Binge Ethanol Leads to Decreased Macrophage Accumulation in Infected Cutaneous Wounds

Sara Hlavin

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LOYOLA UNIVERSITY CHICAGO

BINGE ETHANOL LEADS TO DECREASED MACROPHAGE ACCUMULATION IN INFECTED CUTANEOUS WOUNDS

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
MASTER OF SCIENCE

PROGRAM IN INFECTIOUS DISEASE AND IMMUNOLOGY

BY
SARA KATHLEEN HLAVIN
CHICAGO, ILLINOIS
MAY 2012
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<tr>
<td>BAC</td>
<td>blood alcohol concentration</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CDC</td>
<td>center for disease control and prevention</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming units</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>CXCR2</td>
<td>chemokine cxc receptor 2</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>EGFP</td>
<td>green fluorescent protein expressing</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
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<tr>
<td>FcyR-type II</td>
<td>Fc gamma receptor- type two</td>
</tr>
<tr>
<td>ICAM</td>
<td>intracellular adhesion molecule</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>IL-1β</td>
<td>interleukin 1 beta</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemotactic protein 1</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescent intensity</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>macrophage inflammatory protein- 1 alpha</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>macrophage inflammatory protein- 2 beta</td>
</tr>
<tr>
<td>MIP-2</td>
<td>macrophage inflammatory protein 2</td>
</tr>
<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
</tr>
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<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>MSA</td>
<td>mannitol salt agar</td>
</tr>
<tr>
<td>OCT</td>
<td>optical cutting temperature</td>
</tr>
<tr>
<td>PAMPS</td>
<td>pathogen associated molecular patterns</td>
</tr>
<tr>
<td>PECAM</td>
<td>platelet endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>RPM</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td><em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor- beta</td>
</tr>
<tr>
<td>V-CAM</td>
<td>vascular cell adhesion protein</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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CHAPTER 1
INTRODUCTION

Binge drinking has been found to be the leading cause of preventable death in the United States. In fact, 75% of the drinking done by adults is considered binge drinking (1, 2). Nearly half of all trauma patients presenting to the emergency department have been found to have an elevated blood alcohol concentration (BAC) at the time of admission (3, 4). Moreover, these patients have higher rates of morbidity and mortality than non-alcohol exposed patients with comparable injuries. (5-7). Furthermore, these patients have increased susceptibility to infection (8, 9) and impaired wound healing (7, 10, 11). Because of the high prevalence of ethanol consumption in society and the correlation between ethanol intoxication and disease and death following trauma, many scientific studies have and will be focused on the underlying effects of ethanol on immunity and injury.

The harmful effects of ethanol on the immune system following injury are well documented. Studies have observed impaired immunity in patients who are both acute and chronic users (2). For example, scientific reports dating as far back as 1963 observed abnormal leukocyte responses in chronic ethanol users (12) and it is well known that alcoholics are neutropenic (9). Acute ethanol exposure has also been found to impair both the innate and adaptive immune responses (13, 14) and accounts for the majority of reported cases of ethanol exposure and injury (15). Specific impairments include, but are
not limited to impaired phagocytosis, decreased production of cytokines, and defects in leukocyte responses (16-20).

The defects in immunity caused by ethanol are beginning to be investigated in the context of a healing wound (17). The majority of studies to date have focused on changes in the later phases of wound healing, finding that a single dose of ethanol impairs multiple aspects of both the proliferative and remodeling phases of wound healing (21-24). The inflammatory phase has also been found to be affected by a single dose of ethanol (17), but more investigation is needed to gain a better understanding of the specific effects of ethanol on the innate immune system and how this may lead to increased infection rates. In addition, the majority of people who binge drinking do so an average of four times per month, so it is important to understand how multiple doses of ethanol affect the innate immune system following injury and infection (1).

Among the cells of the innate immune system, neutrophils and macrophages have been found to play a role in the wound healing process (25). Neutrophils are the first infiltrating cell to migrate to the wound site, and once there they begin to clear invading bacteria via phagocytosis and degranulation (26). Neutrophil infiltration is followed by the infiltration of macrophages to the wound site (25). Macrophages have multiple roles in the healing process. First, macrophages take part in the clearance of bacteria and cellular debris by phagocytosis (27). Macrophages also produce multiple growth factors which lead to the later phases of wound healing by stimulating cells that take part in reepithelialization, angiogenesis, and collagen deposition (27, 28). Depletion studies reveal that the ablation of either cell type has a negative effect on the healing of an
infected wound (29-32). Because of this, it is important to understand if and how multiple ethanol exposures can affect these cell types in the context of an infected wound.

*Staphylococcus aureus* (*S. aureus*) is a common skin dermatopathogen. Each year in the United States, *S. aureus* accounts for about 75% of all skin infections seen in emergency departments and 30% of surgical site infections (33). Because of the high prevalence of skin infections with this pathogen, we investigated how ethanol influences bacterial survival and the innate immune response in infected skin wounds. Determining mechanisms by which ethanol exposure negatively regulates the innate immune response following injury and infection may lead to the development of novel therapeutic strategies designed to improve wound care for all patients, including those who had consumed alcohol before they sustained their injuries.

**Hypothesis**

Multiple day binge ethanol exposure reduces leukocyte accumulation at the infected wound site and diminishes the ability of leukocytes to recognize and phagocytose *Staphylococcus aureus* (*S. aureus*), leading to increased bacterial survival.

**Specific Aims**

**Aim 1:** To determine if multi-day ethanol exposure decreases leukocyte accumulation at the site of cutaneous wound infection.

**Rationale:** Both neutrophils and macrophages have been found to play differential, but important roles in the process of wound healing and have also been found to be affected by ethanol exposure. Ablation of macrophages has
been found to be detrimental to the wound healing process (30, 31, 34), while depletion of neutrophils in an infected wound leads to bacteremia (32). To determine if a lack of either neutrophils or macrophages contribute to an increase in bacterial growth or an increase in wound size, we will measure the accumulation of both cell types at the infected wound site.

**Aim 2:** To establish which leukocyte subset(s) phagocytose *S. aureus* in an infected wound and whether multi-day binge ethanol exposure reduces phagocytosis.

**Rationale:** In order to properly clear wound debris and invading bacteria, neutrophils and macrophages need to be able to actively phagocytose. The phagocytic function of both leukocyte subsets has been found to be affected by ethanol exposure (16, 35). To determine if one or both of these cell types is phagocytosing in the current model, we will examine phagocytosis by immunofluorescent staining. We also will determine if ethanol exposure leads to any changes in the number of neutrophils or macrophages that are phagocytosing the invading bacteria.
CHAPTER 2
REVIEW OF THE RELATED LITERATURE

Alcohol

Every year, 80,000 deaths are attributable to excessive alcohol use in the United States, making excessive alcohol use the third leading lifestyle-related cause of death in the nation and the leading cause of preventable death (36). The cost of excessive alcohol consumption in the United States reached $223.5 billion in 2006 and binge drinking accounted for almost three-quarters of these costs (37). Binge drinking is the most common pattern of excessive alcohol use in the U.S. Nearly 15% of the population participating report having binge drinking within the last 30 days and approximately 75% of alcohol consumption by adults is considered binge drinking (1). On top of this, the majority of those who binge drink are not alcohol-dependent or alcoholics (2).

According to the center for disease control and prevention (CDC) binge drinking is defined as the consumption of 4+ (women) or 5+ (men) drinks within a single, two hour period (CDC), or enough alcohol to bring your BAC over 80 mg/dl in a two hour period (NIAAA). Those who take part in excessive drinking increase their risk for both fatal and nonfatal injuries. Binge drinking is also associated with many health problems including alcohol poisoning, sexually transmitted diseases, unintended pregnancy, cardiovascular disease, liver disease, neurological damage, sexual dysfunction, poor control of diabetes, and unintentional and intentional injuries (2, 5, 6, 15, 38).
The process of cutaneous wound healing is a dynamic process that includes three overlapping phases: inflammation, proliferation, and remodeling (25). These phases require the coordination of immune cell infiltration, keratinocyte proliferation, angiogenesis, extracellular matrix deposition and collagen remodeling to ensure proper wound closure (25). Immediately after injury, the inflammatory phase begins. This phase is characterized by an infiltration of platelets, neutrophils, and monocytes into the wound bed (Figure 1). Following wounding, hemostasis is achieved through the release of clotting factors and fibrin. These clotting factors lead to the formation of a fibrin clot, which prevents blood loss and provides a provisional extracellular matrix to which cells can migrate and attach (39). Once hemostasis is achieved, vasomediators and chemotactic factors are released, leading to the recruitment of inflammatory leukocytes to the site of injury (40). The first inflammatory cell that is recruited from the circulating blood to the site of injury is the neutrophil. Neutrophils help clear the wound area of foreign particles and bacteria through the production of reactive oxygen species (26, 41). These cells begin to arrive just minutes after injury and are at peak levels around one day after injury (42, 43). Monocytes infiltrate shortly after and differentiate into activated macrophages, which reach peak levels three days post injury. These activated macrophages continue to participate in host defense through phagocytosis of bacteria and excess neutrophils and through antigen presentation (27). Macrophages also produce multiple cytokines and growth factors which promote macrophage survival and the beginning of tissue formation in wounds (28, 31).
The plethora of growth factors and cytokines produced by macrophages lead into the proliferative phase which is characterized by reepithelialization, angiogenesis, and collagen synthesis (Figure 1) (25). During reepithelialization, which begins a few hours after wounding, keratinocytes switch from an adhesive to a migratory phenotype in order to migrate along the provisional extracellular matrix, separating the wound scab from the viable tissue (44, 45). This migratory phenotype requires the keratinocytes to release collagenase and plasmin to degrade fibrin clots and extracellular matrix (46, 47).

Following the migratory keratinocytes, other keratinocytes at the wound margin begin to proliferate behind the actively migrating cells. These other keratinocytes then return to their normal adhesive phenotype (25, 48).

In order to sustain the newly formed granulation tissue, new blood vessels must form (49). Multiple molecules have been found to induce angiogenesis such as fibroblast growth factor released from macrophages (48) and vascular endothelial growth factor (VEGF) released from keratinocytes (50). These growth factors promote migration of endothelial cells to the injured site and once there, these growth factors stimulate the formation of new blood vessels (51). Once the formation of new granulation tissue in the wound is complete, angiogenesis stops and many of the newly formed blood vessels are degraded through endothelial cell apoptosis (52).

As the proliferative phase continues, fibroblasts begin to synthesize the new extracellular matrix needed to support the cellular growth. Fibroblasts are stimulated by multiple growth factors including fibroblast growth factor (53). Once stimulated, the fibroblasts then migrate toward the center of the wound. As they migrate, the fibrin clot is
degraded and replaced with fibronectin, fibrin, and hyaluronic acid, which make up the early granulation tissue. During this time period, the provisional matrix of granulation tissue is slowly replaced with collagen matrix (48, 54). This collagen matrix is first replaced with collagen type III, followed by the stronger collagen type I. After the deposition of an abundant collagen matrix, the production of collagen by fibroblasts is discontinued (25).

The final remodeling phase of healing is characterized by the slow reorganization of the extracellular matrix proteins (Figure 1). During this time, the deposited collagen is slowly degraded by several proteolytic enzymes (55). This degradation allows for the transition from granulation tissue to scar tissue, a process that also involves new matrix protein synthesis and collagen cross linking (25). This remodeling process leads to a newly organized matrix which is about 70 percent as strong as normal skin (56).

Many murine models are being used to study the wound healing process (17, 21-23, 43, 57, 58). However, there are some key differences between murine and human skin that should be considered when assessing the translational relevance of wound healing studies using mice. The process of wound healing is slightly different in mice and humans. In mice, the panniculosus carnosus, a thin layer of muscle, lies beneath the dermis of the entire body, but in humans, the panniculosus carnosus is only found in the neck (59). This layer of muscle results in quick wound contracture following injury. This is the predominant form of early wound closure in mice (60). On the other hand, wounds in humans only heal by reepithelialization and granulation tissue formation, which results in slower healing compared to mice (57, 59). It is also important to note that mice have a
much thinner epidermis and have fur, while humans have a much thicker epidermis and have limited hair growth. The skin in humans also adheres to the underlying tissue, unlike mouse skin (59). Although there are many similarities in wound healing between mice and humans, it is important to understand the limitations when using a mouse model of wound healing for translational studies.

**Figure 1. Phases of cutaneous wound healing.** Immediately after injury the inflammatory phase begins. This phase is characterized by hemostasis, neutrophil infiltration and macrophage infiltration and usually subsides after a few days. This is followed by the proliferative phases, which begins around day 3 and continues for weeks after injury. This phase is characterized by angiogenesis, reepithelialization, and collagen deposition. The final remodeling phase begins a few weeks after injury, and can continue for months. During this time, collagen is remodeled, leading to increased tensile strength and scar formation.
Leukocyte extravasation refers to the migration of leukocytes from the peripheral blood, to a site injury or infection (61) and involves multiple events. This process begins with the production of chemoattractants released by resident skin cells following wounding. Once a pathogen is recognized or tissue is injured, cytokines such as IL-1 and TNF-α are released (62). These cytokines lead to the expression of cellular adhesion molecules, such as selectins, on the endothelial cells of blood vessels near the site of infection (61).

Once leukocytes have traveled to blood vessels near the infection or injury site, carbohydrate ligands on the surface of leukocytes bind weakly to the selectins on the vessel wall (63). There are three selectins: P-, E-, and L-selectins (61, 63). The weak binding between the selectins and leukocyte causes the leukocytes to slow down and roll along the vessel wall (61). Once the rolling has begun, chemokines are released which activate the rolling leukocytes and the surface integrins switch from a low-affinity to a high-affinity state (64). In this high-affinity state, integrins, such as VLA-4 and LFA-1, bind tightly to complementary receptors expressed on endothelial cells, such as vascular cell adhesion molecule-1 (V-CAM-1) or intracellular adhesion molecules ICAM. Once this attachment occurs, the leukocytes stop rolling (65). At this point the cytoskeleton of the leukocytes is remodeled so they can pass through the gaps between endothelial cells, which is called transmigration, or diapedesis, and is occurs via the use of platelet endothelial cell adhesion molecules (PECAM) (66) and ICAM (67). These proteins are found on the surface of both leukocytes and endothelial cells and pull the cell through the
endothelium (66, 67). However, the mechanism by which transmigration takes place is not well understood. The leukocyte then must penetrate the basement membrane, a process that involves proteolytic digestion of the membrane or mechanical force (61). Finally, once transmigration occurs, leukocytes follow the chemotactic gradient toward the site of injury and/or infection.

**Alcohol Metabolism and its Physiological Effects**

Ethanol is known to affect cellular activity within the body. This effect of ethanol can be directly due to ethanol itself via changes in membrane fluidity, or due to oxidative stress from its metabolic by-products. There are three enzyme systems that are involved in ethanol oxidation: alcohol dehydrogenase (ADH), the microsomal ethanol oxidizing system (MEOS), and catalase (68). Ethanol is predominately oxidized by ADH into acetaldehyde, which is further metabolized into acetate, while the other two pathways are minimally used. When ingested, about 30% of ethanol is metabolized in the gastric mucosa, while the residual ethanol is metabolized in the liver (69). Multiple by products of ethanol such as NADH, acetaldehyde, and acetate can induce tissue damage through the production of reactive oxygen species (ROS), lipid peroxidation, and alterations in signal transduction (70, 71). Acetaldehyde is the primary oxidation product of ethanol and is generated by all three pathways. Acetaldehyde toxicity is mediated by its ability to form protein adducts, which contributes to the decrease in oxygen use, enzyme activity, DNA repair mechanisms, and increase free-radical formation (70, 72).

On the cellular level, ethanol is known to change cell membrane fluidity leading to a disruption in lipid protein interactions and protein conformation (73). Specifically,
acute ethanol exposure leads to more fluid membranes, while chronic ethanol exposure leads to more rigid membranes (74). This change in membrane fluidity is caused by the direct interaction of ethanol with the lipids in the membrane (75). Changes in membrane fluidity can affect cell signaling, an effect that could lead to a dampened immune response, for example (76). Specifically, proteins within the cell membrane, such as Toll-like receptors (TLRs) and a co-receptors may not be able to bind to their targets due to their improper distribution in the cell membrane (77).

Ethanol is also known to have an effect on the body at the systemic level. Specifically, acute ethanol exposure is known to increase systemic glucocorticoid levels (78, 79). Glucocorticoids affect two main body systems: the immune system and the metabolic system. In regards to the immune system, glucocorticoids suppress inflammation (80). Glucocorticoids are hormones, which are activated during a stress-response to internal or external factors. During a stress response, glucocorticoids such as cortisol suppress less essential functions, such as digestion and tissue repair (79). This suppression occurs through the modulation of gene expression mediated by glucocorticoid receptors either by direct or indirect interactions (80). Through these interactions with receptors, glucocorticoids are able to upregulate anti-inflammatory cytokines and downregulate pro-inflammatory cytokines (80).

**Alcohol and Phagocytic Cells**

As mentioned earlier, clinical evidence reveals that, in humans, alcohol has harmful effects on the immune system following injury (5-7, 38). Researchers have observed immune dysregulation in hospital patients following acute and chronic ethanol
exposure (10, 81, 82). For example, alcoholics are known to have a decrease in leukocyte counts (12, 83). This may contribute to higher rates of post-injury infection in intoxicated hospital patients relative to patients with no ethanol exposure (7, 81, 82).

These clinical observations of immune dysregulation by alcohol have been further examined using *in vivo* and *in vitro* models (16, 17, 35, 84-86). Specifically, many studies have focused on how ethanol affects the innate immune response, which is the body’s first line of defense against invading pathogens (25). Two of the main cell types that are involved in the innate immune response after wounding are neutrophils and macrophages. Both of these leukocyte subsets are involved in phagocytosis of invading pathogens and debris (25, 27). Phagocytosis is the process by which the cell membrane engulfs solid particles to form a phagosome within the cell. Phagocytosis begins when bacteria or debris attach to pathogen-associated molecular patterns (PAMPS) on the exterior of the cell (87). Once a particle attaches, the cell engulfs it using the actin-myosin contractile system (88). The phagosome within the cell is then fused with a lysosome leading to degradation of the particle (89).

In a murine model of cutaneous excisional wound healing, mice were given a single dose of ethanol to increase their blood alcohol level to 100-120 mg/dL at the time of injury (17). This single dose of ethanol was found to decrease neutrophil chemoattractants, KC and macrophage inflammatory protein 2 (MIP-2), and the proinflammatory cytokine interleukin 1 beta (IL-1β) in the skin immediately adjacent to the wound within 24 hours of injury. Myeloperoxidase (MPO) levels were also reduced by up to 32% in wounds from ethanol treated mice leading indicating that neutrophil recruitment was impaired by a single dose of ethanol (17). Additionally, *in vivo* binge
drinking models involving a single exposure to ethanol have revealed an increase in blood neutrophil apoptosis in both rats and humans (84). For humans binge drinking was defined as the ingestion of five or more alcoholic drinks within a one hour period; the rats received a 5.5g/kg body weight dose of ethanol via intraperitoneal (i.p) injection. *In vitro* experiments also found a dose-dependent effect of ethanol on apoptosis of human neutrophils following 16 hours of ethanol exposure, with higher levels of ethanol being associated with elevated neutrophil apoptosis (84). Both studies suggest that ethanol reduces neutrophil functionality and leads to elevated neutrophil cell death.

Ethanol has also been found to have a negative effect on macrophage function. Specifically, multiple studies have found a single ethanol exposure leads to impairment of macrophage phagocytosis (16, 35, 85, 86). A study using human blood monocytes demonstrated that, *in vitro*, the presence of ethanol (80mM) inhibited monocyte phagocytic function by up to 67% (85). A reduction was also found in binding of monocytes to Fc gamma receptor-type two (FcγR-type II), a receptor involved in phagocytosis of antibody coated particles (85). Another *in vitro* study using human blood monocytes cultured with 1-3 mg of ethanol per ml found that after incubation for one hour, monocytes showed impaired phagocytosis of IgG-sensitized RBC and non-opsonized *C. albicans* (86). A further *ex vivo* study using alveolar macrophages harvested from mice three hours after they were given 2.2 g/kg of ethanol also showed impairments in phagocytic function (16). When alveolar macrophages were cultured with green fluorescent protein expressing *Pseudomonas aeruginosa* (EGFP-*P. aeruginosa*), macrophages from ethanol treated mice exhibited a 58% decrease in the phagocytic index.
of cells that had bacterial fluorescence in the cytoplasm relative to macrophages from control mice (16).

Impairment in both neutrophil and macrophage phagocytosis resulting from a single ethanol exposure were observed in a mouse model of septic infection (35). Mice received a single dose of 4 or 6 g/kg of ethanol prior to receiving an i.p. injection of *E. coli* to establish an infection. At day 3 post infection, mice exposed to ethanol had up to 100% mortality while control mice had only 30% mortality rate. This mortality coincided with significantly elevated levels of bacteria in the peritoneal cavity. The increased numbers of bacteria could potentially be explained by the finding that, in ethanol treated mice, neutrophils and macrophages in were also found to have a decreased ability to clear invading bacteria (35). Both innate immune cell types were shown to be important for the clearance of invading pathogens. Moreover, this study found that even a single exposure to ethanol can impair the ability of these cells to phagocytose and clear bacteria in the body. This impairment in phagocytic cell functionality could contribute to the elevated rates of infection seen in hospital patients that have alcohol in their system at the time of admission (7, 82).

**Alcohol and Wound Healing**

Nearly half of all trauma patients presenting to the emergency room have an elevated BAC at the time of admission (3, 4). Moreover, these patients have higher rates of morbidity and mortality than non-alcohol exposed patients with comparable injuries (5-7). This includes impaired wound healing (8, 9) and increased susceptibility to infection (7, 10, 11). Alcohol is known to have a harmful effect on cutaneous wound
repair and immune function in both humans and mice (17, 19, 21-23), and having an elevated BAC at the time of injury may contribute to these clinical complications (5-7).

As previously discussed, earlier studies by Fitzgerald et al. have found that a single dose of 1.2 g/kg of ethanol impairs the inflammatory phase of wound healing. Specifically, during the early inflammatory phase, ethanol treated mice had lower levels of neutrophil chemoattractants KC and MIP-2 as well as decreased levels of MPO within excisional wounds up to 24 hours after wounding (17). From these findings it was concluded that ethanol exposure causes impairments in neutrophil recruitment to the wound (17). However, there is a lack of further information examining how ethanol disrupts the rest of the inflammatory phase of wound healing.

Ethanol exposure has also been found to negatively affect multiple aspects of both the proliferative and remodeling phases of wound healing (21-23). Using a murine excisional wound healing model, Radek et al. demonstrated that after a single dose of 1.2 g/kg of ethanol there is a delay in reepithelialization at day 2 post-wounding and a reduction in dermal collagen content in excisional wounds at day 7. In addition, angiogenesis was reduced by up to 61% in ethanol treated mice out to day 14 post-wounding. To determine if the decline in angiogenesis was specific to wound type, wound vascularity in a burn wound model was investigated as well (21). In burn wounds, a single dose of ethanol also led to a decrease in revascularization of the wound (21). Further studies also demonstrated a reduction in the amount of collagen produced in wounds of ethanol treated mice and a shift in the proteolytic balance leading to extracellular matrix degradation and compromised tissue integrity (22). Overall, these findings suggest that a single exposure to ethanol can impair both the proliferative and
remodeling phases of wound healing and these effects can be seen after the ethanol has been cleared from the circulation (21, 22).

Studies have also found that a single high dose of 100mg/dl of ethanol leads to impaired normal human fibroblast proliferation in vitro (24). This was accompanied by a decrease in production of collagen I and lysyl oxidase mRNA. Similar changes were also observed in an in vivo excisional wounding model where mice received 1.4g/kg of ethanol. A 40% decrease in wound breaking strength was found in ethanol-treated mice, as well as reduced lysyl oxidase activity, collagen content, and hyaluronic acid content. Due to the fact that fibroblasts are responsible for the majority of extracellular matrix synthesis, this decline in wound breaking strength is not surprising (24).

**Neutrophils and Wound Healing**

Neutrophils, the first infiltrating leukocyte at the site of injury, play a critical role in defense against foreign pathogens and also in removing contaminated debris (42). Despite these functions, there is a controversy about the necessity of neutrophils in cutaneous wound healing. Excessive release of enzymes, such as elastase and MPO, by neutrophils through the process of degranulation can be damaging to the surrounding tissue, delaying rates of healing and increasing the risk of scar formation (42, 90, 91). Specifically, these granules aid in the microbial killing within phagosomes (91). However, when the granules are released into the extracellular environment in excess, there is potential for host tissue damage by the hydrolytic enzymes (91). Due to these differential effects, the requirement of neutrophil infiltration in the wound healing process is debated.
Neutrophils are recruited to the wound from the peripheral blood in response to chemical gradients. Specifically, chemokines mediate chemoattraction via the chemokine receptor CXC receptor 2 (CXCR2) on neutrophils. Two of the chemokines that induce neutrophil chemotaxis are KC and MIP-2, the murine homologues of IL-8 (58, 92, 93). Inhibition of MIP-2 has also been found to reduce neutrophil influx into the peritoneum in a murine model of peritonitis (92).

Arguing against the necessity of neutrophils for efficient wound healing, in vivo depletion of these cells in mice by, treatment with anti-neutrophil serum prior to injury resulted in more rapid reepithelialization up to day 3 post injury (42). There were also no significant differences in macrophage infiltration. Additionally, neither collagen deposition nor wound breaking strength was affected by a reduction in the neutrophil population (42). This evidence corroborates earlier findings that demonstrate a neutral role for neutrophils in wound healing (94). Following neutrophil depletion and wounding in guinea pigs, no differences were observed in wound debridement or wound repair rates leading to the conclusion that neutrophil infiltration is not essential to wound repair (94). Others have shown that the prolonged presence of neutrophils was detrimental to the healing process. In a murine model of diabetes, prolonged expression of MIP-2 and extended infiltration of neutrophils at the site of injury were associated with impaired wound healing (95).

On the other hand, abundant evidence exists to support neutrophil-dependent wound healing. Mice lacking the CXCR2 receptor, responsible for neutrophil chemotaxis, have impaired neutrophil recruitment to the site of injury. This impairment was accompanied by decreased reepithelialization and angiogenesis (58). Although
neutrophil recruitment was limited, monocyte and macrophage infiltration was enhanced in CXCR2 deficient mice (58), suggesting that the loss of the neutrophil population may alter the interaction between the two cell subsets in the context of wound healing. Moreover, neutrophil depleted mice displayed an increase in ulceration size of infected wounds as well as an increase in bacteremia as compared to mice with an intact neutrophil population (32). These changes coincided with a lack of antibodies specific to the bacterial cell wall in the serum (32). Kim et al. found similar results in infected wounds. When c-kit+ progenitor cells were depleted from the bone marrow and mature neutrophils were blocked using anti-Gr-1, survival rates of infected mice dropped from 80% to 40% at day 2 post infection (29). From these data, it can be concluded that resident macrophages alone are unable to effectively clear invading pathogens. The recruitment of neutrophils to an infected wound is necessary to effectively clear invading pathogens.

**Macrophages and Wound Healing**

Macrophages have been found to be the pivotal immune cell in the context of wound healing, being involved predominately in the inflammatory and proliferative phases of wound repair (25). Their important role is highlighted by the association of impaired macrophage function or alterations in macrophage infiltration with a prolonged inflammatory response, inadequate wound closure and poor restoration of normal cutaneous architecture (25, 30, 34, 90). Although there are resident tissue macrophages in the skin, additional macrophages must be recruited to the site of injury for proper wound repair to take place. Multiple chemoattractants have been found to play a role in macrophage/monocyte recruitment, such as macrophage inflammatory protein-1α (MIP-


$1\alpha$), macrophage inflammatory protein 1-$\beta$ (MIP-1$\beta$), and monocyte chemotactic protein-1 (MCP-1) (40, 96, 97). Specifically, MCP-1 has been found to play a role in monocyte recruitment in incisional and excisional injuries, with peak levels occurring at day one post-injury, prior to maximum macrophage infiltration (98). When MCP-1 was depleted from wounds, a significant 46% decrease in macrophages was observed. On the other hand, when recombinant MCP-1 was added to wounds, a substantial increase in the amount of macrophages was found (98). MCP-1 also seems to play a role in the activation of macrophages. MCP-1 $-/-$ mice were found to have impairments in the later phases of wound healing, but no difference in macrophage recruitment, leading to the conclusion that MCP-1 plays a role in macrophage activation (97).

Multiple studies have revealed the role of macrophages in wound healing by utilizing selective depletion of the macrophage population immediately prior to injury (30, 31, 34). Using transgenic mice which expressed the human diphtheria toxin to specifically ablate macrophages during wound healing, Mirza et al. found there was a significant delay in reepithelialization at day 5 post injury. These mice also exhibited decreased angiogenesis and reduced VEGF production, as well as impaired collagen production and decreased transforming growth factor beta (TGF-$\beta$) production (34). Similarly, using hydrocortisone acetate and antimacrophage serum to eliminate tissue macrophages prior to wounding, Leibovich & Ross saw impairments in wound healing including delayed fibroblast proliferation and inhibition of wound debridement (30). Further studies have investigated the effects of macrophage depletion at different times using inducible diphtheria toxin receptor mice to examine the differential roles of macrophages in the wound healing process (31). Specifically, the absence of
macrophages in the inflammatory phase led to a reduction in the formation of vascularized granulation tissue, impaired reepithelialization, and minimized scar formation (31). In contrast, limiting the number of macrophages in the proliferative phase resulted in severe hemorrhage and transition into the remodeling phase and wound closure did not occur (31). Although there are differences in the models of macrophage depletion, all macrophage depletion prior to and following wounding seem to suggest that the presence of macrophages at the wound site is crucial for all phases of the repair process.

**Conclusion**

Because of the high number of trauma patients that come into the emergency room with an elevated BAC (3, 4), it is important to understand the effect ethanol has on the function of innate immune cells in the setting of cutaneous wound infection. Previous studies have revealed that ethanol exposure prior to injury impairs the early inflammatory response by reducing production of proinflammatory mediators (17), and impedes the later phases of wound repair in non-infected wounds (21-23). There is also evidence that ethanol impairs neutrophil and macrophage function, including phagocytosis and bactericidal activity (16, 17, 35, 85, 86), which may contribute to elevated rates of infection. Determining how ethanol impairs the initial immune response to a cutaneous wound infection may lead to novel therapeutic approaches that could improve the morbidity and mortality rates of intoxicated trauma patients.
CHAPTER 3
MATERIALS AND METHODS

Administration of ethanol, punch wounds, and infection

Male C57BL/6 mice, between 8-10 weeks of age weighing approximately 20 g, were obtained from Jackson laboratories (Bar Harbor, ME). Animals were housed in on a 12-hour light/dark cycle with food and water available *ad libitum*. All experimental procedures and protocols were performed in accordance with Loyola Animal Research Facility Guidelines.

Single and multiple day binge ethanol regimens were used in order to examine differences in bacterial growth. A two-week binge ethanol model was used to mimic binge drinking behavior commonly seen in young adults (99). All mice were administered either saline or ethanol via intraperitoneal (i.p.) injections at 2.2 g/kg body weight in saline (0.3 ml) to achieve a BAC of ~ 300 mg/dl 30 minutes post injection (100) either once (single day binge) or a total of six times (multi-day binge). In the multi-day binge, mice received once daily i.p. injections of saline or ethanol for three consecutive days, followed by four days of rest with no treatment and then either saline or ethanol once daily for an additional three consecutive days (Figure 2). Thirty minutes after the administration of the final or only dose of ethanol or saline, mice were anesthetized, their dorsums were shaved and they received six, 3 mm excisional, full-thickness dorsal
wounds with a dermal punch biopsy (Acuderm) as previously described (17, 21-23). Immediately following injury, 10 µl of saline vehicle or $10^1$-$10^5$ colony forming units (CFU)/10 µl of *S. aureus* Newman strain, were pipetted into each wound in order to achieve a local infection. Back plating was done to determine the amount of bacteria used to infect mice for each individual experiment, and $10^4$-$10^5$ CFU/10 µl of *S. aureus* was used for all experiments. *S. aureus* was chosen as the infecting agent due to the prevalence of this pathogen in skin infections. Each year in the United States, *S. aureus* accounts for about 75% of all skin infection seen in emergency departments and 30% of surgical site infections (33); therefore we used it as our infecting agent.

Following injury and infection, animals were returned to their cages and the cages were placed on heating pads until they were fully awake, after which time they were returned to the animal facility. At 24 and 72 hours post-injury, mice were sacrificed by CO$_2$ inhalation followed by cervical dislocation.

![Figure 2. Model of multi-day binge ethanol followed by cutaneous wound infection.](image)

Tick marks represent days mice were administered ethanol or saline (2.2 g/kg body weight). Arrows represent days the mice were sacrificed.
**Quantification of wound size**

To assess the amount of wound closure, the pelt of the mouse was excised and placed on a flat hockey puck, and pictures were taken from 20 cm above the tissue with a camera (Canon EOS Rebel XT) with a ruler placed within each photograph. Using Photoshop, the number of pixels of each open wound was determined and averaged for each mouse as previously described by Schneider et al (101). Separate groups of mice were sacrificed immediately after wounding to obtain a baseline wound size. The open wound area at each time point was then compared to baseline wounds to calculate the percent of open wound area (101).

**Collection of wound tissue for immunohistochemical and biochemical analyses**

Five mm biopsy punches were used to excise each of the 3 mm punch wounds. One of the six wounds was randomly selected from each mouse and this single wound was utilized for each individual assay (21). For biochemical analysis, wounds were immediately snap frozen in liquid nitrogen and store at -80°C. Wounds for immunohistochemical analysis were frozen in optimal cutting temperature (OCT) media and stored at -80°C. Six μm sections were then made and stored at -80°C for immunofluorescent staining.

**Collection of serum for blood alcohol concentration analysis**

Whole blood was collected via cardiac puncture and put into 1.5 ml microcentrifuge tubes and allowed to sit for 20 minutes. The blood was then centrifuged at 3000 revolutions per minute (rpm) for 20 minutes to obtain the serum. The serum was
then placed into separate tubes and the BAC were measured using the Analox GM7 Micro-Stat.

**Quantification of wound bacteria**

After the wounds were excised, one wound from each mouse was placed in 1 ml of sterile saline and then homogenized in 5 ml tubes. Once there were no more tissue bits left, the homogenized skin was diluted and spread on mannitol salt agar (MSA) plates, which is a selective and differential media specific for *Staphylococci* bacterium due to the high salt concentration. MSA plates also allow for the differentiation between *S. aureus* and *S. epidermidis*, a commensal skin bacteria, due to the mannitol in the agar. *S. aureus* will produce a yellow zone around colonies whereas *S. epidermidis* will not (102). The plates were incubated overnight at 37ºC and the following day plates were counted for the amount of *S. aureus* growth.

**Determination of cutaneous wound chemokine content**

Once skin was obtained from mice, tissue was homogenized in 1ml of a protease inhibitor cocktail (103) and aliquots were frozen at -80ºC prior to assay. MCP-1, KC, and MIP-2 levels were assayed by enzyme linked immunosorbent assay (ELISA) according to manufacturer’s protocols. The amount of protein in the same aliquot used for the ELISA was measured using the BioRad protein assay based on the method of Bradford (BioRad, Hercules, CA) (104). The ELISA results were then normalized by dividing the ELISA results by the amount of protein present in the sample. MCP-1, KC, and MIP-2 data are expressed as the amount of chemokine in pg per mg of total protein.
Quantification of macrophages and neutrophils within the cutaneous wound

To assess the cutaneous wound macrophage and neutrophil content, immunohistochemical staining was performed. MOMA-2, a monoclonal antibody that reacts with the majority of mononuclear phagocytes in tissue (105), served as the primary antibody for macrophages (103). Gr-1, a rat monoclonal antibody that reacts with Ly-6C/G which is expressed on cells of the myeloid lineage served as the primary antibody for neutrophils (106). Following storage in OCT, 6 µm section were cut using a cryostat. Briefly, sections were then fixed to the slide in 4% paraformaldehyde for 15 minutes, and then blocked for nonspecific binding with 10% bovine serum albumin (BSA) and 3% normal goat serum in sterile phosphate buffered saline (PBS) for 1 hour. Slides were then incubated with Gr-1 primary antibody overnight (1:200) followed by a secondary antibody (Alexa Fluor 488 goat anti-rat, 1:500) for 1 hour. Following this, slides were incubated with MOMA-2 primary antibody (1:500) for 1 hour followed by a secondary antibody (Streptavidin conjugated Alexa Fluor 495). Slides were then allowed to dry. Once dry, VectaShield mounting media with 4',6-diamidino-2-phenylindole (DAPI) was added to the slides and they were covered with cover slips (modified from (107)). Quantification of neutrophils and macrophages was determined by using fluorescent microscopy by counting the amount of positively stained cells in ten high-powered fields along the wound edge (40 x) as previously described (108) (Figure 3).

Quantification of wound leukocyte phagocytosis

To assess neutrophil and macrophage phagocytosis in the wound bed, immunohistochemical staining was performed. Slides were stained with either MOMA-2
and anti-*S. aureus* or Gr-1 and anti-*S. aureus*. Briefly, sections were fixed in 4% paraformaldehyde for 15 minutes, and then blocked for nonspecific binding with 10% BSA and 3% normal goat serum in sterile PBS for 1 hour. Slides were then incubated with anti-*S. aureus* biotinylated (1:600) overnight, followed by streptavidin conjugated Alexa Fluor 495 (1:500). Slides were then incubated with either Gr-1 (1:200) overnight followed by Alexa Fluor 488 goat anti-rat (1:500) or MOMA-2 (1:500) for 1 hour followed by or streptavidin conjugated Alexa Fluor 488 (1:500) for 1 hour. Slides were then allowed to dry. Once dry, ProLong Gold antifade reagent with DAPI was added to the slides and they were covered with cover slips (modified from (107)). Fluorescent microscopy was used to quantify the amount of neutrophils and macrophage phagocytosing by counting the amount of co-localized cells in ten high-powered fields along the wound edge (40x) as previously described (108) (Figure 3).

**Statistical analysis**

Data are expressed as mean ± standard error of the mean (SEM) unless otherwise noted. Differences between groups were determined by Student’s t-test. A difference of p<0.05 was considered significant.
Figure 3. Histological image of the wound and surrounding tissue. Arrow points to the wound. Within the box is the wound edge, where the uninjured tissue meets the wound. The area within this box is where all IHC images and quantification are done.
CHAPTER 4
RESULTS

**Blood Alcohol Concentration**

In order to determine if the BAC matches previously published data at the time of injury and infection (100), the BAC was measured 30 minutes following ethanol exposure. Following sacrifice, blood was collected via cardiac puncture and then centrifuged to obtain serum. When the BAC was measured in these mice, the average BAC was 296.68 ± 8.71, which is consistent with previously published data (100).

**Bacterial Counts**

We first elected to determine if either, or both, of our ethanol regimens (single and/or multi-day binge ethanol exposure) lead to a difference in bacterial growth as is seen in the clinical literature (7, 82). In order to examine the amount of bacterial growth, one wound from each mouse was homogenized in sterile saline and analyzed. At 24 hours post-injury, wounds from mice that received multiple doses of ethanol exhibited a 3.6 fold increase in bacterial growth compared to saline controls (Figure 3B). However, mice that received a single dose of ethanol had only a 2.2 fold increase in bacterial growth as compared to saline controls (Figure 3A). Because of the more pronounced difference in bacterial growth in mice with multiple exposures to ethanol, we decided to discontinue use of the single day binge ethanol model. However,
when bacterial growth was analyzed at 72 hours, no statistically significant differences were found between ethanol and saline treated mice (Figure 3C).

Figure 4. Bacterial colonization within the wound bed following cutaneous wound infection. At 24 and 72 hours after ethanol exposure (2.2 g/kg of body weight), injury and infection mice were sacrificed and bacterial colonization was determined by growth on MSA plates. (A) Mice received a single dose of ethanol prior to injury and infection. n=3-5 per group. Data from two experiments (B, C) Mice received 6 doses of ethanol on separate days prior to injury and infection. n=7-12 per group. Data from two experiments. Data represented as mean ± SEM. No statistically significant differences were found.

Wound size

To further examine morphological differences caused by multiple doses of ethanol, we decided to analyze the wound size of mice after they were sacrificed. Pictures were taken of the wounds immediately after the pelt was removed (Figure 4A), and the amount of open wound area was determined in saline and ethanol treated mice. Separate groups of mice were sacrificed immediately after wounding to obtain a baseline wound size. The open wound area at each time point was then compared to baseline wounds to
calculate the percent of open wound area (101). At 24 hours after injury and infection, there was an increased percentage of open wound area in samples obtained from ethanol exposed mice as compared to saline controls. Wounds from saline control mice were 54% open, while wounds from ethanol treated mice were still 74% open (Figure 4B). However, by 72 hours the size of wounds from ethanol treated mice were comparable to the size of wounds from saline treated mice. At 72 hours wounds from saline treated mice were 49% open, while wounds from ethanol treated mice were now 48% open (Figure 4B). Although there was an initial difference in the percentage of open wound area at 24 hours between the two groups, by 72 hours this difference was no longer seen. Because of this, it can be concluded that the difference in wound size at 24 hours is a transient change that is resolved by 72 hours post injury and infection.

**Macrophage and neutrophil accumulation at the infected wound site**

After observing an elevation in bacterial growth and an increase in wound size in multi-day ethanol treated mice at 24 hours, we choose to examine leukocyte infiltration at the wound site. In order to properly clear foreign particles and invading pathogens, both neutrophils and macrophages are needed at the site of injury and infection. Skin sections were stained with anti-Gr-1, a neutrophil marker, and MOMA-2, a macrophage marker. Pictures were then taken in 10 random high power fields along the wound edge for each mouse and the amount of Gr-1 positive and MOMA-2 positive cells were counted (108). Although there is controversy about the necessity of neutrophils in wound healing (42, 94, 95), neutrophil infiltration is a hallmark of the early inflammatory phase (17). While evidence
Figure 5. Percentage of open wound area. At 24 and 72 hours after injury and infection, animals were sacrificed and the percentage of open wound area was determined by analysis using Photoshop. (A) Representative images from wounds at 24 and 72 hours in the saline groups and ethanol groups. (B) The percentage of open wound area was determined by dividing the number of pixels of wounds from saline and ethanol treated mice by the number of pixels from baseline wounds from mice that were immediately sacrificed after injury. Data from two experiments. Data represented as mean ± SEM, n=12-18 per group. *p<0.0001 as compared to saline, by Student’s t-test.
exists that shows neutrophil presence is not important, and maybe even detrimental to the
wound healing process in a noninfected wound (42, 94, 95), their infiltration is important
in order to clear invading pathogens (29, 32). Neutrophil depletion in infected wounds
has been found to increase bacteremia as well as ulceration size (32). Since we saw an
elevation in bacterial growth in wounds from ethanol treated mice relative to control, we
examined neutrophil accumulation at the infected wound site. When examined, no
difference in the amount of Gr-1 positive cells was found between ethanol and saline
control mice (Figure 5A, B). This visual finding was confirmed when the amount of Gr-1
positive cells were counted (Figure 5C).

Studies have shown that the presence of macrophages is important for the wound
healing process. Multiple depletion studies have demonstrated that a lack of macrophages
at the wound site is detrimental to the wound healing process (30, 31, 34). Since we
observed a delay in the reduction of wound size and a trend toward elevated bacterial
growth at 24 hours post injury and infection, we also examined macrophage
accumulation at the infected wound site. When examined, the ethanol treated mice have
fewer MOMA-2 positive cells as compared to saline controls (Figure 5A, B). This was
confirmed when the amount of MOMA-2 positive cells were counted, and there was a
53% decrease in the amount of MOMA-2 positive cells in wounds of ethanol treated mice
as compared to saline controls (Figure 5D).
Figure 6. Macrophage and neutrophil accumulation following cutaneous wound infection. 24 hours after cutaneous wound infection, wounds were examined by fluorescent microscopy with anti-Gr-1, anti-MOMA-2 and DAPI. Ten high power pictures per mouse were taken along the wound edge. Representative images from (A) saline and (B) ethanol groups at 40x. The number of (C) Neutrophils, Gr-1$^+$ cells (green), and (D) Macrophages, MOMA-2$^+$ cells (red) were determined. Data from two experiments. Data represented as mean ± SEM, n=5-9 per group. *p<0.05 as compared to saline, by Student’s t-test. Arrows point to Gr-1$^+$ cells and triangles point to MOMA-2$^+$ cells.
Mean fluorescent intensity of MOMA-2 positive cells

In order to gain additional evidence in support of our finding that ethanol treated mice have a decrease in macrophage accumulation at the infected wound site, we examined the mean fluorescent intensity (MFI) of ten MOMA-2 positive cells in mice from both treatment groups. This was done to determine if the reduction in macrophage accumulation seen was due to down regulation of the cell surface MOMA-2. There was no difference in the MFI of MOMA-2 positive cells between the two groups (Table 1).

<table>
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<th>Table 1: MFI of MOMA-2 positive cells</th>
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<td>MFI</td>
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Using Axiovision 8.1 program, ten MOMA-2+ cells per sample were measured for their MFI using previously taken images. No statistically significant differences were found. Data from two experiments.

Wound chemokine content

Although no differences were observed in neutrophil accumulation, we also decided to measure levels of neutrophil chemoattractants in the injured and infected skin. No differences were observed in levels of KC or MIP-2 between the two groups (Table 2).

To further assess what may be leading to the decrease in macrophage accumulation in wounds from ethanol treated mice, we examined levels of macrophage chemoattractant, MCP-1. MCP-1 recruits monocytes to the site of tissue injury, infection,
and inflammation, and depletion of MCP-1 in wounds was found to reduce the amount of macrophages at the wound site (96, 98). When MCP-1 levels were measured, compared to wounds from saline treated mice, the level of MCP-1 was 26% lower in ethanol treated mice at 24 hours (Table 2).

Levels of all chemokines, KC, MIP-2, and MCP-1, were measured in uninjured control mice. All levels were found to be higher in injured skin of mice as compared to normal, uninjured skin (Table 2).

<p>| Table 2: Levels of MCP-1, KC, and MIP-2 in skin homogenates following cutaneous wound infection |
|---------------------------------|------------|------------|</p>
<table>
<thead>
<tr>
<th>Uninjured Skin</th>
<th>Saline</th>
<th>Ethanol</th>
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<tbody>
<tr>
<td>MCP-1</td>
<td>32.12 ± 8.10</td>
<td>337.45 ± 31.96</td>
</tr>
<tr>
<td>KC</td>
<td>118.07 ± 15.57</td>
<td>2465.88 ± 350.25</td>
</tr>
<tr>
<td>MIP-2</td>
<td>641.89 ± 47.21</td>
<td>5625.84 ± 660.26</td>
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As a control, levels of all chemokine and cytokine levels were measured in uninjured skin from unmanipulated mice. At 24 hours after injury and infection, animals were sacrificed and wounds were homogenized for chemokine and cytokine level analysis. All levels were measured by ELISA and presented as pg/mg total protein. Data shown as mean ± SEM, n=5-9. No statistically significant differences, by Student’s t-test. Data from two experiments.

**Macrophage and neutrophil phagocytosis**

In order for invading leukocytes to properly clear invading pathogens, they need to be able to phagocytose (35). Alcohol has previously been found to impair both neutrophil and macrophage phagocytosis (16, 35, 84, 85). Because of the trend towards
elevated bacterial growth in ethanol treated mice, we elected to examine neutrophil and macrophage phagocytosis via immunohistochemistry (IHC) in parallel. Wound sections were stained for macrophages and *S. aureus* or neutrophils and *S. aureus*. When slides were examined, there was limited bacterial dissemination into the tissue adjacent to the wound. Because of this, no evidence of phagocytosis by either neutrophils or macrophages was found, so no quantification was able to be done (Figure 6). Images show the bacteria are localized in the wound scab (Figure 7)
Figure 7. Phagocytosis immunohistochemical staining in infected cutaneous wounds. 24 hours after injury and infection, wounds were examined by fluorescent microscopy in order to compare how ethanol affects the ability of these cells to phagocytose the bacteria in infected wounds. Images were taken along the wound edge at 40x. In (A) and (B) Gr-1$^+$ cells (neutrophils) are green and in (C) and (D) MOMA-2$^+$ cells (macrophages) are green. S. aureus$^+$ cells are red in all images. In all images yellow represents phagocytosis. Representative image from (A, C) saline group and (B, D) ethanol group. Arrows point to S. aureus$^+$ cells. Data from two experiments.
Figure 8. *S. aureus* localized in the wound. Skin was stained with anti-*S. aureus* and examined by fluorescent microscopy to visualize where the bacteria was located within the wound and surrounding skin. Images were taken of the wound to show where the majority of the *S. aureus* bacteria are located. Images taken at 10x. Dashed lines represent the border of the wound.
CHAPTER 5
SUMMARY AND DISCUSSION

In the United States, more than 38 million adults binge drink on average of 4 times per month (1). 80,000 deaths each year are attributed to excessive drinking, and those that participate in binge drinking have a higher likelihood of fatal as well as nonfatal injuries (36). This can be seen emergency departments, where nearly half of all trauma patients having an elevated BAC at the time of admission (3, 4). These patients with alcohol in their system at the time of injury have increased rates of infection (8) as well as impaired wound healing (7, 10, 11). Because of this, studies have begun to focus on how ethanol impairs the wound healing process (17, 21-23). However, many of these investigations have focused on later phases of wound healing (21-23). In order to gain a more complete understanding of how ethanol exposure impairs wound healing as well as increases rates of infection, the present study focused on the inflammatory phase of the wound healing process.

Not only have previous studies focused on the later phases of wound healing, but they have also examined the effects of ethanol on wound healing using a single ethanol dose model (17, 21-23). Although these studies found ethanol-induced impairments in wound healing using this single dose model, a multiple dose model is more clinically relevant due to the fact that 38 million people binge drink, and do so an average of four
time a month (1). Therefore, using a similar model, the current study examined bacterial
growth in infected wounds from mice exposed to comparable levels of ethanol given
once or several times over a two week period. Using these regimens, it was determined
whether either ethanol exposure paradigm altered the cutaneous response to wound
infection. When bacterial growth was examined in both ethanol exposure regimens at 24
hours post injury and infection, the multi-day binge ethanol exposure had a more
pronounced difference in bacterial growth when comparing ethanol and saline controls.
Hence, the multi-day ethanol regimen was used for the remainder of the experiments.
However, the difference in bacterial growth was not found to be statistically significant in
either ethanol regimen. The ethanol treated mice had a higher amount of variability in
bacterial growth. This could be due to differences in ethanol metabolism in different
mice. Although the i.p. injections are done as similarly as possible, variance in where the
ethanol is injected could cause changes in metabolism, leading to variability in bacterial
growth.

The trend towards elevated bacterial growth in ethanol treated mice was
accompanied by a significant increase in the percentage of open wound area at 24 hours
after injury and infection. The 20% difference in the amount of open wound area in
ethanol treated mice at 24 hours may be due to a delay in the production of growth
factors which promote wound closure. Macrophages produce many growth factors that
promote wound healing (25), and since there are fewer macrophages at the site of injury
and infection in ethanol treated mice, this may be contributing to the difference in wound
size. However, by 72 hours both the bacterial growth and wound size in ethanol treated
mice showed no differences between groups, suggesting that the ethanol-mediated impairments seen at 24 hours in the multi-day binge were transient changes that are resolved 72 hours following injury and infection. It is also important to note that between 24 and 72 hours after injury the wound size of the saline treated mice only showed a 5% reduction in the amount of open wound area. Although there was an initial 45% drop in wound size, the infection may be slowing wound contracture in saline treated mice after 24 hours, meaning that although there is an immediately decrease in wound size, the rate of contracture seems to slow after 24 hours and wounds begin to close by reepithelialization.

Multiple factors could be contributing to this resolution of problems observed with the ethanol exposure. To create a local infection rather than a systemic infection, a lower inoculum of bacteria was used in these experiments compared to doses used in the literature (29, 32, 35, 43). This low bacterial inoculum may not be enough to overwhelm an innate immune response impaired from multiple days of ethanol exposure for an extended period of time. The number of days which the mice received ethanol may also be a factor. Since the majority of people who binge drink do so on a regular basis, on average 4 times a month (1), a longer exposure to alcohol may be needed in order to see a prolonged increase in bacterial growth. It is known that chronic drinkers have decreased blood leukocyte counts (12, 83), so a longer ethanol regimen may lead to excessive bacterial growth due to fewer leukocytes to effectively contain the infection. There are also multiple limitations to the examination of wound size and wound healing in mice. First, it should be noted that the wound healing process is different in humans and mice
Mouse wounds close primarily by contraction during the first 24-48 hours (60), while human wounds heal mainly via reepithelialization (57, 59). Because of this difference, mice wounds heal much more quickly than humans (60). When analyzing wound size in translational studies this needs to be taken into account. Also, the method used to assess wound size is not completely accurate. When pictures are taken of the wounds, the pelt is taken off the mouse in order for wounds to be flat. Because of this, different mice needed to be used to obtain a baseline wound size (101). The percentage of open wound area may not be completely accurate since different mice were used for baseline wound size.

Since we saw a trend toward elevated bacterial growth and a significant increase in wound size at 24 hours after wound infection in ethanol treated mice relative to saline treated controls, we sought to determine what defects in the inflammatory phase of wound healing were leading to these problems. Multiple functions of the innate immune response could be affected by ethanol exposure such as leukocyte infiltration and function, immune cell recruitment, cytokine production, immune cell chemotaxis, bacterial recognition, and immune cell signaling. For this study, we chose to examine neutrophil and macrophage accumulation and phagocytosis. Macrophages have consistently been found to be important to wound healing (30, 31, 34). However, the necessity of neutrophils in the wound healing process is more controversial (29, 32, 42, 92). Even so, in an infected wound, the absence of either of these cells types can be detrimental to the wound healing process (29-32, 34). Because of the importance of
neutrophils and macrophages in wound healing, we chose to examine how ethanol affects their accumulation and phagocytic function during the inflammatory phase.

While the necessity of neutrophils in wound healing is controversial, evidence exists that neutrophils are important in an infected wound (29, 32). Previous studies by Fitzgerald et al. found that a single dose of ethanol led to a decrease in neutrophil chemoattractants, as well as lower MPO levels during the early inflammatory phase of wound healing in a non-infected wound (17). However, no differences in neutrophil accumulation were found by IHC staining in the skin immediately adjacent to the wound (17). Similarly, this study also found no difference in neutrophil accumulation at the wound site. Although there were no differences in the amount of neutrophils at the infected wound site, the ability of these cells to function normally was not addressed. A lack of a respiratory burst by neutrophils could contribute to the trend toward elevated bacterial growth seen in the wounds of ethanol treated mice.

Macrophages have been found to play a consistently important role in the progression of wound healing (30, 31, 34). In this study, ethanol treatment led to a 53% decreased the number of macrophages at the infected wound site. Because of the importance of macrophages in the inflammatory and proliferative phases of wound healing (31), a reduction in the number of macrophages at the wound site could contribute to the elevated bacterial growth and increased wound size. These findings are comparable to depletion studies, where a significant ablation in the amount of macrophages at the wound site led to impairments in all phases of wound healing (30, 31, 34). Since the initial job of macrophages is to clear invading pathogens and debris during
the inflammatory phase (25), in the wounds of ethanol treated mice, there may not be enough mononuclear phagocytes at the wound site to successfully eradicate the pathogen. Macrophages also produce numerous growth factors which directly lead into the proliferative phase and promote wound healing. These growth factors stimulate cells that take part in reepithelialization, angiogenesis, and collagen deposition (25, 40). Because there are fewer macrophages at the infected wound site in the ethanol treated mice relative to saline controls, the levels of growth factors within the wound may be reduced, leading to the larger wound size at 24 hours.

In order for immature monocytes to migrate from the peripheral blood to the infected injury site, a chemotactic signal is needed (96-98). Ethanol treatment was found to reduce wound tissue levels of MCP-1 by 26%, which could explain the 53% reduction in the number of macrophages. MCP-1 has been found to be an important macrophage wound chemoattractant (97, 98). Not only does MCP-1 recruit macrophages to injury sites, but it has also been found to activate them (97). Along with limiting the accumulation of macrophages at the wound site, diminished levels of MCP-1 from ethanol treated mice may result in reduced activation of macrophages, leading to reduced functionality and bacterial clearance, and increased wound size. It is also possible that other key macrophage recruitment signals are attenuated. Other macrophage chemoattractants that have been found to be important in wounds are MIP-1α and MIP-1β (40, 109, 110) and a reduction in either of these molecules could also be contributing to the decrease in macrophages in ethanol treated mice. Reduced expression of macrophage chemokine receptors, such as CCR2, the receptor for MCP-1, could be responsible in part
for the limited recruitment of macrophages to the infected wound site. Without the appropriate expression of chemokine receptors, macrophages are not able to respond to chemotactic signals and migrate from the blood to the infected wound site (111). In order to gain a better understanding of how ethanol treatment affects macrophage accumulation at the infected wound site, the macrophage recruitment factors need to be further examined.

Since we found macrophage recruitment factors are affected by ethanol, potentially leading to a decrease in macrophages at the wound site, it is important to understand how ethanol can mediate the production of these factors. Acute ethanol exposure is known to increase membrane fluidity in cells (74). This change in membrane fluidity can lead to a disruption in proteins within the lipid membrane. Improper distribution or conformation of protein receptors within the membrane could cause inadequate association between receptors. If receptors are unable to associate, the signaling cascade may not begin, causing a lack of chemokine production (76). This cellular effect of ethanol can impair the recognition of invading bacteria. Specifically, S. aureus is recognized via the TLR-2 Myd88 pathway. In order for TLR-2 signaling to begin, TLR-2 must homodimerize or heterodimerize respectively with either TLR-2 or TLR-6 (112). If this dimerization is disrupted by high membrane fluidity, the heterodimer cannot interact with PAMPs and induce a signaling cascade. Without this initial signaling, cells such as keratinocytes at the infected wound area would not be able to produce proinflammatory cytokines or chemoattractants to begin the inflammatory process. Also, neutrophils and macrophages use TLR-2 to recognize PAMPs on S. aureus.
to promote phagocytosis (113). Thus, TLR-2 will be unable to dimerize, and initiate the signaling cascade necessary for neutrophils and macrophages to phagocytose properly.

Furthermore, ethanol is known to have a systemic effect on the body, which can lead to defects in the immune system (80, 114). Acute ethanol is known to increase glucocorticoid levels in the body, which suppress inflammation (78, 115). This suppression of inflammation, specifically proinflammatory cytokines, can cause an impaired immune response to injury. A lack of proinflammatory cytokines, such as TNF-α and IL-1β, can lead to a decrease in chemattractant proteins(17), which in turn can cause a reduction in the number of infiltrating leukocytes. This was seen by Fitzgerald et al. who found a decrease in proinflammatory cytokine IL-1β in ethanol treated mice 6 hours post injury. This reduction in proinflammatory cytokines was potentially involved in suppression of KC and MIP-2 levels as well (17). Although the current study did not measure proinflammatory cytokines, a reduction in cytokines which induce MCP-1 expression could be playing a role. A lack of proinflammatory cytokines could also be affecting leukocyte extravasation. Cytokines IL-1 and TNF-α are both known to induce the expression of selectins on endothelial cells, eventually leading to leukocyte migration to the infected wound site (61). Without these cellular adhesion molecules, neutrophils and macrophages may not be able to migrate to the infected wound site.

In order to confirm the reduction in macrophage accumulation at the infected wound site in ethanol treated animals, the MFI of MOMA-2 positive cells (macrophages) was examined in both saline and ethanol treated mice. Since no differences were found in the MFI of MOMA-2 positive cells between the cells from either treatment group, we are
convinced that ethanol is not triggering a downregulation in MOMA-2 surface expression, and that there is an actual decrease in numbers of macrophages at the wound site. However, in order to further confirm this finding, another macrophage cell marker such as F4/80 could be used for staining.

Ethanol has been found to negatively affect both macrophage and neutrophil function, specifically phagocytosis (16, 35, 85). The ability of both neutrophils and macrophages to phagocytose is crucial for the clearance of invading bacteria and debris during the inflammatory phase of wound healing (25, 35). Visualization of the leukocyte subset(s) that are phagocytosing bacteria \textit{in vivo} in wounds from ethanol treated mice would give a more accurate view of what is happening at the wound site as opposed to using an \textit{ex vivo} phagocytosis assay. However, after examining fluorescently stained skin sections, a minimal amount of bacterial dissemination into the surrounding tissue was observed. The majority of the bacteria were localized in the wound bed. Additionally, photos could not be taken within the wound bed because of the large accumulation of debris and cellular infiltrates. Since a low bacterial inoculum was used to in these studies, it is likely that the majority of the bacteria was contained within the wound bed and was unable to migrate into the adjacent tissue. Even the ethanol treated mice that had elevated bacterial growth had very few bacteria in the surrounding tissue. As a result of this observation, one would need to utilize a different method to assess the phagocytic function of infiltrating leukocytes in order to determine whether the lack of observed phagocytosis might contribute to the elevation in bacterial growth seen at 24 hours post injury and infection in ethanol treated mice.
Although this method to examine phagocytosis was unsuccessful, it is important to understand its limitations. The method used was looking for a fluorescent co-localization between \textit{S. aureus} cells and phagocytic cells, and does not take into account phagocytic cell that have already degraded bacteria. Once the bacteria are internalized and degraded within the cell, they can no longer be fluorescently stained, and therefore, phagocytosis cannot be analyzed using this method. Other methods can be used in order to examine neutrophil and macrophage phagocytosis. One option is accessing phagocytic function of isolated skin wound neutrophils and macrophages by flow cytometry (116). In this method, wound leukocytes are isolated and incubated with opsonized pHrodo-\textit{S. aureus} for one hour. Following this, the phagocytic reaction is stopped, and neutrophils and macrophages are stained (116). Although this method has been used successfully, it does not allow for an examination of neutrophil and macrophage phagocytosis \textit{in vivo}.

In conclusion, in the murine model of multi-day binge ethanol exposure and cutaneous wound infection, at 24 hours after injury and infection, there was a trend towards an elevation in bacterial growth as well as an increase in wound size in ethanol treated mice relative to saline treated controls. A summary of how ethanol is leading to these impairments is depicted in Figure 8. While the number of neutrophils was not affected by ethanol exposure, the number of macrophages at the wound site was decreased potentially due to lower MCP-1 levels. Further research is needed in order to also begin to understand how ethanol is affecting the function of both neutrophils and macrophages during the inflammatory phase of wound healing in the context of an infected wound. Assays that examine the phagocytic function of these cells should be
performed in order to gain a further understanding of how ethanol can regulate the
inflammatory phase of wound healing in an infected wound. The study presented herein
has provided further information about how frequent binge drinking episodes can impact
the innate immune response following a cutaneous wound infection. Because of the
frequency of alcohol consumption and injury (1), it is important to enhance our
understanding of how ethanol exposure affects the wound healing process in order to
improve current therapeutic strategies.
Figure 9. Summary of ethanol induced impairments of the innate immune response following cutaneous wound infection. Immediately following cutaneous wound infection as seen on the left side of the figure, chemoattractants are produced to recruit neutrophils and macrophages from the peripheral blood to the infected wound site in order to clear invading bacteria and debris. Macrophages also produce a plethora of growth factors which promote further wound healing. However, following ethanol exposure as seen on the right side of the figure, decreased levels of MCP-1 within the wound tissue may be leading to a reduction in macrophage number, ultimately causing a decline in bacterial clearance and an increase in wound size.
Quantification of wound closure

Materials:

- Camera
- Photoshop
- Camera stand
- Ruler

Procedure:

1. Remove pelt of mouse and place on hockey puck.
2. Take picture using camera stand 20cm from skin section. Make sure camera is completely zoomed out.
3. Open image in Photoshop.
4. Using lasso tool, outline each wound. Then right click and fill with a color.
5. Using the magic wand tool with the tolerance set at 60, select each individual wound and select histogram and record the number of pixels.
6. Average the number of pixels from the six wounds on each mouse.
7. To determine the amount of closure, compare to a control mouse that was injured and immediately sacrificed.
Quantification of wound bacteria

**Materials:**

- 5 ml snap-cap round bottom tubes
- Sterile PBS
- Homogenizer
- Manitol Salt Agar (MSA) Plates
- Plate spreaders
- Ice
- 96-well plates
- Black permanent marker

**Procedure:**

1. Excise one wound from each mouse and place in individual 5 ml snap-cap round bottom tubes with 1 ml of sterile PBS and keep on ice.
2. Homogenize skin for 30 seconds until no tissue bits are left. Return to ice bucket. Between samples, clean the homogenizer with bleach, ethanol, and then distilled water.
3. Dilute the homogenized skin in 96-well plates and place 20µl of the appropriate dilutions on MSA plates in duplicate and spread with plate spreaders.
4. Incubate plates overnight at 37°C.
5. Count individual *S. aureus* colonies by marking the bottom of the plate with a black permanent marker. Only count colonies that have a yellow ring around them. Record the number of colonies for each plate.
Skin Homogenization for ELISA

Materials:

- 5ml snap-cap round bottom tubes
- 1.2µg syringe filters
- 1cc syringes
- 0.5ml microfuge tubes
- 1.5 microfuge tubes
- Lysis buffer (Cell Lysis Buffer, Factor 1, Factor 2, PMSF)
- Dry ice
- Ice

Procedure:

1. Prepare BioRad Lysis Buffer: 9.9ml Lysis Buffer, 40µl Factor 1, 20µl Factor 2, 40µl PMSF.
   a. Prepare the lysis buffer fresh on the day it is being used
   b. Add PMSF last.
2. Place frozen tissue on dry ice.
3. Add 1.0ml of Lysis Buffer to each tube and keep on ice.
4. Using forceps, remove tissue and place in tube and homogenize for 30 seconds until no tissue bits are left. Return to ice bucket. Between samples, clean the homogenizer with bleach, ethanol, and distilled water.
5. Spin samples at 3000 rmp at 4°C for 5 minutes.
6. Sonicate samples at 30% for 10 seconds or until the sample gets foamy.
   a. Do not allow the sample to get warm. If the sample begins to get warm place on ice and allow to cool before re-sonicating.
7. Centrifuge at 1800 rpm at 4°C for 10 minutes.
8. Using a syringe, suck up the liquid avoiding the pellet and syringe filter the sample into 1.5 ml microfuge tubes and return to the ice bucket.
9. Spin the samples at 2000 rmp for 5 minutes at 4°C.
10. Pipette the samples up and down to mix and aliquot into clean 0.5ml tubes.
11. Store aliquots at -80°C.
ELISA Procedure

Materials:

Procedure:

2. When running skin homogenates for all ELISAs, dilute samples 1:50 in protease inhibitor cocktail.
3. Use the following dilution of standards for the appropriate kit.
   a. MCP-1
      200 pg/ml
      100 pg/ml
      50 pg/ml
      25 pg/ml
      12.5 pg/ml
      3.125 pg/ml
      1.563 pg/ml
      0 pg/ml
   b. KC and MIP-2
      1000 pg/ml
      500 pg/ml
      250 pg/ml
      125 pg/ml
      62.5 pg/ml
      31.25 pg/ml
      15.625 pg/ml
      0 pg/ml
4. Read plate at 450 nm and at 570 nm. Subtract 570 from 450.
**Immunohistochemistry for Gr-1 and MOMA-2 on frozen skin sections**

**Materials:**

- Superfrosted PLUS slides
- PAP pen
- 4% paraformaldehyde in sterile PBS (filtered), 37 degrees
- Sterile PBS (filtered)
- Normal Goat Serum
- BSA
- Gr-1
- Moma-2
- Alexa Fluor 495
- Alexa Fluor 488
- VectaShield with DAPI
- Coverslips

**Procedure:**

1. Allow sections stored at -80°C to dry at room temperature in a humidified chamber for 1-24 hours.
2. Encircle tissue with PAP pen.
3. Fix tissue sections in 4% paraformaldehyde for 15 minutes.
4. Wash with sterile PBS 3 times for 3 minutes
5. Block with 10% BSA and 3% Normal Goat Serum in sterile PBS for 1 hour.
6. Tip slides to remove block. Do not wash. Add Gr-1 (1:200) and incubate for 4°C overnight.
7. Rinse off Gr-1 3 times with sterile PBS for 3 minutes each.
8. Incubate with Alexa Fluor 488 goat α-rat (1:500) diluted in sterile PBS for 1 hour.
9. Rinse 3 times with sterile PBS for 3 minutes each.
10. Incubate with MOMA-2 (1:500) diluted in sterile PBS for 1 hour.
11. Rinse 3 times with sterile PBS for 3 minutes each.
12. Incubate with Streptavidin conjugated Alexa Fluor 495 (1:500) diluted in sterile PBS for 1 hour.
13. Rinse 3 times with sterile PBS for 3 minutes each
15. Place 2-4 drops of Vector Shield mounting media on the slide and cover slip and allow to dry.
16. Once completely dry, store samples at 4°C.
Protein Assay

Materials:

- Dye reagent (Bio-Rad Laboratories)
- Glass test tube
- 96 well flat bottom medium binding non-sterile plate
- 0.5ml microfuge tubes
- BSA standard
- Double distilled water

Procedure:

1. Using 0.5ml microfuge tubes dilute homogenized skin sample in double distilled water (1:50).
2. Add 100µl of diluted skin sample to 96 well plate in duplicate leaving rows 1 and 2 open for standard.
3. In glass test tube add 960µl of double distilled water and 40µl of thawed BSA standard and vortex thoroughly.
4. Add 200µl of standard into wells A1 and A2. Fill the remaining wells in rows 1 and 2 with 100µl of double distilled water.
5. Serial dilute the standard to make a 8 point curve. Starting at 200µg/ml.

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6. Add 25µl of Bio-Rad Protein Assay Dye Reagent into each well while mixing and aspirating. Change pipette tips for each well.
7. Read on the ELISA plate reader at 595 nm.
References


VITA

The author, Sara K. Hlavin was born on November 21, 1988 in Evanston, IL to William and Helen Hlavin. Upon completion of her secondary education in 2006 at Saint Viator High School in Arlington Heights, IL, she entered Augustana College at Rock Island, IL. While attending Augustana College, Sara was a varsity swimmer all four years and started the club water polo team. In May 2010, Sara received a Bachelor of Arts degree in Biology.

In August of 2010, Sara entered the Infectious Disease and Immunology Institute at Loyola University Chicago as a Masters student. In the fall of 2010, Sara began her research on the effects of multi-day binge ethanol exposure on the innate immune response following cutaneous wound infection in the laboratory of Dr. Elizabeth J. Kovacs. In addition to her graduate work, Sara was also a member of the graduate student council at Loyola as a program representative he first year, and was the secretary her second year.