further confirmed the involvement of the UPS in muscle wasting, demonstrating that CBA increased molecules that hinder muscle growth and increased UPS activity in skeletal muscle in SIV-infected animals in the late stages of the disease. Moreover, those studies provided evidence of significant localized inflammatory milieu reflected in increased tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β (LeCapitaine et al. 2011). Together, these findings suggest that inside the skeletal muscles of CBA/SIV macaques there exists a molecular milieu that favors the breakdown of muscle protein and suppresses its creation, while promoting a local proinflammatory state that compromises muscle health, leading to wasting.

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in recovered alveolar macrophages. The mechanism responsible for sustained increase in BAL fluid SIV RNA remains to be elucidated. Because macrophage NF-kB activation was limited to 1 day postinfection, it is possible that transient activation in alveolar macrophages along with the continued alcohol delivery is sufficient to sustain viral replication by these long-lived viral reservoir cells (Blankson et al. 2002). Alternatively, it is plausible that increased viral replication in alcohol-treated macaques occurs in other $CD4^+$ cells in the lung or that NF-kB–independent mechanisms may be operant in pulmonary SIV replication during bacterial infection.

Alcohol/HIV Interactions on the Hematopoietic System

Although most attention has been placed on HIV's destruction of $CD4^+$ T cells, AUD and HIV infection have been shown to independently cause defects in the normal formation of blood cells, a process known as hematopoiesis (Calenda et al. 1992; Yeung et al. 1988). Such changes potentially influence the replacement of cells that play a key role in innate and adaptive immunity. Prior to the development and use of effective ART, HIV patients commonly developed bone marrow pathologies, including dysplasia, impaired erythropoiesis, plasmacytosis, and lymphocytic infiltration into the marrow compartment (Calenda et al. 1992). Lymphocytic infiltration may represent cells recruited through inflammation, resulting in persistent local viral production in the bone marrow. In this regard, HIV has been shown to infect hematopoietic stem and progenitor cells (HSPCs) in a humanized mouse model (Nixon et al. 2013). Thus, bone marrow HSPC-containing proviral DNA may serve as a viral reservoir that, upon activation, may lead to increased local virus-mediated inflammation, further driving recruitment of lymphocytes that support more viral replication. Many of the antiretroviral drugs, including nucleoside reverse transcriptase inhibitors (NRTIs), non-NRTIs (NNRTIs), and protease inhibitors (PIs), largely correct these HIV-induced hematopoietic defects. However, side effects of these drugs are implicated in additional bone marrow pathologies (Baillou et al. 2003; Hernandez-Vallejo et al. 2013). In particular, ART seems to disrupt cells that serve a crucial role in guiding hematopoiesis. For example, PIs have been shown to promote aging and inhibit osteoblastic differentiation of mesenchymal stem cells, which may be a mechanism for reduced bone mineral density observed in PLWHA on ART (Hernandez-Vallejo et al. 2013; Yin et al. 2012). Binge alcohol administration also impairs osteoblastic differentiation, possibly contributing to dysregulated niche architecture. However, the combined effects of HIV, ART, and AUD on the bone marrow hematopoietic niche remain to be explored (Gong et al. 2004).

Alcohol and Adaptive Immune Responses to HIV Infection

As described above, AUD has a significant impact on HIV disease through their direct and indirect pro- and anti-inflammatory effects. However, less is known about the effect of chronic alcohol use on the body's specific, adaptive immune response to the virus and virally infected cells seen in both PLWHA and rhesus macaques infected with SIV. As discussed in more detail in the textbox, "Alcohol's Influence on Wasting Disease," chronic alcohol administration to rhesus macaques increases the plasma viral set point, which is associated with disease progression. These higher viral loads could be due to greater virus production coincident with immune activation or to impaired adaptive host defense.

Studies in SIV-infected macaques consistently show higher levels of viremia in animals receiving alcohol (Bagby 2003, 2006; Kumar 2005; Poonia 2006; Nelson 2013). In humans, alcoholics have been shown to have higher levels of blood T cell activation and higher proportions of memory $CD4^+$ and $CD8^+$ T cells (Szabo et al. 2009). Thus, higher viral loads in alcohol-receiving macaques is likely a reflection of higher turnover of viral target cells in mucosal tissues (Poonia et al. 2005). That said, specific adaptive immune responses to SIV do not seem to be impaired in animals receiving alcohol. In fact, we found that virus-specific $CD8^+$ T cell responses in blood are even higher in alcohol-fed animals, which is likely a reflection of the higher viral loads in these animals (Pahar et al. 2013). Similarly, alcohol use seems to have little influence on viral-specific cellular immune responses in HIV/HCV coinfected patients (Graham et al. 2007).

Although a myriad of immunologic defects have been described in the blood of human drinkers (Szabo et al. 2009) and macaques (Siggins et al. 2009) receiving alcohol, the underlying mechanisms of these defects have yet to be elucidated. In addition, the blood is merely a "window" reflecting only a small part of the immune system as a whole, and the effects of mucosal barrier damage and inflammation may not be adequately reflected in monitoring peripheral blood alone.

Conclusion

Both HIV and alcohol use clearly influence immune function, so it seems logical that, in tandem, they might have an additive influence on disease progression. In fact, research has begun to suggest that alcohol use by people infected with HIV can exacerbate an immune system that already is badly strained. The clearest connection between alcohol, HIV, and immune dysfunction is in the gastrointestinal tract where the damage alcohol does to the mucosal lining of the intestines leads to inflammation, barrier damage, and bacterial leakage, which in turn seems to strengthen HIV's grip. Indeed, alcohol's influence on the body's many mucosal tissues seems to be synergistic with those of HIV,

including the tissues of the intestines, the genital tract, and the lung, where chronic inflammation results in increased local and systemic viral replication, resulting in environments in mucosal tissues that both worsen disease progression, and increase the risks of viral transmission. Much more research remains to be done to clarify the interaction between and among alcohol, HIV, and the immune system and to elucidate the mechanisms involved in this complex interaction.

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Prenatal Alcohol Exposure and the Developing Immune System

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Evidence from research in humans and animals suggest that ingesting alcohol during pregnancy can disrupt the fetal immune system and result in an increased risk of infections and disease in newborns that may persist throughout life. Alcohol may have indirect effects on the immune system by increasing the risk of premature birth, which itself is a risk factor for immune-related problems. Animal studies suggest that alcohol exposure directly disrupts the developing immune system. A comprehensive knowledge of the mechanisms underlying alcohol's effects on the developing immune system only will become clear once researchers establish improved methods for identifying newborns exposed to alcohol in utero.

Key words: Alcohol in utero; prenatal alcohol exposure; fetal alcohol effects; alcohol-related intrauterine disorder; fetal alcohol syndrome; fetal alcohol spectrum disorders; immune system; immune function; fetal development; prenatal development; pregnancy; premature birth

Most Americans are aware that drinking alcohol during pregnancy can injure the developing fetus. Fetal alcohol syndrome (FAS) and fetal alcohol spectrum disorders (FASD), with their developmental, cognitive, and behavioral consequences, probably are the best known dangers (Bakoyiannis et al. 2014; Centers for Disease Control and Prevention [CDC] 2009). However, drinking during pregnancy also can disrupt other areas of fetal development besides the brain, including the developing immune system. Studies in humans and animals suggest that alcohol does, in fact, affect the developing immune system and leads to increased risk of infection and disease in infants exposed to alcohol in utero.

Alcohol's effect on the developing immune system is apparent in infants born at term gestation, with studies showing that these babies are at increased risk of infection when exposed to alcohol in utero. However, premature infants are at even higher risk of infection for multiple reasons. For one, in utero alcohol exposure is associated with premature birth, which independently increases immune-related risks. In addition, animal studies show that alcohol has a direct effect on specific aspects of immune function, particularly in the developing lung. This article will discuss the short and long-term effects of drinking during pregnancy on the immune system of the developing fetus (see the figure for an overview).

Understanding the full extent of alcohol's threat to the developing fetus is critical because, despite increased awareness about the risks of drinking during pregnancy, a significant number of women continue to do so. Based on a large household survey, the CDC estimates that 1 in 13 women drink alcohol during pregnancy (CDC 2012). Studies interviewing women just after birth have found that between 25 and 35 percent of newborns were exposed to alcohol in utero (Gauthier et al. 2005*a*; Lester et al. 2001). Interestingly, and contrary to many traditional biases (Goldberg 1995; Hans 1999), these studies also found that older women and women of higher socioeconomic status were as or more likely to drink during pregnancy than younger, less affluent women (CDC 2012; Gauthier 2005*a*; Hutchinson et al. 2013). Because most studies of maternal alcohol use rely on self-reports, and there remains significant stigma associated with alcohol use during pregnancy, these findings likely underestimate the true extent of this problem.

Risk of Alcohol Exposure in Term Infants

Although full-term babies generally are healthier compared with babies born prematurely, there is some evidence that maternal alcohol exposure can increase the risk of neonatal infection even in term newborns. One study, for example, evaluated neonatal infections in 872 newborns with gestational age greater than or equal to 36 weeks. Infants whose mothers reported any alcohol use, excessive drinking, or smoking during pregnancy were more likely to have an infection than infants whose mothers reported that they abstained from alcohol ingestion or cigarette smoking (Gauthier et al. 2005*a*). When the researchers controlled for race and smoking, infants that were small for gestational age (SGA) and whose mothers used any alcohol had a 2.5-fold increased risk of infection. Excessive alcohol use by the mother in these SGA infants increased the risk of infection three- to fourfold. Even after controlling for low maternal income, smoking, and having a baby that was SGA, the researchers found that the newborns were three times more likely to have a neonatal infection if their mothers drank more than seven drinks per week during pregnancy (Gauthier et al. 2005*a*). This effect was most significant if the alcohol use occurred in the second trimester of pregnancy, a time when the neonatal immune system is developing. These

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findings suggest that maternal alcohol ingestion may increase the risk of potentially serious acute health problems in the postnatal period, even in full-term infants. Risks of alcohol exposure are even more significant for those babies born prematurely. We will therefore focus the remainder of the article on this uniquely vulnerable population.

Alcohol's Link to Premature Birth

Premature infants are at increased risk for a variety of significant medical complications, including respiratory, cardiac, neurological, and gastrointestinal problems as well as infection and infection-related complications. Alcohol consumed during pregnancy, researchers postulate, may exacerbate these problems. In addition, research continues to evaluate the hypothesis that drinking during pregnancy can independently increase the risk of premature birth.

The strength of the potential link between alcohol and premature birth remains under debate, because several studies have failed to demonstrate a significant relationship between alcohol and prematurity (Bailey and Sokol 2008). However, Bailey and Sokol (2008) argue that the suspected link is strengthened if they account for potential flaws in study design, particularly among women who drink heavily or binge drink during pregnancy (Bailey and Sokol 2011). In fact, the data thus far do not demonstrate a link between low-to-moderate drinking during pregnancy and the risk of premature delivery (Bailey and Sokol 2011), but multiple studies demonstrate a two- to threefold increase in the risk of premature delivery for women who drink heavily or binge drink during pregnancy (Kesmodel et al. 2000; Mullally et al. 2011; O'Leary et al. 2009; Sokol et al. 2007). Furthermore, heavy drinkers exhibited a dramatic 35-fold increased risk of delivering their babies extremely prematurely (earlier than 32 weeks) compared with women who did not drink during pregnancy (Mullally et al. 2011; Sokol et al. 2007). Therefore, some authors propose that extreme prematurity is an alcohol-related birth defect (Sokol et al. 2007).

Maternal alcohol use also has been associated with multiple risk factors that independently increase the risk of premature delivery. For example, chorioamnionitis—an inflammation of the fetal membranes due to a bacterial infection—confers a significant risk for preterm labor and premature delivery and also increases the risk of multiple adverse outcomes for premature newborns (Pappas et al. 2014). In multiple reviews, maternal alcohol use significantly increased the risk of chorioamnionitis, with risks ranging from five to more than seven times higher when compared with pregnancies without alcohol exposure (de Wit et al. 2013; Hutchinson et al. 2013). Placental abruption, a dangerous condition when the placental lining separates from the uterus, also increases the risk of premature delivery (Sokol et al. 2007). A large review of risk factors for placental abruption suggested that maternal alcohol ingestion increased the risk of abruption by more than twofold (Martinelli et al. 2012).

Although these findings suggest that maternal alcohol use is a risk factor for premature delivery, identification of alcohol-exposed term and premature newborns using traditional clinical tools is poor in both the well-baby nursery as well as newborn intensive care units (Little et al. 1990; Stoler and Holmes 1999). Given this, in order to accurately determine alcohol's adverse effects on premature newborns, it is paramount to validate biomarkers of alcohol exposure in this already at-risk population. One potential marker is a product of alcohol metabolism called fatty acid ethyl esters, which studies suggest accurately determine alcohol exposure in term newborns and in adults (Bearer et al. 1992, 2005; Best and Laposata 2003; Kulaga et al. 2006; Laposata and Lange 1986). Additional research examining ways to improve the accuracy of identifying alcohol-exposed newborns has evaluated the combination of other products of nonoxidative ethanol metabolism including phosphatidylethanol (PEth), ethyl glucuronide (EtG), and ethyl sulfate (EtS) (Bakhireva et al. 2014; Joya et al. 2012). To date, researchers have investigated these methods only in term pregnancies. They now need to test them in premature newborns exposed to alcohol. Once there is an accurate, safe, and convenient way to identify premature newborns exposed to alcohol, it will enable researchers to determine how prenatal alcohol exposure contributes to the development of common disorders

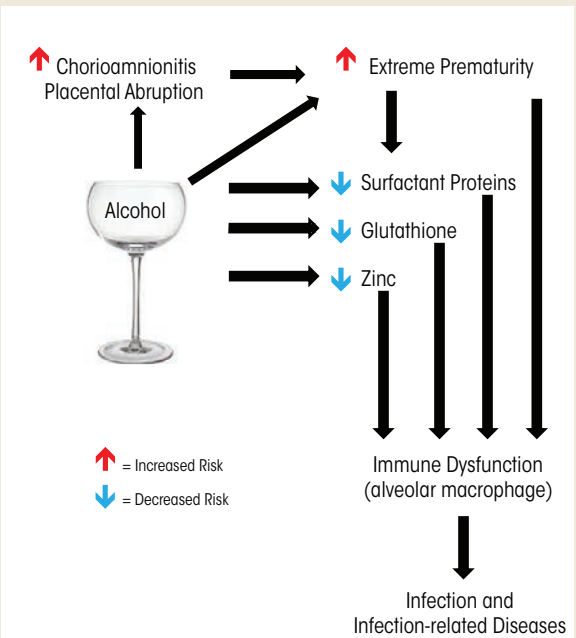

Figure Research suggests that alcohol exposure in utero affects infants' immune function through a variety of mechanisms, including indirectly by increasing the risk of premature birth and directly by influencing immune mediated defenses, particularly in the lungs.

faced by the premature population, including late-onset sepsis (infection), the lung condition bronchopulmonary dysplasia, the gastrointestinal disease necrotizing enterocolitis, and neurodevelopmental delays.

Premature Birth and the Risk of Infection

Despite a lack of biomarkers to specifically identify alcohol-exposed premature infants, research can begin to indirectly link in utero alcohol exposure to increased risk of infections and infection-related illnesses in this population. For all newborns, but particularly those born prematurely, infections play a significant role in illness and mortality (Alarcon et al. 2004; Benjamin et al. 2006; Cordero et al. 2004; Stoll et al. 2010). Even with antibiotic therapy and modern neonatal intensive care, the risk of bacterial infections remains disproportionately elevated in premature newborns and those born within minority groups (Stoll et al. 1998, 2002). Bacterial infection in the premature population increases the risk of a variety of complications including patent ductus arteriosus, in which abnormal blood flow persists between the pulmonary artery and the aorta; necrotizing enterocolitis, in which intestinal tissue becomes diseased and can die; bronchopulmonary dysplasia, a chronic and serious lung condition (Stoll et al. 2002); and neurodevelopmental delays (Adams-Chapman and Stoll 2006; Stoll et al. 2004).

Even as the premature newborn grows, it remains at increased risk for significant problems related to respiratory infections, particularly those of viral origin. Although immunization strategies such as Palivizumab, which aims to prevent serious and often life-threatening lung infections caused by respiratory syncytial virus (RSV), target premature newborns and at-risk newborns with significant lung disease, the growing premature newborn remains at an increased risk for RSV infection, particularly in the lower respiratory tract of the lung (American Academy of Pediatrics 2009; Hall et al. 2009). Furthermore, children born prematurely continue to be at increased risk for severe influenza infections, which adversely affect their long term prognosis (Izurieta et al. 2000; Louie et al. 2006).

Data directly linking in utero alcohol exposure to infections in infants and children are sparse, but some studies suggest an increased risk of neonatal bacterial infection. For example, a small study of children diagnosed with FAS found abnormal lymphocytes and increased rates of bacterial infections such as meningitis, pneumonia, and otitis (Johnson et al. 1981). In addition, hospital stays during the first year of life are approximately three times longer for infants with FAS compared with matched control infants (12.1 days vs. 3.9 days, respectively), with pneumonia being one of the main reasons for hospitalization (Kvigne et al. 2009). Drugs, including alcohol, also potentially increase the risk of maternal to fetal HIV transmission. There is a well-described association between alcohol abuse, the use of other drugs of abuse, and the acquisition and progression of HIV/AIDS among women (Wang and Ho 2011; also see the article by Bagby and colleagues).

The question remains, however, whether alcohol exacerbates the increased risk of infection already occurring in premature infants. To test this, we performed a small case-control analysis of very-low-birth-weight, premature newborns (birth weight less than 1,500 grams). We used social-work interviews to assess maternal alcohol use during pregnancy and found that premature babies exposed to alcohol in utero were 15 times more likely to show signs of early-onset bacterial sepsis than matched premature newborns without in utero alcohol exposure. This risk of early-onset bacterial sepsis with alcohol exposure remained even after we controlled for chorioamnionitis and premature prolonged rupture of membranes (Gauthier 2004). This study suggests that maternal alcohol use during pregnancy increases the risk of infection in the premature newborn, but much investigation still is necessary to fully define the influence of maternal alcohol use on neonatal infection.

Animal models of fetal ethanol exposure play an important role in furthering this research. These models help identify mechanisms underlying alcohol's detrimental effects on immune defense (Gauthier et al. 2005*b*, 2010; Lazic et al. 2007; McGill et al. 2009; Sozo et al. 2009), and they not only support these early clinical findings but also suggest that in utero exposure alters multiple arms of innate immunity in the developing fetal lung, as we discuss below.

Maternal Alcohol Ingestion and Lung Immunity

As mentioned above, viral-mediated respiratory infections can be an ongoing problem for children born prematurely. In particular, they are at increased risk for RSV and influenza. Emerging data from animal research provide insight into mechanisms underlying these findings.

Studies of animals exposed in utero to ethanol suggest that ethanol-induced immune dysfunction persists into adulthood. Specifically, adult animals exposed to ethanol in utero demonstrated impaired adaptive immunity and altered B-cell responses, resulting in increased risk and severity of influenza infection (McGill et al. 2009). Another study (Zhang 2005) demonstrated that in utero ethanol exposure alters the hypothalamic–pituitary–adrenal axis, which in turn results in hyperactivity in stress-induced immunosuppression and increased vulnerability to subsequent infectious illness.

Innate immunity in the lung is impaired in the premature newborn (Bellanti and Zeligs 1995; Hall and Sherman 1992). Growing evidence suggests that in utero ethanol exposure further disrupts multiple arms of innate immunity in the developing lung. Studies in sheep, for example, find that in utero ethanol disrupts immune function by decreasing in the fetal lung surfactant proteins (SP), which also are known as collectins, particularly SP-A and SP-D (Lazic et al. 2007; Sozo et al. 2009). In the lung, these proteins are essential mediators of the local immune response in that

they modulate the function of dendritic and T cells and facilitate the removal of pathogens by the alveolar macrophage (Sorenson et al. 2007).

The alveolar macrophage is the resident inflammatory cell that provides the initial defense against foreign and infectious particles and orchestrates the inflammatory process within the lung (Fels and Cohn 1986; Standiford et al. 1995). Alveolar macrophages reside in the lungs' alveoli and are derived from peripheral circulating blood monocytes (Fels and Cohn 1986; Prieto et al. 1994). As a consequence, anything that affects immune responses of fetal monocytes—for example, exposure to alcohol during pregnancy—may subsequently affect the alveolar macrophage population and the inflammatory environment within the newborn lung (Kramer et al. 2004, 2005).

Furthermore, substances that directly affect alveolar macrophages can therefore affect immunity in the infant lung. Studies in animals find that fetal alcohol exposure decreases the antioxidant glutathione in the fluid lining the alveolar space and within the resident alveolar macrophages (Gauthier et al. 2005*b*). Reductions in glutathione cause oxidative stress in the lung that, in turn, contributes to alveolar macrophage dysfunction and altered alveolar macrophage maturation (Brown et al. 2007; Gauthier et al. 2005*b*, 2010). Other studies in guinea pigs demonstrated that impaired alveolar macrophage function increases the already elevated risk of experimentally induced pneumonia in the newborn pup (Gauthier et al. 2009; Ping et al. 2007). Providing the pregnant guinea pig with the dietary supplement S-adenosylmethionine (SAMe) during ethanol ingestion prevented glutathione depletion in the neonatal lung, protected the neonatal alveolar macrophage from increased reactive oxygen species, improved alveolar macrophage phagocytosis, and decreased the risk of sepsis and pneumonia in the pup. In addition, giving intranasal glutathione treatments to newborn pups exposed in utero to alcohol improved macrophage phagocytosis and diminished lung infections and dissemination of experimentally induced *Group B Streptococcus* pneumonia (Gauthier et al. 2009). These findings support the idea that fetal ethanol exposure causes glutathione depletion in the lung, which in turn decreases the fetal lung's ability to clear infectious particles and increases the risk of respiratory infections.

Research in both humans and animals suggest that zinc depletion also may play a role in dampening immunity in alcohol exposed infants. Zinc is an essential cofactor in approximately 300 enzyme-dependent processes involved in immunity, growth, cell differentiation, and metabolism (Chandra 2002; Uriu-Adams et al. 2010). Studies of global disease burden for 2010 found that a primary risk factor for death in early infancy was bacterial infection linked to zinc insufficiency (Chaffee and King 2012; Lim et al. 2012; Mori et al. 2012). Indeed, zinc is essential for innate and adaptive immune responses (Knoell and Liu 2010; Maggini et al. 2007), and suboptimal concentrations of zinc result in an increased susceptibility to infection as well as exacerbation of existing infections (Prasad 2013). Newborns are at an increased risk for suboptimal zinc concentrations if their mothers have suboptimal zinc pools, and women who abuse alcohol during pregnancy tend to have suboptimal zinc pools (Keen et al. 2010; Picciano 2003). In addition, researchers have shown that decreases in zinc are a potential relative risk factor for FASD, and zinc supplements may protect against some of the adverse effects of prenatal alcohol exposure (Keen et al. 2010; Picciano 2003). Because approximately 50 percent of pregnancies are unintended (Finer and Henshaw 2006), some mothers may continue drinking during at least part of their pregnancy, resulting in significant fetal alcohol exposure and risk of suboptimal zinc concentrations in newborns. Furthermore, because the majority of zinc is transported across the placenta in the third trimester of pregnancy, newborns born prematurely, before zinc transport is complete, also are zinc deficient (Giles and Doyle 2007), which suggests that premature newborns exposed to alcohol in utero may be at an even higher risk of zinc deficiency.

A study in adult rats suggests a possible mechanism for zinc's effect on alcohol-induced alveolar macrophage dysfunction. The study found that chronic ethanol ingestion decreased the zinc levels in alveolar macrophage due to decreased expression of zinc transporters (Mehta and Guidot 2012; Mehta et al. 2011). Equally important, dietary zinc restored zinc pools in the alveolar macrophage and improved phagocytosis. Investigations in fetal ethanol models suggest that similar zinc deficiencies contribute to fetal alveolar macrophage dysfunction in the newborn.

Potential Areas for Further Research

Further research defining the mechanisms underlying alcohol-induced alterations in the immune function of the alcohol-exposed newborn is necessary. In the adult alcohol-exposed lung, alcohol-induced mitochondrial dysfunction significantly contributes to cellular dysfunction and impaired immune response of the alveolar macrophage (Liang et al. 2013, 2014).

Systemically, alcohol alters multiple arms of the immune system. Alcohol-induced increase in intestinal permeability and alterations of the gut microbiome directly contribute to alcohol-associated hepatic inflammation and the progression of liver disease (Chen and Schnabl 2014; Elamin et al. 2013; see also the article by Engen and colleagues). Alcohol-induced changes in gut permeability and the gut's interaction with the liver modulate both lung and liver inflammation in the setting of burn injury (Chen et al. 2014). Antigen presentation and T-cell dysfunction contribute to the complex immune dysfunction of the alcohol-exposed adult (Fan et al. 2011; Gurung et al. 2009). These important mechanisms have yet to be evaluated among fetuses exposed to alcohol in utero. They remain important potential areas of research particularly in the premature newborn, because morbidities such as late onset sepsis, bronchopulmonary

dysplasia, and necrotizing enterocolitis are interrelated (Stoll et al. 2010).

Conclusion

This article highlights evidence from research in humans and animals suggesting that ingesting alcohol during pregnancy can disrupt the fetal immune system and result in an increased risk of infections and disease in newborns and possibly throughout life. It also emphasizes the critical need for more research to illuminate the strength and nature of this link and the mechanisms by which alcohol may influence the developing immune system.

In particular, researchers need more specific and accurate assays for identifying which newborns have been exposed to alcohol in utero, along with methods to determine the extent and timing of such exposure. Such approaches will allow researchers to determine and more precisely measure the influence of alcohol on infections and diseases related to immune system dysfunction. In addition, continued research is needed to clarify the potential link between alcohol and premature birth, particularly extreme premature delivery.

Evidence from studies in animals has begun to provide theories about how alcohol may disrupt the developing immune system. These animal models already have begun to identify molecular mechanisms in the lung that may directly and indirectly lead to an increased risk of respiratory infections. These studies not only point to potential mechanisms of immune system disruption attributed to in utero alcohol exposure but also to possible interventions that might ameliorate the damage to the developing infant.

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Alcohol's Burden on Immunity Following Burn, Hemorrhagic Shock, or Traumatic Brain Injury

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Alcohol consumption contributes to increased incidence and severity of traumatic injury. Compared with patients who do not consume alcohol, alcohol-consuming patients have higher rates of long-term morbidity and mortality during recovery from injury. This can be attributed in part to an impaired immune response in individuals who consume alcohol. Acute and chronic alcohol use can affect both the innate and adaptive immune defense responses within multiple organ systems; the combination of alcohol use and injury results in increased susceptibility to bacterial and viral pathogens. This review examines the major deleterious effects of alcohol on immunity following tissue damage or traumatic injury, with a focus on alcohol's influence on the ability of the immune and major organ systems to fight disease and to repair damaged tissues following injury.

Key words: Alcohol consumption; alcohol use, abuse, and dependence; chronic alcohol use; acute alcohol use; injury; traumatic injury; morbidity; mortality; immune response; impaired immune response; bacterial pathogens; viral pathogens; tissue; organs; disease

The incidence of traumatic injury in alcohol-intoxicated individuals continues to escalate. According to the Centers for Disease Control and Prevention (2012*a*), more than 38 million American alcohol users consume 5 or more drinks on the same occasion (i.e., binge drink) and do so about 4 times per month. This behavior is highly conducive to unintentional or accidental traumatic injury, which according to the National Vital Statistics Reports is the leading cause of years of potential life lost (YPLL) before age 45. Unintentional injury causes more YPLL than that attributed to cancer, intentional injuries, heart disease, and HIV individually (Centers for Disease Control and Prevention 2009). Data from the National Center for Injury Prevention and Control, as well as data derived from prospective and retrospective studies, show that up to 40 percent of victims of traumatic injury have positive blood alcohol concentrations (BAC), with 35 percent presenting with blood alcohol levels above the legal limit of intoxication (Beech and Mercadel 1998).

The severity of trauma, reduced blood flow and oxygen delivery (i.e., hemorrhagic shock, referred to as shock in this article), and tissue injury is greater in intoxicated victims than in sober victims, resulting in higher mortality rates in the alcohol-consuming patient population (Pories et al. 1992). Although immediate mortality from traumatic injury has improved significantly as a result of aggressive resuscitation, long-term morbidity and mortality continue to be unacceptably high during the recovery period. The prevalence of morbidity and mortality is particularly attributable to the altered immune response among impaired patients to subsequent challenges, such as surgery or infection, leading to multiple organ failure (Roumen et al. 1993; Sauaia et al. 1994). Acute alcohol intoxication complicates the initial management of trauma victims and is associated with greater incidences of pneumonia and respiratory distress, requiring ventilator assistance during hospitalization (Gurney et al. 1992; Jurkovich et al. 1992). In addition, major complications including tracheobronchitis, pneumonia, pancreatitis, and sepsis are significantly

increased in patients with high levels of carbohydrate-deficient transferrin (CDT), a marker for alcoholism (Spies et al. 1998). European studies show that, compared with nonalcoholics, alcoholics more frequently develop major complications and require a significantly prolonged stay in the intensive care unit (ICU) following trauma (Spies et al. 1996*a*).

Excessive acute and chronic alcohol consumption has significant effects at multiple cellular levels, affecting both innate and adaptive immune mechanisms (Molina et al. 2010). Both chronic and acute patterns of alcohol abuse lead to impaired immune responses, resulting in increased susceptibility to infectious diseases caused by bacterial and viral pathogens (Brown et al. 2006). Clinical and preclinical studies show that the combined effects of alcohol and injury result in greater immune disruption than either insult alone (Messingham et al. 2002). This article reviews the current understanding of the burden of alcohol on the immune response to three specific traumatic events: burn, shock, and traumatic brain injury (TBI). The major pathophysiological consequences of these injuries on other major organ systems—including the cardiovascular system, pulmonary system, and gastrointestinal tract—are highlighted with emphasis on the contribution of alcohol-induced immunomodulation to postinjury morbidity.

Reestablishment of homeostasis after a traumatic insult involves activation of host defense mechanisms for self-protection against toxic inflammatory processes and tissue repair. Trauma victims frequently are subjected to necessary invasive procedures, such as surgery and anesthesia. In addition, trauma victims frequently are exposed to subsequent challenges, particularly infection. These additional stresses to an already compromised inflammatory and neuroendocrine milieu further contribute to morbidity and mortality in this patient population. Traumatic injury and hemorrhagic shock produce a temporal pattern with early upregulation of pro-inflammatory cytokine[1] gene product expression and with later suppression of stimulated pro-inflammatory cytokine release (Hierholzer et al. 1998; Molina et al. 2001). Together, these alterations lead to generalized immunosuppression, ultimately resulting in an increased susceptibility to infection (Abraham 1993; Ertel et al. 1993).

Alcohol has been shown to affect multiple aspects of the host immune response, contributing to pathological processes (Szabo 1998). For example, alcohol alters the expression and processing of cytokines and a type of cytokine known as chemokines (D'Souza et al. 1989; Standiford and Danforth 1997), the expression of adhesion molecules (Zhang et al. 1999), inflammatory cell recruitment (Patel et al. 1996; Shellito and Olariu 1998) and accumulation, and oxidative capacity of macrophages (Nilsson and Palmblad 1988). The monocyte/macrophage production of cytokines and chemokines, in particular interleukin (IL)-8 and tumor necrosis factor-α (TNF-α), is critical in the regulation of the acute inflammatory host response to infectious challenge. The combined inhibition of pro-inflammatory cytokine production and neutrophil activation and migration to a site of infection has been suggested to contribute to the enhanced susceptibility to infection in alcoholic individuals (Nelson et al. 1991) and to the increased risk of trauma- and burn-related infections associated with alcohol intoxication (Arbabi et al. 1999). Several lines of evidence show that these alcohol-mediated alterations in host defense following injury lead to increased morbidity and mortality from infections during the recovery period (Faunce et al. 2003; Messingham et al. 2002; Zambell et al. 2004). In addition, considerable evidence suggests that the severity of disease processes is greater in intoxicated trauma victims than in nonintoxicated counterparts (Spies et al. 1996*a,b,* 1998). In particular, immunoparalysis characterized by inhibition of stimulated pro-inflammatory cytokine release (Angele et al. 1999) and alterations of both cellular and humoral immunity (Napolitano et al. 1995; Wichmann et al. 1998) have been identified as risk factors for infection and progression to organ injury during the post-traumatic injury period (Abraham 1993; Ertel et al. 1993).

The systemic response to injury is associated with marked activation of neuroendocrine pathways that contribute to cardiovascular adaptation to blood loss, injury, and pain but also exert immunomodulatory effects (Molina 2005). Catecholamines (e.g., dopamine, norepinephrine, and epinephrine), and drugs that mimic their effects (i.e., adrenergic agonists), are especially known to exert important regulatory functions on macrophages as well as on B- and T-lymphocyte cytokine production, proliferation, and antibody secretion; dendritic cell function; cytokine and chemokine release; and nitric oxide (NO) production (Madden et al. 1995). The relevance of these control mechanisms and the implications of their dysregulation have been demonstrated by the high incidence of infection in patients who experience elevated temperature, increased heart rate, and perspiration (i.e., "sympathetic storm") following acute brain trauma and myocardial infarction (Woiciechowsky et al. 1998). Alcohol intoxication produces marked disruption of several neuroendocrine pathways. Disruption of the homeostatic neuroendocrine counterregulatory response to shock impairs hemodynamic stability and recovery, contributing to compromised blood flow and increased end-organ injury (Molina et al. 2013). Specifically, binge alcohol use blunts central neuroendocrine and autonomic activation, and this seems to result from alcohol-accentuated NO production in the periventricular nucleus (PVN) of the hypothalamus (Whitaker et al. 2010). Alcohol-mediated impairment of neuroendocrine counterregulatory responses to traumatic injury not only

[1] Cytokines are proteins involved in cell signaling. They are produced by a variety of cells including immune cells and regulate the immune response.

exacerbates low blood pressure (i.e., hypotension) during hemorrhage but also attenuates blood pressure recovery during fluid resuscitation, leading to significant alterations in blood flow redistribution and notably affecting circulation in the gastrointestinal tract (Wang et al. 1993). Studies have shown that alcohol-intoxicated animals have greater reduction of blood flow to the liver, kidney, and small and large intestines than nonintoxicated animals, following shock and fluid resuscitation (Sulzer et al. 2013). These macro- and microcirculatory changes during trauma and hemorrhage have been implicated in the subsequent development of sepsis and multiple organ failure (Peitzman et al. 1995) and contribute to an increased host susceptibility to infection and tissue injury during recovery (Mathis et al. 2006; Xu et al. 2002). People who abuse alcohol, including both binge and chronic drinkers, have a higher incidence of traumatic injury such as burn, shock, and TBI. The host response to these diverse insults is markedly affected by both patterns of alcohol abuse and some systems—including gastrointestinal, cardiovascular, and pulmonary—are more affected than others according to the specific injury.

Alcohol and Burn Injury

Burn injury is a common type of traumatic injury that affects thousands of people in the United States every year (Bessey et al. 2014). Approximately 50 percent of burn-injured patients have detectable blood alcohol levels at the time of hospital admission (Haum et al. 1995; McGwin et al. 2000), and these patients have more complications, require longer hospital stays, and have greater mortality rates than those with a similar degree of injury who are not intoxicated at the time of injury (McGill et al. 1995). Most morbidity and mortality among patients who survive initial injury is attributed to complications stemming from infection (Baker et al. 1980). Therefore, the pre-burn immunological condition of injured patients affects susceptibility to infection and survival. Several mechanisms contribute to infection in burn patients, including loss of barrier function, changes in normal flora, wound ischemia, and cellular immunosuppression resulting from pro-inflammatory processes. Neutrophil, helper T-cell, and macrophage dysfunction; increased pro-inflammatory cytokine production; and enhanced production of immunosuppressive factors have all been shown to contribute to the pathophysiological response to burn injury (Faunce et al. 1998; Messingham et al. 2000). The mechanisms that contribute to infection in burn patients are influenced by acute and chronic alcohol intoxication and will be discussed below (see figure 1).

Research by Kovacs and colleagues (2008) has offered insight into the combined effects of burn injury and alcohol intoxication on immunity (Bird and Kovacs 2008). Chronic alcohol abuse alone increases the risk for lung infection (Baker and Jerrells 1993), impairs the phagocytic activity of alveolar macrophages and clearance of infectious particles from the airways, and impairs oxidant radicals, chemokine, and cytokine release that are required for microbial killing (Brown et al. 2007; Mehta and Guidot 2012; Molina et al. 2010). Acute alcohol intoxication prior to burn injury significantly suppresses the immune response relative to the insult alone (Faunce et al. 1997) and causes greater suppression of T-cell proliferation and response, reduced IL-2 production, and increased IL-6 production and circulating levels (Choudhry et al. 2000; Faunce et al. 1998). The T-cell and cytokine impairment caused by the combined effect of alcohol and burn injury may further suppress cell-mediated immunity, resulting in even greater susceptibility to infection than burn alone. Alcohol-mediated immunomodulation contributes to tissue injury in target organs as described below.

Gastrointestinal Tract

A multitude of studies have demonstrated that the gut is a reservoir for pathogenic bacteria, which may contribute to increased susceptibility to infections following traumatic injury (Deitch 1990). The intestinal mucosal barrier serves a major role in the local defense against bacterial entry and the translocation of endotoxin to the systemic circulation (Xu et al. 1997). Increased permeability and immune dysfunction indicate the compromised state of the intestinal mucosal barrier to bacterial translocation following trauma (Deitch et al. 1990; Willoughby et al. 1996). Increased intestinal permeability enhances bacterial and endotoxin translocation from the intestinal tract to the systemic circulation, triggering a systemic inflammatory response (Xu et al. 1997). Activated macrophages and lymphocytes release pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6, thereby contributing to tissue injury (Fink 1991). Studies have determined that chronic alcohol consumption disrupts intestinal barrier function and induces gut leak (Li et al. 2008; Tang et al. 2009). In addition, reports have shown a loss of intestinal barrier function followed by an increase in endotoxin and bacterial translocation to the systemic circulation following burn injury alone (Carter et al. 1990; Deitch and Berg 1987; Horton 1994), alcohol intoxication alone (Keshavarzian et al. 1994; Tabata et al. 2002), and burn injury with alcohol intoxication (Choudhry et al. 2002; Kavanaugh et al. 2005; Napolitano et al. 1995). Acute alcohol intoxication at the time of burn injury enhances bacterial growth in the intestine and is reflected in a proportional increase in mesenteric lymph node bacterial count (Kavanaugh et al. 2005). Acute alcohol intoxication also modulates intestinal immune defense by suppressing T-cell proliferation and increasing bacterial accumulation in mesenteric lymph nodes, spleen, and blood, which suggests that T-cell suppression may play a role

in bacterial translocation from the lumen of the gut (Choudhry et al. 2002). Moreover, studies have shown that following shock, trauma, or burn injury, the gut leaks bacteria and pro-inflammatory factors that are carried by the mesenteric lymphatic system, which contributes to acute lung injury (ALI) (Magnotti et al. 1999). The possibility that alcohol exacerbates toxin delivery to the systemic circulation through the lymphatics is supported by studies demonstrating that alcohol regulates the contractile cycle of mesenteric lymphatic vessels modulating the driving force of lymph flow (Keshavarzian et al. 1994; Souza-Smith et al. 2010). Thus, the contribution of gut–lymph to end-organ damage following burn injury and alcohol intoxication may be significant.

Collectively, studies indicate that alcohol consumption preceding burn injury (1) increases gut permeability; (2) enhances intestinal bacterial growth, translocation, and systemic accumulation; and (3) suppresses T-cell proliferation. Further, research supports the concept that the intestine is not only a source of infection but also the site of the initial immune perturbation leading to the development of multiple organ dysfunction or organ failure.

Cardiovascular System

Immediately following a burn injury, the cardiovascular system responds with a decrease in cardiac output (Cuthbertson et al. 2001) as a result of low blood volume and reduced venous return (Kramer et al. 2007). This phase is associated with decreased cardiac contractility, mediated by the release of vasoactive and pro-inflammatory mediators (Williams et al. 2011). Subsequently, there is a surge in counterregulatory neuroendocrine mediators (catecholamines, glucagon, and cortisol) that contribute to the development of a hyperdynamic cardiovascular state—characterized by increased heart rate and cardiac output—and is associated with increased myocardial oxygen consumption and myocardial hypoxia (Williams et al. 2011). These pathophysiological processes enhance oxidative metabolism and increase the risk for free-radical generation, further

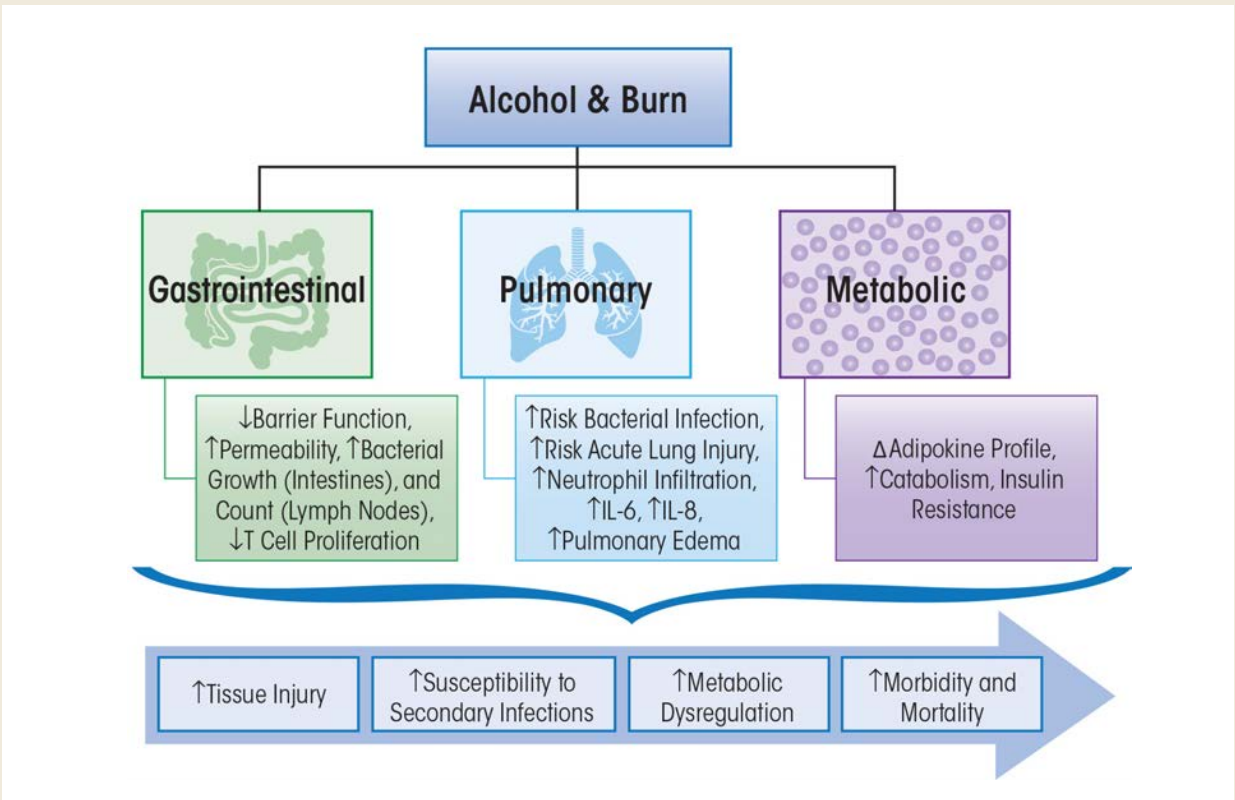

Figure 1 Salient gastrointestinal, pulmonary, and metabolic pathophysiological consequences of alcohol abuse prior to, or at the time of, burn injury. The decrease in gut barrier function leads to increased permeability and bacterial translocation that enhances the risk for bacterial infections and lung injury. Marked alterations in metabolic responses, characterized by altered adipokine profile consistent with increased insulin resistance, collectively contribute to greater morbidity and mortality post–burn injury.

exacerbating the pro-oxidative environment that has been proposed to contribute to impaired wound healing in burn patients (Herndon and Tompkins 2004). Chronic binge alcohol consumption also has been shown to promote a pro-oxidative and pro-inflammatory milieu (Rashbastep et al. 1993), and these factors may further impede wound healing in patients consuming alcohol prior to experiencing burn injury. Additional research is needed to better understand immunomodulation effects following the combined insults of alcohol and burn injury and the mechanisms underlying the more severe outcome of burn injury with alcohol abuse.

Pulmonary System

Adult respiratory distress syndrome (ARDS) is a frequent cause of death in burn patients. The lungs are one of the first organs to fail following traumatic injury (Turnage et al. 2002). Chronic and acute alcohol abuse impair pulmonary host defense to infection, thus increasing the risk of bacterial infection and acute lung injury (Boe et al. 2009; Happel and Nelson 2005). Lung injury as a result of the combination of alcohol intoxication and burn injury may be attributed to the delicate architecture of the lungs combined with other alcohol-related factors, such as bacterial and endotoxin leakage from the gut and a higher risk of contact with pathogens from the circulation and airways (Bird and Kovacs 2008; Li et al. 2007). Previous studies show that the combined insult of acute alcohol consumption and burn injury in mice leads to increased infiltration of the lungs by white blood cells, called neutrophils, and pro-inflammatory cytokine expression of IL-6 (Chen et al. 2013). Systemic and pulmonary IL-6 reflect the inflammatory state of the host and have been shown to be decreased in the absence of Toll-like receptor-4 (TLR-4) and intercellular adhesion molecule-1 (ICAM-1) (Bird et al. 2010). The role of IL-6 in lung injury has been demonstrated in studies in IL-6 knockout mice or following neutralization of IL-6, both of which result in significantly reduced lung inflammation (Chen et al. 2013). Studies also have shown that acute alcohol intoxication at the time of burn injury induces an upregulation of IL-18 production and neutrophil infiltration within the lung compartment, all leading to pulmonary edema (Li et al. 2007).

Metabolism

The post-burn period is characterized by a hypermetabolic state (Pereira and Herndon 2005) consisting of increased oxygen consumption; increased breakdown of glycogen, fats, and proteins; elevated resting energy expenditure and glucose synthesis; and reduced insulin-stimulated glucose uptake into skeletal muscle and adipose tissue (Gauglitz et al. 2009). Previous studies suggest that development of this hypermetabolic state during the post-burn period occurs as a consequence of (1) increased plasma catecholamine and corticosteroid concentrations (Jeschke et al. 2008; Williams et al. 2009; Wilmore and Aulick 1978), (2) increased systemic pro-inflammatory mediator expression, favoring processes that release energy (i.e., catabolic) over those that store energy (i.e., anabolic) (Jeschke et al. 2004), and (3) increased adipose tissue mRNA (Zhang et al. 2008) and protein (Yo et al. 2013) expression of uncoupling protein-1 (UCP-1), enhancing heat production and metabolism. Further, circulating levels of TNF-α, a known anti-insulin cytokine, are increased (Keogh et al. 1990), and the post-burn period can be described as a state of marked insulin resistance (IR) (Gauglitz et al. 2009). Insulin sensitivity has been reported to be decreased by more than 50 percent at 1-week post–burn injury in pediatric patients (Cree et al. 2007) as well as in rodent models of burn injury (Carter et al. 2004). The relevance of insulin levels to overall outcome from burn injury is supported by results from clinical studies showing that exogenous insulin therapy in pediatric burn patients decreased pro-inflammatory cytokines, increased anti-inflammatory cytokines, and increased serum concentrations of insulin-like growth factor-1 (IGF-1) and insulin-like growth factor binding protein-3 (IGFBP-3). Together, these changes could help to preserve organ function and better promote anabolic processes during the post-burn hypermetabolic state (Jeschke et al. 2004). Chronic alcohol consumption decreases insulin responsiveness and can alter insulin signaling through various mechanisms, including increased hepatic protein expression of the gene phosphatase and tensin homologue (PTEN), which directly inhibits insulin signaling through the phosphatidylinositol-5,5-bisphosphate 3-kinase (PI3K)/protein kinase B (Akt) pathway (de la Monte et al. 2012). In addition to the negative regulation of the pathway by PTEN proteins, the enzyme protein tyrosine phosphatase dephosphorylates and decreases activity of important molecules involved in the insulin signaling cascade, potentially contributing to impaired insulin action (Gao et al. 2010; Koren and Fantus 2007). In addition, Lang and colleagues (2014) demonstrated that chronic alcohol consumption reduces Akt and AS160 phosphorylation, reduces membrane localization of glucose transporter type 4 (GLUT-4) protein, and increases serine phosphorylation at serine-307 of insulin receptor substrate-1 (IRS-1), all of which will attenuate insulin-stimulated skeletal muscle glucose uptake and other insulin-mediated anabolic effects (Lang et al. 2014). These negative effects on insulin signaling occurred in conjunction with sustained increases in pro-inflammatory cytokines TNF-α and IL-6 following chronic alcohol exposure (Lang et al. 2014). Thus, both burn injury and chronic alcohol exposure alter metabolic pathways—favoring catabolic and opposing anabolic pathways—possibly resulting in long-lasting alterations in metabolic processes. The metabolic dysregulation

following burn injury is likely to produce more severe consequences in chronic alcohol burn victims. Previous studies assessing nutritional status of alcoholic patients have been discordant, with some studies suggesting that increased alcohol consumption increases the prevalence of malnutrition in alcoholic patients (Hillers and Massey 1985), whereas other studies do not show a role for excessive, or chronic, alcohol consumption in malnutrition (Nicolas et al. 1993; Urbano-Marquez et al. 1989). A study assessing the influences of aging and chronic alcohol feeding in mice on protein synthesis demonstrated that chronic alcohol feeding decreases gastrocnemius muscle protein synthesis, which provides a mechanism for loss of lean body mass (Korzick et al. 2013; Lang et al. 2014). Decreased anabolism during the post-burn period, which itself is a state of heightened catabolic processes, could significantly impair recovery for these alcoholic patients experiencing burn injury. Further, the hypermetabolic state of the post-burn period is thought to contribute to delayed or impaired wound healing, increased susceptibility to infections, and erosion of lean body mass (Pereira and Herndon 2005). Moreover, both binge alcohol consumption (Pravdova and Fickova 2006; You and Rogers 2009) and burn injury (Venkatesh et al. 2009; Wade et al. 2013) can contribute to dysregulation of cytokines secreted by adipose tissue (i.e., adipokines). Recent studies show that mice exposed to a single alcohol binge prior to burn injury have a dramatic increase in pro-inflammatory response and a decrease in anti-inflammatory response in adipose tissue (Qin et al. 2014). The heightened pro-inflammatory response during the post-burn period would be predicted to modulate leptin levels. Thus, recovery from burn injury is likely to be severely impaired in alcoholic individuals as a result of a greater disruption in metabolic processes as well as impairment of host defense mechanisms, leading to greater morbidity and health care costs associated with the management of these patients. Therefore, further investigation is warranted to understand the modulation of the immune system by the combined effect of alcohol and burn that might result in dysregulation of adipose tissue and altered metabolism.

Alcohol and Hemorrhagic Shock

Studies from several investigators have provided evidence that traumatic injury and hemorrhagic shock produce an immediate upregulation of pro-inflammatory cytokine gene product expression (Ayala et al. 1991; Hierholzer et al. 1998). The early pro-inflammatory response is later followed by suppression of stimulated pro-inflammatory cytokine release (Angele et al. 1999; Xu et al. 1998) and alterations of both cellular and humoral immunity (Napolitano et al. 1995; Wichmann et al. 1998), leading to generalized immunosuppression, which ultimately results in an increased susceptibility to infection (Abraham 1993; Ertel et al. 1993). Along with marked alterations in hemodynamic homeostasis and neuroendocrine regulation, immunological derangements and subsequent infections are also a major cause of increased morbidity and mortality following hemorrhagic shock (Livingston and Malangoni 1988; Phelan et al. 2002).

Studies focused on the immune modulatory effects of alcohol exposure following hemorrhagic shock have demonstrated that even 24 hours after the post-hemorrhagic shock, alcohol-intoxicated animals had a marked suppression in cytokine release to an inflammatory challenge (Greiffenstein et al. 2007), affecting the ability to fight secondary infectious challenges. Conversely, findings observed at the tissue level determined that alcohol intoxication enhanced the pro-inflammatory milieu following hemorrhagic shock, priming tissues for injury. The burden of alcohol and hemorrhagic shock on specific target organ systems is discussed below and summarized in figure 2.

Gastrointestinal Tract

Hemorrhagic shock produces similar alterations in gut barrier function to those resulting from burn injury. Alcohol intoxication at the time of hemorrhagic shock further exacerbates hemorrhagic injury-induced gut permeability and leakage (Sulzer et al. 2013). Chronic alcohol consumption has been shown to disrupt intestinal barrier function and induce gut leak (Li et al. 2008; Tang et al. 2009). The combination of greater hypotension and inadequate tissue blood flow (i.e., hypoperfusion) observed in alcohol-intoxicated animals and the increased gut leak observed in alcohol-intoxicated hemorrhaged animals are speculated to contribute to increased host susceptibility to infection and tissue injury during recovery (Molina et al. 2013). Alcohol-intoxicated, hemorrhaged animals have been shown to have greater reduction in hepatic, renal, and intestinal blood flow than that observed in nonintoxicated animals (Sulzer et al. 2013). This reduction in critical organ blood flow was associated with enhanced tissue damage. An additional mechanism that could contribute to tissue injury in the alcohol-intoxicated, hemorrhaged host is the disruption of gut-associated lymphoid tissue function, which has been shown to play a role in other disease states.

Cardiovascular System

Studies using a rodent model of binge-like alcohol consumption prior to hemorrhagic shock have shown that acute alcohol intoxication decreases basal mean arterial blood pressure (MABP), exacerbates hypotension, and attenuates blood pressure recovery during fluid resuscitation (Mathis et al. 2006; Phelan et al. 2002). Following fixed-volume hemorrhage, alcohol-intoxicated animals were significantly more hypotensive throughout the

hemorrhage and resuscitation periods (Mathis et al. 2006). In response to a fixed-pressure (40 mmHg) hemorrhage, a significantly lesser amount of blood was removed from the alcohol-intoxicated animals than controls (Phelan et al. 2002). Similarly, McDonough and colleagues, using a guinea pig model of ethanol exposure prior to hemorrhagic shock (loss of 60% blood volume) and resuscitation, demonstrated that a low dose of ethanol (1 g/kg) decreases MABP and heart rate and exacerbates the metabolic effects of hemorrhagic shock, as shown by increased glucose and lactate concentrations (McDonough et al. 2002). Despite the plethora of previous studies that have examined functional cardiovascular consequence of hemorrhagic shock and hemorrhage with alcohol intoxication, few studies have examined the combined effects of alcohol, hemorrhagic shock, and immune dysfunction on the cardiovascular system. However, exacerbation of pre-existing cardiovascular disease and prolonged recovery are anticipated outcomes of the combined effects of alcohol and hemorrhagic shock, all leading to an impaired immune response.

Pulmonary System

As mentioned previously, alcohol intoxication produces significant dysregulation of the host defense mechanism during the post-injury period. Lung IL-6 and TNF-α are suppressed, while granulocyte-colony stimulating factor (GCSF) mRNA is increased in alcohol-intoxicated, hemorrhaged animals (Mathis et al. 2006; Ono et al. 2004). Moreover, isolated pleural cells and peripheral blood mononuclear cells (PBMCs) from alcohol-intoxicated, hemorrhaged animals display suppressed TNF-α, IL-1β, and IL-6 release following lipopolysaccharide stimulation (Greiffenstein et al. 2007), suggesting greater impairment of humoral immune response than that resulting from hemorrhagic shock alone. The importance of these alterations in host defense mechanisms was demonstrated in animals inoculated with *Klebsiella pneumonia* following hemorrhagic shock. These studies showed suppressed neutrophil response,

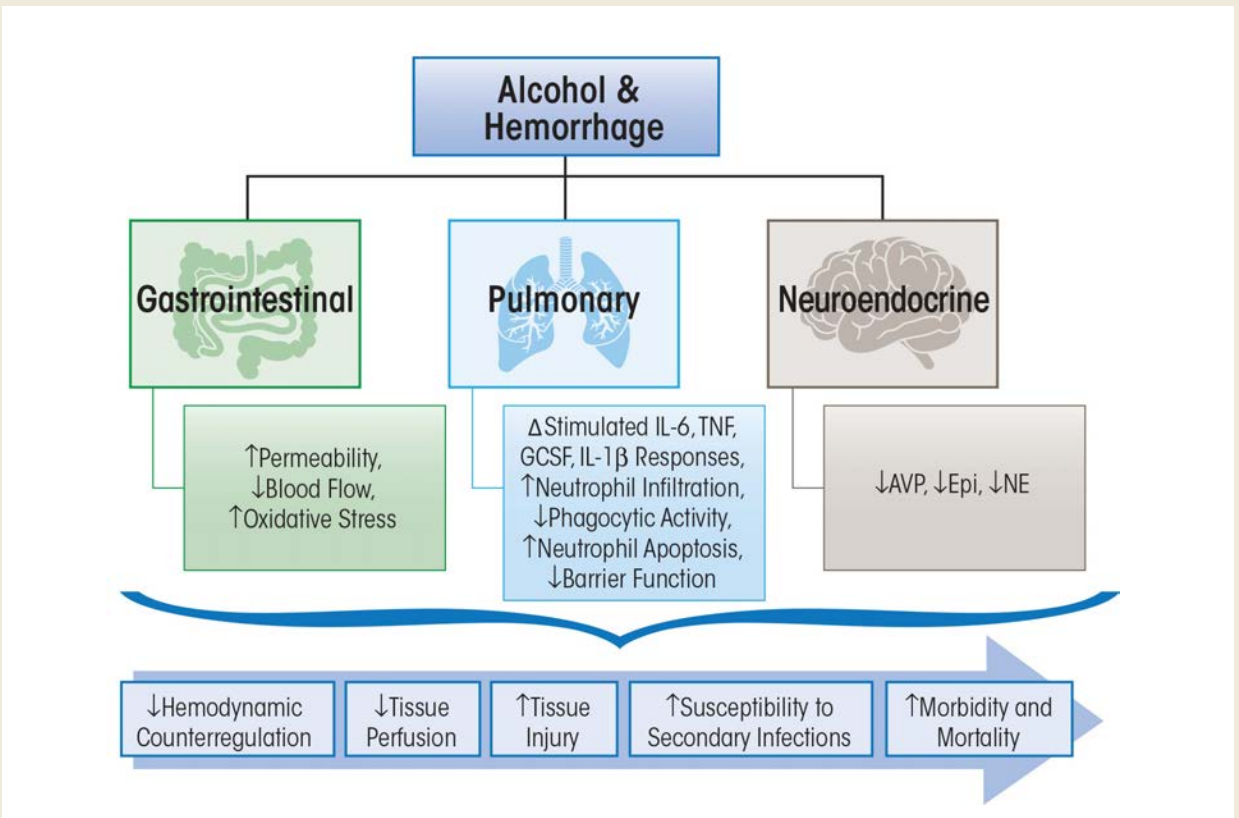

Figure 2 Salient gastrointestinal, pulmonary, and neuroendocrine pathophysiological consequences of alcohol abuse prior to, or at the time of, hemorrhagic shock. The decreased hemodynamic counterregulatory response leads to decreased tissue perfusion, accentuated oxidative stress, and enhanced tissue injury. In addition, the alcohol/hemorrhaged host shows greater susceptibility to secondary infections leading to increased morbidity and mortality during the post-injury period.

decreased phagocytic activity, and increased neutrophil apoptosis in hemorrhaged animals that were alcohol intoxicated at the time of injury (Zambell et al. 2004). This was associated with greater lung bacterial counts and prolonged elevation in TNF-α and IL-6 levels (18 h) post-infection. Furthermore, only 30 percent of alcohol-intoxicated, hemorrhaged animals survived compared with 70 percent survival of dextrose/hemorrhage animals (Zambell et al. 2004). In addition to cytokine dysregulation, alcohol impairs innate barrier functions of the lung by increasing epithelial cell permeability and altering the function of the ciliated epithelium (Elliott et al. 2007; Molina et al. 2010).

Neuroendocrine System

The pathophysiology of traumatic-hemorrhagic injury involves decreased blood volume (i.e., hypovolemia) and hypoperfusion, which results in signaling to central cardiovascular centers aimed at restoring hemodynamic stability through activation of descending autonomic neuroendocrine pathways (Molina 2005). Several mechanisms have been proposed to account for the increased hypotension and impaired hemodynamic stability observed with alcohol intoxication, with one proposed mechanism being blunted neuroendocrine activation. Studies demonstrated that acute alcohol intoxication at the time of injury results in significant attenuated release of counterregulatory hormones and potent vasoconstrictors such as arginine vasopressin (AVP), epinephrine, and norepinephrine in response to fixed-pressure hemorrhage (Phelan et al. 2002). A disruption in the neuroendocrine response with alcohol intoxication at the time of injury is associated with enhanced expression of lung and spleen TNF-α as well as suppression of circulating neutrophil function, which would be expected to enhance the risk for tissue injury (Whitaker et al. 2010). Conversely, Sato and colleagues (2013) demonstrated that alcohol aggravates hemorrhagic shock in a dose-dependent manner not by triggering an immune response but by suppressing hormonal and neurohumoral responses, thereby inhibiting hemodynamic auto-regulation and shortening the survival interval. Thus, both alcohol and hemorrhagic shock have detrimental effects on neuroendocrine responses that are likely to modulate the host immune system in addition to impacting on hemodynamic stability and recovery and accentuating tissue hypoperfusion and end-organ injury.

Alcohol and Traumatic Brain Injury

Traumatic brain injury (TBI) accounts for approximately 50 percent of all trauma-related mortality (Centers for Disease Control and Prevention 2012*b*). TBI affects multiple sectors of the population, and young males have the highest rates of hospital visits and death (Faul et al. 2010). Falls are the first leading cause of TBI, followed by motor vehicle accidents and unintentional trauma sustained during sports activities such as football or boxing. TBI can be categorized as mild, moderate, or severe, and the majority of TBIs sustained in the United States are in the mild category (Centers for Disease Control and Prevention 2012*b*). In addition to the physical dysfunction caused by injury, TBI patients frequently experience lingering psychological symptoms, such as heightened anxiety, depression, sleep disturbances, and pain hypersensitivity (Whyte et al. 1996). These symptoms have been implicated in increased alcohol intake following TBI in humans (Adams et al. 2012). Furthermore, it is well accepted that alcohol consumption increases the risks of sustaining a TBI (Corrigan 1995; Hurst et al. 1994). Nevertheless, a comprehensive understanding of the influences of alcohol on TBI-induced inflammation, recovery from injury, and long-term damage currently is limited and is summarized in the following section (see figure 3).

Neuroinflammation

The pathophysiology of TBI involves a primary mechanical injury followed by a secondary tissue injury resulting from neuroinflammation (Werner and Engelhard 2007). A large percentage of TBI victims show signs of further deterioration following the event (Sauaia et al. 1995). This suggests the induction of a secondary brain injury and immune activation as the key cascades contributing to the pathophysiological processes of the secondary damage (Cederberg and Siesjo 2010). After TBI, a series of events occurs, including the activation of resident immune cells such as astrocytes and microglia, release of pro-inflammatory cytokines and chemokines, upregulation of endothelial adhesion molecules, and recruitment and activation of blood-derived leukocytes across the disrupted blood brain barrier (Feuerstein et al. 1998; Morganti-Kossmann et al. 2001; Ransohoff 2002). An increase in the levels of TNF-α in the serum or cerebrospinal fluid in victims of TBI also has been detected in rodents following closed head injury (Goodman et al. 1990; Ross et al. 1994; Shohami et al. 1994). IL-1β is released after TBI (Fan et al. 1995) and induces nuclear factor-kappa B (NF-κ B), a key transcription factor that regulates the expression of genes encoding cytokines, as well as inducible NO synthase (iNOS), and cyclooxygenase-2 (COX-2) (Blanco and Guerri 2007; Woodroofe et al. 1991; Ziebell and Morganti-Kossmann 2010). Following the rise of early cytokines, the release of IL-6 is associated with increased acute-phase proteins, as well as blood–brain barrier disruption (Kossmann et al. 1995; Shohami et al. 1994; Woodcock and Morganti-Kossmann 2013) and sustained elevation of chemokines such as chemokine (C-C motif) ligand-2 (CCL-2) in the cerebrospinal fluid for as long as 10 days post-injury (Semple et al. 2010). Although early

cytokine release is essential in mediating the reparative processes after injury (Ziebell and Morganti-Kossmann 2010), sustained elevation of pro-inflammatory mediators has been increasingly recognized to play a role in neuropathological changes associated with long-term degenerative diseases (Fan et al. 1995; Lyman et al. 2014). Accordingly, the additional risks of alcohol as a factor contributing to the alterations of TBI-induced neuroinflammatory processes may affect the overall recovery.

Alcohol exerts a profound impact on neuroinflammation. Although there are some conflicting reports in the literature about the role of alcohol on recovery, the major findings are summarized here. Some animal studies suggest that acute alcohol administration prior to TBI leads to an early reduction in the levels of pro-inflammatory cytokines and chemokines in the injured cortex, hippocampus, and hypothalamus, as well as in the serum shortly after TBI (Goodman et al. 2013; Gottesfeld et al. 2002). Recent studies also have confirmed that acute alcohol intoxication at the time of TBI does not exacerbate the expression of pro-inflammatory cytokines and chemokines at 6 hours post-injury. However, results obtained at a later time point (24 hours) show a sustained mRNA expression of IL-1β, TNF-α, IL-6, and CCL-2 following a lateral fluid percussion injury in rodents that were alcohol-intoxicated at the time of TBI (Teng and Molina 2014). Overall, some preclinical studies suggest that acute alcohol treatment prior to TBI may lead to a suppressed release of pro-inflammatory mediators during the early phase post-injury. Thus, the temporal pattern of neuroinflammatory responses and the impact of alcohol intoxication on neuroinflammatory responses are factors to consider when drawing conclusions on the role of alcohol in modulating the outcome from TBIs.

Because the literature surrounding the relationship between acute alcohol intoxication and response to trauma is conflicting, it is important to consider the pattern of alcohol abuse and the

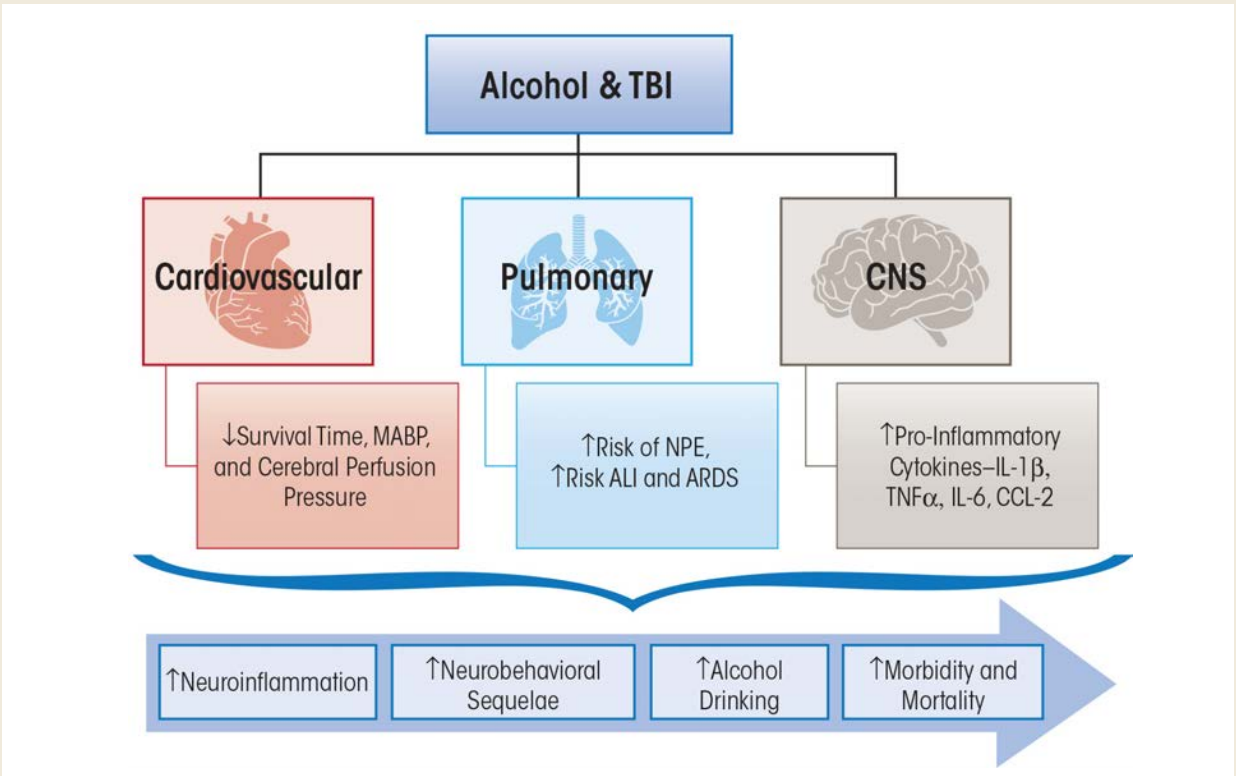

Figure 3 Salient cardiovascular, pulmonary, and central nervous system pathophysiological consequences of alcohol abuse prior to, or at the time of, traumatic brain injury (TBI). The disruption in hemodynamic homeostasis resulting from TBI contributes to decreased cerebral perfusion pressure. The lung is affected through neurogenic mechanisms leading to neuropulmonary edema (NPE) and associated risk for acute lung injury (ALI) and adult respiratory distress syndrome (ARDS). In the brain (CNS), alcohol accentuates neuroinflammation, which is associated with neurobehavioral dysfunction that can potentially promote alcohol drinking. Together, these pathophysiological consequences increase morbidity and mortality from TBI.

model used in different studies. In general, reports in the literature indicate that chronic alcohol exposure produces immune activation in the brain, inducing an enhanced pro-inflammatory state, as evidenced by the presence of CCL-2 and microglial activation in postmortem brains of human alcoholics (He and Crews 2008). Animal studies show that chronic, intermittent binge alcohol administration to rodents results in increased microglial activation and inflammatory cytokine expression in the cortex and hippocampus (Zhao et al. 2013). In addition, Crews and colleagues (2004) have found that chronic alcohol treatment induces expression of inflammatory cytokines such as TNF-α, which further activates resident glial cells to secrete additional pro-inflammatory cytokines and chemokines, resulting in an increased immune activation in the brain. The overall pro-inflammatory effects of alcohol also have been shown by Guerri and colleagues (2007) who reported alcohol-mediated stimulation of TLR-4 and IL-1 receptor signaling pathways, including extracellular regulated-kinase 1/2 (ERK1/2), stress-activated protein kinase/c-Jun N-terminal kinases (JNK), and p38 mitogen-activated protein kinase (MAPK), as well as the expression of NF-kB, activator protein-1 (AP-1), iNOS, and COX-2 in cultured glial cells (Alfonso-Loeches et al. 2010; Fernandez-Lizarbe et al. 2009). The role of TLR4 has been identified in studies where 5 months of chronic alcohol administration increased glial activation and levels of caspase-3, iNOS, COX-2, and cytokines (IL-1β, TNF-α, and IL-6) in the cerebral cortex of wild-type mice but not in the TLR-4–deficient mice (Alfonso-Loeches et al. 2010). Another mediator of alcohol-mediated neuroinflammation is high-mobility group protein B1 (HMGB1), which has been reported to be increased along with TLR-2, TLR-3, and TLR-4 in postmortem brains of human alcoholics (Alfonso-Loeches et al. 2010). Despite a substantial amount of evidence showing increased neuroinflammatory responses to chronic alcohol exposure, there have not been sufficient preclinical studies performed to determine the combined effect of chronic alcohol consumption and TBI on neuroimmune activation. Because both TBI and alcohol can induce inflammation in the brain, we speculate that the combination of the two events would further accentuate neuroinflammation.

Alcohol combined with traumatic injury can significantly affect morbidity and mortality through disruption in host immune responses.

Retrospective studies have revealed that outside of the central nervous system, peripheral organ damage can contribute to the increased mortality rate among TBI patients as a result of cardiovascular, pulmonary, and endocrine dysfunction (Gennarelli et al. 1989; Shavelle et al. 2001). More specifically, TBI patients have an increased incidence of ALI, pulmonary infection, neuroendocrine alterations, and cardiovascular dysfunction during the post-injury period (Vermeij et al. 2013). Although the combined effects of alcohol and TBI and the role of local or systemic immune responses in peripheral organs are understudied, the current knowledge is summarized below (figure 3).

Pulmonary System

ALI, one of the most common nonneurologic complications following TBI, results from acute pulmonary edema and inflammation and can lead to ARDS (Holland et al. 2003; Johnson and Matthay 2010). ALI is characterized by hypoxemia, loss of lung compliance, and bilateral chest infiltrates (Dushianthan et al. 2011). Development of ALI post-TBI has been associated with increased inpatient mortality following injury and worse long-term neurologic outcome in survivors of TBI (Bratton and Davis 1997; Holland et al. 2003). Post-TBI medical interventions including induced systemic hypertension and mechanical ventilation can result in nonneurogenic ALI (Contant et al. 2001; Lou et al. 2013). Development of neurogenic pulmonary edema (NPE) occurs minutes to hours following TBI and typically resolves within days (Bratton and Davis 1997). The possible underlying factors in NPE are the severity of injury leading to increased intracranial pressure and the subsequent increased circulating catecholamines (Demling and Riessen 1990). TBI also is associated with greater incidence of pulmonary infections than that seen following major surgeries, burn injury, and polytrauma (Dziedzic et al. 2004). Clinical reports indicate that over 40 percent of TBI patients with artificial ventilation develop pneumonia and are four times more likely to die from pneumonia (Harrison-Felix et al. 2006). The increased risk of developing pneumonia post-TBI is potentially attributed in part to a systemic immune response syndrome (SIRS) characterized by increased circulating pro-inflammatory cytokines (TNF-α and IL-6) (Keel and Trentz 2005; Kossmann et al. 1995).

The combined impact of alcohol and TBI on pulmonary infections has been minimally investigated. Although, epidemiological studies have shown that in trauma patients, chronic alcohol abuse can independently increase the risk of ALI and ARDS two- to four-fold (Guidot and Hart 2005). In a prospective study of traumatic injury patients with evidence of acute alcohol intoxication or chronic alcohol abuse, chronic alcohol was associated with increased incidence of pneumonia or respiratory failure as a result of its immunosuppressive effects. However, no significant increase in incidence of pneumonia or respiratory failure and

mortality was observed in patients with acute alcohol intoxication with BAC above 100mg/dL (De Guise et al. 2009; Jurkovich et al. 1993). The importance of length and amount of pre-existing alcohol intake and TBI severity may be the key factors in determining a patient's risk for pneumonia. Taken together, the potential effects of chronic alcohol abuse and TBI could potentiate and further increase immunosuppression or immune dysfunction, thus leading to greater susceptibility for pneumonia, ARDS, and ultimately death.

Neuroendocrine System

TBI can lead to a variety of neuroendocrine abnormalities, such as gonadotropin deficiency, growth hormone deficiency, corticotrophin deficiency, and vasopressin alterations (Behan and Agha 2007; Powner and Boccalandro 2008). As a result of the mechanical compression to the pituitary gland or disruption of the pituitary stalk, hypopituitarism can occur and corticotrophin insufficiency is commonly observed after TBI (Agha et al. 2004; Cohan et al. 2005). Excessive alcohol use also has been reported to be associated with neuroendocrine dysfunction, notably in the form of altered regulation of hypothalamic–pituitary–adrenal axis (HPA), resulting in a decreased corticotrophin release (Behan and Agha 2007; Helms et al. 2014). Therefore, it is possible that the combination of alcohol and TBI-induced HPA dysfunction can lead to a dampened cortisol release, which may have an impact on the immune system. Interestingly, a hyperadrenergic state marked by elevated levels of catecholamines can occur after TBI, and alcohol intoxication at the time of TBI has been shown to blunt the sympatho-adrenal activation in a dose-dependent manner (Woolf et al. 1990). Vasopressin has been suggested to play a role in blood brain barrier disruption, edema formation, and the production of pro-inflammatory mediators after TBI (Szmydynger-Chodobska et al. 2010). Vasopressin abnormalities leading to diabetes insipidus or the syndrome of inappropriate anti-diuretic hormone (SIADH) frequently are observed after TBI (Behan and Agha 2007), and acute alcohol intoxication is known to alter AVP release (Taivainen et al. 1995). Whether alcohol intoxication at the time of TBI or during the recovery period from TBI further dysregulates these neuroendocrine mechanisms remains to be examined.

Cardiovascular System

Cardiovascular complications including slow heart rate (i.e., bradycardia), hypotension, electrocardiographic changes, arrhythmias, and increased circulating cardiac enzymes have been reported following TBI (Bourdages et al. 2010; Wittebole et al. 2005). Chronic alcohol abuse alone can lead to alcoholic cardiomyopathy and potentially heart failure (Skotzko et al. 2009), and the underlying etiology has been reviewed (Lang et al. 2005). Several studies by Zink and colleagues (1998*a*,*b*, 2006) focused on the combined effects of acute alcohol intoxication on hemorrhagic shock and TBI in swine, showing decreased survival time, lowered MABP, and reduced cerebral perfusion pressure, which may worsen secondary brain injury. These studies did not investigate alterations in immune function or expression and levels of immune modulators or their actions on cardiovascular function. Overall, the post-TBI cardiovascular complications, including vascular function, have been understudied in both clinical and experimental models of TBI. More specifically, the combined impact of alcohol, TBI, and immune alterations on cardiovascular dysfunction and disease progression has not been examined. A possible prediction is that chronic alcohol-induced immunosuppression would worsen post-TBI cardiovascular complications; and in chronic alcoholics, dilated cardiomyopathy may compound TBI-related cardiovascular complications increasing morbidity and mortality.

Summary

The deleterious effects of alcohol on the immune system in three traumatic injuries are discussed in this review and are summarized in figures 1, 2, and 3. It is evident that, independently, acute or chronic alcohol consumption and traumatic injury negatively modulate the immune system, and the end result is an uncontrolled release of inflammatory mediators. The most important message of this review is the accumulation of evidence that alcohol combined with traumatic injury can significantly affect morbidity and mortality through disruption in host immune responses. Following burn injury, for instance, the risk for infection is greatly increased because of increased gut permeability and increased pro-inflammatory cytokine expression in the lungs (figure 1). Alcohol use following hemorrhage can increase inflammation and oxidative stress in the gut while decreasing lung barrier function and subsequently increasing susceptibility to infection (figure 2). In the central nervous system, alcohol use following TBI can increase neuroinflammation and prolong the recovery period (figure 3). Overall this information is important, because it provides a wealth of evidence that alcohol combined with trauma is a dramatic and preventable cause of increased morbidity and mortality following injury. Mechanistically, two common pro-inflammatory cytokines that are consistently upregulated in all burn injury, hemorrhagic shock, and TBI are TNF-α and IL-6. A fuller understanding of their temporal pattern of expression and downstream effects requires further investigation. Although the studies described in this review have generated important information on the impact of alcohol combined with different types of traumatic injury, and the resultant adverse effects on the immune system, further preclinical

and clinical studies to dissect the complex cascade of immunomodulation following injury are necessary. Specifically, further investigation is warranted to determine the underlying mechanisms involved in immune modulation by acute or chronic alcohol intake and the effects on (1) metabolism and the cardiovascular system following burn, (2) the neuroendocrine system following hemorrhagic shock, and (3) neuroinflammation and the neuroendocrine system following traumatic injury. The responses of the immune system to these inflammatory stimuli are variable and appear to be dependent on the severity of the injury, comorbidities, and the level of alcohol intoxication. Thus, it is necessary to systemically address these variables for translational research to identify potential therapeutic strategies. Furthermore, therapeutic targets for immunomodulation and attenuation of tissue injury in intoxicated and injured patients are likely to reduce morbidity and mortality and improve post-injury quality of life among these patients.

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Macrophages and Alcohol-Related Liver Inflammation

Cynthia Ju, Ph.D., and Pranoti Mandrekar, Ph.D.

Recent studies have suggested that macrophages have a critical role in the development of alcohol-induced inflammation in the liver. To define the precise pathogenic function of these cells during alcoholic liver disease (ALD), it is extremely important to conduct extensive studies in clinical settings that further elucidate the phenotypic diversity of macrophages in the context of ALD. Research to date already has identified several characteristics of macrophages that underlie the cells' actions, including macrophage polarization and their phenotypic diversity. Other analyses have focused on the contributions of resident versus infiltrating macrophages/monocytes, as well as on the roles of macrophage mediators, in the development of ALD. Findings point to the potential of macrophages as a therapeutic target in alcoholic liver injury. Future studies directed toward understanding how alcohol affects macrophage phenotypic switch in the liver and other tissues, whether the liver microenvironment determines macrophage function in ALD, and if targeting of macrophages alleviates alcoholic liver injury, will provide promising strategies to manage patients with alcoholic hepatitis.

Key words: Alcohol consumption; alcoholic liver disease; alcoholic liver injury; alcoholic hepatitis; alcohol-related liver inflammation; liver; immunity; innate immune response; adaptive immune response; macrophage; macrophage phenotypic switch; Kupffer cell

Alcoholic liver disease (ALD) is a complex disease that affects millions of people worldwide and eventually can lead to liver cirrhosis and liver cancer (i.e., hepatocellular carcinoma). Aside from the direct cytotoxic and the oxidative-stress–mediated effects that alcohol and its metabolite, acetaldehyde, exert on hepatocytes, alcohol ingestion activates both the innate and adaptive immune responses in the liver. These responses involve multiple hepatic cell types, including resident macrophages, natural killer cells, natural killer T cells, lymphocytes, and neutrophils. In particular, resident macrophages in the liver, also known as Kupffer cells, are important for clearing pathogens, including bacteria, viruses, immune complexes, bacterial products called endotoxin or lipopolysaccharide (LPS), and tumor cells, from the liver (Jenne and Kubes 2013; Thomson and Knolle 2010). Research tools such as fate mapping, multifocal microscopy, transgenic/reporter mouse models, and next-generation sequencing recently have led to a better understanding of the origins, heterogeneity, and plasticity in the phenotypes and functions of macrophages and their circulating precursor cells (i.e., monocytes).

The activation of circulating monocytes and accumulation of macrophages in the liver are important pathophysiological features in patients with ALD. However, the role of hepatic macrophages in the pathogenesis of ALD has not been fully elucidated. This review will discuss some of the new findings in monocyte/macrophage biology, provide an update of the current studies on the involvement of liver macrophages in ALD, and identify remaining questions to be addressed in order to develop macrophage-targeted therapy for ALD.

Phenotypic and Functional Heterogeneity of Monocytes and Macrophages

Macrophages, which play an important role in the initial innate immune response to infection with pathogens or other insults, fall into two main categories—infiltrating macrophages and tissue-resident macrophages. Infiltrating macrophages are derived from precursor cells called monocytes that circulate throughout the body and are recruited into the tissues when an inflammatory reaction occurs. Tissue-resident macrophages, in contrast, always remain localized within one tissue, serving as sentries and first line of defense against any infection or injury in that tissue.

Monocytes are circulating innate immune cells formed from progenitor cells in the bone marrow; the monocytes then differentiate into numerous subsets of macrophages (Fogg et al. 2006). In both humans and mice, monocytes can be divided into two major subsets—classical and nonclassical—depending on the marker proteins that they exhibit on their surface (Ingersoll et al. 2011; Sunderkotter et al. 2004; Ziegler-Heitbrock 2007) (see table 1). In humans, monocytes (all of which express $CD115^{+}$) are divided into the two major subsets based on their CD14 and CD16 expression, as well as on their expression of markers called CCR2 and CX3/CR1:

- The predominant subset, representing 90 percent of circulating monocytes, is the classical subset characterized by the marker combination $CD14^{hi}CD16^{-}$ and $CCR2^{+}/CX3CR1^{lo}$.

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- The less abundant nonclassical monocyte subset further can be divided into two groups characterized by the marker combinations $CD14^{dim}CD16^{+}$ and $CCR2^{-}$/$CX3CR1^{hi}$/$CCR5^{hi}$ (nonclassical monocytes) or $CD14^{hi}CD16^{+}$ and $CCR2^{-}$/$CX3CR1^{hi}$/$CCR5^{hi}$ (intermediate monocytes).

Analogous to their human counterparts, murine subsets include classical $Ly6C^{hi}$ monocytes, which are similar to the human $CD14^{hi}CD16^{-}$ monocytes, and nonclassical $Ly6C^{lo}$ monocytes, which are similar to human $CD14^{dim}CD16^{+}$ monocytes. These human and murine cells patrol in the blood vessels until they are recruited to the organs in case of an injury or insult. Although the gene-expression profiles related to activation and trafficking have been well conserved between murine and human monocytes, the ratios of various subsets can differ between mice and humans. Therefore, it is important to be careful and take these differences into consideration when extending experimental murine monocyte studies to human disease.[1]

Resident macrophages can be found in a variety of tissues, such as the brain, skin, lungs, liver, and spleen. Tissue-resident macrophages exhibit a large diversity of phenotypes and functions, based on their tissue of residence, raising the question of the origin of these cells (Davies et al. 2013). Recent fate-mapping studies have revealed that embryonic yolk sac and/or fetal liver progenitor cells are the source of many tissue-resident macrophages, such as those in the liver (i.e., Kupffer cells), skin, and central nervous system (i.e., microglia) (Gomez Perdiguero and Geissmann 2013). Tissue-resident macrophages are defined as a heterogeneous population of immune cells important for maintaining the homeostatic function of the specific tissue (Davies et al. 2013). Whether tissue macrophages are self-renewing or continuously replenished from the bone marrow still is a matter of debate. However, overwhelming evidence suggests that bone-marrow–derived circulating monocytes can be recruited to the site of injury early during inflammation in tissues, where they differentiate into macrophages. Classical and nonclassical monocytes are recruited in a sequential fashion, depending on the nature of insult (e.g., infection or infarction) and the injured tissue. Additionally, both resident macrophages and recruited monocytes reportedly are capable of self-renewal induced by certain cytokines, such as interleukin (IL)-4 (Jenkins et al. 2011).

The Kupffer cells in the liver are the largest population of tissue-resident macrophages and largely contribute to inflammatory reactions in the liver. The innate immune function of Kupffer cells not only is critical in the body's response to liver injury but also is crucial in tolerogenic responses to antigens in the liver. Kupffer cells are located in the hepatic sinusoids and fall into two major subsets (Klein et al. 2007):

[1] Comprehensive transcriptomic analysis on unique human and murine immune-cell gene expression during differentiation, activation, and tissue-specific localization is available from project consortia such as ImmGen (www.immgen.org) and InnateDB (www.innatedb.ca).

Table 1 Monocyte Populations of Human and Mouse Origin

Monocytes		Markers	Function
Human	Classical	$CD14^{hi}CD16^{-}CCR2^{+}CX3CR1^{lo}$	Phagocytosis and inflammatory effectors
	Intermediate	$CD14^{hi}CD16^{+}CCR2^{-}CX3CR1^{hi}$	Inflammatory effectors
	Nonclassical	$CD14^{dim}CD16^{+}CCR2^{-}CX3CR1^{hi}$	Patrolling, antiviral role
Mouse	Classical	$CD11b^{+}Ly6C^{hi}CCR2^{+}CX3CR1^{-}$	Inflammatory effectors
	Nonclassical	$CD11b^{+}Ly6C^{lo}CCR2^{-}CX3CR1^{+}$	Patrolling, tissue repair

- Radiosensitive macrophages that are replaced rapidly by hematopoietic precursors and are important in inflammatory reactions; and
- Radioresistant, long-lived Kupffer cells that do not participate in inflammatory foci.

Mouse models frequently are used to investigate various aspects of macrophage function. However, as with the monocyte precursors, differences in the characteristics of murine and human macrophages exist that must be taken into account when using mice as preclinical models of disease (Mestas and Hughes 2004). For example, murine and human macrophages can differ in the expression of surface molecules called Toll-like receptors (TLRs) that are involved in macrophage activation, in their responses to immune activators, and in their production of nitric oxide.

Macrophage Polarization

Macrophages have a unique ability to alter their phenotypes and, thus, their functions, depending on tissue microenvironmental cues, such as the presence of cytokines, growth factors, pathogen-associated molecular pattern molecules (PAMPs), and damage-associated molecular pattern molecules (DAMPs). This process is known as polarization and results in the emergence of two macrophage phenotypes labeled M1 and M2 macrophages. M1 macrophages primarily have proinflammatory effects. For example, classically activated M1 macrophages help mediate the initial defense against intracellular bacteria and viruses; in addition, they are important for the response to a tissue injury. The M1 macrophages produce proinflammatory and stress mediators and cytokines, such as IL-1, tumor necrosis factor alpha (TNFα), interferon γ, IL-12, IL-18, nitric oxide, and reactive oxygen species (ROS), and can activate adaptive immune responses (Jouanguy et al. 1999; Shaughnessy and Swanson 2007). Once the infection or injury is

controlled, macrophages convert to an anti-inflammatory, tissue-restorative phenotype in order to reign in excessive tissue-damaging inflammatory responses (Benoit et al. 2008; Noel et al. 2004). These cells usually are referred to as alternatively activated macrophages (M2) and help promote the resolution of inflammation as well as tissue repair (Sica and Mantovani 2012) (see figure 1). They can be distinguished from the M1 macrophages by the presence of high levels of several marker proteins (e.g., Fizz1, Mrc1, Ym1, and Arg1) (Gordon 2003; Mantovani et al. 2002).

The functional heterogeneity of macrophages is reflected in their differential, sometimes opposing, roles in various diseases (Sica and Mantovani 2012). For example, whereas M1 cells are essential for eliminating bacteria and viruses during acute infection, a dysregulated M1 response can result in collateral tissue damage. Thus, the proinflammatory function of M1 macrophages contributes to conditions such as autoimmune diseases (e.g., arthritis and multiple sclerosis) and metabolic diseases (e.g., insulin resistance, diabetes, and atherosclerosis). Similarly, although M2 macrophages often are associated with tissue repair and immune regulation, excessive M2 responses can contribute to chronic diseases such as atopic dermatitis, asthma, and tissue fibrosis. Additionally, diseases characterized by changes in the phenotype of the cells over time resulting from changes in the tissue environment also may be accompanied by a switch in macrophage phenotype (i.e., macrophage plasticity). For example, during early stages of cancer, tumor-associated macrophages resemble the classically activated M1 cells, which promote anti-tumor immune responses. As the tumor progresses, however, these tumor-associated macrophages switch to a regulatory phenotype that suppresses anti-tumor immunity and facilitates tumor growth (Allavena et al. 2008). As another example, adipose-tissue macrophages in nonobese individuals primarily exhibit a wound-healing phenotype, with little production of proinflammatory cytokines. In obese patients, however, the adipose-tissue macrophages switch to a proinflammatory M1-like phenotype characterized by cytokine production that leads to insulin resistance (Zeyda and Stulnig 2007).

Although it is convenient to divide macrophages into M1 and M2 cells, it is important to note that this division is oversimplified. The M1 and M2 macrophages only represent the two extremes of a full spectrum of phenotypes, and within either category there are subpopulations with different phenotypes and functions. For example, the M2 cells can be classified into at least two subtypes, wound-healing and immune-regulatory macrophages (Edwards et al. 2006). The wound-healing macrophages develop in response to the cytokines IL-4 and IL-13 that are released by various types of leukocytes. Compared with M1 macrophages, these cells produce much lower levels of proinflammatory cytokines,

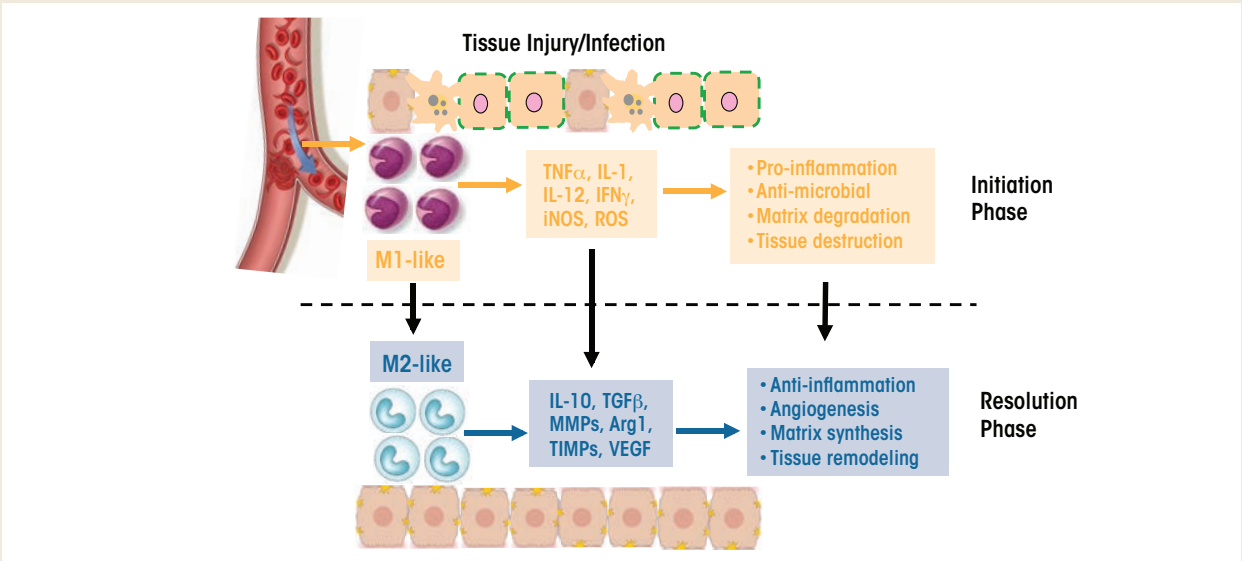

Figure 1 Schematic representation of macrophage plasticity and its involvement in tissue injury. Macrophages recruited to the site of an injury or infection during the initiation phase of the inflammatory reaction have an M1 phenotype. They produce proinflammatory and stress mediators and cytokines, such as tumor necrosis factor α (TNFα), interleukin (IL)-1 and -12, interferon γ (IFNγ), an enzyme generating nitric oxide (iNOS), and reactive oxygen species (ROS). These macrophages have proinflammatory and antimicrobial effects and lead to matrix degradation and tissue destruction. During the resolution phase of the injury, these M1 macrophages are converted into an M2 phenotype with a different cytokine and chemokine repertoire, including IL-10, transforming growth factor β (TGF-β), matrix metalloproteinases (MMPs), arginase 1 (Arg1), tissue inhibitors of metalloproteinases (TIMPs), and vascular epithelial growth factor (VEGF). These M2 macrophages have anti-inflammatory effects and promote blood-vessel formation (angiogenesis), matrix synthesis, and tissue remodeling.

ROS, and nitric oxide but higher levels of molecules that promote tissue regeneration and wound healing (e.g., mannose receptors, extracellular matrix components, and factors regulating matrix remodeling). Conversely, immune-regulatory macrophages arise during late stages of the adaptive immune response or in response to stress-induced upregulation of glucocorticoids. These macrophages are characterized by the production of high levels of IL-10. Factors that induce the generation of immune-regulatory macrophages include immune complexes, prostaglandins, apoptotic cells, adenosine, histamine, and adiponectin. Unlike the wound-healing macrophages, the regulatory macrophages do not induce extracellular matrix remodeling.

The plasticity of macrophage phenotypes is controlled by various intracellular molecular mechanisms, including signaling proteins, transcription factors, and epigenetic events. For example, activation of macrophages via TLRs and interferon receptors, which induces a signaling mechanism involving a molecule called STAT1,[2] steers their polarization toward the M1 phenotype (Qin et al. 2012). Conversely, alternative activation via IL-4/IL-13 and STAT6-mediated mechanisms generates the M2 phenotype (Daley et al. 2010; Moreno et al. 2003; Stolfi et al. 2011). Other M2-like phenotypes are induced via IL-10/STAT3 and IL-3/STAT5 signaling mechanisms (Sica and Mantovani 2012). Another important regulator of macrophage polarization is the enzyme JNK, which phosphorylates STAT6 (Shirakawa et al. 2011). Obese mice deficient in a JNK activator called MLK3 lack M1 macrophage polarization, suggesting a role for JNK in activation of the M1 phenotype (Gadang et al. 2013). IRF proteins, which modulate the transcription of certain genes, also are important regulators of macrophage polarization. For example, IRF5 activity promotes IL-12 gene transcription and is associated with an M1 phenotype, whereas repression of IRF5 induces IL-10, resulting in an M2 phenotype (Krausgruber et al. 2011). Similarly, activation of a regulatory protein complex called Notch/IRF8 leads to M1 polarization (Xu et al. 2012), whereas activation of M-CSF/IRF4 leads to M2 polarization. Another family of proteins called SOCSs also serves as essential regulators of macrophage polarization, with the specific cytokine stimulus and SOCS isoform involved determining whether the cells attain an M1 or M2 phenotype. Thus, the presence of IL-4 acting on SOCS1/STAT1 induces an M1 phenotype (Whyte et al. 2011), whereas interferon γ acting in concert with TLR can induce SOCS3/STAT3 and result in M2 macrophage polarization (Arnold et al. 2014). Various receptors located in the cells' nucleus, such as molecules called PPARγ, PPARδ, Krupple like factor-4, and c-myc also contribute to macrophage polarization downstream of the IRF/STAT-SOCS pathway (Zhou et al. 2014). Finally, regulatory processes that affect DNA structure and gene expression without altering the DNA sequence (i.e., epigenetic mechanisms) promote the induction of an M2 phenotype and inhibit M1-characteristic genes (Banerjee et al. 2013; Satoh et al. 2010). These epigenetic regulators include such factors as histone demethylase, Jumonji D3, and microRNA let-7c.

In ALD, macrophage imprinting and polarization to M1 or M2 phenotypes is influenced by cytokine mediators in the liver. The detailed investigation of pathways activated by cytokines and stress proteins in the liver during ALD will provide insights into the polarization of resident versus infiltrating liver macrophages.

Macrophages in ALD

Significance of Macrophages in Clinical ALD

Macrophages seem to play a central role in ALD. In fact, recent findings suggest the coexistence and complex inter-

Table 2 Complete Names of Enzymes and Other Molecules Mentioned in This Article and Their Abbreviations

Abbreviation	Complete Name
CCR2	C-C chemokine receptor 2
CD	Cluster of differentiation
CX3/CR1	C-X3-C motif chemokine receptor 1
ERK	Extracellular-signal–regulated kinase
IKK	Inhibitor of nuclear factor kappa-B kinase
IL	Interleukin
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
JNK	C-jun N-terminal kinase
LPS	Lipopolysaccharide
MCP	Monocyte chemoattractant protein
M-CSF	Macrophage colony-stimulating factor
MIP	Macrophage inflammatory protein
MLK	Mixed lineage kinase
PD-1	Programmed cell death protein 1
PPAR	Peroxisome proliferator-activated receptor
SOCS	Suppressor of cytokine signaling
STAT	Signal transducers and activators of transcription
TGF	Transforming growth factor
TIM-3	T-cell immunoglobulin mucin-3
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha

[2] For a list of the complete names of this and other molecules mentioned in this article, see table 2.

actions of different types of macrophages in ALD (Lee et al. 2014). Thus, immunohistochemical analyses of liver samples from patients with alcoholic steatohepatitis identified macrophages that express receptors and cytokines commonly associated with M1 cells, as well as markers associated with M2 cells. Numerous other analyses have indicated that macrophage function may be clinically correlated with disease state in patients with alcoholic hepatitis and fibrosis, as follows:

- Increased macrophage numbers have been reported in both early (i.e., fatty liver) and late (i.e., hepatitis and cirrhosis) stages of ALD (Karakucuk et al. 1989), although no clear correlation exists between macrophage numbers and disease severity.

- The levels of chemokines involved in monocyte recruitment, particularly MCP-1, MIP-1α, and MIP-1β, were increased in the liver of patients with ALD (Afford et al. 1998).

- In analyses of gene-expression profiles, the expression of inflammatory genes was higher in macrophages from patients with alcohol-related cirrhosis than in macrophages from patients with Hepatitis C virus–related cirrhosis (Tapia-Abellan et al. 2012).

- Factors that imply monocyte activation, such as neopterin and leukocyte-function–associated antigen 3, were elevated in ALD patients (Luna-Casado et al. 1997).

- Circulating monocytes from ALD patients express TNFα receptors and spontaneously produce TNFα. When stimulated by LPS, they release even higher levels of TNFα (Gobejishvili et al. 2006; Zhang et al. 2001). Highly elevated TNFα levels in the blood, in turn, are associated with poorer outcomes in patients with acute alcoholic hepatitis (Bird et al. 1990). In some cases, normal levels of the anti-inflammatory cytokine IL-10 were linked to a failure to inhibit the excessive production of TNFα (Le Moine et al. 1995).

- Patients with alcoholic hepatitis and/or cirrhosis exhibit elevated levels of other cytokines (e.g., IL-6, IL-8, and IL-18) and chemokines produced by circulating monocytes and liver macrophages (Afford et al. 1998; Fisher et al. 1999). These increased cytokine levels are correlated with clinical outcomes (Khoruts et al. 1991; McClain and Cohen 1989).

- Global gene-expression profiling of liver samples from patients with alcohol-related cirrhosis demonstrated unique gene-expression patterns that differed between early and late stages of cirrhosis. Genes expressed at much higher levels in early than late stage of cirrhosis included those related to macrophage activation, proliferation, and migration (Lederer et al. 2006), emphasizing the role of macrophages in the progression of ALD.

Additional clinical studies evaluating macrophages and circulating monocytes from human patients at different stages of ALD are needed to understand the precise functional contributions of monocytes/macrophages to disease progression.

Role of Kupffer Cells in ALD

Kupffer cells are liver-resident macrophages that are activated through the CD14/TLR4 receptor complex in response to increased intestinal translocation of LPS during prolonged alcohol consumption and which may contribute to alcohol-induced liver injury. Animal studies have revealed that acute and chronic ethanol administration are associated with signs of CD14/TLR4 activation of macrophages in the liver, including upregulation of CD14 as well as increased production of TNFα, MCP-1, and ROS (Enomoto et al. 2001). Furthermore, depletion of liver macrophages through various approaches prevented alcohol-induced liver inflammation (Koop et al. 1997; Petrasek et al. 2012), confirming that the cells are needed to induce liver injury.

Researchers have investigated how alcohol consumption may trigger Kupffer-cell activation. ROS production may be one of the mechanisms contributing to increased sensitization of Kupffer cells to LPS in the alcoholic liver (Thakur et al. 2006). During prolonged alcohol exposure, Kupffer cells produce ROS, likely mediated by induction of an enzyme involved in alcohol metabolism in the liver (i.e., cytochrome P450 2E1) (Kono et al. 2000). The crucial role of ROS production in Kupffer-cell activation also was demonstrated in studies in which rats were pretreated with an agent that inhibits an enzyme essential for ROS production (i.e., NADPH oxidase). This pretreatment normalized ROS production in alcohol-fed rats as well as reduced phosphorylation of the signaling molecule ERK1/2 and inhibited production of the proinflammatory cytokine TNFα in Kupffer cells (Kono et al. 2000; Thakur et al. 2006).

Another essential component in alcohol-mediated Kupffer-cell activation is the CD14/TLR4 receptor complex. LPS-induced activation of this receptor complex on Kupffer cells triggers downstream signaling kinases (i.e., IRAK and IKK), ultimately leading to the induction of the proinflammatory cytokines TNFα, IL-6, and MCP-1. Consistent with this model, Kupffer cells from alcohol-fed mice are sensitized to LPS and exhibit increased LPS responses, leading to higher levels of TNFα (Nagy 2003) and MCP-1 (Mandrekar et al. 2011). Enhanced expression of multiple TLRs also can contribute to ROS-mediated Kupffer-cell sensitization in the alcoholic liver (Gustot et al. 2006). Hritz and colleagues (2008) and Inokuchi and colleagues (2011) confirmed the importance of TLR4 expression on Kupffer cells and bone-marrow–derived immune cells in ALD. However, it is unclear whether liver-resident Kupffer-cell–specific TLR4 is the only TLR

contributing to alcohol-mediated pathogenesis, and this issue requires further investigation using mice deficient in macrophage-specific TLR4. Nevertheless, the findings to date suggest that both alcohol-induced ROS and increased Kupffer-cell sensitization to endotoxin, which lead to enhanced proinflammatory responses, are major players in Kupffer-cell activation in ALD.

Inhibition of Kupffer-cell activation and reduction of proinflammatory cytokines—particularly inhibition of proinflammatory cytokine production by Kupffer cells—has been a major focus of efforts to alleviate ALD. For example, it may be possible to reverse Kupffer-cell sensitization by treating alcohol-exposed Kupffer-cell primary cultures with adiponectin, an anti-inflammatory adipokine (Thakur et al. 2006). Treatment with globular adiponectin prevents LPS-stimulated TNFα expression in Kupffer cells by activating the IL-10/STAT3/hemoxygenase-1 pathway and inducing M2 macrophages (Mandal et al. 2010, 2011). M2 macrophages, in turn, seem to be associated with reduced or limited liver injury, because in current drinkers with mild liver injury and steatosis, M2 macrophages are predominant, whereas patients with severe liver injury exhibit M1 macrophages (Wan et al. 2014). Another possible approach to ALD treatment may involve the desensitization of alcohol-exposed Kupffer cells by increasing IL-10 levels. The alcohol-induced decrease in IL-10 has been shown to contribute to the sensitization of macrophages, and studies in IL-10–deficient mice found increased alcohol-mediated proinflammatory cytokine production (Hill et al. 2002). Recent studies also have indicated an IL-10–mediated protective effect via activation of TLR3 in alcoholic liver (Byun et al. 2013).[3] Selective targeting of TLR signaling pathways in Kupffer cells likely will provide better insights into the contribution of the balance between pro- and anti-inflammatory cytokine production in ALD.

Hepatic Infiltrating Macrophages in ALD

Tissue-resident macrophages, such as Kupffer cells in the liver, not only protect against pathogens but also help nourish and maintain the cells (i.e., exert trophic functions) and ensure tissue homeostasis. However, under stress conditions caused by infection or by inflammation in the absence of infection (i.e., sterile inflammation), additional monocytes infiltrate the damaged tissue and differentiate into macrophages that help clear the pathogens, remove dead cells and cell debris, and restore tissue homeostasis. In fact, in many disease models (e.g., peritoneal inflammation) the tissue macrophages that have been described actually are derived from such infiltrating monocytes (Ghosn et al. 2010).

Studies of acute and chronic liver injuries also have demonstrated the hepatic recruitment of monocytes. For example, acute treatment of mice with carbon tetrachloride (CCl_4), which causes liver damage, results in an influx of infiltrating macrophages that can increase the total number of hepatic macrophages tenfold (Karlmark et al. 2009). A recent study in mice with chronic CCl_4-induced liver fibrosis demonstrated that infiltrating macrophages played an important role in the progression and regression of the fibrosis (Ramachandran et al. 2012). Similarly, in a mouse model of acetaminophen-induced liver injury, infiltrating macrophages recruited during the recovery phase contributed substantially to tissue repair (Holt et al. 2008).

Chronic alcohol-induced liver disease also is mediated and likely propagated by infiltrating immune cells, because chronic ethanol administration can cause accumulation of infiltrating macrophages in the liver of mice (Wang et al. 2014). The infiltrating macrophages consist of two subsets—Ly-6C^{hi} and Ly-6C^{low} cells—with distinct genetic profiles. The Ly-6C^{low} cells exhibit an anti-inflammatory and tissue-protective phenotype, expressing low levels of proinflammatory cytokines and high levels of anti-inflammatory molecules that may be involved in tissue repair (Arnold et al. 2007; Nahrendorf et al. 2007). Conversely, the Ly-6C^{hi} cells exhibit a proinflammatory tissue-damaging phenotype; however, upon phagocytosis of apoptotic hepatocytes, they seem to switch to a Ly-6C^{low} phenotype (Wang et al. 2014). The two subsets of infiltrating macrophages coexist and exhibit distinct, and sometimes opposite, functions in many models of inflammatory tissue injury. In a model of kidney injury, bone marrow Ly-6C^{hi} monocytes were recruited to the injured kidney, where they differentiated into functionally distinct Ly-6C^{low} cells (Lin et al. 2009). In the livers of animals with CCl_4-induced fibrosis, Ly-6C^{low} infiltrating macrophages, which were derived from the Ly-6C^{hi} cells, were important for resolving inflammation and fibrosis and restoring tissue homeostasis (Ramachandran et al. 2012). Studies of the contribution of infiltrating-macrophage subsets in myocardial infarction also have demonstrated sequential recruitment of Ly-6C^{hi} and Ly-6C^{low} cells into the tissue. The proinflammatory Ly-6C^{hi} cells, which infiltrate the tissue during the early phase of injury, have proteolytic and phagocytic functions. At a later phase of the myocardial infarction, Ly-6C^{low} cells are recruited that possess attenuated inflammatory properties and are involved in tissue repair by promoting blood-vessel formation (i.e., angiogenesis) and activation of heart muscle cells (i.e., myofibroblasts) (Nahrendorf et al. 2007).

In humans, an increase in the number (i.e., expansion) of the nonclassical CD14^{+}CD16^{+} monocytes, which correspond to the Ly-6C^{low} infiltrating macrophages, occurs in a variety of inflammatory diseases, including rheumatoid arthritis, atherosclerosis, asthma, atopic eczema, pancreatitis, and alveolar proteinosis. Nonclassical CD14^{+}CD16^{+} monocytes also expand in the circulation and liver of patients with chronic liver disease, suggesting their involvement in the progression of liver inflammation and fibrogenesis (Ziegler-Heitbrock 2007).

[3] In addition to TLR3 and TLR4, increased expression of TLR2 and TLR8 has been identified by immunohistochemistry in liver biopsies from alcoholic hepatitis patients (Lee et al. 2014).

Macrophage Mediators in ALD

The heterogeneous populations of both resident and infiltrating macrophages present in the liver have multiple functions that are relevant to ALD (see figure 2):

- They can serve as antigen-presenting cells that display foreign molecules on their surface, thereby triggering adaptive immune responses.
- They may exhibit liver proteins that have been modified by malondialdehyde-acetaldehyde (i.e., malondialdehyde-acetaldehyde adducts) (Willis et al. 2002). This modification can change or impair the protein's functions. In patients with ALD, these adducts also may be associated with the presence of autoantibodies.
- They normally produce antimicrobial peptides and mediators and have microbial killing activities; however, these functions may be compromised during ALD.
- Through activation of TLR-mediated signaling, they may lead to increased expression of immunoinhibitory receptors called PD-1 and TIM-3 on T cells, thereby impairing antimicrobial activity in patients with alcoholic hepatitis (Markwick et al. 2015).
- Certain subpopulations (e.g. Ly6C^{hi} infiltrating macrophages) produce a variety of proinflammatory mediators, including ROS, reactive nitrogen species, proinflammatory cytokines, and chemokines, thereby causing tissue damage.

Among the mediators identified, the cytokine TNFα has been extensively studied not only in patients with alcoholic hepatitis but also in animal models of ALD (Bird et al. 1990). The analyses found that mice lacking the TNFα receptor were protected from ALD (Yin et al. 1999); moreover, antibodies against TNFα were able to ameliorate alcohol-induced liver injury (Iimuro et al. 1997). Both of these findings indicate that TNFα is crucial in the pathophysiology of ALD. The role of IL-6 in ALD also has been widely investigated. Alcohol-fed, IL-6–deficient mice showed increased liver injury, suggesting a protective role for IL-6 (El-Assal et al. 2004). Additional analyses demonstrated that IL-6 reduces or increases inflammation in ALD in a cell-type–specific manner and exerts its effects via the STAT3 signaling molecule (Horiguchi et al. 2008), confirming the significant contribution of the IL-6/STAT3 axis in the development of ALD.

Other macrophage mediators involved in ALD include the chemokines IL-8, MCP-1, and MIF, which either inhibit leukocytes or help recruit them to the sites of injury and inflammation (Barnes et al. 2013; Mandrekar et al. 2011). Whereas IL-8 induces neutrophil infiltration, MCP-1 and MIF, which primarily are produced by Kupffer cells and infiltrating macrophages, facilitate the recruitment of additional monocytes/macrophages in ALD. Chemokines also induce the activation of stellate cells, which helps promote disease progression to liver fibrosis. Thus, the recruitment

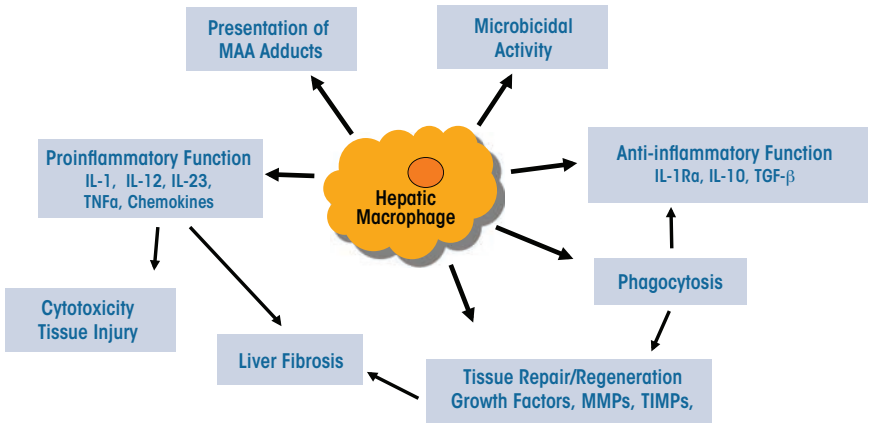

Figure 2 Macrophage functions in alcoholic liver disease. Macrophages fulfill a variety of functions in the context of alcoholic liver disease, including both proinflammatory and anti-inflammatory functions, depending on the state of the disease. These activities include the production of proinflammatory cytokines (e.g., interleukin [IL]-1, -12, and -23; tumor necrosis factor alpha [TNFα]) and chemokines, as well as of anti-inflammatory cytokines (e.g., IL-10, IL-1 receptor a [IL-1Ra], and transforming growth factor beta [TGF-β]). Other relevant activities include presentation of malondialdehyde-acetaldehyde (MAA) adducts and microbicidal and phagocytotic activity, as well as tissue repair and regeneration through the production of growth factors, matrix metalloproteinases (MMPs), and tissue inhibitors of metalloproteinases (TIMPs).

of inflammatory cells sets off a vicious cycle in which inflammatory and stellate cells stimulate one another, leading to fibrosis and cirrhosis (Karlmark et al. 2009). Given the central role that MCP-1 and MIF seem to play in ALD, chemokines can be considered likely therapeutic targets for this condition (Mandrekar et al. 2011; Seki et al. 2009).

Other macrophage mediators in addition to cytokines and chemokines include the complement system and adipokines such as adiponectin and leptin. The C3 and C5 complement systems are activated in macrophages during early phases of murine ALD and contribute to disease initiation and progression (Roychowdhury et al. 2009). Adipokines, in contrast, seem to negatively regulate macrophage function in murine ALD. Identification of novel macrophage mediators that can regulate polarization and thus influence development and progression in ALD is needed.

As liver injury progresses, macrophages also are needed to clear dead cells or cellular debris by phagocytosis, which is a critical step for successful resolution of inflammation and promotion of tissue repair. As a result of phagocytosis, macrophages begin to produce anti-inflammatory cytokines, such as IL-10 and TGF-β (Henson and Bratton 2013; Korns et al. 2011; Xiao et al. 2008), as well as growth factors and tissue-remodeling mediators that have proinflammatory effects. Thus, a recent immunohistochemistry study observed robust TGF-β expression in macrophages of liver samples from alcoholic hepatitis patients (Lee et al. 2014). When liver injury persists, however, the chronic inflammation and tissue-repair processes can lead to tissue fibrosis. Insufficient oxygen supply to the tissue (i.e., hypoxia) may be a factor in this process, because liver tissue hypoxia has been observed after chronic ethanol feeding (Arteel et al. 1996). Hypoxia causes stabilization and activation of proteins called hypoxia-inducible factors (HIFs), which regulate multiple pathways that control cell survival, proliferation, and metabolism. Macrophages are known to accumulate in large numbers within hypoxic areas of injured tissues (Murdoch et al. 2005). In a mouse model of liver injury, chronic liver injury induced macrophage expression of HIF1α, which promotes fibrosis by regulating the production of profibrogenic mediators (Copple et al. 2012; Mehal 2012).

Oxidative-stress–mediated activation of macrophages and subsequent production of cytokines that influence macrophage polarization are major contributors to inflammation in ALD.

ALD Therapy—Are Macrophages a Plausible Target?

Regardless of disease stage, abstinence from alcohol has been the most effective treatment in ALD. However, patients often lack motivation and compliance, leading to relapse. Another approach includes aggressive nutritional and anti-oxidant therapies using zinc (Kang and Zhou 2005), vitamins, and S-adenosylmethionine to restore nutritional status in alcoholic cirrhosis, albeit with limited beneficial outcomes. Alternative therapies using silymarin and betaine also have been suggested for future clinical trials in ALD (Frazier et al. 2011). Anti-inflammatory treatments targeting macrophage function, such as treatment with corticosteroids, pentoxyfylline, or anti-TNFα antibodies, also have been evaluated for ALD patients for more than 30 years. Success, however, has been limited to date. Clinical trials using glucocorticoids in patients with acute alcoholic hepatitis showed minor benefits but ultimately were terminated because of a heightened risk of sepsis and gastrointestinal bleeding (Maddrey et al. 1978). Subsequent studies evaluated the effects of therapy with specific anti-TNFα antibodies, again with limited success. Consequently, the need for the development of effective strategies for patients with alcoholic hepatitis and cirrhosis remains unfulfilled.

To address this need, researchers also are assessing a variety of strategies to target macrophages in preclinical murine ALD studies. These strategies often use cytokine inhibitors or intracellular mediators to regulate cytokine production, with some promising results:

- Approaches targeting alcohol-induced IL-1β signaling in macrophages using an IL-1 receptor antagonist (e.g., anakinra) have yielded a reduction in alcohol-induced inflammatory responses in murine liver (Petrasek et al. 2012).
- Studies using globular adiponectin to induce IL-10 production in Kupffer cells via the enzyme heme oxygenase-1 alleviated murine ALD (Mandal et al. 2010). Induction of this enzyme in liver macrophages by modulating carbon monoxide availability in the liver also had beneficial effects in mouse models of ALD (Bakhautdin et al. 2014).
- Efforts centering on the MCP-1 and MIF produced by Kupffer cells and infiltrating macrophages in the mouse alcoholic liver identified these chemokines as effective targets (Barnes et al. 2013; Mandrekar et al. 2011).
- Strategies targeting stress-induced heat-shock protein 90 with specific inhibitors—an approach currently assessed in clinical trials for cancer—helped ameliorate ALD by inhibiting macrophage inflammatory responses in murine liver (Ambade et al. 2014).

These studies collectively support clinical evaluations of macrophage-targeting therapies in alcoholic-hepatitis patients. Clinical research combining biologics, small-molecule drugs, and antioxidant therapies targeting macrophage function and phenotype may provide lasting therapeutic efficacy in alcoholic hepatitis and cirrhosis.

Conclusion and Perspectives

As in many chronic inflammatory diseases, macrophages have emerged as critical players and perhaps a therapeutic target in ALD. However, depleting all hepatic macrophages will not be an effective approach because of the heterogeneity

and phenotype diversity of these cells; the specific populations to be targeted for maximum benefit remain to be determined.

There are many questions that still need to be addressed with regard to the role of hepatic macrophages in ALD development. For example, how do infiltrating monocytes differentiate within the liver during ALD? What are the tissue-environmental cues and molecular-signaling pathways that drive the reprogramming of infiltrating macrophages in the alcoholic liver? Another important question is how the number of infiltrating macrophages is controlled after tissue homeostasis is reestablished. Do excess cells undergo apoptosis or do they emigrate? Moreover, in-depth knowledge of the molecules and pathways that control and regulate the phenotype and functions of hepatic macrophages is critical for developing therapeutic strategies to treat ALD. For example, the functions of Ly-6C^{low} infiltrating macrophages in tissue repair and wound healing can be utilized to prevent chronic liver inflammation during the early phase

Glossary

Adipokine: A bioactive factor produced and secreted by fat (adipose) tissue that can modulate the function of other tissues.

Alveolar proteinosis: A chronic lung disease characterized by the filling of the *alveoli* with a protein-like material that prevents ventilation of the affected area; results in shortness of breath, coughing, chest pain, weight loss, and spitting up of blood.

Alveoli: Sac-like structures in the lungs where the gas exchange between the inhaled air and blood takes place.

Autoantibody: An immune molecule (i.e., antibody) formed in response to, and acting against, one of the individual's own normal tissue constituents.

Chemokine: Any of a family of small *cytokines* that induce the movement of *leukocytes* (e.g., to the site of an infection).

Complement system: A complex system of about 20 distinct proteins, their receptors, and related regulatory proteins that induce the destruction (i.e., lysis) of cells during an immune response as well as regulate various other biologic functions (e.g., *phagocytosis*).

Cytokine: Any of the non-antibody proteins released by one type of immune cell on contact with a specific antigen that acts as a mediator between cells (e.g., in the generation of an immune response).

Damage-associated molecular pattern molecules (DAMPs): Molecules that can initiate an immune response in response to cell or tissue damage (i.e., as part of a noninfectious inflammatory response).

Endotoxin: Toxic molecule associated with the membranes of certain bacteria that are released when the cells are disrupted and have numerous biologic effects (e.g., fever, altered resistance to bacterial infection, shock); endotoxins are composed of lipopolysaccharides (LPS).

Epigenetic: Pertaining to mechanisms that alter the activity of genes without changing their DNA sequences (e.g., by chemically modifying the DNA or altering the accessibility of the DNA for regulatory proteins).

Fate mapping: An experimental approach to determine the origins of various tissues in the adult organism from the embryonic structures and to track the development of specific cells through several developmental stages.

Leukocytes: Any of variety of white blood cells, such as monocytes or lymphocytes.

Macrophage polarization: Process during which macrophages acquire specific characteristics and functions in response to external signals (e.g., certain *cytokines*); the two main types of polarized macrophages are M1 (classically activated) and M2 (alternatively activated) macrophages, each of which produces specific *cytokines* and induces specific immune responses.

Malondialdehyde: An organic compound formed during the degradation of lipids by reactive oxygen species (e.g., during alcohol metabolism, which results in formation of reactive oxygen species); malondialdehyde can interact with certain DNA building blocks, forming DNA adducts, which can induce mutations in the DNA.

Pathogen-associated molecular pattern molecules (PAMPs): Molecules that can initiate an immune response in response to infection with a pathogen (i.e., as part of an infectious inflammatory response).

Phagocytosis: Process by which a cell (e.g., a macrophage) takes up microorganisms or cell fragments in membrane-enclosed vesicles in which the engulfed material is killed and digested.

Steatohepatitis: Condition in which fat droplets accumulate in the liver (e.g., as a consequence of alcohol misuse) with simultaneous inflammation of the liver.

Stellate cell: Cell type found in the liver with a characteristic star-like shape that is mainly responsible for fat storage in the liver as well as for collagen production; source of excess collagen produced during hepatitis.

Tolerogenic: Capable of inducing immunologic tolerance (i.e., lack of a reaction to a molecule that would normally trigger an immune response).

of ALD. The conversion of the proinflammatory tissue-damaging Ly-6C^{hi} infiltrating macrophages to anti-inflammatory tissue restorative Ly-6C^{low} cells can serve as a target for treatment of advanced stages of ALD, such as alcoholic steatohepatitis, and has been suggested in human and mouse studies (Singal et al. 2013). Inhibition of macrophage- mediated inflammation is already being used as a therapeutic option in other conditions; for example, agents such as statins, thiazolidinedione, and n-3 fatty acids, which can prevent macrophage-mediated inflammation, are a preferred strategy in diabetes treatment (Ji et al. 2009; Methe et al. 2005; Ramirez et al. 2008; Yeop Han et al. 2010). These therapies also warrant evaluation for their effects in attenuating liver injury and inflammation in alcoholic steatohepatitis.

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The Role of Innate Immunity in Alcoholic Liver Disease

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The innate immune system represents the first-line response to invading microbes, tissue damage, or aberrant cell growth. Many of the proteins and cells involved in innate immunity are produced by, and reside in, the liver. This abundance in immune cells and proteins reflects the liver's adaptation to various immune challenges but also makes the organ particularly vulnerable to alcohol's effects. Heavy alcohol consumption may produce leakage of microbes and microbial products from the gastrointestinal tract, which quickly reach the liver via the portal vein. Exposure to these immune challenges and to alcohol and its breakdown products dysregulates the liver's normally fine-tuned immune signaling pathways, leading to activation of various cellular sensors of pathogen- or damage-associated molecular patterns. The ensuing expression of pro-inflammatory cytokines (e.g., tumor necrosis factor α [TNFα], interleukin [IL]-8, and IL-1β) results in cellular dysfunction that contributes to alcoholic liver disease (ALD). Investigations into the roles of the various components of liver innate immunity in ALD have begun to uncover the molecular basis of this disease. Further progress in this area may help inform the development of interventions targeting the innate system to augment current treatments of ALD. These treatments could include antibodies against pro-inflammatory cytokines, use of anti-inflammatory cytokines, or suppression of alcohol-induced epigenetic regulators of innate immunity.

Key words: Alcohol use, abuse and dependence; heavy alcohol drinking; alcohol effects and consequences; alcoholic liver disease; liver; gastrointestinal tract; immunity; innate immune system; immune cells; cytokines; chemokines; inflammation

Heavy consumption of alcohol poses a well-known health risk worldwide. Alcohol's effects on health and well-being are numerous and include injuries and fatalities resulting from alcohol-induced incapacitation. Moreover, chronic and heavy alcohol consumption affects the integrity and function of vital tissues and organs, causing slow but significant structural and functional damage over time. One of alcohol's principal actions is damage to the liver, the primary organ for its metabolism. As a result, some 90 percent of heavy drinkers (i.e., those drinking 60 g or more of alcohol per day)—and even some who drink less—develop fatty liver (i.e., steatosis) (O'Shea et al. 2009). Up to one-third of heavy drinkers may incur more extensive liver injury, including alcoholic hepatitis, scarring (i.e., fibrosis), cirrhosis, or liver cancer (Gao et al. 2011). Moreover, about 70 percent of individuals who develop alcoholic hepatitis will progress to cirrhosis (Schwartz and Reinus 2012). The spectrum of alcohol-induced liver injuries ranging from steatosis to cirrhosis, defined here as alcoholic liver disease (ALD), is therefore a major cause of liver impairment worldwide (Gao et al. 2011).

A major contributor to ALD is alcohol-induced activation of liver innate immunity, precipitating disorders ranging from localized and transient inflammation to widespread hepatocellular injury and tissue damage (Cohen and Nagy 2011; Gao et al. 2011; Orman et al. 2013; Seki and Schnabl 2012; Wang et al. 2012). Given the pivotal role of the innate immune system in protecting the liver against foreign agents, it may seem surprising that some of the worst outcomes of alcohol-induced liver disease are the result of activation of innate immune cells. But, in fact, recent studies have revealed that alcohol induces immune activation, which drives the progression of ALD.

Innate immunity comprises chemical-physical barriers (e.g., epidermal cells, mucous membranes, and pH), as well as cellular defenses against any invading microbe or agent the immune system perceives as dangerous to the body's cells and tissues (Gao et al. 2011). These cellular defenses, which

include both immune cells (e.g., macrophages and dendritic cells) and proteins (e.g., cytokines), normally are well balanced to sense and respond to harmful agents while avoiding unnecessary immune activation. Alcohol disrupts this balance, triggering immune responses that result in inflammation (Gao et al. 2011; Seki and Schnabl 2012; Szabo et al. 2011; Wang et al. 2012). Continued high alcohol intake fuels a multistage process in which alcohol-induced liver damage advances along a continuum of steatosis, inflammation, and fibrosis, to the final stage, cirrhosis, marked by widespread tissue deformation and damage (Gao et al. 2011; Orman et al. 2013; Seki and Schnabl 2012; Wang et al. 2012).

It has been known for some time that alcohol consumption triggers inflammation of the liver, but how alcohol brings about this disease state has long remained unclear. More recently, researchers have uncovered key roles of Toll-like receptors (TLRs), whose activation during alcohol exposure results in upregulation of pro-inflammatory cytokines (e.g., tumor necrosis factor α [TNFα] and interleukin [IL]-1β) and chemokines (e.g., monocyte chemoattractant protein [MCP]-1). Moreover, these immune responses result in production of reactive oxygen species (ROS), epigenetic changes, and infiltration of tissues with circulating monocytes and neutrophils (Gao et al. 2011; Petrasek et al. 2013; Seki and Schnabl 2012; Szabo et al. 2011; Wang et al. 2012).

Although the exact molecular mechanisms through which alcohol activates innate immune cells are not entirely understood, there is increasing evidence for the close relationship between the effects of alcohol on the gastrointestinal (GI) tract and injury to the liver. Heavy alcohol consumption changes the composition of microbial communities in the GI system, tipping the balance toward more pathogenic species. Recent observations in animal models suggest that these changes are involved in promoting ALD (Yan and Schnabl 2012). Alcohol also seems to disrupt the structural integrity of the gut, causing release of bacteria and bacterial products into the circulation, which activates innate immune responses (Rao 2009; Seki and Schnabl 2012; Yan and Schnabl 2012). Because the GI tract is closely connected to the liver via the portal vein, the liver is a focal point for these alcohol-induced, gut-derived immune challenges.

Receptors located on resident immune cells in the liver (i.e., Kupffer cells; see sidebar, "Liver Cell Types and Their Roles in ALD") sense and transmit these immune challenges. These receptors are specifically adapted to the high-challenge environment of the liver, and this adaptation contributes to the decreased responsiveness to immune challenges (i.e., liver tolerance) in healthy individuals (Petrasek et al. 2013; Seki and Schnabl 2012). However, alcohol's effects on the gut and on immune cells, such as Kupffer cells, reduce liver tolerance and thus increase the potential for persistent inflammation. For example, microbial metabolites and cellular products released in response to the damage caused by alcohol and its metabolites activate cell surface (e.g., TLR4) and intracellular (e.g., nucleotide-binding oligomerization domain [NOD]-like) receptors (Cohen et al. 2011; Petrasek et al. 2013). This activation triggers the expression of pro-inflammatory genes, secretion of cytokines, and recruitment of various immune cells.

Additional findings suggest that alcohol exposure leads to heritable changes in how genes are expressed (e.g., epigenetic regulation) (Curtis et al. 2013). These long-lasting changes in gene expression may shift production of immune cells from anti- to pro-inflammatory cells and may induce other cellular changes that promote inflammation and ALD. Alcohol consumption also destabilizes reduction and oxidation processes (i.e., the redox balance) in the liver (Cohen et al. 2011), leading to increased production of destructive ROS that damage tissues and thus activate innate immune cells in the organ.

The goal of this review is to highlight recent advances in efforts to unravel the role of innate immunity in ALD. The following sections will focus on knowledge gleaned from recent studies of the roles of innate immune cells, proteins, and pathways in the development and progression of ALD. Although ALD is a human disease, much of the current knowledge of the role of innate immunity in ALD has been inferred from animal and in vitro cellular models of alcohol exposure. The significant degree of conservation in innate immune pathways from mouse to human bolsters the idea that many, if not most, findings in these animal and cellular models can be extrapolated to people. However, most of the information from the animal and cellular models discussed in this review awaits confirmation in studies with human subjects. The article will also explore how this knowledge may be used for treating and managing this disease.

The Natural History of ALD

Approximately 30 percent of people who regularly consume large amounts of alcohol have a significantly increased risk for developing ALD (Lucey et al. 2009; O'Shea et al. 2010), which becomes chronic and progressively worse if alcohol consumption continues unchecked (Gao et al. 2011). The disease typically commences with the development of fatty liver (i.e., hepatic steatosis); with continued heavy alcohol consumption, steatosis may transition to inflammation, resulting in tissue damage and fibrosis (see figure 1). Ultimately, chronic ALD results in extensive organ damage and disease characterized by necrosis (i.e., cirrhosis), and in about 2 percent of cases, cancer (i.e., hepatocellular carcinoma) may develop (Orman et al. 2013; Schwartz and Reinus 2012). Alcoholic hepatitis—an acute manifestation of ALD that may coincide with clinical signs of fatty liver (in which case it is termed

alcoholic steatohepatitis) (Lucey et al. 2009)—may occur at any stage of the disease process and significantly predisposes patients to developing cirrhosis.

The first stage in ALD, hepatic steatosis, involves several processes. Alcohol's metabolism generates an overabundance of the metabolic intermediate nicotinamide adenine dinucleotide in its reduced form (NADH), which stimulates the synthesis of excess fatty acids in the liver (Lieber 2004). In addition, recent evidence has shown significant involvement of innate immune pathways in steatosis (Mandrekar et al. 2011). This evidence points to substantial crosstalk between metabolic and immune pathways and highlights the multifactorial nature of this initial stage. Steatosis typically resolves with abstinence from alcohol in people who have no other conditions (e.g., obesity) that promote steatosis. However, continued alcohol use may lead to alcoholic hepatitis, a moderate to severe disorder arising from acute alcohol-induced inflammation for which no highly effective treatment currently is available.

Chronic alcohol use may also lead to the development of fibrosis (Hernandez-Gea and Friedman 2011), characterized by the generation of scar tissue composed of extracellular matrix proteins, such as collagens. As

Liver Cell Types and Their Roles in ALD

Kupffer Cells

Kupffer cells are macrophages located in the liver sinusoids. They usually are among the first cells exposed to alcohol-induced, microbe-derived immunogenic challenges originating from the gut, including lipopolysaccharides (LPSs) and peptidoglycans. Kupffer cells have a dual role in mediating pro-inflammatory responses and moderating these responses through expression of anti-inflammatory cytokines. They express Toll-like receptors (TLRs), including TLR4, TLR2, TLR3, and TLR9, which, on contact with LPS, lipoteichoic acid (a component of the cell walls in Gram-positive bacteria), viral RNA, and CpG-island DNA, respectively, trigger pro-inflammatory response pathways. For example, in response to LPS stimulation, Kupffer cells produce inflammatory cytokines (e.g., tumor necrosis factor α [TNFα], interleukin [IL]-1β, IL-6, and IL-10) and several chemokines through the central regulator of inflammation, nuclear factor κB (NF-κB). Secretion of IL-12 and IL-18 activates production of interferon γ (IFN-γ) in natural killers cells, and production of transforming growth factor β 1 (TGF-β1) and ROS contribute to alcohol-induced fibrogenesis in liver tissues. Kupffer cells contribute to liver tolerance by expressing anti-inflammatory IL-10 on exposure to LPS.

Hepatic Stellate Cells

Hepatic stellate cells (HSCs) are activated by liver damage and express TLR2, TLR4, and TLR9, which respond to lipoteichoic acid, LPS, and CpG DNA, respectively. TLR stimulation in HSCs results in expression of IL-6, TGF-β1, and monocyte chemotactic protein (MCP-1). On activation, HSCs differentiate into myofibroblasts, representing the major producers of extracellular matrix, which contributes to fibrosis.

Hepatocytes

Hepatocytes are epithelial cells and the major cell type of the liver. They significantly contribute to elimination of inflammation-inducing LPS from circulating blood. LPS uptake by hepatocytes requires activity of the TLR4–CD14–MD-2 complex. Hepatocytes are the target for TNFα released by Kupffer cells in response to alcohol and LPS exposure and may undergo apoptosis or necrosis in response to TNFα receptor activation.

Hepatic Dendritic Cells

Activation of TLR9 and TLR7 on or in specialized plasmacytoid dendritic cells results in production of IFN-α. Conventional dendritic cells of the liver respond to LPS or lipoteichoic acid via activation of TLR4 or TLR2 by producing TNFα, IL-12, or IL-6.

Biliary Epithelial Cells

Biliary epithelial cells express TLRs 1 through 10 and exhibit activation of NF-κB expression and TNFα expression after stimulation with high doses of alcohol-induced LPS.

Sinusoidal Endothelial Cells

Sinusoidal epithelial cells (SECs) line the hepatic sinusoids and express TLR4-CD14 along with TLR9. Exposing SECs to LPS downregulates NF-κB activation, CD54 expression, and leukocyte adhesion. In these cells, LPS tolerance is not controlled via TLR4 expression, and the role of SECs in uptake of LPS in the liver is unclear.

in steatosis, both aberrant metabolic processes and activation of immune responses play roles in the development and progression of fibrosis. Acetaldehyde generated during the oxidative breakdown of alcohol inhibits certain immune cells (i.e., natural killer cells) that normally moderate fibrosis by inducing apoptosis in activated hepatic stellate cells (HSCs) (Hernandez-Gea and Friedman 2011; Orman et al. 2013). In addition, cytokines secreted by Kupffer cells, as well as inflammatory scar–associated macrophages recruited from the periphery (Ramachandran and Iredale 2012), activate quiescent HSCs, resulting in the development and proliferation of extracellular matrix–producing myofibroblasts, whose activity precipitates fibrosis.

About 10 to 20 percent of patients with fibrosis who continue to heavily consume alcohol progress to the final stage of ALD, cirrhosis (Orman et al. 2013). This disease stage is characterized by widespread damage to the liver, including fibrotic deformation of tissues and blood vessels, as well as necrosis of cells. The main features of cirrhosis are the formation of nodules of varying sizes, which signify localized regeneration of lost tissues, and the obstruction of blood vessels, which causes portal hypertension. Release of immunogenic cellular debris from necrotic liver cells and the loss of the liver's ability to clear microbial and other pro-inflammatory metabolites from the circulation results in unremitting stimulation of innate immune pathways. As a result, cirrhosis generally is associated with a poor prognosis, with a median survival time of about 10 years. Further, liver cancer (i.e., hepatocellular carcinoma) is seen in about 2 percent of patients with cirrhosis (Orman et al. 2013).

Innate Immunity and ALD

As mentioned above, various innate immune cells and their actions play prominent and complex roles in the initiation and progression of ALD. The oxidative breakdown of alcohol by dedicated alcohol dehydrogenases and by cytochrome P450 monooxygenases generates ROS that may damage proteins, lipids, and other cellular structures. In addition, alcohol's breakdown metabolite, acetaldehyde, exerts toxic effects on cellular structures and DNA (Wang et al. 2012). The ROS- and acetaldehyde-induced cell damage activates innate immune cells, triggering an inflammatory reaction even in the absence of invading pathogens (i.e., sterile inflammation) (Kubes and Mehal 2012). Sterile inflammation results from activation of pro-inflammatory pathways in immune and other cells carrying receptors for detecting damage-associated molecular patterns (DAMPs; also called alarmins). These molecules are released by stressed or necrotic cells, such as hepatocytes damaged by alcohol or its breakdown products. These immune pathways are essential for clearing damaged cells and cellular debris from tissues; however, their persistent activation by alcohol leads to repeated cycles of cell damage and

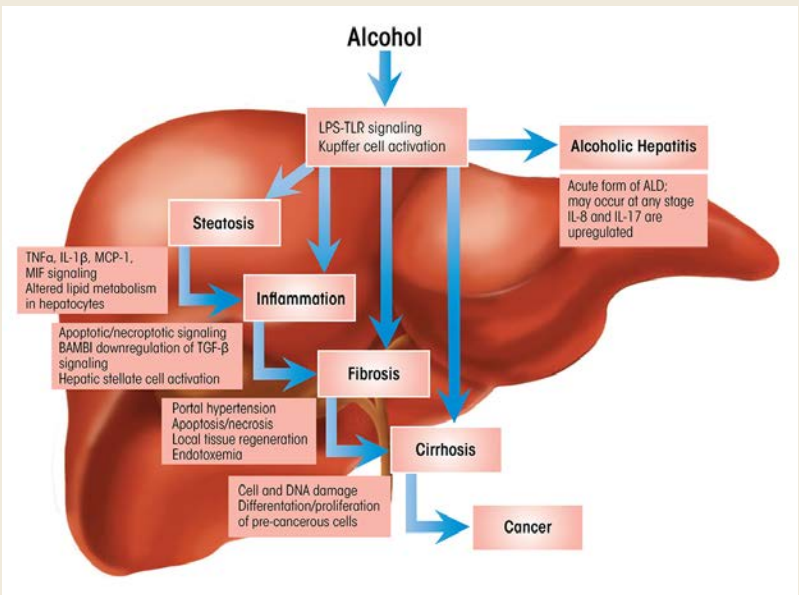

Figure 1 The role of innate immunity in the natural history of alcoholic liver disease (ALD). Heavy alcohol consumption causes release of bacterial products (i.e., lipopolysaccharides [LPSs]) from the gut into the bloodstream. These LPSs lead to activation of liver innate immunity by stimulating Toll-like receptor 4 (TLR 4) signaling on Kupffer cells and hepatocytes. The damaging effects of alcohol and its metabolism on cells trigger additional immune responses. Steatosis and inflammation in hepatocytes represent the early stages of ALD; continued alcohol-induced inflammation leads to apoptosis/necroptosis in hepatocytes. Downregulation of BMP and activin membrane–bound inhibitor (BAMBI) and increased transforming growth factor β (TGF-β) signaling activate hepatic stellate cells, which differentiate into myofibroblasts causing fibrosis. About 10 to 20 percent of patients with ALD (about 70 percent of patients with alcoholic hepatitis) progress to cirrhosis. Differentiation and proliferation of precancerous liver cells present in cirrhosis lead to cancer in about 10 percent of cirrhosis patients. Acute alcohol-induced inflammation (i.e., alcoholic hepatitis), characterized by high levels of pro-inflammatory cytokines (e.g., interleukin [IL]-17 and IL-8), may occur at any stage of ALD and, in severe cases, may cause death in about 50 percent of patients.

resultant stimulation of innate immune cells, causing chronic inflammation of the liver.

Chronic alcohol intake also has more indirect effects that play a major role in ALD. Excessive alcohol consumption changes the composition of microbes found in the gut (i.e., the gut microbiome) and seems to contribute to loss of tight cellular connections in the small intestine (Rao 2009). This alcohol-induced breach of the gut barrier causes release of immunogenic compounds, primarily bacterial lipopolysaccharide (LPS, also known as endotoxin) (Bode et al. 1987) and other cell-wall constituents like peptidoglycans and microbial DNA, into the circulation. LPS is a major trigger of pro-inflammatory pathways, and its role in inflammation of the liver and stimulation of innate immunity is well established (Petrasek et al. 2013; Rao 2009; Seki and Schnabl 2012). Unmethylated CpG–containing DNAs released from bacterial cells have also emerged as a significant activator of liver innate immunity (Petrasek et al. 2013; Seki and Schnabl 2012).

In addition, alcohol depletes the levels of *S*-adenosylmethionine (SAM) (Lieber 2000) a universal methyl donor important for epigenetic regulation of transcription. Intragastric feeding of SAM in rats diminished the activity of alcohol-activated innate immune pathways (Oliva et al. 2011), highlighting the potential role of SAM in moderating innate immune responses.

Effects on Kupffer Cells and the Complement System

Kupffer cells are the resident macrophages of the liver and have key functions in innate immunity. Because of the crucial role Kupffer cells play in defending the liver against pathogens, they are among the first immune cells to respond to alcohol-induced surges of microbial metabolites, such as LPS. These bacterial products engage with the TLR4 receptors on the Kupffer cells. LPS-induced TLR4 activation stimulates the production of cytokines, including TNFα, IL-6, and IL-1β, and of chemokines, such as KC (CXCL1), MIP-2 (CXCL2), MCP-1 (CCL2), and RANTES (Gao et al. 2011; Mandrekar and Szabo 2009; Petrasek et al. 2013; Szabo et al. 2011). Secretion of these molecules from Kupffer cells, in turn, activates a pro-inflammatory cascade affecting processes in other liver cells. For example, tumor necrosis factor α (TNFα) secreted by activated Kupffer cells interacts with TNFα receptors on hepatocytes (see figures 2 and 3). TNFα receptor activation, in turn, contributes to steatosis and, ultimately, to necrosis and apoptosis of the hepatocytes that normally clear LPS and other xenobiotic compounds from the liver (Gao et al. 2011). Alcohol also affects macrophage plasticity—the environmentally determined activation to either classical pro-inflammatory (i.e., M1) or alternative anti-inflammatory (i.e., M2) macrophages. Alcohol represses activation to the M2 phenotype and thus skews macrophage distributions toward the pro-inflammatory M1 state (Louvet et al. 2011; Mandal et al. 2011).

The complement system is a major antimicrobial defense pathway that straddles both innate and adaptive immunity. Most of the proteins in this system are produced in the liver. Alcohol activates complement pathways (Cohen et al. 2010) and, in heavy drinkers, can also compromise complement action by impairing liver function. Complement activation results in the production of C3a and C5a anaphylatoxins—short peptides of the complement system. Through interactions with C3a and C5a receptors, these anaphylatoxins trigger the production of pro-inflammatory innate immune proteins, such as cytokines, in leukocytes and thus contribute to inflammation (Cohen et al. 2011) (see figures 2 and 4).

Expression and Activation of TLRs

TLRs are expressed on many cells of the liver, including Kupffer cells, endothelial cells, dendritic cells, biliary epithelial cells, HSCs, and hepatocytes. The expression of the TLRs and their level of responsiveness on these different cells normally are adjusted to promote appropriate reactions to immune challenges and prevent misplaced and potentially damaging responses (Petrasek et al. 2013). Alcohol exposure turns up the dial of this finely tuned TLR network, heightening TLR responses to external and internal triggers, such as pathogen-associated molecular patterns (PAMPs) and DAMPs, respectively (Petrasek et al. 2013; Seki and Schnabl 2012).

TLR4 plays a very prominent role in alcohol-induced inflammation, activating two distinct signaling pathways—the myeloid differentiation primary response gene 88 (MyD88)-dependent pathway and the MyD88-independent pathway (Petrasek et al. 2013; Seki and Schnabl 2012; Wang et al. 2012). Engagement of the MyD88-dependent TLR pathway triggers expression of nuclear factor kappa B (NF-κB), a central transcriptional regulator of immune responses and pro-inflammatory pathways. TLR4-mediated, MyD88-dependent signaling also promotes mitogen-activated protein kinase (MAPK)-induced production of cytokines, including TNFα. The MyD88-independent pathway proceeds via a different major adaptor protein, TIR domain–containing adapter-inducing interferon-β (TRIF), and results in production of interferon regulatory factor 3 (IRF3), type 1 interferons (IFNs), and pro-inflammatory cytokines.

Experiments in rodent models of ALD have demonstrated that TLR4–TRIF signaling plays an essential role in alcohol-induced activation of TLR4 in Kupffer cells (Hritz et al. 2008; Mandal et al. 2010*b*) (see figure 2). Moreover, TLR4 signaling in both immune cells (i.e., Kupffer cells) and nonimmune cells involved in tissue repair (i.e., HSCs) is required for the development of alcoholic hepatitis and fibrosis (Inokuchi et al. 2011). Co-receptor proteins (e.g., cluster of differentiation [CD] 14 and myeloid differentiation [MD] 2) influence the

responsiveness of TLR4 to receptor ligands, such as LPS.

Cytokines and Chemokines in ALD

Cytokines are relatively small proteins (i.e., less than 30 kDa in size), many of which are produced by various cells in response to injury or contact with pathogens. Patients with ALD often have elevated levels of various cytokines (see sidebar, "Key Cytokines and Hormonal Peptides in ALD") (Szabo et al. 2011). The cytokines are key components of the innate immune system, facilitating cell-to-cell communication and regulating proliferation and maturation of cell populations in response to immune challenges and environmental changes. Cytokines engage with cells via specific receptors on the cellular surfaces, sometimes triggering their own (i.e., autocrine) production in the cells or amplifying or inhibiting the activities of other cytokines. These interactive cytokine networks play an indispensable role in mediating innate immune responses, and their relative contributions define the different types and outcomes of these responses. A subset of cytokines, the chemokines, recruit immune cells, such as neutrophils and lymphocytes, to sites of injury or infection; thus, chemokines are often involved in pro-inflammatory signaling pathways.

As can be expected from alcohol's effect on the innate immune cells of the liver, such as Kupffer cells, and on LPS-activated TLR signaling, heavy alcohol consumption stimulates the production of many cytokines. One of the major cytokines in ALD and one of the first to be associated with the condition is TNFα (McClain and Cohen 1989). TNFα is expressed early in response to alcohol exposure, and its production coincides with liver damage; moreover, abolishing its expression in animal models of ALD mitigates liver injury (Gao 2012; Wang et al. 2012). These observations underscore that TNFα's prominent pro-inflammatory role in ALD and its activity significantly contributes to alcohol-induced liver damage.

Interleukins are also among the pro-inflammatory cytokines implicated in ALD. For example, TNFα, along with other NF-κB–induced agents, stimulates the expression of IL-8, whose levels are greatly increased in alcoholic hepatitis (Sheron et al. 1993). IL-17 is also upregulated in ALD, and although its activity is lower than that of TNFα, it seems to play a role in both inflammation and fibrosis of the liver (Lemmers et al. 2009).

Alcohol exposure also induces expression of anti-inflammatory cyto-

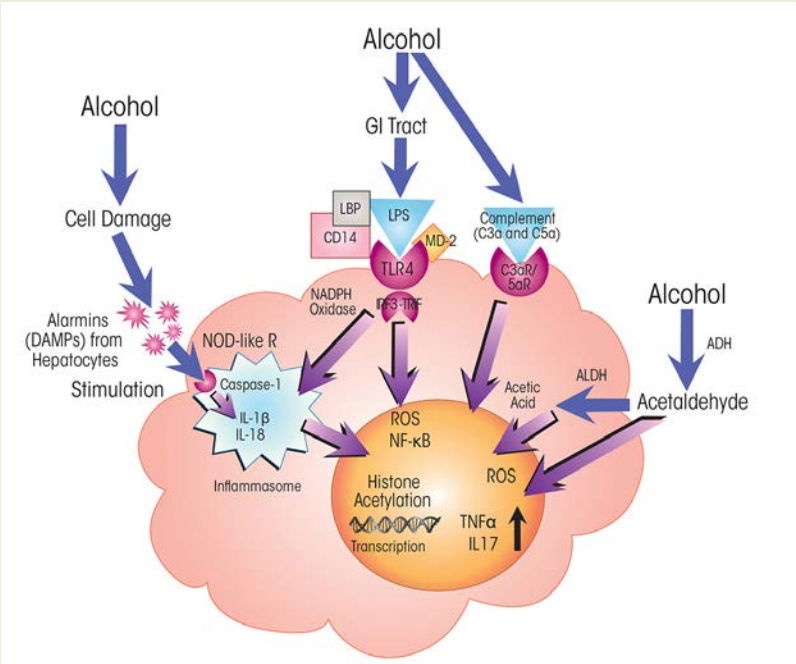

Figure 2 Alcohol's effects on pro-inflammatory pathways in liver macrophages (i.e., Kupffer cells). Excessive alcohol consumption increases the permeability of the gastrointestinal (GI) tract, exposing Kupffer cells to bacterial endotoxin (i.e., lipopolysaccharide [LPS]). LPS is bound by LPS-binding protein (LBP), enabling engagement with Toll-like receptor 4 (TLR 4) and activating the myeloid differentiation primary response (MyD) 88–independent signaling pathway involving interferon regulatory factor 3 (IRF3) and TIR domain–containing adapter-inducing interferon-β (TRIF). IRF3–TRIF signaling induces production of reactive oxygen species (ROS) by nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and activates nuclear factor κB (NF-κB) and histone acetylation, which trigger transcription of genes for several pro-inflammatory cytokines (i.e., tumor necrosis factor α [TNFα] and interleukin [IL]-17). Alcohol's breakdown to acetaldehyde and acetate also stimulates ROS signaling and cytokine production. In addition, IRF3–TRIF signaling and detection of damage-associated molecular patterns (DAMPs or alarmins) released from hepatocytes after alcohol exposure stimulate the inflammasome, a multiprotein complex containing caspase 1, which cleaves and thus activates another pro-inflammatory cytokine, IL-1β. Alcohol activates complement, generating anaphylatoxins C3a and C5a, which dock with their cognate receptor on Kupffer cells, further stimulating cytokine production.

NOTES: ADH = alcohol dehydrogenase; ALDH = acetaldehyde dehydrogenase; C3a/C5a R = C3a/C5a receptor; CD14 = cluster of differentiation 14 protein; MD-2 = myeloid differentiation 2 protein; NOD-like R = nucleotide-binding oligomerization domain–like receptor.

kines. For example, three interleukins—IL-6, IL-10, and IL-22—activate signal transducer and activator of transcription 3 (STAT3), a transcriptional regulator of an array of genes involved in immunity and cellular defenses and differentiation (Gao 2012; Wang et al. 2012). IL-22 binds to specific receptors on epithelial cells and on hepatocytes and triggers the expression of anti-apoptotic and anti-oxidative stress genes while repressing genes involved in lipid production (Gao 2012; Wang et al. 2012). It often remains unclear whether the expression of anti-inflammatory cytokines reflects a compensatory response of the immune system to the alcohol-induced upregulation of the pro-inflammatory cytokines or to the cell damage alcohol produces.

Interestingly, although IL-6, IL-10, and IL-22 all stimulate STAT3, only IL-22 seems to have solely anti-inflammatory effects protecting against acute and chronic liver damage (Park et al. 2011). IL-6 and IL-10, in contrast, have dual roles as both pro- and anti-inflammatory proteins in ALD (Gao 2012; Wang et al. 2012). Their specific effects seem to be determined by the cell type affected and the stage of ALD. For example, IL-6 increases the expression of pro-inflammatory cytokines in Kupffer cells (Gao 2012), but its activity also protects hepatocytes. Recent findings suggest that alcohol-induced oxidative stress stimulates the expression of IL-6, promoting senescence in hepatocytes, which, in turn, makes cells more resistant to steatosis and apoptosis (Wan et al. 2014). IL-10 blocks the activation of TNFα and complement, thus reducing expression of pro-inflammatory pathways; but it also checks expression of IL-6, thus limiting liver regeneration afforded by IL-6–induced upregulation of expression of liver-protective genes (Gao 2012).

Chemokines also play critical roles in alcohol-induced inflammation. For example, the levels of MCP-1 (also known as CCL2) are elevated in patients with ALD, and upregulated MCP-1 expression is also observed in Kupffer cells and hepatocytes of alcohol-fed mice (Mandrekar et al. 2011). Feeding alcohol to MCP-1–deficient mice results in less steatosis, lower expression of pro-inflammatory cytokines (i.e., of TNFα, IL-1β, and IL-6), and lower levels of oxidative stress than in wild-type mice (Mandrekar et al. 2011). Moreover, MCP-1 is required for activating cytokine expression in response to LPS (Mandrekar et al. 2011). In patients with ALD, increased MCP-1 expression is associated with increased disease severity and elevated levels of the pro-inflammatory cytokine IL-8 (Dégre et al. 2012). One major role of MCP-1 in ALD is to recruit neutrophils to inflamed liver tissues. However, because circulating neutrophils in the ALD patients lack MCP-1 receptors (Dégre et al. 2012), the exact mechanisms by which MCP-1 exerts its control over neutrophil movement remain to be elucidated.

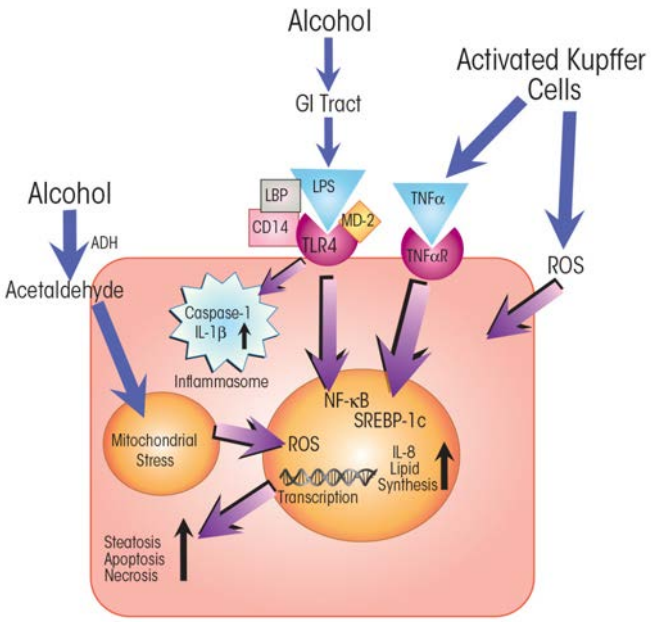

Figure 3 Alcohol's direct effects on activity and viability of parenchymal liver cells (i.e., hepatocytes) and on immune-cell signaling to hepatocytes. Excessive alcohol consumption increases the permeability of the gastrointestinal (GI) tract, exposing hepatocytes to bacterial endotoxin (i.e., lipopolysaccharide [LPS]). LPS is bound by LPS-binding protein (LBP), enabling engagement with Toll-like receptor 4 (TLR4) and activation of pro-inflammatory signaling pathways. TLR4 signaling activates expression of nuclear factor κB (NF-κB), which, along with reactive oxygen species (ROS) generated in mitochondria (as a result of exposure to the toxic alcohol-breakdown product acetaldehyde) and Kupffer cells, activates transcription of pro-inflammatory cytokines (i.e., IL-8). Tumor necrosis factor α (TNFα) produced by activated Kupffer cells stimulates sterol regulatory element–binding protein 1c (SREBP-1c), which triggers expression of genes in lipid synthesis, in turn initiating the development of abnormal fat deposition (i.e., steatosis). The combined action of lipid synthesis and upregulated expression of pro-inflammatory cytokines may spur programmed cell death (i.e., apoptosis) and necrosis, resulting in alcohol-induced loss of hepatocytes from tissues

NOTES: ADH = alcohol dehydrogenase; CD14 = cluster of differentiation 14 protein; MD-2 = myeloid differentiation 2 protein; TNFαR = TNFα receptor.

Nevertheless, these results connect chemokines to lipid metabolism in the liver and suggest that MCP-1 plays a major role in alcohol-induced liver inflammation by activating several pro-inflammatory cytokines in response to common triggers of ALD and by promoting neutrophil infiltration into liver tissues.

Barnes and colleagues (2013) recently demonstrated that macrophage migration inhibitory factor (MIF)—a multifunctional pro-inflammatory cytokine and chemokine, which also has some hormonal features—has a critical role in both the early and chronic stages of liver injury in a mouse model of ALD. MIF-deficient mice are protected against several of alcohol's effects on the innate immune system, including inflammation, and also against hepatocyte damage and apoptosis. Similar to MCP-1, MIF plays a role in alcohol-induced lipid accumulation in liver cells (Barnes et al. 2013), suggesting a role for both chemokines in regulating lipid metabolism directly or indirectly. The findings lend further support to existing evidence that links innate immune pathways and proteins to the regulation of fundamental metabolic processes.

The effect of the hormonal peptide adiponectin on innate immunity, specifically on anti-inflammatory cytokine production and activity, is also worth noting. Adiponectin is secreted by fat cells (i.e., adipocytes) and has been shown to alleviate steatosis, inflammation, and liver damage in animal models (Xu et al. 2003). Recent evidence suggests that adiponectin moderates alcohol-induced production of pro-inflammatory TNFα and promotes expression of IL-10 (Mandal et al. 2010*a*). Because IL-10 activates STAT3, its activation by adiponectin lowers inflammation by stimulating STAT3-induced expression of anti-inflammatory genes in myeloid cells, such as Kupffer cells. In addition, adiponectin stimulates heme oxygenase 1 (HO-1), which suppresses the pro-inflammatory TLR4-dependent/

Key Cytokines and Hormonal Peptides in ALD

Tumor Necrosis Factor α

Tumor necrosis factor α (TNFα) is a major pro-inflammatory cytokine whose levels are increased in the blood and liver of individuals with alcoholic liver disease (ALD). TNFα expression is regulated by the transcription factor nuclear factor kappa B (NF-κB). It is upregulated in macrophages (i.e., Kupffer cells) as well as in circulating monocytes in response to Toll-like receptor 4 (TLR4) activation by bacterial endotoxin (i.e., lipopolysaccharide [LPS]) and by the breakdown products of alcohol, acetaldehyde and acetic acid. TNFα induces necrosis and apoptosis in hepatocytes, thus contributing to inflammation in ALD. TNFα repression by the phosphodiester-inhibitor pentoxifylline and by treatment with TNFα antibody alleviates TNFα-induced liver damage in mice and improves the short-term survival of ALD patients, respectively, but increases the risk for infections in ALD patients.

Interleukin 1β

Interleukin-1β (IL-1β) along with type I IL-1 receptor (IL-1R1), and IL-1 receptor antagonist (IL-1Ra), is an important regulator of the IL-1 signaling complex. This complex plays a critical role in alcohol-induced hepatic steatosis, inflammation, and damage. IL-1β activation is mediated through the inflammasome, a multiprotein complex in macrophages that senses and transduces endogenous danger signals via IL-1β cleavage by caspase-1. IL-1β increases the activity of pro-inflammatory monocyte chemotactic protein (MCP-1) in hepatocytes and contributes to increased TLR4-dependent pro-inflammatory signaling in macrophages.

IL-6

IL-6 has both pro- and anti-inflammatory activities. It increases expression of pro-inflammatory cytokines in macrophages and decreases necrosis-associated inflammation in hepatocytes, which aids recovery from injury and facilitates tissue regeneration. Along with IL-10 and IL-22, IL-6 activates signal transducer and activator of transcription 3 (STAT3), which controls expression of a set of genes involved in innate immunity and in cell survival and differentiation. IL-6 release from M2 macrophages induces senescence and blocks apoptosis and steatosis in hepatocytes in the early stage of alcohol-induced liver injury in mice. IL-6 activates STAT3 in sinusoidal endothelial cells of the liver, thereby increasing cell survival. IL-6 levels, along with those of IL-8 and IL-10, are increased in patients with ALD who have no clinical signs of liver disease.

IL-8

IL-8 is released from injured hepatocytes and has important pro-inflammatory roles as a chemokine that recruits neutrophils to sites of inflammation. Its expression is

MyD88-independent pathway in Kupffer cells (Mandal et al. 2010*b*). Alcohol-induced oxidative stress decreases secretion of adiponectin by adipocytes (Tang et al. 2012), which links oxidative stress to decreased levels of liver-protective, anti-inflammatory hormones, representing yet another mechanism by which alcohol perturbs liver innate immunity.

Another small peptide, ghrelin, which is produced mainly in the gut but also in the liver, has been shown to promote antifibrotic and hepatoprotective effects in both animals and humans with hepatic fibrosis (Moreno et al. 2010). Ghrelin decreases activation of NF-κB in hepatocytes, which attenuates apoptotic signaling in these cells. It also limits expression of collagen-α1 and TGF-β1 but not of NF-κB and IL-8 in HSCs, indicating that ghrelin protects liver tissues mainly by suppressing fibrogenic activities in liver cells (Moreno et al. 2010).

Activation of the Inflammasome and IL-1β Expression

Recent observations by Petrasek and colleagues (2012) in a mouse model of alcoholic hepatitis support significant involvement of another important cytokine, IL-1β—which has roles in rheumatoid arthritis and autoimmune disorders—in alcohol-induced inflammation. The researchers show that the IL-1β signaling complex is essential for the initiation of alcohol-induced inflammation and progression to liver fibrosis. IL-1β is activated through the inflammasome, a large protein assembly composed of NOD-like receptor proteins that sense alarmins, the caspase-1 (Casp-1) protein (an enzyme that cleaves other proteins to activate them), and an apoptosis-associated speck-like CARD-domain-containing (ASC) protein (Szabo and Csak 2012) (see figures 2 and 3). The inflammasome is activated in Kupffer cells of alcohol-fed mice (Petrasek et al. 2012) as well as in hepatocytes exposed to LPS and fatty acids (Csak et al. 2011). It is stimulated by intracellular signals such as alarmins, which engage with

Key Cytokines and Hormonal Peptides in ALD *(continued)*

induced by TNFα via activation through NF-κB. IL-8 levels are greatly increased in people with acute alcoholic hepatitis but are only moderately upregulated in those with cirrhosis. IL-8 levels, along with those for IL-6 and IL-10, are elevated in individuals with alcoholism who have no signs of liver disease.

IL-10

IL-10 is a strong suppressor of inflammation by preventing production of the pro-inflammatory cytokines TNFα, IL-1β, and IL-6 in macrophages. However, its anti-inflammatory, hepatoprotective effects are contingent on the expression of other cytokines, and its inhibitory effect on IL-6 expression can delay liver regeneration and increase steatosis. IL-10 expression is moderately to highly increased in ALD and, along with that of IL-6 and IL-8, is also upregulated in alcoholic patients without signs of liver disease. IL-10 acts only on immune cells expressing its cognate receptors and facilitates sustained activation of the transcription factor STAT3 in Kupffer cells, thus inhibiting inflammation. IL-10 also inhibits fibrosis.

IL-17

IL-17 is a recently discovered, pro-inflammatory chemokine whose levels are increased in people with ALD. It is produced by monocytes and T cells and plays an important role in recruiting neutrophils to inflamed liver tissues. It may act in concert with TNFα to activate NF-κB, thereby inducing expression of other pro-inflammatory cytokines. IL-17's main targets are hepatic stellate cells (HSCs), in which it induces production of pro-inflammatory IL-8. IL-17 is also thought to be involved in the development of fibrosis.

IL-22

IL-22 is an anti-inflammatory cytokine whose expression limits steatosis and liver damage. The IL-22 targets are hepatocytes in which it activates the transcription factor STAT3. The antioxidant, antiapoptotic, and antisteatotic actions of IL-22 make it a promising target of interventions for treating ALD with minimal side effects because of the restricted distribution of IL-22 receptors.

Adiponectin

Adiponectin is an adipokine, a peptide hormone, whose secretion from fat cells (i.e., adipocytes) is inhibited by alcohol. Adiponectin increases fatty acid oxidation and thus suppresses steatosis. It also decreases expression of the pro-inflammatory cytokine TNF-α in macrophages (i.e., Kupffer cells) by inducing expression of heme oxygenase 1 (HO-1), which decreases TLR4/MyD88-independent signaling, and by increasing polarization to anti-inflammatory M2 macrophages. In addition, adiponectin upregulates expression of anti-inflammatory IL-10.

pattern-recognition domains of the NOD-like receptors.

Inflammasome activity in Kupffer cells promotes Casp-1–mediated cleavage, and thus activation, of IL-1β (Petrasek et al. 2012). Blocking IL-1β activity strongly decreases liver inflammation and damage (Petrasek et al. 2012). These findings further amplify the view that IL-1β acts as a pro-inflammatory cytokine in immune cells in ALD and that IL-1β signaling through the inflammasome is required for alcohol-induced liver injury. Coupled with the observations of Mandrekar and colleagues (2011) on MCP-1, these findings also establish a link between innate immune activity and steatosis. This association is further supported by the observation that steatosis can be prevented when innate immune responses are experimentally abrogated by checking IL-1β or MCP-1 activity. Additional investigations into pro-inflammatory signaling in the methyl-choline deficiency model of non-alcoholic steatohepatitis have indicated that activation of the inflammasome and generation of IL-1β are independent of TLR4 but contingent on the MyD88-dependent pathway (Csak et al. 2014).

Cytokine Effects on Hepatocytes and HSCs

Alcohol-induced increases in LPS stimulate TNFα production and its release primarily from Kupffer cells (McClain and Cohen 1989; Wang et al. 2012). In addition, alcohol sensitizes other liver cells to TNFα's actions (An et al. 2012). One distinct role of TNFα is to induce programmed cell-death pathways (i.e., apoptosis and necroptosis) by binding to and activating death receptors on cells. Activation of these receptors results in the expression of pro-apoptotic or necroptotic mediators (e.g., caspases and receptor-interacting proteins). Alcohol thus has profound effects on cell viability by activating the expression of cytotoxic cytokines and increasing cell sensitivity to the actions of these cytokines. For instance, whereas hepatocytes in healthy people are not highly sensitive to TNFα activation of death receptor pathways, alcohol primes cells for the TNFα-mediated stimulation of cell-death pathways (Pastorino and Hoek 2000).

TNFα binds to and activates two receptors—TNF-R1 and TNF-R2—and the outcome of their activation hinges on the relative levels of stimulation of three main pathways: a pro-apoptotic pathway, a pro-necroptotic pathway, and a cell-survival pathway (Vanden Berghe et al. 2014). Binding to TNF-R1 activates apoptotic and necrotic pathways through a signaling cascade involving multiple proteins, including TNF-R1–associated death domain protein (TRADD); TNF receptor–associated factor 2 (TRAF2); and caspases 8, 3, and 7. TNF-R1 activation also may result in the stimulation of a cell-survival pathway involving NF-κB. In contrast, activation of TNF-R2 stimulates only cell survival (Malhi et al. 2010).

Apoptosis is an important regulatory mechanism for controlling the size of cell populations (e.g., neutrophils) or to avoid unchecked proliferation of abnormal cells. However, apoptosis triggered via the action of alcohol-induced TNFα on, for example, hepatocytes can severely impair liver function. Recent evidence also indicates that chronic ethanol feeding can activate necroptotic cell-death pathways in hepatocytes. Mice in which an important necroptotic regulator—receptor-interacting protein kinase (RIP)-3—is inactivated are protected from alcohol-induced liver injury (Roychowdhury et al. 2013). Hepatocytes are the major cell type in the liver and, along with Kupffer cells, eliminate most of the LPS from circulation. Widespread apoptosis and necrosis of hepatocytes therefore increase levels of circulating LPS, which in turn fuels further inflammation through activation of TLR4 on Kupffer cells and ultimately escalates the release of pro-apoptotic TNFα. This helps explain why patients with severe liver disease (i.e., cirrhosis) often show high levels of LPS in the blood (i.e., endotoxemia), resulting in sepsis (Bode et al. 1987).

Persistent alcohol-induced activation of cytokines in immune cells such as Kupffer cells also promotes fibrosis. Production and secretion of transforming growth factor (TGF)-β and of platelet-derived growth factor (PDGF) from Kupffer cells or from inflammatory scar–associated macrophages activates HSCs (see figure 4), triggering them to develop into myofibroblasts. Moreover, HSCs may be further activated by engulfing apoptotic hepatocytes. HSCs express a number of receptors involved in innate immunity, including the C5a receptor, whose ligand, C5a, is a potent mitogen that may stimulate HSC migration (Das et al. 2014). HSCs also express TLR4, which senses LPS. Although LPS alone cannot activate HSCs, its action via TLR4 downregulates expression of BMP and activin membrane-bound inhibitor (BAMBI), thereby sensitizing the cells to activation by TGF-β (Liu et al. 2014; Seki et al. 2007). BAMBI is a pseudoreceptor, which in quiescent HSCs diminishes TGF-β's activity by binding to it without triggering intracellular TGF-β signaling. As shown by Seki and colleagues (2007), decreased BAMBI expression sensitizes HSCs to TGF-β activation, and LPS-induced TLR4 activation stimulates chemokine secretion along with recruitment and activation of Kupffer cells. Moreover, LPS activates TLR4 signaling in HSCs through the MyD88-dependent NF-κB pathway, demonstrating involvement of this key innate immune pathway in fibrosis and recruitment of immune cells. NF-κB directly binds the BAMBI promoter, along with histone deacetylase (HDAC) 1, thus repressing BAMBI expression and promoting TGF-β activation (Liu et al. 2014).

Activated HSCs produce and secrete extracellular matrix proteins (i.e., collagens), resulting in fibrogenesis (Hernandez-Gea and Friedman 2011;

Seki and Schnabl 2012). Although fibrogenesis is essential for normal tissue repair, its dysregulation by recurrent activation of cytokines acting on HSCs precipitates inflammatory fibrosis. This stage of ALD involves the formation of scar tissue, which interferes with normal tissue function and often results in portal hypertension (Hernandez-Gea and Friedman 2011). Another cytokine pathway involving HSCs and leading to fibrosis in ALD patients involves IL-17. This cytokine, which is produced by peripheral blood cells (i.e., Th17 lymphocytes) in patients with alcoholic hepatitis and cirrhosis, stimulates its cognate receptors on HSCs (Lemmers et al. 2009). In response, the cells secrete IL-8 and growth-related oncogene (GRO)-α, recruiting neutrophils to their tissue, resulting in localized pro-inflammatory immune-cell infiltrates and fibrosis scores closely correlated with IL-17 levels.

Together, these findings indicate that cytokines produced by immune cells, such as TNFα produced by Kupffer cells and IL-17 produced by Th-17 lymphocytes, play a major role in ALD by affecting the viability and function of hepatocytes and by activating quiescent HSCs to produce excess extracellular proteins. These observations thus provide critical insight into the molecular processes and mechanisms that produce liver damage and fibrosis in ALD.

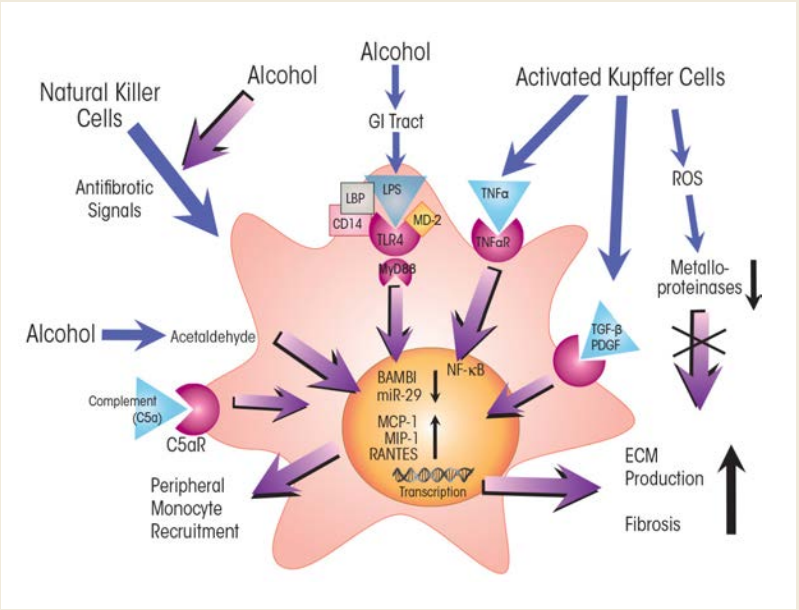

Figure 4 Alcohol's effects on fibrogenic pathways in hepatic stellate cells (HSCs). HSCs are quiescent liver cells that, on stimulation by pro-inflammatory proteins and other agents, differentiate into myofibroblasts to repair damaged tissues. Excessive alcohol consumption increases the permeability of the gastrointestinal (GI) tract, exposing HSCs to endotoxin (i.e., lipopolysaccharide [LPS]). LPS is bound by LPS-binding protein (LBP), enabling engagement with Toll-like receptor 4 (TLR 4) and activating the myeloid differentiation primary response (MyD88)-dependent pathway. MyD88 signaling decreases expression of BMP and activin membrane–bound inhibitor (BAMBI), a pseudoreceptor that suppresses responses to transforming growth factor β (TGF-β; secreted by activated Kupffer cells). Thus, alcohol-induced TLR4–MyD88 signaling increases the HSCs' responsiveness to TGF-β. microRNA 29 (miR-29) inhibits the production of extracellular matrix (ECM), and its downregulation by MyD88 signaling therefore increases ECM deposition. TLR4–MyD88 signaling in HSCs—along with complement 5a and exposure to the alcohol-breakdown product acetaldehyde and platelet-derived growth factor (PDGF) and tumor necrosis factor α (TNFα) secreted from activated Kupffer cells—upregulates the expression of various chemokines (i.e., monocyte chemotactic protein [MCP-1], macrophage inflammatory protein 1 [MIP-1], and regulated on activation, normal T cell expressed and secreted [RANTES]). These chemokines recruit macrophages (i.e., Kupffer cells and scar-associated macrophages) and other immune cells to the site where HSCs reside (i.e., the liver perisinusoidal space or space of Disse). These signals spur the differentiation of HSCs into myelofibroblasts that produce and secrete ECM, leading to liver fibrosis. In addition, Kupffer cell–produced ROS inhibit activities of metalloproteinases, which normally degrade ECM and thus inhibit fibrosis.

NOTES: C5aR = C5a receptor; CD14 = cluster of differentiation 14 protein; MD-2 = myeloid differentiation 2 protein; TNFαR = TNFα receptor.

Epigenetic Effects

Alcohol exerts additional effects on the innate immune system, for example, by producing epigenetic changes in the expression of genes for pro- and anti-inflammatory pathways (Curtis et al. 2013). Epigenetic changes affect the activity at gene promoters or entire gene regions and can have long-term and even heritable effects on gene expression without altering the underlying DNA sequence. Three main mechanisms operate in epigenetics:

- DNA methyltransferases, using SAM as methyl donor, methylate a cytosine nucleotide at CpG-rich regions (i.e., CpG islands) in the DNA of gene promoters, which decreases expression of the downstream genes.
- Methylation, acetylation, phosphorylation, ubiquitination, or sumoylation of the proteins around which DNA is coiled (i.e., histones) alters accessibility

of the transcriptional proteins to the DNA.

- Some investigators are now extending the concept of epigenetics to include transcriptional regulation by microRNAs (miRNAs). These molecules regulate the expression of mRNAs with which they share similar sequences (Curtis et al. 2013).

The study of alcohol's effects on epigenetic regulation and of the mechanisms by which alcohol exerts these effects has been a rapidly emerging field over the past decade. Insight gleaned from initial studies has shown that alcohol can interfere with the fundamental processes of epigenetic regulation in people with ALD as well as in animal models of the disease or in cultured human cells exposed to alcohol or its metabolic byproducts (reviewed by Kruman and Fowler 2014; Mandrekar 2011; Shukla and Lim 2013).

Studies in animals and in human cells lines have demonstrated that alcohol and LPS increase the expression of microRNA-34a, which helps alleviate alcohol-induced apoptosis in hepatocytes and biliary epithelial cells by targeting caspase 2 and sirtuin 1. The elevated expression of miRNA-34a is the result of an alcohol-induced decrease in methylation (i.e., hypomethylation) at a CpG island in the miRNA-34a promoter (Meng et al. 2012).

Alcohol also alters the cellular levels of SAM and of histone acetyltransferases (HATs) and deacetylases (HDACs), whose activities make DNA more or less accessible, respectively, to gene transcription. For example, the histone deacetylase HDAC1 has been shown to play a critical role in the silenced expression in HSCs of the fibrosis-attenuating protein BAMBI (see figure 4) (Liu et al. 2014). In addition, chemical inhibition of HDAC activity seems to reduce inflammation by reversing an alcohol-induced perturbation in macrophage polarization that results in a greater proportion of pro-inflammatory (M1) macrophages (Curtis et al. 2013). Oxidative stress caused by alcohol metabolism also triggers epigenetic changes, and alcohol-induced release of LPS and activation of TLR4 affects both HAT and HDAC activities, resulting in epigenetic changes in DNA regions containing genes for pro-inflammatory cytokines (Curtis et al. 2013).

Approaches for Resolving Alcohol-Induced Liver Inflammation

Standard interventions for treating ALD depend on the stage and severity of the disease and typically include counseling abstinence from alcohol use; administration of corticosteroids (to inhibit alcohol-induced, pro-inflammatory pathways) and nutritional support for alcoholic hepatitis; and, in advanced cases, liver transplantation (Gao and Bataller 2011; Orman et al. 2013). Although rates of disability and death caused by ALD remain high despite these interventions, such treatments can significantly improve quality of life and avert early death caused by ALD. Overturning earlier assumptions about the persistence of alcohol-induced liver damage, recent studies have reported that some of the tissue injuries present even in the advanced stages of ALD, such as fibrosis, are reversible (Hernandez-Gea and Friedman 2011). This makes the discovery of new treatments that can augment existing ones even more urgent.

Recognition of the central role of innate immunity in ALD has spurred research into modulating the activity of key immune cells and cytokines. To this end, the discovery of the central role of TNFα in promoting inflammation in ALD prompted studies in which antibodies against TNFα were used to alleviate alcohol-induced inflammation. TNFα antibodies indeed significantly dampen liver inflammation (Gao 2012), but because TNFα is critical to fighting microbial pathogens, this approach often increases the risk for serious infections in ALD patients.

The limitations of the above approach highlight that cytokine-based interventions will need to be carefully calibrated according to the activity profile and radius of action of each cytokine to minimize or prevent adverse effects. For instance, exploitation of the hepatoprotective properties of IL-6 is limited by the abundance of IL-6 receptors in many tissues, potentially resulting in off-target effects. However, as proposed by Gao (2012), using IL-6 in ex vivo treatment of donor livers to reverse minor organ damage (e.g., steatosis) before transplantation into ALD patients could have some utility.

Current approaches for interventions in vivo focus on those immune regulators that target only a few cells or tissues. As discussed previously, IL-22 has an array of hepatoprotective activities, including antioxidant, antimicrobial, and antiapoptotic effects. Moreover, expression of the IL-22 receptor, IL-22R, is confined to epithelial cells, such as hepatocytes. This has led to the proposition that combining the use of IL-22 with anti-inflammatory corticosteroids and TNFα inhibitors could offset the immunosuppressing effects of these two agents and promote recovery of liver tissues (Gao 2012). However, because evidence from animal models suggests that IL-22 may play a role in the development of hepatic carcinoma (Park et al. 2011), such use would be restricted to ALD patients who do not have cirrhosis (which may contain precancerous cells) or liver cancer (Gao 2012).

The discovery of the role of epigenetic factors in the development of ALD and their effects on immune cells and responses opens the way to possible interventions that target key epigenetic regulators and processes in ALD. For example, because HDAC1 seems to make HSCs more receptive to the fibrosis-inducing action of TGFβ (Liu et al. 2014), inactivation of HDAC1 via antibodies or chemical agents may

augment current treatments for halting or reversing fibrosis in patients with ALD. In addition, chemical inactivation of HDACs involved in alcohol's effects on macrophage polarization to pro-inflammatory M1 macrophages may help reduce inflammation, steatosis, and fibrosis in tissues. Thus, HDAC inhibitors or activators of HATs that prevent or reverse the effects of HDACs may someday prove useful in the treatment of ALD.

Finally, technological advances to sequence and analyze DNA of patients has helped identify key genetic variants such as single-nucleotide polymorphisms (SNPs) in genes involved in liver diseases (Guo et al. 2009; Singal et al. 2014). Although in its early stages and not yet fully extended to the specific etiology of ALD, genetic profiling of ALD patients for SNP variants in genes involved in innate immune pathways could help identify patients vulnerable to advanced stages of ALD (e.g., cirrhosis) (Guo et al. 2009). Such personalized-medicine approaches could significantly improve the success and cost-effectiveness of current treatments and spur development of new interventions for ALD.

Conclusions

Innate immunity plays a central role in ALD, and recent studies have uncovered several pivotal molecular mechanisms underlying alcohol's effects on the immune system of the liver. Excessive consumption of alcohol alters the characteristics and composition of the microbiome in the GI tract and increases translocation of bacteria and bacterial products, such as LPS and peptidoglycans, from the gut via the portal system to the liver. This increased influx of LPS, along with the direct effects of alcohol on immune cells and liver tissues, activates innate immune pathways. This activation occurs via stimulation of TLRs and through sensors of cell damage on or in the immune cells of the liver, such as Kupffer cells. These processes lead to the production of several pro-inflammatory cytokines (e.g., of TNFα, IL-1β, IL-8, and IL-17), triggering steatosis in hepatocytes and inducing fibrogenic pathways in HSCs. Moreover, production of chemokines, such as MCP-1 and MIF, leads to the infiltration of liver tissues by monocytes, neutrophils, and dendritic cells whose activities can further increase inflammation and impede recovery. Alcohol and its metabolic breakdown products acetaldehyde and acetate, along with ROS produced during alcohol metabolism, generate oxidative stress and affect epigenetic regulation that trigger activation of pro-inflammatory pathways such as polarization to M1 macrophages.

The insight gleaned from these complex interactions and pathways may provide the impetus for devising treatments using pro- or anti-inflammatory cytokines that act on defined cell types or employing agents that control epigenetic regulators to expand currently available interventions for treating ALD.

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The author declares that she has no competing financial interests.

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The Gastrointestinal Microbiome

Alcohol Effects on the Composition of Intestinal Microbiota

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The excessive use of alcohol is a global problem causing many adverse pathological health effects and a significant financial health care burden. This review addresses the effect of alcohol consumption on the microbiota in the gastrointestinal tract (GIT). Although data are limited in humans, studies highlight the importance of changes in the intestinal microbiota in alcohol-related disorders. Alcohol-induced changes in the GIT microbiota composition and metabolic function may contribute to the well-established link between alcohol-induced oxidative stress, intestinal hyperpermeability to luminal bacterial products, and the subsequent development of alcoholic liver disease (ALD), as well as other diseases. In addition, clinical and preclinical data suggest that alcohol-related disorders are associated with quantitative and qualitative dysbiotic changes in the intestinal microbiota and may be associated with increased GIT inflammation, intestinal hyperpermeability resulting in endotoxemia, systemic inflammation, and tissue damage/organ pathologies including ALD. Thus, gut-directed interventions, such as probiotic and synbiotic modulation of the intestinal microbiota, should be considered and evaluated for prevention and treatment of alcohol-associated pathologies.

Key words: Alcohol consumption; alcohol use, abuse, and dependence; alcohol use disorder (AUD); alcoholic liver disease (ALD); microbiota; intestinal microbiota; microbiota analyses; gastrointestinal microbiome; dysbiosis; probiotics; synbiotics

It has been estimated that approximately 2 billion people worldwide drink alcohol on a daily basis, with more than 70 million people having a diagnosed alcohol use disorder (World Health Organization 2004). Globally, alcohol use is the fifth leading risk factor for premature death and disability among people between the ages of 15 and 49 (Lim et al. 2012). Excessive alcohol consumption in the United States accounts for 80,000 deaths yearly (Centers for Disease Control and Prevention 2004) and is the third leading preventable cause of death in the United States (Mokdad et al. 2004). In addition, the Centers for Disease Control and Prevention (CDC) found that in 2006, excessive drinking cost the United States more than $224 billion (Bouchery et al. 2011). In a subgroup of alcoholics, alcohol consumption is linked with tissue injury and organ dysfunction, including alcoholic liver disease (ALD) (Purohit et al. 2008), increased risk of developing cancer (Seitz and Stickel 2007), abnormal function of the immune system that increases the risk of acute and chronic infections (Szabo and Mandrekar 2009), pancreatitis (Chowdhury and Gupta 2006), heart disease (Liedtke and DeMuth 1975), and disruption of the circadian clock (Spanagel et al. 2005). The observation that only some alcoholics develop alcohol-induced pathology indicates that, although alcohol is necessary, it is not sufficient to cause organ dysfunction. Consequently, factors other than the toxicity of alcohol are involved in generating health complications, one of which may be alcohol-induced changes in intestinal microbiota composition and/or function.

The intestinal microbiota is classified as the total collection of microbial organisms (bacteria and microbes) within the gastrointestinal tract (GIT). It contains tens of trillions of microorganisms, including at least 1,000 different species of known bacteria, the vast majority of which belong to the phyla *Firmicutes* and *Bacteroidetes* (Ley et al. 2008). The metagenome is the collection of all the different genes found within the gut microbiome; the GIT microbiome contains more than 3 million unique genes, outnumbering the number of human genes 150 to 1 (Proctor 2011). The GIT and the intestinal microbiota display a symbiotic relationship. The microbiota contributes to the extraction of energy from food and synthesis of vitamins and amino acids, and helps form barriers against pathogens (Tappenden and Deutsch 2007). Disruption of intestinal microbiota homeostasis—called dysbiosis—has been associated with inflammatory bowel disease (IBD) (Hold et al. 2014), irritable bowel syndrome (IBS) (Kassinen et al. 2007), celiac disease (Nadal et al. 2007), food allergies (Kuvaeva et al. 1984), type 1

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diabetes (Wen et al. 2008), type 2 diabetes (Larsen et al. 2010), cancer (Schwabe and Jobin 2013), obesity (Turnbaugh et al. 2006), and cardiovascular disease (Harris et al. 2012). Although it is unclear whether dysbiosis is the cause or the result of these diseases, factors that contribute to the development and progression of many of these diseases are known to influence the GIT microbiota.

Dysbiosis can be caused by environmental factors commonly encountered in Western societies, including diet (David et al. 2014), disruption of circadian rhythms (Voigt et al. 2014), and alcoholic beverage consumption (Mutlu et al. 2009; Yan et al. 2011) (figure 1). It is well-established that diet influences intestinal microbiota composition and diversity (David et al. 2014) (figure 1). Diets high in fat alter intestinal microbiota (Cani et al. 2007), as do "Western" diets, comprising high fat and high sugar (Turnbaugh et al. 2008). The consequence of diets high in fat or sugar may contribute to the development of obesity and liver injury (Frazier et al. 2011), as well as IBD, IBS, celiac disease, type 1 and type 2 diabetes, food allergies, and cardiovascular disease (Brown et al. 2012; Manzel et al. 2014), at least in genetically susceptible individuals. Alcohol is another dietary disruptor of the intestinal microbiota. A limited number of studies have examined the effects of alcohol on the microbiota in rodents (Mutlu et al. 2009; Yan et al. 2011) and humans (Bode et al. 1984; Chen et al. 2011; Mutlu et al. 2012; Queipo-Ortuno et al. 2012). These changes seem to be relevant for alcohol-associated pathologies because interventions known to alter the intestinal microbiota diminish some alcohol-associated pathologies such as liver disease (Bull-Otterson et al. 2013; Liu et al. 2004; Mutlu et al. 2009).

In this review, we examine alcohol-induced effects on microbiota and how interventions targeted at normalizing alcohol-induced dysbiosis may mitigate some of the detrimental effects of alcohol.

Analyzing the Intestinal Microbial Community

Before we can understand the influence of alcohol on the GIT microbiota, we need to understand a bit about how researchers measure these microorganisms and evaluate changes in their populations. In fact, it is difficult to directly

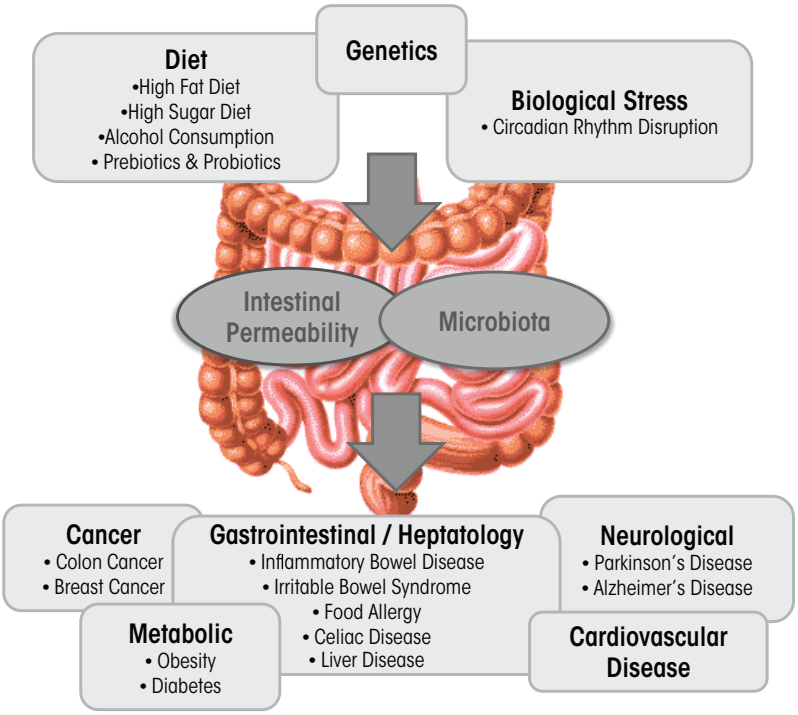

Figure 1 Disruption of intestinal microbiota homeostasis (dysbiosis) has been associated with these diseases (shown above). In addition, dysbiosis can be caused by environmental factors commonly encountered in Western societies, including diet, genetics, disruption of circadian rhythms, and alcoholic beverage consumption. Dysbiosis also can be prevented or treated with probiotics and prebiotics.

measure microbial communities such as those within the GIT because of a number of confounding factors. For one, microorganisms maintain incredible genetic diversity but house this diversity in an extraordinarily limited array of cellular morphologies (Woese 1987). In addition, microorganisms have redundant functional capabilities, share divergent functional capabilities with closely related microorganisms, have the potential for high metabolic diversity within single microbial lineages, and are extraordinarily difficult to isolate under laboratory conditions. Taken together, these confounding factors compel researchers to use molecular tools—tools that examine DNA and RNA—to analyze these complex communities. These tools fall into two broad categories: polymerase chain reaction (PCR)–based targeted approaches and shotgun sequencing approaches (figure 2), which we explain in detail in the sidebar.

Because it is exceedingly difficult to obtain microbial samples from different locations in the GIT, researchers overwhelmingly extract the genomic DNA they need to analyze the GIT microbiota from mucosa-associated colonic tissue biopsies and from fecal samples. However, using these samples assumes that the colonic tissue and feces are a suitable proxy for the GIT. A study (Stearns et al. 2011) addressed this issue in an analysis of microbiota community structure in mouth, stomach, duodenum, colon, and stool, via gastroscopy and colonoscopy from four healthy individuals. When examined in the context of the entire GIT, colonic tissue and fecal samples were most similar to each other in all individuals. However, the community composition was substantially altered in colon and fecal samples from the same individual: three of four individuals had a much reduced level of microorganisms from the phylum *Bacteroidetes* in fecal samples. This led to a substantially altered ratio of *Firmicutes* to *Bacteroidetes,* a ratio that has been used as a diagnostic parameter in studies of disease (see sidebar). Eckburg and colleagues (2005) also found a similar divergence between GIT colonic tissue and fecal microbiota. Thus, although colonic tissue and fecal samples will continue to serve as common, imperfect proxies for GIT microbiota, they should not be considered a perfect representation of the entire GIT microbial community, which undergoes dramatic changes from the stomach to colon (Stearns et al. 2011). No obvious solution is available, leaving only highly invasive sampling techniques as a mechanism to collect samples from multiple locations of the GIT.

Alcohol-Induced Effects and Implications on the Intestinal Microbiota

The study of alcohol's effects on the structure and activity of GIT microbiota still is in its infancy, particularly compared with other alcohol-induced effects. The literature reviewed below demonstrates that alcohol consumption leads to quantitative and qualitative dysbiosis in the intestinal microbiota of rodents and humans (table 1). These studies demonstrate alterations in the dominant bacterial taxa from the phyla *Bacteroidetes* and *Firmicutes* and, in several studies, an increase in bacteria from the phylum *Proteobacteria*.

Rodent Models

Studies in mice and rats find both alcohol-induced bacterial overgrowth and dysbiosis. In one study, C57BL/6 mice were intragastrically fed alcohol (30.9 g/kg per day; 40 percent of their total daily calories from alcohol) for 3 weeks and compared with control mice intragastrically fed an isocaloric liquid diet. The alcohol-fed mice developed ALD, which was associated with small intestinal bacterial overgrowth and dysbiosis in the cecum—the beginning of the large intestine (Yan et al. 2011). In particular, the GIT microbiota of alcohol-treated mice showed a decrease in *Firmicutes* and an increase in the relative abundance of *Bacteroidetes* and *Verrucomicrobia*, among other bacteria (table 1). In comparison, the GIT microbiota of control-fed mice showed a relative predominance of bacteria from the phylum *Firmicutes*. In a separate study, Sprague-Dawley rats intragastrically fed alcohol daily (8 g/kg per day) for 10 weeks showed altered colonic mucosa–associated bacterial microbiota composition leading to ileal and colonic dysbiosis (Mutlu et al. 2009). In prior studies, Sprague-Dawley rats developed intestinal oxidative stress, intestinal hyperpermeability, endotoxemia, and steatohepatitis by the 10th week of alcohol treatment (Keshavarzian et al. 2009), suggesting that changes in the microbiota may be contributing to the alcohol-induced effects on the intestine and liver. Intestinal dysbiosis may potentially contribute to the pathogenesis of liver disease by altering intestinal barrier integrity, resulting in intestinal hyperpermeability, as well as increased production of proinflammatory factors that could both promote liver pathology.

Humans

Chronic alcohol consumption in humans also causes bacterial overgrowth and dysbiosis. One study using culture-based methods, for example, found alcohol-induced alterations, including small intestine bacterial overgrowth of both aerobic and anaerobic bacteria in the jejunum (Bode et al. 1984). Another study showed that alcohol consumption alters the composition of mucosa-associated microbiota in human sigmoid biopsies taken from alcoholics with and without ALD as well as healthy control subjects (Mutlu et al. 2012). In this study, the researchers used 16S rRNA gene sequencing to assess the microbiota. They found that the microbial community was significantly altered—containing a lower abundance of *Bacteroidetes* and a higher abundance of *Proteobacteria*—in a subgroup of alcoholics with and without liver disease (table 1). Other studies show that dysbiotic microbiota in alcoholics also correlates with a high level of endotoxin in the blood, indicating that dysbiosis may contribute to intestinal hyperpermeability and/or the increased translocation of gram-negative microbial bacterial products

Methods for Analyzing the Gastrointestinal Microbiota

To understand the results of microbiota analyses, it can help to understand a bit about the methods researchers use. As mentioned in the main article, researchers tend to use techniques that look for DNA and RNA related to specific microorganisms. To do that, they typically use one of two techniques: polymerase chain reaction (PCR) and shotgun sequencing. Here, we explain in general terms how each method is used to analyze GIT microbiota.

PCR

To successfully use PCR, researchers needed to find an appropriate gene target that would be common enough among microorganisms so they could use a known segment for searching but different enough so that they could individuate among microorganisms. They quickly selected ribosomal RNA (rRNA) genes (Pace 1986; Woese 1987). Ribosomal RNAs are essential for protein synthesis within all cells and therefore their genes have many features that make them desirable for determining the makeup of complex microbial communities. In particular, the genes contain regions of DNA that are highly variable among species and so can serve as a kind of identifier; but they also contain regions that are highly conserved, or the same among many species, and are therefore suitable for the development of broad-range PCR primers that use snippets of known DNA to search for specific genes. As a result of these features, rRNA genes have become the "gold standard" for molecular analyses, and they are typically analyzed using PCR-based techniques coupled with indirect fingerprinting or direct sequencing, including with next-generation sequencing (NGS). To profile GIT microbial communities using rRNA gene analysis, researchers typically extract genomic DNA from mucosa-associated colonic biopsies and fecal matter. They then use PCR to amplify the DNA, creating what are called "amplicons," using primers targeting conserved regions of the small subunit (SSU or 16S) rRNA gene from all bacteria and some-

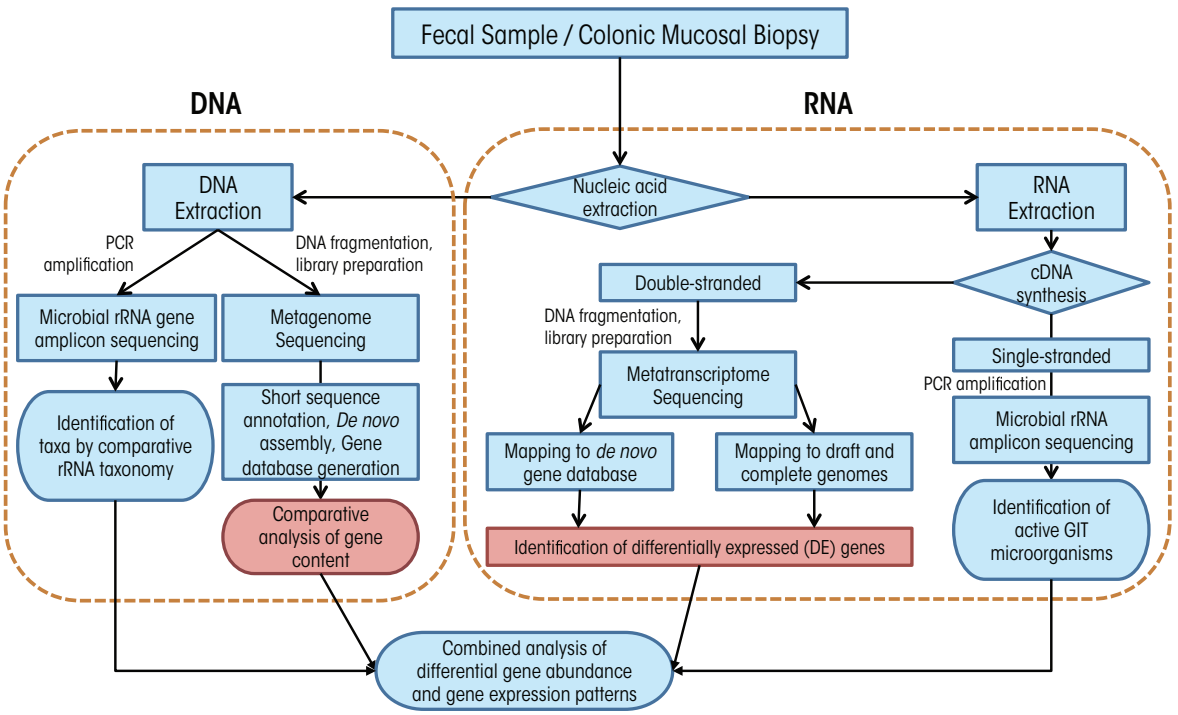

Figure 2 Basic pipeline for amplicon-based and shotgun sequencing approaches to the interrogation of GIT microbial communities. Nucleic acids can be interrogated independently to characterize the community structure and gene content of total (DNA) and active (RNA) microbial communities or combined to examine how shifts in microbiota are correlated with changes in community gene expression patterns.

Methods for Analyzing the Gastrointestinal Microbiota (*continued*)

times archaea. The researchers then sequence these PCR amplicons after suitable preparation for the chosen sequencing platform (Langille et al. 2013). Whereas it was previously common to have clone libraries on the order of 100 sequences per sample, it is more typical with NGS approaches to have sequence libraries of 10,000 to 100,000 sequences per sample. A suite of bioinformatics tools has been developed to process this high-throughput data such as RDP (Cole et al. 2005), mothur (Schloss et al. 2009), and QIIME (Caporaso et al. 2010).

Because of limitations inherent in the analysis of a structural gene, such as the rRNA gene that is common to all organisms, this method should be viewed as the first step in a multi-tiered approach to the analysis of microbial communities. The following are some limitations: (1) rRNA gene sequencing does not provide definitive physiological information about an organism; (2) for DNA-based methods, the presence of an organism's rRNA gene does not guarantee that the organism is active in the studied system at the time of sampling; (3) variation in the number of rRNA genes among bacterial lineages distorts the true diversity of microorganisms in an environmental sample; and (4) difficulty in species- and strain-level phylogenetic resolution among some taxa, depending upon the region of rRNA gene analyzed. Nonetheless, for large studies with many samples, a preliminary screen using this method is often suitable for identifying large-scale shifts in microbial community structure and for identifying statistically significant changes in the relative abundance of organisms between groups or treatments.

That said, the interpretation of results from the analysis of microbial community composition using DNA-based methods can be confounded by the presence of DNA from dead, dormant, or weakly active organisms contributing little to overall microbial community function. To circumvent these limitations, researchers can directly target rRNAs instead of rRNA genes. In such an approach, researchers extract total RNA from an environmental sample and reverse transcribe this RNA using either a random primer mix or a gene-specific "reverse" primer matching the rRNA (figure 2). This process generates single-stranded complementary DNA (cDNA), which is then used as a template for PCR and sequencing with domain-level primer sets as is done with genomic DNA. As microbial RNA is labile and degrades rapidly if not continually produced, rRNA analysis reflects only active microorganisms, and the relative abundance of rRNAs represents the relative activity of organisms in the system. Although rRNA analysis still does not provide an explicit link to physiology for most organisms, such analyses may find stronger correlation to measured functions at the time of sampling. Microbial RNA degrades rapidly, and for GIT colonic tissue and fecal samples, the time delay until RNA can be extracted may result in a serious distortion of active organisms and gene expression patterns from in situ. Thus, animal model systems in which animals are killed for sampling may be more suitable for RNA studies as mRNAs and ribosomes can be preserved rapidly for downstream analyses.

Shotgun Metagenomic and Metatranscriptomic Sequencing

Although amplicon sequencing approaches are extremely useful for GIT microbiota community characterization, they are limited by the need to have some known DNA sequences to look for. Therefore, to detect novel genes and gene variants, it is necessary to have sequencing approaches that do not depend on such information. Researchers use so-called "shotgun" sequencing approaches (figure 2) to circumvent the need for a priori sequence information through the use of molecular manipulations of nucleic acids to attach known sequences for priming of sequencing reactions to unknown sequences. Shotgun sequencing approaches, in which no a priori selection of a region or gene of interest is performed, provides a holistic view of microbial communities, gene content, and expression patterns. However, low-abundance taxa or those with small genomes, like viruses, may be swamped out by high-abundance or large genome organisms and may benefit from targeted amplification approaches.

Two techniques are used for more detailed assessments of GIT microbiota functional capabilities: In shotgun metagenomics, total genomic DNA is fragmented and sequenced directly (Qin et al. 2010), and in shotgun metatranscriptomics, fragmented messenger RNAs are sequenced directly (Perez-Cobas et al. 2013). These techniques can provide data to identify active organisms and metabolic activities at the time of sampling (metatranscriptome) and to directly link community function to specific microbial lineages, even at the species or subspecies level (metagenome and metatranscriptome). Such in-depth analyses can

Methods for Analyzing the Gastrointestinal Microbiota (*continued*)

identify key GIT microbiota community members, identify essential genes associated with the GIT microbiota, and improve metabolic modeling to predict the physiology of dominant organisms in environments undergoing global changes (Greenblum et al. 2012; Karlsson et al. 2013; Qin et al. 2010). Metagenome sequencing can provide much more detailed taxonomy of communities based on genes other than rRNAs, particularly at the species and strain level (Morowitz et al. 2011; Poretsky et al. 2014). In particular, GIT microbiota analyses of disease states and obesity have found widespread application (Greenblum et al. 2012; Karlsson et al. 2012, 2013; Manichanh et al. 2006; Qin et al. 2012). A full survey of the methods for analysis of metagenomic data is beyond this review; however, many recent articles provide deeper overviews (Cho and Blaser 2012) and describe suitable pipelines (Huson et al. 2007; Meyer et al. 2008; Treangen et al. 2013; Zakrzewski et al. 2013).

Although powerful, these approaches are limited by many factors:

- High cost attributed to heavy sequence demand;
- Insufficiently robust reference databases to provide suitable annotation to all recovered gene fragments;
- High microbial diversity in the GIT, which leads to limited coverage of most organisms aside from highly abundant organisms;
- High transcript abundance of housekeeping genes; and
- High computer memory and computational demand for analysis.

Because of the relatively high cost of shotgun sequencing approaches relative to amplicon sequencing approaches (typically about 20 to 30 times higher cost), researchers must carefully tailor their project goals to the appropriate molecular methodology. In a tiered sequencing approach, researchers perform amplicon sequencing on all samples and use their analysis of amplicon data to select critical or representative samples for deeper sequence analysis.

Considerations for Nucleic Acid Extraction

Analysis of gastrointestinal tract (GIT) microbiota communities presents several features worthy of consideration. In particular, researchers take the majority of samples from feces and mucosa-associated colonic tissue biopsies. Traditionally, extraction of nucleic acids from mammalian feces generated nucleic acid templates of poor purity. However, new extraction protocols and commercial kits have largely removed nucleic acid purity as a limitation to downstream molecular analyses (Claassen et al. 2013; Ó Cuív et al. 2011). Indeed, many manufacturers produce kits specifically for GIT colonic tissue and fecal DNA extraction (e.g., Mo Bio PowerFecal® DNA Isolation Kit; Qiagen QIAamp DNA Stool Mini Kit; Zymo ZR Fecal DNA MiniPrep kit; Epicentre ExtractMaster™ Fecal DNA Extraction Kit). Although many of these extraction kits have similar chemistry, other features of the kits may be critical to the maximum recovery of genomic DNA from GIT colonic tissue and feces and to minimize distortion of the GIT microbiota community as a result of differential lysis of different types of microbial cells.

Mammalian GIT microbiota communities are dominated by bacteria from two phyla: *Bacteroidetes* and *Firmicutes* (Ley et al. 2008), and researchers have used the ratio of these phyla as a diagnostic parameter. For example, Mariat and colleagues (2009) observed dramatic age-related changes in the ratio of *Firmicutes* and *Bacteroidetes* (F/B) in feces from healthy individuals, and the ratio has been broadly utilized in studies of obesity, with greater numbers of *Firmicutes* in obese patients (Ley et al. 2006). That said, sampling processing procedures can affect this ratio because the phylum *Firmicutes* consists of mostly gram-positive bacteria with thick cell walls that can make them difficult to lyse, thus high-energy lysis steps (e.g., bead-beating) are important in extraction protocols. In addition, lytic enzymes such as lysozyme, mutanolysin, and lysostaphin can be used individually or in combination to enhance lysis of difficult-to-lyse organisms (Yuan et al. 2012). One study (Bahl et al. 2012) demonstrated that freezing of fecal samples prior to DNA extraction can alter the F/B ratio, with enhanced relative abundance of *Firmicutes* after freezing. As a result of these issues, it may be difficult to easily compare directly between studies of fecal samples processed under different conditions. Likewise, protocols should be carefully considered and rigorously adhered to in order to provide reproducible handling for each sample.

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from the intestinal lumen into systemic circulation (Mutlu et al. 2009; Rimola 1991). Similarly, 16S rRNA gene analysis of fecal microbiota from human subjects with hepatitis B or alcohol-related cirrhosis shows a reduction in *Bacteroidetes* and an increase in *Proteobacteria* and *Fusobacteria*, compared with healthy control subjects (table 1) (Chen et al. 2011). At a finer taxonomic resolution, this study also shows a significant increase in potentially dangerous bacteria from the families *Prevotellaceae*, *Enterobacteriaceae, Veillonellaceae,* and *Streptococcaceae* in subjects with alcoholic cirrhosis, compared with subjects with hepatitis B cirrhosis and with control subjects. The prevalence of potentially pathogenic bacteria in patients with cirrhosis may affect prognosis, something supported by previous research (Guarner et al. 1997; Liu et al. 2004). Other lower resolution studies find that the relative abundance of bacteria from the phylum *Bacteroidetes* decreases as those from the phylum *Proteobacteria* increase and that individuals with cirrhosis exhibit a unique increase in *Fusobacteria* (Chen et al. 2011; Mutlu et al. 2012). Overall, alcoholics and cirrhosis patients demonstrate microbial communities enriched in *Proteobacteria* of the class *Gammaproteobacteria* and *Firmicute* of the class *Bacilli.* In contrast, *Firmicutes* of the class *Clostridia* are depleted in alcoholics but are not significantly changed in the cirrhosis group, with the exception of *Veillonellaceae,* which is increased and *Lachnospira,* which is decreased (table 1). These findings suggest that microbiota community differences between alcoholics and alcoholics with cirrhosis (e.g., *Fusobacteria, Clostridia*) may contribute to the development of liver disease or may be a biomarker indicating liver disease (figure 3). Future studies will need to determine the cause-and-effect relationship of the microbiota community structure and liver disease.

Although alcohol can cause intestinal dysbiosis, some alcoholic beverages contain compounds that may favorably alter the GIT microbiota community composition. A study showed the effects of dietary polyphenols on the human GIT microbiota in human healthy control subjects who consumed red wine (272 mL per day), de-alcoholized red wine (272 mL per day), or gin (100 mL per day) for 20 days and had their total fecal DNA assessed from stool collected at baseline and after treatment (Queipo-Ortuno

Table 1 Changes in the Intestinal Microbiome Associated With Alcohol in Rodent Models and Humans

Reference	Tested Organism	Experimental Condition	Methodology	Major Taxa Altered in Presence of Alcohol [a,b]	Major Finding
Yan et al. 2011	Mouse	3-week alcohol-fed mice/control isocaloric liquid	•16S rRNA gene amplicon sequencing (pyro-sequencing) •Mouse cecum	↑***Verrucomicrobia* phylum:** ↑*Akkermansia* genus ↑***Bacteroidetes* phylum:** ↑*Bacteroidetes* class, ↑*Bacteroidales* order, ↑*Bacteroides* genus, ↑*Porphyromonadaceae* family ↓***Firmicutes* phylum:** ↓*Lactococcus*, ↓*Pediococcus*, ↓*Lactobacillus*, and ↓*Leuconostoc* genus	Alcohol-fed mice have GIT microbial community composition significantly altered from control mice indicating dysbiosis.
Mutlu et al. 2009	Rat	10-week alcohol-fed rats/control isocaloric dextrose	•Length heterogeneity PCR (LH-PCR) •Ileal and colonic rat mucosa tissue		Alcohol-fed rats have GIT microbial community composition significantly altered from control rats. Dysbiosis may be an important mechanism of alcohol-induced endotoxemia.
Mutlu et al. 2012	Human	•Alcoholics with and without alcoholic liver disease/healthy patients	•16S rRNA gene amplicon sequencing (pyro-sequencing) •Mucosa sigmoid biopsies	↑***Proteobacteria* phylum:** ↑*Gammaproteobacteria* class ***Firmicutes* phylum**: ↑*Bacilli* & ↓*Clostridia* class ↓***Bacteroidetes* phylum:** ↓*Bacteroidetes* class ***Verrucomicrobia* phylum:** ↓*Verrucomicrobiae* class	Human chronic alcohol use is associated with changes in the mucosa-associated colonic bacterial composition in a subset of alcoholics from healthy controls. Dysbiotic microbial community alteration correlated with high level of serum endotoxin.
Chen et al. 2011	Human	•Cirrhotic/healthy patients •Alcoholic cirrhotic/healthy patients •Hepitits B virus cirrhosis/alcoholic cirrhotic patients	•16S rRNA gene amplicon sequencing (pyro-sequencing) •Fecal samples	↑***Proteobacteria* phylum:** ↑*Gammaproteobacteria* class: ↑*Enterobacteriaceae* family ***Firmicutes* phylum:** ↑*Bacilli* class: ↑*Streptococcaceae* family; *Clostridia* class: ↑*Veillonellaceae* and ↓*Lachnospiraceae* family ↑***Fusobacteria* phylum:** ↑*Fusobacteria* class ↓***Bacteroidetes* phylum:** ↓*Bacteroidetes* class ***Bacteroidetes* phylum:** ↑*Prevotellaceae* family	Fecal GIT microbial community composition significantly altered in patients with cirrhosis compared with healthy individuals. **Prevotellaceae* was enriched in alcoholic cirrhosis patients when compared with HBV cirrhosis patients and healthy controls.
Queipo-Ortuno et al. 2012	Human	Healthy patients 20-day intake of either red wine, de-alcoholized red wine, or gin	•Quantitative real-time PCR •Fecal samples	**Red wine** ↑***Proteobacteria* phylum:** (↓Gin) ↑***Fusobacteria* phylum:** (↑De-Alcoholized) (↓Gin) ↑***Firmicutes* phylum:** (↓Gin) ↑***Bacteroidetes* phylum:** (↓Gin) **Red wine** ↑*Enterococcus* genus (↑De-Alcoholized) (↓Gin) ↑*Prevotella* genus (↑De-Alcoholized) (↓Gin) ↑*Bacteroides* genus (↑De-Alcoholized) (↓Gin) ↑*Bifidobacterium* genus (↑De-Alcoholized) (↓Gin) ↑*Bacteroides* uniformis species: (↑De-Alcoholized) (↓Gin) ↑*Eggerthella lenta* species (↑De-alcoholized) (↓Gin) ↑*Blautia coccoides-Eubacterium rectale* species (↑De-alcoholized) (↓Gin) ↓*Clostridium* genus (↓De-Alcoholized) (↑Gin) ↓*Clostridium histolyticum* species (↓De-alcoholized) (↑Gin)	Red wine consumption, compared to de-alcoholized red wine and gin, significantly altered the growth of select GIT microbiota in healthy patients.This microbial community composition could influence the host's metabolism. Also, polyphenol consumption suggests possible prebiotic benefits, due to the increase growth of *Bifidobacterium.*
Bode et al. 1984	Human	Alcoholic/hospitalized control patients	•Aerobic and anaerobic bacterial culture incubation •Jejunum aspirates	↑**Gram-negative anaerobic bacteria** ↑**Endospore-forming rods** ↑**Coliform microorganisms**	Chronic alcohol abuse leads to small intestinal bacterial overgrowth, suggesting dysbiosis may contribute to functional and morphological abnormalities in the GIT.

NOTES: [a] A comparison of bacterial Taxa either ↑, increased or ↓, decreased relative to the presence of alcohol. [b] Taxonomy was updated using the NCBI Taxonomy Browser.

et al. 2012). Red wine polyphenol significantly increases the abundance of *Proteobacteria*, *Fusobacteria*, *Firmicutes* and *Bacteroidetes*, whereas gin consumption significantly decreases these same bacterial phyla (table 1). De-alcoholized red wine consumption significantly increases *Fusobacteria,* and gin consumption increases *Clostridium* abundance compared with de-alcoholized and red wine (table 1). Red wine and de-alcoholized red wine consumption increases the abundance of *Bifidobacterium*, a bacterium that has been shown to be beneficial in the GIT (Gibson et al. 1995). Thus, it seems that polyphenol consumption is associated with an increase in bacteria that are known to promote GIT health, whereas alcohol consumption alone may be damaging to the microbiota balance. The significant decrease of *Clostridium* associated with the consumption of red wine polyphenols suggests that polyphenols may have an inhibitory effect on the growth of *Clostridium,* which has been linked to the progression of colonic cancer and the onset of IBD (Guarner and Malagelada 2003). These results indicate that polyphenol consumption may be used as a dietary intervention to alter the microbiota in a specific way. In addition, daily moderate consumption of red wine polyphenols increases the growth of *Bifidobacterium*, which could be associated with positive prebiotic effects of GIT microbiota, production of beneficial organic acids, and the growth inhibition of pathogenic bacteria (Gibson et al. 1995). Also, as an important consideration to evaluating alcohol-induced effects on the GIT microbiota, differences attributed to the type of alcohol consumption may be contributing to intra- and interstudy variability.

Whether alcohol-induced dysbiosis contributes to the pathogenesis of diseases, such as ALD or alcohol-related cirrhosis, is undetermined. Future studies will need to determine the biological, functional, and clinical significance of the dysbiotic intestinal microbiota composition in alcohol-related disorders.

From Dysbiosis to Disease

Once alcohol disrupts the intestinal microbiota, both the microbiota and microbiome may increase susceptibility to pathological changes (Lozupone et al. 2012). The majority of the reviewed studies indicate an association between alcohol-induced intestinal bacterial overgrowth and dysbiosis and the development/progression of ALD and cirrhosis. Indeed, disrupted intestinal barrier function, which is associated with alcohol consumption, in combination with alcohol-induced bacterial overgrowth and dysbiosis, could be highly relevant for the development of alcohol-induced liver pathology, including nonalcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH), and ALD. Studies show that alcohol consumption disrupts the intestinal barrier (Keshavarzian et al. 1999) via increasing oxidative stress burden in the intestine, which in turn disrupts tight junctions and promotes intestinal hyperpermeability (Rao et al. 2004). Increased intestinal hyperpermeability allows proinflammatory/pathogenic microbial products, including endotoxin (e.g., lipopolysaccharide [LPS] and peptidoglycan), to translocate from the intestinal lumen to the liver via the portal vein (Frazier et al. 2011). Exposure to these bacterial products causes inflammation in the liver, which may work in conjunction with the direct effects of alcohol to cause ALD (Schnabl and Brenner 2014). This translocation of viable bacterial products during bacterial overgrowth or alcohol-induced dysbiosis may significantly contribute to end-stage liver disease observed in alcohol cirrhosis patients and may therefore contribute to the mortality of cirrhotic patients by inducing infection (Schnabl and Brenner 2014).

Interventions to Normalize Alcohol-Induced Intestinal Dysbiosis

Research in rodents and humans has begun to investigate whether alcohol-induced intestinal dysbiosis and its consequences may be reversible with probiotic and synbiotic interventions (table 2). Probiotics are live microorganisms that, when taken by the host, have beneficial effects on the host beyond their simple nutritive value (Ewaschuk and Dieleman 2006). Synbiotics are a combination of probiotics and prebiotics—nondigestible fibrous compounds, such as oats, that stimulate the growth and activity of advantageous bacteria in the large intestine.

Probiotics, especially *Lactobacillus rhamnosus* GG (LGG), have several beneficial effects on intestinal function, including stimulating intestinal development and mucosal immunity, ameliorating diarrhea, prolonging remission in ulcerative colitis and pouchitits, reducing intestinal oxidative stress, and maintaining or improving intestinal barrier function (Bruzzese et al. 2004; Ewaschuk and Dieleman 2006; O'Hara and Shanahan 2006; Resta-Lenert and Barrett 2003; Sartor 2004; Tao et al. 2006; Versalovic 2007). Synbiotics have been demonstrated to favorably alter liver metabolism in alcohol-fed animals (Martin et al. 2009).

Studies in rodents demonstrate that both probiotics and prebiotics prevent alcohol-induced dysbiosis. A study in Sprague-Dawley rats that had consumed alcohol (8 g/kg per day) daily for 10 weeks showed that intragastrically feeding them probiotic LGG (2.5×10^7 live once daily) or prebiotic oats (10 g/kg) prevented alcohol-induced GIT dysbiosis (Mutlu et al. 2009). The rats given the interventions had microbiota composition profiles similar to that of control rats that were intragastrically fed an isocaloric dextrose diet for 10 weeks. This finding corresponds to results obtained in an ALD rodent model demonstrating that LGG attenuates endoxtemia and alcoholic steatohepatitis (Nanji et al. 1994). Furthermore, LGG and oat supplementation ameliorates alcohol-induced intestinal oxidative stress, intestinal hyperpermeability, and liver injury in rodent models of alcohol steatohepatitis (Forsyth et al. 2009; Tang et al. 2009). In another study, researchers orally fed C57BL/6 mice the Lieber-DeCarli diet with or without alcohol

Table 2 Changes in the Intestinal Microbiota Associated With Alcohol and Probiotic or Synbiotic Intervention in Rodent Models and Humans

Reference	Tested Organism	Experimental Condition	Methodology	Major Taxa Altered in Presence of Alcohol [a,b]	Major Finding
Mutlu et al. 2009	Rat	**10 week:** Control isocaloric dextrose-fed rats/alcohol-fed rats **1 week (at week 10):** Alcohol + LGG-fed rats/ alcohol + oat-fed rats/ dextrose + oat-fed rats	• Length heterogeneity PCR (LH-PCR) • Colonic rat mucosa tissue		Alcohol-fed rats have GIT microbial community composition significantly altered from control rats. Both probiotic (LGG) and prebiotic (oats) intervention prevented alcohol-induced dysbiosis, at week 10 in the colonic mucosa tissue of rats.
Bull-Otterson et al. 2013	Mice	**6 week:** Alcohol-fed mice/control isocaloric maltose dextrin-fed mice **3 week (at weeks 6–8):** Alcohol + LGG-fed mice	• 16S rRNA gene amplicon sequencing (pyro-sequencing) • Fecal mice samples	**Alcohol induced:** ↑***Proteobacteria* phylum:** ↑*Alcaligenes* genus ↑***Artinobacteria* phylum:** ↑*Corynebacterium* genus ***Firmicutes*:** ↑*Aerococcus,* ↑*Listeria,* ↑*Acetivibrio,* ↑*Clostridiales,* ↑*Allobaculum,* ↑*Lactobacillus* genus ↓***Bacteroidetes* phylum:** ↓*Bacteroides,* ↓*Parabacteroides,* ↓*Tannerella,* ↓*Hallella* genus ↓***Firmicutes* phylum:** ↓*Lachnospiraceae,* ↓*Ruminococcaceae* genus **Alcohol + LGG:** ↓***Proteobacteria* phylum:** ↓*Alcaligene* genus ↓*Artinobacteria* phylum: ↓*Corynebacterium* genus ↑***Bacteroidetes* phylum** ↑↑↑***Firmicutes* phylum:** ↑*Lactobacillus,* ↑*Ruminococcaceae* genus	Alcohol-fed mice have fecal GIT microbial community composition significantly altered from control mice. Probiotic (LGG) treatment prevented alcohol induced dysbiosis expansion. LGG reversed the expansion of the *Proteobacteria* and *Actinobaceria* phyla, which could play a pathogenic role in the development of alcoholic liver disease. *Firmicutes* expanded greatly in the alcohol + LGG–fed group.
Liu et al. 2004	Human	**30-day treatment:** • Cirrhotic with MHE + synbiotic or prebiotic or placebo/ control patients **Subgroup:** • Sober alcoholics 2 weeks & etiology is alcohol-cirrhosis	• Quantitative bacterio-logical culture • Fecal samples	**Cirrhotic with MHE:** ↑ *Escherichia coli* species ↑ *Staphylococcus* genus **Cirrhotic with MHE + synbiotic** ↓ *Escherichia coli* species ↓ *Staphylococcus* genus ↓ *Fusobacterium* genus ↑*Lactobacillus* genus **Cirrhotic with MHE + prebiotic** ↓ *Escherichia coli* species ↓ *Fusobacterium* genus ↑*Bifidobacterium* genus	Cirrhotic patients with MHE were found to have significant fecal overgrowth of potentially pathogenic gram-negative (*E. coli*) and gram-positive (*Staphylococcus*) aerobic microbiota. After 30 days of synbiotic or prebiotic treatment, supplementation reduced *E. coli, Staphylococcus,* and *Fusobacterium* and increased *Lactobacillus* (Synbiotic) and *Bifidobacterium* (prebiotic) organisms in feces of cirrhotic patients with MHE.

NOTES: [a] A comparison of bacterial Taxa either ↑, increased or ↓, decreased relative to the presence of alcohol. [b] Taxonomy was updated using the NCBI Taxonomy Browser.

(5% vol/vol) for 6 weeks and gave a subset of the mice 1 mL of LGG (bacterial density 1×10^9 cfu/mL) orally each day for 6 to 8 weeks (Bull-Otterson et al. 2013). Similar to other findings, the alcohol-fed mice demonstrated a decrease in the abundance of *Bacteriodetes* and *Firmicutes* and an increase in *Proteobacteria* and *Actinobacteria* (table 2). However, probiotic LGG supplementation prevented this alcohol-induced dysbiotic intestinal microbiota composition, especially increasing *Firmicutes*, including *Lactobacillus*. Other studies find that LGG prevents alcohol-induced intestinal hyperpermeability, endotoxemia, and liver injury (Wang et al. 2011, 2013), supporting the notion that LGG may be a therapeutic approach to decrease the development of ALD.

Studies in humans show similar results. One study examined Minimal Hepatic Encephalopathy (MHE) patients with cirrhosis who typically have substantial alterations in their GIT microbiota composition caused by the overgrowth of the potentially pathogenic *Escherichia coli* and *Staphylococcal* species (table 2). Following 30 days of synbiotic and prebiotic treatments, these patients had significantly reduced viable counts of potentially pathogenic GIT microbiota with a concurrent significant increase in fecal content of *Lactobacillus* species (table 2) (Liu et al. 2004). Half of the patients receiving synbiotic treatment also exhibited a significant reduction in blood ammonia levels, endotoxemia, and reversal of MHE, when compared with control subjects. These improvements in MHE correlate with similar findings showing that probiotic supplementation improved hepatic encephalopathy (HE) in patients with cirrhosis (Macbeth et al. 1965). Interestingly, probiotic LGG supplementation prevents alcohol-induced dysbiosis of the intestinal microbial community, and leads to an increase in *Firmicutes,* particularly of the genus *Lactobacillus*. Furthermore, in an U.S. Food and Drug Administration phase I study, the administration of probiotic LGG to cirrhotic patients with MHE (most of whom had Hepatitis C–induced cirrhosis) found that LGG significantly reduces dysbiosis, tumor necrosis factor (TNF)-α, and endotoxemia in comparison to placebo (Bajaj et al. 2014). In addition, LGG shows beneficial changes in the stool microbial profiles and significant changes in metabolite/microbiota correlations associated with amino acid, vitamin, and secondary bile-acid metabolism in comparison to MHE cirrhotic patients randomly assigned to placebo. In a comparison of the synbiotic and prebiotic treatment to cirrhotic patients with MHE in the study above, probiotic LGG does promote beneficial microbiota; however, it does not increase *Lactobacillus* and does not improve cognitive function in the patients for this randomized clinical trial. Thus, taken together, probiotics

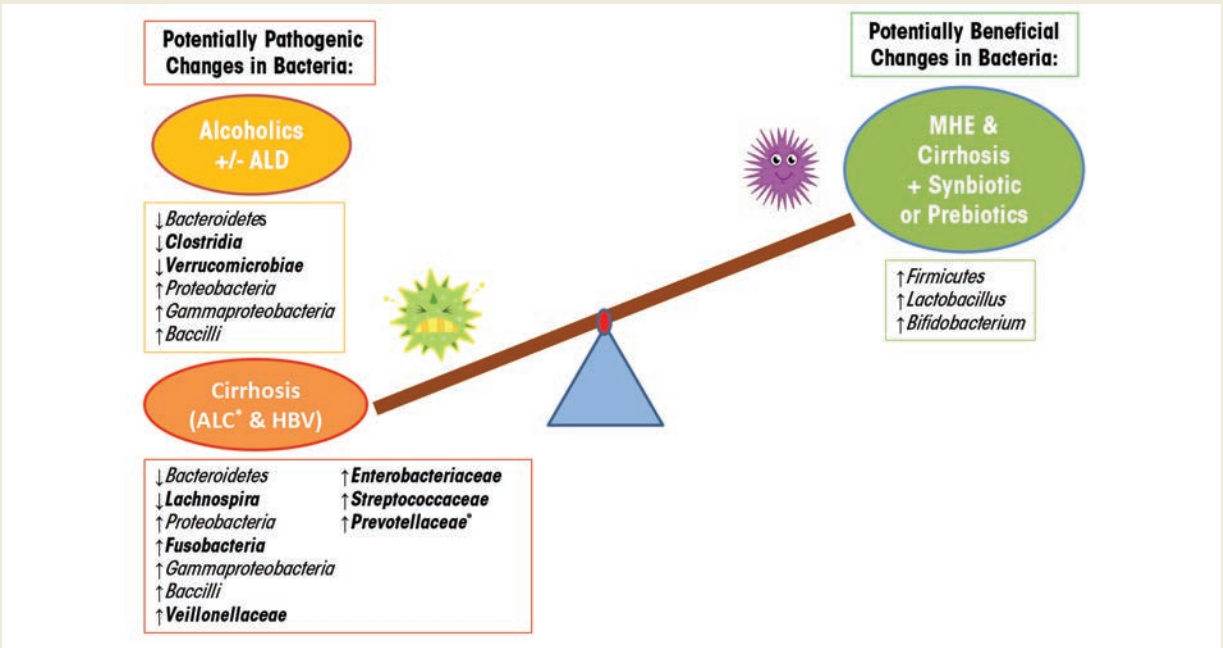

Figure 3 Alcohol-induced imbalances in the microbiome of the gastrointestinal tract (dysbiosis) have been associated with promoting potentially pathogenic changes in bacteria in alcoholics with and without liver disease and in patients with cirrhosis caused by hepatitis B or alcohol. Both alcoholic and cirrhosis patients demonstrate similar dysbiotic microbiota changes, except for the bacteria indicated, suggesting that these dysbiotic bacterial differences could contribute to liver disease or may be a biomarker indicating liver disease. Using synbiotics and prebiotics to treat Minimal Hepatic Encephalopathy patients with cirrhosis, significantly improved their GIT microbiota, suggesting that the same treatment may benefit patients with alcohol-induced dysbiosis.

and/or synbiotics may be a viable approach in humans to alter the GIT microbiota to a more favorable profile to improve clinical outcomes (figure 3).

Therapeutic Intervention for Treating Alcohol-Induced Intestinal Dysbiosis

The therapeutic intervention studies in this review indicate that in ALD rodent models and MHE alcohol-cirrhosis humans, probiotic and synbiotic intervention increases *Lactobacillus* and *Bifidobacterium* (table 2). These findings suggest that the intestinal microbiota play a role in attenuating alcohol-induced dysbiosis and liver injury. In addition, the modulation of intestinal microbiota could be a viable therapeutic strategy to prevent or normalize alcohol-induced dysbiosis and which would be expected to have beneficial effects on alcohol-induced liver injury as well as other inflammatory-mediated diseases resulting from chronic alcohol consumption.

Evidence suggests that probiotic and synbiotic interventions can not only reverse alcohol-induced dysbiosis but can improve the pathogenesis symptoms of the GIT and liver in ALD. Treatment with probiotics prevents or significantly decreases alcohol-induced intestinal permeability (Forsyth et al. 2009; Wang et al. 2012), intestinal oxidative stress and inflammation of the intestine and liver (Forsyth et al. 2009), TNF-α production (Wang et al. 2013), and expression of intestinal trefoil factor and its transcriptional regulator hypoxia-inducible factor-2α (HIF-2α) (Wang et al. 2011) and attenuates endotoxemia and alcoholic steatophepatitis (Nanji et al. 1994) in rodent models and in humans with ALD. Probiotics also restore stool microbiota community structure and liver enzymes in ALD human patients (Kirpich et al. 2008). In addition, prebiotic oat supplementation prevents alcohol-induced gut leakiness in an ALD rat model by preventing alcohol-induced oxidative tissue damage (Tang et al. 2009). Thus, these studies suggest that probiotics (e.g., *Lactobacillus*) transform the intestinal microbiota community composition, which may prevent alcohol-induced dysbiosis, intestinal permeability, bacterial translocation, endotoxemia, and the development of ALD. Transformation of the intestinal microbiota may be a therapeutic target for the treatment of intestinal barrier dysfunction and the development of ALD.

Clinical studies suggest that probiotic consumption of *Lactobacilli, Bifidobacteria,* and *Lactocooci* are effective for the prevention and treatment of a diverse range of disorders (Snydman 2008). History shows that probiotic consumption is safe in healthy people but must still be taken with caution in certain patient groups, including premature neonates, people with immune deficiency, people with short-bowel syndrome, people with central venous catheters, the elderly, and people with cardiac disease (Boyle et al. 2006; Snydman 2008). Clinical trials show that the effects of probiotics are variable depending on age, health, and disease state. Probiotic use also has its concerns. It presents a major risk of sepsis (Boyle et al. 2006) and has been associated with diseases such as bacteremia or endocarditis, toxic or metabolic effects on the GIT, and the transfer of antibiotic resistance in the gastrointestinal flora (Snydman 2008). In addition, the many properties of different probiotic species vary and can be strain specific. Therefore, the effect of new probiotic strains should be carefully analyzed in clinical trials before assuming they are safe to market as a potential therapeutic treatment.

Glossary

Dysbiosis: Dysbiosis is a term used to describe a microbial imbalance on or inside the body, commonly within the digestive tract where it has been associated with illness.

Endotoxemia: The presence of endotoxins in the blood, where endoxins are toxic substances bound to the cell wall of certain bacteria.

Polymerase Chain Reaction (PCR): A biochemical technology used to amplify a single or a few copies of a particular piece of DNA, generating millions of copies of that DNA sequence. Among other uses, the technique allows researchers to make enough copies of a piece of DNA to sequence it. PCR requires "primers" or small snippets of DNA that match a piece of the DNA researchers are attempting to replicate.

Tumor necrosis factor-alpha (TNF-α): A type of cytokine, or cell-signaling protein that can cause cell death.

Future Directions

Chronic alcohol consumption causes intestinal dysbiosis in both rodent models and humans. Dysbiosis in the intestinal microbiota may contribute to the pathogenesis of liver disease by altering intestinal barrier function leading, for example, to gut leakiness, the production of proinflammatory/pathogenic microbial products, and/or liver metabolic pathways. Further investigation into intestinal microbiota composition in alcoholism is necessary to identify new diagnostic as well as therapeutic targets to prevent alcohol-associated diseases, such as ALD. Such therapeutic avenues could include probiotics, prebiotics, synbiotics, or polyphenols to alleviate the symptoms associated with alcohol disorders. Thus, understanding the effect of alcohol on intestinal microbiota composition, may lead to a better understanding of its future functional activity, with the ultimate goal to restore intestinal microbiota homeostasis.

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The authors declare that they have no competing financial interests.

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The First Line of Defense

The Effects of Alcohol on Post-Burn Intestinal Barrier, Immune Cells, and Microbiome

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Alcohol (ethanol) is one of the most globally abused substances, and is one of the leading causes of premature death in the world. As a result of its complexity and direct contact with ingested alcohol, the intestine represents the primary source from which alcohol-associated pathologies stem. The gut is the largest reservoir of bacteria in the body, and under healthy conditions, it maintains a barrier preventing bacteria from translocating out of the intestinal lumen. The intestinal barrier is compromised following alcohol exposure, which can lead to life-threatening systemic complications including sepsis and multiple organ failure. Furthermore, alcohol is a major confounding factor in pathology associated with trauma. Experimental data from both human and animal studies suggest that alcohol perturbs the intestinal barrier and its function, which is exacerbated by a "second hit" from traumatic injury. This article highlights the role of alcohol-mediated alterations of the intestinal epithelia and its defense against bacteria within the gut, and the impact of alcohol on intestinal immunity, specifically on T cells and neutrophils. Finally, it discusses how the gut microbiome both contributes to and protects the intestines from dysbiosis after alcohol exposure and trauma.

Key words: Alcohol use, abuse, and dependence; alcohol consumption; alcohol exposure; alcohol effects and consequences; burns; immunity; immune cells; microbiome; intestine; gut; intestinal lumen; intestinal barrier; bacteria; sepsis; organ failure; trauma; T cells; neutrophils; dysbiosis; human studies; animal models

Each year 2.5 million people die from alcohol abuse and its related morbidities worldwide, making alcohol related deaths among the highest preventable causes of death, and the greatest cause of premature death and disability in men between ages 15 and 59 (World Health Organization 2011). Alcohol abuse predisposes individuals to life-threatening conditions such as alcoholic liver disease (ALD), acute respiratory distress syndrome (ARDS), sepsis, and multiple organ failure (MOF) (Bird and Kovacs 2008; Molina et al. 2003; Purohit et al. 2008). Further, studies show that intoxication often plays a role in physical injury (Pories et al. 1992). Data demonstrate that a majority of patients admitted to the hospital for traumatic injury have detectable blood alcohol levels at the time of admittance (Grobmyer et al. 1996; Jones et al. 1991; Maier 2001; McGill et al. 1995; McGwin et al. 2000; Silver et al. 2008). These patients generally require more extensive care than patients who have not been drinking. They more frequently require surgical intervention, experience higher susceptibility to infection, and have longer hospital stays (Silver et al. 2008). Supporting these observations, experimental data suggest that alcohol at the time of trauma results in more severe pathology in animal models (Choudhry and Chaudry 2008; Messingham et al. 2002; Molina et al. 2003, 2013). As a result, researchers estimate that in the United States alone, trauma and alcohol-related expenses to society total $185 billion annually (Li et al. 2004).

The disruptions to human biology that underlie the association between alcohol and these conditions bear exploring. The intestine, where alcohol first meets with digestive and immune mechanisms, is a primary source of alcohol-related pathologies. Here, alcohol and its metabolites encounter the physical barrier lining the gut that prevents invading pathogens from moving into the body. They also come into contact with a particularly complex frontier where the immune system

must distinguish between commensal bacteria that normally colonize human intestines, and foreign microbes that cause disease. Any disruption of these systems by alcohol certainly could contribute to inflammatory states in the body that may in turn lead to serious conditions such as sepsis and MOF.

In support of these possibilities, data have shown that acute alcohol exposure negatively affects the function of the intestines, and this is exacerbated by a second traumatic insult such as burn injury (Akhtar et al. 2009, 2011; Li et al. 2008*a*, 2009, 2011, 2012; Rendon et al. 2012, 2013, 2014). The consequences of disruptions to the intestinal barrier, immune cells, and microbiome (see Glossary) can be observed within 24 hours following injury, and likely contribute to the life-threatening complications mentioned above. Thus, understanding how both acute and chronic alcohol exposure disrupt the homeostatic gastrointestinal tract is paramount. This article will review relevant studies examining the role of gut epithelia in defense against pathogenic bacteria within the gut and the impact of alcohol on intestinal immunity, highlighting T cells and neutrophils. Finally, it will review how the gut microbiome plays a role in maintenance of gut barrier integrity following alcohol exposure and trauma.

Intestinal Anatomy and Histology

To fully understand the intricate relationships among the gut barrier, immune system, and microbiome, gastrointestinal (GI) anatomy requires review. The spatial relationships established between the lumen and barrier of the gut are essential for the proper function of the GI tract in digestion and nutrient absorption. The GI tract is a continuous tube that begins at the mouth and ends at the anus. The small and large intestines function mainly to absorb nutrients and water, and this review will focus on these organs.

The small intestine is divided into three regions: the duodenum, jejunum, and ileum, respectively. At the distal end of the ileum lies the cecum, which connects the small and large intestines. From the cecum, the large intestine (colon) is composed of four regions: the ascending, transverse, descending and sigmoid colon, respectively, terminating in the rectum and anus. The small and large intestines are held in place to prevent twisting by the mesentery, which also contains the mesenteric lymph nodes (MLNs). As shown in figure 1, the small and large intestines at the histological level contain a barrier of mucous and epithelial cells that block the translocation of bacteria in the lumen to sites in the body beyond the intestines. Just below the intestinal epithelia lies a layer of loose connective tissue called the lamina propria (LP), which connects the surface mucosal epithelium to the basement muscularis mucosae. The LP also contains a large number of intestinal immune cells. In addition, specialized regions within the small intestine called Peyer's patches (PPs) serve as lymphoid follicles, where naïve immune cells differentiate into a variety of mature immune cell subsets.

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When a pathogen invades through the gut, the intestinal barrier and the immune cells in it mount a response to prevent infection. However, the picture gets more complex because of the gut microbiome, the mix of commensal bacterial species colonizing the lumen. The immediate proximity of the intestinal immune cells to the bacteria within the lumen presents a major challenge for homeostatic regulation. Thus, the interactions between the immune cells, intestinal barrier, and lumenal microbiome are of major interest in all areas related to pathology associated with the intestines. Alcohol modulates all of these components, and a disruption of any one can result in serious disease and/or infection that can affect all regions of the body.

The Homeostatic Intestinal Physical Barrier

Looking more closely at the meeting point of the lumen with the intestinal wall, the intestinal physical barrier consists of a layer of mucus and epithelial cells that line the lumen and provide a crucial first line of defense against pathogens. Starting from the lumen, the first component of the physical barrier is a mucus layer. Mucus offers protection from the lumenal bacterial content and also lubricates the intestinal walls for passing bile (Bollinger et al. 2006; Groschwitz and Hogan 2009; Peterson and Artis 2014; Valatas and Kolios 2009). Immediately below the mucus layer, a single layer of epithelial cells forms a second barrier featuring tight junction protein complexes that adhere adjacent cells to each other (Peterson and Artis 2014; Ulluwishewa et al. 2011). The body maintains this barrier by regulating the proliferation and apoptosis of epithelial cells (Peterson and Artis 2014). Together, the mucus layer and epithelial cells of the intestinal barrier minimize interactions of inflammatory host immune cells with the lumenal bacteria.

Mucus Layer

The mucus layer is a key component of the physical barrier and is formed by a glycoprotein, mucin (mainly mucin-2). Goblet cells found in the intestinal epithelial layer secrete mucin (Kim and Ho 2010). Mucin contains a glycosylated peptide backbone, which creates an incredibly viscous mucus layer effective at preventing pathogen

penetration (Hartmann et al. 2013). Recently, a study found that the small intestine has a porous mucus layer that allows uptake of mucin-2 (MUC2) by intestinal dendritic cells (DCs) (see "Primer on the Immune System" in this issue). DCs containing MUC2 were able to generate anti-inflammatory responses through β-catenin and NFκB-mediated mechanisms, giving rise to a newly identified homeostatic role for the intestinal mucosa (Shan et al. 2013).

Epithelial Layer

The mucus layer is not impenetrable, however, and the tight junction complexes between the epithelial cells below the mucus layer play a crucial role in providing a second level of protection. Tight junctions (figure 2) are multi-protein complexes consisting of transmembrane, scaffold, and adaptor proteins, which play an indispensable part in the maintenance of barrier function (Ivanov 2012). The proteins of tight junctions form a paracellular seal and function as a selectively permeable barrier between adjacent epithelial cells. They allow nutrients from food to pass out of the lumen while blocking passage of bacteria. Among the transmembrane proteins making up tight junctions are occludin, claudins, tricellulin, and junctional adhesions (Ulluwishewa et al. 2011). Although the function of occludin proteins is unknown, they are not essential for tight junction formation but appear instead to be instrumental in the regulation of the junctions (Balda and Matter 2008; Forster 2008; Groschwitz and Hogan 2009). Claudins are a family of both tissue- and cell-type–specific proteins considered to be the main structural components of the tight junctions. A third class of proteins found in tight junctions are junction-associated adhesion molecules (JAMs); however, little is known about their contribution to tight junction function and assembly (Balda and Matter 2008;

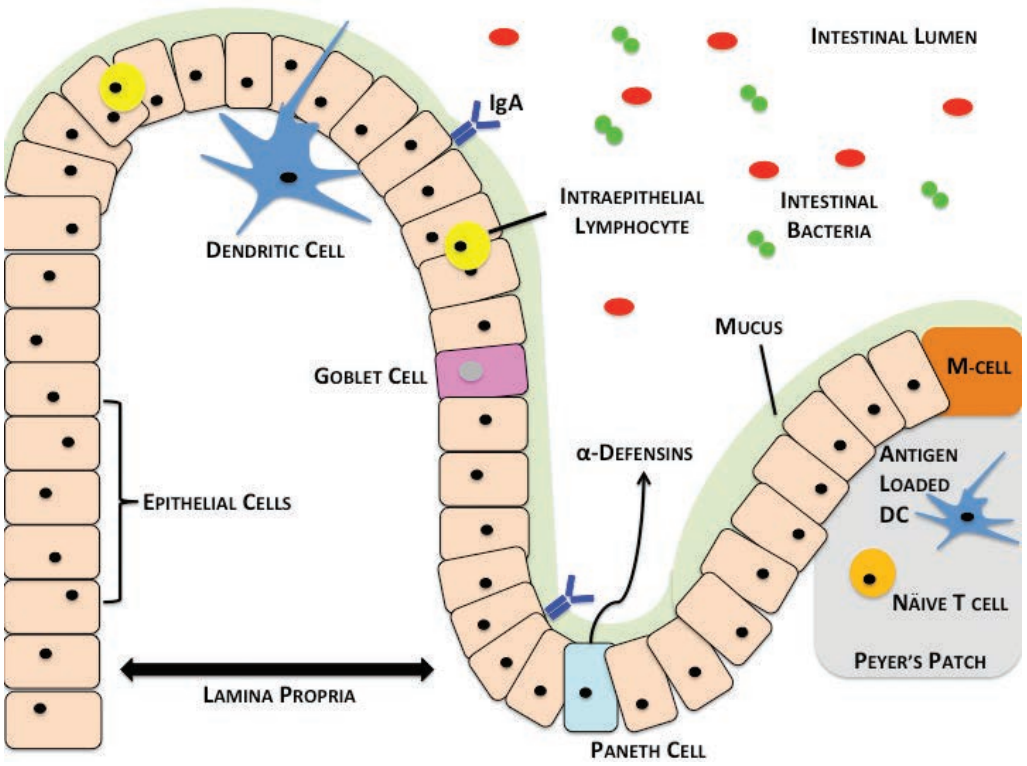

Figure 1 Overview of the intestinal barrier, immune cells, and microbiome. Lumenal bacteria (red and green) are relegated to the lumen of the intestine by the intestinal barrier composed of the mucus (green), which contains IgA bound antibodies (blue) and epithelial cells. The epithelial-cell layer contains intraepithelial lymphocytes (yellow) and mucin-secreting goblet cells (pink). At the base of the intestinal crypts lie Paneth cells (light blue), which secrete alpha-defensins. Directly below the epithelial layer lies the lamina propria. Dendritic cells sample the lumenal bacterial contents and migrate to Peyer's patches (gray) within the small intestine, where they interact with T cells (orange). M cells allow the passage of antigens into Peyer's patches for uptake by resident antigen presenting cells.

Forster 2008; Groschwitz and Hogan 2009).

In addition to the transmembrane proteins that constitute the paracellular barrier, tight junctions also contain a complex system of adaptor molecules and scaffold proteins that mediate crosslinks between the transmembrane proteins and the actin cytoskeletons within epithelial cells. Besides forming tight junctions, intestinal epithelial cells themselves constitute a dynamic community of cells. The crypt-villus axis (see Glossary) allows constant regeneration of cells by differentiation and migration of cryptic stem cells to maintain barrier integrity. This balance of apoptosis and proliferation enables normal intestinal barrier function (Peterson and Artis 2014).

Intestinal Physical Barrier Following Alcohol Exposure and Trauma

Disruptions in either the intestinal mucus or epithelial barrier can result in pathogenic bacterial translocation. This can lead to systemic infections, sepsis, and multiple organ failure, which underscores the importance of maintaining barrier integrity (Choudhry et al. 2000, 2004; Napolitano et al. 1995). Alcohol exposure can cause disruptions in all components of the intestinal barrier (Farhadi et al. 2003; Keshavarzian and Fields 2003). Such alterations may subsequently lead to an increase in bacterial translocation and infection among hospitalized trauma patients who have detectable blood alcohol levels at the time of their admittance (Bird and Kovacs 2008; Maier 2001; McGill et al. 1995; Molina et al. 2013; Silver et al. 2008; Valatas and Kolios 2009). Researchers have started to identify alcohol's specific effects on different parts of the physical barrier.

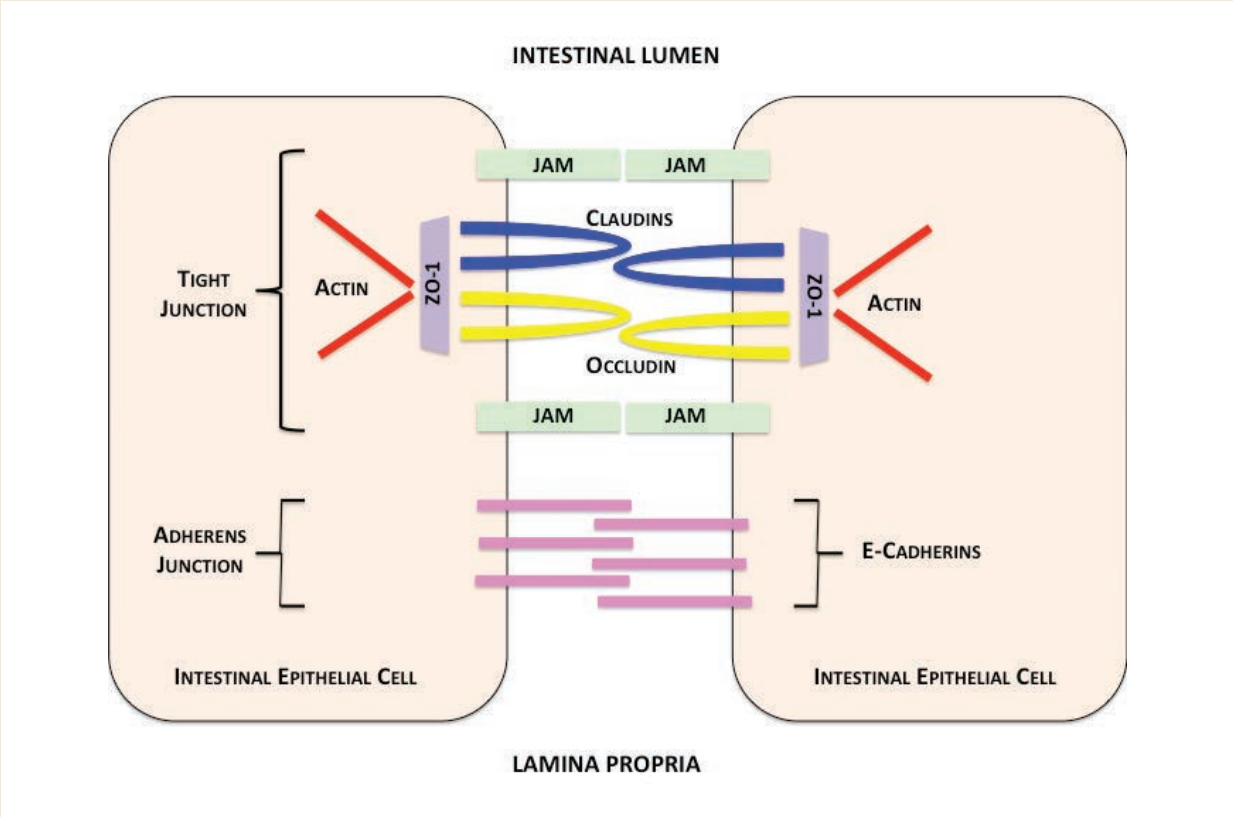

Figure 2 Intestinal epithelial-cell junctions. Contents within the intestinal lumen are prevented from passing between epithelial cells by apical tight-junction complexes. Tight junctions are composed of claudin proteins (blue) and regulated by occludin proteins (yellow). Claudin and occludin proteins are transmembrane proteins attached to an adaptor molecule, zonula occludins protein 1 (ZO-1) (purple), which anchors tight-junction proteins to intracellular actin (red). Alcohol causes disruption of occludin and ZO proteins by an unknown mechanism. Junctional adhesion molecules (JAMs) (green) also support tight-junction interactions. Intestinal epithelial cells are further supported by adherens molecules, including E-cadherins (pink), which also contribute to cell–cell contact. These junctions allow selective separation of the intestinal lumen (top) and lamina propria (bottom).

As the first line of defense against pathogenic organisms within the intestinal lumen, the mucus layer and its alteration by alcohol exposure are of particular research interest. Grewal and Mahmood (2009) investigated the role of chronic alcohol exposure on mucin production in a rat model. They demonstrated that prolonged alcohol exposure (25 to 56 days) resulted in increased mucin production. This study also discovered that several components of the mucin biochemical composition were altered following prolonged alcohol exposure. Modulation of glycosylation and enzymatic activity within the mucus layer could potentially affect the barrier's integrity, as these sites could begin to harbor adherent pathogenic bacteria (Van Klinken et al. 1995). In contrast to this finding, others have shown that chronic alcohol exposure results in decreased mucin production in the intestines of rats (Slomiany et al. 1997, 2000). Furthermore, Hartmann and colleagues (2013) demonstrated that MUC2 knockout mice are less susceptible to bacterial overgrowth and translocation following chronic alcohol exposure and are thus less prone to alcoholic liver disease. These findings suggest a relationship between alcohol exposure and mucus production. Further investigation will be required to establish the effects of alcohol on mucin production and to elucidate the mechanism by which alcohol alters the intestinal mucus layer.

Not surprisingly, alcohol and trauma also disrupt the integrity of tight junction complexes between intestinal epithelial cells (Choudhry et al. 2002; Li et al. 2008*a*; Tang et al. 2009). An in vitro study showed that Caco-2 human intestinal epithelial cells exposed to a daily regime of alcohol demonstrated a reduction in membrane localization of the adherens protein ZO-1. Furthermore, allowing the alcohol-treated cells to "recover" from alcohol exposure by culturing them for 2 weeks in alcohol-free media improved ZO-1 localization (Wood et al. 2013). Studies by Rao and colleagues have also demonstrated that acetaldehyde, a metabolite of alcohol, results in similar disruption of occludin and ZO-1 proteins by altering their phosphorylation status (Atkinson and Rao 2001; Dunagan et al. 2012; Rao 2008). Another study conducted by Ma and colleagues (1999) using Caco-2 cells showed identical perturbation of ZO-1 proteins. The study further demonstrated that alcohol activates an enzyme, myosin light-chain kinase (MLCK), that phosphorylates myosin regulatory light-chain (MLC), promoting its interaction with actin to cause cytoskeletal sliding (Ma et al. 1999). This interaction is important in tight junction function and may be one cause of the alcohol-related disruption of tight junctions in intestinal epithelial cells (Groschwitz and Hogan 2009). Zahs and colleagues (2012) examined the role of MLCK in gut barrier disruption following combined binge alcohol exposure and burn injury. They showed that the combination of alcohol intoxication and burn injury results in both elevated MLCK and phosphorylated MLC and decreased co-localization of both occludin and ZO-1. Such changes could alter barrier permeability.

In an in vivo study of acute alcohol exposure and burn injury in rats, Li and colleagues (2012) showed that the combined insult resulted in a significant reduction in phosphorylation and expression of occludin and claudin-1, which was correlated with increased epithelial cell apoptosis. Yoseph and colleagues (2013) further demonstrated that the combination of chronic alcohol and cecal ligation and puncture (CLP)-sepsis resulted in elevated intestinal epithelial apoptosis as well as decreased proliferation of cells compared to CLP-sepsis alone. Clearly, exposure to alcohol and trauma greatly affects all components of the intestinal physical barrier through changes in mucosal production and biochemical structure, disruptions of tight junction protein complexes, and increasing susceptibility to apoptosis in epithelial cells. The mechanisms by which alcohol and trauma cause these alterations are just beginning to be elucidated. Future work will focus on how to prevent such disruptions.

The Intestinal Immune System

Beyond the physical barrier, the next line of defense against invading pathogens is the immune system within the gut, which has the most difficult task in the body. Not only does it protect the host from invading pathogens, but it also maintains homeostasis with the vastly diverse microbiome within the intestinal lumen. The immune system must distinguish between commensal and pathogenic bacteria so that it does not mount a damaging autoimmune inflammatory response. The immune cells that carry out these tasks comprise parts of both innate and acquired immune functions. They can be found in all areas of the intestines, especially in regions called gut associated lymphoid tissue (GALT). GALT includes the gut epithelium, PPs, MLNs, and LP (Choudhry et al. 2004; Mowat and Viney 1997). Intestinal T cells are found in GALT sites and exist closely with antigen presenting cells (APCs), such as DCs and macrophages, that aid in T cell differentiation and activation (figure 3). Scientists are beginning to define the roles of macrophages and DCs in gut immune functions following alcohol exposure or trauma, as well as the initial innate immune responses that occur following these insults. These immune cells activate or suppress one another using highly complex chemical signaling pathways that researchers are beginning to uncover. Alcohol could produce disruptive effects at any point along these pathways (see figure 3).

Innate Immunity

A key part of the innate immune response, neutrophils, or polymorphonuclear leukocytes (PMNs), make up a significant portion of the innate immune cells present in humans. They play integral roles in initial responses to infection including degranulation and phagocytosis (Amulic et al. 2012).

It appears that one of the main functions of gut neutrophils under homeostatic conditions is to prevent the translocation of bacteria across the epithelial barrier (Choudhry et al. 2002; Kuhl et al. 2007; Li et al. 2008*b*). In addition, IL-17 cytokine released by activated T cells known as Th17 cells supports an inflammatory immune response through recruitment of neutrophils (Hundorfean et al. 2012). It is important to note that the role of neutrophils under pathologic conditions in the intestines remains unclear. In models of inflammatory bowel disease (IBD), different studies have shown neutrophils to be beneficial (Kuhl et al. 2007; Zhang et al. 2011), harmful (Kankuri et al. 2001; Natsui et al. 1997), or indifferent (Yamada et al. 1991). Interestingly, understanding of the function of neutrophils within the intestines of mice and humans has diverged slightly as studies show that murine neutrophils secrete defensins (see Glossary), whereas human neutrophils do not (Ganz 2003; Ouellette and Selsted 1996; Risso 2000).

Neutrophil Activity Following Alcohol Exposure and Trauma

Following alcohol intoxication and trauma, neutrophil infiltration increases into different organs, including the lungs and intestines (Akhtar et al. 2009; Bird et al. 2010; Li et al. 2008*b*; Scalfani et al. 2007). Although the role of neutrophils is unclear in disease models such as IBD, neutrophils appear to have detrimental effects after alcohol exposure and trauma (Li et al. 2008*b*). Several studies have found that the inflammatory microenvironment following alcohol exposure and/or trauma may allow neutrophils to exacerbate tissue damage in numerous organs including intestine (Amin et al. 2007*a,b*; Bird and Kovacs 2008; Li et al. 2007, 2008*a,* 2011). Studies in animal models provide details surrounding neutrophil activity after alcohol intoxication and trauma. These publications show that not only are neutrophils recruited by the pro-inflammatory cytokines IL-6 and IL-18, but they also have a prolonged presence at the injury sites (Akhtar et al. 2009; Scalfani et al. 2007; Zahs et al. 2013). Scientists do not know whether IL-6 and/or IL-18 directly recruit neutrophils, or whether these cytokines signal through other molecules such as monocyte chemoattractant-1 (MCP-1) or myeloperoxidase (MPO) (Li et al. 2011; Rana et al. 2005). They also do not know what role alcohol plays in neutrophil recruitment. However, previous work showed that alcohol intoxication leads to increased recruitment of neutrophils to the intestine following ischemic injury (Tabata and Meyer 1995). One proposal suggests that this may occur through upregulation of intestinal ICAM-1 expression following ischemic/reperfusion injury (Olanders et al. 2002). Once at the injury site, neutrophils secrete superoxide anions that kill any invading pathogens entering through the compromised intestinal barrier (Li et al. 2008*b*, 2011). Although this response is helpful at initially protecting from invading pathogens, prolonged neutrophil responses mediate tissue damage in multiple organs under inflammatory conditions (Fukushima et al. 1995; Partrick et al. 2000). Further studies will be necessary to determine how neutrophils respond following alcohol exposure, and also how they mediate the subsequent adaptive immune response.

Adaptive Immunity

T lymphocytes form a large part of the adaptive immune response in the intestine. Under homeostatic conditions, the balance between inflammatory and immunosuppressive T cells is maintained through cell-to-cell cytokine signaling. Although the intestines contain a large and diverse population of T lymphocytes, the major subsets of resident T cells within the gut include Th1, Th2, Th17, and T-regulatory (Treg) cells (Belkaid et al. 2013). The default T cell response in the intestines under normal conditions is immunosuppressive. This occurs through the production of TGF-β, primarily by APCs, which drives Treg development (figure 3). In addition to TGF-β, IL-4 production drives Th2 cell development and B cell IgA antibody production. IgA also maintains gut homeostasis, in part by regulating the microbiome (Weaver et al. 2006).

The production of these immunomodulatory cytokines largely depends on resident DCs that sample the lumenal contents at the epithelial barrier (Cerovic et al. 2014). DCs decipher commensal and pathogenic bacterial antigens to modulate appropriate T-cell development by a mechanism now under investigation (Cerovic et al. 2014). Naïve $CD4^+$/Foxp3-T cells within GALT are driven toward specific T-cell phenotypes, depending upon the milieu of extrinsic factors present. Once activated, these T cells release cytokines to generate an immune response. Development of the Th1 phenotype depends on cytokines including IL-12, which is augmented by the presence of IL-18. IL-12 binds to its cognate receptor (IL-12R), which results in downstream signaling through the transcription factors STAT4 and T-box protein 21 (T-bet) (Amsen et al. 2009). Interestingly, recent reports show that STAT4 and T-bet may act in unison to drive Th1 differentiation. Thieu and colleagues (2008) have described a role for STAT4 in chromatin remodeling that promotes *Ifng* gene transcription by T-bet to drive Th1 differentiation. This signaling is initiated following antigen recognition on MHC-II molecules, whereupon Th1 cells secrete the cytokines IFN-γ and lymphotoxin alpha (LT-α), a member of the pro-inflammatory TNF family (Weaver et al. 2006). Some have hypothesized that Th1 cells may play a role in regulating innate mucosal responses; however, further investigation must confirm this (Belkaid et al. 2013). As mentioned above, other cytokines such as TGF-β keep development of Th1 cells in

check under homeostatic conditions. TGF-β plays an important role in preventing the differentiation of naïve T cells into inflammatory phenotypes (Sansonetti and Di Santo 2007).

Th17 cells form the other major inflammatory T cell subset found in the intestines. Intestinal Th17 development also depends heavily on the cytokine milieu. It is largely driven by the presence of IL-6. More recent studies have implicated IL-23 in Th17 differentiation, but it appears that IL-23 may only augment Th17 differentiation as opposed to being an essential component (Maynard and Weaver 2009). IL-6 and IL-23, which are mainly produced by DCs and macrophages, signal through their cognate receptors on naïve $CD4^+$ T cells, which in turn signal through the ROR-γT transcription factor. ROR-γT transcription drives Th17 cells to produce a host of different cytokines including IL-17A, IL-17F, IL-21, and IL-22 (Maloy and Kullberg 2008). Many contrasting studies have been published regarding the roles of Th17 cytokines. Although IL-17A and IL-17F are generally present under inflammatory conditions (Ahern et al. 2010; Leppkes et al. 2009; Wu et al. 2009; Yang et al. 2008), scientists have also observed contradictory protective roles of IL-17A in models of IBD (Yang et al. 2008). Fewer studies have examined the actions of IL-21 and IL-22, but both cytokines seem to play a protective role in epithelia regeneration following injury (Maloy and Kullberg 2008; Sonnenberg et al. 2010). Although it is clear that Th17 cells play an essential part in modulating intestinal inflammatory immune responses, more studies will be needed to elucidate their specific functions in homeostatic and diseased conditions within the intestines.

Balancing the inflammatory T cells within the intestines, modulatory T cells are an important subset made up

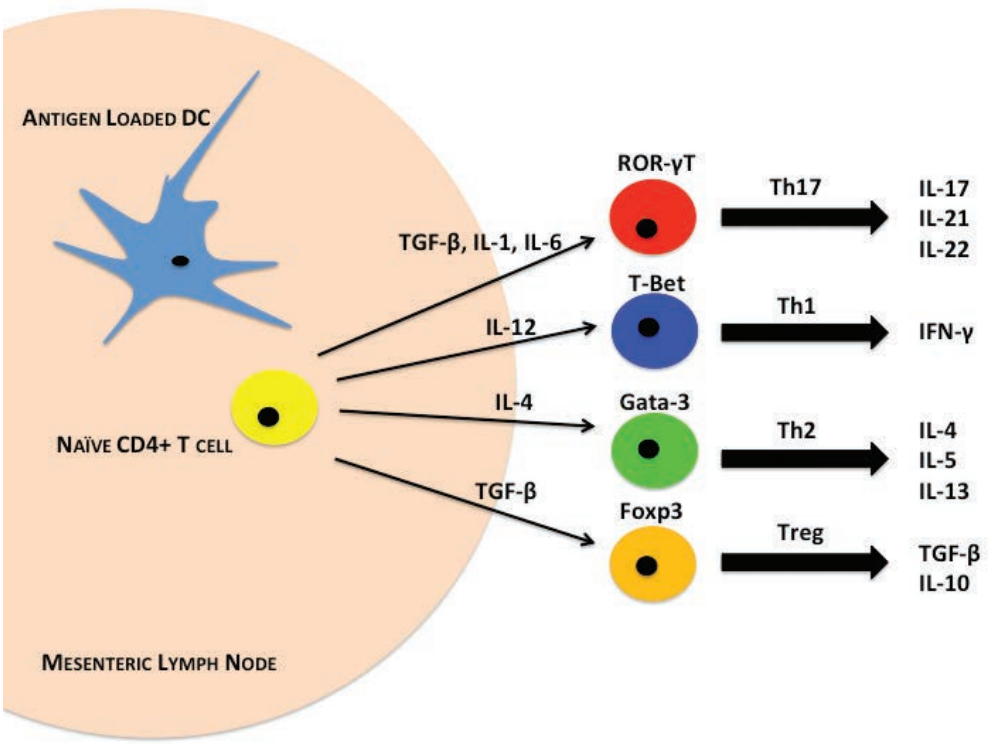

Figure 3 Intestinal $CD4^+$ T-cell differentiation. Antigen-loaded dendritic cells (DCs) interact with naïve $CD4^+$ T cells (yellow) in mesenteric lymph nodes through MHC-II molecules. DCs secrete different cytokines depending on this interaction. Following alcohol and burn injury, antigen-presenting cells (APCs) such as DCs may have a significantly altered cytokine expression profile. The cytokine profiles present lead to the expression of different transcription factors that promote differentiation of T cells into either Th17 (red), Th1 (blue), Th2 (green), or Treg (orange) phenotypes. These T-cell subsets secrete different cytokines that lead to inflammatory or immunosuppressive immune responses. Combined alcohol and burn injury has been shown to suppress T-cell cytokines including interferon (IFN)-γ, interleukin (IL)-17, and IL-22 from T cells.

of Th2 and Treg cells. Antigen-loaded DCs that have sampled the lumenal contents release IL-4 to drive the differentiation of Th2 cells. Activation of the IL-4 receptor leads to downstream signaling through the transcription factor STAT6, which mediates the expression of another transcription factor, Gata3 (Ansel et al. 2006). Gata3 plays a major role in mediating production of key Th2 cytokines IL-4, IL-5, and IL-13. Gata3 also prevents Th1 differentiation through its inhibitory effects on IL-12 receptor and STAT4 signaling (Amsen et al. 2009; Ansel et al. 2006). One of the most important roles for Th2 cells in the maintenance of gut homeostasis is their interaction with B cells to aid in the development of IgA-producing plasma cells. IgA antibodies function to regulate homeostasis of the microbiome, as well as act as a first line of immune defense against pathogens in the GI lumen. They are by far the most highly expressed class of antibodies in the intestines of humans (Mantis et al. 2011).

Treg cells also serve a critical function in modulating the immune responses within the intestines. Populations of Tregs within the gut derive both from thymic $CD4^+CD25^+Foxp3^+$ precursors that migrate to the gut, as well as from the gut itself, where resident naïve $CD4^+$ T cells are preferentially driven towards a Treg phenotype by TGF-β, IL-10, and Foxp3 expression (Fontenot et al. 2005). Studies show that the recognition of self-antigens presented by DCs initiates Treg activation (Hsieh et al. 2006; Nishikawa et al. 2005; Watanabe et al. 2005). After sampling the lumenal contents in the intestine, DCs migrate to MLNs where some present self-antigens on MHC-II molecules to naïve $CD4^+$ T cells. Activation of T-cell receptors by self-antigens stimulates Foxp3 signaling to drive anti-inflammatory TGF-β and IL-10 secretion. In this regard, Tregs are able not only to inhibit inappropriate inflammatory responses to these self-antigens by Th1 and Th17 cells, but also to drive Th2 and subsequent IgA production to maintain intestinal homeostasis. More recent observations have demonstrated that T cell lineages can interconvert, specifically Treg-to-Th17 and Th17-to-Th1 (Lee et al. 2009; Zhou et al. 2009). In light of these studies, it is important to highlight that while each subset of T cells found in the intestines plays a crucial role in balancing homeostasis, these relationships are dynamic and can be altered by changes within the intestinal environment, such as those following alcohol exposure.

Intestinal T Cells Following Alcohol Exposure and Trauma

Surprisingly, few studies in the current literature have examined the effects of alcohol specifically on intestinal immunity. However, alcohol has significant, well-documented impacts on immune cells at sites outside the intestine, including in the spleen, thymus, and on circulating lymphocytes (Curtis et al. 2013; Ippolito et al. 2013; Messingham et al. 2002). Intestinal studies suggest that alcohol may have inflammatory effects, and subsequently compromise the intestine's ability to prevent bacteria from passing into the body.

Of course, an important consideration in studying the effects of alcohol on immune function is the nature of the alcohol exposure (acute vs. chronic). The authors examined the effects of alcohol exposure in an acute model, which is followed by a second traumatic burn injury. In this model, mice are given a single dose of alcohol to produce a blood alcohol level of 90–100 mg/dL 4 hours after alcohol administration, at which time they are given a full thickness ~12.5% total body surface area dorsal scald burn. Findings demonstrate that alcohol intoxication or burn injury alone does not cause significant changes to immune profiles within the gut in the first 24 hours. However, combined alcohol and burn injury lead to great perturbations resulting in high levels of inflammation accompanied by neutrophil infiltration, T-cell suppression, and bacterial translocation (Brubaker et al. 2013; Li et al. 2008*a,b,* 2011, 2012, 2013; Rendon et al. 2012, 2013, 2014; Zahs et al. 2013). These results clearly demonstrate that alcohol intoxication leads to greater susceptibility to secondary insults by sensitizing the immune system through an unknown mechanism.

Studies from the authors' laboratory also show a decrease in Th1 cells, particularly in MLNs, paired with decreases in IL-12 following alcohol intoxication and burn injury (Choudhry et al. 2002; Li et al. 2006). Intriguingly, restoration of IL-12 following alcohol and burn treatment restores Th1 profiles of the cytokines IFN-γ and IL-2 via an ERK-dependent pathway (Li et al. 2009). IL-12 is largely produced by resident APCs, and thus alcohol intoxication and burn injury may have both direct (i.e., on T cells) and indirect (on APCs) effects on Th1 function. Diminished Th1 effector cells present following alcohol intoxication and burn injury may allow bacteria and other pathogens to progress across the intestinal barrier. However, future studies will further address the signaling pathway(s) involved.

The authors also examined the effect of alcohol and traumatic burn injury on intestinal Th17 cells. They previously discovered a decrease in IL-23 and the Th17 effector cytokines IL-17 and IL-22 in PPs following alcohol and burn (Rendon et al. 2014). Due to the decreased presence of IL-23, they examined the effects of adding IL-23 following alcohol and burn injury (Rendon et al. 2014). Interestingly, IL-23 restored IL-22 production in an aryl hydrocarbon receptor (AhR)–dependent fashion, but IL-23 had no effect on IL-17 levels. These data give new insight into the role of IL-23 in mediating Th17 IL-22 responses, but not IL-17 responses. Like Th1 cells, the suppression of Th17 cells in the context of the alcohol/burn model may mean enhanced susceptibility to bacterial translocation and infection. Future studies will further examine

the role of both Th1 and Th17 cells and their functions following alcohol intoxication and trauma. Th2 and Treg activity following alcohol and burn injury also has not been well studied.

Another research group published recent studies examining the effects of alcohol on intestinal immunity in the context of chronic alcohol exposure followed by sepsis (Yoseph et al. 2013). Studies performed in this model showed disruptions in intestinal permeability similar to those in the studies discussed above. In addition, a significant increase in $CD4^+$ production of IFN-γ and TNF-α was observed in alcohol-treated mice compared with controls (Yoseph et al. 2013). Interestingly, studies of non-alcoholic human sepsis patients have shown lower levels of IFN-γ and TNF-α production in the spleen, which highlights the fact that local and systemic immune responses may differ greatly regardless of the presence of alcohol (Boomer et al. 2011).

Only a few studies in the literature have examined the effects of alcohol alone on intestinal immunity (Sibley and Jerrells 2000). An early study by Lopez and colleagues (1997) examined the effects of both acute and chronic alcohol exposure on PPs. They observed a significant decrease in the total number of cells within PPs of mice given a brief alcohol exposure of 5 weeks. In a more chronic exposure model, mice receiving alcohol for 19 weeks showed both a significant decrease in total PP cells, as well as a significant reduction of T and B cells present in PPs (Lopez et al. 1997). This study was important in demonstrating that alcohol administration affects the mucosal immune system, particularly PPs, suggesting that alcohol may thus affect T-cell differentiation within the intestines.

A more recent study demonstrated that alcohol exposure causes disruption of the epithelial barrier in the stomach and upper intestines (Bode and Bode 2003). It has been reported than even a single dose of alcohol at binge consumption levels can result in epithelial barrier disruptions within the gut (Bode and Bode 2005).

Glossary

AhR: Aryl Hydrocarbon Receptor: Transcription factor that drives Th17 cell differentiation.

β-Catenin: Transcription factor involved heavily in cell adhesion regulation.

CD(4/8): Cluster of differentiation: proteins expressed on the surface of cells used to identify specific cell phenotypes.

Crypt-Villus Axis: The plane that exists from the base of intestinal crypts to the tops of the villi. Epithelial cells divide from stem cells at the base of crypts and migrate to the tops of villi as they mature.

C-Type Lectins: Carbohydrate binding proteins with a diverse range of functions, including mounting immune responses against pathogens.

Defensins: Small proteins secreted by paneth cells that mediate defense against harmful microbes.

Dysbiosis: Any perturbation in the normal intestinal microbiota.

Extracellular Signal–Related Kinase (ERK): Signaling molecules that transmit a variety of intracellular signaling following activation.

Foxp3: Transcription factor that drives regulatory T cell differentiation.

Gata3: Trans-acting T-cell-specific transcription factor involved in the development of Th2 cells.

Glycosylation: A post-translational modification that involves the attachment of a carbohydrate to the specific region of a protein to enhance its function.

Intracellular Adhesion Molecule-1 (ICAM-1): Expressed mainly on endothelial cells and immune cells to mediate migration from circulation into tissues.

Microbiome: The entire makeup of bacteria that inhabit the intestines.

Nuclear Factor Kappa–Light-Chain Enhancer of Activated B Cells (NFκB): Transcription factor considered to be the master regulator of inflammation.

Retinoic Acid-Related Orphan Receptor Gamma T (ROR-γT): Transcription factor that mediates Th17 development.

Sepsis: Life-threatening whole-body inflammatory response in order to fight systemic infection.

Signal Transducer and Activator of Transcription (STAT): Following receptor activation, STAT family proteins mediate transcription events to drive specific gene expression.

T-Box Transcription Factor (T-bet): Transcription factor the mediates development of Th1 T cells.

Zonula Occludins Protein 1 (ZO-1): Adherens transmembrane junction proteins linking to the actin cytoskeleton to occludin and claudin proteins support tight junctions.

Interestingly, in an acute model of alcohol exposure, mice displayed higher numbers of Treg cells in the LP in response to barrier disruption (Boirivant et al. 2008). These results contrast with studies of chronic alcohol exposure that show increased levels of inflammatory neutrophil, Th1, and Th17 activation and production of IL-17A, IFN-γ, IL-1, and TNF-α (Bode and Bode 2005; Koivisto et al. 2008) Thus, acute alcohol exposure may result in suppression of inflammation, allowing pathogens past the intestinal barrier, while chronic exposure may produce an inflammatory state. In addition, one report with human subjects showed increases in IgA antibody production coupled with increases in TNF-α and IL-8 production in chronic alcoholics (Koivisto et al. 2008). Chronic alcohol consumption studies have reported significant effects on the liver and connected the inflammatory conditions observed in the intestines with alcoholic liver disease (Bode and Bode 2005; Koivisto et al. 2008).

Microbiota and Intestine Immune Homeostasis Following Alcohol and Burn Injury

The adaptive T-cell response provides a critical component of pathogen protection, and innate responses conducted mainly by neutrophils also play a large role in maintaining intestinal homeostasis. Importantly, however, both of these immune responses are shaped by their interactions with the intestinal microbiome. The intestinal immune system encounters more antigens than any other part of the body. Therefore, the recognition of "self" and "non-self" antigens is critical to discriminate the harmless commensal microbiota and food antigens from harmful pathogenic microbes. In part, this equilibrium is established by the balance of effector T cells discussed earlier. Antigens from the intestinal microbiota presented in GALT by APCs shapes this balance of Treg/Th17 cells, which drives pro- or anti-inflammatory signaling.

In addition to affecting the T-cell balance, the composition of the intestinal microbiota facilitates development of lymphoid organs and directs immune cell responses and production of effector cytokines. Studies using germ free mice—that is, mice devoid of any microbes—reveal that these mice are more susceptible to colonization by pathogenic microbes; have small and undeveloped lymphoid organs; and show reductions in $CD4^+$ and $CD8^+$ T-cells, IgA secretion, and production of antimicrobial peptides (AMPs) including β-defensins and C-type lectins such as Reg3γ (Bouskra et al. 2008; Cash et al. 2006; Zachar and Savage 1979). Further, following combined alcohol and burn injury, Reg3β and Reg3γ are significantly decreased in the small intestines of wild-type mice (Rendon et al. 2013). Together, these findings suggest that following alcohol intoxication and injury, bacterial overgrowth and translocation may be partially mediated through the inhibition of AMPs.

Several recent studies demonstrate that certain bacterial species have specific effects on immune system balance. The commensal microbes, it turns out, are essential for regulating immune physiology and the innate and adaptive immune systems. One commensal, *Bacteroides fragilis,* produces an immunomodulatory molecule called polysaccharide A (PSA), which regulates the Th1 and Th2 balance and directs Treg development to protect against intestinal inflammation (Mazmanian et al. 2005; Round and Mazmanian 2010; Round et al. 2011; Xu et al. 2003). Mazmanian and colleagues (2005) showed that therapeutic treatment with PSA led to the production of anti-inflammatory IL-10 and alleviated intestinal inflammation in various models of IBD. Segmented filamentous bacteria (SFB), a group of Gram-positive bacteria, attach to small intestine epithelial cells and lead to the production of serum amyloid A (SAA). SAA then stimulates dendritic cells in the LP to secrete IL-6 and IL-23, which promotes Th17 cell differentiation and maturation (Ivanov et al. 2009). Littman's laboratory and coauthor Ivanov and their team showed that germ-free mice have reductions of Th17 cells in the small intestine, but that levels could be restored by colonizing mice with feces taken from germ-free, SFB mono-colonized mice (Ivanov et al. 2009). Furthermore, they determined the specific membrane bound antigenic proteins of SFB that direct Th17 production (Yang et al. 2014). This bacterial group is also necessary for the secretion of IgA (Wu et al. 2011). Nevertheless, overgrowth of this bacterium may upset the Th17/Treg balance in favor of overactive Th17 cells. This shift can potentially lead to autoimmune diseases: inflammatory bowel disease, arthritis, and multiple sclerosis (Lee et al. 2011; Wu et al. 2010).

The Intestinal Microbiota Following Alcohol Exposure and Trauma

Unexpectedly, few studies in the current literature have examined the effects of alcohol exposure on the microbiome within the intestines. A recent study examining the effects of chronic daily alcohol consumption found dysbiosis—a microbial imbalance—in the colons of rats after 10 weeks (Mutlu et al. 2009). Others have correlated microbial dysbiosis to alcoholic liver disease and demonstrated that administration of probiotics reduces hepatic inflammation associated with it (Mutlu et al. 2009; Wang et al. 2013). The work done by the authors showed that combined alcohol intoxication followed by traumatic burn injury results in a significant increase in bacterial translocation across the intestinal barrier (Choudhry et al. 2002; Kavanaugh et al. 2005; Li et al. 2012; Rendon et al. 2013), and this work is supported by a previous study (Napolitano et al. 1995).

However, the long-term impact of alcohol on different microbiota and the host's health and immune function remains to be shown. Classification of the healthy intestinal microbiome is clinically necessary for determining how alcohol may alter the microbiota composition and lead to disease development and progression. Thus, whether bacterial translocation after alcohol and trauma is related to changes in the microbiome remains largely unknown. Furthermore, studies are needed to establish whether changes in the biome have any role in epithelial barrier disruption following alcohol and burn injury.

Future Directions and Perspectives

Taken together, the range of effects alcohol has on the intestines is extremely broad and alters all levels of intestinal homeostatic regulation. In parallel, alcohol exposure predisposes its users to more complications following major injury and trauma; however, the underlying mechanisms remain largely unexplored. Although studies have demonstrated that alcohol modulates the various components of the intestinal barrier, making any causal connections between these effects and complication from trauma requires more study. The balance of inflammatory and immunosuppressive T cells can be skewed following alcohol exposure. Current research suggests inflammatory conditions are mediated through both neutrophil infiltration and Th17 recruitment leading to tissue damage within the intestines. Whether alcohol influences this also needs to be explored. Many studies now show roles for the intestinal microbiome in developing the immune profiles within the intestines. Although few studies have explored whether alcohol exposure alters the composition of the microbiome, it is not far-fetched to hypothesize that this is likely the case. Together, the authors believe that the largest gap in the field remains the lack of mechanistic support for the changes observed following alcohol exposure with and without burn trauma. More studies are needed to understand the molecular signaling pathways mediating changes in the barrier, immune system, and biome to give a clearer understanding of the relationship between these components and how they overlap after alcohol and burn injury.

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Alcohol's Effects on Lung Health and Immunity

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It has long been known that people with alcohol use disorder (AUD) not only may develop physical dependence but also may experience devastating long-term health problems. The most common and identifiable alcohol-associated health problems include liver cirrhosis, pancreatitis, cardiomyopathies, neuropathies, and dementia. However, the lung also is adversely affected by alcohol abuse, a fact often overlooked by clinicians and the public. Individuals with AUD are more likely to develop pneumonia, tuberculosis (TB), respiratory syncytial virus (RSV) infection, and acute respiratory distress syndrome (ARDS). Increased susceptibility to these and other pulmonary infections is caused by impaired immune responses in people with AUD. The key immune cells involved in combating pulmonary conditions such as pneumonia, TB, RSV infection, and ARDS are neutrophils, lymphocytes, alveolar macrophages, and the cells responsible for innate immune responses. Researchers are only now beginning to understand how alcohol affects these cells and how these effects contribute to the pathophysiology of pulmonary diseases in people with AUD.

Key words: Alcohol use, abuse, and dependence; alcohol use disorder; immunity; impaired immune response; innate immune response; lung disorders; pneumonia; tuberculosis; respiratory syncytial virus infection; acute respiratory distress syndrome; pulmonary infection; neutrophils, lymphocytes, alveolar macrophages; pathophysiology

People have been drinking alcoholic beverages for millennia, and alcohol consumption has played an important role throughout human history, being linked to ancient and modern religions, early medicine, and social occasions and celebrations. Although alcohol consumption is socially accepted across many cultures, heavy and prolonged alcohol intake can lead not only to physical dependence but also to devastating long-term health problems. An estimated 18 million Americans have alcohol use disorder (AUD), including alcoholism and harmful drinking (National Institute on Alcohol Abuse and Alcoholism [NIAAA] 2014). NIAAA (2014) has established guidelines for low-risk drinking that are age and gender specific. Thus, for men ages 21–64, low-risk drinking is defined as consumption of no more than 4 drinks per day or 14 drinks per week. For women, as well as for men ages 65 and older, drinking levels for low-risk drinking are defined as no more than 3 drinks per occasion or 7 drinks per week. Exceeding these daily or weekly drinking limits significantly increases the risk of developing AUD and problematic health outcomes (NIAAA 2014).

The most common health problems associated with AUD are liver cirrhosis, pancreatitis, damage to the heart muscles (i.e., cardiomyopathies), nerve damage (i.e., neuropathies), and dementia (Lieber 1995). However, the lung also is adversely affected by alcohol abuse, a fact that often is overlooked by clinicians and the public. For example, it is clear that heavy drinkers are more likely to have pneumonia (Jellinek 1943; Samokhvalov et al. 2010), tuberculosis (TB) (Borgdorff et al. 1998; Buskin et al. 1994; Kline et al. 1995; Narasimhan et al. 2013), respiratory syncytial virus (RSV) infection (Jerrells et al. 2007), and acute respiratory distress syndrome (ARDS) (Moore et al. 2003; Moss et al. 1996). In recent years, researchers have come to better understand the pathophysiology of lung injury in individuals with AUD and the role that alcohol's effects on lung immune responses play in this process. This review focuses on these four common pulmonary conditions associated with AUD and their pathophysiologic lung immune responses.

Bacterial Pneumonia

One of the most common and deadliest conditions afflicting individuals with AUD is bacterial pneumonia.

Dr. Benjamin Rush, the first Surgeon General of the United States, described some of the earliest links of alcohol abuse to pneumonia over two centuries ago, reporting that pneumonia was more common in drinkers than nondrinkers (Jellinek 1943; Rush 1810). Two centuries later, the correlation between alcohol abuse and lung infections still remains strong. According to the Centers for Disease Control and Prevention (CDC), people who abuse alcohol are 10 times more likely to develop pneumococcal pneumonia and 4 times more likely to die from pneumonia than nondrinkers (Lujan et al. 2010).

Pneumococcal pneumonia, caused by the bacterium *Streptococcus pneumoniae,* is the most common type of pneumonia in both healthy individuals and heavy alcohol users (Ruiz et al. 1999). In addition, the incidence of infections with *Klebsiella pneumoniae* also is increased in people with AUD and seems to cause disproportionate rates of lung infection and high mortality in this population (Feldman et al. 1990; Limson et al. 1956). Regardless of the bacterial pathogen causing the infection, dysfunction of the host's immune responses to bacterial pneumonia, particularly those involving macrophages in the lungs (i.e., alveolar macrophages) and neutrophils, is an important contributor to the pathogenesis of the disease in people with AUD. The alveolar macrophages eliminate pathogens by ingesting them—a process known as phagocytosis—whereas neutrophils are involved in inflammatory responses.

Alveolar macrophages are the first line of defense in lung cellular immunity. These phagocytic cells ingest and clear inhaled microbes and foreign particles from the lungs. The release of cytokines and chemokines by these cells, in turn, mediates the influx of neutrophils into the lungs that occurs in response to infection. Chronic alcohol exposure significantly interferes with alveolar macrophage function. Prolonged alcohol consumption impairs the cells' phagocytic capacity (Joshi et al. 2005, 2009), release of cytokines and chemokines (D'Souza et al. 1996), and release of neutrophil chemoattractants (Craig et al. 2009). Although alveolar macrophages are the primary residential innate immune cells and play a pivotal role in the clearance of bacterial and viral pathogens, understanding of and research on their specific function in the context of heavy alcohol consumption and AUD still is lacking. It is clear, however, that prolonged alcohol consumption alters the pathophysiology and key factors involved in neutrophil-driven lung immunity in response to *S. pneumoniae* infection. Thus, studies have shown that exposure to alcohol impairs neutrophil recruitment (Gluckman and MacGregor 1978), weakens phagocytosis of pathogens by neutrophils (Boe et al. 2001; Jareo et al. 1995), and reduces neutrophil production and release of neutrophils into circulating blood (Melvan et al. 2011; Siggins et al. 2011). The following paragraphs outline the data supporting these deleterious effects of heavy alcohol consumption on neutrophil function in the context of *S. pneumoniae* lung infections.

Neutrophils are the earliest immune effector cells recruited to the site of inflammation during a bacteria-triggered inflammatory response. In the case of pneumonia, neutrophil recruitment to the lung is a critical early step in the host's immune response. In the early stages of infection, circulating neutrophils are recruited to sites of inflammation by a gradient of inflammatory mediators, including proinflammatory cytokines and chemokines. Neutrophils traverse the cells lining the blood vessels (i.e., vasculature endothelial cells) into the space between the lung cells (i.e., the interstitial space of the lung). From there, they migrate into the airspace within the alveoli to the sites of microbial invasion. Once in the alveolar space, neutrophils ingest, degrade, and remove invading pathogens (Nathan 2006). This neutrophil-recruitment process is impaired by alcohol; even brief alcohol exposure decreases neutrophil recruitment to infected sites (Astry et al. 1983). For example, alcohol studies in rodents infected with aerosolized *Staphylococcus aureus* or *Proteus mirabilis* have demonstrated that alcohol intoxication decreases bacterial clearance in conjunction with decreased pulmonary neutrophil recruitment (Astry et al. 1983). Similarly, Boe and colleagues (2001) found that alcohol-exposed rats had decreased pulmonary neutrophil recruitment for up to 18 hours following *S. pneumoniae* challenge; after that, however, neutrophil recruitment remained elevated even 40 hours post-challenge compared with nondrinking rats. This observation suggests that in individuals with heavy alcohol exposure, the host neutrophils arrive late at the infected lung but stay longer (Sisson et al. 2005). Impaired neutrophil recruitment also has been reported in human volunteers with blood alcohol concentrations (BACs) of 0.10 percent and 0.24 percent (Gluckman and MacGregor 1978)—that is, even at BACs that only slightly exceed the threshold for legal intoxication in the United States (i.e., 0.08 percent). These findings highlight that alcohol intoxication impairs neutrophil recruitment into infected tissues and the lung and also hinders neutrophil clearance from the lung.

The alcohol-induced dysregulation of lung neutrophil recruitment and clearance is only part of the problem in people with AUD, because alcohol also has harmful effects on other aspects of neutrophil functioning. However, alcohol's effects on neutrophil phagocytosis and pathogen killing are less clear than the effects on neutrophil recruitment, and the findings to date are inconclusive. Thus, some studies indicate that alcohol has no effect on neutrophil phagocytosis or pathogen killing (Nilsson et al. 1996; Spagnuolo and MacGregor 1975), whereas other studies demonstrate that acute alcohol exposure impairs functional activities of neutrophils. For example, Davis and colleagues (1991) found that alcohol-fed rats failed to clear bacteria from the lungs and had increased

mortality. Some of this discrepancy likely is related to differences in the bacterial pathogens studied. Thus, Jareo and colleagues (1995) noted impaired neutrophil killing of selected strains of *S. pneumoniae* in vitro and a complete absence of killing of other bacterial strains in alcohol-exposed animals. In human studies, BACs as low as 0.2 percent (i.e., approximately 2.5 times the legal intoxication level) impaired neutrophil degranulation and bactericidal activity (Tamura et al. 1998).

In addition to neutrophil recruitment to infected areas and reduced neutrophil-killing potential, production of these cells also is affected. In healthy individuals, the bone marrow produces approximately 120 billion neutrophils per day (Cartwright et al. 1964; von Vietinghoff and Ley 2008). Moreover, bone-marrow neutrophil production is significantly increased 24 to 48 hours after a systemic bacterial infection (Melvan et al. 2011). Several studies observed decreased numbers of neutrophils in people with AUD. Alcohol exposure suppresses neutrophil production by the bone marrow and other blood cell–producing (i.e., hematopoietic) tissues (Melvan et al. 2011; Raasch et al. 2010; Siggins et al. 2011). This decreased neutrophil proliferation may account for the decreased number of neutrophils found in the lungs during the host response to pneumonia following alcohol consumption. Alcohol primarily suppresses neutrophil production by interfering with the actions of granulocyte colony-stimulating factor (G-CSF), which is the principal driver of neutrophil production, maturation, and function in the bone marrow and inflamed tissues (Bagby et al. 1998). G-CSF levels normally increase in situations where more neutrophils are needed. Thus, G-CSF levels rise significantly within 3 hours of pulmonary bacterial infections, peaking at 12 hours, and plateauing around 18 hours post-infection within the lung and systemic circulation. Additional studies have demonstrated that alcohol-consuming animals are more likely to succumb to *S. pneumoniae* within 2 to 4 days following infection compared with their nondrinking counterparts (Boe et al. 2001). Alcohol-induced suppression of G-CSF–driven neutrophil production combined with impaired bacterial clearance likely account for the high severity and mortality of bacterial infections among the alcohol-fed mice observed in these studies.

Because of the key role of G-CSF in neutrophil regulation, investigators have hypothesized that alcohol-induced neutrophil dysfunction can be prevented by pretreatment with G-CSF (Nelson et al. 1991). Indeed, pre-treatment of alcohol-consuming mice with G-CSF for 2 days before *K. pneumoniae* infection increased neutrophil recruitment compared with that of control animals not receiving G-CSF. In addition to increased neutrophil recruitment, the pre-treated animals also exhibited improved bacterial killing and decreased mortality (Nelson et al. 1991). The findings indicate that G-CSF can prevent alcohol-induced deficits in neutrophil-dependent pulmonary defenses by increasing neutrophil production and bacterial killing function.

In summary, in the context of lung bacterial infections, alcohol impairs neutrophil recruitment (Gluckman and MacGregor 1978), reduces pathogen killing through phagocytosis (Boe et al. 2001; Jareo et al. 1995), and decreases neutrophil production and release of neutrophils into circulating blood (Melvan et al. 2011; Siggins et al. 2011). Pretreatment with G-CSF ameliorates alcohol-induced neutrophil dysfunction, including impairments in neutrophil recruitment and bacterial killing.

Tuberculosis

Bacterial pneumonia is not the only infectious disease with an increased risk among people with AUD. Lung infections with *Mycobacterium tuberculosis*, the underlying pathogen of TB, also occur at higher rates in this population (Jellinek 1943; World Health Organization [WHO] 2014). TB is the second-leading cause of death worldwide, accounting for 1.3 million deaths in 2012. The disease is spread from person to person through the air, when infected people cough, sneeze, speak, or sing, thereby releasing *M. tuberculosis* into the air (WHO 2014). Interestingly, not everyone infected with *M. tuberculosis* becomes sick. The infection can remain latent for years while the host's immune system is able to combat it. The infected individual will have no symptoms and is not infectious to others. However, latent TB may become active when the immune system is weakened. Alcohol abuse is therefore a risk factor for active TB (Borgdorff et al. 1998; Buskin et al. 1994; Kline et al. 1995; Narasimhan et al. 2013).

Although TB is treatable with antibiotics, the prevalence of multidrug-resistant tuberculosis (MDRTB) is on the rise and has been reported worldwide (WHO 2014). One of the main factors increasing the prevalence of MDRTB is noncompliance by patients who do not complete their normal 6-month treatment regimen, leading to the emergence of drug-resistant *M. tuberculosis*. A recent study of MDRTB in South Africa reports that of 225 patients diagnosed with MDRTB, only 50 percent were cured or completed treatment. Treatment default rates were highest among alcohol users (Kendall et al. 2013). Other countries also report similar TB treatment defaults in individuals with AUD, resulting in poorer treatment outcomes and increased mortality rates (Bumburidi et al. 2006; Jakubowiak et al. 2007). Along with noncompliance, people with AUD have compromised lymphocytes, which are among the main immune components combating TB infections. The three main types of lymphocytes are natural killer (NK) cells, T cells, and B cells. Chronic alcohol intake modulates the functions of all three of these lymphocyte populations (Cook 1998; Lundy et al. 1975; Meadows et al. 1992; Spinozzi et al. 1992; Szabo 1999).

NK cells do not need previous exposure to their target cells to recognize, bind to, and destroy these targets (e.g., cancer and virus-infected cells) (Vivier et al. 2008). In a mouse model, NK cells also become activated during the early response to *M. tuberculosis* infection and produce interferon γ (INF-γ), an important cytokine that stimulates cell-mediated immunity (Junqueira-Kipnis et al. 2003). Alcohol consumption in mice reduces the in vitro killing capacity of NK cells compared with control animals not exposed to alcohol (Meadows et al. 1992).

Chronic alcohol intake impairs not only the killing capacity of NK cells but also diminishes normal functioning of various types of T cells, which primarily mediate the immune response to TB (Gambon-Deza et al. 1995). (For more information on the types of T cells, see the textbox.) Alcohol exposure affects T-cell function through a variety of pathways:

- People with AUD often have reduced numbers of lymphocytes (i.e., lymphopenia), alterations in the T-cell compartments (Cook 1998; Szabo 1999; Tonnesen et al. 1990), decreased response to substances that stimulate cell division (i.e., mitogen-stimulation response) (Spinozzi et al. 1991), and impaired delayed-type hypersensitive responses (Lundy et al. 1975).[1]

- Chronic alcohol consumption interferes with the proper presentation of pathogen-derived molecules (i.e., antigens), which is required for T- and B-cell activation (Ness et al. 2008).

- Alcohol-exposed T cells have a reduced capacity to produce IFN-γ compared with control cells (Chadha et al. 1991).

- Alcohol-fed mice infected with TB exhibit decreased numbers of the two main subtypes of T cells (i.e., $CD4^+$ and $CD8^+$ T cells) as well as decreased proliferation of these cells compared with control mice (Mason et al. 2004).

IFN-γ–producing (i.e., type 1) T cells mediate immune reactions that are responsible for fighting not only *M. tuberculosis* infections but also infections by other bacterial pathogens, such as *K. pneumoniae* (Greenberger et al. 1996; Moore et al. 2002). Infection with *K. pneumoniae* induces time-dependent release of IL-12 from T cells, which in turn drives T cell IFN-γ production. This chain of reactions is disrupted by alcohol, because the levels of both IL-12 and IFN-γ were decreased in alcohol-exposed mice infected with *K. pneumoniae* (Zisman et al. 1998). These deficits could account for decreased clearance of these bacteria from the lungs. In addition to this flawed type-1 (Th1) response, the lungs of alcohol-fed rodents exhibit increased amounts of the inflammatory cytokine IL-10, which also may contribute to impaired lung clearance because normalizing IL-10 levels within the pulmonary system improves bacterial lung clearance (Greenberger et al. 1995).

B cells are responsible for the second arm of the immune response (i.e., the humoral immunity) that is mediated not by specific cells but by immune molecules (i.e., antibodies) produced and secreted by B cells in response to exposure to a pathogen. These antibodies consist of molecules called immunoglobulins (Igs). There are different types of Igs (e.g., IgA, IgM, and IgG) that all have specific functions during the immune response. Alcohol exposure in the context of TB also affects this arm of the immune response. Thus, although the total number of circulating B cells does not differ significantly between people with and without AUD, people with AUD have elevated levels of circulating IgA, IgM, and IgG (Spinozzi et al. 1992). In the lungs of people with AUD, however, Ig levels are reduced as determined by bronchoalveolar lavage (BAL) (Spinozzi et al. 1992). Replacement IgG therapy only

Types of T Cells

T cells are an important part of the immune system and fulfill a variety of functions in defending the organism against various pathogens. To do this, T cells are divided into different subgroups that all have specific functions. The two main subgroups are T helper cells and cytotoxic T cells. T helper cells, as the name implies, assist other immune cells in various ways. These T cells are characterized by the presence of a molecule called CD4 on their surface and therefore also are called $CD4^+$ cells. When they become activated, $CD4^+$ cells secrete various cytokines to facilitate different types of immune responses. Depending on the exact cytokines they produce, they can be further classified. For example, type 1 $CD4^+$ cells are characterized by the secretion of interferon γ (IFN-γ); they act primarily against pathogens that are found within cells. Conversely, type 2 $CD4^+$ cells do not produce IFN-γ but various types of interleukins. These cells act primarily against pathogens that are found outside the cells.

The other main subgroup of T cells, the cytotoxic T cells, has CD8 molecules on their surfaces. They are therefore also known as $CD8^+$ cells. These T cells directly destroy virus-infected and tumor cells.

[1] Delayed-type hypersensitivity responses are excessive immune reactions that occur only a few days after the body has been exposed to the pathogen. These responses are not mediated by immune molecules produced by B cells (i.e., antibodies) but by T cells.

partially restored Ig levels in these people, although it decreased the rates of pulmonary infections (Spinozzi et al. 1992).

RSV Infection

Although much of the attention concerning lung infections in people with AUD has been focused on bacterial infections, these individuals also have an increased susceptibility to viral airway infections. RSV is one of the most common lower respiratory tract viral pathogens and is a major cause of respiratory infections in children. Although RSV infections once were thought to be limited to children, it is now clear that RSV also is a serious problem in older people, patients with chronic obstructive pulmonary disease (COPD), and people with AUD. Prolonged alcohol exposure alters the first line of the innate cellular defense, the mucociliary apparatus, against invading pathogens such as RSV. This defense system propels inhaled particles, microbes, toxins, and debris out of the lungs and airways with the help of the fine hairs (i.e., cilia) on the cells that line the respiratory tract.

Alcohol has unique effects on the ciliated airways because it is rapidly and transiently absorbed from the bronchial circulation directly across the ciliated epithelium of the conducting airways. It then is vaporized into the airways and excreted during exhalation. However, when the exhaled air cools as it reaches the trachea, the alcohol vapor condenses and is dissolved back into the fluid in periciliary airway lining (George et al. 1996). This recycling of alcohol vapor continually subjects the conducting airways to high concentrations of alcohol (George et al. 1996), which modify airway-epithelium host defenses by altering cytokine release, barrier function (Simet et al. 2012), and cilia function (Sisson 1995; Sisson et al. 2009; Wyatt and Sisson 2001).

As is the case with other organs, alcohol's specific effects on the conducting airways depend on the route, dose, and length of the exposure (Sisson 2007). Early studies found that direct exposure of the ciliated airways to very high and nonbiologically relevant alcohol concentrations (i.e., 4 to 10 percent or 0.8–3.2 M) interfere with the movement of the cilia (i.e., cause ciliostasis) in a concentration-dependent manner (Nungester and Klepser 1938; Purkinjie and Valentine 1835). More recent studies have established that biologically relevant alcohol concentrations have very focused and specific effects on the lung airways. Over the past two decades, studies demonstrated that brief exposure to modest alcohol concentrations triggers generation of nitric oxide (NO) in the airway epithelial cells. This NO production stimulates a signaling pathway that involves the enzyme guanylyl cyclase, which produces a compound called cyclic guanosine monophosphate (cGMP). cGMP, in turn, activates cGMP-dependent protein kinase (PKG), followed by activation of the cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA). Activation of this dual kinase signaling pathway results in faster cilia beat frequency (CBF) in cilia briefly exposed to a moderate alcohol dose compared with controls (Sisson 1995; Sisson et al. 2009; Stout et al. 2007; Wyatt et al. 2003). More recent studies demonstrated that this rapid and transient alcohol-induced increase in NO levels was triggered by the alcohol-induced phosphorylation of heat shock protein 90 (HSP90) (Simet et al. 2013*b*). Upon phosphorylation, HSP90 increases its association with endothelial nitric oxide synthase (eNOS) in cilia, which then activates the cyclase–kinase cascade, resulting in increased CBF (Simet et al. 2013*b*). These findings are counterintuitive to the conventional wisdom that alcohol interferes with lung host defenses because stimulation of CBF should protect the lung; however, the clinical observation is that heavy alcohol exposure impairs lung host defenses. Indeed, that is just the first part of the story.

In contrast to brief alcohol exposure, prolonged alcohol exposure completely desensitizes lung airway cilia such that they can no longer beat faster when exposed to inhaled pathogens. This cilia-desensitization effect is known as alcohol-induced cilia dysfunction (AICD). In AICD, prolonged alcohol exposure results in failure to stimulate CBF, thereby desensitizing cilia to activating agents such as beta agonists (Wyatt and Sisson 2001). AICD likely results from decreased HSP90/eNOS association, which in turn attenuates the NO-stimulated cGMP/cAMP-dependent kinase activation pathway (Simet et al. 2013*a*; Wyatt and Sisson 2001). Alternatively, AICD may be related to oxidant-driven eNOS uncoupling, because AICD can be prevented in alcohol-drinking mice by concurrently feeding the animals dietary antioxidants, such as Procysteine™ or N-acetylcysteine (Simet et al. 2013*a*).

Regardless of the exact underlying mechanism, the consequence of alcohol-induced impairment in airway ciliary function is increased susceptibility to airway bacterial and viral infections, such as RSV. For example, Jerrells and colleagues (2007) demonstrated that alcohol-fed mice are inefficient in clearing RSV from the lungs. In addition, the alcohol-consuming mice exhibited enhanced and prolonged RSV infection compared with nondrinking RSV-infected animals. RSV infection itself causes a significant loss of ciliated cells from the airway epithelium and the remaining cilia beat more slowly compared with control cells from uninfected epithelia (Slager et al. 2006). This ciliary slowing is regulated by the activation of another signaling protein called protein kinase Cε (PKCε); moreover, once PKCε becomes inactivated again, the ciliated cells detach from the epithelium (Slager et al. 2006). It is unknown how concurrent alcohol exposure impacts these consequences of RSV infection. In summary, these

studies demonstrate that alcohol exposure compromises innate defenses against viral pathogens such as RSV in part by disrupting airway ciliary function.

ARDS

People with AUD who experience any type of lung injury—be it caused by infections with bacteria, TB-causing *M. tuberculosis*, or viruses or by noninfectious events such as trauma, pancreatitis, or burns—are at high risk for developing ARDS. The syndrome is characterized by endothelial and alveolar epithelial barrier dysfunction, severe inflammation, and surfactant dysfunction.[2] During ARDS, robust lung inflammation results in increased accumulation of fluid and inflammatory cells in the alveolar spaces. This causes impaired gas exchange in the lung, resulting in decreased oxygenation of the blood and multiple organ failure caused by the insufficient oxygen levels. ARDS is a life-threatening complication that develops in response to several events, including lung infection, non-lung sepsis, aspiration of stomach contents, trauma, and/or inhaled toxins. Among the most common causes of ARDS are bacterial pneumonia and an associated severe inflammatory response (i.e., alveolar sepsis). Alcohol abuse also has been identified as an independent risk factor that increases the odds of at-risk individuals to develop ARDS (Moss et al. 1996). Indeed, ARDS is two to four times more common in individuals with AUD than in non-AUD individuals (Moss and Burnham 2003).

One of the central features of ARDS is an impaired barrier function of the alveolar epithelial and endothelial cells.[3] Studies on the effect of alcohol alone on alveolar barrier function have revealed that chronic alcohol intake alters physical barrier properties within alveoli (Guidot et al. 2000). Interestingly, alveolar cells from ethanol-fed rats had increased expression of sodium channels in the membrane facing the interior of the alveoli (i.e., the apical membrane). This up-regulation of sodium channels may counteract the increased paracellular leak from the blood space into the alveolar airspace observed in the lungs of alcoholic subjects, and may explain why prolonged alcohol intake, in the absence of inflammation, does not result in fluid accumulation in the lungs (i.e., pulmonary edema) (Guidot and Hart 2005). However, these alcohol-fed rats had diminished airway clearance when challenged with saline, even in the absence of an inflammatory challenge (Guidot et al. 2000). These data suggest that the alveolar epithelium actually is dysfunctional after alcohol exposure, even though it seems normal and is able to regulate the normal air–liquid interface by enhancing sodium channels at the apical surface. In the presence of an inflammatory reaction, the compensatory mechanism likely becomes overwhelmed, resulting in greater susceptibility to barrier disruption and flooding of the alveolar space with protein-containing fluid.

One of the molecules involved in disrupting epithelial integrity is the cytokine transforming growth factor β_1 (TGF-β_1). Studies in rats that had been fed alcohol for a prolonged period of time found that expression of inactive TGF-β_1 protein doubled in lung tissue compared with nondrinking animals; however, there was no evidence of TGF-β_1 release or activation in the absence of an infection (Bechara et al. 2004). Nevertheless, alcohol-fed rats released five times more activated TGF-β_1 into the alveolar airspaces than did nondrinking rats in the presence of bacterial toxins in their blood (i.e., during endotoxemia). Additional studies using alveolar epithelial cell layers derived from these alcohol-fed rats found that this permeability defect was inhibited by neutralizing antibodies to TGF-β_1 (Bechara et al. 2004). Together, these data suggest that prolonged alcohol intake increases TGF-β_1 levels, which during inflammatory responses can be released and activated in the alveolar space, where it can directly impair epithelial barrier properties (Guidot and Hart 2005).

Another fundamental component contributing to alcohol's effects on the lungs is oxidative stress and the resulting alterations in alveolar macrophage function. As mentioned previously, alveolar macrophages are key components of both innate and acquired immunity against invading pathogens in the lung. After mucociliary clearance, these cells are the next line of cellular defense against invading pathogens through their phagocytic, microbiocidal, and secretory functions (Rubins 2003). Chronic alcohol ingestion decreases alveolar macrophage function by inhibiting the release of cytokines and chemokines as well as other factors essential for microbial killing and immune response (Franke-Ullmann et al. 1996; Omidvari et al. 1998). Alcohol-induced alveolar macrophage dysfunction likely occurs primarily as a result of alcohol-induced increases in oxidative stress, which is reflected by depletion of the antioxidant glutathione (GSH) in BAL fluid (Brown et al. 2007; Yeh et al. 2007). Impaired secretion of granulocyte monocyte colony-stimulating factor (GM-CSF) by type II alveolar cells likely also contributes to alcohol-induced oxidative stress (Joshi et al. 2005).

The alcohol-associated oxidative stress in the lungs is related at least in part to alcohol-driven changes in NADPH oxidase (Nox) enzyme function and GSH depletion. Nox enzymes generally promote oxidative stress, whereas antioxidants such as GSH help protect the cells against oxidative stress. Increased levels of Nox enzymes (e.g., Nox_4) and decreased GSH pools are emerging as significant components of the processes through which alcohol induces oxidative stress

[2] Surfactant is a lipoprotein complex produced by alveolar cells that covers alveoli and helps ensure proper lung function.

[3] The epithelial cells line the alveolar surface that faces the inside (or airspace) of alveoli, whereas the endothelial cells line the surface that faces the outside of the alveoli and the surrounding blood vessels.

that then causes alveolar macrophage dysfunction. As mentioned previously, chronic alcohol intake increases the levels of activated TGF-β_1, which then upregulates and activates Nox_4 (Brown and Griendling 2009). Nox_4 activation in turn leads to activation of Nox_1 and Nox_2, both of which cause production of reactive oxygen species (ROS) in the alveolar macrophages (Yeligar et al. 2012). At the same time, chronic alcohol consumption depletes levels of GSH in the lungs. Both of these processes promote chronic oxidative stress, which then impairs alveolar macrophage functions (Brown et al. 2004, 2007; Holguin et al. 1998; Yeh et al. 2007). Thus, both cellular-based microbial lung clearance and alveolar macrophage cell viability are decreased after chronic alcohol exposure and the resulting increase in oxidative stress (Velasquez et al. 2002). This role of alcohol-induced oxidative stress in macrophage dysfunction has been demonstrated in animal models in which chronic alcohol-drinking mice had decreased levels of GSH and increased levels of Nox enzymes and Nox-associated proteins in alveolar macrophages (Yeligar et al. 2012, 2014).

The identification of alcohol-driven oxidative stress as a contributor to alveolar macrophage dysfunction has led to promising antioxidant treatment approaches aiming to prevent alcohol-induced lung conditions in rodent models of prolonged alcohol consumption. For example, oral GSH treatment in alcohol-drinking mice was able to restore GSH pools, reverse alcohol-induced Nox increases, and restore alveolar macrophage function (Yeligar et al. 2012, 2014). Other studies have demonstrated that treatment with GSH precursors such as Procysteine™, N-acetylcysteine, or s-adenosylmethionine was able to improve alveolar macrophage phagocytosis (Brown et al. 2007) and promote differentiation of interstitial macrophages into mature alveolar macrophages (Brown et al. 2009) during chronic alcohol ingestion. These results suggest that GSH is a vital component in restoring alcohol-induced alveolar macrophage function by decreasing Nox proteins and restoring GSH pools.

Studies also have analyzed the role of GM-CSF in alcohol-induced oxidative stress and impaired lung immunity. GM-CSF is secreted by type II alveolar cells and is required for terminal differentiation of circulating monocytes into mature, functional alveolar macrophages (Joshi et al. 2006). The levels of GM-CSF are reduced in chronic alcohol-drinking mice (Joshi et al. 2005). Studies have shown that mice that have been genetically modified to no longer produce GM-CSF (i.e., GM-CSF knockout mice) exhibit a variety of changes contributing to impaired lung immune responses, including impaired surfactant expression, clearance, and phagocytosis; decreased expression of GM-CSF receptor; and impaired alveolar macrophage development (Dranoff et al. 1994; Joshi et al. 2005; Trapnell and Whitsett 2002). Conversely, overexpression of GM-CSF in genetically modified (i.e., transgenic) mice causes increased lung size, excessive growth (i.e., hyperplasia) of alveolar epithelial cells, and improved surfactant protein removal from the alveolar space (Ikegami et al. 1997). Other studies using a rat model of chronic alcohol consumption found that although the levels of GM-CSF in the alveolar space were not affected by alcohol exposure, the expression of GM-CSF receptors was significantly decreased in the membranes of alveolar macrophages (Joshi et al. 2005). Chronic alcohol intake also decreased alveolar binding of PU.1, a transcription factor responsible for GM-CSF activation. When the animals were treated with recombinant GM-CSF, alveolar macrophage bacterial phagocytic capacity, GM-CSF receptor expression, and PU.1 nuclear binding were restored (Joshi et al. 2005). These studies offer the groundwork for understanding the importance of GM-CSF within the lung for the maturation and host immune function of the alveolar macrophage as well as the deleterious impact of chronic alcohol use on these processes.

As these experimental studies have demonstrated, chronic alcohol intake exerts a detrimental effect on the function of alveolar macrophages, an important cell type involved in limiting ARDS risk and severity. Restoration of GM-CSF following alcohol exposure, replenishing of GSH pools, and normalization of Nox enzymes restore alveolar macrophage functions. The use of recombinant GM-CSF and antioxidants potentially could improve alveolar macrophage function in people with AUD. Preventing the pathophysiological consequences of lung injury, including excessive inflammation, and the resulting pulmonary edema and insufficient oxygen supply (i.e., hypoxia) in the tissues associated with ARDS remains the goal of research on alcohol-enhanced ARDS.

Summary

For centuries, it has been known that people with AUD are more likely to have pulmonary infections such as pneumonia and TB. Over the past two decades, it has become clear that other conditions such as RSV and ARDS also are linked to high-risk alcohol consumption. Even with the development of antibiotics, vaccinations, health education, and preventative medicine, a strong correlation still exists among heavy alcohol consumption, pulmonary infections, and ARDS. Over the past 30 years, however, research has vastly enhanced our understanding of the pathophysiology of the immunocompromised "alcoholic lung." This includes new insight into the mechanisms that cause the harmful effects of heavy alcohol intake on neutrophils, lymphocytes, airway ciliary function, and alveolar macrophages, all of which contribute to the prolonged and often more severe pulmonary diseases observed in people who abuse alcohol. Armed with a better understanding of the lung pathophysiology unique to the

heavy drinker, clinicians now are better prepared to combat these diseases through various treatment regimens. Preclinical models suggest that antioxidant nutritional supplements may prevent alcohol-induced lung oxidative stress, allowing mucociliary clearance and alveolar macrophage functions to be preserved. Promising animal studies also show that restoration of normal G-CSF, IgG, and GM-CSF levels could permit normal lung recovery following infection and injury in individuals with AUD. These disease- and cell-associated studies offer hope for novel preventative and therapeutic options for restoration of a normal lung immune response in people with AUD.

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Impact of Alcohol Abuse on the Adaptive Immune System

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Alcohol exposure, and particularly chronic heavy drinking, affects all components of the adaptive immune system. Studies both in humans and in animal models determined that chronic alcohol abuse reduces the number of peripheral T cells, disrupts the balance between different T-cell types, influences T-cell activation, impairs T-cell functioning, and promotes T-cell apoptosis. Chronic alcohol exposure also seems to cause loss of peripheral B cells, while simultaneously inducing increased production of immunoglobulins. In particular, the levels of antibodies against liver-specific autoantigens are increased in patients with alcoholic liver disease and may promote alcohol-related liver damage. Finally, chronic alcohol exposure in utero interferes with normal T-cell and B-cell development, which may increase the risk of infections during both childhood and adulthood. Alcohol's impact on T cells and B cells increases the risk of infections (e.g., pneumonia, HIV infection, hepatitis C virus infection, and tuberculosis), impairs responses to vaccinations against such infections, exacerbates cancer risk, and interferes with delayed-type hypersensitivity. In contrast to these deleterious effects of heavy alcohol exposure, moderate alcohol consumption may have beneficial effects on the adaptive immune system, including improved responses to vaccination and infection. The molecular mechanisms underlying ethanol's impact on the adaptive immune system remain poorly understood.

Key words: Alcohol use, abuse and dependence; alcohol use disorder; heavy drinking; beneficial moderate alcohol consumption; ethanol consumption; prenatal alcohol exposure; alcoholic liver disease; immune system; adaptive immune system; immune response; growth and development; infection; T cells; B cells; lymphocyte; immunoglobulin; vaccinations; cancer; pneumonia; HIV; hepatitis C virus; tuberculosis; human studies; animal models

In the United States, alcohol use disorder (AUD) is the third-leading cause of preventable death. It is associated with increased susceptibility to bacterial pneumonia; viral infections, such as HIV and hepatitis C virus (HCV); and increased postoperative morbidity and mortality. This increased susceptibility is mediated in part by functional alterations in various cells of the immune system. The immune system is broadly divided into two branches: innate and adaptive immunity. The innate immune system represents the first line of host defense and is necessary for inducing the adaptive immune response. The adaptive immune system can be subdivided further into cellular and humoral immunity. The main components of cellular immunity are CD4 and CD8 T cells. CD4 T cells play a critical role in the activation and differentiation of macrophages, CD8 T cells, and B cells. CD8 T cells, on the other hand, are essential for eliminating cells infected with intracellular pathogens, as well as cancer cells. Humoral immunity is mediated by B cells, which produce antibodies to eliminate extracellular microorganisms and prevent spread of infections. This review will summarize the impact of chronic heavy drinking or AUD as well as of moderate alcohol consumption on adaptive immunity and discuss future areas of research in this rapidly evolving field.

Impact of AUD on T Cells

Effects on T-Cell Numbers, Phenotype, and Activation

T cells constitute a diverse population of lymphocytes that develop in the bone marrow and mature in the thymus. Each T cell expresses a unique T-cell receptor (TCR) that confers specificity

for one particular foreign molecule (i.e., antigen). Early studies already had indicated that chronic alcohol abuse (i.e., for 12 to 15 years) resulted in reduced numbers of peripheral T cells (Liu 1973; McFarland and Libre 1963). More recent studies confirmed this observation and showed that the lack of lymphocytes (i.e., lymphopenia) was as severe in people who engaged in a short period of binge drinking as it was in individuals who drank heavily for 6 months (Tonnesen et al. 1990). Interestingly, abstinence for 30 days was sufficient to restore lymphocyte numbers back to control levels (Tonnesen et al. 1990). Similar findings were obtained in animal models, where the number of T cells in the spleen decreased in mice fed a liquid diet (i.e., Lieber-DeCarli diet) containing 7 percent ethanol for as little as 7 days (Saad and Jerrells 1991) or 6 percent ethanol for 28 days (Percival and Sims 2000). Likewise, adult male Sprague-Dawley rats consuming liquid diets containing up to 12 g ethanol/kg/day for 35 days exhibited significantly reduced absolute numbers of T cells (Helm et al. 1996).

In addition to reducing T-cell numbers, chronic alcohol exposure disrupts the balance between different T-cell types (i.e., T-cell homeostasis), leading to a shift toward a memory phenotype. Specifically, people who had consumed 30.9 ± 18.7 alcoholic drinks/day for approximately 25.6 ± 11.5 years exhibited a decreased frequency of naïve (i.e., $CD45RA^+$) CD4 and CD8 T cells, as well as an increased frequency of memory T cells (i.e., $CD45RO^+$) (Cook et al. 1994). Another study conducted in humans with self-reported average alcohol consumption of approximately 400 g/day also found an increase in the percentage of both $CD45RO^+$ memory CD4 cells and CD8 cells (Cook et al. 1995). These observations were confirmed in animal models. Thus, studies in C57BL/6 mice demonstrated that chronic ethanol consumption (20 percent ethanol in water for up to 6 months) decreased the frequency of naïve T cells and increased the percentage of memory T cells (Song et al. 2002; Zhang and Meadows 2005). This loss of naïve T cells could result from decreased T-cell production in the thymus; increased cell death (i.e., apoptosis) of naïve T cells; or increased homeostatic proliferation. Additional analyses detected evidence that T-cell proliferation in the spleen was increased in alcohol-consuming mice (Zhang and Meadows 2005). Together, these observations suggest that chronic alcohol consumption results in lymphopenia, which can increase homeostatic proliferation and accelerate conversion of naïve T cells into memory T cells (Cho et al. 2000).

Alcohol consumption also influences T-cell activation both in humans and in mouse models (Cook et al. 1991, 1995). For example, alcoholics who had consumed approximately 23 ± 9 drinks/day for 27.0 ± 11.5 years exhibited significantly elevated numbers of activated CD8 T cells immediately after admission for detoxification, which persisted after 4 to 10 days of abstinence (Cook et al. 1991).[1] The percentage of activated CD8 T cells expressing both human leukocyte antigen (HLA)-DR and CD57 also was increased in alcoholics with self-reported average alcohol consumption of approximately 400 g/day (Cook et al. 1995). An increase in these cells could contribute to the chronic inflammation observed in alcoholic patients, because human CD57-expressing T cells can rapidly produce the pro-inflammatory cytokine interferon gamma (IFN-γ) after stimulation through their TCR, without requiring a second signal, as is the case with other cells (Song et al. 2001). Similar findings were described in mouse models. Thus, C57BL/6 or BALB/c mice that consumed 20 percent ethanol in water for up to 6 months showed a greater frequency of activated T cells, increased rapid IFN-γ response, and heightened sensitivity to low levels of TCR stimulation, with no requirement for a second signal (Song et al. 2002; Zhang and Meadows 2005).

The effects of chronic alcohol exposure are not limited to phenotypic changes in T cells but also include T-cell functions. One study in mice investigated the impact of ethanol or wine consumption on T-cell migration by dividing the animals in three groups (i.e., receiving water, 6 percent ethanol in water, or alcohol in the form of wine adjusted to 6 percent ethanol with water) and injecting them with bacterial molecules called lipopolysaccharides (LPS) or endotoxin, which can activate the immune response. Among other reactions, LPS injection normally triggers lymphocyte migration out of the circulation and into tissues and the lymphatic system (Percival and Sims 2000). In water- or wine-consuming mice, LPS injection, as expected, led to a 50 percent reduction in the number of lymphocytes in the peripheral blood, indicating their mobilization into tissues. In contrast, the ethanol-consuming mice exhibited no change in the frequency of certain circulating lymphocytes (i.e., CD3 cells) after LPS injection, suggesting that chronic alcohol consumption may potentially impair the ability of lymphocytes to migrate out of circulation (Percival and Sims 2000). One potential explanation for the lack of detrimental effects of wine in this experiment could be the presence of phytochemicals in wine that may be able to overcome ethanol's harmful impact on immunity.

Effects on T-Cell Apoptosis

Activated T cells normally undergo apoptosis if they receive a second activation stimulus within a short interval. This process is known as activation-induced cell death (AICD) and is important to maintain T-cell homeostasis and self-tolerance (Alderson et al. 1995). Experiments done in an immortalized line of human

[1] T-cell activation was assessed by measuring the expression of human leukocyte antigen (HLA)-DR on the patient's CD8 cells. HLAs are proteins found on the surface of various cells that present antigens to the TCR on T cells to induce an immune response.

T lymphocyte cells used in cancer research (i.e., Jurkat cells) found that exposure to different concentrations of ethanol (i.e., 25, 50, 100, 150, 200 mM) for 24 hours resulted in decreased cell viability in a dose-dependent manner. Furthermore, ethanol exposure decreased expression of the anti-apoptotic molecule Bcl-2 and promoted expression of the pro-apoptotic molecule BAX in the cells. These findings suggest that ethanol pretreatment can sensitize T cells to AICD (Kapasi et al. 2003). Similarly, in vitro exposure of peripheral T cells to a physiologically relevant concentration of 25mM ethanol significantly enhanced the activation of a protein that mediates apoptosis (i.e., caspase-3) as well as promoted DNA fragmentation (which is a hallmark of apoptosis) when the cells were stimulated (Kelkar et al. 2002). In vivo studies in humans confirmed these observations, demonstrating that binge drinking (i.e., consuming 5 to 7 drinks within 90 to 120 minutes) promoted T-cell apoptosis and decreased Bcl-2 expression (Kapasi et al. 2003).

Another mechanism contributing to ethanol-induced apoptosis in human T cells could involve down-regulation of the vitamin D receptor (VDR). VDR normally reduces expression of a signaling molecule called renin angiotensin (RAS) (Li et al. 2004). Lowered RAS levels in turn induce dysregulation of the mitochondria (Kimura et al. 2005) and enhance production of reactive oxygen species (ROS) that can damage various molecules in the cells (Iuchi et al. 2003). Both mitochondrial dysregulation and ROS production promote apoptosis. Naïve human T cells produce low levels of VDR, but expression is increased to moderate levels in activated T cells (Irvin et al. 2000). Human T cells incubated in vitro with variable concentrations of ethanol (0, 10, 25, and 50mM for 24 hours) showed a reduced expression of the VDR, accompanied by increased expression of RAS and ROS as well as increased T-cell death (Rehman et al. 2013). Additional analyses demonstrated that ethanol exposure promoted apoptosis by inducing breaks in the DNA of the T cells. This damage to the DNA most likely was mediated by ROS generation in response to RAS activation. Treatment with a compound that activates the VDR (i.e., a VDR agonist) restored the T cell's VDR expression, down-regulated RAS expression as well as ROS generation, and thus preserved T-cell survival (Rehman et al. 2013).

In summary, these studies suggest that chronic alcohol abuse in humans and animal models results in lymphopenia, increased T-cell differentiation and activation, and reduced migration (see figure 1). Chronic activation of the T-cell pool may alter the T cells' ability to expand and respond to pathogenic challenges (potentially by inducing a state of unresponsiveness, or anergy, of the T cells), place the T cells under increased regulatory control, or lead to their elimination through increased sensitivity to AICD. These changes in turn compromise the organism's ability to respond to pathogens and contribute to increased susceptibility to infections.

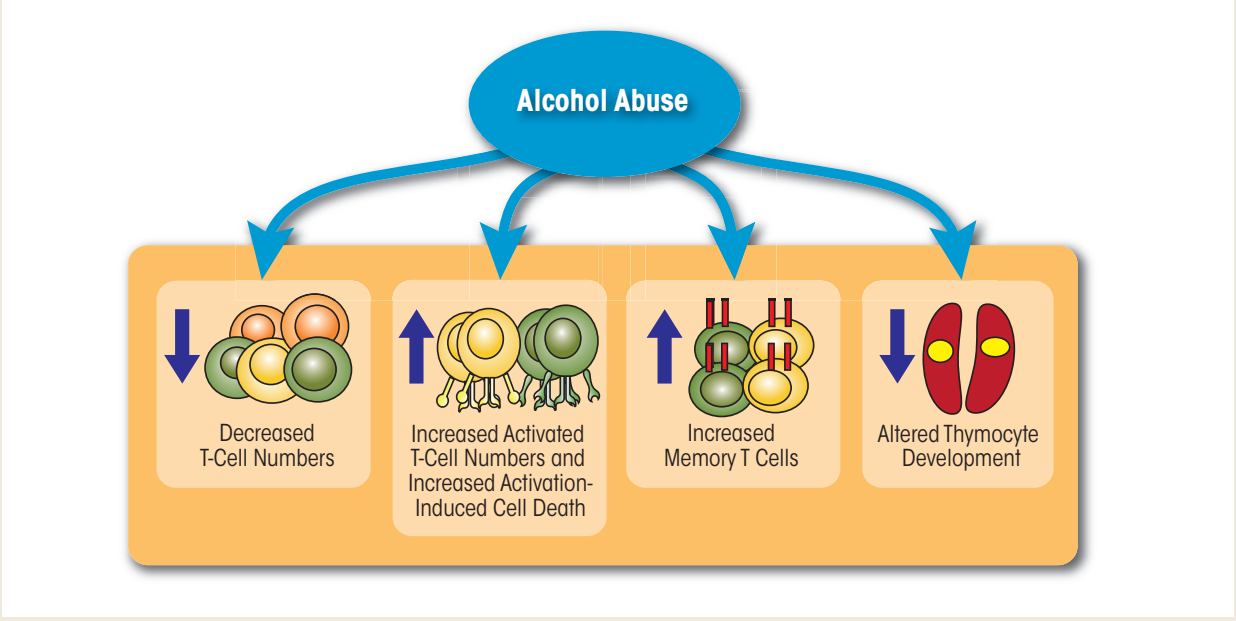

Figure 1 Alcohol abuse affects both the number and function of T cells. Chronic alcohol consumption decreases the number of circulating T cells, increases the number of activated T cells, accelerates differentiation of T cells to a memory phenotype, and interferes with thymocyte development.

Impact of AUD on B cells

Effects on B-Cell Numbers and Phenotype

B cells are lymphocytes that originate in the bone marrow and mature in the spleen. They produce proteins called immunoglobulins (Igs) located either on the cell surface (where they are referred to as B-cell receptors [BCRs]) or secreted in the form of antibodies. Like T cells, each B cell expresses a unique BCR that only binds to a specific antigen. B cells and the antibodies they secrete mediate both T-cell–dependent and T-cell–independent immune responses, depending on the specific class of antibody released. Similar to what has been observed for T cells, alcoholics (90 to 249 drinks/month) exhibit lower B-cell numbers than do moderate (30 to 89 drinks/month) or light drinkers (<9 drinks/month) (Mili et al. 1992; Sacanella et al. 1998). The loss of circulating B cells is particularly severe in patients with alcoholic liver disease (ALD) consuming 164.9 to 400 grams of alcohol/day on average (Cook et al. 1996; Matos et al. 2013).

The loss of peripheral B cells primarily seems to affect certain subpopulations of cells. B cells can be divided into two main subtypes that produce different types of Igs and other proteins and respond to particular types of antigens:

- B-1 B cells, which primarily seem to respond to polysaccharide antigens, such as bacterial LPS. They can be divided further into the B-1a subset, which produces broadly reactive IgM antibodies, and the B-1b subset, which is important for T-cell–independent responses.
- B-2 B cells, which are considered "conventional" B cells. They produce high-affinity antibodies and, unlike B-1 B cells, B-2 B cells can develop into long-lived memory B cells that are critical in protection from subsequent infection with the same pathogens (i.e., form an immunological memory).

The alcohol-related decrease in peripheral B cells primarily seems to be mediated by a decrease in the frequency of the B-2 B cells. The number of B-1a cells also seems to decline, but this decrease is accompanied by a relative increase in the percentage of B-1b cells (Cook et al. 1996). The loss of B-2 cells may explain why alcoholics often cannot respond adequately to new antigens. The relative increase in B-1b cells also may lead to autoantibody production, especially of the IgM and IgA classes (which is discussed below).

Effects on Circulating Immunoglobulin Levels

Igs mediate a critical part of the adaptive immune response. There are five classes of antibodies:

- IgD is present in small amounts in blood and serum and signals naïve B cells to be activated.
- IgM is the first antibody produced during an immune response and is responsible for agglutination and antibody-dependent cytotoxicity.
- IgG is abundant in blood, lymph fluid, cerebrospinal fluid, and peritoneal fluid and can activate the complement system to facilitate phagocytosis of microorganisms.
- IgA is present primarily in mucosal secretions and prevents pathogens from attaching to and penetrating epithelial surfaces.
- IgE is widely found in the lungs, skin, and mucous membranes and plays an important role in allergic, anti-parasitic, and type 1-hypersensitivity responses.

Although chronic alcohol consumption leads to reduced B-cell frequency, it also results in increased production of Igs. Alcoholic patients with ALD (i.e., hepatic fibrosis or cirrhosis) who had consumed the equivalent of 10 oz. of 100-proof ethanol/day for 10 years showed elevated IgA and IgG levels compared with controls or alcoholics without ALD (Smith et al. 1980). Similarly, both IgA and IgM levels were increased in heavy drinkers (90 to 249 drinks/month) compared with light (<9 drinks/month) or moderate drinkers (30 to 89 drinks/month) (Mili et al. 1992). Other studies determined an increase in IgE levels with chronic alcohol consumption (>100g/day for a minimum of 5 years), which was associated with an increased prevalence of pollen allergies (Gonzalez-Quintela et al. 1999, 2003; Hallgren and Lundin 1983). Furthermore, IgE levels decreased gradually with length of abstinence, suggesting a strong correlation between alcohol consumption and IgE serum levels (Gonzalez-Quintela et al. 1995; Hallgren and Lundin 1983).

These clinical observations were confirmed with cultured cells as well as in rodent studies. Treatment of a mouse cell line (i.e., A78-G/A7 hybridoma cells) with different concentrations of ethanol (25, 50, 100, and 200mM) for 48 hours resulted in a linear increase in IgM levels (Muhlbauer et al. 2001). Moreover, spontaneous IgA synthesis by peripheral blood mononuclear cells (PBMCs)—a mixed population of various white blood cells that also includes B cells—was higher in PBMCs isolated from alcoholic patients with liver disease compared with controls (Wands et al. 1981). IgA concentrations also were increased in a layer (i.e., the lamina propria) of the mucous membranes lining the intestine of adult female Wistar rats after acute ethanol administration (4g/kg intraperitoneally) for 30 minutes (Budec et al. 2007). Recent studies suggest that the increase in IgA levels may be mediated by an ethanol-induced elevation of the enzyme neuronal nitric oxide synthase (nNOS) in the animals' intestine, because inhibition of nNOS before ethanol injection suppressed the IgA increase (Budec et

al. 2013). However, additional studies are needed to fully uncover the mechanisms that underlie increased Ig production while B-cell numbers are reduced. Potential mechanisms include increased Ig production by mature B cells (i.e., plasma cells), which have not yet been examined, and increased levels of antigens in the body, either because of higher rates of infection or potentially because of higher production of antigens derived from the body itself (i.e., autoantigens) as a result of organ damage.

Interestingly, ALD patients have increased concentrations (i.e., titers) of circulating antibodies directed against liver-specific autoantigens (McFarlane 2000). Thus, patients with active alcohol-induced hepatitis have antibodies directed against liver-specific membrane lipoprotein, with titers correlating with disease severity (McFarlane 1984). Antibodies against the ethanol-metabolizing enzyme alcohol dehydrogenase also have been found in 50 percent of patients with ALD and were associated with alcoholic hepatitis (Ma et al. 1997). Furthermore, anti-phospholipid antibodies could be observed in up to 80 percent of patients with alcoholic hepatitis or cirrhosis, as well as in heavy drinkers (>80g/day for more than 1 year) with milder liver damage (Chedid et al. 1994). Finally, about 70 percent of patients with advanced ALD have elevated levels of antibodies directed against compounds formed in the body during metabolic processes (i.e., lipid peroxidation) that occur as a result of alcohol-induced ROS production (Mottaran et al. 2002). These compounds, which include malondialdehyde, 4-hydroxynonenal, and lipid hydroperoxides, are readily detectable in the serum and liver of ALD patients and alcohol-fed rodents (Albano 2006). These observations suggest that ethanol-induced organ damage could stimulate auto-antibody production, leading to overall increased concentration of circulating antibodies.

Additional studies conducted with mice indicate that the development of antibodies against antigens generated as a result of lipid peroxidation is associated with the hepatic expression of proinflammatory cytokines and development of fatty liver (i.e., steatohepatitis) (Ronis et al. 2008). Similarly, studies in humans have shown that elevated levels of antibodies directed against lipid-peroxidation products are associated with elevated levels of the proinflammatory cytokine tumor necrosis factor-α in the blood (Vidali et al. 2008). The exact mechanisms by which these autoantibodies lead to increased production of proinflammatory factors remain to be elucidated. It is possible that antibody-mediated opsonization and tissue destruction result in inflammatory cytokine production by various immune cells that ingest the marked antigens (i.e., phagocytic cells or natural killer [NK] cells).[2]

Together, the above studies demonstrate that chronic alcohol consumption simultaneously is associated with reduced B-cell numbers and increased Ig levels, including Igs directed against liver autoantigens and byproducts of oxidative damage (see figure 2). The presence of these antibodies contributes to increased production of inflammatory cytokines, which in turn may accelerate and/or exacerbate liver damage.

Impact of AUD on Lymphocyte Development

Effects on Thymocytes

Thymocytes are progenitor cells in the thymus that entered a selection and maturation process called thymopoiesis to become T cells. Alcohol exposure can interfere with this process. Thus, when pregnant female C57BL/6J mice consumed a liquid diet in which 25 percent of the calories were derived from ethanol from gestational day 1 to day 18, the offspring exhibited reduced thymocyte numbers (Ewald 1989; Ewald and Frost 1987; Ewald and Walden 1988). This reduction in thymocyte numbers may be mediated by ethanol-associated activation of the hypothalamic–adrenal–pituitary axis and increased glucocorticoid levels (Hiramatsu and Nisula 1989).[3] Glucocorticoids long have been recognized as a potential cause for shrinkage (i.e., atrophy) of the thymus through induction of apotopsis (Reichert et al. 1986). This hypothesis is supported by findings that a single dose of ethanol results in increased levels of endogenous glucocorticoids and thymic atrophy (Han et al. 1993). Furthermore, administration of a glucocorticoid antagonist can block thymic atrophy and DNA fragmentation indicative of apoptosis in 8- to 12-week-old female mice consuming a solution of 20 percent ethanol in water (Han et al. 1993). However, another study showed that ethanol-fed animals whose adrenal glands had been removed (i.e., which had been adrenalectomized) and which therefore could no longer produce glucocorticoids still had fewer thymocytes than control adrenalectomized animals (Jerrells et al. 1990). This suggests that glucocorticoids are responsible for some, but not all, of the ethanol-induced thymic dysfunction.

Alcohol also activates an enzyme acting at the thymocyte membrane called adenylate cyclase, which increases the intracellular concentration of cyclic AMP (Atkinson et al. 1977). cAMP has multiple regulatory functions in the cell, and increased cAMP levels can stimulate DNA fragmentation, leading to thymocyte apoptosis (McConkey et al. 1990). In studies conducted in vitro using a controlled model of thymocyte differentiation known as fetal thymus organ culture, exposure to 0.2 or 0.4 percent ethanol for 5 days resulted in generation of fewer total thymocytes and increased

[2] Opsonization is a process by which a pathogen or other antigen is covered with antibodies and thereby marked for ingestion and destruction by other immune cells (i.e., phagocytic cells).

[3] The hypothalamic–adrenal–pituitary axis is a hormonal system that primarily is involved in the stress response. Activation of this system culminates in the production and release of corticosteroid (i.e., cortisol in humans and corticosterone in rodents) from the adrenal glands, which then act on various tissues to mediate the stress response.

thymocyte apotopsis in a dose-dependent manner compared with control cultures (Bray et al. 1993). Finally, exposure to ethanol concentrations of 0.4 to 2 percent had a more profound effect on apoptosis of cultured thymocytes than on mature T cells (Slukvin and Jerrells 1995). All of these studies demonstrate that ethanol interferes with normal thymocyte function and maturation into T cells in a variety of ways.

Effects on B-Cell Development

Numerous analyses also have evaluated the effects of ethanol exposure on the development of B cells. As described above for thymopoiesis, the offspring of pregnant mice that from gestational day 1 to day 18 consumed a liquid diet in which 25 percent of calories were derived from ethanol exhibited decreased numbers of both immature and mature B cells in the spleens directly after birth. Moreover, these B-cell subpopulations did not recover to normal levels until 3 to 4 weeks of life (Moscatello et al. 1999; Wolcott et al. 1995). Other studies were conducted using a precursor cell type called oligoclonal-neonatal-progenitor (ONP) cells, which in vitro can differentiate either into B lymphocytes or into other white and red blood cells (i.e., myeloid cells), depending on the cytokines to which they are exposed. ONP cells isolated from newborn mice that had been exposed to alcohol in utero demonstrated a greatly reduced capacity to respond to interleukin (IL)-7 and commit to the B lineage, whereas their response to the growth factor granulocyte–monocyte colony-stimulating factor (GM-CSF) and commitment to the myeloid lineage was not affected (Wang et al. 2006). Similarly, ONP cells isolated from newborn mice and cultured in vitro in the presence of 100 mM ethanol for 12 days failed to respond to IL-7 and commit to the B lineage, suggesting intrinsic defects (Wang et al. 2011). Additional investigations demonstrated that alcohol affects ONP cell differentiation into B lineage at a late stage by down-regulating the expression of several transcription factors (e.g., EBF and PAX5) and cytokine receptors, such as the IL-7 receptor (IL-7Ra) (Wang et al. 2009).

As described earlier for adult humans, alcohol can lead to increases in Ig levels during development, even if the numbers of mature B cells decrease. Thus, maternal alcohol consumption during pregnancy (12 mg/week for most of the pregnancy) increased IgE levels in the umbilical cord blood of the infants (Bjerke et al. 1994).

Taken together, all these findings suggest that in utero exposure to ethanol may increase the risk for infections during early childhood or adulthood as a result of alcohol-induced defects in B-cell and T-cell development. Indeed, in utero exposure to ethanol resulted in a significant reduction in T-cell and B-cell responses to various antigens that did not recover to control levels until 4 to 5 weeks of life.

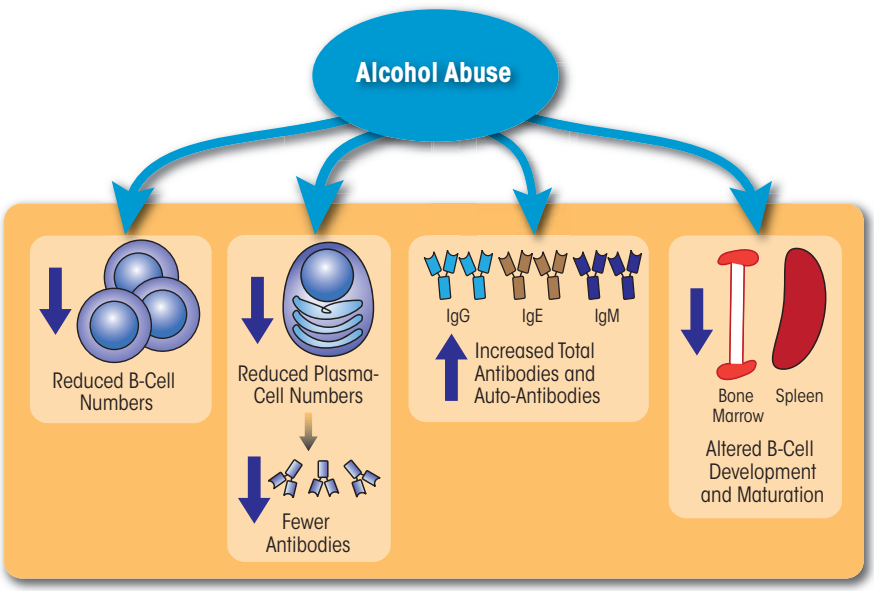

Figure 2 Alcohol abuse impairs both the number and function of B cells. Chronic alcohol consumption reduces B-cell numbers, decreases antigen-specific antibody responses, increases the production of auto-antibodies, and interferes with B-cell development and maturation.

In contrast, ethanol exposure did not significantly affect the development of the lytic functions of NK cells (Wolcott et al. 1995).

Impact of AUD on Adaptive Immune Responses

Responses to Infections

Alcohol abuse has been associated with increased incidence and severity of community-acquired pneumonia (Happel and Nelson 2005; Zhang et al. 2002), HIV infection (Mbulaiteye et al. 2000; Rasch et al. 2000; Stein et al. 2000; Welch 2000), HCV infection (Seronello et al. 2010; Zhang et al. 2003), and infection with *Mycobacterium tuberculosis* (Hedemark et al. 1995; Hudolin 1975; Kline et al. 1995; Panic and Panic 2001; Sabot and Vendrame 1969). This increased susceptibility could be caused by alcohol-induced alterations in lymphocyte numbers and function or by AUD-related enhanced behavioral or environmental exposure to these pathogens.

Analyses of animal models can help delineate the contribution of behavioral and immunological changes to the increased susceptibility to infection. Indeed, experiments in a mouse model of influenza A infection showed that animals that had consumed 18 to 20 percent ethanol for 4 to 8 weeks exhibited an impaired influenza-specific CD8 T-cell response. Specifically, mice in the ethanol group exhibited a decrease in the number of influenza-specific CD8 T cells (Meyerholz et al. 2008).[4] Influenza A virus infections increasingly are recognized as an important agent in community-acquired pneumonia. Because influenza-specific effector CD8 T cells play a central role in the elimination of influenza-infected cells (Epstein et al. 1998), a reduced T-cell response could lead to increases in the incidence and severity of community-acquired pneumonia (Horimoto and Kawaoka 2005). Finally, adult mice exposed to ethanol only during gestation and nursing exhibited increased influenza-associated morbidity and mortality, increased numbers of virus particles in the lungs, and decreased numbers of both B cells and influenza-specific CD8 T cells in the lungs following influenza infection (McGill et al. 2009).

Researchers also have investigated the molecular and cellular mechanisms underlying increased susceptibility to HIV associated with chronic drinking using animal models. In one approach, rhesus macaques were administered either alcohol or a sugar solution with the same calorie content directly into the stomach. When both groups of animals were infected with the primate equivalent of HIV (i.e., simian immunodeficiency virus [SIV]) by the rectal route, higher SIV loads were observed in the alcohol-consuming animals. In addition, alcohol-consuming animals exhibited a higher CD4:CD8 T-cell ratio in part of the intestine (i.e., the duodenum) compared with control animals (Poonia et al. 2006). Because intestinal CD4 T cells are the major target cells in HIV and SIV infections (Veazey et al. 2001), an increased percentage of CD4 T cells in the gut of alcohol-consuming macaques could be the reason for the higher SIV loads observed in these animals (Poonia et al. 2006). In addition, CD8 T-cell responses play a critical role in controlling HIV infections and eliminating infected cells; therefore, the decrease in CD8 T cells could lead to impairment in anti-HIV responses (Betts et al. 2006).

The increased susceptibility to *M. tuberculosis* was confirmed in a mouse study where consumption of a liquid ethanol diet for 9 weeks (serum alcohol levels = 39 mg/dL) resulted in significantly higher bacterial burden in the lung (Mason et al. 2004). Further analyses also identified blunted CD4 T-cell responses (i.e., reduced proliferation as well as IFN-γ and IL-2 production by the cells) as well as decreased CD8 T-cell numbers in draining lymph nodes of alcohol-consuming mice compared with control mice (Porretta et al. 2012).

Responses to Vaccination

Because alcoholics are at increased risk for hepatitis B (HepB) infections, immunization with a HepB vaccine is recommended. However, the magnitude of the response to the vaccination (i.e., the production of antibodies) is lower in alcoholics compared with nonalcoholic control subjects (Nalpas et al. 1993), patients with other drug dependencies (Hagedorn et al. 2010), or patients with chronic liver disease caused by HCV or unknown causes (i.e., cryptogenic liver disease) (Roni et al. 2013), with the lowest responses found in alcoholics with liver disease. Another study (Rosman et al. 1997) demonstrated that the impaired antibody response in alcoholic patients (i.e., with consumption levels of 230 ± 16 g/day ethanol for 26.4 ± 1.8 years) can be improved by doubling the dose of HepB vaccine from 10 µg to 20 µg at 0, 1, and 6 months. Similar results also were obtained in animal models. Thus, mice that were chronically fed ethanol generated a weaker antibody response following vaccination with HCV compared with control mice (Encke and Wands 2000). Abstinence partially restored antibody responses against hepatitis antigens in a mouse model (Encke and Wands 2000).

Additional studies in rodents assessed the effects of alcohol on the effectiveness of bacillus Calmette-Guérin (BCG) vaccination, which protects against tuberculosis. The studies found that when animals consumed ethanol before BCG vaccination, they were not protected against a subsequent pulmonary challenge with *M. tuberculosis*. In contrast, mice that consumed ethanol after the BCG vaccination were protected against a subsequent *M. tuberculosis* challenge (Porretta et al. 2012). Taken together, these data

[4] Similarly, chronic consumption of 18 percent ethanol in water for 31 weeks resulted in impaired antigen-specific CD8 T-cell responses following inoculation with *Listeria monocytogenes* (Gurung et al. 2009).

suggest that chronic ethanol exposure interferes with immunity to new antigens but not with immunity established before alcohol consumption.

Cancer Risk

Alcohol-related alterations of immune surveillance also have been implicated in the development of cancer (Poschl and Seitz 2004). Reduced cell-mediated immunity was proposed as a potential explanation for the high incidence of head and neck cancer observed in alcoholic patients (Lundy et al. 1975). However, these studies are difficult to interpret, because several factors affect antitumor immunity in human alcoholics, including malnutrition, vitamin deficiencies, and liver cirrhosis. The impact of alcohol on NK cells, which are the first responders against tumor-forming cells, has been investigated in mouse models. Those studies showed decreased cytolytic activity of NK cells in C57BL/6 mice consuming 20 percent ethanol for 4 weeks; however, no differences existed in the metastasis of B16-BL6 melanoma cells in alcohol-consuming and control animals (Meadows et al. 1993). Another study using different tumor cells (i.e., MADB106 mammary adenocarcinoma cells) demonstrated that ethanol administration 1 hour before tumor inoculation suppressed NK-dependent destruction of tumor cells, resulting in a 10-fold increase in the number of lung metastases in Fischer 344 rats (Ben-Eliyahu et al. 1996). The presence of ethanol in an in vitro culture of spleen cells also suppressed NK cell cytotoxic activity against MADB106 tumor cells (Yirmiya et al. 1992).

Delayed-Type Hypersensitivity

Another aspect of cell-mediated immunity that is affected by ethanol consumption is the delayed-type hypersensitivity (DTH) response. DTH refers to a cutaneous T-cell–mediated inflammatory reaction that takes 2 to 3 days to develop. It is mediated by CD4 T helper cells, specifically the Th1 subpopulation. The data on alcohol-induced alterations in DTH responses are limited. One early study (Lundy et al. 1975) showed defects in cell-mediated immunity in male alcoholic patients admitted for detoxification, in response both to a new antigen and to an antigen to which they had previously been exposed. A more recent study (Smith et al. 2004) reported that a negative correlation existed between the amount of alcohol consumed by the participants and the size of DTH skin test responses to a specific antigen (i.e., keyhole limpet hemocyanin). Similar results were described in rodent models. For instance, genetically modified BALB/c mice that carried a TCR specific for the ovalbumin peptide and were fed a diet containing 30 percent ethanol-derived calories exhibited decreased antigen-specific Th1 responses (Waltenbaugh et al. 1998). Similarly, C57BL6 mice fed a liquid diet in which ethanol provided 27 percent of the total calories generated significantly decreased DTH responses to a T-cell–dependent antigen (i.e., sheep red blood cells) (Jayasinghe et al. 1992). The reduced DTH response and accompanying decrease in IL-12 and IFN-γ cytokine production are thought to result in part from ethanol-mediated depletion of the antioxidant glutathione in antigen-presenting cells (Peterson et al. 1998).

Effects of Moderate Ethanol Consumption on Adaptive Immunity

The discussion in the preceding sections centered primarily on the effects of chronic alcohol abuse on the immune system. In contrast with these generally detrimental effects, data from several studies suggest that moderate alcohol consumption may exert beneficial effects on the adaptive immune system. For example, healthy volunteers who had a 30-day alcohol abstinence period before drinking moderately (i.e., 330 mL beer per day for women and 660 mL for men) for 30 days showed significant increases in a variety of variables associated with adaptive immune responses (e.g., CD3 subsets; secretion of IL-2, IL-4, IL-10, and IFN-γ by mitogen-stimulated PBMCs; and levels of IgG, IgM, and IgA in the blood) (Romeo et al. 2007).

Several studies also have reported improved responses to vaccination and infection in both humans and animal models of moderate alcohol consumption. A study exploring the impact of alcohol consumption on the incidence of colds among 391 subjects intentionally exposed to 5 different respiratory viruses showed that moderate alcohol consumption (i.e., 1 to 2 drinks/day) was associated with decreased incidence of colds in nonsmokers (Cohen et al. 1993). Similarly, people who consumed a moderate amount of wine (i.e., 3.5 glasses), and especially red wine, had a reduced incidence of the common cold compared with nondrinkers (Takkouche et al. 2002). In a rat model, low to moderate ethanol doses resulted in a greater delayed cutaneous hypersensitivity response and improved clearance of *Mycobacterium bovis,* whereas high ethanol doses were associated with a reduced response and decreased bacterial clearance (Mendenhall et al. 1997). Finally, in a rhesus macaque model, animals that voluntarily consumed moderate amounts of ethanol (1.3 to 2.3 g/kg/day) showed an improved response to a vaccine to which the animals had been exposed before (i.e., recall vaccine response) compared with controls (Messaoudi et al. 2013).

The mechanisms by which moderate alcohol consumption might exert these beneficial effects are only beginning to emerge. In a study examining the impact of moderate alcohol consumption on gene-expression patterns in blood cells (Joosten et al. 2012), young men consumed either 100 mL vodka with 200 mL orange juice or only orange juice daily during dinner for 4 weeks. After this period, the moderate-drinking participants exhib-

ited down-regulation of a transcription factor (i.e., NF-Kappa B), modulation of pathways of antigen presentation, altered B- and T-cell receptor signaling, and reduced IL-15. Furthermore, the plasma levels of various proinflammatory signaling molecules (e.g., positive acute phase protein ferritin, α1-antitrypsin, and cytokines such as an IL-1 receptor agonist and IL-18) were significantly reduced, whereas anti-inflammatory proteins such as adiponectin were increased after moderate alcohol consumption (Joosten et al. 2012).

Summary

Studies over the last 30 years have clearly demonstrated that chronic ethanol abuse impairs the functions of both T cells and B cells. Chronic alcohol consumption results in lymphopenia with a loss in circulating T cells and B cells. The decrease in T cells is accompanied by increased homeostatic proliferation, which in turn leads to increased T-cell differentiation, activation, and conversion to the memory phenotype. Impairment in T-cell recruitment also was observed in mouse models of chronic alcohol exposure. Despite reduced B-cell numbers, people with AUD exhibit increased serum concentration of IgA, IgG, and IgE. This increase in circulating Igs correlates with increased levels of antibodies directed against liver antigens and byproducts of oxidative damage. Finally, alcohol exposure in utero significantly interferes with the development of T cells and B cells, which ultimately might increase risk for infections during adulthood. In contrast to the devastating effects of chronic alcohol abuse, a few studies have shown that moderate alcohol consumption increases the number of T cells; improves T-cell cytokine production; and enhances immune response to vaccines in humans, nonhuman primates, and rodents.

The molecular mechanisms underlying the dose-dependent impact of ethanol on immunity remain poorly understood. Most studies have been carried out in vitro using primary cells or cell lines in the presence or absence of ethanol. The use of cultured cells presents several advantages, such as the ability to precisely control the amount and duration of ethanol exposure, the relatively low cost, the ease of culturing large quantities of cells, and the ease of manipulating nutrients in the media and regulating gene expression. However, in vitro studies alone cannot reveal the underlying mechanisms of immune modulation by ethanol, because immune cells carry out their functions in a multicellular environment. Therefore, in vivo studies also are necessary. These studies frequently use rodent models, which allow researchers to use an abundance of reagents to characterize the immune response and to access genetically modified animals (i.e., transgenic and knockout strains) that facilitate mechanistic studies. However, alcohol consumption is not voluntary in these models. Thus, there is a pressing need to conduct additional studies using clinical samples or animal models that more faithfully mirror the complexity of human alcohol consumption, metabolism, and immune responses. Macaques are genetically diverse and, like humans, consume alcohol voluntarily and exhibit a wide spectrum of drinking patterns. Additionally, their long lifespan facilitates the study of the effects of long-term alcohol consumption, and their large size allows simultaneous longitudinal sampling from various body tissues and organs that harbor immune cells (e.g., blood, lung, and gut). The tools available to study immunity in nonhuman primate models also are becoming more sophisticated. However, the high cost associated with nonhuman primate models remains an obstacle for large-scale studies.

Future studies should leverage the different models to uncover the molecular mechanisms underlying the dose-dependent impact of alcohol on immune function by investigating changes in gene expression patterns (Mayfield and Harris 2009). Such approaches should also investigate the contributions of noncoding RNAs, such as microRNAs (miRNAs), and epigenetic modifications, which are known to regulate gene expression patterns (Curtis et al. 2013; Sato et al. 2011). miRNAs are small, single-stranded, noncoding RNAs that bind within one end of the target genes and prevent the generation of functional proteins from these genes by either destabilizing the mRNAs generated from the genes, preventing the translation of the genetic information in the mRNA into a protein, or both (Ambros 2004; Bartel 2004; Filipowicz et al. 2008). A single miRNA can target hundreds of mRNA transcripts, and a single mRNA transcript simultaneously can be targeted by more than one miRNA, ensuring fine-tuned and/or redundant control over a large number of biological functions. Epigenetic modifications are chemical changes that occur within a genome without changing the DNA sequence. These changes include direct addition of a methyl group to DNA (i.e., DNA methylation) or chemical modifications of the proteins (i.e., histones) around which DNA is wrapped, such as acetylation, methylation, and phosphorylation (Holliday 2006; Hsieh and Gage 2005; Murrell et al. 2005). Both regulatory mechanisms related to miRNA and epigenetic mechanisms are interrelated (see figure 3). Thus, several miRNAs themselves are regulated epigenetically but also are capable of targeting genes that control epigenetic pathways (e.g., polycomb group-related genes and histone deacetylase). Studies have identified ethanol-mediated changes in both miRNA abundance (Miranda et al. 2010; Pietrzykowski 2010) and epigenetic modifications within PBMCs (Biermann et al. 2009; Bleich and Hillemacher 2009; Bonsch et al. 2006). Other investigators have described ethanol-induced epigenetic modifications (i.e., alterations in histone acetyltransferases and histone deacetylases)

in liver cells (i.e., hepatocytes) in rodent models of binge drinking and ALD (Bardag-Gorce et al. 2007; Choudhury et al. 2010; Park et al. 2005; You et al. 2008). However, very few studies have examined ethanol-induced changes in gene expression and regulation within specific immune-cell subsets. Moreover, none of the studies have conducted a comprehensive integrated analysis of mRNA, miRNA, and epigenetic expression patterns in the same cell(s) before and after alcohol consumption. Integrating gene expression patterns with gene regulation could reveal novel insight into specific pathways that are dysregulated with alcohol abuse and could explain the increased susceptibility to infection. These insights could lead to interventions to restore immunity, such as reversing changes in histone modifications and DNA methylation patterns or modulating expression levels of miRNAs. In addition, such studies could reveal the pathways that are modified by moderate alcohol consumption to enhance immune response to vaccination.

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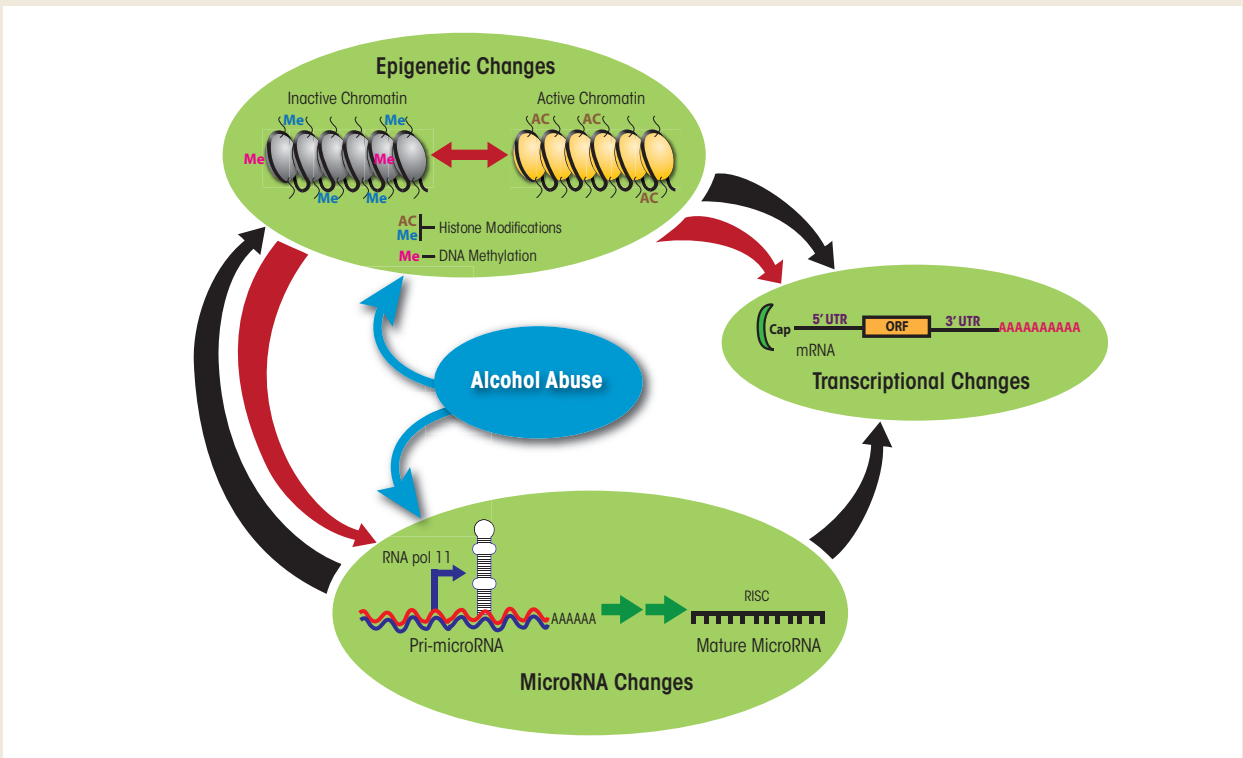

Figure 3 Alcohol modulates gene expression—that is, the generation of mRNAs and, ultimately, functional proteins from the DNA template—through changes in noncoding microRNA (miRNA) levels and epigenetic modifications. These epigenetic modifications, which include methylation of the DNA as well as modifications (e.g., acetylation and methylation) of the histone proteins around which the DNA is wound, determine whether the complex of DNA and histones (i.e., the chromatin) is in an active or inactive conformation. Such epigenetic changes can promote (red arrow) or inhibit (black arrow) the expression of mRNAs as well as promote the expression of certain miRNAs (including the processing of precursor molecules called pri-micro RNA into mature miRNA). Conversely, miRNAs can inhibit the actions of the methylation machinery and expression of proteins involved in histone modifications as well as can interfere with the transcription of mRNAs.

NOTE: 3′ UTR = 3′ untranslated region; 5′ UTR = 5′ untranslated region; ORF = open reading frame; RISC = RNA-induced silencing complex, a complex of miRNA and proteins.

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Alcohol Use As a Risk Factor in Infections and Healing

A Clinician's Perspective

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Physicians have recognized for more than a century that alcohol use is associated with infections and that alcoholics are especially at risk for pneumonia. Clear evidence now indicates that alcohol has a systemic effect on every organ. This review first presents a clinical case to describe a patient with immunity issues complicated by alcohol use—a setting familiar to many clinicians. This is followed by a description of the molecular mechanisms that explain the secondary immune deficiency produced by alcohol in the host, focusing mostly on the gut and lower respiratory mucosal immunity. The goal of this review is to increase awareness of the new mechanisms being investigated to understand how alcohol affects the human immune system and the development of new strategies to attenuate adverse outcomes in the affected population.

Key words: Alcohol use; alcohol use and misuse; alcoholism; alcoholics; infection; immunity; immune system; pneumonia; molecular mechanisms; secondary immune deficiency; gut; lower respiratory mucosal immunity

Alcohol use and misuse have been part of human society for centuries. Early physicians recognized since the 1800s that alcohol produced not only impairment of the senses but also higher predisposition for tuberculosis. William Osler, the father of scientific medicine, reported in 1905 that patients who misused alcohol had higher predisposition to pneumonia (Osler 2001).

Between 2006 and 2010 in the United States, excessive alcohol consumption resulted in approximately 88,000 deaths (data based on 11 U.S. States), and the median alcohol-attributable death rate was 28.5 per 100,000 population (Gonzales et al. 2014). Furthermore, the potential years of life lost attributed to alcohol (estimate of the average years people would have lived if they had not died prematurely) averaged 2.5 million years annually from 2006 through 2010 (Gonzales et al. 2014). Importantly, the majority of alcohol-related deaths and potential life lost were among working-age adults (20- to 64-year-olds). In addition, the estimated cost of excessive drinking was $223.5 billion in 2006, from which the majority represented loss of productivity, valued at $161.3 billion, followed by increased health care costs and criminal justice costs of $21 billion each (Bouchery et al. 2011).

Patients with a history of heavy acute or chronic alcohol use have higher rates of hospitalizations (Smothers and Yahr 2005), longer hospital stays (de Wit et al. 2011), risk for major complications when they also suffer polytrauma (like pneumonia, bleeding disorders, and withdrawal syndrome) (Spies et al. 1996), increased mortality (de Wit et al. 2011), higher intensive care unit admissions, and greater postoperative complications when they are admitted to hospitals and when they need surgery (Delgado-Rodriguez et al. 2003), compared with patients with no history of alcohol use. The

following section illustrates the scope of these problems with a clinical case.

Clinical Case

A 42-year-old white male presented to the emergency department with a 3-day history of fevers, shortness of breath, and a mucus-producing cough. He reported a history of hypertension, smoking half a pack to one pack of cigarettes per day, and drinking four to six beers per day. (Although not reported, the specific size of each beer is 22 ounces. Thus, the patient consumed 88 to 132 ounces of beer per day, an equivalent of approximately 7 to 11 standard drinks per day). He reported no history of lung disease, such as chronic obstructive pulmonary disease or asthma.

The patient received immediate attention as a result of having reduced oxygen supply. Oxyhemoglobin saturation, a measure of oxygen in the blood, was low (89 percent). His blood pressure was 102/72 mmHg and heart rate 118 beats per minute. This blood pressure was well below the patient's baseline pressure. The patient appeared ill and had shortened speech pattern. No jugular venous distention was present. His cardiac examination was normal and the lung examination revealed dullness to percussion at the right base, along with bronchial breath sounds and increased resonance of vocal sounds (i.e., egophony) over the right lung base. The patient's abdomen was non-distended and non-tender, he had normal bowel sounds, and his liver edge was 5 centimeters below the right costal margin. His extremities revealed no clubbing, skin discoloration as a result of low oxygen (i.e., cyanosis), or edema, and his neurological exam was remarkable for a patient who was alert and oriented to time, place, and location. The patient moved all his extremities spontaneously.

Laboratory data revealed elevated white blood cell count (i.e., leukocytosis) to 15,600 cells per microliter with 13 percent bands, toxic granulations, and mild low blood platelet count (i.e., thrombocytopenia) to 123,000 cells per microliter. Electrolytes were remarkable for low potassium levels (i.e., hypokalemia) to 3.2 millimoles per liter, which were repleted. Arterial blood gas was consistent with the buildup of carbon dioxide in the lungs (i.e., acute respiratory acidosis) and a low level of oxygen in the blood (i.e., hypoxemia). A chest X-ray revealed dense alveolar consolidation of the right lung base. The patient was found to have severely low blood pressure because of his ongoing infection and inflammatory response (i.e., septic shock) and overly active proteins that control blood clotting (i.e., disseminated intravascular coagulation) attributed to pneumonia.

The patient received supplemental oxygen and oxyhemoglobin saturation increased from 89 percent to 95 percent. Broad-spectrum antibiotics were initiated within 2 hours after the patient arrived at the emergency department. Despite administration of 2.5 liters of normal saline, the patient's blood pressure further decreased to 92/54 mmHg. As a result, a central venous catheter was placed, and norepinephrine was given to raise the patient's blood pressure. The patient was transferred from the emergency department to the medical intensive care unit. However, shortly after arrival to the medical intensive care unit, oxyhemoglobin saturation decreased to 86 percent, and the patient continued to have a difficult time breathing. He was emergently intubated with an endotracheal tube because of acute respiratory failure, and mechanical ventilation was initiated. A repeated chest X-ray revealed progression of air-space disease: It involved both lungs and all four quadrants. The patient was diagnosed with acute respiratory distress syndrome, and ventilator settings were adjusted to decrease tidal volume to 6 milliliters per kilogram ideal body weight. The patient also required increasing amounts of positive end expiratory pressure to 14 centimeters of water and fraction of inspired oxygen 70 percent. Over the ensuing 2 to 3 days, the patient's blood flow improved slowly, his norepinephrine dose was decreased, and leukocytosis resolved. Blood cultures grew cephalosporine-sensitive *Klebsiella pneumoniae*.

Although the patient's clinical condition improved, his neurocognitive function did not. He was given sedatives to facilitate mechanical ventilation. However, he required high doses of the depressant midazolam, and his mental status fluctuated from being unresponsive to sitting upright in bed attempting to remove the endotracheal tube. On hospital day 2, the patient developed delirium. The presence of the endotracheal tube prevented the patient from expressing himself verbally, and the medical team had very limited ability to communicate with him. The team was unable to determine whether the patient had a headache, nausea, or experienced hallucinations or the feeling of insects crawling on one's skin (i.e., formication), signs of alcohol withdrawal. Rather, the medical team was limited to evaluating gross motor movements, behavior, and facial expressions. The patient's fevers and rapid heart rate (i.e., tachycardia) persisted for days, and the physicians were unable to determine whether these were attributed to ongoing infection and inflammatory response (i.e., sepsis) or if the patient was developing superimposed alcohol withdrawal. Administration of midazolam was based on motor behaviors and a modified Clinical Institute Withdrawal Assessment for Alcohol (CIWA-Ar). Realizing its limitations in the nonverbal critically ill patient, physicians were left attempting different strategies for management of sedation. They increased the dose of midazolam, only to find that the patient became overly sedated and unresponsive. Empiric trials of opioids were attempted in order to determine if the patient was experiencing pain, but again this strategy did not seem to improve the patient's neurocognitive function. Next, haloperidol, an antipsychotic, was administered to control agitation

and perhaps aided in management of delirium. The team continued to attempt different strategies to manage his fluctuating agitation and sedation, including attempts to limit midazolam administration in favor of administration of haloperidol. However, after approximately 7 days, the patient was more alert and cooperative, but it was not clear what led to the improvement. He remained delirious but was able to follow commands. His septic shock resolved, and mechanical ventilation was removed.

Discussion

This case illustrates the complexities of managing critically ill patients with alcohol use disorder (AUD), which is common in such patients and may occur in 40 percent of hospitalized patients. Critically ill patients with AUD have unique problems, including needs for higher doses of sedatives and increased risk of requiring mechanical ventilation (de Wit et al. 2007*a*). Patients with AUD are at increased risk of developing pneumonia and sepsis. In addition, pneumonia encompasses a larger geographic area of the lung in patients with AUD compared with patients without AUD (de Roux et al. 2006; Fernandez-Sola et al. 1995; Ruiz et al. 1999; Saitz et al. 1997; Torres et al. 1991). This patient population also has prolonged fevers and slower radiographic resolution of airspace disease. Furthermore, acute respiratory distress syndrome, which carries a mortality greater than 30 percent, is more common among patients with AUD who experience sepsis (Moss et al. 1996). Sepsis also is more severe and has a higher mortality in this patient population (O'Brien et al. 2007). Patients with AUD may not only experience higher severity of illness but also are at increased risk of developing neurocognitive complications, such as delirium. The patient in the case scenario developed agitation, which could have been a result of a variety of etiologies, including dyspnea, anxiety, pain (from the endotracheal tube, lying in bed, or from procedures), delirium induced by critical illness, alcohol withdrawal, or delirium tremens (de Wit et al. 2007*b*). Despite the high prevalence of these disorders among critically ill patients, only limited data are available to aid physicians in managing this patient cohort so as to optimize patient outcome (Awissi et al. 2013; Sarff and Gold 2010). For instance, the management of alcohol withdrawal has not changed much over the last two decades and is centered in supportive care (Sarff and Gold 2010), electrolyte replenishment, vitamin supplementation (especially thiamine), benzodiazepine administration, and consideration of alternative agents such as propofol and beta blockers. Benzodiazepines (usually midazolam and lorazepam) are routinely administered during the course of mechanical ventilation to treat agitation. However, benzodiazepines also are linked to the development of delirium, a condition associated with increased mortality (Ely et al. 2004).

Effects of Alcohol on the Mucosal Immune System

For more than a century, physicians have noticed that alcohol produces abnormalities in the host defenses. As early as the late 1800s, scientists performed studies to determine how ethanol produced detrimental effects in humans. Human studies have provided important epidemiological data demonstrating an association between alcohol consumption and risk of infection. This increased risk of infection has been attributed to alcohol's effect on the immune system. The following sections focus mainly on the effects of alcohol on gut, lung, and skin mucosal immunity.

Effects on Mucosal Gut (Intestinal) Immunity

The intestinal mucosa plays an important role in alcohol metabolism, as the epithelial surface incorporates alcohol into the blood system by passive diffusion, which accounts for approximately 80 percent of alcohol absorption (Norberg et al. 2003). The other 20 percent is absorbed through the gastric mucosa.

A recent study described the effects of acute alcohol binge drinking on gut homeostasis in healthy human study participants (Bala et al. 2014). Higher levels of alcohol (about 90 mg/dl), which occurred 1 hour after oral ingestion of vodka (40 percent ethanol), correlated with higher expression of serum bacterial DNA as measured by the abundance of 16S rDNA (a conserved genetic component of several bacteria that should not be present in the blood in healthy people). Furthermore, endotoxin, which is produced by Gram-negative organisms, was increased in serum as early as 30 minutes after alcohol ingestion and persisted at high levels for 3 hours after ingestion. These data suggested that even acute episodes of alcohol intoxication provoked abnormalities in the gut epithelium. These findings are supported by studies performed in rats, which showed evidence of a mild increase of endotoxemia after one dose of acute alcohol ingestion (Rivera et al. 1998). Thus, it is critical to understand not only how chronic alcohol consumption increases endotoxin levels in the host but also how acute alcohol intoxication affects translocation of bacteria or microbial products. One known mechanism involves alcohol's role in increasing permeability of the intestine, leading to bacterial translocation and higher levels of the molecules found in the outer membrane of Gram-negative bacteria (i.e, lipopolysaccharide) in the blood (Bode and Bode 2005; Schaffert et al. 2009; Szabo and Bala 2010).

The exact mechanisms underlying alcohol's role in increasing gut permeability and/or transient endotoxemia are not clearly elucidated, but recent studies have suggested some possible causes (see figure). Cytochrome P4502E1 is an enzyme present in the liver and is involved in the metabolism

and oxidation of alcohol, fatty acids, and foreign compounds (Cederbaum 2010). CYP2E1 is the most highly expressed isoform of the CYP450 cytochrome enzymes and is highly expressed not only in the liver but also in the small intestine and colon. In addition, an acute model of alcohol binge intoxication in mice shows that alcohol can induce the expression of intestinal CYP2E1 and that this induction is correlated with higher endotoxemia and translocation of liver bacteria. These outcome measures were substantially reduced, however, in Cyp2e1^{-} null mice (genetically modified mouse in which Cyp2e1 gene is inactive) compared with wild-type control mice, suggesting that CYP2E1 is essential for development of gut leakiness. The deleterious effects of alcohol-induced CYP2E1 were ameliorated with treatment with the antioxidant N-acetylcysteine (Abdelmegeed et al. 2013).

Activated CYP2E1 can produce oxidative stress and tissue damage as a result of reactive oxygen species, and this oxidative stress can damage intestinal barrier function. In vitro studies have shown that acute exposure for 2 to 4 hours of Caco-2 cells (human colon adenocarcinoma cell line) to 43 mM of alcohol increased CYP2E1, which correlated with higher intestinal permeability (measured by trans-electrical epithelial resistance). Reducing the expression of CYP2E1 (i.e., knock-down) by small-interfering RNA reversed alcohol-induced alteration in cell permeability (Forsyth et al. 2013). Furthermore, CYP2E1 metabolism of alcohol and its oxidative stress products induced reduction and oxidation-sensitive circadian CLOCK (clock circadian regulator) and PER2 (period circadian clock 2) protein expression in intestinal epithelial cells (Caco-2 cells). The induction of CLOCK and PER2 promoted intestinal hyperpermeability. Thus, alcohol can disrupt circadian rhythms at the level of gene transcription in the intestine. Circadian clock genes are those that depend on day and light and feeding patterns. Growing evidence suggests that alcohol disrupts circadian rhythmicity, probably by intestinal-derived lipopolysaccharide (Voigt et al. 2013). Inhibition of CYP2E1 protein expression stopped intestinal hyperpermeability as well as induction of CLOCK and PER2 (Forsyth et al. 2013) and knocking out Clock or Per2 in intestinal epithelial cells also ameliorated alcohol-induced intestinal hyperpermeability (Swanson et al. 2011).

In the case of chronic ethanol models, the disruption of intestinal permeability is highly correlated with the development of alcoholic liver disease (Mathurin et al. 2000; Szabo 2015; Wang et al. 2014). That is, bacterial translocation causes high endotoxin levels in the circulation, which induces the production

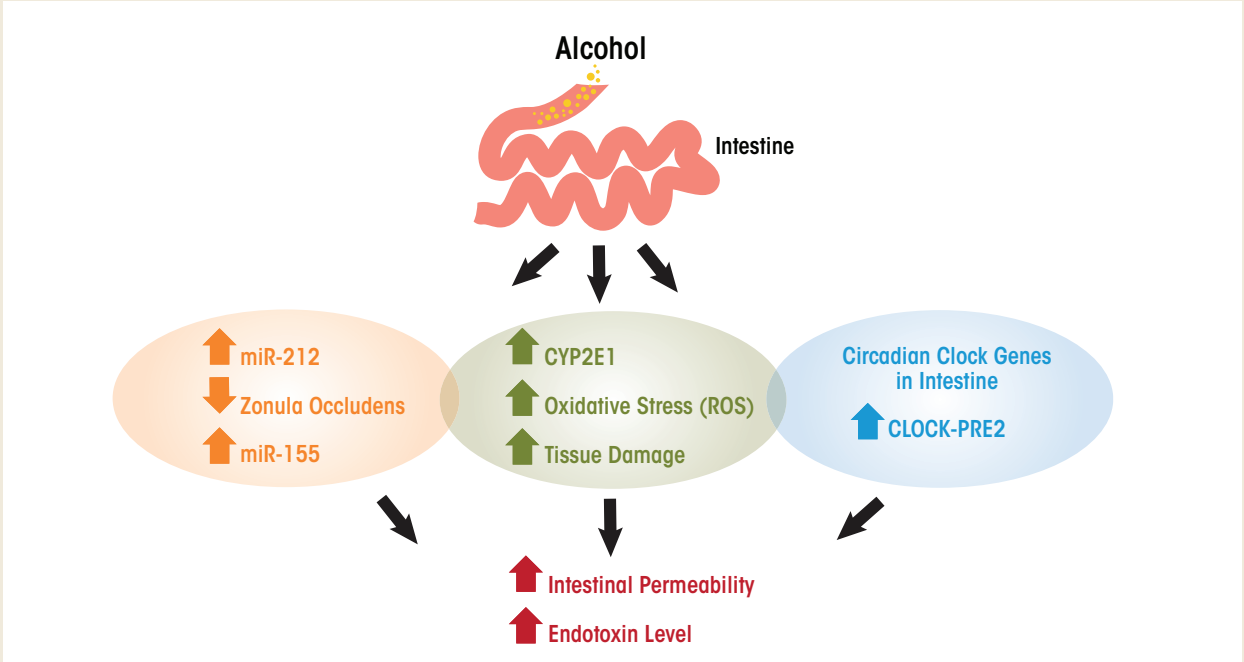

Figure Alcohol use increases intestinal permeability and endotoxin levels. The mechanisms include increases in microRNA miR-212 levels, which decrease gene expression within the zonula occludens, resulting in increases to miR-155 that produce intestinal inflammation. Alcohol induces expression of the enzyme CYP2E1, increasing reactive oxygen species, which damage tissue through increases in oxidative stress. Alcohol also increases the expression of circadian clock genes that alter intestinal permeability.

of cells that regulate inflammation (i.e., tumor necrosis factor-alpha [TNF-α]) by Kupffer cells in the liver. This chain of events can then lead to alcoholic liver disease (Bode and Bode 2005). Furthermore, bacterial overgrowth in the small intestine probably contributes to higher endotoxin blood levels (Fouts et al. 2012). One other possible mechanism of increased endotoxin levels upon alcohol intake is through the upregulation of microRNAs, which are small noncoding RNAs involved in modulating protein synthesis, likely by inhibiting translation of mRNA (the coding RNA). Most tissues produce specific microRNAs, and an increasing amount of research over the last 10 years supports their role in protein expression and regulation. That said, research also shows that microRNAs are affected by alcohol intake. Thus, high levels of specific microRNAs are associated with gut leakiness. In particular, the microRNA miR-212 inhibits gene expression of zonula occludens gene (ZO-1), an important gene that regulates the formation of tight junctions in the gut lumen, especially in the colon (Tang et al. 2008). Furthermore, chronic alcohol intake increases tissue-specific microRNA miR-155, which contributes to alcohol-induced small bowel inflammation and alteration of gut barrier integrity in mouse small intestine. This has been shown by research in which miR-155 knockout mice, despite chronic alcohol administration, were protected from higher serum endotoxin levels and preserved their regenerating islet-derived protein III beta (Reg3b) protein expression, an antimicrobial peptide that plays a role in intestinal barrier integrity, unlike wild-type mice (Lippai et al. 2014). In addition, research using a non-human primate model of chronic alcohol consumption (Asquith et al. 2014) found that expression of miR-155 was correlated with alcohol administration. The increase in miR-155 among alcohol-exposed primates was inversely correlated with the production of immune cell-signaling proteins (i.e., cytokines) by colonic T cells.

Research with a chronic alcohol model in rhesus macaques has found a detrimental effect on immune cells in the gut. The alcohol-exposed group had a smaller percentage of immunity-boosting TNF-α^+ cluster of differentiation 8 ($CD8^+$) cells in the layer of intestinal tissue beneath the epithelium (i.e., lamina propria) compared with non–alcohol-exposed animals (Asquith et al. 2014). The study also found that alcohol-exposed animals had a decreased percentage of intraepithelial immune cells known as $IL17^+$ $INF\gamma^+$ $CD4^+$ T cells from the jejunum and a decreased percentage of $IL17^+$ $IFN\gamma^+$ $CD8^+$ T cells from the lamina propria in the ileum. Alcohol also has been associated with the profound loss of gut-associated lymphoid tissues in a mouse model of Salmonella typhimurium infection (Sibley and Jerrells 2000). Both mesenteric lymph nodes and Peyer's patches lymphocytes, important immune system components, were decreased from day 3 to day 7 in the alcohol-fed mice group, compared with the pair-fed and chow controls. The alcohol-fed mice exhibited higher bacteria levels in the liver and intestinal tract compared with pair-fed control animals (Sibley and Jerrells 2000). Thus, this body of evidence shows the deleterious effects and mechanism of action by which alcohol perturbs mucosal gut immunity.

Effects on Mucosal Lung Immunity

The effects of alcohol intake undermine immune defenses in both the upper and lower airways. This review will focus mainly on mucosal lung immunity of the lower airways. The alveolar macrophage is an important immune cell affected by alcohol consumption. Alveolar macrophages have the receptor for the granulocyte-macrophage colony-stimulating factor (GM-CSF), which is important for terminal differentiation of fetal monocytes in the lung into mature alveolar macrophages (Guilliams et al. 2013; Schneider et al. 2014). Interaction of GM-CSF with its receptor leads to the nuclear binding of the transcription factor PU.1, which is important for alveolar macrophage gene regulation and development (Bonfield et al. 2003). In chronic alcohol-fed rats, the alveolar macrophage displayed decreased membrane expression of the GM-CSF receptor as well as impaired bacterial ingestion (i.e., phagocytic activity) (Joshi et al. 2005). These effects were reversed with recombinant GM-CSF, which restores GM-CSF signal responsiveness and innate function in alveolar macrophages of alcohol-fed rats.

Moreover, alcohol activates nicotinamide adenine dinucleotide phosphate (NADPH) oxidases upon oxidative stress in the alveolar macrophages. When activated, these enzymes can produce reactive oxygen species (i.e, superoxide, hydrogen peroxide). Specifically, chronic alcohol intake increases the level of NADPH oxidase 1 (Nox1), NADPH oxidase 2 (Nox2), and NADPH oxidase 4 (Nox4) at the transcriptional and protein levels in alveolar macrophages of animal models and human alveolar macrophages from alcoholic study participants (Yeligar et al. 2012). In addition to increased oxidative stress, chronic alcohol use results in the depletion of important alveolar antioxidants like glutathione or its precursors. In one study, restoration of glutathione through its precursor N-acetylcysteine into the alveolar environment improved alveolar phagocytic function and decreased alveolar damage in animal models (Yeligar et al. 2014).

Zinc is another important element impaired by alcohol intake. Joshi and colleagues (2009) found that alcohol-fed animals had lower zinc levels in the alveolar compartment compared with control animals and that this level did not correlate with zinc blood levels, which were within normal range. A study performed in human study participants corroborated the

finding that alveolar macrophages from alcoholics had lower intracellular zinc levels than nonalcoholic study participants (Mehta et al. 2013). Furthermore, these human macrophages had decreased phagocytic activity when exposed to *Staphylococcus aureus* in vitro. Intracellular zinc levels and phagocytic activity were improved when alveolar macrophages were treated with Zinc and procysteine, a glutathione precursor (Mehta et al. 2013).

Alcohol also has been found to affect lung immunity through other mechanisms. These include alterations in the recruitment of white blood cells (i.e., neutrophils) into the alveolar space, impairment of neutrophil movement in response to infection, and decreased activation of proteins that induce an immune response (Boe et al. 2001, 2003). These findings have been observed in rodent models of acute alcohol intoxication with *S. pneumoniae* and *K. pneumoniae* lung infection (Boe et al. 2001; Quinton et al. 2005). Further investigation into the impaired recruitment of neutrophils to the alcoholic lung upon infection has revealed that alcohol enhances the phosphorylation of the transcription factor signal transducer and activator of transcription 3 (STAT3) in nucleated bone marrow cells, blunting hematopoietic precursor cell response (i.e., formation of immune cells) (Siggins et al. 2011) against pneumococcal infection in a mouse model of acute chronic alcohol intake.

Furthermore, alcohol seems to produce abnormalities and decreased numbers in natural killer (NK) cells, which are decreased in mouse models of alcohol consumption (Blank et al. 1993). In research with a mouse model, Zhang and Meadows (2008) reported that chronic alcohol impaired the release of NK cells from the bone marrow, which translates into decreased bone marrow–derived NK cells in the spleen and higher percentages of thymus-derived NK cells (Zhang and Meadows 2008). The alcohol-induced imbalance of NK cells may be disadvantageous for the host because thymus-derived NK cells have less cytolytic capacity and more cytokine production properties. The observation that alcoholic patients have predisposition to viral infections like cytomegalovirus (Arase et al. 2002; Bekiaris et al. 2008) and influenza as well as certain tumors may be related to NK-cell dysfunction. In a mouse model of chronic alcohol intake, the populations of NK cells in the spleen were decreased at 1 week compared with controls, which accounted for decreased cytotoxic activity. This difference was attributed to decreased percentage and decreased absolute number of the NK T cells NK1.1^{+} and CD3^{-} negative cells (marker of NK T cells). However, the groups did not differ in number or percentages at 8 weeks post–alcohol intake. A decrease in the NK subtype Ly49H^{+}, CD11b^{+},CD27^{-} was observed 10 weeks after alcohol consumption. This subtype has been involved with predisposition to cytomegalovirus infections in a mouse model. Thus, it seems that alcohol may affect selective populations of NK cells in a time-dependent manner (Ballas et al. 2012).

Effects on Mucosal Skin Immunity

Like any other organ in the human body, the skin is also affected by alcohol intake. Alcoholism is associated with higher rates of wound infection and delay in wound closure. It is associated with increased risk for *Staphylococcus aureus* infection, including methicillin-resistant *Staphylococcus aureus*, *Streptococcus pyogenes,* and *Vibrium vulnificus*.

Ethanol seems to impair dermal fibroblast function, which plays a role in wound healing. Dermal fibroblasts display proliferative responses along with secretion of growth factors. In vitro studies of human fibroblasts exposed to alcohol demonstrated a reduction in dermal wound breaking strength (immature wound) (Ranzer et al. 2011). Although human skin differs in cellular components compared with other mammalian species, mouse models of skin infection and alcohol consumption have helped researchers understand alcohol's damaging effects on the skin. One study found that mice had 30 to 50 percent fewer epidermal immune cells (i.e., Langerhans cells) after 4 weeks of chronic alcohol consumption (Ness et al. 2008). This effect is likely to account for decreased immune surveillance once the host encounters a pathogenic organism in the skin.

In the mouse epidermis, a type of resident skin T cell known as dendritic epidermal T cells (DETCs) are prompt to respond to skin injury, participate in wound healing (Jameson et al. 2002), and fight against tumor formation. These resident T cells have a gamma delta T-cell receptor (γδ TCR) and do not need antigen presentation or major histocompatibility complex (MHC) class molecules to mature to have an effector function. In the mouse, DETCs are exclusively restricted to the epidermis and are absent in other tissues, peripheral circulation, or lymph nodes. DETCs also display receptors and molecules (e.g., junctional adhesion molecule-like [JAML] protein, NK group 2, member D [NKG2D], cluster of differentiation 69 [CD69]) to facilitate their crosstalk with other cells in the network upon skin stress or damage. Inhibition of JAML leads to decreased γδ T-cell induction and delayed wound healing (Witherden et al. 2010).

Chronic ethanol intake can also affect skin T cells in mouse models. DETCs are significantly decreased in ethanol-fed mice compared with water-fed controls and ethanol-fed mice show significant depletion of dermal γδ T cells compared with controls. Furthermore, dermal γδ T17 cells have decreased interleukin-17 production following administration of the immunosuppressive drug anti-CD3 monoclonal antibody (Parlet and Schlueter 2013). So it seems that skin T-cell populations are affected by ethanol and that T cells

that express the γδTCR are more affected, whereas those that express the αβTCR seem to be unaffected.

Some differences between human and mouse skin need to be considered, however. For instance, DETCs are only found in the mouse epidermis, in which they represent 98 percent of $CD3^+$ T cells. In the human epidermis, by contrast, $\alpha\beta^+$ and $\gamma\delta^+$ T cells (mostly Vδ1, a subset of gamma delta cells) are represented equally. Nevertheless, the mechanisms studied in the mouse models bring up questions that can be studied in human cells and these studies may help to reveal novel pathways by which ethanol impairs human skin immunity.

As illustrated above, patient care is clearly complicated by alcohol-induced immunity issues. The mechanisms described explain alcohol's role in causing immune deficiency in the gut and respiratory mucosa. With greater awareness of these mechanisms, researchers and clinicians will have a better understanding of how alcohol affects the human immune system, leading to the eventual development of new strategies to reduce adverse outcomes in the affected population.

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Primer on the Immune System

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The human body regularly encounters and combats many pathogenic organisms and toxic molecules. Its ensuing responses to these disease-causing agents involve two interrelated systems: innate immunity and adaptive (or acquired) immunity. Innate immunity is active at several levels, both at potential points of entry and inside the body (see figure). For example, the skin represents a physical barrier preventing pathogens from invading internal tissues. Digestive enzymes destroy microbes that enter the stomach with food. Macrophages and lymphocytes, equipped with molecular detectors, such as Toll-like receptors (TLRs), which latch onto foreign structures and activate cellular defenses, patrol the inside of the body. These immune cells sense and devour microbes, damaged cells, and other foreign materials in the body. Certain proteins in the blood (such as proteins of the complement system and those released by natural killer cells, along with antimicrobial host-defense peptides) attach to foreign organisms and toxins to initiate their destruction.

When a pathogenic organism or toxin does gain a foothold in the

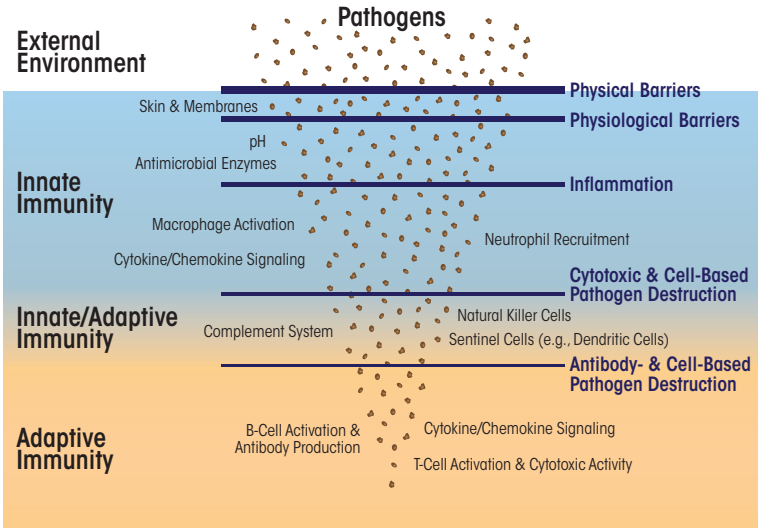

Figure Overview of the immune system. Innate immunity encompasses several non-specific protective mechanisms against infection, including physical and physiological barriers, cells (e.g., macrophages and neutrophils) that detect and attack other cells carrying pathogen-associated molecular patterns, and small proteins that signal pathogen invasion (i.e., cytokines and chemokines) or short peptides that directly attach to and restrict microbial pathogens. The adaptive immune system comprises specialized cells (e.g., B and T cells) and proteins (i.e., antibodies) that detect and eliminate specific pathogens and also uses cytokine/chemokine signaling to recruit additional immune cells. Several cells in adaptive immunity (i.e., memory B and T cells) can store immune memory of a pathogenic invasion. The complement system, along with natural killer cells and dendritic cells, straddles both innate and adaptive immunity.

body, the defenses furnished by the innate immune system are reinforced by those of the adaptive immune system. Compared with innate immunity, adaptive immunity is a more evolved and complex system consisting of both cells and proteins. These adaptive immunity agents specifically target and destroy the invading pathogen. Within days or weeks, the adaptive immune system manufactures antibodies tailored to the pathogenic invader to halt its spread. This process, known as the humoral response or antibody-mediated immune response, relies on specific cell types, called B cells, which produce antibodies. In parallel, this response activates lymphocytes, including T cells, programmed with information to detect surface molecules specific to the invader—a second type of adaptive immunity called cellular immunity. A hallmark of adaptive immunity is that it can store—via production of specialized T and B cells—a memory of the pathogen's unique molecular structures allowing for a more rapid response to future invasions by the same pathogen.

The expanded glossary below presents the main features of and mechanisms and players in the innate and adaptive immune systems that are relevant to this special issue of *Alcohol Research: Current Reviews*.

The Innate Immune System

Responses of the innate immune system to acute or persistent infection or injury typically manifest as inflammation. The primary purpose of the inflammation is to contain the infection, enable rapid access of immune cells and proteins to the infection site, and promote healing once the pathogen(s) has been cleared. This process involves multiple cytokines and types of immune cells. Many of the cells of the innate immune system are phagocytes: cells that ingest other cells or cellular debris through a process called phagocytosis, which neutralizes harmful agents. In phagocytosis, the immune cells engulf microorganisms or foreign particles and inactivate them in an intense chemical shower of reactive oxygen species called the respiratory burst.

Innate immune cells have various functions, including the following.

Granulocytes are white blood cells (i.e., leukocytes) characterized by the presence of granules in their cytoplasm. Granulocytes include the following cell types:

Neutrophils are the most abundant granulocytes and also the most abundant type of white blood cell, reaching concentrations of up to 5 million cells per milliliter in the blood. Neutrophils normally circulate in the blood and, upon injury or infection, quickly move to the affected site. They thereby follow chemical signals consisting of *cytokines* and *chemokines* to the site where they are among the first immune cells to arrive. Neutrophils detect pathogens via TLRs and directly attack them, for example, through phagocytosis. Neutrophils also release extracellular traps composed of DNA and antimicrobial peptides that ensnare and kill microbes. Thus, neutrophils represent an important first-line defense against invading microbes.

Basophils originate from bone marrow and circulate in the blood; they are the least abundant white blood cells. Upon activation by proteins, they move to an injured or infected site. Similar to *mast cells*, basophils also sometimes cause inflammatory responses such as allergic reactions. Basophils release the anticoagulant heparin and the vasoactive compounds histamine and serotonin, which reduce blood clotting and contribute to wound swelling typical of inflammations, respectively.

Eosinophils develop and mature in bone marrow and then also circulate in the blood. They are activated, for example, by lymphocytes of the adaptive immune systems, and they are crucial for combating larger parasites that cannot be phagocytosed, such as protozoans. Eosinophils also help fight other types of infections.

Mast cells reside in connective tissues and mucous membranes and aid in wound healing and also in defending against pathogens. When activated by pathogens or allergens such as pollen, mast cells rapidly release protein-carrying granules rich in both histamine and heparin, molecules involved in inflammation. Mast cell activation often underlies adverse immune responses such as allergies, arthritis, and anaphylactic shock.

Monocytes are the largest cells of the innate immune system. They mature in bone marrow and then circulate through the blood. Half of them are stored in the spleen and the other half in other locations throughout the body. Monocytes are precursors for two other innate immune system cells: *macrophages* and *dendritic cells*.

Macrophages are cells that search for and phagocytose pathogens. Upon exiting blood and entering tissues, *monocytes* develop into macrophages. They help remove excess, damaged, or dead cells marked by surface proteins for elimination. "Resident" macrophages inhabit specific locations or organs that are prone to infections, such as the lungs and liver, or serve in hubs, such as the spleen, for rapid deployment to injured or infected sites. Examples

include Kupffer cells, macrophages residing in the liver, and microglia, residing in the central nervous system. Macrophages carry on their surface several TLRs that are activated by pathogen- or damage-associated molecular patterns—this activation stimulates the macrophages to phagocytose pathogens or damaged cells or to secrete *cytokines* to activate and recruit additional immune cells. Macrophages contribute to wound healing, help control immune responses and other cells of the innate immune system, and also stimulate adaptive immunity (see below).

Dendritic cells act as messengers between the innate and adaptive immune systems. They reside in tissues exposed to the external environment, including the skin and the linings of the nose, lungs, stomach, and colon. Like *neutrophils* and *macrophages*, they detect foreign invaders via TLRs. Upon encountering a pathogen, dendritic cells ingest (i.e., endocytose) it or its products and attach pieces of the pathogen (i.e., antigens) to their cell surface on a protein assembly called the major histocompatibility complex II (MHC II). The dendritic cells then migrate to the lymph nodes where they activate *T cells* and *B cells* by presenting the pathogen's antigens to them. Dendritic cells are the most potent of several types of antigen-presenting cells, which effectively jumpstart the adaptive immune response.

Natural killer cells (NK cells) rapidly respond to the presence of virus-infected and tumor cells and destroy them with proteolytic enzymes and cytotoxic proteins that destabilize the cells' membranes and induce apoptosis. NK cells recognize stressed cells in the absence of the chemical triggers other immune cells need to mount an immune response. Although traditionally classified along with innate immune cells, some evidence of immunological memory in NK cells (see table) suggests that these cells are also affiliated with adaptive immunity.

The complement system consists of more than 30 blood-borne proteins produced in the liver. These proteins help or "complement" the killing of pathogens by antibodies. The complement system triggers a biochemical cascade in which foreign cells are first opsonized (i.e., coated) with complement proteins, weakening or rupturing (i.e., lysing) their cell walls. The action of complement also attracts other immune cells such as *macrophages* and *neutrophils*, along with antibodies, to the site of infection.

Table Components of the Immune System

Innate Immunity	Adaptive Immunity
Immune responses are largely non-specific, e.g., via Toll-like receptors (TLRs)	Immune responses specifically target pathogens via its antigens detected by specific immune cell receptors
Comprises a variety of defense mechanisms, i.e., physical/physiological barriers, lytic enzymes, reactive oxygen species, isolation of diseased tissues, and cell- and protein-mediated immunity	Involves mainly cell- and protein-mediated immunity
Immediate response to pathogenic challenge	Lag time between pathogen detection and response
No immunological memory (with some evidence for immune memory in NK cells)	Activation leads to immunological memory
Often underlying chronic inflammation in allergies and degenerative diseases (e.g., Alzheimer's disease, rheumatoid arthritis)	Often underlying autoimmune diseases in which self/nonself recognition is impaired, causing adaptive immune cells to attack the body's own cells (e.g., in type I diabetes, autoimmune hepatitis)
Present in all eukaryotes (including plants, which, however, use different mechanisms and molecules in innate immunity)	Present in jawed vertebrates with emerging evidence of related immune mechanisms in jawless vertebrates and some invertebrates

The Adaptive Immune System

The cells and structures of all organisms display unique antigens, which are molecules characteristic only to them. During the development of the immune system, adaptive immune cells originating from lymphocytes differentiate to recognize specific antigens, and the entire complement of this antigen specificity enables recognition of all possible antigens. As rearrangements within the genes in the immune cells occur during this developmental process, antigens present in the host (self-antigens) interact with the emerging cell population to eliminate those adaptive immune cells that would attack the host, while retaining only those cells that will target any non–self-antigens.

The functions of cells of the adaptive immune system are as follows.

B lymphocyte cells display an enormous variation in the cells they target—the blood and lymphatic systems contain millions of B cells, produced early in the body's development, which differ in the type of antibody they produce in response to the antigens they recognize. Each B cell carries a cell-surface receptor designed to fit a specific antigen on the pathogen. B cells scan for pathogens (such as viruses and bacterial toxins), and on encountering a pathogen whose antigen fits its receptor, a B cell will start to make copies of itself (i.e., proliferate). The proliferating B cells grow into a colony of plasma cells producing and secreting antibodies that block the pathogen from gaining access to healthy cells. After the infection has resolved, some of these plasma cells may persist for 50 years or longer as memory B cells, which contribute to immunological memory and can respond quickly by producing antibodies if they encounter the same pathogen again.

T lymphocyte cells mainly target cells of the body that have been invaded by pathogens such as viruses, or that show abnormal molecular patterns on their surface associated with cancerous growth or necrosis. T cells do not produce antibodies and they mature in the thymus. They may be broadly divided into three groups: helper T cells, cytotoxic T cells, and regulatory T cells.

Helper T cells (also known as $CD4^+$ cells) represent a key cell type in adaptive immunity and consist of four groups. Th1 and Th2 cells are involved in defenses against intra- and extracellular pathogens and in autoimmune and allergic responses, respectively; a recently identified helper T cell, Th17, represents another $CD4^+$ cell group involved in neutralizing extracellular microbes and also has been shown to be involved in chronic inflammation and autoimmune disease. Regulatory T (Treg) cells represent a fourth group that helps to check responses of effector T cells and suppress pro-inflammatory pathways—for example, when an infection has resolved. These cells also keep the immune system in check when there is no infection, preventing immune cells from attacking the normal cells of the body.

The T helper cells do not attack pathogens directly, but activate other immune system cells, including *B cells*, *killer T cells*, and *macrophages*. They are activated by antigens presented on, for example, *dendritic cells* and *B cells*. Each helper T cell, derived from cells produced early in the body's development by the above-mentioned differentiation mechanism, has T-cell receptors on its surface that recognize a specific antigen attached to MHC II of the presenting cells. On encountering an immune cell that presents an MHC II–bound antigen that matches the helper T cell's receptor, the helper T cell is activated and begins to proliferate. Some of these proliferating cells become memory helper T cells that contribute to immunological memory and respond quickly to future infections by the same pathogen. The others become effector helper T cells, which release *cytokines* to attract other immune cells, such as *macrophages*, *B cells*, and *cytotoxic T cells*, or regulate the activity of these cells.

Cytotoxic or killer T cells (also known as $CD8^+$ cells) search for and destroy cells infected with viruses or other pathogens or for cells that are damaged or abnormal such as cancer cells. Like *helper T cells*, cytotoxic T cells have T-cell receptors. These receptors bind to MHC I, a protein complex that is present on the surface of all cells in the body. When a microbe or virus infects a cell or a cell becomes cancerous, fragments of damaged proteins are transported to the cell surface and are presented on MHC I. A cytotoxic T cell whose receptor fits an antigen presented on MHC I binds to the antigen, resulting in activation of the T cell. Activated cytotoxic T cells begin to proliferate into memory cytotoxic T cells or effector cytotoxic T cells. The latter cells bind to MHC I on the antigen-presenting cells and destroy them, whereas the former contribute to immunological memory of the activation event.

Signaling in Innate and Adaptive Immunity

Cytokines are small proteins that help immune cells to communicate; they are secreted from immune cells on contact with a pathogen- or damage-associated molecular pattern or with an antigen. Many cells of the innate and adaptive immune systems release cytokines, which activate or suppress the activity of other immune cells by binding to specific receptors on these cells. Cytokines help regulate virtually all immune processes, affect the balance between humoral and cellular immunity, and help control the growth and maturation of many immune cells. They include *chemokines*, *interferons*, *interleukins*, and *tumor necrosis factor*.

Chemokines represent cytokines whose action on the receptors of immune cells (i.e., leukocytes) promotes movement (i.e., chemotaxis) toward the source of the chemokines; chemokines thus attract the immune

cells to, for example, sites of inflammation or injury.

Interferons are cytokines released by cells (especially leukocytes) interacting with viruses, other pathogens, or toxic proteins; they bind to and activate specific receptors on neighboring cells. This activation leads to increased transcription of genes for proteins that increase the cells' resistance to viral infection. Interferons also inhibit activation of *B cells* and increase the cytotoxicity of *NK cells*. Interferons are represented by three distinct classes (α, β, and γ), each of which is characterized by specific functions and is produced by specific cells (e.g., by leukocytes, fibroblasts, and *lymphocytes*).

Interleukins (*ILs*) are produced by leukocytes, *lymphocytes*, and even non-immune cells (in some circumstances). ILs include both cytokines and *chemokines*. Low concentrations of these proteins mainly facilitate localized communication among leukocytes in inflammation, such as promoting the production of chemokines to recruit additional immune cells. At higher concentrations, some ILs (e.g., IL-1) enter the blood stream and act as endocrine hormones, producing fever and stimulating production of immune proteins in the liver.

Tumor necrosis factor α (*TNF*α) is a major pro-inflammatory cytokine. It primarily is produced by macrophages and promotes inflammation both during infection and in dysregulated immune responses, such as those active in degenerative diseases (e.g., arthritis). By binding to and activating its specific cell receptor, TNFR (i.e., cluster of differentiation 120 [CD120]), TNFα activates several transcription factors such as nuclear factor κB, which upregulates expression of pro-inflammatory genes. TNFα also induces cell death (i.e., apoptosis) and necrosis in some cell types.

Conclusion

The innate and adaptive immune systems have distinct roles in combating infections and pathogenic cells, and both systems have some modest functional overlap. Whereas innate immunity represents a relatively non-specific and first-line defense against microbes and parasites, adaptive immunity encompasses a highly evolved assemblage of sophisticated defense mechanisms that specifically target groups of related or individual pathogens. The innate immune system blocks entry of pathogens by physical (e.g., skin) and physiological (e.g., pH, nucleases, proteases, and host-defense peptides) means. If a pathogen succeeds in breaching these initial barriers, detection of the pathogen by innate immune cells stimulates inflammation that attempts to isolate infected cells and tissues and to inactivate the invading pathogen. If this initial inflammatory response does not eliminate the pathogen, the adaptive immune system comes into play.

The cells of the adaptive immune system translocate to the site of infection and begin to inactivate, for example, free virus particles (by way of *B cells*) and to destroy virus-infected or damaged cells (by way of *T cells*), or help eliminate other pathogens such as bacteria, fungi, or larger parasites. Formation of B and T memory cells then guards against future attack by the same pathogen.

Current research still is untangling the complex interactions between these two immune systems and studying the functions of the many proteins and chemical signals involved.

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Alcohol's Effect on Host Defense

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Alcohol affects many organs, including the immune system, with even moderate amounts of alcohol influencing immune responses. Although alcohol can alter the actions of all cell populations involved in the innate and adaptive immune responses, the effect in many cases is a subclinical immunosuppression that becomes clinically relevant only after a secondary insult (e.g., bacterial or viral infection or other tissue damage). Alcohol's specific effects on the innate immune system depend on the pattern of alcohol exposure, with acute alcohol inhibiting and chronic alcohol accelerating inflammatory responses. The proinflammatory effects of chronic alcohol play a major role in the pathogenesis of alcoholic liver disease and pancreatitis, but also affect numerous other organs and tissues. In addition to promoting proinflammatory immune responses, alcohol also impairs anti-inflammatory cytokines. Chronic alcohol exposure also interferes with the normal functioning of all aspects of the adaptive immune response, including both cell-mediated and humoral responses. All of these effects enhance the susceptibility of chronic alcoholics to viral and bacterial infections and to sterile inflammation.

Key words: Alcohol effects and consequences; alcohol consumption; alcohol exposure; acute alcohol exposure; chronic alcohol exposure; alcohol use pattern; alcoholic liver disease; pancreatitis; immunity; immune system; immune response; innate immune response; adaptive immune response; immunosuppression; bacterial disease; viral disease; inflammatory response; proinflammatory response; anti-inflammatory; infection; inflammation

Alcohol has been the most common substance of use and abuse in human history. Moderate amounts of alcohol are enjoyed for its anxiolytic effects; however, its addictive properties can lead to chronic, excessive alcohol use and alcohol use disorder. In addition to its commonly recognized behavioral effects, alcohol affects many organs, including the immune system that controls the body's defense against infectious pathogens (e.g., bacteria and viruses) and other harmful agents. Chronic alcohol use is associated with significant alterations in the immune system that predispose people to viral and bacterial infections and cancer development. In general, severe chronic alcoholics are considered immunocompromised hosts. Although moderate alcohol use has less obvious clinical effects on the immune system, both in vitro and in vivo studies indicate that even moderate amounts of alcohol and binge drinking modulate host immune responses.

This review gives a general overview of the immune effects of alcohol. However, it is important to realize that many aspects of alcohol consumption and its effects on immunity and host defense have not yet been fully elucidated. For example, the pattern of alcohol consumption (e.g., occasional binge drinking versus chronic heavy drinking) may affect the immune system in different ways that are yet to be explored.

Overview of the Immune System

The immune system serves to defend the host from pathogens and to prevent unwanted immune reactions to self. This defense involves coordinated complex interactions between two arms of the immune system—the innate and the adaptive immune responses. Innate immunity provides immediate responses to pathogen-derived or nonpathogen–associated (i.e., sterile) danger signals and results in activation of proinflammatory cytokines and/or Type I interferons, regardless of the underlying cause and without the body having encountered the pathogen before. Adaptive immunity, in contrast, which only sets in after a certain delay, is specific to the pathogen or

antigen and requires an initial encounter with the pathogen or antigen to activate the response.

The innate immune response usually involves inflammatory reactions and/or production of reactive oxygen species (ROS) and other signaling molecules. Once the pathogen is eliminated, the innate immune response is resolved, allowing restoration of immune homeostasis (Miyake and Kaisho 2014; Mogensen 2009; Newton and Dixit 2012). The key cell types in innate immunity are a variety of white blood cells, including neutrophils, monocytes/macrophages, dendritic cells (DCs), and natural killer (NK) cells (table 1). In addition, the innate immune system has various soluble components, including the following:

- Cytokines, such as interleukin-(IL-)1, IL-6, or tumor necrosis factor alpha (TNFα), are produced by innate immune cells as part of the initial response and induce and support a full-fledged inflammatory response.

- Interferons mainly are produced by virus-infected cells and can induce an antiviral response in neighboring cells. Based on the receptors with which they interact they can be divided into three classes (Type I to Type III), of which the Type I interferons primarily are involved in the antiviral response.

- The complement system, a group of small proteins that mainly are produced in the liver and then released into the blood, recognize specific molecules on the surface of pathogens and help other immune molecules (i.e., antibodies) and immune cells (i.e., phagocytic cells) to identify and eliminate the pathogens from the organism. Thus, complement molecules can cover the pathogen in a process called opsonization, which enhances phagocytosis of the pathogen; attract macrophages and neutrophils to the pathogen; help rupture the membranes of foreign pathogens, and induce clustering and binding of pathogens.

The innate immune system is activated when the involved cells recognize certain immune danger signals. This recognition occurs through molecules called pattern recognition receptors, which include Toll-like receptors (TLRs), helicase receptors, and Nod-like receptors (NLRs).[1] These receptors are strategically located on the cell surface or within the cells, where they can sense pathogen-derived signals, such as certain proteins, bacterial products called lipopolysaccharides (LPS), peptidoglycans, DNA, RNA, and numerous metabolic or other signals (Kawai and Akira 2009; Meylan et al. 2006; Seki and Brenner 2008). TLRs, NLRs, and helicase receptors are expressed on innate immune cells as well as on the functional cells (i.e., parenchymal cells) in most organs; however, activation of pattern recognition receptors triggers proinflammatory cytokine induction most robustly in the immune cells.

In addition to producing proinflammatory cytokines, innate immune cells (particularly DCs and monocytes) are necessary to present pathogen-derived molecules (i.e., antigens) to adaptive immune cells so as to trigger or facilitate adaptive immune responses. These adaptive immune cells include T cells, B cells, and natural killer T cells (NKTs), which must cooperate in a controlled manner to mount an effective response (Castellino and Germain 2006; Mitchison 2004). T cells in turn fall into several different categories, including helper T cells, also known as $CD4^+$ cells; cytotoxic T cells, also called $CD8^+$ cells; Th17 cells; and regulatory T (Treg) cells (table 1). As the name implies, helper T cells help control the activity of other immune cells by producing and secreting various cytokines.

[1] NLRs can be classified into four subfamilies based on their specific structure at one end of the molecules. These families are called NLRA, NLRB, NLRC, and NLRP. Each of these subfamilies may comprise several members (e.g., NLRP1, NLRP2, etc.).

Depending on the specific cytokines they produce, helper T cells can further be subdivided into two types with specific functions:

- Th1 cells, which initiate a cell-mediated immune response against intracellular pathogens by activating macrophages or cytotoxic T cells that then destroy the pathogen. The Th1 cells primarily induce their effects by releasing interferon gamma (IFN-γ), which promotes inflammatory responses.

- Th2 cells, which initiate a humoral immune response against extracellular pathogens that is mediated by proteins (i.e., immunoglobulins [Igs]) produced by B cells. The Th2 cells induce their effects primarily by releasing a variety of interleukins, some of which have anti-inflammatory effects.

Th17 cells also can be considered a type of helper T cells characterized by the production of interleukin 17. Their main function is to defend against pathogens at epithelial and mucosal barriers. Finally, Treg cells serve to limit and suppress the immune response to prevent overreaction of the immune system as well as immune reactions against self-antigens.

B cells are characterized by the production of antibodies comprised of Igs. Various types of Igs (e.g., IgA, IgG, IgM) are produced at different times during an infection or in response to a range of antigens that have specific roles in the adaptive immune response.

In contrast to the innate immunity, which can be induced by any kind of antigen, adaptive immune responses are specific to individual antigens. In other words, each T cell or B cell can be activated only by one specific antigen. An antigen-specific T-cell response is initiated by interactions between antigen presenting cells (such as DCs) and naïve T cells and is optimized by engagement of co-stimulatory molecules and

cytokines for antigen-specific T-cell activation (Mogensen 2009; Newton and Dixit 2012). The initial activation triggers a memory response in the form of memory B cells that remain in the circulation for long periods and can respond quickly when they encounter that antigen a second time to mount a stronger, more rapid response.

Table 1 Cells Involved in the Innate and Adaptive Immune Responses

Cell Type	Characteristics	Functions
Innate Immune Responses		
Dendritic Cells	Have a roughly star-shaped form with several arms	Present antigens to other immune cells Stimulate T-cell responses Produce interferons (IFNs) Produce cytokines and reactive oxygen species (ROS)
Monocytes/Macrophages	Have a kidney-shaped nucleus Monocytes are precursors of macrophages; mature into macrophages when they enter the tissues Specific subtypes of macrophages reside in the tissues (Kupffer cells in the liver, microglial cells in the brain, alveolar macrophages in the lungs)	Destroy pathogens by phagocytosis Produce cytokines and ROS
Natural Killer (NK) Cells		Destroy cells infected with viruses and intracellular pathogens
Neutrophils	Have a multi-lobed nucleus	Destroy pathogens by phagocytosis Produce ROS
Adaptive Immune Response		
CD4 T Cells	Express CD4 glycoprotein on their cell surface	Helper T cells that activate B cells and macrophages
Th1 Cells	Primarily act by secreting IFN gamma (IFNγ)	Initiate cell-mediated immune response; have proinflammatory effects
Th2 Cells	Primarily act by secreting various interleukins	Initiate a humoral immune response; have some anti-inflammatory effects
Th17 Cells	Subtype of CD4 T cells Produce interleukin (IL)-17	Involved in recruitment, activation, and migration of neutrophils Provide defense against pathogens at mucosal and epithelial barriers
CD8 T Cells	Express CD8 glycoprotein on their cell surface	Cytotoxic T cells Destroy virus-infected and tumor cells
Treg Cells	Formerly known as suppressor T cells	Inhibit T-cell responses to prevent excessive immune reactions
B Cells		Produce antibodies Form memory cells
NKT Cells	Share properties of NK cells and T cells	Produce multiple cytokines Can perform functions ascribed to both helper and cytotoxic T cells

The complexity of the innate and adaptive immune responses are increased further by the fact that different subsets of immune cells may reside in specific organs, such as the liver, lungs, brain, skin, bones, or muscles. This complex structure of the immune system with its multitude of different cells with diverse functions allows the organism to defend itself properly against the hugely diverse pathogens it may encounter, without endangering its own cells. At the same time, it makes it much more difficult to investigate and understand the impact of external influences, such as acute or chronic alcohol exposure, on the body's immune responses.

Alcohol's Effects on the Immune System

Alcohol can modulate the activities of all of these cell populations by affecting the frequency, survival, and function of most of these cells, thereby interfering with pivotal immune responses. However, unlike other mechanisms that cause classical immunocompromised states, such as HIV or tuberculosis infection, alcohol use typically results in a subclinical immunosuppression that becomes clinically significant only in case of a secondary insult. For example, chronic alcohol consumption increases the risk and severity of chronic infections with HIV; hepatitis C virus (HCV); or *Mycobacterium tuberculosis*, the bacterium that causes tuberculosis, and promotes post-trauma immunosuppression (for more information, see the articles in this issue by Bagby and colleagues, by Dolganiuc, by Molina and colleagues, and by Simet and Sisson).

Emerging evidence also suggests that alcohol may affect immune functions by altering the balance and interactions between the host immune system and the entirety of microorganisms found in the host (i.e., the host microbiome). This microbiome is composed of the normal microorganisms found in and

on the body (i.e., commensal microorganisms), which are needed for the body's normal functioning, and disease-causing pathogens. Increasing evidence suggests that alcohol may modulate the composition of pathogenic and commensal organisms in the microbiome of the gut, oral cavity, skin, and other mucosal surfaces (Chen and Schnabl 2014; Leclercq et al. 2014*a,b*). These alcohol-induced changes could have clinical significance because the composition of the microbiome sends important pathogenic as well as homeostatic signals for the functions of host immunity. For example, chronic alcohol use is associated with changes in the gut microbiome, both increasing the microbial content in the first part of the large intestine (i.e., cecum) and changing the abundance of different types of microorganisms in the gut (Chen and Schnabl 2014; Fouts et al. 2012; Yan et al. 2011). This may alter the levels of LPS released by certain types of bacteria in the gut, which can contribute to inflammation in alcoholic liver disease as well as in liver cancer (i.e., hepatocellular carcinoma) (Chassaing et al. 2014; Gao et al. 2011; Szabo and Bala 2010). (For more information, see the articles in this issue by Hammer and colleagues and by Engen and colleagues).

Alcohol and Innate Immunity

Alcohol modulates the function of nearly all components of the innate immune system, but the specific effects on inflammatory cell responses depend on the pattern of alcohol exposure (i.e., acute or chronic). In human monocytes or mouse macrophages, acute alcohol results in a decrease in TLR responses (i.e., TLR tolerance), which attenuates particularly production of the TNFα in response to a subsequent LPS stimulation (Bala et al. 2012; Mandrekar et al. 2009). However, this initial inhibitory effect of acute alcohol on monocytes and macrophages is transient, and repeated alcohol exposure (such as in chronic alcohol use) leads to loss of TLR4 tolerance; instead, the cells become more responsive to LPS stimulation, a process known as sensitization (Mandrekar and Szabo 2009; Mandrekar et al. 2009). Even a single episode of binge drinking can have measurable effects on the innate immune system, inducing a transient proinflammatory state within the first 20 minutes after alcohol ingestion, followed by an anti-inflammatory state 2 to 5 hours after alcohol ingestion (Afshar et al. 2015).

Both TLR tolerance and sensitization of monocytes and macrophages are associated with the production of specific sets of signaling molecules. The molecular signatures of alcohol-induced TLR tolerance and sensitization, respectively, have been well described and involve downstream components of the TLR-induced signaling cascades. This includes activation of molecules called IRAK-M, IRAK1/4, Bcl-3, and NF-κB, all of which regulate proinflammatory cytokine activation (Bala et al. 2012; Mandrekar and Szabo 2009; Mandrekar et al. 2009). (For a list of the full names of these and other molecules mentioned in this article, please see table 2.) The proinflammatory effect of prolonged alcohol exposure has been demonstrated in response to molecules (i.e., ligands) that activate TLR4, TLR3, and TLR2 receptors (Bird et al. 2010; Fernandez-Lizarbe et al. 2013; Goral and Kovacs 2005; Oak et al. 2006; Pruett et al. 2004).

It is increasingly evident that sensitization of proinflammatory pathways to activation in monocytes and macrophages after chronic alcohol use has biological and clinical significance. It is known that alcohol-mediated sensitization of immune cells to gut-derived LPS is a major component in the pathogenesis of alcoholic liver disease and alcoholic pancreatitis (Choudhry

Table 2 Full Names of Molecules Mentioned in the Article

AIM2	Absent in melanoma 2
ASC	Apoptosis-associated speck-like protein containing a CARD
Bcl-3	B-cell lymphoma 3-encoded protein
ERK	Extracellular signal–regulated kinase
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IRAK1/4	Interleukin-1 receptor–associated kinase 1/4
IRAK-M	Interleukin-1 receptor–associated kinase M (restricted to monocytes/macrophages)
LPS	Lipopolysaccharide
NF-κB	Nuclear factor "kappa-light-chain-enhancer" of activated B cells
NLR	Nod-like receptor
NLRP	Nod-like receptor, subfamily P
STAT	Signal transducer and activator of transcription
TGFß	Transforming growth factor beta
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha

et al. 2002; Keshavarzian et al. 1994; Nolan 2010; Szabo et al. 2010, 2011). In fact, in acute alcoholic hepatitis, the severity of clinical outcome and death correlates with serum levels of the proinflammatory cytokines, particularly TNFα (Frazier et al. 2011; McClain et al. 2004). (For more information on the role of innate immunity in the pathogenesis of alcoholic liver disease, see the articles in this issue by Nagy and by Mandrekar and Ju.) Chronic alcohol use also promotes inflammation in the small bowel, brain, lungs, and other organs, suggesting that common mechanisms may underlie the proinflammatory effects of alcohol. The exact triggers for alcohol-induced inflammation in the different tissues are yet to be identified. Importantly, deficiency in TLR4, the major sensor of LPS, attenuates inflammation induced by chronic alcohol use in the liver, brain, and intestine (Hritz et al. 2008; Lippai et al. 2013*a,b*, 2014). However, LPS increase was not found in the brain, suggesting that other ligands and/or alcohol itself may activate TLR4 (Alfonso-Loeches et al. 2010; Lippai et al. 2013*b*).

In addition to direct induction of chemokines and most proinflammatory cytokines by TLR activation, activation of the inflammasome was detected in the liver, brain, and intestine after chronic alcohol use (Orman et al. 2013; Szabo and Lippai 2014). The inflammasome is a multiprotein intracytoplasmic complex that comprises a sensor (e.g., NLRP1, NLRP3, NLRC4, or a protein called AIM2) and adapter molecules (e.g., a molecule called ASC). This protein complex can be activated by a variety of sterile danger signals (Tsuchiya and Hara 2014). Activation of the inflammasomes results in induction of caspase-1, an enzyme needed to form mature secreted IL-1β or IL-18. Recent studies have demonstrated inflammasome activation and IL-1β induction in the liver, brain, and intestine after chronic alcohol administration in mice (Alfonso-Loeches et al. 2010; Lippai et al. 2013*a,b*, 2014; Orman et al. 2013). These findings are biologically significant, because administration of a recombinant IL-1 receptor antagonist that blocks signaling via the IL-1 receptor can attenuate alcohol-induced liver disease and cerebral inflammation (Petrasek et al. 2012). These observations demonstrate that chronic alcohol administration results in inflammation and leads to a vicious cycle of upregulation of the inflammatory cascade. Future studies are needed to evaluate whether disruption of this vicious cycle would be sufficient to attenuate and or prevent chronic alcohol-induced tissue damage in various organs.

Alcohol also interferes with the body's normal mechanisms that help control the innate immune response and prevent excessive inflammatory reactions. These mechanisms include the induction of anti-inflammatory cytokines, such as IL-10 and transforming growth factor beta (TGFß) (Ouyang et al. 2011; Sanjabi et al. 2009). Again, the specific effects depend on the duration of alcohol exposure. Thus, whereas acute alcohol exposure increases both IL-10 and TGFß production in monocytes and macrophages, chronic alcohol exposure mostly is associated with decreased IL-10 production or prevents appropriate increases in IL-10 levels to counterbalance the overproduction of proinflammatory cytokines (Byun et al. 2013; Järveläinen et al. 1999; Mandrekar et al. 2006; Norkina et al. 2007; Pang et al. 2011).

Alcohol exposure may modify not only cytokine secretion but also the overall function of monocytes and macrophages. These cells exhibit remarkable plasticity that allows them to change their phenotype from a proinflammatory (M1) phenotype that inhibits cell proliferation and can cause tissue damage to an alternatively activated type (M2) that has anti-inflammatory and tissue-repair capacity (Italiani and Boraschi 2014). This process is known as polarization. Interestingly, in alcoholic liver disease, which would be expected to be characterized by the presence of primarily proinflammatory M1 macrophages, the numbers of both M1 and M2 macrophages are increased. An alcohol-induced shift toward M2-type cells may have some beneficial effects by destroying pro-inflammatory M1 macrophages (Wan et al. 2014); however, the fibrogenic effect of M2 macrophages that leads to the formation of scar tissue also can damage liver function. Further studies on the effects of alcohol on monocyte/macrophage polarization may reveal potential therapeutic interventions for alcohol-induced immunomodulation.[2]

Neutrophils represent another important innate immune cell type affected by alcohol. Studies found that alcohol increases ROS production by neutrophils; however, their phagocytic capacity, which is important in antibacterial defense, was decreased by alcohol administration (Gandhi et al. 2014; Karavitis and Kovacs 2011).[3] Interestingly, recruitment of neutrophils to the liver is a characteristic of the pathology of acute alcoholic hepatitis. Recent studies suggested that the increase in the numbers of neutrophils in the liver correlates with survival in acute alcoholic hepatitis (Altamirano et al. 2014); however, the role of neutrophils in this process is not yet fully understood.

The pattern-recognition receptors (i.e., TLRs, NLRs, and helicase receptors) found on innate immune cells play a pivotal role particularly in the defense against viral infections. These receptors recognize viral nucleic acids (i.e., DNA and RNA) and mount an immediate response mediated by interferons (Stetson and Medzhitov 2006; Takeuchi and Akira 2009). Production of interferons in monocytes is induced by activation of var-

[2] Recent studies indicate that the distinction between M1 and M2 macrophage populations may not be as clear cut because the markers that have delineated these two populations have become blurred and because macrophage polarization falls along a spectrum between the historic M1 and M2 phenotypes (Martinez and Gordon 2014; Murray et al. 2014; Xue et al. 2014). Thus, the concept of macrophage polarization currently is an evolving area.

[3] Alcohol alters not only phagocytosis mediated by neutrophils but also phagocytosis by macrophages.

ious TLRs and helicase receptors. The actions of interferons within the cells, in turn, are mediated by regulatory molecules called signal transducers and activators of transcription (STATs), a family of transcription factors that regulate the expression of certain immune genes. Alcohol interferes with these processes at multiple levels. Thus, both acute and chronic alcohol inhibit induction of Type-I interferons via TLR3, TLR7/8, or TLR9 or by helicase receptors in monocytes (Pang et al. 2011; Pruett et al. 2004). Alcohol also impairs Type-I interferon-receptor signaling by inhibiting STAT signaling (Norkina et al. 2008; Plumlee et al. 2005).

Alcohol's Effects on Adaptive Immunity

Many studies have evaluated the effects of chronic alcohol on adaptive immune responses, and this research is reviewed in more detail in the article by Pasala and colleagues in this issue. Chronic alcoholics have impaired T-cell responses; moreover, the balance between Th1 and Th2 responses is shifted toward a predominance of Th2-type responses (Fan et al. 2011; Lau et al. 2006; Szabo 1999). Consistent with this, chronic alcoholics exhibit an increase in IgA and a relative decrease in IgG antibodies, which play a role in antibody-dependent cell-mediated immune responses (Massonnet et al. 2009; Nouri-Aria et al. 1986). Other studies have noted a greater-than-normal abundance of Th17 cells in people with alcoholic liver disease (Lafdil et al. 2010; Ye et al. 2011). Specific aspects of the adaptive immune response also are affected. For example, even a single dose of alcohol may impair antigen-specific T-cell activation. Thus, in human monocytes and myeloid DCs, alcohol inhibits the cells' antigen-presentation function as well as their capacity to induce antigen-specific (Mandrekar et al. 2009) and general T-cell activation (Szabo et al. 2001).

Alcohol's Effects on Maturation and Development of Immune Cells From Precursors

Alcohol abuse has an adverse effect on hematopoiesis and can cause leukopenia, granulocytopenia, and thrombocytopenia in humans (Latvala et al. 2004). Acute alcohol can block differentiation or maturation of granulocytes (i.e., granulopoiesis) during infections (Zhang et al. 2009). Examination of the bone marrow from alcoholic patients has shown vacuolated granulopoietic progenitors with a significantly reduced number of mature granulocytes (Yeung et al. 1988). Alcohol intoxication also can suppress the myeloid proliferative response by inhibiting the Stem Cell Antigen-1/ERK pathway during bacterial infection (Melvan et al. 2012). Chronic alcohol consumption also affects the NKT cell populations that play important immunoregulatory roles. Thus, alcohol consumption enhances immature NKT (iNKT) cell proliferation and maturation in the thymus and increases IFN-γ–producing iNKT cells (Zhang et al. 2015). In vivo activation of iNKT cells induces a Th1-dominant immune response and enhances the activation of DCs as well as NK cells, B cells, and T cells in alcohol-consuming mice (Zhang et al. 2015).

DCs, which are the major cell type linking the innate and adaptive immune response, also are affected by alcohol intoxication. Acute alcohol exposure alters function and cytokine production in human monocyte-derived myeloid DCs (Szabo et al. 2004*a*). Chronic alcohol consumption in humans causes alterations in the immunophenotype of DCs and decreased production of IL-1β and TNFα (Laso et al. 2007). Studies in rhesus macaques have helped elucidate the effects of alcohol on DC development in hematopoietic tissues and the functional activities of the DCs (Siggins et al. 2009). In these studies, chronic alcohol exposure decreased the pools of myeloid DCs in the bone marrow and peripheral blood. Alcohol also suppressed expression of the co-stimulatory molecule CD83 during DC maturation, which may attenuate the ability of DCs to initiate T-cell expansion (Siggins et al. 2009).

Alcohol-Induced Modulation of the Host Defense Against Different Pathogens

It has been known for decades that chronic alcoholic individuals have increased susceptibility to infections (Sternbach 1990; Szabo 1999). This increased susceptibility to both viral and bacterial infections has been attributed to alcohol's general immunosuppressive effects, and animal models of chronic alcohol use and infections repeatedly have confirmed this (Jerrells et al. 1994, 2007). In addition, chronic alcoholics seem to be vulnerable to inflammatory reactions not associated with pathogenic infections (i.e., sterile inflammation).

Viral Infections

Most evidence for alcohol-associated increases in susceptibility to infection comes from studies of human viral infections, such as HCV, hepatitis B virus (HBV), HIV, and pulmonary viral infections. Such investigations have yielded the following findings:

- The prevalence of HCV infection is higher in individuals with chronic alcohol use than in the general population. Alcohol exposure and HCV interact at several levels. For example, alcohol exposure augments HCV replication by altering the levels of a molecule that supports HCV replication (i.e., microRNA-122) in liver cells (i.e., hepatocytes) (Hou et al. 2013). Moreover, alcohol and HCV synergistically impair antiviral immunity by interfering with the function of antigen-presenting cells, altering the activity and frequency of Treg cells, and modifying production of Type-I

Glossary

Antibody: Immune molecule (protein) produced by *B cells* that recognizes foreign molecules that have entered the body (i.e., *antigens*), binds to these molecules, and marks them for destruction by the body's immune system.

Antigen: Any molecule that can bind specifically to an *antibody* and can induce an immune response.

B Cells: One of the two main types of lymphocytes involved in the adaptive immune response; when activated by interacting with a specific *antigen*, they differentiate into specific subtypes and begin to produce *antibodies* that recognize the specific *antigen*.

Cell-Mediated Immune Response: Part of the adaptive immune response that is mediated by various populations of *T cells*.

Chemokine: Small proteins that serve as chemoattractants, stimulating the migration and activation of cells, particularly phagocytic cells and lymphocytes; they have a central role in inflammatory responses.

Cytokine: Any of a group of molecules, produced primarily by immune cells, that regulate cellular interactions and other functions; many cytokines play important roles in initiating and regulation inflammatory reactions.

Dendritic Cell: A type of immune cell involved in the innate immune response that are characterized by a branched morphology; dendritic cells can bind to *antigens* and present these antigens to *T cells*, thereby initiating an adaptive immune response.

Granulocytopenia: Condition in which the number or proportion of certain white blood cells (i.e., granulocytes) in the blood is lower than normal; granulocytes, which are characterized by the presence of small, enzyme-containing vesicles (i.e., granules) in the cytoplasm, are part of the innate immune system.

Helicase Receptors: A class of proteins that act as intracellular pattern recognition receptors and play a central role in the innate immune system; they recognize the presence of viruses in the cells and initiate antiviral responses.

Hematopoiesis: The entirety of the processes through which the different blood cells are formed.

Humoral Immune Response: Part of the adaptive immune response that is mediated by various populations of *B cells* and the *antibodies* they produce.

Inflammasome: A complex comprised of several proteins that is a component of the innate immune system and is responsible for activation of inflammatory processes; it promotes the maturation of several inflammatory *cytokines.*

Leukopenia: Condition in which the number or proportion of white blood cells (i.e., leukocytes) in the blood is lower than normal.

Macrophage: A type of immune cell that ingests foreign particles and micro-organisms in a process called *phagocytosis* and which synthesizes *cytokines* and other molecules involved in inflammatory reactions.

Natural Killer (NK) Cell: A type of immune cell involved in the innate immune response that can kill certain harmful cells, particularly tumor cells, and contributes to the innate immune response to cells infected with viruses or other intracellular pathogens.

Neutrophil: A type of immune cell involved in the innate immune response that engulfs and kills extracellular pathogens in a process called *phagocytosis*.

Nod-Like Receptors (NLRs): A class of proteins that act as pattern recognition receptors and play a central role in the innate immune system; they are embedded in the membrane of various immune and nonimmune cells and can recognize certain bacterial molecules, thereby initiating the immune response to the bacteria.

Opsonization: The process by which *antibodies* bind to a pathogen, thereby marking it for destruction by *phagocytosis*.

Parenchymal Cells: The cells in an organ that comprise the functional part of the organ (e.g., the hepatocytes in the liver).

Phagocytosis: Internalization or engulfment of particles or cells by specific cells (i.e., phagocytes), such as such as *macrophages* or *neutrophils*.

T Cells: One of the two main types of lymphocytes involved in the adaptive immune response after activation through the interaction with a specific *antigen*. T cells can be divided into several subgroups that support other immune cells (helper T cells), kill invading pathogens or infected cells (cytotoxic T cells), or help turn off the adaptive immune response (regulatory T cells).

Thrombocytopenia: Condition in which the number or proportion of platelets (i.e., thrombocytes) in the blood is lower than normal.

Toll-Like Receptors (TLRs): A class of proteins that act as pattern recognition receptors and play a central role in the innate immune system; they are embedded in the membrane of *macrophages* and *dendritic cells* and can recognize molecules derived from pathogens, thereby initiating the immune response to those pathogens.

interferons (Dolganiuc et al. 2003; Plumlee et al. 2005; Szabo et al. 2004*b*). In patients with liver disease caused by chronic HCV infection, chronic alcohol use is an independent risk factor for development of advanced liver disease and cirrhosis (Corrao and Arico 1998; Szabo 2003).

- Chronic HBV infection affects about 240 million people worldwide (Centers for Disease Control and Prevention 2013). Research has shown that alcohol use accelerates the progression of liver disease caused by chronic HBV infection to liver fibrosis and hepatocellular cancer (Gao and Bataller 2011; Zakhari 2013). However, the cellular and molecular mechanisms by which alcohol and HBV interact still await further investigations.

- Studies on the effect of alcohol on HIV infectivity in humans have yielded conflicting results. However, the combined immunosuppressive effects of alcohol use and advanced HIV infection (AIDS) are well established (Muga et al. 2012; Szabo and Zakhari 2011).

- In pulmonary viral infections, it is unclear whether alcohol increases susceptibility to influenza infections or adversely affects the outcome of established infections. However, in animal models of pulmonary infections, alcohol administration is associated with adverse clinical parameters and increased lung damage (Boé et al. 2009; Zhang et al. 2008).

Bacterial Infections

Bacterial infections can be either systemic or localized to a specific organ, such as the lungs. Alcohol use has negative effects on all types of pulmonary bacterial infections. For example, infections with *Mycobacterium tuberculosis* are more severe in chronic alcoholics, and alcohol use is associated with systemic dissemination of tuberculosis (Rehm et al. 2009; Szabo 1997). Furthermore, infections with *Klebsiella pneumoniae* and *Streptococcus pneumoniae*, common causes of pneumonia in humans, are more common in alcoholics compared with the nonalcoholic general population (Bhatty et al. 2011; Jong et al. 1995). Alcohol-induced dysfunction of specific immune cells contributes to severe pneumonias in this population. For example, the function of alveolar macrophages is impaired because of alcohol-induced changes in cytokine profiles as well as in the levels of ROS and antioxidants that result in oxidative stress (Liang et al. 2012; Mehta and Guidot 2012). Recruitment and function of neutrophils in alcoholic individuals also are increased, resulting in increased tissue damage in the lung alveoli (Dorio and Forman 1988; Kaphalia and Calhoun 2013).

Not only chronic alcohol abuse but also acute alcohol exposure can impair immune response to pulmonary infections. For example, acute intoxication in humans with blood alcohol levels of 0.2 percent can severely disrupt neutrophil functioning and their ability to destroy bacteria (Tamura et al. 1998). Studies in laboratory animals have confirmed the adverse effects of acute alcohol exposure on pulmonary infections. Thus, acute alcohol exposure in animals that were then infected with *S. pneumoniae* impaired lung chemokine activity in response to the infection, which resulted in reduced recruitment of immune cells into the lungs, decreased bacterial clearance from the lungs, and increased mortality (Boé et al. 2001; Raasch et al. 2010). The effects of both acute and chronic alcohol exposure on the immune responses in the lungs and thus on susceptibility to pulmonary infections are discussed in more detail in the article by Simet and Sisson.

Sterile Inflammation

Inflammatory reactions (i.e., innate immune responses) can be induced not only by invading pathogens but also by danger signals resulting from damage to the body's own cells. Elucidation of the immune processes occurring in response to damaged self also may offer a better understanding of the proinflammatory effects of alcohol in various organs (e.g., liver or brain). One example of this is the relationship between gut-derived bacterial LPS, alcohol exposure, and inflammatory reactions. Although gut-derived LPS clearly has a role in alcoholic liver disease, it is equally clear that LPS alone does not cause alcoholic liver disease. Many other conditions associated with increased levels of gut-derived LPS in the systemic circulation, such as HIV infection or inflammatory bowel disease, do not involve liver disease (Caradonna et al. 2000; Marchettia et al. 2013). Furthermore, inflammatory reactions can occur in the brain after alcohol use, even in the absence of detectable LPS in the brain (Lippai et al. 2013*a*; Szabo and Lippai 2014). These observations suggest that although gut-derived LPS can promote tissue inflammation, another alcohol-induced component is required as well. Thus, it seems that alcohol exposure directly leads to the release of sterile danger signals from parenchymal cells in different tissues, which in turn result in the activation of inflammatory cells via TLRs and NLRs. These alcohol-induced sterile danger signals include a wide variety of molecules, such as high-mobility-group protein B1 (HMGB1), heat shock proteins, adenosine triphosphate (ATP), and potassium ions (Rock and Kono 2008; Sangiuliano et al. 2014).

It is now thought that alcohol-induced sterile danger signals contribute to the proinflammatory cytokine activation seen after chronic alcohol use in various organs (e.g., liver, intestine, and brain). This hypothesis also is supported by findings that in hepatocytes, alcohol exposure results in a rapid induction of apoptosis, which precedes induction of inflammatory cytokines (Caradonna et al. 2000; González-Reimers et al. 2014; Marchettia et al.

2013; Petrasek et al. 2013). Additional evidence for the role of sterile inflammatory signals in alcohol-induced inflammation and tissue damage comes from findings that HMGB1 is increased both in the liver and brain after chronic alcohol exposure (Crews et al. 2013; Csak et al. 2014; Lippai et al. 2013*a,b*). Finally, NLRs, specifically NLRP3 and NLRP4, have been found to be involved in alcoholic liver inflammation. Given the role of NLRs in sensing endogenous danger molecules, this observation further supports the notion that alcohol-induced tissue inflammations is caused at least partially by alcohol-induced danger signals.

Summary

As this review has indicated, alcohol exposure, and particularly chronic alcohol use, has profound effects on all aspects of the body's immune responses, including both innate and adaptive immunity. These effects can impair the body's defenses against a wide range of pathogens, including viruses and bacteria, as well as against damaged self and can affect tissues and organs throughout the body (see the figure). Thus, alcohol's effects on innate immune responses seem to promote inflammatory reactions, which may contribute to tissue damage in a variety of organs. Alcohol-related impairments of adaptive immune responses render the organism more vulnerable to viral and bacterial infections, contributing to more severe or accelerated disease progression. In addition, dysregulation of normal immune responses may contribute to such conditions as alcoholic liver disease and pancreatitis, altered gut permeability and gastrointestinal inflammation, neuroinflammation in the brain, and the development of cancer (see the article by Meadows and Zhang).

The following articles in this journal issue look at various aspects of alcohol's impact on innate and adaptive immune responses in more detail. They will also further explore the consequences of alcohol-induced

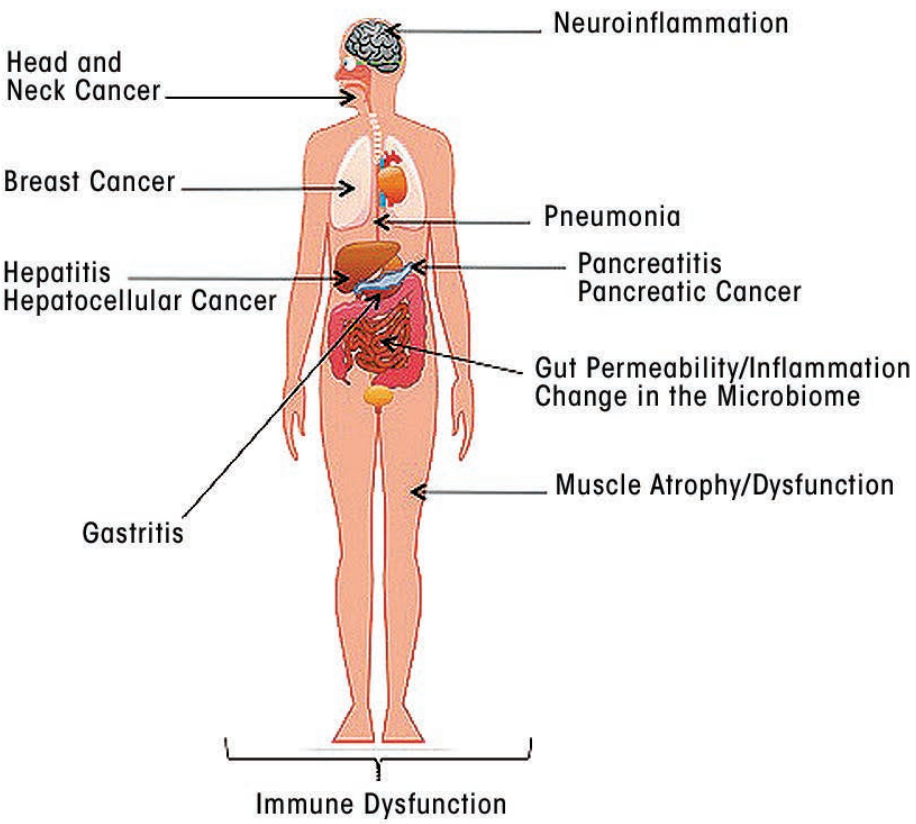

Figure Overview of alcohol's effects on human health that are associated with alcohol-induced dysfunction of the immune system.

disturbances of immune function on a variety of specific conditions, including liver disease, lung disease, cancer, traumatic injury, and bacterial and viral infections. Together, these articles will highlight the pivotal role that alcohol's effects on immune function play in the overall morbidity and mortality associated with excessive alcohol use.

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Alcohol and the Immune System

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Clinicians have long observed an association between excessive alcohol consumption and adverse immune-related health effects such as susceptibility to pneumonia. In recent decades, this association has been expanded to a greater likelihood of acute respiratory stress syndromes (ARDS), sepsis, alcoholic liver disease (ALD), and certain cancers; a higher incidence of postoperative complications; and slower and less complete recovery from infection and physical trauma, including poor wound healing.

This issue of *Alcohol Research: Current Reviews* (*ARCR*) summarizes the evidence that alcohol disrupts immune pathways in complex and seemingly paradoxical ways. These disruptions can impair the body's ability to defend against infection, contribute to organ damage associated with alcohol consumption, and impede recovery from tissue injury. It is our hope that a greater understanding of the specific mechanisms through which alcohol exerts its effects on the immune system may lead to development of interventions to prevent, or at least mitigate, the negative health consequences of alcohol misuse.

Contributors to this issue of *ARCR* lay the groundwork for understanding the multilayered interactions between alcohol and immune function by presenting an overview of the immune system (see the article by Spiering) and by reviewing current research on the effects of alcohol on innate immunity (see the article by Nagy) and on adaptive immunity (see the article by Pasala and colleagues). As reviewed by Szabo and Saha, alcohol's combined effects on both innate and adaptive immunity significantly weaken host defenses, predisposing chronic drinkers to a wide range of health problems, including infections and systemic inflammation. Alcohol's widespread effects on immune function also are underscored in the article by Gauthier, which examines how in utero alcohol exposure interferes with the developing immune system in the fetus. This exposure increases a newborn's risk of infection and disease; additional evidence suggests that alcohol's deleterious effects on immune development last into adulthood.

The gastrointestinal (GI) system is typically the first point of contact for alcohol as it passes through the body and is where alcohol is absorbed into the bloodstream. One of the most significant immediate effects of alcohol is that it affects the structure and integrity of the GI tract. For example, alcohol alters the numbers and relative abundances of microbes in the gut microbiome (see the article by Engen and colleagues), an extensive community of microorganisms in the intestine that aid in normal gut function. These organisms affect the maturation and function of the immune system. Alcohol disrupts communication between these organisms and the intestinal immune system. Alcohol consumption also damages epithelial cells, T cells, and neutrophils in the GI system, disrupting gut barrier function and facilitating leakage of microbes into the circulation (see the article by Hammer and colleagues).

These disruptions to the composition of the gut microbiota and to gut barrier function have important implications beyond the intestinal system.

For example, Nagy discusses how the leakage of bacterial products from the gut activate the innate immune system in the liver, triggering inflammation that underlies ALD, a condition that affects more than 2 million Americans and which eventually may lead to liver cirrhosis and liver cancer. Infection with viral hepatitis accelerates the progression of ALD, and end-stage liver disease from viral hepatitis, together with ALD, is the main reason for liver transplantations in the United States. The article by Dolganiuc in this issue explores the synergistic effects of alcohol and hepatitis viruses on the progression of liver disease as well as alcohol consumption's injurious effect on liver antiviral immunity. Mandrekar and Ju contribute an article that homes in on the role of macrophages in ALD development, including recent insights into the origin, heterogeneity, and plasticity of macrophages in liver disease and the signaling mediators involved in their activation and accumulation.

In addition to pneumonia, alcohol consumption has been linked to pulmonary diseases, including tuberculosis, respiratory syncytial virus, and ARDS. Alcohol disrupts ciliary function in the upper airways, impairs the function of immune cells (i.e., alveolar macrophages and neutrophils), and weakens the barrier function of the epithelia in the lower airways (see the article by Simet and Sisson). Often, the alcohol-provoked lung damage goes undetected until a second insult, such as a respiratory infection, leads to more severe lung diseases than those seen in nondrinkers.

In a clinical case study reviewed in this issue, Trevejo-Nunez and colleagues report on systemic and organ-specific immune pathologies often seen in chronic drinkers. In such patients, alcohol impairs mucosal immunity in the gut and lower respiratory system. This impairment can lead to sepsis and pneumonia and also increases the incidence and extent of postoperative complications, including delay in wound closure. HIV/AIDS is a disease in which mucosal immunity already is under attack. Bagby and colleagues review substantial evidence that alcohol further disrupts the immune system, significantly increasing the likelihood of HIV transmission and progression.

Alcohol–immune interactions also may affect the development and progression of certain cancers. Meadows and Zhang discuss specific mechanisms through which alcohol interferes with the body's immune defense against cancer. They note, too, that a fully functioning immune system is vital to the success of conventional chemotherapy. The clinical management of all of these conditions may be more challenging in individuals who misuse alcohol because of coexisting immune impairment.

Alcohol consumption does not have to be chronic to have negative health consequences. In fact, research shows that acute binge drinking also affects the immune system. There is evidence in a number of physiological systems that binge alcohol intake complicates recovery from physical trauma (see the article by Hammer and colleagues). Molina and colleagues review research showing that alcohol impairs recovery from three types of physical trauma—burn, hemorrhagic shock, and traumatic brain injury—by affecting immune homeostasis. Their article also highlights how the combined effect of alcohol and injury causes greater disruption to immune function than either challenge alone.

Not only does the immune system mediate alcohol-related injury and illness, but a growing body of literature also indicates that immune signaling in the brain may contribute to alcohol use disorder. The article by Crews, Sarkar, and colleagues presents evidence that alcohol results in neuroimmune activation. This may increase alcohol consumption and risky decisionmaking and decrease behavioral flexibility, thereby promoting and sustaining high levels of drinking. They also offer evidence that alcohol-induced neuroimmune activation plays a significant role in neural degeneration and that the neuroendocrine system is involved in controlling alcohol's effects on peripheral immunity.

Much progress has been made in elucidating the relationship between alcohol consumption and immune function and how this interaction affects human health. Continued advances in this field face several challenges, however. The regulation of immune function is exceedingly complex. Normal immune function hinges on bidirectional communication of immune cells with nonimmune cells at the local level, as well as crosstalk between the brain and the periphery. These different layers of interaction make validation of the mechanisms by which alcohol affects immune function challenging. Significant differences between the immune system of the mouse—the primary model organism used in immune studies—and that of humans also complicate the translation of experimental results from these animals to humans. Moreover, the wide-ranging roles of the immune system present significant challenges for designing interventions that target immune pathways without producing undesirable side effects.

By illuminating the key events and mechanisms of alcohol-induced immune activation or suppression, research is yielding deeper insights into alcohol's highly variable and sometimes paradoxical influences on immune function. The insights summarized in this issue of *ARCR* present researchers and clinicians with opportunities to devise new interventions or refine existing ones to target the immune system and better manage alcohol-related diseases.

Epigenetic Targets for Reversing Immune Defects Caused by Alcohol Exposure

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Alcohol consumption alters factors that modify gene expression without changing the DNA code (i.e., epigenetic modulators) in many organ systems, including the immune system. Alcohol enhances the risk for developing several serious medical conditions related to immune system dysfunction, including acute respiratory distress syndrome (ARDS), liver cancer, and alcoholic liver disease (ALD). Binge and chronic drinking also render patients more susceptible to many infectious pathogens and advance the progression of HIV infection by weakening both innate and adaptive immunity. Epigenetic mechanisms play a pivotal role in these processes. For example, alcohol-induced epigenetic variations alter the developmental pathways of several types of immune cells (e.g., granulocytes, macrophages, and T-lymphocytes) and through these and other mechanisms promote exaggerated inflammatory responses. In addition, epigenetic mechanisms may underlie alcohol's ability to interfere with the barrier functions of the gut and respiratory systems, which also contribute to the heightened risk of infections. Better understanding of alcohol's effects on these epigenetic processes may help researchers identify new targets for the development of novel medications to prevent or ameliorate alcohol's detrimental effects on the immune system. **KEY WORDS: Alcohol consumption; alcohol exposure; alcoholism; chronic drinking; binge drinking; epigenetics; epigenetic mechanisms; epigenetic targets; DNA code; immune system; immune cells; innate immunity; adaptive immunity; infections; inflammation; gut; respiratory system; acute respiratory syndrome (ARDS); liver cancer; alcoholic liver disease (ALD)**

Extensive clinical and experimental data suggest that alcohol consumption has dose-dependent modulatory effects on the immune system that influence the two arms of the immune response (i.e., innate and adaptive immune responses). In many other organ systems, such as the brain and liver, alcohol consumption has been shown to alter factors that can modify gene expression without changing the DNA code (i.e., epigenetic modulators) and which play critical roles in mediating alcohol's effects. However, very few studies have focused on the effects of alcohol-mediated epigenetic alterations on immunity. Because chronic alcohol consumption is correlated with an exacerbated state of chronic inflammation (which is part of the innate immune response), researchers can apply knowledge of how epigenetic factors are dysregulated in inflammatory and autoimmune disorders to identify potential epigenetic targets that can be used to develop therapies for treating alcohol-abusing patients. This review summarizes how inflammatory mediators and both innate and adaptive immune responses are modulated by moderate, binge, and chronic alcohol consumption. The discussion further identifies and highlights exciting potential avenues to explore epigenetic regulation of these immune responses.

Epigenetics: An Overview

All cells within an organism carry identical genetic information in the form of DNA, yet a multitude of individual cell types arises during the course of development. These individualized cellular morphologies, characteristics, and functions result from the unique gene expression profiles of the different cell types. Regulation of gene expression profiles is critical not only during development, but also for cellular

proliferation, differentiation, environmental adaptation, stress, and immune responses throughout the individual's lifetime and is largely dependent on epigenetic mechanisms. An epigenetic trait is a stably heritable observable characteristic (i.e., phenotype) that results from changes in a chromosome without alterations in the DNA sequence (Berger et al. 2009). Epigenetic regulation can involve a variety of chemical modifications of the DNA (e.g., methylation) and the histone proteins around which the DNA is wrapped (e.g., methylation, acetylation, phosphorylation, ubiquitinylation, ADP-ribosylation, and sumoylation), as well as the actions of small molecules called noncoding microRNAs (miRNAs). Of these mechanisms, higher-than-normal DNA methylation (i.e., hypermethylation) and miRNAs generally are correlated with decreased protein production through gene-silencing mechanisms and posttranscriptional regulation (Carthew and Sontheimer 2009). Age, environment, and exposure to drugs and other toxins (e.g., alcohol) can directly influence the epigenetic profile of the organism (Feil and Fraga 2012).

It is well documented that alcohol exposure prior to an injury or infection dampens the immune system, resulting in a range of adverse outcomes, such as delaying infection clearance, extending hospital stays, and increasing morbidity and mortality compared with nonintoxicated patients (for a review, see Messingham et al. 2002). This has led to the development of the "two-hit hypothesis," where the first hit (i.e., alcohol exposure) exaggerates the organism's physiological responses to the second hit (i.e., injury or infection). Epigenetic memory may be a contributing factor in this process.

So how does the epigenetic memory work? Throughout evolution, eukaryotic cells have adapted so that a vast amount of genetic material has become organized and compacted into the nucleus by forming a higher-order structure known as chromatin. The basic building block of chromatin is the nucleosome, which comprises 147 base pairs of DNA wrapped around a core of eight small histone proteins. Nucleosomes undergo dynamic relaxation and condensation in the nucleus, a process requiring the activities of two sets of molecules:

- ATP-dependent chromatin remodeling complexes that physically tighten or loosen histone–DNA contacts; and
- Epigenetic modifying enzymes that add or remove posttranslational covalent modifications from the tails of the histone proteins, thus either allowing or preventing access of nuclear factors to the DNA that are needed for gene transcription.

Also known as the histone code, the intricate combination of covalent modifications on the histones directly influences DNA–histone binding by altering electrical charge and providing a specific docking signal for recruitment of chromatin-modifying complexes and transcriptional machinery to either block or promote active gene transcription (Jenuwein and Allis 2001; Strahl and Allis 2000). Some covalent modifications are typically associated with the same effect on transcription; for example, histone acetylation generally is associated with active gene transcription (Turner 2000). The effects of histone methylation are much more complex. Thus, the degree of methylation (i.e., mono-, di-, or trimethylation); the particular histone protein, and, more specifically, lysine residue(s) being modified (e.g., H3K4,[1] H3K9, H3K27, H3K36, H3K79, or H4K20); and the degree of chromatin condensation (i.e., condensed heterochromatin versus relaxed euchromatin) all play a role. Likewise, the part of the gene where the DNA or histone modification occurs (i.e., the genomic location)—that is, whether it occurs in a promoter, enhancer, or the gene body—influences whether a gene will be actively transcribed after lysine methylation (Bannister and Kouzarides 2005; Heintzman et al. 2007; Martin and Zhang 2005).

Specific enzymes are responsible for adding or removing acetyl or methyl moieties from histone tails. Histone acetyl transferases (HATs) and histone deacetylases (HDACs) add and remove acetyl groups, respectively. Similarly, methylation is tightly regulated by enzymes that add methyl groups to (i.e., methyltransferases) or remove methyl groups from (i.e., demethylases) specific lysine residues (Shilatifard 2006). So far, 18 HDACs have been identified and subdivided into four classes. Classes I, II, and IV require Zn^{2+} for enzymatic activity, whereas class III HDACs, also known as sirtuins, utilize a mechanism that requires the cofactor nicotinamide adenine dinucleotide (NAD^+) (Shakespear et al. 2011).

Several approaches may potentially be used to prevent or correct the epigenetic effects of alcohol consumption, such as alcohol-mediated immune defects. For example, inhibition of HDACs by molecular HDAC inhibitors (HDACis), alteration of DNA methylation on cytosine residues, or miRNA modulation all represent branches of possible therapeutic targets for restoring immune defects caused by alcohol exposure. These approaches will be discussed later in this review.

Epigenetics and Alcohol

Beverage alcohol (i.e., ethanol) is predominantly metabolized by the enzymes alcohol dehydrogenase (ADH), cytochrome p450 (CYP 450), and aldehyde dehydrogenase (ALDH) in the liver (Dey and Cederbaum 2006). This process produces oxidative metabolites such as acetaldehyde, acetate, acetyl-CoA, and reactive oxygen species (ROS), as well as nonoxidative products, such as phosphatidylethanol (PEth) and fatty acid ethyl ester (FAEE) (Best and Laposata 2003; Shukla and Aroor 2006; Shukla et al. 2001). Many of these products or metabolites can

[1] The standard nomenclature for histone modifications is to indicate both the histone protein (e.g., histone 3) and the specific amino acid affected. For example, in H3K4, the fourth lysine (abbreviated as K) of histone 3 is affected by epigenetic modification.

induce tissue-specific epigenetic changes (Choudhury and Shukla 2008; Shukla and Aroor 2006). Ethanol exposure leads to epigenetic alterations through several mechanisms, including enhancing the enzymatic activity of HATs; altering substrate availability for histone acetylation, DNA, and histone methylation; or by influencing miRNA production. For example, studies found the following:

- Ethanol exposure enhances the activity of a HAT called p300 in the liver of rats fed a chronic ethanol diet, which leads to heightened histone acetylation (Bardag-Gorce et al. 2007).

- Elevated ROS levels resulting from ethanol metabolism increase histone H3 acetylation in liver cells (i.e., hepatocytes) (Choudhury et al. 2010).

- Chronic alcohol exposure can mediate a shift in the ratio of reduced NAD^+ (NADH) to NAD^+, and this reduced redox state suppresses the activity of the redox-sensitive HDAC, SIRT1, thus augmenting histone acetylation in rats (Bardag-Gorce and French 2007; You et al. 2008).

- Ethanol metabolism dramatically increases production of acetyl-CoA, which is used in histone acetylation by HATs; consequently, ethanol exposure and metabolism amplifies the amount of substrate available for histone acetylation (Yamashita et al. 2001).

- Ethanol exposure causes dysregulated methionine metabolism, resulting in diminished production of a molecule called S-adenosylmethionine (SAMe), which serves as a methyl-group donor for both DNA and histone methylation (Lu and Mato 2005; Mason and Choi 2005; Shukla and Aroor 2006).

- Chronic ethanol exposure decreases the levels of the antioxidant glutathione, which serves as the predominant scavenger of ROS in the liver (Choudhury and Park 2010; Lu et al. 2000); this glutathione reduction leads to both regionally and globally reduced DNA methylation (i.e., hypomethylation) (Lee et al. 2004; Lertratanangkoon et al. 1997).

- Chronic ethanol exposure in rats leads to inhibition of a set of reactions called the ubiquitin–proteasome pathway, which helps eliminate molecules that are defective or no longer needed from the cell. This inhibition of the ubiquitin–proteasome pathway likely alters protein turnover of transcription factors and histone-modifying enzymes and is associated with epigenetic alteration at a specific lysine residue (K9) of histone H3 (i.e. increased H3K9-ac and reduced H3K9-me2) as well as DNA hypomethylation (Oliva et al. 2009).

- Acetylation of H3K9 also is associated with increased ADH1 expression in cultured rat hepatocytes treated with 100 mM ethanol for 24 hours, suggesting that ethanol (and its metabolites) may amplify ethanol metabolism (Park et al. 2005).

Through the various mechanisms discussed above, alcohol consumption can lead to multifactorial, dose-dependent, and tissue-specific epigenetic effects. For example, cultured primary rat hepatocytes demonstrated a dose- and time-dependent histone-acetylation response to ethanol exposure. Thus, cells treated with 5–100 mM ethanol for 24 hours exhibited a maximal, eightfold increase in H3K9-ac levels at 24 hours following treatment with the highest ethanol concentration (Park et al. 2003). Furthermore, histone acetylation seemed to be selective for the H3K9 residue, because acetylation of other H3 lysines (i.e., K14, K18, and K23) was unaffected by ethanol exposure (Park and Lim 2005; Park and Miller 2003). Similar findings were obtained in hepatic stellate cells (Kim and Shukla 2005). Finally, another study (Pal-Bhadra et al. 2007) found that hepatocytes treated for 24 hours with 50 mM and 100 mM ethanol also exhibited altered histone methylation status, resulting in increased H3K4 dimethylation (H3K4-me2) and decreased H3K9 dimethylation (H3K9-me2). However, unlike histone lysine acetylation, which was restored to baseline levels 24 hours after ethanol withdrawal in cultured hepatocytes, changes in histone lysine methylation status were not reversed and may provide a long-term epigenetic memory (Pal-Bhadra et al. 2007).

Ethanol metabolites, including acetaldehyde and acetate, also could cause H3K9-specific acetylation in rat hepatocytes. Interestingly, the signaling pathways activated by acetate and ethanol seemed to modulate H3K9-ac via different mechanisms. Thus, certain molecules (i.e., inhibitors of enzymes known as mitogen-activated protein kinases) prevented acetylation by ethanol but had no effect on the acetate-dependent formation of H3K9-ac (Park and Lim 2005). In addition to acetylation, ethanol and acetaldehyde exposure also promotes phosphorylation of histone H3 at serines 10 and 28 (Lee and Shukla 2007). Whereas ethanol exposure lead to similar phosphorylation levels at both serine 10 and serine 28, acetaldehyde generated greater phosphorylation at serine 28 than at serine 10 (Lee and Shukla 2007). These studies indicate that the complexity of ethanol-induced epigenetic changes increases even further when taking into account that ethanol metabolites also trigger epigenetic effects that may differ from those produced by ethanol exposure.

Rat models of acute/binge and chronic alcohol exposure have been utilized to examine the relationship between epigenetic gene regulation and alcohol exposure in vivo. In one of those models, a single dose of ethanol diluted in sterile water resulting in a concentration of 6 grams ethanol per kilogram bodyweight

(g/kg) was injected directly into the stomachs of 8-week-old male Sprague-Dawley rats. This high-dose binge-alcohol exposure model was used to compare H3K9 modification status across 11 different tissues at 1, 3, and 12 hours following ethanol exposure (Kim and Shukla 2006). The investigators found that in the testes, this alcohol exposure caused robust global increases in H3K9-ac at 1 hour but not at later time points. In contrast, in the lung and spleen robust increases in H3K9-ac were apparent at all three time points. In the liver, no marked elevation in H3K9-ac was observed at early (i.e., 1- or 3-hour) time points, but a profound elevation occurred at 12 hours. In addition, in the blood vessels, pancreas, colorectum, stomach, heart, brain, and kidney, no change in H3K9-ac was observed at any time-point tested. Finally, methylation of H3K9 was not altered in any tissue (Kim and Shukla 2006).

Other investigators evaluated changes in gene expression levels after chronic ethanol treatment using in vivo models. One of these models is the Tsukamoto-French rat model of alcoholic liver disease (Tsukamoto et al. 1985), in which male Wistar rats were fed a liquid diet containing a constant amount of alcohol (13 g/kg/day) for 30 days using an intragastric feeding tube. This treatment, which resulted in a 6- to 10-day cyclic pattern of urinary alcohol level (UAL) peaks (about 500 mg%) and troughs (about 100 mg%) (Bardag-Gorce and French 2002), allowed the investigators to compare gene expression profiles at high and low blood alcohol levels (BALs) by microarray analyses. These analyses identified dramatic changes in gene expression levels in the livers of the alcohol-treated rats. Overall, approximately 1,300 genes were dysregulated between BAL cycles (French et al. 2005), prompting additional studies aimed at elucidating the epigenetic contribution of alcohol-mediated transcriptional dysregulation in the liver and other tissues (Bardag-Gorce and French 2007; Kim and Shukla 2006; Park and Lim 2005). Furthermore, UAL peaks were associated with increased levels of the HAT, p300, which specifically transfers acetyl groups to H3K9 residues. This finding at least partially explains the selective H3K9 acetylation observed both in vitro and in vivo in correlation with ethanol exposure (Bardag-Gorce and French 2007). Finally, studies assessing the effects of changes in epigenetic mechanisms resulting from inhibition of the ubiquitin–proteasome pathway (using a drug called PS-341) or from chronic ethanol exposure in rats using the Tsukamoto-French model found increases in H3K9-ac levels, decreases in H3K9-me2 levels, and increased p300 levels in liver nuclear extracts (Oliva and Dedes 2009). These findings suggest that chronic ethanol exposure alters transcriptional regulation of a plethora of genes through many mechanisms that affect epigenetic modulators.

Understanding the role of nutrients in regulating epigenetic modifications will provide insight into potential dietary supplementation in chronic alcohol-abusing patients.

In summary, both acute/binge and chronic alcohol exposure can result in tissue- and cell-specific patterns of epigenetic responses. Future studies to determine the precise role of alcohol-mediated chromatin modifications hopefully will identify new epigenetic targets and pathophysiological mechanisms for regulating gene expression in diseases associated with alcohol consumption. The factors contributing to altered epigenetic modifications arising from acute versus chronic alcohol exposure may differ, because chronic alcohol exposure has been strongly correlated with nutrient deficiencies and a shift in the redox state. This implies that potential therapeutic interventions targeting epigenetic modifiers may need to differ depending on the degree of alcohol consumption. Furthermore, understanding the role of nutrients in regulating epigenetic modifications will provide insight into potential dietary supplementation in chronic alcohol-abusing patients.

Alcohol and the Immune System

A recent report from the Centers for Disease Control and Prevention (CDC) stated that alcohol abuse in the form of binge drinking (defined by the CDC as four or more drinks for women and five or more drinks for men in a short period of time) is the third-leading preventable cause of death in the United States, resulting in more than 80,000 deaths each year and enormous economic costs (i.e., more than $220 billion in 2006) (CDC 2012). A significant, positive correlation exists between the duration and amount of alcohol consumed and the risk for developing several serious medical conditions, including acute respiratory distress syndrome (ARDS) (Boe et al. 2009; Moss et al. 1999); liver cancer (i.e., hepatocellular carcinoma) (McKillop and Schrum 2009; Yamauchi et al. 1993); and alcoholic liver disease (ALD), which encompasses cirrhosis, hepatitis, and fibrosis (Gramenzi et al. 2006; Mann et al. 2003). Binge and chronic consumption (defined as more than eight drinks per day) renders patients more susceptible to various types of infection, such as hepatitis C virus infection in the liver and opportunistic infections in the respiratory system (e.g., ARDS and pneumonia), and advances the progression of HIV infection, largely through dysregulated immune responses (Baliunas et al. 2010; Bhatty et al. 2011; Prakash et al. 2002; Romeo et al. 2007*b*; Zhang et al. 2008) (figure 1).

The mammalian immune system is an elaborate network of molecules and cells that identify, combat, and eliminate harmful agents; it can be divided into two branches: innate and adaptive immunity. The innate immunity is

present from birth, whereas the adaptive immunity develops over the organism's life course with the continuous exposure to pathogens and other potentially harmful compounds.

The Innate Immune Response

Following pathogen or toxin exposure, the ancient innate immune response is responsible for immediate recognition, rapid attack, and destruction of foreign intruders and involves inflammatory reactions. Innate immune cells carry special molecules called Toll-like receptors (TLRs) on their surface that recognize and bind highly conserved structures on bacterial, fungal, or viral surfaces, including peptidoglycan, flagellin, zymosan, and lipopolysaccharide (LPS, also known as endotoxin) (Janeway and Medzhitov 2002). The innate-immune cells also activate the adaptive immune response by digesting the foreign intruders and then presenting certain molecules derived from these pathogens (i.e., antigens) on their surface for recognition by adaptive immune cells. This antigen presentation, which initiates the adaptive immune response and provides a "memory" of the initial recognition of the antigen, allows for a rapid immune response if the same infection occurs again in the future.

An important subset of innate immune cells are macrophages; they eliminate pathogens by a process called phagocytosis[2] and then present pathogen-derived molecules on their surface to activate

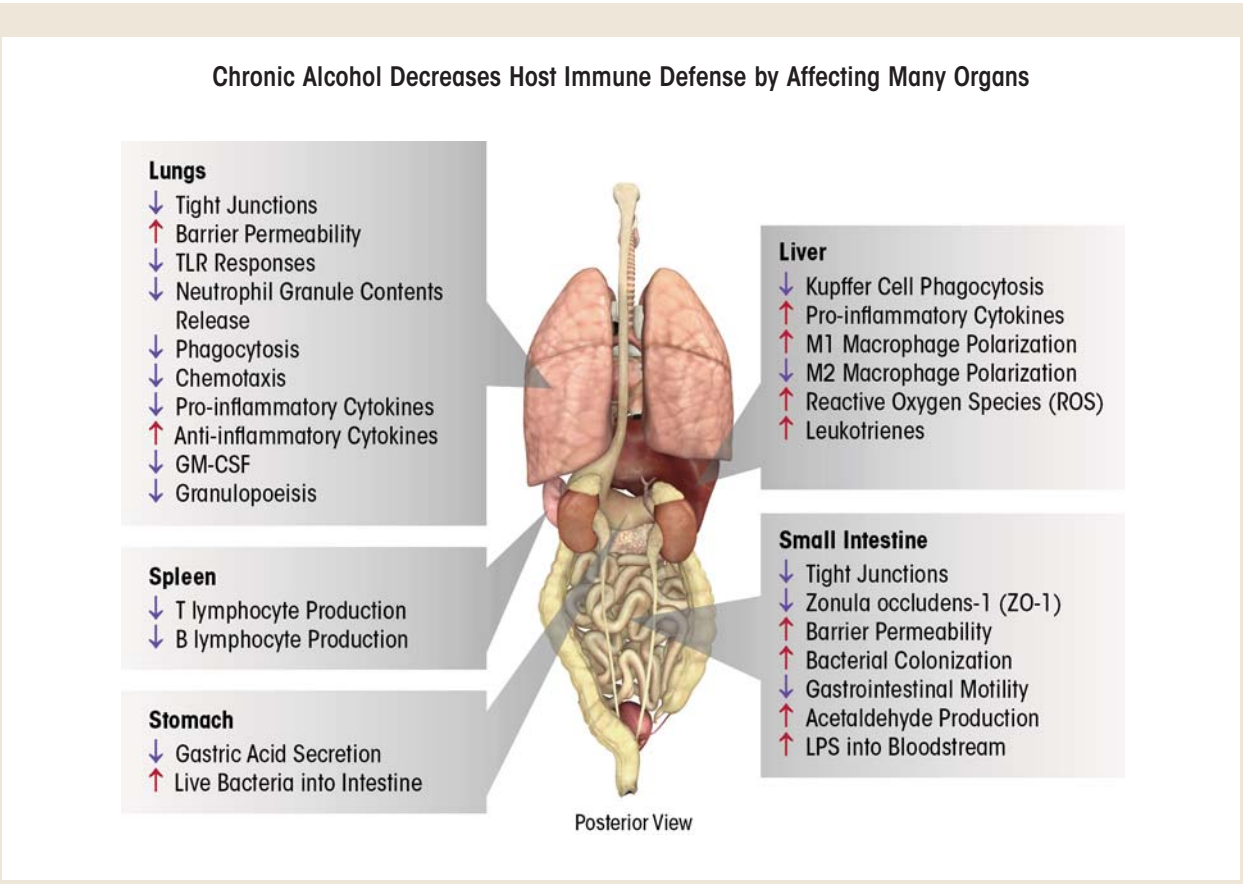

Figure 1 Chronic alcohol exposure causes immune dysfunction through effects on multiple organs. In the lungs, excessive inflammation causes tissue damage, increasing barrier permeability, and dampening many cellular immune responses, such as recognizing bacteria (through toll-like receptors [TLRs]), attacking pathogens (through phagocytosis), decreasing production of granulocytes (i.e., granulocytopenia) as well as their migration (i.e., chemotaxis), and altering important signaling and recruiting molecules (e.g., GM-CSF and chemokines). In the spleen, alcohol consumption affects immunity by decreasing T- and B-lymphocyte production. In the stomach, alcohol decreases gastric acid levels, allowing live bacteria to pass into the small intestine. Combined with decreased gastrointestinal motility, a byproduct of alcohol metabolism (i.e., acetaldehyde) increases intestinal barrier permeability by weakening cell–cell junctions, and allows bacterial toxins (i.e., lipopolysaccharide [LPS]) to pass into the bloodstream. LPS damages the liver, leading to excessive release of pro-inflammatory cytokines, leukotrienes, and ROS into the circulation. In addition, alcohol in the liver can alter macrophage (Kupffer cell) polarization and decrease phagocytosis.

adaptive immune cells. Macrophages can have alternate names based on their anatomical location; for example, macrophages residing in the liver are called Kupffer cells. Furthermore, macrophages can be subdivided into two groups based on their functional phenotype (Martinez et al. 2008) (see table 1):

- Classically activated (M1) macrophages, whose activation results in a proinflammatory response.
- Alternatively activated (M2) macrophages, whose activation results in an anti-inflammatory response.

After challenge to the immune system occurs (e.g., an infection), macrophages are generated by the maturation of precursor cells called monocytes. During this process, the macrophages can become either M1 or M2 macrophages; this is called macrophage polarization. The ratio of M1 to M2 macrophages changes depending on the presence of a variety of factors; this variability is known as macrophage plasticity and allows the organism to modulate the immune response. Accordingly, controlling macrophage plasticity is critical to first battle pathogens and then resolve the resulting inflammation to prevent tissue damage. Alcohol exposure skews macrophage polarization towards M1 (i.e., towards inflammation) in the liver (Louvet et al. 2011; Mandal et al. 2011), resulting in deleterious consequences (figure 2).

Dendritic cells (DCs) are an additional component of the innate immune response. They have an important role in linking the innate and adaptive branches of the immune system. To this end, the DCs exhibit proteins called major histocompatibility complex (MHCs) on their surface. With the MHC proteins, DCs present antigens to other cells that are part of the adaptive immune system—that is, B and T lymphocytes (also known as B and T-cells). DCs mature following stimulation by whole bacteria or LPS or after exposure to various signaling molecules, such as interleukin 1β (IL-1β), granulocyte macrophage colony–stimulating factor (GM-CSF), and tumor necrosis factor-α (TNFα) (Winzler et al. 1997). The mature DCs migrate to lymphoid organs to prime and activate naïve T-cells (Lee and Iwasaki 2007). Activated T-cells then complete the immune response by producing and releasing specific signaling molecules (i.e., cytokines) that will stimulate other innate immune cells or interact with B-cells, leading to the development of immune molecules (i.e., antibodies). Mature DCs also secrete high levels of IL-12 (Reis e Sousa et al. 1997), enhancing both innate and adaptive immune responses (summarized in table 2).

Alcohol consumption has a variety of effects on innate immune cells. For example, alcohol decreases the phagocytic activity of monocytes, macrophages, Kupffer cells, microglia, and DCs and diminishes their capacity to present antigens and produce the molecules necessary for microbe killing. In addi-

[2] During phagocytosis, the macrophage engulfs the foreign pathogen, thus ingesting it into the cell, where it is degraded and eliminated.

Table 1 Macrophages, Alcohol, and Potential Epigenetic Targets

	Subtype	Factors Contributing to Activation[1]	Major Roles Following Activation[1]	Defects Caused by Chronic Alcohol [2]	Potential Epigenetic Targets
Macrophages	M1 (Classical)	IFNγ Microbes	Engulf necrotic cells, toxic substances, and pathogens ↑ pro-inflammatory cytokines and reactive oxygen species (ROS) for direct pathogen killing and recruitment of other immune cells	Leads to predominant M1 polarization[3] Kupffer cells sensitized to endotoxin stimulation[3,4] ↑ Pro-inflammatory cytokines ↓ Phagocytic activity[5] ↓ Capacity to present antigen[6]	miR-155 promotes M2 polarization[7] Histone lysine demethylase, JmjD3, promotes transcription of M2-specific genes[8,9]
	M2 (Alternative)	Parasites Cytokines released by Th2, NK, basophils	↑anti-inflammatory cytokines Promote angiogenesis Promote wound healing	Macrophage polarization skewed towards M1 phenotype[3]	

SOURCES: [1] Gordon and Taylor, 2005 , [2] Goral et al., 2008 , [3] Thakur et al., 2007, [4] Mandrekar and Szabo, 2009, [5] Karavitis and Kovacs, 2011, [6] Szabo et al., 1993, [7] Ruggiero et al., 2009, [8] De Santa et al., 2007, [9] Satoh et al., 2010.

tion, alcohol alters expression of other proteins (i.e., pathogen pattern recognition receptors) on their cell surface that are required for cell–cell interactions among immune cells (for reviews, see (Goral et al. 2008; Karavitis and Kovacs 2011; Romeo and Warnberg 2007*b*). Furthermore, the levels of a type of immune cell called granulocytes often are very low in alcoholics with severe bacterial infections, which has been strongly correlated with increased mortality (Perlino and Rimland 1985). Finally, rodent models have demonstrated that following infection, alcohol significantly decreased both phagocytic activity and production of the signaling molecule granulocyte colony-stimulating factor (G-CSF) in a TNFα-dependent manner (Bagby et al. 1998) as well as blocked differentiation or maturation of granulocytes (i.e., granulopoiesis) (Zhang et al. 2009).

The Adaptive Immune Response

B-cells, T-cells, and antigen-presenting cells (APCs) are key players of the adaptive immune response. Like DCs, APCs present antigen to B and T-cells that have not yet been activated (i.e., naïve B and T-cells), contributing to their maturation and differentiation. Naïve T-cells are classified based on expression of specific proteins on their surface called cluster of differentiation (CD) proteins. Two of those proteins important in distinguishing different T-cell populations are CD4 and CD8. T-cells carrying the CD8 protein (i.e., $CD8^+$ cells) ultimately gain the ability to recognize and kill pathogens (i.e., become cytolytic T-cells). Conversely, $CD4^+$ T-cells give rise to several T helper (Th) cell subsets, including Th1, Th2, and Th17 cells, that will produce mutually exclusive groups of cytokines which help mount specific immune responses by stimulating other immune cells (Zygmunt and Veldhoen 2011) (table 3). Alcohol exposure can promote the development of Th2 cells over the other helper-cell populations. This shift in T helper differentiation towards Th2 is correlated strongly with defective immune responses as well as increased rates of infection, morbidity, and mortality (Cook et al. 2004; Romeo and Warnberg 2007*b*).

Epigenetic Modulation May Reverse M1 Polarization Caused by Chronic Alcohol Consumption

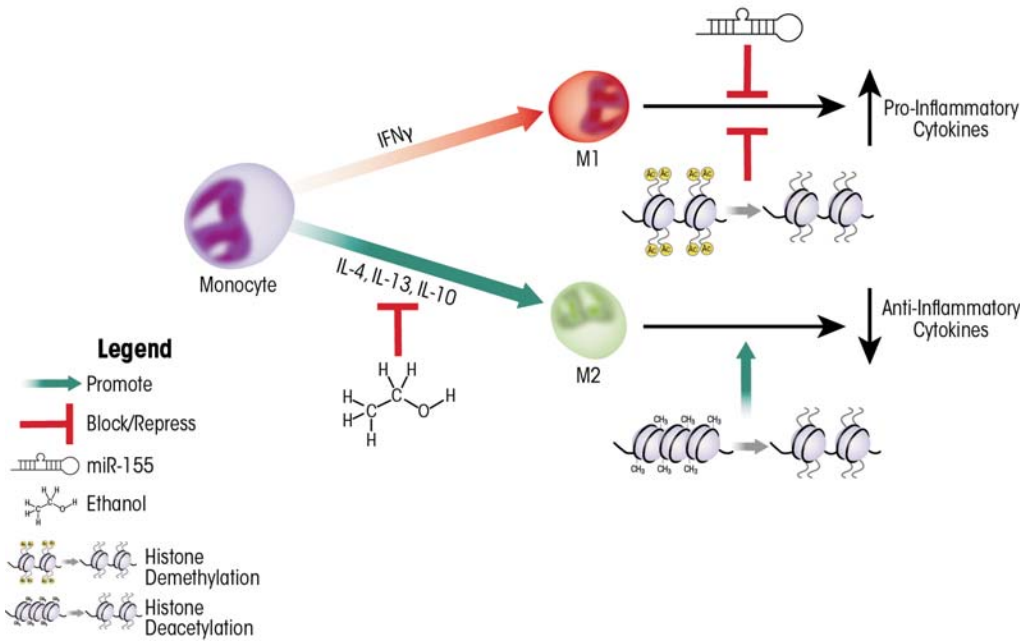

Figure 2 Chronic alcohol consumption skews macrophage polarization toward an M1 (i.e., pro-inflammatory) phenotype, leading to excessive or prolonged inflammation. Two approaches using epigenetic modulators—microRNA 155 (miR-155) and histone deacetylase inhibitors—can potentionally reverse protein translation or gene transcription of M1 pro-inflammatory cytokines. Another type of enzyme—histone lysine (H3K27) demethylases—increase transcription of M2 anti-inflammatory cytokines. Factors that increase protein levels or enhance activity of H3K27 demethylases therefore may potentially be utilized to promote M2 polarization.

The Effects of Alcohol Exposure on Innate Immune Cells and the Potential Role of Epigenetics

Epigenetics Play a Crucial Role in Innate Immune-Cell Differentiation and Maturation

During the early stages of blood cell formation (i.e., hematopoiesis), the developing cells fall into one of two developmental paths: the myeloid lineage, which includes granulocytes and monocytes (which then further differentiate into macrophages or DCs), and the lymphoid lineage, which includes B- and T-lymphocytes. This myeloid versus lymphoid lineage commitment corresponds with global and reduced DNA methylation, respectively (Ji et al. 2010). During infection, alcohol suppresses the development and maturation of granulocytes (i.e., granulopoiesis) (Zhang et al. 2009). Factors that increase DNA methylation, and therefore promote myeloid cell commitment, may serve as potential therapeutic targets for increasing granulocyte populations. Similarly, epigenetic factors play a crucial role in regulating monocyte terminal differentiation into DCs. Proper functioning of monocyte cells requires the expression of CD14, because it recognizes and binds LPS. DCs, however, do not utilize CD14, but instead require CD209 (DC-SIGN). Therefore, when monocytes differentiate into DCs, they lose expression of *CD14,* which is correlated with loss of epigenetic modifications associated with active transcription, including H3K9-Ac and H3K4me3. Concurrently, epigenetic changes occur within the *CD209* locus, leading to increased *CD209* transcription. The increase in *CD209* transcription is associated with loss of epigenetic modifications typically associated with transcriptional silencing, including DNA methylation and formation of H3K9me3 and H3K20me3 (Bullwinkel et al. 2011). In the future, therapeutics that specifically target epigenetic modifications within the *CD14* or *CD209* loci may be designed to direct monocyte terminal differentiation towards one particular cellular fate (Bullwinkel et al. 2011).

Epigenetic Regulation of Macrophage Polarization

Alcohol alters macrophage polarization in the liver—that is, it alters the normal ratio of M1 to M2 macrophages. Chronic alcohol exposure sensitizes Kupffer cells to LPS stimulation, leading to prolonged and predominant M1 polarization and the exacerbated release of pro-inflammatory cytokines (Mandrekar and Szabo 2009; Thakur et al. 2007). This shift in macrophage polarization is reversible, because recent studies demonstrated that a hormone produced by adipose cells (i.e., adiponectin), can shift Kupffer cells isolated from chronic alcohol-exposed rat livers towards M2 polarization (Mandal and Pratt 2011).

Another potential strategy for shifting Kupffer cell polarization is the use of therapeutic reagents that target epigenetic modifiers because epigenetic processes play central roles in the regulation of immune-system functions. For example, one critical mechanism to restore the internal balance (i.e., homeostasis) of the immune system in response to infection involves miRNA-dependent post-transcriptional regulation. Researchers found that expression of one specific miRNA called miR-155 was dramatically increased when macrophages derived from the bone marrow were stimulated by LPS. This enhanced miRNA expression served to fine-tune the expression of pro-inflammatory mediators and promote M2 polarization (Ruggiero et al. 2009). Similarly, ethanol exposure also can affect miR-155 expression. When a specific macrophage cell line (i.e., the RAW 264.7 macrophage cell line) was treated with 50 mM ethanol (corresponding to a BAL of 0.2 g/dl, which commonly is observed in chronic alcoholics), miR-155 expression was significantly enhanced (Bala et al. 2011). Ethanol treatment prior to stimulation with LPS further augmented miR-155 production, and a linear, significant correlation existed with increased TNFα production, likely because miR-155 increased TNFα mRNA stability (Bala and Marcos 2011). Finally, a murine model of ALD confirmed increased miR-155 and TNFα levels in Kupffer cells isolated from ethanol-treated animals compared with control animals, suggesting that miR-155 is an important regulator of TNFα in vivo and likely contributes to the elevated TNFα levels often observed in chronic alcoholics (Bala and Marcos 2011).

Besides ethanol-induced production of miR-155, histone modifications also can regulate macrophage polarization. As mentioned earlier, macrophages and other innate immune cells carry TLRs on their surface that can interact with LPS and other molecules, leading to the activation of the TLRs. Studies have demonstrated that when TLR4 was stimulated by LPS, histone acetylation and H3K4 tri-methylation (both of which are associated with active gene transcription) occurred in DNA regions encoding several pro-inflammatory cytokines (Foster et al. 2007; Takeuch and Akira 2011). Macrophage stimulation using the cytokine IL-4 and LPS also induced expression of an H3K27 histone lysine demethylase enzyme called Jumonji Domain Containing-3 (JmjD3/Kdm6b), causing transcription of specific M2-associated genes (De Santa et al. 2007; Satoh et al. 2010). The role of this demethylase is further supported by studies using cultured cells or mice in which specific genes were inactivated (i.e., knockout mice) that demonstrated that JmjD3/Kdm6b activity was not required for mounting antibacterial M1 responses, but was essential for M2 responses following exposure to a molecule (i.e., chitin) found in fungi and other parasites (Bowman and Free 2006; Satoh and Takeuchi 2010). Taken together, these findings suggest that epigenetic regulation of factors that specifically alter macrophage polarization may be able to shift and/or restore the normal M1/M2

physiological balance in alcohol-exposed patients (also see table 1 and figure 2).

The Effects of Alcohol Exposure on Adaptive Immunity and the Potential Role of Epigenetics

The Potential Role of Epigenetics in Reversing Th2 Polarization

Alcohol exposure impairs IL-12 production by DCs and IL-23 production by macrophages, thereby skewing T helper cell commitment towards a Th2 lineage (Happel et al. 2006; Mandrekar et al. 2004). Lysine methylation at histone H3K27 plays an important role in regulating transcription of the *IL-12* gene and thereby regulating DC activation (Wen et al. 2008). Accordingly, the development and use of drugs that target H3K27-specific histone methyltransferases or demethylases to treat diseases associated with alcoholism are a promising, future endeavor (see table 2).

T-cell production also is modulated by alcohol consumption, but at least some of the effects may be both gender- and dose-dependent. For example, moderate daily consumption of one beer by women or two beers by men for 30 days caused significantly higher abundance of $CD3^+$ T-cells in women, but not in men (Romeo et al. 2007*a*). Conversely, in male mice, chronic alcohol exposure was correlated with decreased $CD4^+$ and $CD8^+$ T-cells in the spleen and thymus (Saad and Jerrells 1991) and increased free (i.e., soluble) CD8 in the blood. This soluble CD8 can bind T-cell receptors, block activation by APCs, and thus impede viral clearance (Jerrells et al. 2002), indicating a way through which chronic alcoholism can impair the immune response. These findings indicate that drugs that can enhance cytokine production by the limited, inefficient T-cells found in alcoholics may restore the immune response. HDACis may be one such approach because histone deacetylation inhibits transcription of the gene encoding IL-4 (i.e., *Il4*) and inhibition of deacetylation accordingly could promote IL-4 production (Valapour et al. 2002). Drugs targeting DNA methylation also may be beneficial because DNA methylation plays an important role in regulating the transition of naïve T-cells to either Th1 or Th2 cell fates. Specifically, when naïve T-cells transition into Th2 cells, certain regions of the Il4 loci (specifically the 5' region) become hypomethylated. Conversely, when transitioning to Th1 cells, the 3' region of *Il4* becomes hypermethylated, demonstrating that a highly complex system of methylation/demethylation mediates T helper cell differentiation (Lee et al. 2002; Mullen et al. 2002). Treatment of T-cell lines with an agent called 5-azacytidine, which inhibits DNA methylation, leads to the production of cytokines not normally produced by these cells, including IL-2 and IFNγ (Ballas 1984; Young et al. 1994). This effect may help to restore the defective Th1 response in patients abusing alcohol (also see table 3 and figure 3).

The Effects of Chronic Alcohol and Inflammation and the Potential Role for Epigenetics

Chronic alcoholism is correlated with excessive or prolonged inflammation, caused in part through an overactive innate immune response and elevated oxidative stress (Khoruts et al. 1991). Studies have demonstrated that circulating levels of the pro-inflammatory cytokines TNFα, IL-1β, and IL-6 were much higher in alcoholics than in healthy nondrinkers (Khoruts and Stahnke 1991). The higher circulating levels of these cytokines resulted from increased production of pro-inflammatory cytokines by circulating monocytes and resident tissue macrophages, including Kupffer cells (for a review, see Cook 1998). These cells were also more sensitive to stimulation by LPS, which further exacerbated TNFα secretion and contributed to cytotoxicity (Schafer et al. 1995). The increased sensitivity to LPS stimulation partially was caused by decreased production of the anti-inflammatory cytokine, IL-10, which negatively regulates TNFα

Table 2 Dendritic Cells, Alcohol, and Potential Epigenetic Targets

Factors Contributing to Activation[1]	Major Roles Following Activation[2]	Defects Caused by Chronic Alcohol	Potential Epigenetic Targets
Whole bacteria LPS IL-1β GM-CSF, TNFα	Migrate to lymphoid organs and present antigens to naïve T and B lymphocytes ↑IL-12 to enhance innate and adaptive immunity[5]	↓ IL-12 production[3]	Histone lysine methylation (H3K27) controls transcription of the IL-12 gene[4]

SOURCES: [1] Winzler et al., 1997 , [2] Lee and Iwasaki, 2007, [3] Reis e Sousa et al, 1997, [4] Mandrekar et al., 2004, [5] Wen et al., 2008.

secretion by monocytes (Le Moine et al. 1995). Thus, chronic alcohol exposure disrupts the delicate and precise regulation of inflammatory regulators.

To assess alcohol's effects on the inflammatory responses of macrophages, researchers have used a human monoblastic cell line, MonoMac6, which has many features of mature macrophages and has been used to model Kupffer cell responses (Zhang et al. 2001). Preliminary studies demonstrated that prolonged (i.e., 7 day) exposure of these cells to high-dose (86 mM) ethanol dramatically enhanced pro-inflammatory cytokine responses following LPS stimulation and was correlated with increased histone H3 and H4 global acetylation, as well as elevated acetylation of specific cytokine gene promoters, including those encoding *IL-6* and *TNF* (Kendrick et al. 2010). This increased acetylation was dependent upon conversion of ethanol to its metabolites, acetate and acetyl-coA, by two enzymes called acetyl-coenzyme A synthetase short-chain family members 1 and 2 (ACSS1 and ACSS2) and also was associated with a significant decrease in HDAC activity (Kendrick and O'Boyle 2010). Interestingly, unlike with rat hepatocytes and hepatic stellate cells, no global modulation of histone acetylation was observed with acute ethanol treatment (Kendrick and O'Boyle 2010).

ACSS1 and ACSS2 only are activated for acetate and acetyl-CoA formation during ethanol metabolism but not during normal sugar metabolism that also results in acetyl-CoA generation. Therefore, they represent an exciting potential therapeutic target for reducing the exacerbated inflammatory response observed with chronic alcohol exposure because their depletion should not alter normal cellular metabolism and energy generation. Another potential approach to restoring cytokine homeostasis may be to reduce proinflammatory cytokine transcription by administering drugs that increase HDAC recruitment to actively transcribed chromatin (e.g., theophylline), thereby counteracting the decreased HDAC activity induced by chronic ethanol exposure (Kendrick and O'Boyle 2010).

Although drugs that modulate epigenetic targets have not yet been used specifically to treat alcohol-induced inflammation, research of other inflammatory and autoimmune diseases suggest that epigenetic modulation plays a critical role in regulating the inflammatory cytokine network (Ballestar 2011; Halili et al. 2009; Rodriguez-Cortez et al. 2011). Accordingly, agents that normalize this epigenetic modulation (e.g., HDACis) are a promising therapy for the treatment

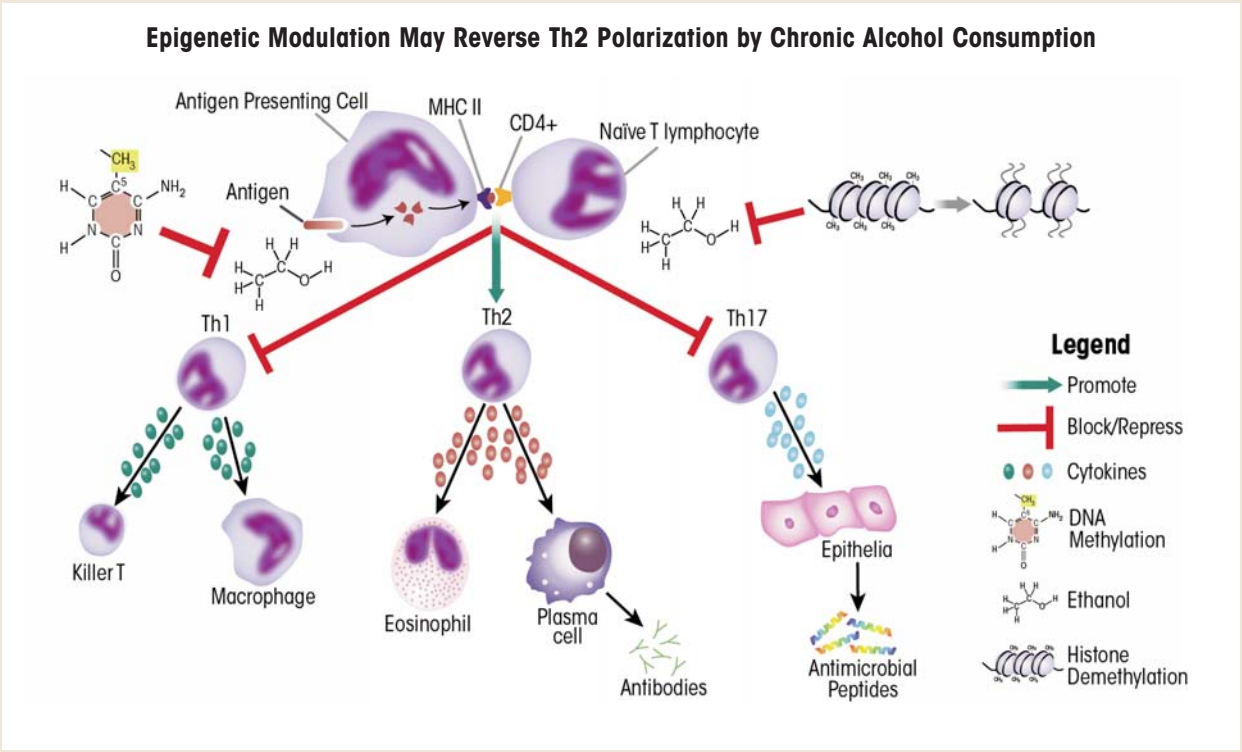

Figure 3 Alcohol-induced T helper cell polarization towards a Th2 phenotype suppresses immune responses. Alcohol decreases IL-12 production by antigen presenting cells, resulting in fewer naïve T-cell differentiating into Th1 cells, and blocks the release of IL-23 from macrophages, thereby preventing Th17 differentiation. Methylation of DNA or histones (H3K27) may reverse Th2 polarization.

of inflammatory and autoimmune diseases, including the exacerbated inflammation observed with chronic alcohol exposure. HDACis are efficacious in animal models of inflammatory bowel disease, septic shock, graft-versus-host disease, and rheumatoid arthritis (Bodar et al. 2011; Halili and Andrews 2009; Joosten et al. 2011; Reddy et al. 2004, 2008). Furthermore, the HDACi vorinostat has been used in clinical trials for reducing the severity of graft-versus-host disease in patients with bone marrow transplants (Choi and Reddy 2011), and the HDACi givinostat has been studied for the treatment of several other inflammatory conditions. These HDACis originally were developed to increase transcription of genes that induce cell death (i.e., apoptosis) of malignant cells. The doses of HDACi required to diminish inflammatory processes, however, are dramatically lower than the doses required for cancer treatment, and minimal side effects have been reported (Dinarello 2010; Vojinovic and Damjanov 2011). The importance of lysine acetylation as a regulatory mechanism has been supported by a study characterizing the entirety of all proteins that are acetylated in the human body (i.e., the human lysine acetylome). This study identified 1,750 proteins that could be acetylated on lysine side chains, including proteins involved in diverse biological processes, such as the processing of mRNAs (i.e., splicing), cell-cycle regulation, chromatin remodeling, and nuclear transport (Choudhary et al. 2009). In fact, protein acetylation may be as important as phosphorylation in governing cellular processes (Choudhary and Kumar 2009; Kouzarides 2000). For example, acetylation of proteins in the fluid filling the cell (i.e., the cytosol) can either activate or block essential signaling cascades and may partially explain how low-dose HDACi treatment decreases the production of pro-inflammatory cytokines (Dinarello et al. 2011).

It is important to note that the development of selective HDACis may be complicated by the fact that most HDACs are components of multi-protein complexes, which often include other HDACs (Downes et al. 2000; Fischle et al. 2001). Therefore, it is possible that inhibition of one HDAC inadvertently may alter the activity of other HDACs present in the complex. It also is likely that some functional redundancy exists among HDACs as well as within the biological inflammatory pathways they regulate. Moreover, the role of individual HDACs is tissue and cell-type specific; accordingly, development of specific HDACi molecules for treatment of each particular inflammatory disease will require cell- or tissue-targeting components.

Alcohol Abuse and Leaky Barriers

Another important component of the innate immune system are the epithelial cells that line the outer surfaces of exposed tissues, such as the skin, respiratory, gastrointestinal (GI), and urogenital tracts. These cells provide a physical barrier that impedes pathogen invasion by forming strong intercellular associations (Tam et al. 2011; Turner 2009). Another critical function of epithelial cells in the innate immune system is their production of cytokines and chemokines in response to pathogen

Table 3 T-Cells, Alcohol, and Potential Epigenetic Targets

T-Cells Subtype	Major Roles Following Activation by Specific Antigen-Presenting–Cell Interaction	Defects Caused by Chronic Alcohol	Potential Epigenetic Targets
$CD8^+$Cytolytic T-cells	Direct pathogen killing	↓ $CD8^+$ production in spleen and thymus[1] ↑ soluble CD8→ blocks APC activation[2]	
$CD4^+$T helper 1 (Th1)	↑ IFNγ → activates macrophages and cytolytic T-cells	↓ $CD4^+$ production in spleen and thymus[1] ↓IL-12 production by DC→ ↓Th1 lineage specification[3]	↓ DNA methylation →↑transcription of the gene coding for IFNγ (*Ifng*)[4]
$CD4^+$T helper 2 (Th2)	↑ IL-4, IL-5, IL-13 → activates eosinophils ↑antibody production by plasma cells Important for humoral immunity and allergic response	↓ $CD4^+$ production in spleen and thymus[1] ↓$Th1^+$ and ↓Th17→ Th2 predominates	↑ DNA methylation → ↓ transcription of gene coding for IL-4 (Il4)[5] ↑ histone acetylation → ↓ Il4 transcription[6]
$CD4^+$T helper 17 (Th17)	↑ IL-17, IL-17F, IL-21, IL-22, IL-23, IL-26→ ↑Antimicrobial peptides Important for mucosal barrier maintenance and immunity	↓ CD4+ production in spleen and thymus[1] ↓IL-23 production by macrophages→ ↓Th17 lineage specification[7]	

SOURCES: [1] Saad and Jerrells, 1991, [2] Jerrells et al., 2002, [3] Mandrekar et al., 2004, [4] Young et al., 1994, [5] Lee et al., 2002, [6] Valapouret al., 2002, [7] Happel et al., 2006.

detection. (Elias 2007; Izcue et al. 2009; Parker and Prince 2011; Quayle 2002; Schleimer et al. 2007; Tracey 2002). Alcohol abuse is strongly correlated with defective, leaky barriers, particularly in the GI and respiratory tracts (Bhatty and Pruett 2011; Purohit et al. 2008).

The Effect of Alcohol on the Gut and the Potential Role of Epigenetics

Chronic alcohol consumption increases microbial colonization and LPS accumulation in the small intestine by decreasing gastric acid secretion in the stomach and delaying GI motility (Bienia et al. 2002; Bode and Bode 1997; Bode et al. 1984). The intestinal epithelial barrier must allow water and nutrients to pass freely, yet prevent transfer of larger macromolecules. Whereas the epithelial cells themselves are impermeable to substances dissolved in water (i.e., hydrophilic solutes), the space between the cells (i.e., paracellular space) must be sealed to maintain this barrier function. A leaky intestinal barrier is deleterious because it allows transfer of potentially harmful macromolecules and bacterial products (e.g., LPS) into the blood and lymph (Rao 2009). If it reaches the liver, LPS can target multiple cell types there, including Kupffer cells, neutrophils, hepatocytes, sinusoidal endothelial cells, and stellate cells (Brun et al. 2005; Duryee et al. 2004; Hoek and Pastorino 2002; Paik et al. 2003). Activation of these cells results in the release of pro-inflammatory mediators, such as ROS, leukotrienes, chemokines, and cytokines (e.g., TNFα and IL-1β), thereby directly contributing to liver damage and prolonged inflammation in chronic alcohol-abusing patients (Albano 2008; Brun and Castagliuolo 2005; Khoruts and Stahnke 1991; McClain et al. 2004).

The multifactorial contributions of chronic alcohol consumption to the development of ALD largely have been deciphered using rodent models. For example, investigators demonstrated a direct translocation of LPS across the gut mucosa in rats continuously administered alcohol directly into the stomach for 9 weeks (Mathurin et al. 2000). Other studies using mice in which the TNF-receptor 1 (TNF-R1) was removed (i.e., TNF-R1 knockout mice) and that were treated continuously with alcohol for 4 weeks determined that the alcohol-induced presence of LPS in the blood (i.e., endotoxemia) led to the release of TNFα from Kupffer cells, that in turn played a direct role in ALD (Yin et al. 1999). TNFα production is negatively regulated by H3K9 methylation (Gazzar et al. 2007), indicating that histone methylation can play a role in regulating inflammatory processes. This observation suggests that the prolonged inflammatory state associated with chronic alcohol exposure partially may be controlled by drugs targeting H3K9-specific demethylase enzymes.

Although alcohol itself does not alter intestinal permeability, one of the products of alcohol metabolism (i.e., acetaldehyde) increases barrier permeability in a dose-dependent manner (Basuroy et al. 2005) by disrupting intercellular connections, including both tight and adherens junctions (Atkinson and Rao 2001). One of the critical proteins ensuring the functionality of tight junctions is called zonula occludens 1 (ZO-1), and disrupted ZO-1 complexes are strongly correlated with increased intestinal barrier permeability (Walker and Porvaznik 1978). Interestingly, studies using a human intestinal cell line called Caco-2 found that ZO-1 production is regulated by microRNA-212 (miR-212). When these cells were cultured in the presence of 1 percent alcohol for 3 hours, they contained 71 percent less ZO-1 compared with cells not treated with alcohol. Moreover, the expression of miR-212 increased with alcohol treatment in a concentration-dependent manner; thus, cells treated with 1 percent alcohol for 3 hours had 2-fold higher expression of miR-212. These changes corresponded with defective tight junction morphology. Importantly, studies of colon samples taken from patients with ALD found significantly increased miR-212 expression compared with healthy control subject, and this increase paralleled a decrease in ZO-1. These findings demonstrate that miR-212 may play an important role in leaky intestinal barriers in ALD patients (Tang et al. 2008).

The Effect of Alcohol on the Respiratory System and the Potential Role of Epigenetics

Mucosal organ leakiness also contributes to respiratory infections, partially by altering tight junctions between epithelial cells lining the air sacs in lungs where gas exchange occurs (i.e., the alveoli) (Simet et al. 2012). This leaky barrier provides the ideal opportunity for bacteria normally found in the body (i.e., commensal bacteria), such as *Streptococcus pneumoniae,* to invade the tissues and become pathogenic (Bhatty and Pruett 2011). In fact, alcohol consumption is correlated with increased incidence of community-acquired pneumonia, with approximately 50 percent of adult pneumonia patients reporting a history of alcohol abuse (Goss et al. 2003). Furthermore, alcohol abuse worsens complications from pneumonia (Saitz et al. 1997) and increases mortality (Harboe et al. 2009) in a dose-dependent manner (Samokhvalov et al. 2010). Alcohol also shifts the cytokine balance in the lung, contributing to the development of ARDS (Boe and Vandivier 2009; Crews et al. 2006; Moss and Steinberg 1999).

When an infection occurs, neutrophils and monocytes are recruited to the lungs (Goto et al. 2004). Upon activation, monocytes differentiate into alveolar macrophages, which play a crucial role in the clearance of *S. pneumoniae* (Goto and Hogg 2004). Rodent models have demonstrated that chronic alcohol exposure contributed to increased infection susceptibility by causing mucosal organ leakiness, as well as defective leukocyte recruitment and decreased neutrophil maturation, adhesion, chemotaxis, and phagocytosis.

These changes partly resulted from faulty production of important signaling molecules, including G-CSF, GM-CSF, IL-8, IL-6, macrophage inflammatory protein (MIP-2), and CXC chemokine cytokine-induced neutrophil chemoattractant (CINC) (Boe et al. 2001). Alcohol also affected anti-inflammatory mediators by increasing the production of IL-10 and TGF-β (Boe and Vandivier 2009). Furthermore, chronic alcohol exposure inhibited the responses of $CD8^+$ T-cells , which increased the morbidity and mortality associated with influenza virus infection (Meyerholz et al. 2008), and decreased IFNγ production following infection with *Klebsiella pneumoniae* (Zisman et al. 1998) in murine models.

Several strategies targeting epigenetic regulatory mechanisms may be effective in the treatment of alcohol-induced lung infections. For example, therapies that restore neutrophil recruitment to infected lungs through regulation of cytokine production would be beneficial. In support of this notion, it was demonstrated that pretreatment with G-CSF prior to alcohol exposure and *K. pneumoniae* infection was protective in mouse models (Nelson et al. 1991). Targeting miRNAs for treatment of inflammatory lung diseases, such as ARDS, offers an additional, novel therapeutic approach because the production of several miRNAs, including miR-9, miR-146a, miR-147, miR-148, and miR-152, was induced by LPS stimulation in mouse lungs (Bazzoni et al. 2009; Liu et al. 2009, 2010; Nahid et al. 2009; Taganov et al. 2006; Tili et al. 2007; Zhou et al. 2011). Several of these upregulated miRNAs created a negative feedback loop to prevent excessive production of pro-inflammatory cytokines, therefore contributing to immune regulation and homeostasis (Bazzoni et al. 2009; Liu et al. 2009, 2010). Although most research focused on understanding the role of miRNAs in inflammatory lung disease has been performed using animal models, future studies using human cell lines, tissues, and eventually patient samples clearly are warranted.

Summary

The relationship between alcohol exposure and altered immune responses is complex. Chronic alcohol abuse is correlated with increased susceptibility to infection and causes tissue damage from an overactive innate immune response, excessive oxidative stress, and exacerbated or prolonged inflammation. Alcohol exposure has tissue- and immune cell-type–specific effects, such as influencing cell recruitment to infected or inflamed tissue, altering cytokine and chemokine production and secretion, skewing differentiation towards a particular cell fate or preventing cell replication, impairing antigen presentation, interfering with phagocytosis and granulopoiesis, or inducing apoptosis. Although the specific role of epigenetic modulation in this alcohol-induced immune dysregulation has not yet been determined, research in related fields strongly suggests that experimental and clinical studies are warranted. ■

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Circadian Disruption

Potential Implications in Inflammatory and Metabolic Diseases Associated With Alcohol

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Circadian rhythms are a prominent and critical feature of cells, tissues, organs, and behavior that help an organism function most efficiently and anticipate things such as food availability. Therefore, it is not surprising that disrupted circadian rhythmicity, a prominent feature of modern-day society, promotes the development and/or progression of a wide variety of diseases, including inflammatory, metabolic, and alcohol-associated disorders. This article will discuss the influence of interplay between alcohol consumption and circadian rhythmicity and how circadian rhythm disruption affects immune function and metabolism as well as potential epigenetic mechanisms that may be contributing to this phenomenon. **KEY WORDS: Alcohol consumption; alcohol-related disorders; disease factors; risk factors; circadian disruption; circadian rhythm; circadian clock; immune function; metabolism; inflammatory diseases; metabolic diseases; epigenetic mechanisms**

Circadian Disruption and Society

The circadian clock is a sophisticated mechanism that functions to synchronize (i.e., entrain) endogenous systems with the 24-hour day in a wide variety of organisms, from simple organisms such as fungi up to the complex mammalian systems. Circadian rhythms control a variety of biological processes, including sleep/wake cycles, body temperature, hormone secretion, intestinal function, metabolic glucose homeostasis, and immune function. Functional consequences of modern-day society, such as late-night activity, work schedules that include long-term night shifts and those in which employees change or rotate shifts (i.e., shift work), and jet lag are substantial environmental disruptors of normal circadian rhythms. Fifteen percent of American workers perform shift work (Bureau of Labor Statistics 2005), indicating the pervasiveness of circadian disruption as a normal part of modern-day society. This change from the diurnal lifestyle of our ancestors to one that is more prominently nocturnal results in misalignment between natural rhythms based on the 24-hour day and behavioral activity patterns (i.e, circadian misalignment). Circadian misalignment has a significant detrimental effect on cell, tissue, and whole-organism function. These alterations can manifest in humans as chronic health conditions, such as metabolic syndrome,[1] diabetes, cardiovascular disease, cancer, and intestinal disorders (Karlsson et al. 2001; Morikawa et al. 2005; Schernhammer et al. 2003; Penev et al. 1998; Caruso et al. 2004). The increased prevalence of diseases associated with circadian disruption underscores the need to better understand how circadian disruption can wreak havoc in so many different ways throughout the body.

[1] Metabolic syndrom is a combination of disorders that can lead to diabetes and cardiovascular disease, characterized by abnormal levels of fat and/or cholesterol in the blood and insulin resistance.

Central and Peripheral Circadian Rhythms

The master or central circadian clock (i.e., "pacemaker") is located in the suprachiasmatic nucleus (SCN) in the anterior hypothalamus in the brain (Turek 1981) (see figure 1). The SCN is regulated by light stimulating retinal ganglion cells in the eye (Berson et al. 2002), and it is by this mechanism that light directs central circadian rhythms. Circadian rhythms are found in nearly every cell in the body, including the periphery, encompassing the immune system, heart, adipose tissue, pancreas, and liver (Allaman-Pillet et al. 2004; Boivin et al. 2003; Storch et al. 2002;

Yoo et al. 2004; Zvonic et al. 2006). The SCN synchronizes circadian rhythms found in the periphery (figure 2A) via several mechanisms, including communication with nerve cells that influence visceral functions such as digestion, heart rate, etc., via direct release of the hormones oxytocin and vasopression into the general vasculature or indirectly via release of local signals that affect the release of hormones from the anterior pituitary gland (i.e., neuroendocrine and autonomic neurons) (Buijs et al. 2003). In addition, peripheral circadian rhythms can be regulated by external factors other than central light-entrained rhythms. For instance, abnormal feeding patterns can cause peripheral circadian rhythms (i.e., in the intestine and liver) to become misaligned with central rhythms if feeding is out of synch with the normal 24-hour pattern, a phenomenon that can be observed in both animals and humans (see figure 2B). Peripheral tissues express self-sustained rhythms that are able to function independent of the central clock in the SCN. For example, following SCN lesion that terminates central circadian rhythmicity, peripheral circadian clocks continue to demonstrate rhythmicity; however, peripheral rhythms become desynchronized from each other over time (Yoo et al. 2004) (see figure 2C). This internal misalignment is particularly detrimental because peripheral circadian clocks directly regulate up to 5 to 20 percent of the genome (i.e., so-called clock-controlled genes) (Bozek et al. 2009). Furthermore, reports indicate that 3 to 20 percent of the entire genome demonstrates 24-hour oscillations in gene expression, including genes critical for metabolic processes. This observation suggests that although not directly controlled by the circadian clock, genes are influenced as a consequence of rhythmic changes in transcription factors and transcriptional (i.e., the process of creating a complementary RNA copy of a sequence of DNA) and translational (i.e., when RNA is used to produce a specific protein) modifiers (i.e., proteins controlling the levels and activity of various processes including lipid metabolism and glucose synthesis) (Panda et al. 2002).

At the cellular level, circadian rhythms originate from self-sustained, autoregulated, cyclic expressions of clock genes, which constitute the molecular clock. The molecular circadian clock consists of transcriptional activators and repressors—that is, proteins that stimulate and repress the production of proteins, respectively, in a cyclic process that is approximately 24-hours in duration (Reppert and Weaver 2002). The molecular circadian cycle is initiated when the transcriptional activators Clock and Bmal1 (Bunger et al. 2000) combine (i.e., heterodimerize) to stimulate the transcription of target circadian genes including period (*Per*) and cryptochrome (*Cry*) (i.e., *Per1* to *Per3* and *Cry1* and *Cry2*) as well as a host of other clock-controlled genes. When PER and CRY proteins accumulate in the cytosol, they heterodimerize and translocate to the nucleus where they act as transcriptional repressors to terminate CLOCK-BMAL1–mediated transcription, thus ending the molecular circadian cycle (van der Horst et al. 1999) (see figure 3). The cycle is further regulated by additional proteins, including the enzyme sirtuin 1 (SIRT1), a histone deacetylase that modifies circadian proteins or DNA by removing acetyl groups to alter gene expression. SIRT1 is sensitive to levels of the coenzyme nicotinomide adenine dinucleotide (NAD^+), making NAD availability a potential regulator of the molecular circadian clock (Grimaldi et al. 2009). The details of this oscillating cycle are found elsewhere (Reppert and Weaver 2002).

Demonstrating the importance of the molecular circadian clock, mutations of the core circadian clock components can have a devastating effect on the function of the circadian clock. This is true for both *Bmal1* (Bunger et al.

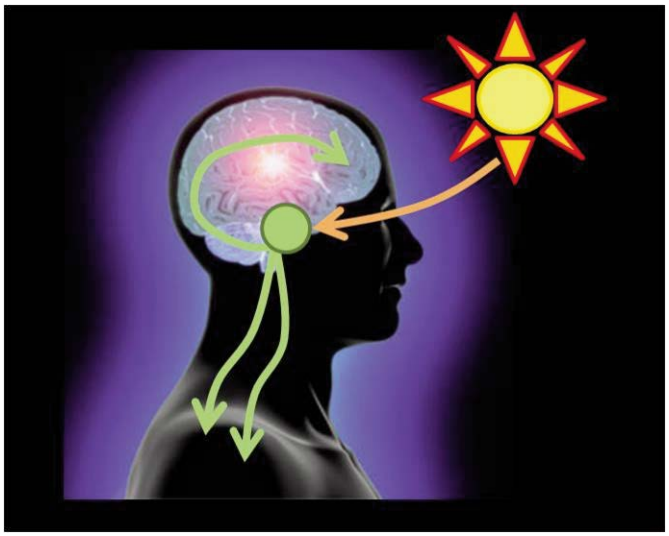

Figure 1 The suprachiasmatic nucleus (SCN) is the central circadian pacemaker. The SCN is located in the hypothalamus and is regulated by light signals from the eye. The SCN then affects a wide variety of physiological and behavioral outcomes.

2000) and *Clock* (Oishi et al. 2006). Likewise, molecular perturbation of the circadian clock (i.e., altering the *Clock, Bmal1, Per1, Per2, Cry1,* or *Cry2* expression via genetic manipulations including deleting or mutating the gene of interest to affect the levels of functional protein produced) disrupts normal circadian behavioral rhythms (Antoch et al. 1997; Bunger et al. 2000; van der Horst et al. 1999; Zheng et al. 2001). This article will discuss the influence of alcohol on circadian rhythms and how circadian-rhythm disruption affects immune function and metabolism, significant factors for alcohol-associated poor health outcomes. It also will discuss potential epigenetic mechanisms by which circadian disruption and alcohol may establish long-term changes in gene expression, resulting in adverse health outcomes.

Alcohol and Circadian Rhythmicity

Circadian organization and stable circadian rhythms are vital for optimal health as numerous diseases are associated with circadian-rhythm disruption. Environmental factors such as shift work or jet lag are obvious disrupters of circadian rhythmicity. However, other environmental factors, such as alcohol consumption and the timing of food intake, can profoundly disrupt and disorganize circadian rhythmicity, which can be observed on behavioral, cellular, and molecular levels.

Alcohol Disrupts Behavioral and Biological Circadian Rhythms

Alcohol has a dramatic effect on circadian rhythms. These circadian abnormalities include disrupted sleep/wake

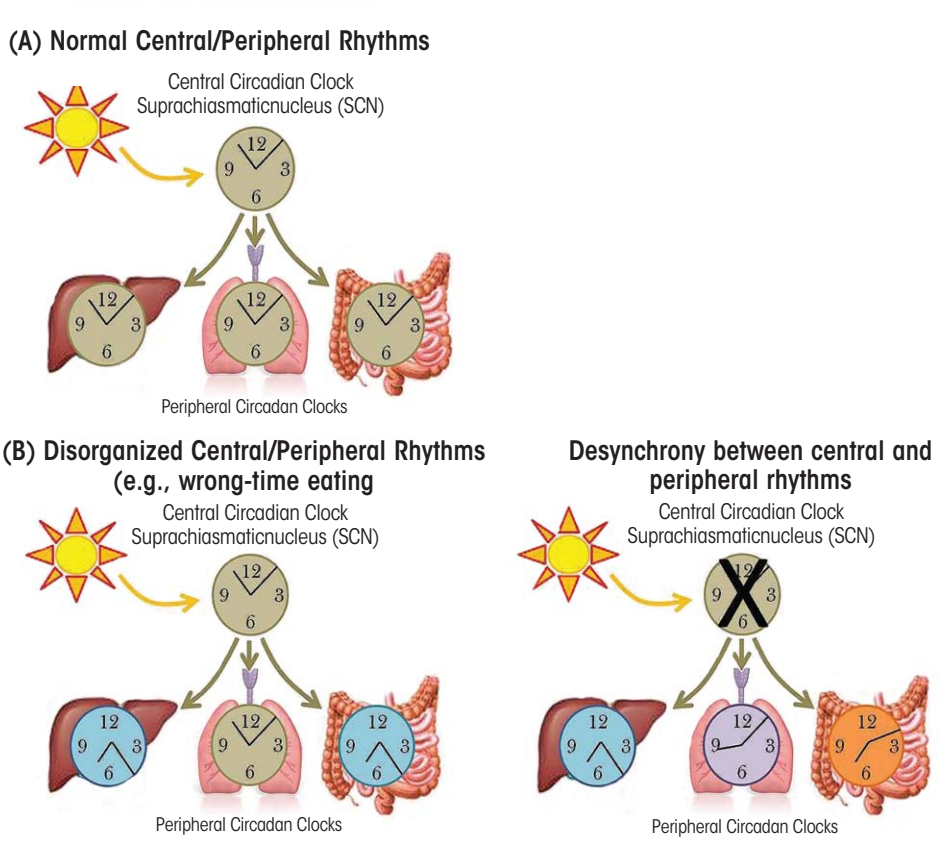

Figure 2 Central and peripheral circadian rhythms. **(A)** Under normal conditions, the central circadian clock in the suprachiasmatic nucleus which is entrained by light, then regulates peripheral circadian clocks. **(B)** Wrong-time eating can cause misalignment between the central circadian clock (entrained by light) and the peripheral circadian clocks entrained by food (illustrated here are intestine and liver). **(C)** When the central circadian clock is disrupted (e.g., due to lesion) peripheral circadian clocks will continue to cycle but will gradually become more misaligned with each other.

cycles in humans (Brower 2001; Imatoh et al. 1986) as well as disrupted circadian responses to light and abnormal activity patterns in rodents (Brager et al. 2010; Rosenwasser et al. 2005). The changes observed in behavioral patterns and responses to light may be the consequence of alcohol-induced disruption of normal tissue/organ function and neuroendocrine function. For example, normal cyclic patterns associated with body temperature (i.e., thermoregulation) (Crawshaw et al. 1998), blood pressure (Kawano et al. 2002), and characteristics of biochemical circadian rhythms including glucose and cholesterol rhythms (Rajakrishnan et al. 1999) are significantly affected by alcohol consumption. In addition, the circadian-driven production of hormones including melatonin (i.e., an endocrine hormone that is important in circadian entrainment) in rats (Peres et al. 2011) and humans (Conroy et al. 2012), corticosterone (i.e., a steroid hormone produced by the adrenal gland that responds to stress and regulates metabolism) (Kakihana and Moore 1976), and pro-opiomelanocortin (i.e., a polypeptide hormone that is a precursor to several hormones) (Chen et al. 2004) are disrupted by alcohol consumption. Alcohol-induced changes such as these have a profound impact on the functioning of a wide variety of peripheral organs and biological processes, which are dependent upon central circadian synchronization for proper function.

Alcohol Disrupts the Molecular Circadian Clock

Not surprisingly, the changes observed in the behavioral and biological systems also are observed on the molecular level as a disrupted molecular circadian clock, an effect that is evident both in vitro and in vivo. Exposure of intestinal epithelial cells (i.e., Caco-2 cells, a widely used model of the human intestinal barrier) to alcohol increases the levels of circadian clock proteins CLOCK and PER2 (Swanson et al. 2011). Likewise, alcohol-fed mice have disrupted expression of *Per1–Per3* in the hypothalamus (Chen et al. 2004), human alcoholics demonstrate markedly lower expression of *Clock, BMAL1, Per1, Per2, Cry1,* and *Cry2* in peripheral blood mononuclear cells (i.e., immune cells) compared with nonalcoholics (Huang et al. 2010), and in humans alcohol consumption is inversely correlated to BMAL1 expression in peripheral blood cells (Ando et al. 2010). The alcohol-induced changes seem to have long-lasting effects on the circadian clock, particularly when the exposure occurs early in life, which may be the consequence of epigenetic modifications (discussed below). For example, neonatal alcohol exposure in rats disrupts normal circadian-clock expression levels and expression patterns over a 24-hour period (i.e., rhythmicity) (Chen et al. 2006; Farnell et al. 2008). These examples illustrate the ability of alcohol to have profound and long-lasting effects on clock-gene expression in multiple organs and tissues.

Feed-Forward Cycle: Alcohol Promotes Circadian Disruption and Circadian Disruption Promotes Alcohol Consumption

Interestingly, circadian-clock disruption can promote alcohol consumption, which can further exacerbate this cycle. For example, *Per2* mutant mice exhibit increased alcohol consumption compared with wild-type counterparts (Spanagel et al. 2005), an effect attributed to altered reinforcement systems leading to enhanced motivation to consume alcohol. This may explain why humans with circadian disruption are more prone to substance abuse disorders (Trinkoff and Storr 1998). This phenomenon also sets up a potentially devastating cycle in which circadian disruption drives alcohol consumption, which further exacerbates circadian disruption.

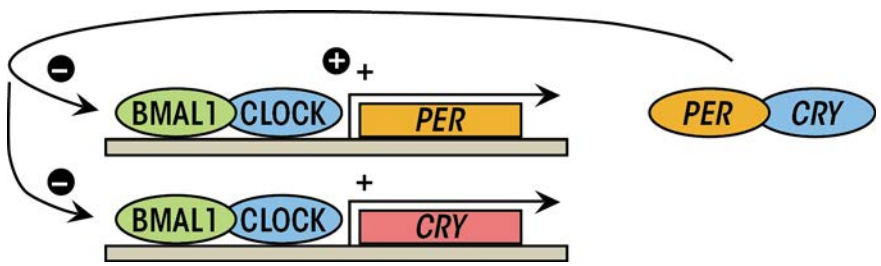

Figure 3 The molecular circadian clock. Transcription of the clock-controlled genes, including *Per* and *Cry* is initiated by the heterodimerization and binding of BMAL1 and CLOCK (the positive limb of the molecular circadian clock). Once sufficient amounts of PER and CRY have been produced, they dimerize and inhibit further BMAL1/CLOCK-mediated transcription (the negative limb of the molecular circadian clock).

Mechanisms of Alcohol-Induced Circadian Disruption

The mechanisms by which alcohol disrupts circadian rhythmicity are likely a consequence of alcohol metabolism and alcohol-induced changes in intestinal barrier integrity.

Consequences of Alcohol Metabolism

Alcohol is metabolized via several mechanisms, including the enzymes catalase, alcohol dehydrogenase (ADH), and cytochrome P450 (CYP2E1) (Lu and Cederbaum 2008). Although alcohol metabolism most prominently occurs in the liver, other tissues such as the stomach, intestine, and brain also play a role in this process. One consequence of alcohol metabolism that is particularly relevant for alcohol-induced disruption of circadian rhythmicity is a shift in the cellular NAD^+/NADH ratio. SIRT1, which regulates the molecular circadian clock, is highly sensitive to the cellular NAD^+/NADH ratio. Therefore, a perturbation in the availability of NAD^+ (e.g., as a consequence of alcohol metabolism by ADH or as a consequence of aldehyde metabolism by acetaldehyde) would be one mechanism by which alcohol could disrupt the molecular circadian clock and resulting circadian rhythms.

Alcohol, the Intestine, and Inflammation

Another mechanism by which alcohol can exert a negative influence on circadian rhythmicity is by promoting intestinal hyperpermeability. Alcohol disrupts intestinal barrier integrity in vitro (Swanson et al. 2011), in rodents (Keshavarzian et al. 2009), and humans (Keshavarzian et al. 1994, 1999). Intestinal hyperpermeability allows luminal bacterial contents such as endotoxin (e.g., lipopolysaccharide (LPS) to translocate through the intestinal epithelium into the systemic circulation. Endotoxin can disrupt circadian rhythms. LPS administered to rodents impairs the expression of *Per* in the heart, liver, SCN, and hypothalamus (Okada et al. 2008; Yamamura et al. 2010) and suppresses clock gene expression in human peripheral blood leukocytes (Haimovich et al. 2010). Thus, intestinal-derived LPS may be one mechanism by which alcohol disrupts circadian rhythmicity. In addition, LPS elicits a robust immune response in the periphery (Andreasen et al. 2008), and systemic inflammation disrupts normal circadian rhythmicity (Coogan and Wyse 2008). For example, tumor necrosis factor α (TNFα), a cytokine produced in response to endotoxins, disrupts normal locomotor behavior and sleep/wake cycles and alters expression of the molecular circadian clock in the liver (Cavadini et al. 2007). Thus, there are several plausible mechanisms by which alcohol-induced effects on the intestine may disrupt central and peripheral circadian rhythms.

Intestinal-derived LPS may be one mechanism by which alcohol disrupts circadian rhythmicity.

It is clear that alcohol-induced effects on the intestine are highly detrimental to circadian rhythmicity. Interestingly, the reverse also is true in that the molecular circadian clock in the intestine influences alcohol-induced effects. Intestinal circadian rhythms are largely driven by feeding patterns (Hoogerwerf et al. 2007; Scheving 2000) and even the apical junctional complex (AJC) proteins, which regulate tight junctions (and thus intestinal permeability), are clock controlled in the kidney (Yamato et al. 2010). Alcohol exposure increases intestinal circadian gene expression, and knocking out *Clock* or *Per2* in intestinal epithelial cells (i.e., Caco-2 cells) prevents alcohol-induced intestinal hyperpermeability (Swanson et al. 2011). Taken together, alcohol—via metabolism products or intestine effects including endotoxemia and systemic inflammation—disrupts intestinal circadian rhythms, an effect that can further exacerbate internal misalignment.

Circadian Rhythms and Immune Function

The immune system demonstrates robust circadian rhythmicity with daily variations in immune parameters, including lymphocyte proliferation, antigen presentation, and cytokine gene expression (Fortier et al. 2011; Levi et al. 1991). These rhythms seem to be sensitive to perturbations in circadian homeostasis, with differential effects depending on the cell type, model system, and outcome measure. For example, inhibition of *Per2* in natural killer (NK) cells (part of the innate immune system) decreases the expression of the immune effectors granzyme-B and porforin (i.e., critical cytotoxic components) (Arjona and Sarkar 2006*a*). Despite these changes, selective reduction of *Per2* in NK cells does not effect NK rhythmic production of the cytokine interferon-γ (IFNγ), which is important for the formation and release of reactive oxygen species. In contrast, whole-animal *Per2*-deficient mice have drastically disrupted IFNγ rhythms (Arjona and Sarkar 2006*b*). The IFNγ rhythmic disruption in *Per2*-deficient mice but not after selective reduction of *Per2* in isolated NK cells would be expected if IFNγ is dependent upon other circadian parameters, such as circadian fluctuations in hormones or temperature. Indeed, rhythmic hormones such as glucocorticoids and melatonin, which are significantly affected by circadian disruption, modulate immune function (Dimitrov et al. 2004; Srinivasan et al. 2005). *Per2*-deficient mice also demonstrate blunted LPS-induced septic shock compared with wild-type mice (Liu et al. 2006), indicating a

functional change that has important biological implications. These studies demonstrate the significant disturbances that can occur as a consequence of a disrupted molecular circadian clock.

In addition to genetically manipulating circadian homeostasis, environmentally disrupting circadian rhythms also negatively affects immune function. For example, loss of regular sleep/wake cycles alters the normal circadian rhythmicity observed in immune cells (Bryant et al. 2004; Vgontzas et al. 2004) and increases the susceptibility to infections (Everson 1993; Mohren et al. 2002). Indeed, chronically shifting light/dark cycles in mice augments LPS-induced immune response, resulting in greater mortality compared with non–circadian-disrupted mice (Castanon-Cervantes et al. 2010).

Taken together, these studies provide evidence that circadian disruption can significantly, and typically negatively, influence immune function. Therefore, alcohol-induced circadian disruption may be a susceptibility factor for immune dysregulation, which may promote alcohol-associated inflammatory processes. Furthermore, the altered response to LPS has particular relevance in light of the alcohol-induced effects on intestinal permeability.

Circadian Rhythms and Metabolic Syndrome

Although only a few metabolic genes are direct targets of circadian genes (Noshiro et al. 2007; Panda et al. 2002), the direct targets do include many transcription factors and other modulators of transcription and translation. These clock-controlled genes include factors regulating lipid and cholesterol biosynthesis, carbohydrate metabolism, oxidative phosphorylation, and glucose levels (Oishi et al. 2003; Panda et al. 2002).

Eating is an environmental factor that selectively affects peripheral circadian rhythmicity in the intestine and liver. Feeding at the incorrect time (e.g., late-night eating for humans) can result in internal circadian misalignment. For example, restricted feeding paradigms in which animals only have access to food during inappropriate times (i.e., during the light cycle for nocturnal rodents) results in misalignment between central light-entrained circadian rhythms (i.e., in the SCN) and peripheral food-entrained circadian rhythms, including those in the liver (Damiola et al. 2000). Recent studies suggest that this internal misalignment scenario is linked to weight gain, obesity, and metabolic syndrome. Indeed, mice fed during the inappropriate time gain more weight (Arble et al. 2009; Salgado-Delgado et al. 2010) than mice fed during appropriate time, despite similar activity levels and caloric intake (Arble et al. 2009). This phenomenon also is observed in humans; people who skip breakfast and have eating patterns shifted toward late-night eating tend to be more overweight than those who consume food during more appropriate time periods (Berkey et al. 2003; Ma et al. 2003).

Feeding at the incorrect time (e.g., late-night eating for humans) can result in internal circadian misalignment.

Genetic abnormalities in the molecular circadian clock also are associated with metabolic disorders, including obesity, metabolic syndrome, and diabetes (Scott et al. 2008; Woon et al. 2007). For example, *Clock* mutant mice, which have disrupted circadian rhythms (Vitaterna et al. 1994), are obese and demonstrate characteristics of metabolic syndrome such as high cholesterol levels and high blood glucose (Turek et al. 2005). *Bmal1* mutant mice also have disrupted circadian rhythmicity (Bunger et al. 2000), disrupted adipogenesis (Shimba et al. 2005), and demonstrate markers of metabolic syndrome (e.g., higher levels of triglycerides and glucose) (Marcheva et al. 2010; Rudic et al. 2004). Similarly, mutations in *Cry* genes disrupt hormonal rhythms (Fu et al. 2005; Yang et al. 2009) and *Cry* mutants show markers of metabolic syndrome (Okano et al. 2009). It should be noted that although some of these mutant mice demonstrate disrupted locomotion and feeding behaviors (i.e., wrong-time feeding), the abnormalities seem to be attributable to mutations in the circadian clock machinery rather than to appropriate feeding times because mice (e.g., *Bmal1* mutant mice) that do exhibit normal activity/feeding patterns still exhibit markers of metabolic syndrome (Lamia et al. 2008; Marcheva et al. 2010).

In addition to these effects of circadian rhythms on indices of metabolism, it is also important to consider the effect of circadian disruption on the immune system because chronic inflammation is a prominent feature associated with metabolic syndrome. Thus, the immune dysfunction that occurs upon circadian rhythm disruption may be a predisposing or exacerbating factor for metabolic syndrome.

Epigenetic Alterations: Circadian Rhythm Disruption and Alcohol

Epigenetics is the study of stable changes in gene expression that do not involve DNA sequence modifications but rather are the consequence of processes such as DNA methylation, histone modification (i.e., acetylation, methylation, phosphorylation, ubiquitinylation, ADP-ribosylation, and sumoylation), and noncoding microRNAs (miRNAs). These changes in gene expression are critical to optimize cellular function and for cellular development and differentiation. However, epigenetic changes also occur in response to environmental changes,

including circadian rhythm disruption and alcohol use.

Shift work (i.e., chronic circadian disruption) is associated with an increased incidence of cancer. Potential mechanisms for this relationship include changes in melatonin levels and levels of circadian clock genes (Straif et al. 2007). However, epigenetics also may influence circadian rhythm disruption and thereby affect cellular function. Indeed, long-term shift work affects promoter methylation of the circadian genes *Clock* and *Cry2* (Zhu et al. 2011) with increased methylation of *Clock* (Hoffman et al. 2010*a*) and decreased methylation of *Cry* (Hoffman et al. 2010*b*) observed in cancer patients. Epigenetic changes also occur as a consequence of chronic circadian disruption in the promoter regions of genes encoding glucocorticoid receptors (important for hypothalamic–pituitary–adrenal axis function), TNFα (a cytokine critical for cell functioning and inflammation), and IFNγ (Bollati et al. 2010). Changes such as these may play a critical role in how chronic circadian disruption promotes cancer, inflammation, and metabolic disorders.

In addition to circadian-disruption–induced epigenetic changes, alcohol consumption is also associated with epigenetic modifications. Alcohol-induced DNA acetylation is observed in vitro in rat hepatocytes (Park et al. 2003), in vivo in rat hepatic stellate cells (Kim and Shukla 2005, 2006), lung, spleen, and testes (Kim and Shukla 2006). Similar to the increased cancer risk associated with chronic circadian disruption, alcohol-induced epigenetic changes are associated with the development of cancer. Indeed, colorectal cancer in high-alcohol– consuming humans is associated with high levels of promoter hypermethylation of several relevant genes when compared with low- or no-alcohol– consuming counterparts with colorectal cancer (van Engeland et al. 2003; Giovannucci et al. 1995). Similarly, alcohol-consuming individuals with head and neck cancer have hypermethylated gene promoters for specific genes of interest compared with non-alcohol–drinking individuals (Puri et al. 2005) and alcohol-dependent humans have hypermethylation of liver and peripheral blood cell DNA. Thus, it seems that both circadian disruption and alcohol consumption can affect long-term changes in gene expression via epigenetic modifications that may impact a wide variety of health outcomes.

Summary and Future Directions

Circadian rhythms are a prominent and critical feature of cells, tissues, organs, and behavior that help an organism function most efficiently and anticipate things such as food availability. Therefore, it is not surprising that disrupted circadian rhythms or misalignment between central and peripheral circadian rhythms predispose and/or exacerbate a wide variety of diseases, including alcohol-associated disorders. One environmental factor that has been shown to have a disruptive effect on circadian rhythms is alcohol consumption. This disruption occurs at the molecular levels (i.e., changes in the expression levels of the circadian clock genes), also affects tissues and organs (e.g., changes in the cyclic pattern of hormones), and leads to overt behavioral changes. Thus, in the context of alcoholism, disrupted circadian rhythms may create a positive feedback loop that markedly exaggerates alcohol-induced immune/inflammatory-mediated diseases by (1) negatively influencing immune function and (2) promoting alcohol consumption that leads to further circadian-rhythm disruption. These changes are highly relevant because circadian-rhythm disruption has a substantial impact on immune function, which in turn has important implications for a wide variety of pathological conditions, including metabolic syndrome. A better understanding of how circadian rhythms influence such a wide variety of systems and bodily functions and how environmental factors such as alcohol use influence these processes is vital to our ever more circadian-disrupted society.

A better understanding of the mechanisms by which circadian disruption affects health outcomes such as cancer, inflammation, metabolic disease, and alcohol-induced pathology is critical. This information may lead to the development of chronotherapeutic approaches to prevent and/or treat a wide variety of conditions that are promoted or exacerbated by circadian-rhythm disruption and may lead to better risk stratification for individuals who are at risk for developing chronic conditions. Going forward, characterizing the epigenetic modifications that occur during chronic circadian disruption may be critical for understanding not only how disruption affects an individual but also how these modifications are passed on to offspring, which may influence the health of future generations. Thus, the issue of circadian disruption is vitally important for the health and well-being of current and future generations. ■

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Immune Function Genes, Genetics, and the Neurobiology of Addiction

Fulton T. Crews, Ph.D.

The neuroimmune system (i.e., the immune system and those components of the nervous system that help regulate immune responses), and in particular the innate immune system, play a role in the development of addictions, including alcoholism, particularly in the context of stressful situations. Certain cells of the neuroimmune system are activated both by stress and by environmental factors such as alcohol, resulting in the induction of genes involved in innate immunity. One of the molecules mediating this gene induction is a regulatory protein called nuclear factor-κB, which activates many innate immune genes. Innate immune gene induction in certain brain regions (e.g., the frontal cortex), in turn, can disrupt decision making, which is a characteristic of addiction to alcohol and other drugs. Likewise, altered neuroimmune signaling processes are linked to alcohol-induced negative affect and depression-like behaviors and also regulate alcohol-drinking behavior. Moreover, the expression of several genes and proteins involved in innate immunity is enhanced in addicted people. Finally, specific variants of multiple innate immune genes are associated with the genetic risk for alcoholism in humans, further strengthening the connection between increased brain innate immune gene expression and alcohol addiction. KEY WORDS: Other drug dependence; alcoholism; addiction; causes of alcohol and other drug use; genetic factors; environmental factors; neurobiology; neuroimmune system; immune system; innate immune system; innate immune genes; immune function genes; nuclear factor-κB; stress; decision making; depression

The nervous system and the immune system interact closely to regulate the body's immune responses, including inflammatory responses. Accordingly, the term "neuroimmune system" refers to the immune system and those components of the nervous system that help regulate immune responses and also encompasses the hormones and other signaling molecules that convey signals between the immune and nervous systems. Part of the neuroimmune system is the innate immune system—a network of cells and the signaling molecules they release that are present from birth and form the first line of the body's defense system, including such responses as inflammatory reactions. This article summarizes the role that the neuroimmune system and genes encoding components of the innate immune system play in the development of addiction, including alcoholism.

Neuroimmune Signaling, Drug Abuse, and Stress

Neuroimmune signaling influences the responses and functions of a variety of body systems, including the digestive (i.e., enteric) system, sensory pathways, and the hormonal axis known as the hypothalamic–pituitary–adrenal (HPA) axis, which is involved in the body's stress response and also plays a role in addiction to alcohol and other drugs (AODs).[1] Immune cells called monocytes and monocyte-like cells in the brain (e.g., microglia) are sensitive key cells involved in neuroimmune signaling. When the immune system is stimulated or tissue damage occurs, these cells go through multiple stages of activation, which at the molecular level are reflected by the activation of a cascade of innate immune genes (Graeber 2010). These responses of the monocytes and microglia involve the production and secretion of signaling molecules, including inflammation-promoting (i.e., proinflammatory) cytokines and chemokines, such as monocyte chemotactic protein (MCP)-1, tumor necrosis factor α (TNFα), and interleukin 1β (IL1β). In the brain, microglial activation contributes to the activation of another type of cell called astroglia, or astrocytes, which, like microglia, show multiple stages of neuroimmune activation. In the microglia, the different stages of activation are accompanied by morphological changes. Thus, these cells change from their resting state with multiple branches (i.e., the ramified form) to a less branched, bushy morphology after mild activation and a rounded morphology after strong activation (i.e., when major brain cell death occurs). Chronic alcohol treatment induces mild, bushy microglial activation as well as mild astrocyte activation (see figure 1).

Activated glia show increased production of a wide range of proteins. For example, they produce and secrete increased amounts of proteases as well as of proteins found in the space between cells (i.e., extracellular matrix proteins). In addition, they generate increased amounts of proteins called toll-like receptors (TLRs) that play a role in alcohol-induced depressed mood and negative emotions (see below) and

[1] Among the main molecules involved in the HPA system are the glucocorticoids (e.g., cortisol), and cycles of stress as well as AOD abuse lead to elevated basal glucocorticoid levels and promote addiction (Armario 2010).

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show enhanced activity of enzymes known as oxidases that promote inflammatory reactions (e.g., nicotinamide adenine dinucleotide phosphate [NADPH] oxidases [NOX], cyclooxygenase [COX], and inducible nitric oxide synthases [iNOS]).

Microglia easily can become activated, and the initial stages of activation are characterized by the secretion of signaling molecules, slight morphological changes, and increased production of molecules involved in immune responses (i.e., major histocompatibility complex [MHC]) as well as of TLRs (Graeber 2010). Activation of microglia and astrocytes also increases proinflammatory agents, including TNFα, that alter the transmission of nerve signals (i.e., neurotransmission), including signal transmission mediated by the excitatory neurotransmitter glutamate. Likewise, studies have suggested that alcoholism is related to excessive glutamate levels (i.e., a hyperglutamate state). In the outer layer of the brain (i.e., the cerebral cortex), chronic alcohol-induced neuroimmune activation leads to a hyperglutamate state that reduces cortical function (figure 2). One mechanism contributing to this hyperglutamate state involves TNFα, which acts to reduce the activity of glutamate transporters[2] in the astrocytes (Zou and Crews 2005). Similarly, beverage alcohol (i.e., ethanol) has been shown to inhibit glutamate transport (Zou and Crews 2006). This blockade of glutamate transporters increases glutamate levels outside the cells and particularly in the space between two neurons where nerve signals are transmitted (i.e., the synapse), resulting in excessive neuronal activity (i.e., hyperexcitability). TNFα also stimulates the production of certain proteins found on signal-receiving neurons that interact with glutamate (i.e., the AMPA glutamate receptors) (Beattie et al. 2010). Increases in synaptic glutamate receptors and glutamate concentrations cause hyperexcitability that disrupts the normal concentration of the brain's response to a specific area of the cortex (i.e., cortical focus), thereby reducing cortical function. Through these mechanisms, monocytes, microglia, and astrocytes progressively become activated by stress and environmental factors, including ethanol, resulting in the induction of genes that encode proteins involved in the innate immune response.

Stress and Drug Abuse Increase Transcription of Innate Immune Genes

Stress and AODs, as well as sensory and hormonal signals, activate a regulatory protein (i.e., transcription factor[3]) called nuclear factor κ–light-chain enhancer of activated B cells (NF-κB) that is produced in large amounts (i.e., is highly expressed) in monocytes and microglia. Although NF-κB is found in most cells, it is the key transcription factor involved in the induction of innate immune genes in microglia and other monocyte-like cells. A wide range of stimuli, such as stress, cytokines, oxidative free radicals, ultraviolet irradiation, bacterial or viral molecules, and many other signaling molecules, increase binding of NF-κB to specific sequences of the DNA. This binding increases the transcription of many genes, particularly those encoding signaling molecules (e.g., chemokines and cytokines) and enzymes (e.g., oxidases and proteases) (figure 3). Studies found that ethanol can increase the binding of NF-κB to its corresponding DNA sequences both in the brains of living organisms (Crews et al. 2006) and in cultured brain slices obtained from a brain area called the hippocampal–entorhinal

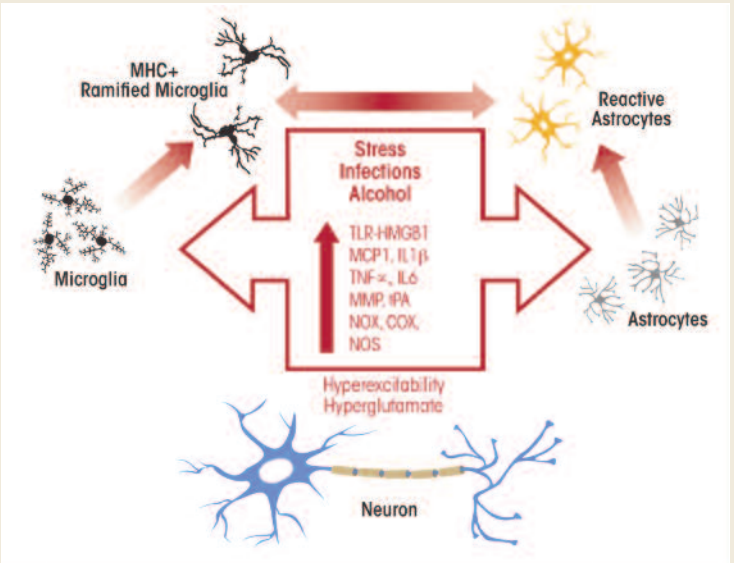

Figure 1 Activation of microglia and astrocytes by alcohol in the brain. Microglia and astrocytes undergo multiple stages of activation that include characteristic changes in morphology. Resting microglia become ramified microglia with that express molecules called major histocompatibility complex (MHC) on their surface. Similarly, astrocytes begin to show markers of reactive astrocytes. Alcohol-induced glial activation is associated with increased expression of innate immune genes, including increased expression of the chemokine monocyte chemoattractant protein-1 (MCP1); the cytokines tumor necrosis factor-α (TNFα), interleukin-1 β (IL-1β) , and interleukin-6 (IL-6); the proteases matrix metalloproteinase (MMP) and tissue plasminogen activator (TPA); and the oxidases nicotinamide adenine dinucleotide phosphate oxidase (NOX), cyclooxygenase (COX), and nitric oxide synthetase (NOS). The alcohol-induced activation of glial innate immune genes increases neuronal hyperexcitability (Crews et al. 2011).

[2] Glutamate transporters are proteins that shuttle glutamate released by nerve cells (i.e., neurons) into the space between cells back into the neuron; this is essential to terminate transmission of a nerve signal and thus ensure appropriate regulation of neuronal activity.

[3] Transcription factors are proteins that are necessary for a set of reactions called transcription, which is the first step of the process during which the genetic information encoded in the DNA is used as a template for the generation of functional proteins.

cortex (HEC) (Zou and Crews 2006). These and other studies also have indicated that ethanol increases transcription of NF-κB target genes, including the genes encoding the following:

- MCP-1;
- Certain proinflammatory cytokines, such as TNFα, IL-1β, and IL-6;
- Certain proinflammatory oxidases, such as iNOS (Zou and Crews 2010), COX-2 (Knapp and Crews 1999), and NOX (Qin et al. 2008); and
- Certain proteases, such as TNF–converting enzyme (TACE) and tissue plasminogen activator (Zou and Crews 2010).

Not only ethanol but also chronic stress increases brain NF-κB activation (Koo et al. 2010; Madrigal et al. 2002), as well as the levels of cytokines, prostaglandin,[4] and COX-2 (Madrigal et al. 2003), all of which have proinflammatory effects. Although acute stress–induced responses, such as elevated glucocorticoid levels, are anti-inflammatory by blocking NF-κB production, chronic elevation of glucocorticoid levels during cycles of stress and/or AOD abuse reverses these anti-inflammatory effects and indeed results in proinflammatory NF-κB activation in the frontal cortex (Munhoz et al. 2010). Thus, activation of NF-κB is a common molecular mechanism through which stress and AODs can induce innate immune genes.

Addiction and Neuroimmune Signaling

Alcoholism is a progressive disease related to repeated episodes of alcohol abuse that reduce the brain's behavioral control and decision-making ability; at the same time, increasing habitual

[4] Prostaglandins are lipid compounds that are produced by almost all cells in the body and have a variety of important physiological effects, including the regulation of inflammatory reactions.

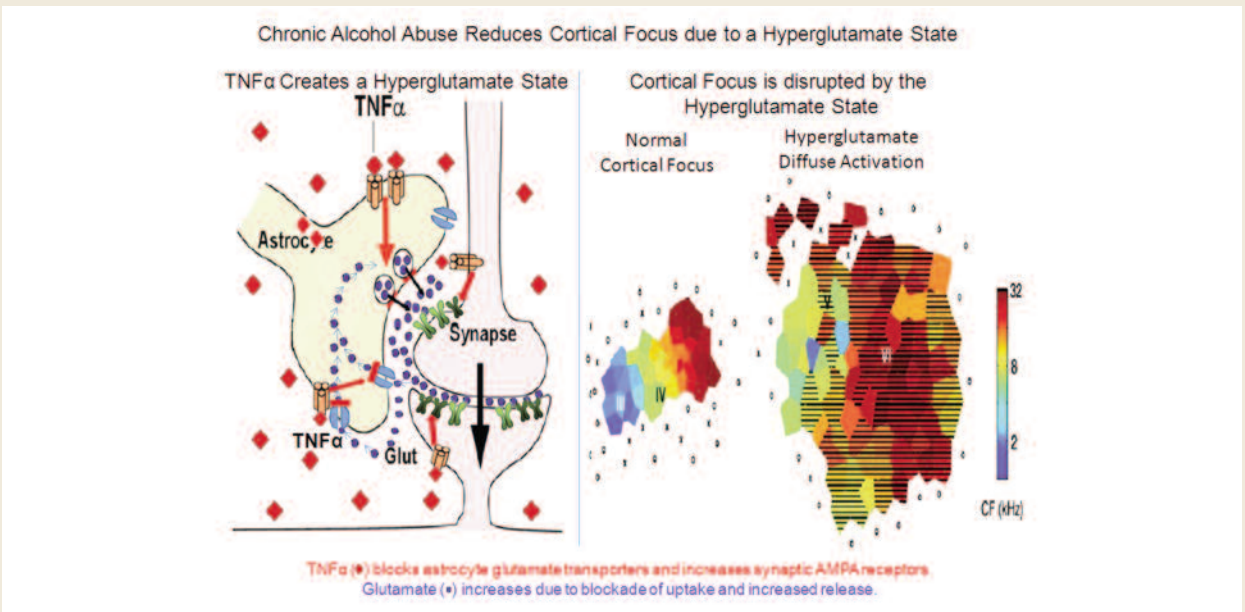

Figure 2 Mechanisms of alcohol-induced excessive glutamate activity in the cortex and loss of cortical focus. Ethanol-induced activation of microglia and astrocytes increases the levels of proinflammatory cytokines, including tumor necrosis factor-alpha (TNFα). (Left panel) TNFα creates a state characterized by excess activity of the neurotransmitter glutamate (i.e., a hyperglutamate state). Thus, TNFα reduces the levels of the primary glutamate transporters, GLT-1, in the astrocytes, in the cerebral cortex, and inhibits glutamate transport, possibly through induction of TNFα and other proinflammatory genes. As a result, glutamate levels outside the neurons, and particularly at the synapse, increase, resulting in a hyperglutamate state. In addition, TNFα increases the levels of certain molecules that interact with glutamate (i.e., AMPA receptors). All these processes causes excessive neuronal excitability. (Right panel) Hyperexcitability disrupts cortical focus. The left image shows the response of a normal adult auditory cortex to a series of tones with a frequence of 2-32 kHz colorized as blue to red. The response to a specific tone involves activation of a specific focal cortical region, which likely relates to the ability to distinguish specific tones of sounds. The right image shows the disrupted hyperglutamate-state–like response to sound that involves the entire auditory cortex without specific tonal areas of focus. The hyperglutatamate state increases cortical excitability, which in turn decreases function because it results in loss of focal activation and likely loss of tonal discrimination. In alcoholism, the hyperglutamate state most strongly affects the frontal cortex, which may disrupt decision making as well as attention and behavioral control mechanisms.

SOURCE: Image in right panel adapted from Chang and Merzenich (2003).

urges combined with increasing bad feelings (i.e., negative affect) promote continued drinking. Frontal cortical brain regions that designate attention and motivation, using information to predict the result of actions (Schoenbaum and Shaham 2008), play a role in addiction development. Frontal cortical dysfunction often is investigated using reversal-learning tasks. In reversal learning, the subject first learns to make one choice (e.g., responding to the black objects in a series of black and white objects) and then has to learn to reverse this choice (e.g., to respond to the white objects). Thus, the initially expected responses suddenly are considered wrong, requiring the subject to exhibit flexible behavior in response to outcomes that do not match those predicted by preceding cues (Stalnaker et al. 2009).

In behavioral studies, poor performance on such tasks is supposed to reflect the inability of drug-addicted individuals to learn new healthy behaviors and avoid the negative consequences of their drug consumption. Such learning and/or changes in behavior require signals from the frontal cortex to indicate the value of decisions. Studies found that binge drinking induces persistent deficits in reversal learning in rats (Obernier et al. 2002; Pascual et al. 2007) and in adult mice following a model of adolescent binge drinking (Coleman 2010). Other investigators similarly have demonstrated that cocaine use results in abnormally slow reversal learning, even though initial learning is normal (Calu et al. 2007; Schoenbaum et al. 2004). Specifically, human cocaine and alcohol addicts exhibit dysfunctional decision making in reversal-learning tasks that probe cognitive flexibility (Bechara et al. 2002). Lesions in the frontal cortex cause reversal-learning deficits comparable to those induced by chronic drug abuse (Schoenbaum et al. 2006). The persistence of addiction matches the persistent increases in innate immune gene activation (Qin et al. 2007, 2008) and loss of behavioral flexibility. Thus, it is thought that innate immune gene induction in the frontal cortex disrupts decision making consistent with addiction (Crews et al. 2011).

Addiction to alcohol, opiates, and stimulant drugs involves both changes in attention–decision making and increased temporal lobe anxiety–negative affect urgency. Addiction-induced negative affect and depression-like behaviors also are linked to neuroimmune signaling because neuroimmune signals can alter moods. For example, a compound called lipopolysaccharide (LPS) that can induce brain innate immune genes causes depression-like behavior that mimics components of addiction-like negative affect. LPS naturally binds with one of the TLRs (i.e., TLR4) and this interaction results in NF-κB activation, ultimately leading to the induction of innate immune genes. In humans, LPS infusions reduce reward responses and increase depressed mood (Eisenberger et al. 2010). Likewise, when patients with cancer or viral infections are treated with agents such as interferon and IL that influence innate immune genes, they may experience severe depression as a major adverse effect (Kelley and Dantzer 2011). Innate immune activators such as LPS, chemokines, and cytokines can mimic the amplification of depressed mood that occurs during repeated cycles of drug abuse or stress (Breese et al. 2008). All of these observations further support the link between neuroimmune signaling and mood as well as the role of neuroimmune signaling as a key component of addiction neurobiology. Of interest, chronic alcohol leads to withdrawal anxiety in normal mice

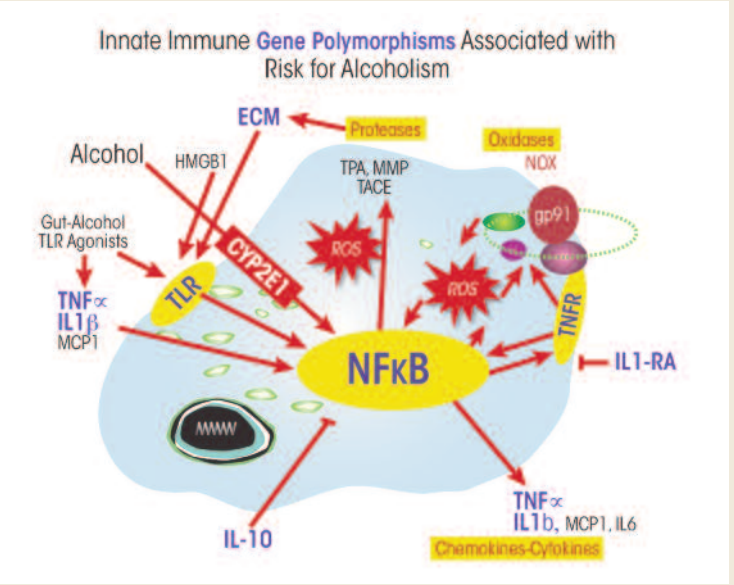

Figure 3 Innate immune gene polymorphisms associated with risk for alcoholism. The schematic shows a representative astrocyte or microglial cell. Genes associated with genetic risk for alcoholism are in light blue. Nuclear factor κ-lightchain–enhancer of activated B cells (NF-κB) is a key transcription factor involved in induction of innate immune genes that is sensitive to reactive oxygen species (ROS). These ROS are generated by the enzyme CYP2E1 during alcohol metabolism, and certain DNA sequences (i.e., polymorphisms) in the CYP2E1 gene are associated with alcoholism. CYP2E1 is highly expressed in monocyte-like cells, which are activated when CYP2E1 metabolizes alcohol. The ROS formed during this process activate proinflammatory NF-κB responses. Chronic ethanol treatment increases CYP2E1 expression in the brain, particularly in astrocytes.The resulting elevated ROS levels activate NF-κB–mediated transcription of innate immune genes, and this response may be amplified in the presence of certain NF-κB polymorphisms (i.e., NF-κB1). Certain variants of other genes also are associated with alcoholism, including polymorphisms of T NFα, interleukin-10 (IL-10), interleukin-1 receptor antagonist (IL-1RA), and other components of the IL-1 gene complex, as well as of certain proteins in the space surrounding the cells (i.e., extracellular matrix proteins [ECM]).

tent with the hypothesis that innate immune activation drives negative affect and associated anxiety responses. Thus, the anxiety–depression negative affect that contributes to addiction occurs with increased brain neuroimmune signaling.

Neuroimmune signaling also regulates alcohol drinking behavior. Genetic comparisons among different strains of rats and mice found that addiction-like drinking behavior was associated with increased levels or activity of NF-κB, its regulatory proteins, and multiple innate immune genes (Mulligan et al. 2006). Furthermore, induction of innate immune genes resulted in increased ethanol consumption, whereas inactivation of such genes reduced drinking behavior (Blednov et al. 2005, 2011*b*). Thus, across genetically divergent strains of mice, innate immune responses to LPS corresponded to increases in ethanol consumption (Blednov et al. 2005, 2011*b*). In fact, even a single injection of LPS was able to produce a long-lasting increase in ethanol consumption (Blednov et al. 2011*a*) that corresponded to sustained increases in brain innate immune gene expression (Qin et al. 2007). These studies identified several innate immune molecules (e.g., β2-microglobulin, cathepsins, and CD14, a key innate immune signaling protein) as important for regulating drinking behavior. Thus, innate immune gene induction may underlie the progressive loss of behavioral flexibility, increasing negative affect, and increased alcohol drinking associated with repeat episodes of alcohol abuse and alcoholism.

Activity of Innate Immune Genes Is Increased in the Addicted Brain

Direct analyses of changes in the activity or levels of various proteins in the brains of alcoholics and other drug addicts also can provide insight into the neurobiology of addiction. Such studies found the following:

- Postmortem studies of the brains of human alcoholics indicate that the innate immune chemokine MCP-1 is increased severalfold in multiple brain regions (Breese et al. 2008). Consistent with this, chronic alcohol treatment of mice (Qin et al. 2008) or of cultured brain slices from the rat hippocampus (Zou and Crews 2010) also increases expression of MCP-1 and other innate immune genes.

- Proteins that serve as markers of microglial activation are increased across the alcoholic brain (He and Crews 2008).

- Consistent with alcoholism being related to neuroimmune signaling, postmortem studies of gene expression in the brains of human alcoholics found increased levels of a subunit of NF-κB; moreover, 479 genes targeted by NF-κB showed increased expression in the frontal cortex of alcoholics (Okvist et al. 2007).

- Postmortem analyses of alcoholic human brain gene expression found innate immune activation of cell adhesion and extracellular membrane components of innate immune gene signaling (Liu et al. 2006).

Thus, the findings of several studies of gene or protein expression are consistent with increased neuroimmune signaling in the brains of addicted individuals.

Polymorphisms of Innate Immune Genes and Genetic Risk of Addiction

Genetic factors account for approximately 50 percent of the risk of alcohol dependence (Schuckit 2009). Multiple genes linked to innate immune function also have been linked to the risk for alcoholism (see figure 3). DNA variations (i.e., polymorphisms) at specific locations on the chromosomes result in gene variants (i.e., alleles) that differ in their function or activity and thereby may increase or reduce the risk of alcoholism. For example, polymorphisms in the gene encoding an enzyme called CYP2E1, which is involved in ethanol metabolism, have been associated with the risk for alcoholism (Webb et al. 2010). In the body, CYP2E1 is highly expressed in monocyte-like cells; ethanol metabolism by CYP2E1 leads to the activation of these cells. Specifically, CYP2E1-mediated ethanol metabolism causes an increased production of highly reactive molecules called reactive oxygen species (ROS) within the monocytes that activate proinflammatory NF-κB responses (Cao et al. 2005) (see figure 3). In the brain, ethanol exposure leads to increased CYP2E1 expression, particularly in astrocytes (Montoliu et al. 1994, 1995), which likely contributes to astrocyte activation of NF-κB transcription during chronic alcohol exposure.

Human genetic association studies also have directly linked certain polymorphisms of the genes encoding NF-κB to alcohol dependence (Edenberg et al. 2008; Flatscher-Bader et al. 2005; Okvist et al. 2007). For example, polymorphisms in a precursor gene called *NF-kB1* that encodes one of the subunits of the transcription factor (i.e., the NF-κB p50 subunit) and which is important for activation of transcription have been associated with the risk for alcoholism (Edenberg et al. 2008). Likewise, alleles of the proinflammatory cytokine TNFα that result in increased TNFα expression have been linked to alcoholism and alcoholic liver disease (Pastor et al. 2000, 2005; Powell et al. 2000). Another genetic linkage exists between certain alleles of the anti-inflammatory, NF-κB–inhibiting cytokine IL-10 and alcoholism (Marcos et al. 2008). Additional genetic evidence regarding innate immune genes and the risk for alcoholism comes from polymorphisms of the gene encoding a molecule called the IL-1 receptor antagonist as well as from multiple other alleles of the IL-1 gene complex (Saiz et al. 2009).

In general, gene polymorphisms associated with increased risk of alcoholism tend to increase proinflammatory responses. For example, alcohol exposure may increase the expression of proinflammatory cytokines or individuals at risk of alcohol dependence may carry alleles associated with decreased anti-

inflammatory cytokine secretion. Thus, multiple innate immune gene polymorphisms are associated with genetic risk for alcoholism in humans, consistent with the assumption that increased brain innate immune gene expression contributes to the neurobiology of alcohol addiction.

Summary

The findings summarized in this article link innate immune gene induction to addiction and alcoholism. Monocytes, microglia, and astrocytes are sensitive to AODs and stress, with repeated AOD use causing progressive innate immune gene induction that parallels changes in decision making, mood, and alcohol consumption. Stress and AODs activate NF-κB transcription in the brain, which in turn enhances expression of proinflammatory NF-κB target genes. As a result, molecules related to the innate immune response, such as the chemokine MCP-1, the proinflammatory cytokines TNFα, IL-1β, and IL-6; the proinflammatory oxidases iNOS, COX, and NOX (Qin et al. 2008); and proinflammatory proteases are found following chronic ethanol treatment. Postmortem analyses of human alcoholic brain also have demonstrated increased expression of innate immune genes, which can disrupt cognition, mood, and drug consumption and is consistent with addition-like behavior. Finally, polymorphisms of genes involved in the innate immune responses influence the risk for alcoholism. These studies suggest that innate immune genes contribute to alcoholism and may be involved in the genetic risk for alcoholism. ■

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