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Role of the Interleukin-22 and STAT3 Signaling Pathway in Gut Barrier Maintenance Following Intoxication and Burn Injury

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ROLE OF THE INTERLEUKIN-22 AND STAT3 SIGNALING PATHWAY IN GUT BARRIER MAINTENANCE FOLLOWING INTOXICATION AND BURN INJURY

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LIST OF ABBREVIATIONS

aGVHD	Acute Graft-Versus-Host Disease
ALD	Alcoholic Liver Disease
ALT	Alanine Aminotransferase
AMP	Antimicrobial Peptide
ANOVA	Analysis of Variance
APC	Allophycocyanin
AUD	Alcohol Use Disorder
BAC	Blood Alcohol Concentration
BP	Binding Protein
CINC	Cytokine-induced neutrophil chemoattractant
CLP	Cecal-Ligation and Puncture
CXCL	Chemokine Ligand
DAPI	(4’,6-Diamidino-2-Phenylindole, Dihydrochloride)
DC	Dendritic Cell
DSS	Dextran Sodium Sulfate
DTT	Dithiothreitol
E-cadherin	Epithelial cadherin
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor

ELISA	Enzyme-linked Immunosorbant Assay
ERK	Extracellular Signal-Regulated Kinase
F-actin	Filamentous Actin
FBS	Fetal Bovine Serum
FISH	Fluorescent in situ Hybridization
FITC	Fluorescein Isothiocyanate
GALT	Gut Associated Lymphoid Tissue
GI	Gastrointestinal
HBSS	Hank's Balanced Salt Solution
H&E	Hematoxylin and Eosin
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HRP	Horseradish Peroxidase
ICAM	Intracellular Adhesion Molecule
IEC	Intestine epithelial cell
Ig	Immunoglobulin
IGF	Insulin-like Growth Factor
IHC	Immunohistochemistry
IL	Interleukin
ILC	Innate Lymphoid Cell
i.p.	Intraperitoneal
Jak	Janus Kinase
JAM	Junction-associated adhesion molecule
LP	Lamina Propria

LPS	Lipopolysaccharide
LTi	Lymphoid Tissue Inducer
MAPK	Mitogen-Activated Protein Kinase
M-cell	Microfold cell
MCP-1	Monocyte Chemoattractant Protein 1
MIP-2	Macrophage Inflammatory Protein 2
MLC	Myosin regulatory light-chain
MLCK	Myosin ligh-chain kinase
MLN	Mesenteric Lymph Node
MOF	Multiple organ failure
MPO	Myeloperoxidase
NIAAA	National Institute of Alcohol Abuse and Alcoholism
NK	Natural Killer
NKT	Natural Killer T
OCT	Optimal Cutting Temperature
PBS	Phosphate Buffered Saline
PIK	Permeant Inhibitor of Kinase
PP	Peyer’s Patches
PVDF	Polyvinylidene Difluoride
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
Reg	Regenerating Islet-Derived Protein
rmIL-22	Recombinant Mouse Interleukin-22
ROS	Reactive Oxygen Species

rmIL-22	Recombinant Mouse Interleukin-22
RPMI	Roswell Park Memorial Institute medium
rRNA	Ribosomal Ribonucleic Acid
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
SOCS	Suppressor of Cytokine Signaling
SSU	Small Subunit
STAT	Signal Transducer and Activator of Transcription
TBSA	Total Body Surface Area
Th	T-helper cell
TLR	Toll-like Receptor
Tyk	Tyrosine Kinase
YAMC	Young Adult Mouse Colon
ZO	Zonula Occludins

CHAPTER ONE

INTRODUCTION

Alcohol (ethanol) remains one of the most abused substances worldwide, creating a vast economic burden on society, and causing a host of health and behavioral problems in its users. In addition to the mental impairments associated with intoxication, alcohol consumption also leads to risky behavior and increases the risk of accidents and/or traumatic injuries, such as burn injury. Epidemiologic studies have demonstrated that up to 50% of patients in burn units have detectable levels of blood alcohol at the time they are admitted for treatment. Additionally, patients who are intoxicated at the time of injury have a worsened prognosis than patients who have not been drinking, demonstrated by longer hospital stays, and increased incidence of infection, sepsis, and multiple organ failure (MOF).

In addition to infections in the burn wound itself, the intestines are the largest reservoir of bacteria in humans, and thus represent one potential source from which bacteria or bacterial endotoxins may be able to initiate systemic infection and inflammation following alcohol and burn injury. Previous studies supporting this notion from many groups have demonstrated the intestinal barrier breaks down following alcohol intoxication and/or burn injury. Therefore, studying the effects of intoxication and burn injury in the context of the intestines may give insight into how to prevent systemic inflammation, sepsis, and multiple organ failure in burn patients.

The epithelial barrier of the intestines represents the first barrier to prevent resident microbes and pathogens within the intestinal tract from invading extra intestinal host tissues. Due to the extremely close proximity of the microbiome and intestinal epithelial cells, the intestinal barrier is tightly regulated to prevent autoimmune responses. In addition, the intestinal epithelial barrier is the fastest regenerating barrier in the human body, completely renewing every 3-5 days, demonstrating further need for tight regulation of proliferation and apoptosis in intestinal epithelial cells. Many molecules are involved in modulating these homeostatic processes, however, one cytokine that has been shown to be key for intestinal barrier maintenance is interleukin-22 (IL-22).

IL-22, a member of the IL-10 family, is a unique cytokine in that it is produced by a number of different immune cells, but its receptor is only found on cells of non-hematopoietic origin, such as epithelial cells. Once bound to its receptor, IL-22 has been shown to signal through several different downstream molecules, however, the signal transducer and activator of transcription factor-3 (STAT3) is the best described. IL-22 has many functions when present in the intestine, which include promoting mucus secretion, enhancing epithelial stem cell proliferation, and increasing anti-microbial peptide secretion from intestine epithelial cells (IECs). Previous work has demonstrated that IL-22 administration at the time of resuscitation mitigates intestinal leakiness and Gram-negative bacterial overgrowth one day following alcohol and burn injury. However, how IL-22 functions to do this following alcohol and burn injury remains unknown.

This led us to our central hypothesis that <u>IL-22 utilizes STAT3 signaling in intestinal epithelial cells to promote barrier regeneration and restore the intestinal microbiome following alcohol and burn injury.</u> To test our central hypothesis, we designed three specific aims to address these

current gaps in the field. **Aim 1** profiles changes to the small intestine epithelial barrier following the combined injury. **Aim 2** evaluates if exogenous administration of recombinant IL-22 following alcohol and burn injury restores the disrupted epithelial barrier. Finally, **Aim 3** elucidates the signaling mechanism by which IL-22 restores gut barrier integrity following alcohol and burn injury.

To examine how IL-22 protects the intestinal barrier, we administered recombinant mouse IL-22 via intraperitoneal (i.p.) injection at the time of resuscitation. Intestinal barrier damage was measured in total tissue by immunohistochemistry (IHC) for morphological changes, immunofluorescence to examine changes in cell junction proteins and numbers of proliferating/apoptosing cells, and ELISA to quantify the presence of inflammatory mediators including IL-6, KC, and IL-18. Intestinal epithelial cells were isolated and analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) for changes in gene expression of antimicrobial peptides, tight junctions, mucins, and janus kinase (Jak)/STAT signaling pathway molecules. Finally, changes to the microbiome were assessed by performing both fluorescent in situ hybridization (FISH) and 16S rRNA qRT-PCR for total and Enterobacteriaceae. Together, these studies give insight into how the intestinal barrier is damaged on a molecular level following alcohol and burn injury, and the mechanism by which IL-22 is protective to the intestine following the combined insult. Our findings present a potential new option for patient therapy in burn units, and may also translate to others with conditions that damage the intestinal barrier.

CHAPTER TWO

REVIEW OF RELATED LITERATURE: EFFECTS OF ALCHOL AND BURN INJURY ON THE INTESTINAL BARRIER AND MICROBIOME

Epidemiological and Clinical Consequences of Alcohol and Burn Injury.

Alcohol (ethanol) abuse represents a major source of health and economic burden in society. Each year 2.5 million people die from alcohol abuse and its related causes, making alcohol-related deaths one of the highest preventable causes of death in the world, and the highest cause of pre-mature death and disability in men between ages 15 and 59 [1]. In addition to the increased risk of mortality, alcohol abuse predisposes individuals to engage in more risky behaviors, which result in more traumatic injuries, including burn injury. Burn injury alone represents a major clinical issue in the United States. Over one million burn injuries are reported annually, 40,000 of which require hospitalization. Studies have demonstrated that half of patients in burn units have detectable blood alcohol levels at the time of admittance [2-7]. These patients generally require more frequent surgical intervention, exhibit higher susceptibility to infection, and experience longer hospital stays [2]. As a result, it is estimated that in the United States trauma and alcohol related expenses on society total $185 billion annually [8].

While chronic alcohol abuse remains a large problem in the United States, a majority of alcohol is consumed in a binge-like fashion [9]. The National Institute of Alcohol Abuse and Alcoholism (NIAAA) defines binge drinking as any pattern of drinking that raises blood alcohol concentration (BAC) to 0.08 g/dL or higher [10]. One possible explanation for the prevalence of

traumatic injuries in binge drinkers versus alcoholics is that alcoholics learn to function with high BAC levels. In contrast, binge drinkers are more sensitive to a high BAC as they do not regularly function with high levels of alcohol in their circulation, which makes them more likely to incur traumatic injuries, including burn injuries.

As the largest burn center in Illinois, Loyola University Chicago Medical Center provides an ideal environment to develop better treatments and post-burn care regimens for patients. Burn injuries range from superficial, small body surface area burns to large, life-threatening burns. According the American Burn Association, scald injury (the model our laboratory uses to administer burn injury) is the second most common source of burn injury and accounted for over 30% of the burns reported in the United States between 2003-2013 [11]. Severity of burn injury is classified into four different categories, first degree to fourth degree, by how deep the burn penetrates beneath the skin [12]. First-degree burns only affect the very outer layer of skin, the epidermis, and do not require medical treatment. Fourth degree burns are the most severe burns, where the injury extends into muscle and bone. Our model of burn injury is a third degree burn injury, meaning the burn penetrates the full-thickness of the epidermis and into the underlying dermis tissue. Serious burn injuries, even small surface area burns, can have a large detrimental impact on many organs. Co-morbidities, such as alcohol intoxication at the time of injury, can significantly worsen post-burn pathogenesis, and likely contribute to sepsis and multiple organ failure [13]. However, current treatment regimens for patients receiving post-burn care do not take into account the confounding effects alcohol intoxication at the time of injury. Thus, one of the overarching goals of the work in our laboratory is to demonstrate that blood alcohol levels can drastically alter post-burn pathogenesis, and should be considered by physicians when implementing treatment following burn injury.

Extra-intestinal Pathophysiology of Alcohol and Burn Injury.

Burn injury induces an enormous amount of stress throughout the body, and to our knowledge, a serious burn injury (>10% TBSA, 3rd degree or worse) affects every organ in the body to some extent. Immediately following burn injury, fluid shifts out of circulation, termed third spacing, causing systemic edema, ischemia, and inflammation [14]. Additionally a state of shock, inadequate perfusion of blood into tissues, is common amongst patients with serious burn injuries [15]. In this regard, proper fluid resuscitation is the most important goal of acute post-burn care [16], in addition to managing pain and preventing infection immediately following the injury. In spite of large improvements in post-burn care over the last half-century, many peripheral organs are still susceptible to large amounts of stress and damage following burn injury, especially when co-morbidities such as alcohol intoxication are present [13].

Under life-threatening conditions, our bodies are equipped with mechanisms to protect our vital organs; lungs, brain, and heart. However, serious burn injury still takes a large toll on these vital organs. Heart rate increases drastically following injury to try to maintain blood pressure drops that result due to fluid shifts [17]. Studies in mice have shown that just three hours after a 25% TBSA burn injury, inflammatory markers such as IL-17 can be found in cardiac tissues [18]. As a result of stress on the cardiovascular system, anoxic brain injury has been observed to account for as high as 16% of deaths resulting from burn injury in patients [19]. Though the effects on the nervous system following burn injury are significant, the same study found that respiratory failure accounted for nearly one third of deaths associated with burn injury.

Respiratory distress accounts for nearly one third of deaths following burn injury [19]. Finding how alcohol worsens post-burn pathogenesis in the lungs is of the utmost importance,

and has been one of the primary focuses of Dr. Elizabeth Kovacs. Many of her studies have aimed to elucidate how alcohol preceding a burn injury worsens inflammation and leukocyte accumulation in the lungs. Initial findings demonstrated that mice receiving a ~1.2g/kg gavage of alcohol 30 minutes before a ~15% TBSA burn injury experienced elevated levels of neutrophils and the inflammatory marker macrophage inflammatory protein-2 (MIP-2) for prolonged periods of time compared to mice receiving burn injury alone [20] . Similar studies in rats receiving alcohol and burn injury showed elevated levels of inflammatory markers including IL-18, cytokine-induced neutrophil chemoattractants (CINC-1/CINC-3), myeloperoxidase (MPO), and tissue edema [21].

Interestingly, there appear to be several mechanisms leading to pulmonary inflammation and leukocyte accumulation. In the rat study above, it was demonstrated that inhibiting IL-18 through either caspase-1 inhibitor or anti-IL-18 neutralizing antibodies prevented excessive inflammation observed following the combined injury [21]. Other studies in mice using the same model of alcohol and burn injury have shown
that inhibition of IL-6 using knockout mice or neutralizing antibodies, also prevents damage in the lungs [22].

While many studies have focused on pulmonary inflammation, a number of studies have also examined the role of leukocyte accumulation in the presence of a bacterial infection induced in the lungs. Administration of *Pseudomonas aeruginosa* into the trachea of mice following alcohol and burn injury showed that two days after injury and infection, mice receiving alcohol and burn injury had increased leukocytic infiltration, increased MIP-2 levels, and decreased pulmonary oxygen saturation compared to mice receiving alcohol and burn injury alone [23, 24] . Mechanistically, this appears to be dependent on Toll-like receptor 4 (TLR4) signaling, as

TLR4 knockout mice given *P. aeruginosa* infection following alcohol and burn injury had lower pulmonary IL-6 and KC levels, as well as decreased numbers of neutrophils [25]. One of the most interesting studies showed direct links between the lungs and the gut-liver axis following alcohol and burn injury. This study showed that preventing gut-barrier leakiness in mice using the specific myosin light chain kinase inhibitor membrane-permeant inhibitor of kinase (PIK) mitigated triglyceride accumulation and IL-6 levels in the liver and prevented alveolar edema and pulmonary neutrophil accumulation [26]. The illumination of this link that exists between organs following alcohol and burn injury has been hallmark in beginning our understanding of how systemic inflammation may stem from the gut and progress to other organs.

Due to its role in alcohol metabolism, the response of the liver following alcohol and burn injury remains one of the most important and interesting realms of research in the field. In addition to its role in alcohol metabolism, the liver has direct connections to the heart and lungs, as well as the intestines via the biliary and hepatic portal systems, termed collectively as the gut-liver axis [26]. In this regard, studying the hepatic response may allow further connections to be drawn about how the systemic inflammatory response may spread from one organ system to another following the combined insult.

Similar to the lungs, the liver experiences large amounts of inflammation within the first 24 hours following alcohol and burn injury. An early study looking at the effects of alcohol and burn injury on the livers of mice showed that mice with BACs of 100 or 300 mg/dL at the time of 12-15% TBSA burn had elevated levels of IL-6 and reactive oxygen species (ROS) compared to mice receiving alcohol or burn injury alone [27]. A supporting study that examined cytokine profiles and MPO activity in various organs of mice one day following alcohol and a 12-15% TBSA burn injury revealed that the liver had increased levels of IL-10, MPO, IL-18, MCP-1, and

edema [28]. Interestingly, insulin treatment has been shown to mitigate hepatic damage following alcohol and burn injury. A study in mice given a 12.5% TBSA injury preceded by alcohol intoxication showed the injury increased both liver and circulating levels of alanine aminotransferase (ALT), and induced steatosis in the livers of animals. Insulin treatment following the injury prevented elevation of hepatic and serum ALT levels, and drastically reduced steatosis in the liver [29]. Together, these data suggest that glycemic control following alcohol and burn injury may be one method to prevent damage to the live.

The mechanisms by which alcohol and burn injury affect the liver are still largely unknown. In a review by Chen *et al.*, several intra-hepatic mechanisms are proposed to contribute to the hepatic response following the combined injury [30]. First, the authors discuss the role of Kupffer cells, which become hyperactivated following alcohol and burn injury due to interaction with bacteria and bacterial products such as lipopolysaccharide (LPS). As a result, Kupffer cells produce large amounts of pro-inflammatory cytokines that result in an inflamed state in the liver. Next, the authors propose that oxidative stress, steatosis, and epigenetic modifications all contribute to stress induced on the liver following alcohol and burn injury. Finally, Chen *et al.* also highlight the close relationships between the liver and the intestines, citing studies that demonstrate the immediate damage done to the liver and intestines is very similar, and probably directly related.

Intestinal Homeostasis.

Before discussing the details of how alcohol and burn injury affects the intestines, it is important to first understand the structure and functions of the tissues and cells within the intestinal tract. The spatial relationships established between the lumen and barrier of the gut are absolutely essential for the proper function of the gastrointestinal (GI) tract in digestion and

nutrient absorption. The GI tract is a continuous tube that begins at the stomach and ends at the anus. The focus of this research is confined to the small intestine and large intestine (colon). The small intestine is divided into three regions proximally to distally: the duodenum, jejunum, and ileum, respectively. At the 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 proximally to distally: the ascending, transverse, descending and sigmoid colon, respectively, and terminates with 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). The small and large intestines at the histological level contain a barrier of mucous and epithelial cells that block the translocation of lumenal bacteria to extra-intestinal sites. Just below the intestinal epithelia lies a layer of loose connective tissue, the lamina propria (LP), which connects the surface mucosal epithelium to the basement muscularis mucosae. The LP also contains a large number of the intestinal immune cells. Additionally, specialized regions within the small intestine called Peyer's patches (PP) serve as lymphoid follicles. The immediate proximity of the intestinal immune cells and the bacteria within the lumen present a major challenge for homeostatic regulation. Thus, the interactions between the immune cells, intestinal barrier, and the lumenal microbiome are of major interest in all areas related to pathology associated with the intestines. In order to understand how the intestinal barrier breaks down following alcohol and burn injury, an understanding of the components that make up the physical barrier of the intestinal wall and how they are regulated is important.

The intestinal physical barrier consists of a layer of mucus and epithelial cells that line the lumen of the intestines, and provide a crucial first line of defense against pathogens (**Figure 1**). Starting from the lumen, the first component of the physical barrier is a mucus layer. Mucus

provides protection from the luminal bacterial content, and also lubricates the intestinal walls for passing bile [31-34]. Immediately below the mucus inflammatory host immune cells with lumenal bacterial antigens. Disruptions in either the intestinal mucus or epithelial barrier can result in pathogenic bacterial translocation, which can lead to systemic infections, sepsis, and multiple organ failure, underscoring the importance of maintaining barrier integrity [36-38]. The mucus layer is a key component of the physical barrier of the intestine, and is formed by a glycoprotein, mucin (mainly mucin-2 in rodent intestine), which is secreted by goblet cells found in the intestinal epithelial ayer [39]. Mucin contains a glycosylated peptide backbone, which causes the mucus layer to be incredibly viscous and effective at preventing pathogen penetration [40]. The mucus layer is not impenetrable however, and the tight junction complexes between the epithelial cells below the mucosa play a crucial role in providing a second level of protection. The intestinal wall is lined with a layer of columnar epithelial cells that serve many functions key to maintaining gut homeostasis. First, intestinal epithelial cells (IECs) aid in the absorption and metabolism of nutrition ingested by the host [41]. IECs contain microvilli on their apical surface to drastically increase the surface area of the intestines, which aid in their absorptive capacity [42]. IECs also maintain a barrier between the lumen of the intestine and extra-intestinal sites. While many epithelial cells serve these primary roles, there also exist several populations of specialized IECs. Goblet cells are mucus-secreting cells that lay down the mucin layer that lines the intestinal lumen [39]. Paneth cells are specialized anti-microbial peptide (AMP) secreting cells that aid in regulating the microbiome [43-45].

Finally, enteroendocrine cells are a diffuse population of cells that play a major role in sensing the intestine luminal content. These cells have apical physiochemical receptors that signal the release of peptides and hormones from their basolateral surfaces to mediate autocrine

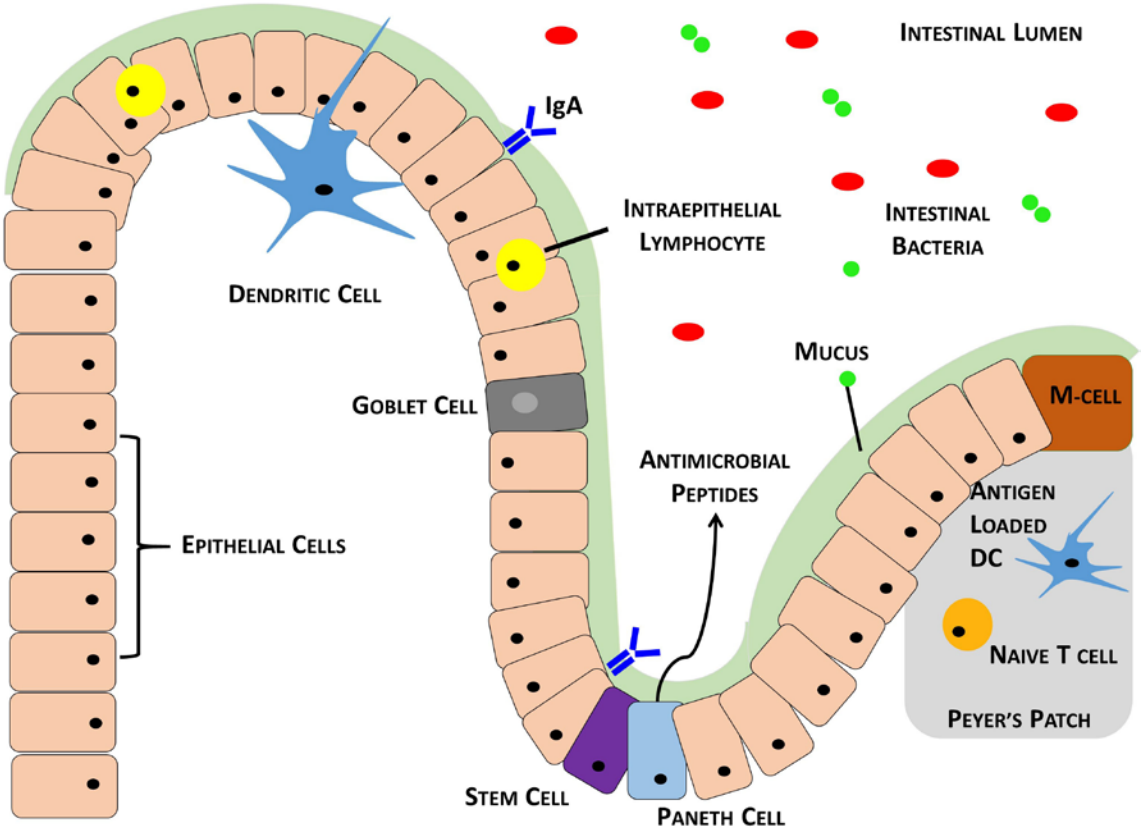

Figure 1. Overview of intestinal barrier, immune cells, and microbiome. Lumenal bacteria (red and green) are relegated to the lumen of the intestine by the intestinal barrier comprised of the mucus (green), which contains IgA bound antibodies (blue), and epithelial cells. The epithelial cell layer contains 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 (grey) 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.

and/or paracrine signaling [46]. All of these specialized epithelial cells must be renewed during the turnover of intestinal epithelial cell generation. Tight junctions (**Figure 2**) are multi-protein complexes consisting of transmembrane, scaffold, and adaptor proteins, and play an indispensable role in the maintenance of barrier function [47]. The proteins of tight an indispensable role in the maintenance of barrier function [47]. Tight junctions form a paracellular seal and function as a selectively permeable barrier between adjacent epithelial cells. Tight junctions consist of several transmembrane proteins including occludin, junctional adhesions, and claudins [35]. While the function of occludin is currently unknown,it is understood that it does not mediate tight junction formation, but appears to be instrumental in the regulation of the junctions [32.48. 49]. Claudins are a family of proteins that are both tissue and cell type specific, and are considered to be the main structural components of the tight junctions.

Occludin and claudin proteins are directly linked through their C-terminus into anchor proteins called zonula occludins (ZO) proteins [50, 51]. ZO proteins are indispensable to the formation of tight junctions, as they act as the bridge between the transmembrane occludin/claudin proteins and the actin cytoskeletion [50, 51]. ZO proteins are regulated through phosphorylation events mediated by (MLCK) [52]. MLCK phosphorylates MLC, which initiates

MLC to associate with the actin cytoskeleton and cause cytoskeletal sliding. As a result, tight junction complexes are disrupted and permeability between IECs increases. As a whole, intestinal epithelial cells constitute a dynamic community of cells. The crypt-villus axis, that is, the migration of new epithelial cells to up the villus generated from stem cells that reside in the crypts, 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 [31].

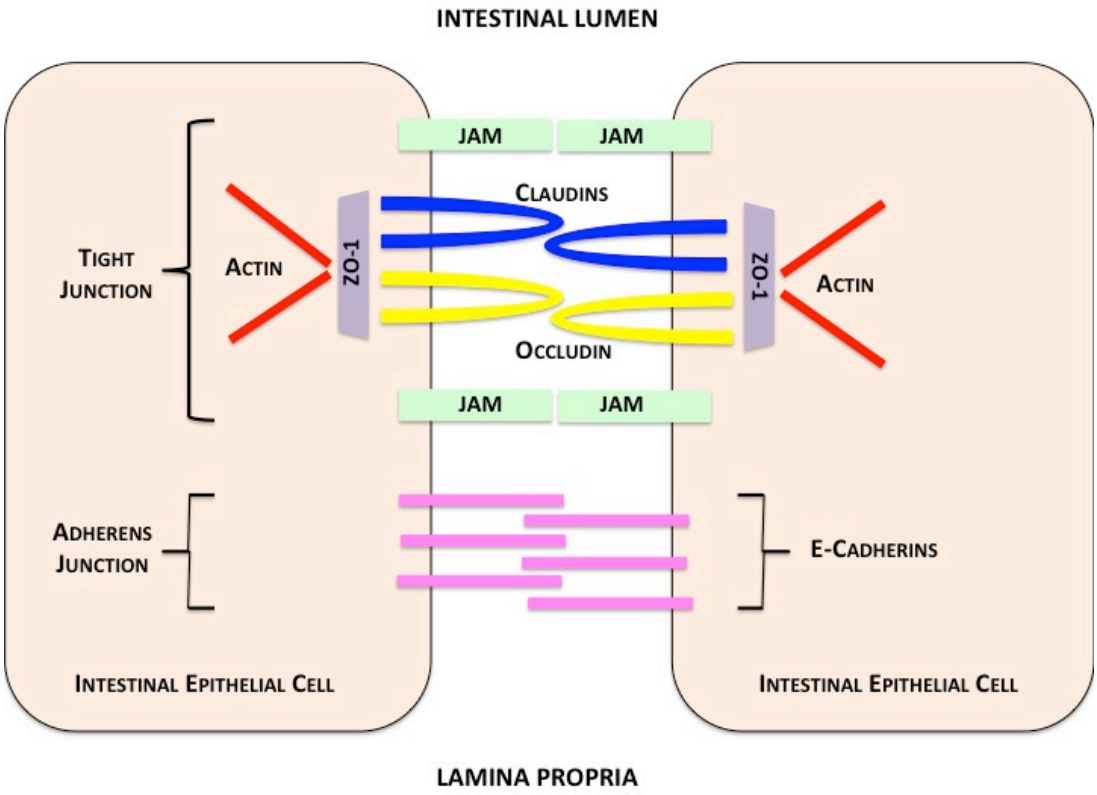

Figure 2. Intestine epithelial cell junctions. Contents within the intestinal lumen are prevented from passing between epithelial cells by apical tight junction complexes. Tight junctions are composed of claudin proteins (blue) and regulated by occludin proteins (yellow). Claudin and occludin proteins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

Relative to every other epithelial barrier in the body, IECs have an extremely high turnover rate, renewing every 3-5 days [53]. Many levels of regulation are necessary due to the high proliferation of newly made cells, and high apoptosis rate in cells that are being sloughed off to make room for newly generated IECs. Intestinal epithelial cells are generated from stem cell progenitors that sit at the base of intestinal crypts. This population of stem cells is long-lived, and retains specific cell surface markers that distinguish them from differentiated epithelial cells [54-56]. These stem cells are multi-potent, and can give rise to every specialized type of epithelial cells present in the intestines discussed above.The proliferation of IECs is dependent on several locally produced key growth factors including epidermal growth factor (EGF), keratinocyte growth factor, insulin-like growth factor-1 (IGF-1), and glucagon-like peptide 2 [57] . In addition, several hormones produced locally by enteroendocrine cells promote IEC proliferation including neurotensin and cholecystokinin [58].

Intestinal Pathophysiology of Alcohol and Burn Injury.

As the first line of defense against pathogenic organisms within the intestinal lumen, alcohol-mediated alterations of the mucus layer are of particular research interest. *Grewal & Mahmood* investigated the role of chronic alcohol exposure on mucin production in a rat model where they demonstrated prolonged alcohol exposure (25-56 days) resulted in increased mucin production [59]. Interestingly, this study also demonstrated that several components of mucin biochemical composition were altered following prolonged alcohol exposure. Modulation of glycosylation and enzymatic activity within the mucus layer could potentially affect the barrier integrity, as these sites could gain the ability to harbor adherent pathogenic bacteria [60]. Other studies that support these findings have discovered not only changes in the biochemical properties of mucin, but also decreased mucin production in the intestines of rats following

chronic alcohol intoxication [61, 62]. In other animal studies, *Hartmann et al.* showed data in a mucin-2 knockout mouse model suggesting that chronic alcohol increases the intestinal mucus content [40]. Despite an observed increase in intestinal permeability, bacterial overgrowth and translocation following alcohol exposure was decreased. Taken together, these findings suggest a relationship between alcohol exposure and mucus production, but the mechanism(s) of changes in mucin production due to alcohol exposure is remains unknown.

Not surprisingly, alcohol and trauma also disrupt the integrity of tight junction complexes between intestinal epithelial cells [63, 64]. An *in vitro* study showed Caco-2 human intestinal epithelial cells exposed to a daily regime of 0.2% alcohol demonstrated a reduction in ZO-1 adherens protein membrane localization. Furthermore, allowing the alcohol treated cells to "recover" from alcohol exposure by culturing for two weeks in alcohol free media improved ZO-1 localization [65]. Supporting this finding, a study conducted by *Ma et al.* in Caco-2 cells showed identical effects on ZO-1 proteins, and additionally looked into the molecular mechanism. The group found that alcohol activates an enzyme, myosin light-chain kinase (MLCK), which phosphorylates myosin regulatory light-chain (MLC), promoting actin interaction [66]. This interaction is important in tight junction function, and may be one cause of the disruption of tight junctions in intestinal epithelial cells [32]. *Zahs et al.* examined the role of MLCK in gut barrier disruption following combined binge alcohol exposure and burn injury in mice. They showed the combination of alcohol intoxication and burn injury results in both elevated MLCK and phosphorylated MLC compard to ethanol or burn injury alone, and a decrease in co-localization of both occludin and ZO-1 [67]. In another *in vivo* study of acute alcohol exposure and traumatic burn injury in rats, *Li et al.* showed the combined insult resulted in a significant reduction in phosphorylation and expression of occludin and claudin-1 compared

to sham vehicle animals, and correlated this with increased epithelial cell apoptosis [68]. *Yoseph et al.* further demonstrated that the combination of chronic alcohol and cecal ligation and puncture (CLP)-sepsis in mice resulted in elevated intestinal epithelial apoptosis as well as decreased proliferation compared to ethanol or CLP alone [69]. As a whole, data from many groups clearly show a correlation between alcohol exposure/trauma and tight junction disruption. Indeed, exposure to alcohol and trauma greatly affect 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. However, the mucous and epithelial layer of the intestinal tract represent just a fraction of the components that make up the barrier. Immune cells that reside within the intestines play a crucial role in intestinal homeostasis, as the gut has been suggested to be home to the largest number of immune cells in humans.

Unexpectedly, there are few studies in the current literature that 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 in the colon of rats after 10 weeks [70]. Others have correlated microbial dysbiosis in mice and rats to alcoholic liver disease, and demonstrated that administration of probiotics reduces hepatic inflammation associated with it [70, 71]. Our laboratory has previously shown in both mice and rats that combined alcohol intoxication followed by traumatic burn injury results in a significant increase in bacterial translocation across the intestinal barrier [63, 68, 72, 73], and this work is supported by a previous study [38]. However, the long-term impact of alcohol on these different microbiota and the host's health and immune function remains to be shown. Classifying the healthy intestinal microbiome is clinically necessary for determining how alcohol may alter the

microbiota composition and lead to disease development and progression. 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.

Interleukin-22 (IL-22), a member of the IL-10 family of cytokines, is a 22kDa cytokine that is 80% homologous between mice and humans [74]. IL-22 is produced by a number of immune cells including T cells, class three innate lymphoid cells (ILC3s), LTi cells, NK and NKT cells, and recent studies have even suggested that dendritic cells (DCs) and neutrophils can produce IL-22 as well [75, 76] . IL-22 is unique from other cytokines in that its receptor, made up of the IL-22 receptor-1 (IL-22R1) and IL-10 receptor-2 (IL-10R2) subunits, is only found on cells of non-hematopoietic origin in both rodents and humans [75, 76]. This is a notable quality of IL-22, because it means that IL-22 signals on cells (e.g. epithelial cells) that are different from the ones that produce it mentioned above. Once bound to its receptor, downstream phosphorylation events, mainly on Jak1 and Tyk2, lead to activating phosphorylation on STAT3 [75]. Phosphorylated STAT3 homodimerizes and translocates to the nucleus where it acts as a transcription factor for a host of different genes (**Figure 3**). In the context of the small and large intestines, the IL-22 receptor complex is found on IECs, where it has been shown to elicit a number of downstream effects. IL-22 has been shown to promote mucin secretion from goblet cells, enhance AMP production from Paneth cells, and stimulate stem cells within the crypts to proliferate and regenerate the intestinal epithelial barrier [76].

In nearly all models of disease and injury in the intestine, IL-22 has been proposed to be a protective molecule. These rodent models include inflammatory bowel disease, graft-versus-host disease, and alcohol and burn injury [73, 75-79]. Interestingly, IL-22 has been shown to promote

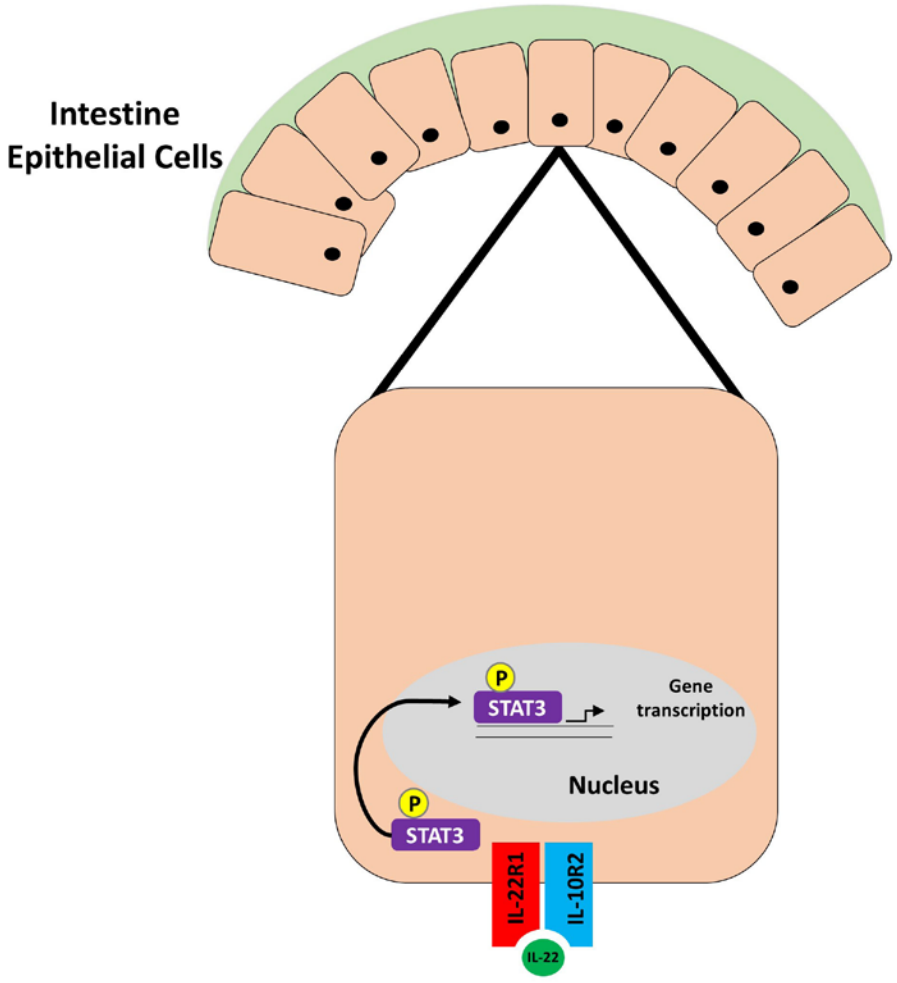

Figure 3. IL-22 and STAT3 signaling in intestine epithelial cells. IL-22 (green) is produced by many immune cells including T cells, natural killer cells, and innate lymphoid cells. Intestine epithelial cells constitutively express the IL-22 receptor 1 (IL-22R1, red) and the IL-10 receptor 2 (IL-10R2, blue). Upon binding to the IL-22 receptor complex, phosphorylation events take place mainly through Jak1 and Tyk2 (not shown), leading to downstream phosphorylation of STAT3 (purple). Once phosphorylated, STAT3 homo-dimerizes and translocates to the nucleus. Once bound to its promoter, STAT3 can transcribe a host of genes for anti-microbial peptides, cell proliferation, and mucins.

inflammation in mouse models of psoriasis and in the presence of specific types of bacterial infections (e.g. *Toxoplasma gondii*), demonstrating that the microenvironment may play a significant role in the type of response IL-22 elicits [80, 81].A previous study from our laboratory has shown that IL-22 levels in small intestinal tissue are suppressed following alcohol and burn injury in mice [73]. In this study, mice were given intraperitoneal treatment of 1mg/kg recombinant mouse IL-22 in their resuscitation fluid to examine if restoration of IL-22 would be protective following injury. The study showed that mice that received alcohol and burn injury as well as IL-22 had significantly less intestinal permeability and culturable Gram-negative bacterial from both total small intestine tissue as well as small intestine luminal content. This was paired by a rescue of the AMPs regenerating islet-derived protein-3β (Reg3β) and Reg3γ in small intestine tissue. Together, these data suggest that IL-22 treatment immediately following alcohol and burn injury is protective to the intestines, however, the mechanism by which IL-22 elicits this protection were not investigated. Therefore, a significant part of the current project is to illuminate the signaling mechanism IL-22 uses to protect the intestine following alcohol and burn injury.

Conclusions.

The current body of literature describing the effects of alcohol and burn injury clearly demonstrates the global systemic effects that the combined insult causes. While our lab has illuminated many detriments to the intestines following alcohol and burn injury, the mechanism(s) by which alcohol and burn injury causes gut barrier disruption and dysbiosis remains unknown. The largest gaps in our current knowledge, in our opinion, are the largely unknown effects of the combined injury on the intestinal epithelial barrier and microbiome. Elucidating how these two facets, the epithelial barrier and microbiome, are altered following

alcohol and burn injury may provide insight into how the intestines may contribute to the systemic inflammatory response, sepsis, and/or multiple organ failure. Furthermore, these studies have the potential to further our understanding of the connections between the intestines, liver, and lungs following injury, and may provide insight into how therapies intended to improve intestinal barrier function may also benefit or harm other organs. Finally, we aim to begin understanding the intimate relationships between the intestinal immune system, epithelial barrier, and microbiome, as the interactions between these three components of the intestine likely have direct relationships in the intestinal, and perhaps systemic response, following alcohol and burn injury.

CHAPTER THREE

THE EFFECTS OF ALCOHOL INTOXICATION AND BURN INJURY ON THE EXPRESSION OF CLAUDINS AND MUCINS IN THE SMALL AND LARGE INTESTINES

Abstract.

Alcohol intoxication at the time of burn injury exacerbates post-burn pathogenesis. Recent findings suggest gut barrier integrity is compromised after combined alcohol and burn insult, which could contribute to these complications. Tight junction proteins and mucins play critical roles in keeping the gut barrier in tact. Therefore, the goal of this study was to examine the effects of alcohol and burn injury on claudin and mucin expression in the intestines. We also evaluated if the combined insult differentially influences their expression in the small and large intestines. Male C57BL/6 mice were given a single dose of 2.9 g/kg alcohol prior to a ~12.5% body area burn. One and three days following injury, we profiled expression of several tight junction, mucin, and bacterial 16S rRNA genes in small and large intestine using qPCR. We observed >50% decrease in expression of claudin-4 and claudin-8 genes in both ileal and colonic epithelial cells one day after injury. Claudin-2 was significantly upregulated, and occludin was down-regulated in small intestine one day following injury. Mucin-3 expression was substantially elevated (>50%) in small intestine, whereas mucin-2, and mucin-4 were considerably diminished in the colon (>50%) one day following injury. Most parameters were normalized to sham levels on day three, except for mucin-3 and claudin-8, which remained decreased in large intestine. Neither alcohol nor burn alone resulted in changes in tight junction or mucin gene expression compared to shams. We observed large increases in the family of

Gram-negative bacteria, Enterobacteriaceae, in both small (150-fold increase) and large (75-fold increase) intestines one day following injury. Our results indicate that alcohol and burn injury affects tight junction and mucin expression in the small and large intestines, which may be mediated through local changes in the intestinal microbiome, leading to the development of gut barrier leakiness following alcohol and burn injury.

Introduction.

Alcohol (ethanol) abuse remains a serious problem worldwide. A study from the World Health Organization reported that alcohol attributed deaths exceeded three million in 2012 [1]. One of the leading consequences of intoxication is the increased risk factor it poses for the occurrence of a traumatic injury [82]. According to the American Burn Association, burn injury alone leads to 40,000 patient hospitalizations annually in the United States [11]. Data have shown that approximately 50% of hospitalized burn patients have detectable blood alcohol levels at the time of their admission (2, 4). A host of studies have shown the combination of alcohol and burn injury results in worsened patient prognosis including increased susceptibility to infection, sepsis, multiple organ failure, and death [2, 83]. The intestines are the major reservoir of bacteria within the host, and disruptions of the intestinal barrier could exacerbate any of these sequelae [73, 84]

Our lab has previously demonstrated that there is an increase in intestinal permeability following combined alcohol and burn injury in mice and rats [64, 73, 85]. Gut leakiness following the combined insult is paired with significant increases in pro-inflammatory mediators including IL-6, IL-18, and MCP-1 mesured from total intestinal tissues homogenates [28, 85, 86]. In addition, bacterial translocation to extra-intestinal sites, such as the mesenteric lymph nodes, has been observed as early as 24 hours post-injury [73]. Others have demonstrated gut

leakiness and immune dysregulation following burn injury, which may perpetuate the ability of bacteria and other pathogens to extravasate from the intestines following traumatic injury [87, 88]. Moreover, models of alcoholic liver disease (ALD) have shown that gut-derived bacterial endotoxins can contribute to ALD progression [89], further demonstrating the clear necessity for gut barrier maintenance.

In healthy individuals, proper intestinal function is accomplished through the formation and maintenance of a selective barrier formed by tight junctions which allow the intestinal wall to retain commensal microbes to the lumen, while allowing the passage of nutrients out of the gut. Previous studies from our laboratory have demonstrated significant reductions in the expression and phosphorylation status of occludin and claudin-1 in the ileum of rats following alcohol and burn injury [68]. Literature from other laboratories has shown significant disruptions in occludin and ZO-1 protein expression in mouse ileum following the combined injury [67, 90]. However, studies in occludin knockout mice have suggested that occludin may not be essential for the maintenance of epithelial barrier integrity [91], and that claudin-1, -3, -4, -5, and -8 are most involved in strengthening the intestinal barrier [35, 92]. Of these, claudin-4 and -8 are highly expressed in the ileum and colon of mice, and dysregulation of either could contribute to the leakiness observed following injury [92].

In addition to changes in tight junction protein expression, the mucus layer serves to protect barrier integrity by limiting the amount of interaction between luminal contents and intestinal epithelial cells. The mucus layer becomes more prominent moving distally down the intestinal tract, likely due to the correlating increase of commensal bacteria. In murine small and large intestine, the most prevalent mucin is mucin-2 [39], and aberrant mucin-2 profiles have been observed in diseased states such as inflammatory bowel disease [93]. In addition, a

reduction in mucin-2 has also been correlated with increased ability of pathogens to cross the intestinal barrier [94]. As such, alterations in mucin expression as a result of intestinal inflammation parallels with the severity of pathogenesis and serve as an excellent marker to assess intestinal damage and leakiness. Changes in junction and mucin proteins may contribute to gut leakiness following alcohol and burn injury, which can result in multiple organ failure.

Therefore, we profiled mucus and tight junction gene expression in mouse ileum and colon epithelial cells one and three days following alcohol and burn injury. We hypothesized that both mucin and tight junction expression would be decreased in both the small and large intestine one day following alcohol and burn injury. Our results demonstrate that both ileal and colonic epithelial cells have decreased tight junction and mucin gene expression, and that goblet cell presence and mucin glycoprotein expression is reduced in colonic tissue one day following injury. These changes correlate with large a marked increase in the Gram-negative family, Enterobacteriaceae, in both the small and large intestine feces at this time point. Taken together, our results indicate that alcohol and burn injury affects many components of the small and large epithelial barriers, and this may be significant in the development of gut barrier leakiness following alcohol and burn injury.

Materials and Methods.

Animals. C57BL/6 male mice 10-12 weeks old (23-25g body weight) were obtained from Charles River Laboratories and maintained in animal housing facilities at Loyola University Chicago Health Sciences Division, Maywood, Illinois, USA.

Murine Model of Binge Alcohol and Burn Injury. For our animal experiments, we used the well-established mouse model of alcohol and burn injury that we have described previously [28]. Mice were randomly assigned into one of the four experimental groups: sham

injury + vehicle (water), sham injury + alcohol, burn injury + vehicle, or burn injury + alcohol. On the day of injury, mice were gavaged with 400µl of 25% alcohol in water (2.9 g/kg) to mimic a significant alcohol binge. Animals in the vehicle groups were gavaged with 400µl of water. Four hours following gavage, mice were anesthetized by intraperitoneal injection with a ketamine hydrochloride/xylazine cocktail (~80 mg/kg and 1.2 mg/kg, respectively). The dorsal surfaces of mice were shaved, and animals were placed in a prefabricated template exposing approximately 12.5% total body surface area calculated using Meeh's formula as described by Walker and Mason [95]. Animals in burn groups were immersed in 85-87°C water bath for ~7 seconds to induce a full-thickness scald burn injury. Sham animals were placed in a 37°C water bath for 7 seconds. Immediately following burn or sham injury, mice were given 1.0 mL normal saline resuscitation by intraperitoneal injection. Animals were returned to their cages and allowed food and water *ad libitum*. All experiments were conducted in accordance with the guidelines in the Animal Welfare Act and were approved by the Institutional Animal Care and Use Committee at Loyola University Health Sciences Division.

Intestine Epithelial Cell Isolation. One or three days following injury, mice were humanely euthanized. Isolation of intestinal epithelial cells was performed as described previously by Weigmann *et al.* [96]. Small and large intestines were removed from the peritoneal cavity. For small intestine studies, the distal 10cm of the small intestine was separated from the remainder of the small intestine for analysis. The entirety of the colon was harvested for analysis. The tissues were cut longitudinally and placed in ice cold PBS + 1% penicillin/streptomycin cocktail. Following two washes in PBS + pen/strep, tissues were placed in a digestion solution containing 5% heat-inactivated fetal bovine serum (FBS), 1% HEPES, 1% pen/strep, 0.5% gentamicin, 5mM EDTA, and 1mM dithiothreitol (D.T.T.) in Hank's Balanced Salt Solution

(HBSS) at 37°C. Tissues were placed in a 37°C incubator and shaken on a rotator at 250 rpm for 20 minutes. Tissues were vortexed to separate the epithelial cells from the tissue and passed through a 100 μm filter. Cells were counted on a hemocytometer to determine epithelial cell purity (≥90%). Intestinal epithelial cells were then processed for downstream applications.

Epithelial Cell RNA Isolation and cDNA Synthesis. RNA isolation was performed using a RNeasy Mini Kit (Qiagen, Valencia, CA) described previously [73]. Genomic DNA was removed by DNase digestion using an RNase-free-DNase Set (Qiagen). Concentration of isolated RNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Bannockburn, IL). Only samples with a 260/280 ratio of ≥2.0 were used for cDNA synthesis. cDNA synthesis was performed using a High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA) and reactions were run on a Veriti 96-well Fast Thermocycler (Life Technologies).

Real-Time PCR. Expression of occludin, claudin-2, claudin-4, claudin-8, E-cadherin, mucin-1, mucin-2, mucin-3, and mucin-4 mRNA levels were analyzed by qPCR using TaqMan primer probes and TaqMan Fast Advanced Master Mix (Life Technologies). Target gene Ct cycle values were normalized to housekeeping control beta actin Ct values. Data were calculated using the ΔΔCt method, and all groups were expressed relative to the sham + vehicle group [97].

Alcian Blue Staining. Sections of the distal ileum (1 cm) and proximal colon (1 cm) were fixed in Carnoy's Solution. Tissue sections were prepared by the Loyola University Chicago Health Sciences Division Tissue Processing Core. Briefly, tissues were cleared in 100% ethanol and embedded in paraffin as described previously [98]. 5 μm sections were cut, stained with alcian blue to stain for mucin expression in the ileum and colon, and cover slipped for analysis. Five images of five separate microscopic fields were taken at 200X total

magnification for each animal and images were processed using Adobe Photoshop CS3

Isolation of Fecal Genomic DNA. At the time of euthanasia, fecal samples from the distal 5 cm of the ileum and the complete colon were sterilely collected. Wet weight of feces was obtained followed by isolation of fecal genomic DNA using a QIAmp DNA Stool Isoaltion Kit (Qiagen) per the manufacturer's instructions, and included incubation at 95°C to improve bacterial cell lysis. Genomic DNA purity and concentration was obtained using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

16S rRNA Bacterial Quantitative Real-time PCR. Real-time PCR analysis using specific primer sets targeting small subunit (SSU) 16s rRNA of total bacteria and Enterobacteriaceae were used to quantify the fecal microbiome as described previously [99, 100]. Primer sequences were: 340F (ACTCCTACGGGAGGCAGCAGT) and 514R (ATTACCGCGGCTGCTGGC) for total bacteria, and 515F (GTGCCAGCAGCCGCGGTAA) 826R (GCCTCAAGGGCACAACCTCCAAG) for Enterobacteriaceae (Invitrogen). Standard curves for PCR quantification were generated using 10-fold serial dilutions of bacterial SSU ribosomal copy number from *Blautia producta* (Prevot) Liu et al. (*Ruminococcus productus*) genomic DNA (ATCC, Manassas, VA) for total bacteria, and *Escherichia coli* genomic DNA (ATCC) for Enterobacteriaceae. PCR was run using 1X iTaq Universal SYBR Green Supermix (Bio-Rad) with a total of 300nM of forward and reverse primers per reaction. Reactions were run as follows: 95°C for 3 min, 40 cycles of 95°C for 15s, followed by data collection at 63°C (Bacteria) or 67°C (Enterobacteriaceae) for 1 min. Reactions were performed on a Step One Plus Real-Time PCR instrument (Applied Biosystems). Because changes in total bacteria 16S rRNA were observed in alcohol + burn samples, Enterobacteriaceae was expressed at the relative copy number per gram of feces. All groups are expressed as fold-increase in copy number relative to

the sham + vehicle treated group

Statistics. The data, wherever applicable, are presented as means + SEM and were analyzed using analysis of variance (ANOVA) with Tukey's post-hoc test or Student's t test (GraphPad Prism6). A p-value of <0.05 was considered statistically significant.

Results.

Previous data from our laboratory have demonstrated alterations in both the expression and phosphorylation status of occludin and claudin-1 in the ileum of rats one day following alcohol and burn injury [68]. However, many different claudins are expressed within murine intestine, and may play a significant role in maintaining the gut barrier following alcohol and burn injury. To further expand the underlying mechanisms of gut leakiness following the combined insult, we performed gene expression analysis of several key tight junction markers including claudin-2, claudin-4, and claudin-8. Real-time PCR data obtained from isolated intestinal epithelial cells from the distal ileum of mice demonstrate significant reductions in occludin (60% reduction), and claudin-8 (76% reduction) one day following alcohol and burn injury compared to sham animals (**Figure 4A**). Claudin-4 levels were significantly decreased (50% reduction) following alcohol and burn injury when analyzed by student's t test ($p=0.0001$). Claudin-2, a leaky claudin that is permissive to water and ions, was significantly elevated (80%) relative to sham mice (**Figure 4A**). All mRNA levels returned to sham levels three days following injury, indicating that gene expression in the small intestine is able to recover following injury (**Figure 4B**).

In spite of the large number of studies examining the effects of alcohol, trauma, or disease states on tight junctions in the colon, no studies to our knowledge have examined the effects of combined alcohol and burn injury in the context of the large intestine. As in the ileum,

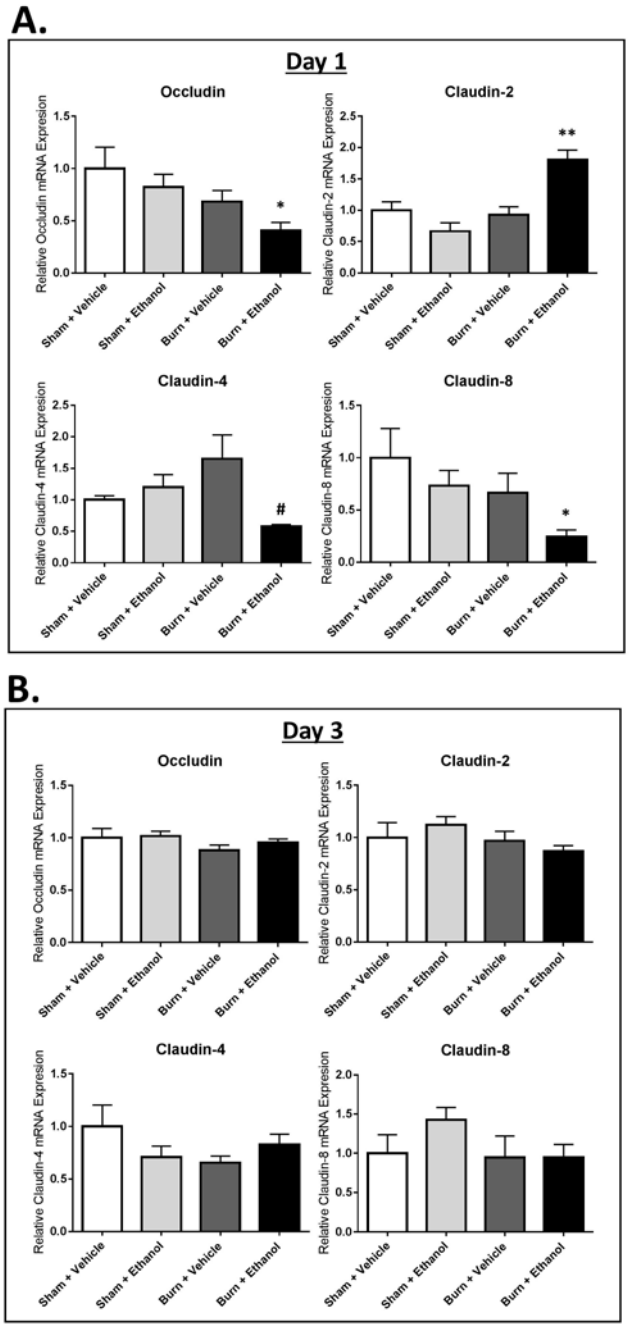

Figure 4. Tight junction mRNA profiles in small intestine ileum epithelial cells one and three days following alcohol and burn injury. RNA was isolated from small intestine epithelial cells and gene expression was examined using qPCR to examine expression of several tight junction genes. **A.)** Significant reductions in occludin, claudin-4, and claudin-8 were observed one day following combined alcohol and burn injury. Claudin-2 was expressed at higher levels than in sham vehicle controls. **B.)** All tight junction mRNA levels were normalized three days post-injury ($n = 3\text{-}8$, $^{*}p < 0.05$, $^{**}p < 0.01$ by ANOVA, $\#p < 0.05$ by student's t test compared to sham + vehicle). Data are from one representative experiment and expressed relative to the sham vehicle group.

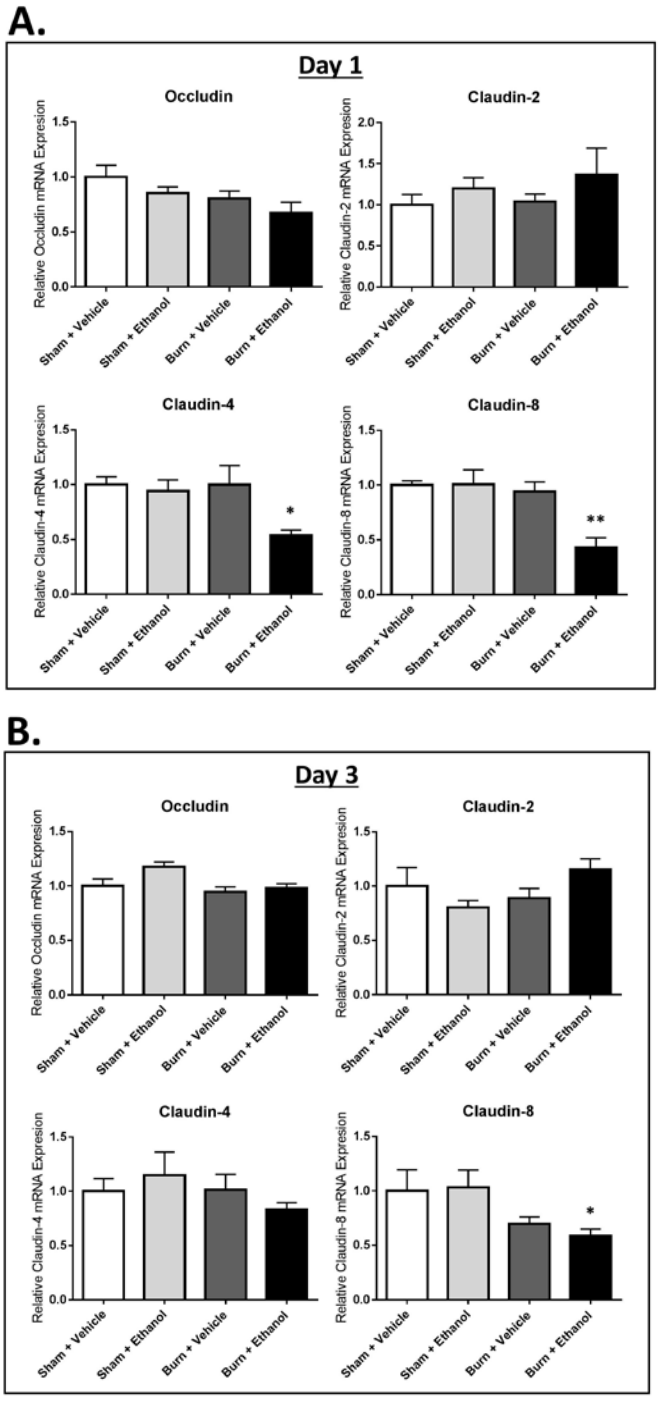

Figure 5. Tight junction mRNA profiles in large intestine epithelial cells one and three days following alcohol and burn injury. RNA was isolated from large intestine epithelial cells and gene expression was examined using qPCR to examine expression of several tight junction genes. **A.)** Significant reductions in claudin-4, and claudin-8 were observed one day following combined alcohol and burn injury. **B.)** All tight junction mRNA levels were normalized three days post-injury with the exception of decreased claudin-8 expression (n = 3-8, *$p < 0.05$, **$p < 0.01$ by ANOVA compared to sham + vehicle).

mRNA expression from isolated colonic epithelial cells was quantified by qPCR on day one and day three post-injury (**Figure 5**). Similar decreases in claudin-4 (46% reduction) and claudin-8 (57% reduction) were observed in colonic epithelial cells one day following the combined injury (**Figure 5A**). A trend of a decrease in occludin ($p = 0.07$ by student's t test) was also observed, but no significant changes in claudin-2 gene expression were seen in the colon. Similar to the results observed in the ileum, all gene expression returned to sham levels three days following injury with the exception of claudin-8 (41% reduction, **Figure 5B**).

To determine if adherens junctions were altered following combined alcohol and burn injury, E-cadherin gene expression was analyzed in ileal and colonic epithelial cells as above. One day following the combined insult, a trend of a decrease in E-cadherin gene expression ($p = 0.0519$ by student's t test) was observed in colon epithelial cells (**Figure 6B**). However, E-cadherin expression was unchanged in ileal epithelial cells one day after alcohol and burn injury (**Figure 6A**).

In addition to tight junction complexes between intestinal epithelial cells, the mucus layer serves as a buffer between the epithelial cells and the luminal microbiome. Thus, we sought to profile key mucin gene expression from isolated distal small intestine and colon epithelial cells following alcohol and burn injury. Data in **Figure 7A** demonstrate significant elevations in mucin-3 (76%) are observed in the distal ileum at one day following the combined insult. However, mucin-2 levels were significantly decreased (20%) compared to sham + vehicle animals by student's t test ($p = 0.02$). A trending decrease in mucin-4 (44%, $p = 0.06$ by student's t test) was also observed in the small intestine one day following injury. Similarly to tight junction gene expression, mucin mRNA expression in ileum of all groups is similar to the sham vehicle group three days after injury with the exception of mucin-1 (**Figure 7B**).

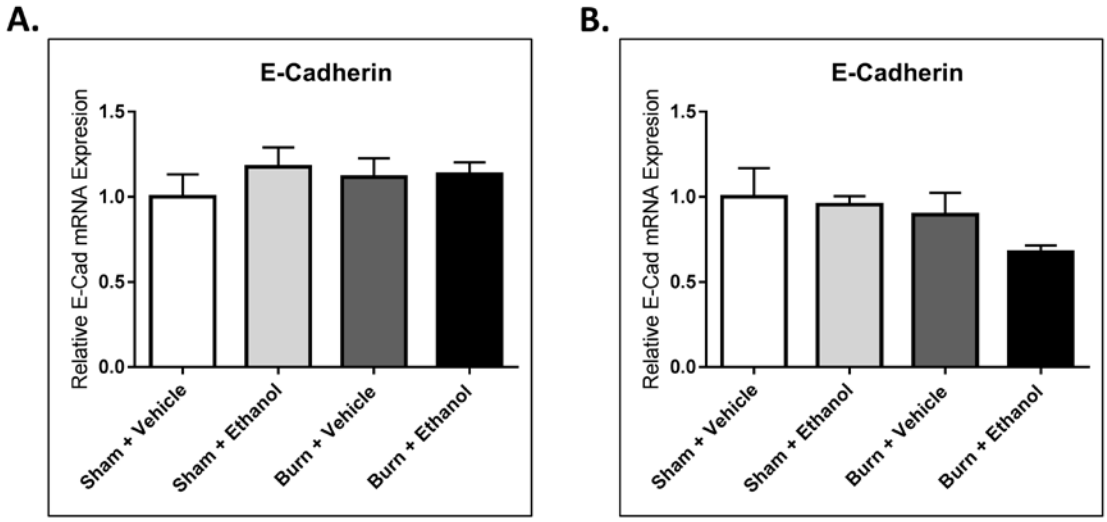

Figure 6. Adherens mRNA profiles in small and large intestine epithelial cells one day following alcohol and burn injury. RNA was isolated from small and large intestine epithelial cells and gene expression was examined using qPCR to examine expression of E-cadherin. **A.)** No observed changes in E-cadherin expression was observed between any groups in ileum epithelial cells. **B.)** A trend of a decrease ($p = 0.0519$ by student's t test) was observed between sham + vehicle and alcohol + burn large intestinal epithelial cells one day following the combined insult (n = 5-9 animals/group).

The most notable differences in mucin gene expression were observed in colonic epithelial cells one day following injury (**Figure 8A**). Both mucin-2, the most highly expressed mucin in mouse intestine, as well as mucin-4 were significantly reduced 51% and 54%, respectively. Three days following injury, mucin gene expression returns to levels similar to sham vehicle in all groups, except in the case of mucin-1 ($p = .03$, student's t test) and mucin-3 (**Figure 8B**).

To validate the changes in mucin gene expression observed by qPCR, we performed alcian blue histological stains on both distal small intestine and colon tissues. Alcian blue stains acidic mucins, which are ubiquitously expressed in both murine small and large intestines. Representative images from all groups are shown (**Figure 9**). Data demonstrate slightly

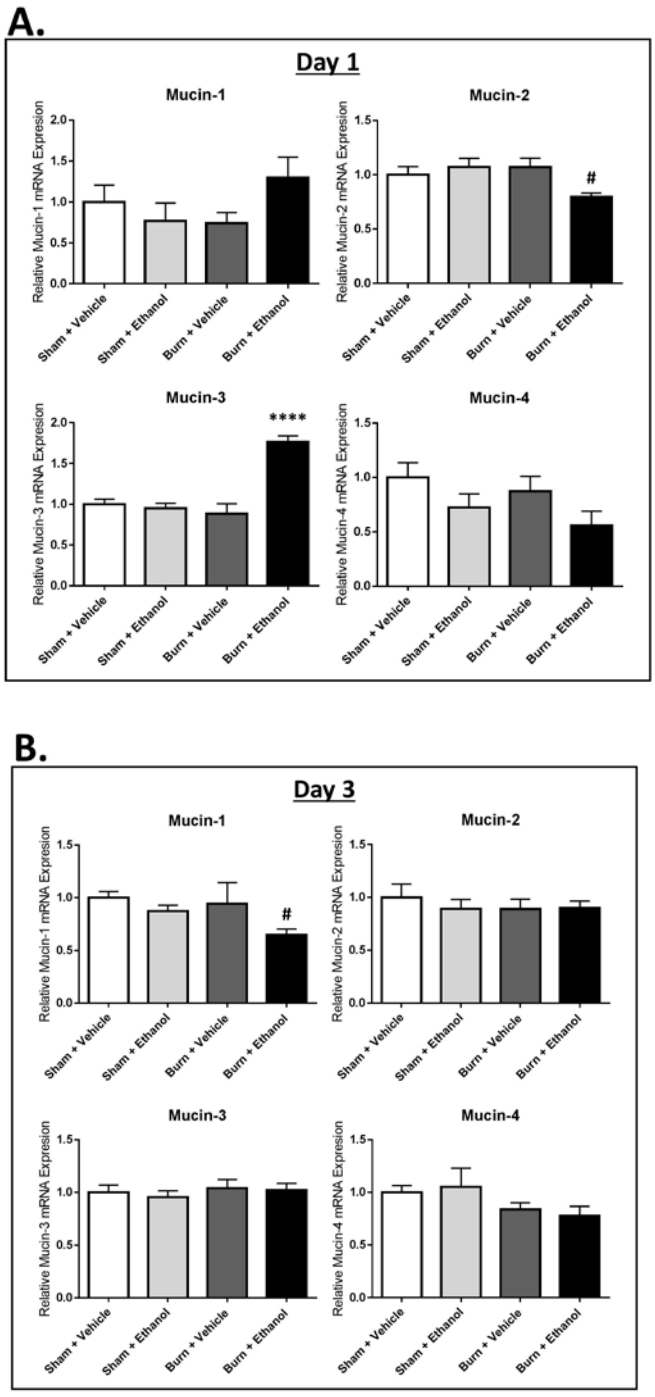

Figure 7. Small intestine ileum epithelial cell mucin-2 profiles reduced following combined alcohol and burn injury. RNA was isolated from small intestine epithelial cells and gene expression was examined using qPCR to examine expression of several mucin genes. **A.)** mRNA levels of mucin-3 were observed to be significantly elevated, while mucin-2 levels were diminished one day following the combined insult. **B.)** All mucin mRNA levels returned to sham levels three days post-injury except for mucin-1 ($n = 3\text{-}8$, ****$p < 0.0001$ by ANOVA, #$p < 0.05$ by student's t test compared to sham + vehicle).

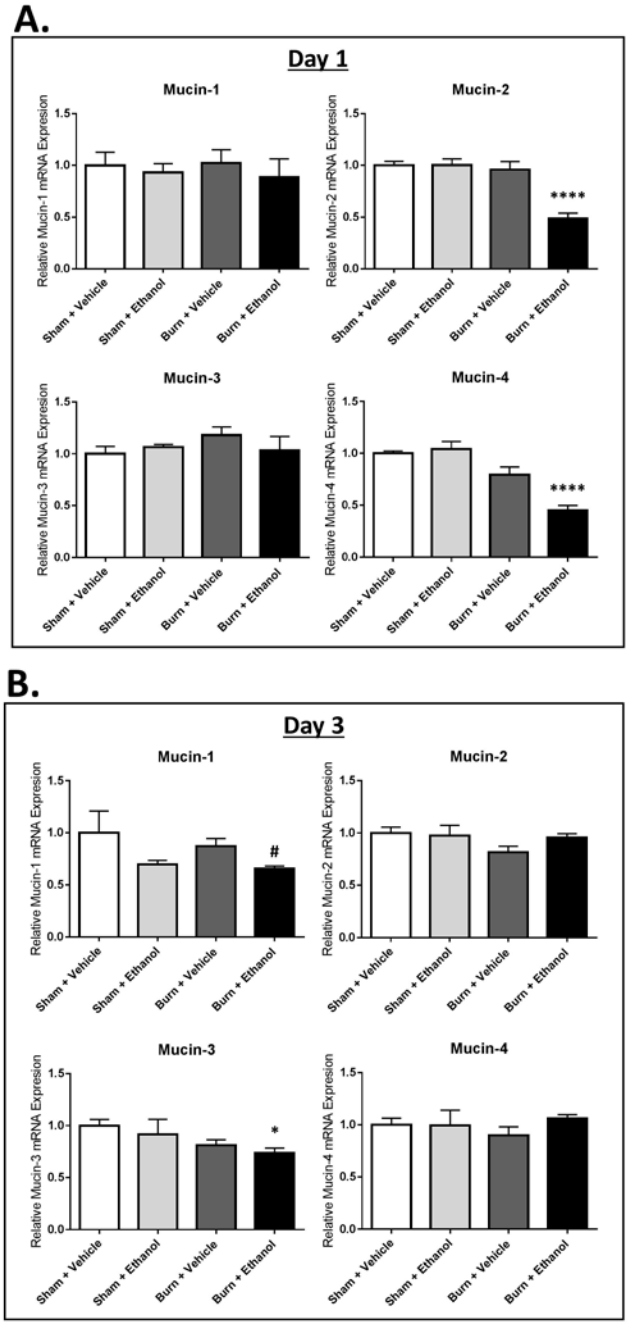

Figure 8. Significant reductions in large intestine epithelial cell mucin profiles following combined alcohol and burn injury. RNA was isolated from large intestine epithelial cells and gene expression was examined using qPCR to examine expression of several mucin genes. **A.)** Large decreases in both mucin-2 and mucin-4 were observed in the large intestine one day post-injury. **B.)** Mucin gene levels were largely restored by three days following combined alcohol and burn injury. However, significant decreases were observed in mucin-3 analyzed by ANOVA, and also in mucin-1 analyzed by student's t test (n = 3-8, *$p < 0.05$, ****$p < 0.0001$ by ANOVA, #$p < 0.05$ by student's t test compared to sham + vehicle).

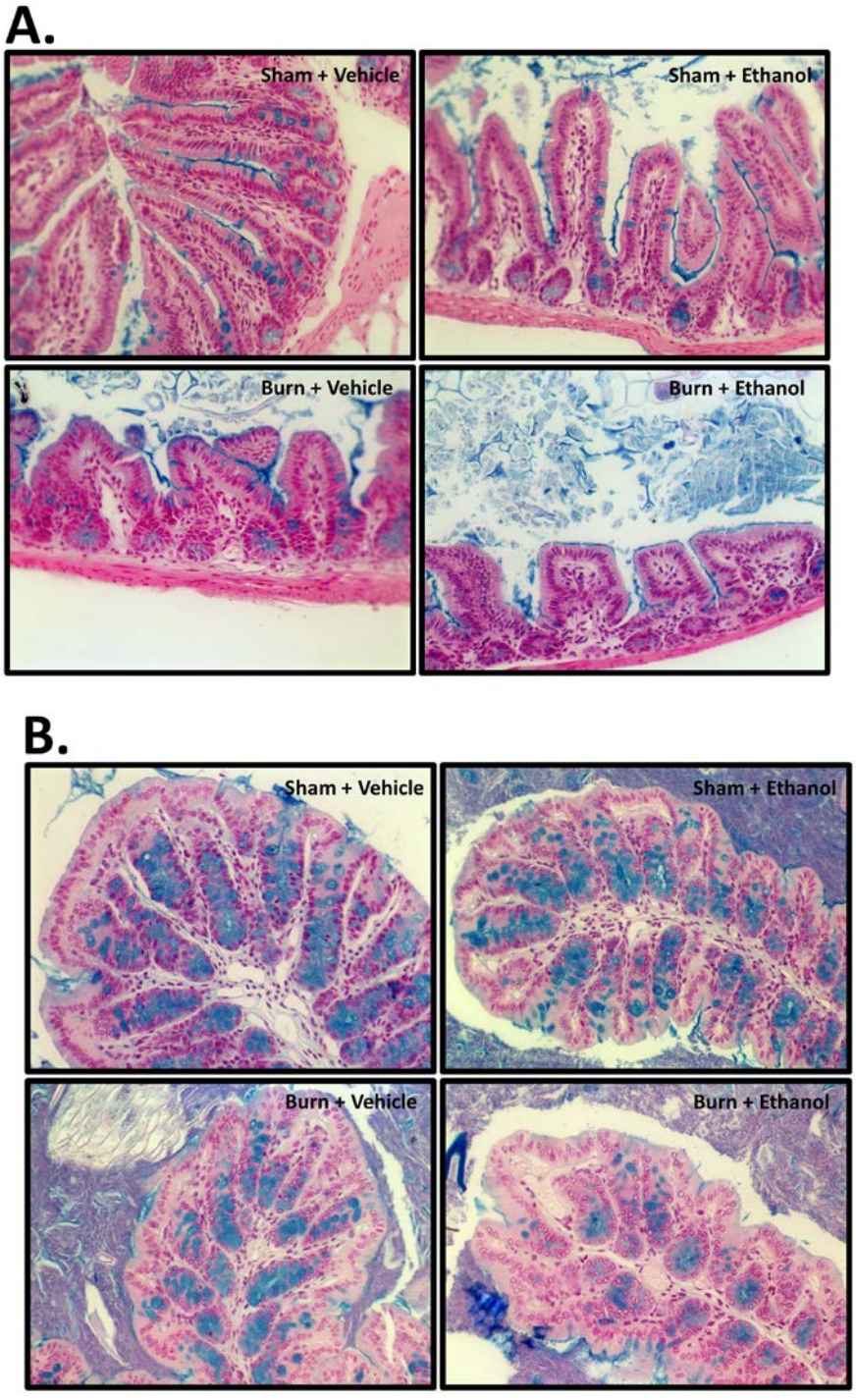

Figure 9. Significant reduction in large intestine mucus protein expression following combined alcohol and burn injury. Representative alcian blue staining one day following alcohol and burn injury was performed on **A.)** distal ileum and **B.)** proximal colon sections. Substantial reductions in the amount of mucin glycoprotein expression and goblet cell presence was observed in five out of eight mice receiving alcohol and burn injury in the large intestine compared to shams (n = 5-8).

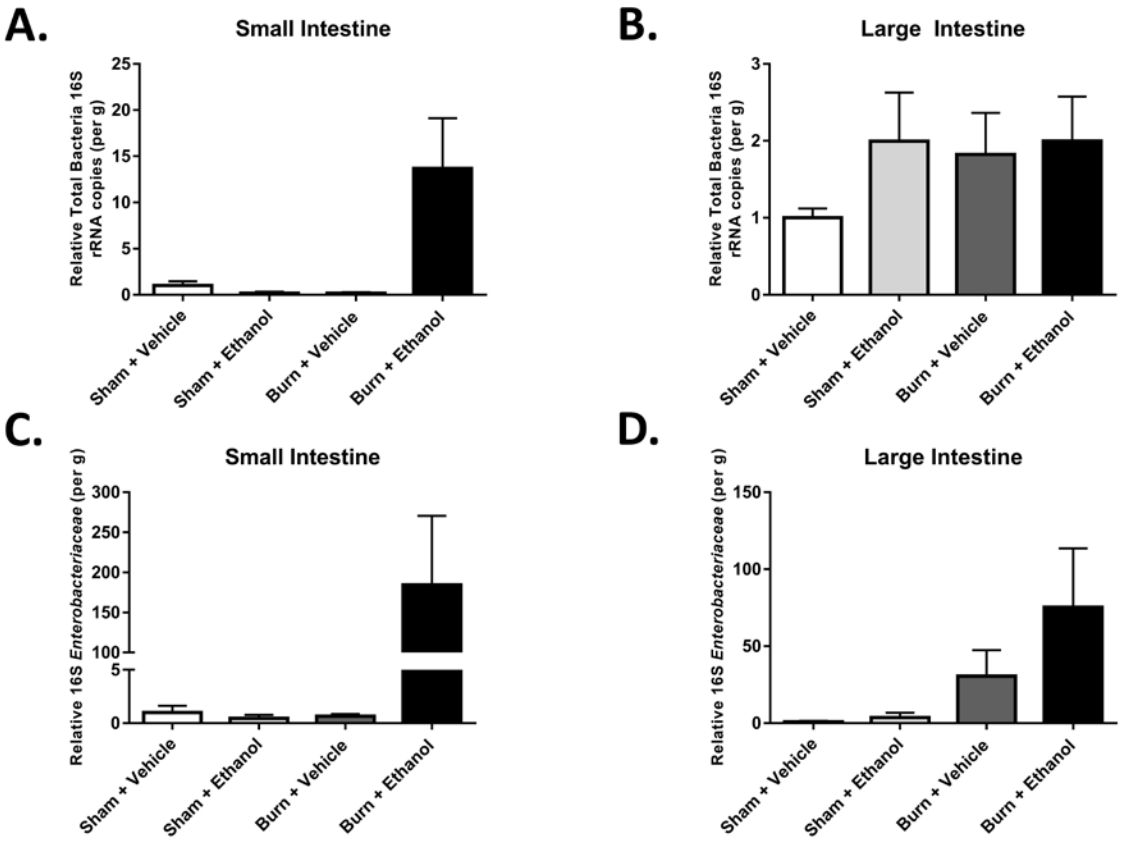

Figure 10. Large increases in Enterobacteriaceae in small and large intestine feces one day following alcohol and burn injury. Changes in total bacteria and Enterobacteriaceae populations within the small and large intestine feces one day following injury were analyzed using domain (total bacteria) and family (Enterobacteriaceae) specific primers by qRT-PCR. Data are expressed as the copy number of 16S rRNA per gram of feces relative to the sham + vehicle group in the **A.)** ileum and **B.)** colon. Enterobacteriaceae was highly increased in both the **C.)** small and **D.)** large intestine feces one day following injury (n = 5-8).

diminished goblet cell presence in ileum of alcohol + burn mice. No demonstrable changes were observed in mucin expression in small intestine one day after injury (**Figure 9A**). However, substantial reductions of mucin expression and goblet cell number were observed in the colon of five out of eight animals one day after receiving combined alcohol and burn injury compared to sham animals (**Figure 9B**).

Feces were isolated from distal ileum and colon one day following injury. DNA was

isolated and both total bacteria and Enterobacteriaceae family populations were analyzed by quantitative real-time PCR (**Figure 10**). We observed large (13.6-fold) increases in total bacteria in the feces from the distal ileum one day following injury (**Figure 10A**). No changes were observed in the total bacteria populations in the colon (**Figure 10B**). However, extremely large increases in the Gram-negative family Enterobacteriaceae were observed in both the small (184-fold) and large (75-fold) intestines relative to sham + vehicle mice one day following alcohol and burn injury (**Figure 10C-D**). In addition, we also observed large increases in Enterobacteriaceae in the large intestine following burn injury alone (30-fold) (**Figure 10D**). While these increases were not statistically significant due to high variability, changes in the microbiome are clearly visible following alcohol and burn injury.

Summary.

In the current study, we observed significant decreases (>50% reduction) in claudin-4 and claudin-8 gene expression in both the small and large intestines one day following alcohol and burn injury compared to sham vehicle animals. Additionally, claudin-2 was significantly up-regulated (50% increase) in small intestine epithelial cells one day following injury compared to mice in the sham vehicle group. Levels of all claudin genes were restored in both the small intestine and colon three days post-injury, with the exception of decreased claudin-8 expression in the colon. While we observe a restoration of most of these genes by three days following injury, it is plausible that bacteria and other pathogens are able to quickly invade even in very acute lapses in intestinal barrier integrity following injury. Our lab has generated supporting data showing bacteria present in mesenteric lymph nodes of alcohol burn injured mice one day following injury [73]. We further found that only mucin-2 was statistically reduced in ileal epithelial cells compared to sham animals. In contrast, mucin-2 and -4 gene expression were

significantly decreased in the colon one day following injury. A considerable reduction in alcian blue staining in the colon of mice receiving the combined injury compared to shams one day following injury supported these observations. These results were not surprising considering the high level of mucin expression in the colon of healthy animals. Finally, we observed large increases in Enterobacteriaceae in both intestines one day following injury compared to all other experimental groups.

CHAPTER FOUR

INTERLEUKIN-22 PREVENTS MICROBIAL DYSBIOSIS AND PROMOTES INTESTINAL BARRIER REGENERATION FOLLOWING ACUTE INJURY

Abstract.

Intestine barrier disruption and bacterial translocation can contribute to sepsis and multiple organ failure- leading causes of mortality in burn-injured patients. Additionally, findings suggest alcohol (ethanol) intoxication at the time of injury worsens symptoms associated with burn injury. We have previously shown that interleukin-22 (IL-22) protects from intestinal leakiness and prevents overgrowth of Gram-negative bacteria following alcohol and burn injury, but how IL-22 mediates these effects has not been established. Here, utilizing a model of alcohol and burn injury, we show that the combined insult results in a significant loss of proliferating cells within small intestine crypts and increases of Enterobacteriaceae, despite elevated anti-microbial peptides (AMPs) Reg3β, Reg3γ, and lipocalin-2. IL-22 administration substantially restored numbers of Ki-67 positive cells within crypts, further increased AMP transcript levels in intestine epithelial cells, and resulted in significant reduction of Enterobacteriaceae in the small intestine. Knockout of signal transducer and activator of transcription factor-3 (STAT3) in intestine epithelial cells resulted in complete loss of IL-22 protection, demonstrating STAT3 is required for intestine barrier protection following alcohol combined with injury. Together, these findings suggest IL-22/STAT3 signaling is critical to gut barrier integrity and targeting this pathway may be of beneficial clinical relevance following burn injury.

Introduction.

Trauma, including burn injury has been shown to result in gut barrier disruption and bacterial translocation, which can cause systemic inflammation, sepsis, and multiple organ failure in burn-injured patients [67, 68, 84, 101-104]. Co-morbidities including age, pre-existing disease, and alcohol intoxication at the time of trauma, negatively impact patient prognosis and recovery. Several studies have demonstrated that approximately half of patients admitted to burn units have detectable blood alcohol levels, and that these patients exhibit higher rates of infection, longer hospital stays, and greater incidence of mortality [2, 82, 105]. We have shown that alcohol intoxication exacerbates the suppression of intestinal T cells and potentiates small bowel barrier disruption and Gram-negative bacterial overgrowth compared to sham vehicle mice within one day after burn injury [73, 106]. More recently, we discovered changes to the microbiome in our rodent model of burn injury directly reflect the changes observed in the microbiome of hospitalized burn patients [100]. Microbial dysbiosis and gut leakiness, paired with suppressed immune cell functions, may represent significant confounding factors to post-burn pathogenesis, and restoring these factors may drastically improve recovery.

Interleukin-22 (IL-22) can protect the intestinal barrier by promoting mucin production, epithelial cell proliferation, and anti-microbial peptide secretion (e.g. Reg3β/Reg3γ) [107]. Downstream signaling is limited to epithelial cells as the IL-22 receptor, IL-22R1, is only expressed on cells of non-hematopoietic origin [108]. The primary producers of IL-22 in the intestine are type-3 innate lymphoid cells (ILC-3), and T-helper(Th)-17 and Th-22 cells that reside in gut-associated lymphoid tissue (GALT) [76]. We have previously shown a significant reduction (>50% decrease) in IL-22 production from T cells in GALT following the combined injury [106]. Intraperitoneal treatment with IL-22 in resuscitation fluid following alcohol and

burn injury resulted in substantially reduced gut barrier leakiness, and mitigated Gram-negative bacterial overgrowth [73]. While IL-22 was protective following the combined insult, the mechanism of this protection remains unknown.

IL-22 mediates its effects in intestinal epithelial cells via multiple signaling pathways, including the activation of mitogen-activated protein kinases/extracellular signal-related kinase (MAPK/ERK) and Janus kinase (Jak)/STAT pathways [76]. The activation of STAT3, in particular, has been shown to be sufficient for IL-22 mediated protection in a variety of systems, including alcoholic liver disease, hepatitis, and graft-versus-host disease [77, 79, 109]. While IL-22 generally appears to be protective in various models of inflammatory bowel disease [78, 110], it has also been shown to trigger inflammation in other autoimmune disorders such as rheumatoid arthritis [111]. For example, IL-22 from both natural killer cells and T cells conferred intestinal protection in a murine model of dextran sulfate sodium (DSS) colitis [110]. Conversely, IL-22 promoted detrimental inflammation via MAPK signaling in a model of rheumatoid arthritis [111]. Understanding the various mechanisms and effects of IL-22 in different pathological settings, particularly in acute versus chronic conditions, remains an important gap in the field.

The goal of this study was to examine the role of the IL-22/STAT3 signaling pathway in maintenance of gut barrier integrity following the acute combined insult of alcohol and burn injury. We hypothesized that IL-22 will protect the small intestine from microbial dysbiosis and epithelial barrier disruption via STAT3 signaling in intestine epithelial cells following the combined insult. Wild-type and VillinCre $STAT3^{-/-}$ knockout mice displayed similar increases of Enterobacteriaceae and reduction in the number of proliferating intestinal epithelial cells within the small intestine crypts following the combined insult of alcohol and burn injury.

Administration of IL-22 at the time of resuscitation mitigated the increase in Enterobacteriaceae, and restored intestine epithelial cell proliferation in wild-type animals. However, IL-22-mediated protection was lost in $STAT3^{-/-}$ knockout mice. Together, these findings support the suggestion that IL-22 is protective to the intestine, and that STAT3 signaling may be a relevant pathway to target for clinical therapies to protect the intestinal epithelial barrier and microbiome following injury.

Materials and Methods.

Animals. Male 10-12 week old (~23-25g body weight) C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA). Intestine epithelial cell specific VillinCre $STAT3^{flox/flox}$ knockout (henceforth referred to as "$STAT3^{-/-}$") mice were a generous gift from Dr. Bin Gao at the National Institute of Alcohol Abuse and Alcoholism (NIAAA), and were re-derived at Jackson Laboratories (Bar Harbor, ME). Mice were housed at Loyola University Chicago Health Science Division, Maywood, IL, USA, in accordance with IACUC regulations.

Alcohol/Burn Injury and IL-22 Treatment. Mice were subjected to alcohol and burn injury as described previously [28]. Briefly, mice were randomly separated into six different experimental groups: sham vehicle, sham alcohol, burn vehicle, burn alcohol, sham + IL-22, burn alcohol + IL-22. As shown in **Figure 1A,** mice received 400 µl of 25% alcohol (~2.9g/kg) or vehicle (water) by oral gavage. Four hours following gavage when blood alcohol was approximately ~100 mg/dL, mice were anesthetized with ~80 mg/kg ketamine hydrochloride and ~1.2 mg/kg xylazine, and their dorsum was shaved. Mice were then placed in a pre-fabricated template to expose ~12.5% of their total body surface area. Mice receiving burn injury were submersed in 85-87°C water for 7 seconds, resulting in a full-thickness burn. Sham animals were immersed in 37°C water for 7 seconds. Mice were immediately given 1 mL intraperitoneal

normal saline resuscitation injection with or without 1mg/kg recombinant mouse IL-22 (GenScript, Piscataway Township, NJ), and returned to their cages and allowed food and water *ad libitum*. One day following the alcohol gavage, animals were humanely euthanized.

Tissue Staining and Immunofluorescence. For hematoxylin and eosin (H&E) staining, sections of ileum were fixed in 10% formalin. Tissues were processed, sectioned, and stained by the Loyola University Health Science Division Tissue Processing Core. Ki-67 staining was performed on tissues frozen in optimal cutting temperature (O.C.T.) medium. 5μm sections were made on a Cryostar NX50 Cryosectioner (Thermo Fisher Scientific, Waltham, MA) and mounted on glass slides. Ki-67 antibody (Cell Signaling, Danvers, MA) and Alexa-488 conjugated secondary antibody (Thermo Fisher Scientific) were used. Tissues were counterstained using ProLong Gold Antifade Reagent with DAPI (Thermo Fisher Scientific) to visualize nuclei. Images were obtained on a Zeiss Axiovert 200m microscope (Zeiss, Jena, Germany) at 200X total magnification. Brightness and contrast of images were adjusted using Photoshop CS3. Villus height was measured using Axiovision software (Zeiss). Intestinal epithelial cell proliferation was quantified by counting the number of Ki-67 positive staining cells per crypt in ileum tissue. Tissue sections were blinded and a minimum of 3 randomly obtained images at 200X magnification per animal were counted, and are represented as number of Ki-67 positive cells/crypt. Data presented are the average of two independent experiments.

Fluorescent in situ Hybridization (FISH). Small intestine sections were taken from the ileum and fixed in 10% formalin. Paraffin blocks and slides with 5μm sections were prepared by the Loyola University Health Science Division Tissue Processing Core. Fluorescent in-situ hybridization was performed as described previously with small adjustments [112]. Slides were deparaffinized, dried, and incubated with the indicated probes at a final concentration of 1 ng/μl

in hybridization buffer (0.9M NaCl, 20mMTris-HCL, pH 7.5, 0.1% SDS) and left to incubate overnight at 50°C in a humidified slide box. Probe sequences were as follows [100]: Universal bacterial probe EUB338: Alexa 555 5'-GCTGCCTCCCGTAGGAGT -3' Enterobacteriaceae probe ENTBAC 183: Alexa 488 5'-CTCTTTGGTCTTGCGACG -3' (Thermo Fisher Scientific). Following incubation, slides were washed 3 x 15' in prewarmed hybridization buffer at 50°C, air dried, and mounted using ProLong Gold Antifade Reagent with DAPI (Thermo Fisher Scientific). Sections were imaged using a Zeiss Axiovert 200m microscope and processed using Axiovision software (Zeiss).

Quantitative Analyses of Fecal Microbiome. Real-time PCR was used to quantify bacterial ribosomal small subunit (SSU) 16S rRNA gene abundance, as described previously [100]. Primers targeting SSU rRNA genes of microorganisms at the domain level (Bacteria) and at the family level (Enterobacteriaceae) were used. Primers included 340F: (ACTCCTACGGGAGGCAGCAGT) and 514R: (ATTACCGCGGCTGCTGGC) for domain-level analyses and 515F: (GTGCCAGCMGCCGCGGTAA) and 826R: (GCCTCAAGGGCACAACCTCCAAG) for Enterobacteriaceae analyses (Thermo Fisher Scientific). 10-fold dilution standards were made from purified genomic DNA from reference bacteria. Reactions were run at 95°C for 3', followed by 40 cycles of 95°C for 15" and a 63°C (Bacteria) or 67°C (Enterobacteriaceae) for 60" using a Step One Plus Real-Time PCR instrument (Applied Biosystems, Foster City, CA).

Intestinal Epithelial Cell Isolation. Intestinal epithelial cells were isolated from the small intestine as described previously [96]. Briefly, the distal 10 cm of the small intestine was removed, opened longitudinally, and placed in ice cold PBS. Epithelial cells were then disrupted from the tissue by vigorous shaking in a digestion solution containing 5% heat-inactivated fetal

bovine serum (FBS), 1% HEPES, 1% pen/strep, 0.5% gentamicin, 5mM EDTA, and 1mM dithiothreitol (D.T.T.) in 1X Hank's Balanced Salt Solution (HBSS) at 37°C.

Real-time PCR Gene Analysis. Anti-microbial peptide transcript levels were measured using TaqMan Gene Expression Assay. Briefly, RNA was isolated from intestinal epithelial cells using a Qigaen RNEasy Kit per the manufacturer's instructions (Qiagen, Hilden, Germany). Reverse transcription was performed using a High Capacity Reverse Transcription Kit (Applied Biosystems), and real-time PCR were performed per manufacturer's instructions. Primers for Reg3β, Reg3γ, lipocalin-2, and β-actin were obtained from Applied Biosystems.

Enzyme-Linked Immunosorbent Assay. Blood was collected by cardiac puncture and allowed to clot at room temperature for 30 minutes. Serum was separated from red blood cells by centrifugation at 8000xg for 10 minutes. IL-22 ELISA (eBioscience, San Diego, CA) and IL-22BP ELISA (LifeSpan Biosciences, Seattle, WA) were performed per the manufacturer's instructions. Lipocalin-2 ELISA (R&D Systems, Minneapolis, MN) was performed on protein isolated from small intestine epithelial cells per the manufacturer's instructions. STAT3 DNA binding activity was quantified by subjecting total protein lysates from small intestine to a TransAM STAT3 DNA Binding ELISA (Active Motif, Carlsbad, CA) per the manufacturer's instructions.

Flow Cytometry. Isolated small intestine epithelial cells were stained with antibodies against cytokeratin-FITC (Abcam, Cambridge, United Kingdom ab78478) and mouse IL-22 receptor-APC (R&D Systems, FAB42941A). Cells were then run on a CantoII Flow Cytometer (BD Biosciences, Franklin Lakes, NJ) and subsequently analyzed using FlowJo Software (FlowJo, Ashland, OR).

Western Blotting. Protein isolated from ileum intestinal epithelial cells was separated by

SDS-PAGE and transferred to a PVDF membrane. Membranes were probed using STAT3 (clone 124H6), pSTAT3 Y705 (clone D3A7), and and β-actin (Cell Signaling). An HRP-conjugated secondary (Cell Signaling) was used for detection. Membrane was exposed on a ChemiDoc (BioRad, Hercules, CA) and densiometry was quantified using ImageLab software (BioRad). Bands were normalized to actin, followed by calculation of the pSTAT/STAT3 ratio.

YAMC STAT3 Activation Assay. Young adult mouse colon (YAMC) cells were seeded in a 12-well tissue culture plate to form a 100% confluent monolayer. Cells were then cultured with serum from one representative animal from each experimental group, 1000 pg/mL recombinant IL-22, or RPMI for 20 minutes. Following incubation, cells were lysed and pSTAT3 levels were measured by Western blot as described above.

Statistics. Data from sham vehicle and alcohol + burn animals from independent experiments were combined and presented as the averages of those experiments. Comparisons within groups were analyzed using a One-Way ANOVA with a Tukey post-hoc test. Comparisons between C57BL/6 wildtype and VillinCre STAT3$^{-/-}$ knockout mice were performed using a student's t test. All analysis was done using GraphPad Prism software (GraphPad, La Jolla, CA). A confidence level of $p < 0.05$ was considered statistically significant. Significance is represented throughout the manuscript as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Results.

We first examined the effects of ethanol, burn, and the combined insult on small intestine morphology. Hematoxylin and eosin staining of distal small intestine sections (**Figure 11B**) showed a significant amount of villus blunting (widening), and villus height shortening one day following both burn alone and the combined injury compared to sham vehicle (**Figure 11C).**

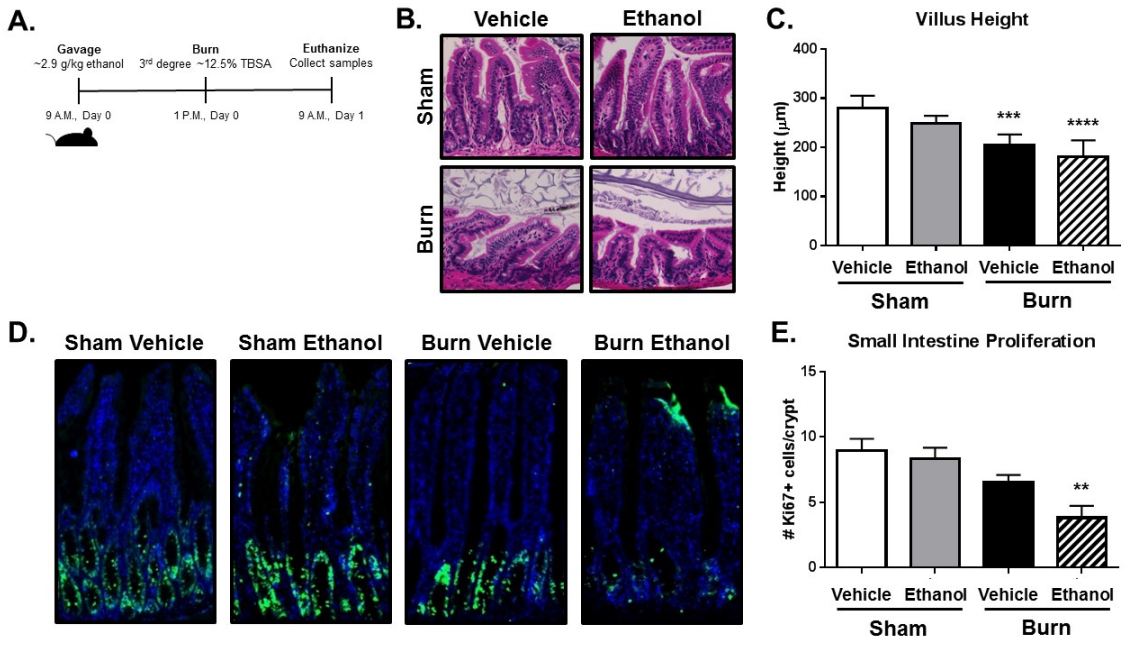

Figure 11. Ethanol and burn injury damages intestine morphology and reduces proliferation. Schematic of the combined ethanol and burn injury model (A). H&E staining of distal ileum sections from four experimental groups (B) and the respective villus height measurements (C). Analysis of proliferation within small intestine crypts using Ki-67 staining (D) and quantification represented as the number of Ki-67+ cells per crypt, (E). n = 3-8 animals per group, ***p < 0.001, **** p < 0.0001 by One-Way ANOVA with Tukey post-hoc compared to sham vehicle.

which supports our previous findings in rats [118]. We next wanted to quantify the proliferative capacity of epithelial cells within the small intestine to see if barrier regeneration was hindered. Immunofluorescent staining using Ki-67 showed significant decreases in the number of actively proliferating cells within the crypts of the small intestine following alcohol and burn injury compared to either alcohol or burn injury alone (**Figures 11D-E**). These data collectively demonstrate that burn injury results in a significant loss to villus morphology and IEC proliferative capacity, which is worsened in the presence of ethanol.

Previous data from our laboratory has demonstrated that there is a significant increase in both total bacteria and *Enterobacteriaceae* in the small intestine luminal content following the combined ethanol and burn injury [113]. Therefore, we sought to quantify the ability of small

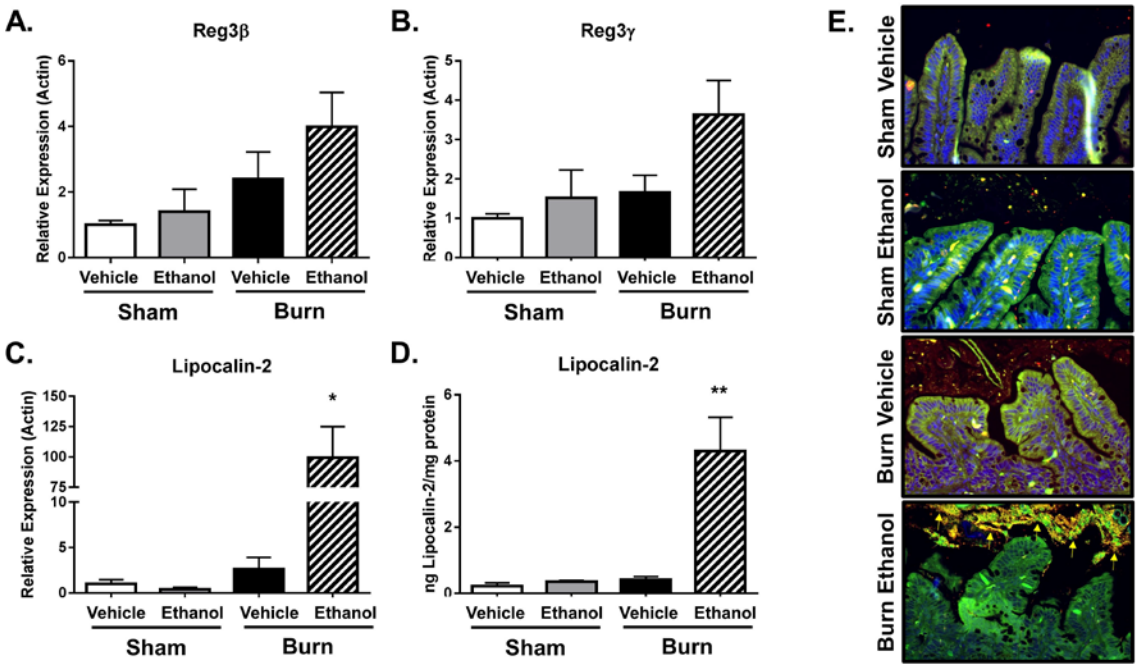

Figure 12. Combined injury results in overgrowth of *Enterobacteriaceae* despite increased epithelial cell AMPs. Real-time PCR analysis of AMP transcripts for Reg3β, Reg3γ, and lipocalin-2 following the combined injury, sham (A-C). Lipocalin-2 ELISA from total small intestine tissue (D), n = 3-11 animals per group. Specific fluorescent probes were used to perform FISH staining on total bacteria (red labeled probe) and *Enterobacteriaceae* (green labeled probe) on sections from distal ileum (E). *Enterobacteriaceae* appear yellow (arrows) due to the overlap of total bacteria and *Enterobacteriaceae* probes. FISH staining from one representative experiment, n = 3-6, *$p < 0.05$, **$p < 0.01$, by One-Way ANOVA with Tukey post-hoc compared to all groups.

intestine epithelial cells to produce anti-microbial peptides following the combined injury. While there were no significant differences between groups in Reg3β or Reg3γ transcript expression, lipocalin-2 transcripts were increased following the combined injury compared to sham animals (**Figures 12A-C**). We chose to focus on lipocalin-2 protein expression, as lipocalin-2 has been shown to preferentially target the Gram-negative *Enterobacteriaceae* family by sequestering iron required for bacterial growth [114]. Lipocalin-2 protein levels in small intestine tissue were also significantly elevated compared to all other groups (**Figure 12D**). Finally, we performed fluorescent in situ hybridization (FISH) using probes targeting specific regions of the 16S

ribosomal gene in order to examine the proximity of total bacteria (red labeled probe) and *Enterobacteriaceae* (green labeled probe) to intestinal villi following the injury,. Bacterial proximity to intestinal villi is one subjective way to correlate the ability of bacteria and endotoxins to cross the intestinal epithelial barrier into systemic or lymphatic circulation. We observed large populations of *Enterobacteriaceae* (yellow arrows) in close proximity to the villi in the small intestines of mice receiving the combined insult compared to all other experimental groups (**Figure 12E**). These observations suggest that even in the presence of elevated anti-microbial peptide production from epithelial cells, *Enterobacteriaceae* are still able to preferentially expand.

We have previously established that IL-22 production from T cells in gut associated lymphoid tissue (GALT) is significantly reduced following the combined injury [106]. In addition, administration of recombinant IL-22 was shown to reduce intestine leakiness, suggesting that IL-22 may be beneficial following acute injury [73]. We hypothesized that we could restore proliferation and further promote an anti-microbial defense by administering recombinant IL-22 at the time of resuscitation. Data in **Figures 13 A-B** show that treatment of mice with a single dose of IL-22 resulted in near complete restoration of the number of Ki67-positive cells within the crypts of the small intestine following the combined insult of ethanol and burn injury.Additionally, IL-22 further increased the expression of Reg3β, Reg3γ, and lipocalin-2 transcripts, and lipocalin-2 protein in the small intestine epithelial cells compared to mice receiving only saline resuscitation (**Figures 14A-D**).

To further delineate the mechanism underlying IL-22 mediated protection of the intestine barrier, we used intestine epithelial cell specific $STAT3^{-/-}$ knockout mice. STAT3 has been shown to mediate the beneficial effects of IL-22 in other models of chronic inflammatory

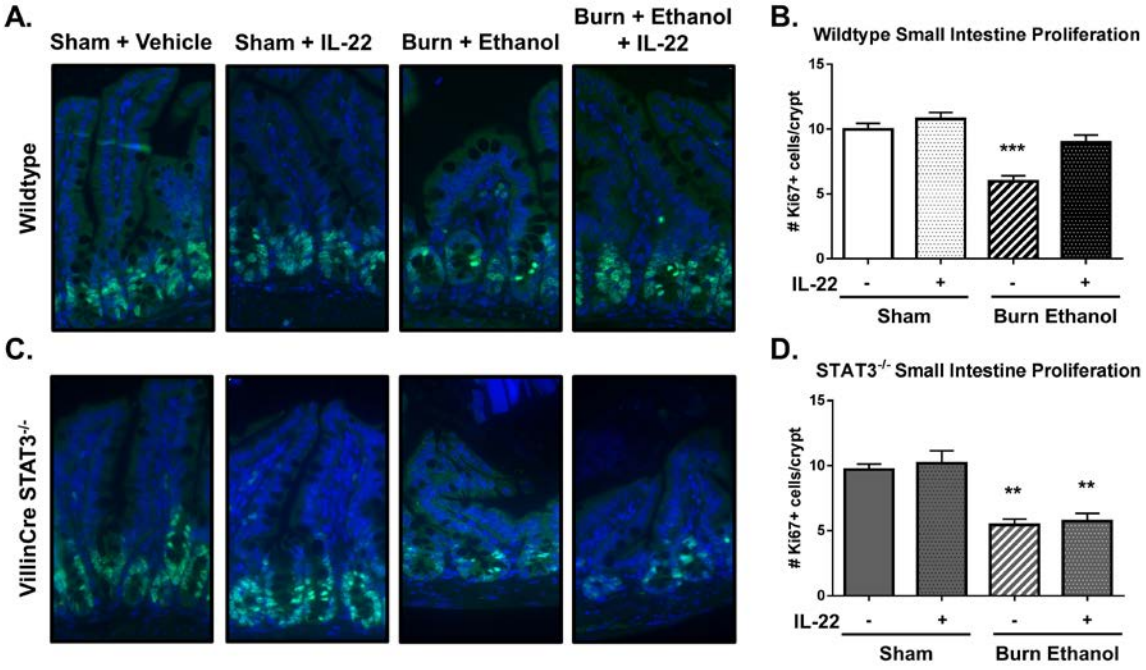

Figure 13. IL-22 mediated restoration of proliferation is dependent on STAT3 in intestine epithelial cells. Ki-67 staining on distal ileum sections of wildtype (n = 7-13), ***$p < 0.001$ by One-Way ANOVA with Tukey post-hoc compared to all groups, and VillinCre $STAT3^{-/-}$ knockout (n = 3-5) **$p < 0.01$ by One-Way ANOVA with Tukey post-hoc compared to sham groups. Wildtype data are expressed as the mean of two independent experiments. Quantification was blinded and expressed as the number of Ki-67+ proliferating cells per crypt.

conditions such IBD [115]. To determine if IL-22 was mediating acute protective effects through STAT3, we administered IL-22 in STAT3 deficient mice and assessed the proliferative capacity and AMP production from small intestine epithelial cells. IL-22 administration to $STAT3^{-/-}$ mice did not rescue the proliferation of epithelial cells within the crypts of the small intestine following the combined injury (**Figures 13 C-D**). Additionally, $STAT3^{-/-}$ mice were unable to generate any anti-microbial peptide response following injury, or treatment with IL-22 relative to wildtype mice (**Figures 14 E-H**). These data together demonstrate that exogenously administered IL-22 utilizes STAT3 in intestinal epithelial cells to promote both barrier regeneration and anti-microbial peptide production following acute injury.

We sought to determine if IL-22 and STAT3 signaling plays any role in the maintenance

of intestinal microbiome following ethanol and burn injury. We performed quantitative real-time PCR on 16S ribosomal RNA of both total bacteria and *Enterobacteriaceae* on small intestine lumenal content. Animals were housed in the same room and fed the same diet for a minimum of two weeks before beginning experiments to assimilate microbial exposure. Our results showed very large increases in both total bacteria (182-fold in wildtype, 208-fold in $STAT3^{-/-}$ knockout) and *Enterobacteriaceae* (1119-fold in wildtype, 3513-fold in $STAT3^{-/-}$ knockout) copies from the lumenal content of mice receiving the combined injury compared to shams (**Figures 15 A-B**). Administration of IL-22 immediately after injury impressively mitigated these large increases in total bacteria (no change) and *Enterobacteriaceae* (5-fold increase) copies in wildtype mice. However, IL-22 was unable to rescue the large increases in either total bacterial (463-fold increase) or *Enterobacteriaceae* (9383-fold increase) populations in the $STAT3^{-/-}$ knockout mice (**Figures 15 A-B**). Taken together, these data support our hypothesis that IL-22 promotes intestine barrier and mitigates microbial dysbiosis following ethanol and burn injury, however these IL-22 protective effects are dependent on STAT3 signaling in epithelial cells.

Finally, we assessed the levels of circulating IL-22 as verification that IL-22 was indeed present in our mice receiving treatment. We were surprised to find IL-22 in circulation of mice receiving ethanol and burn injury alone. While IL-22 levels are elevated following the combined injury, we believe the amount of circulating IL-22 may not be sufficient to provide protective benefits to the intestines. An even more interesting observation was the extremely elevated levels of IL-22 in mice receiving both the combined injury and IL-22 treatment (**Figure 16C**). There was no difference in IL-22 receptor expression on intestine epithelial cells following ethanol and burn injury compared to shams (**Figure 16 A-B**). We further examined if this IL-22 was potentially sequestered by IL-22BP, however, no differences were observed in the expression of

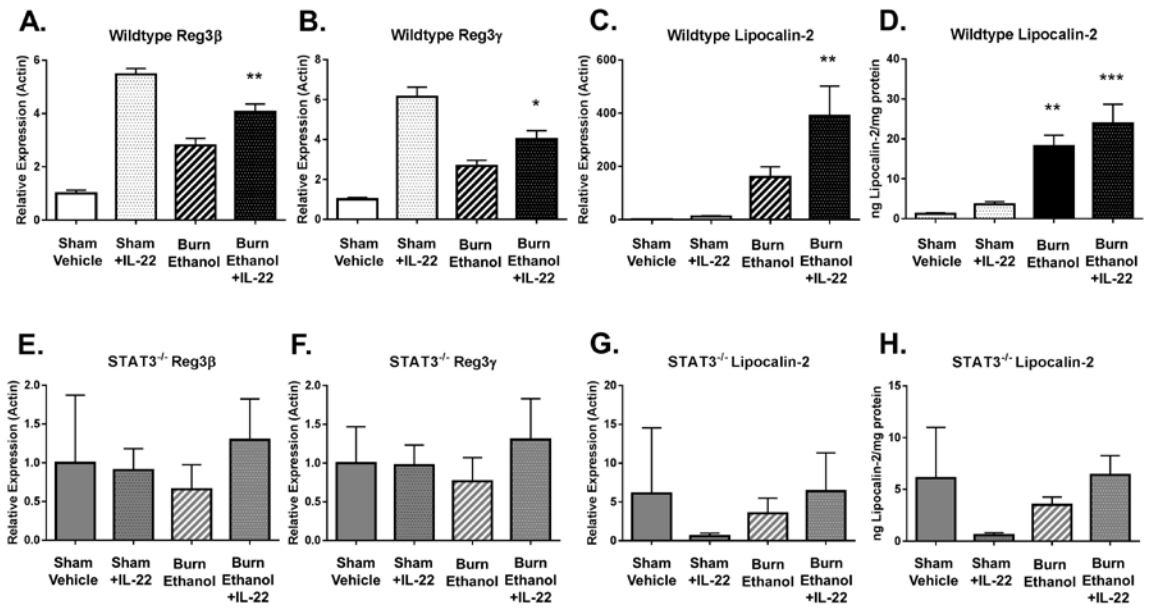

Figure 14. IL-22 treatment further increases AMP production via STAT3 signaling. Real-time PCR analysis of anti-microbial peptide gene expression following injury and IL-22 treatment. RNA from ileum epithelial cells was analyzed for expression of (A, E) Reg3β, (B, F) Reg3γ, and (C,G) lipocalin-2 transcripts in wild-type mice, (n=4-10) (A-D) and VillinCre STAT3$^{-/-}$ knockout mice, sham vehicle (n =3-8) (E-H). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, by One-Way ANOVA with Tukey post-hoc compared to untreated sham vehicle.

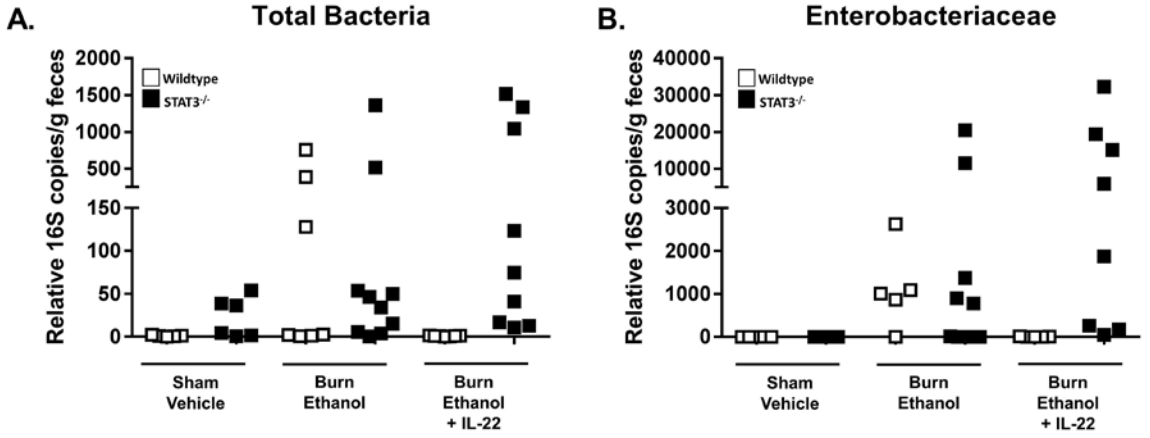

Figure 15. IL-22 prevents overgrowth of Gram-negative *Enterobacteriaceae* through intestine epithelial cells STAT3 signaling. Real-time PCR 16S rRNA sequencing of small intestine luminal content in wild-type (black) and VillinCre STAT3$^{-/-}$ knockout (gray) mice. Primers were specific for total bacteria (A) and *Enterobacteriaceae* (B). Data are combined from 2 independent experiments.

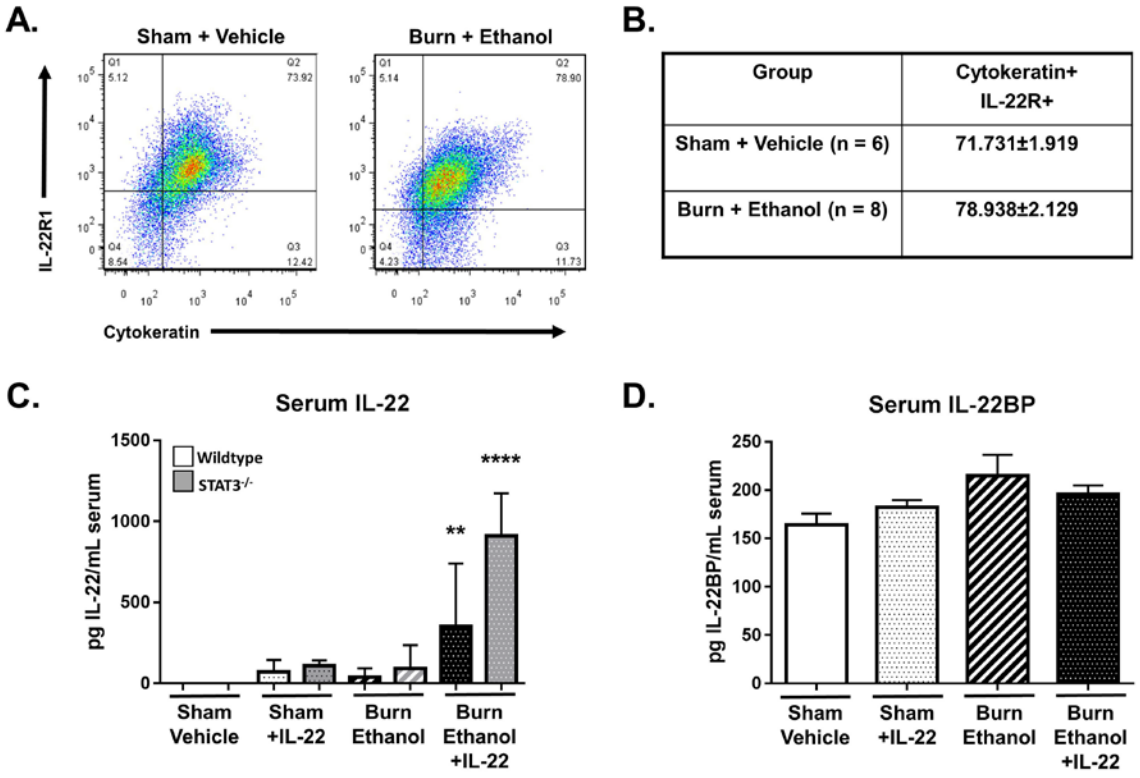

Figure 16. IL-22 treatment results in large amounts of IL-22 present in circulation following the combined injury. Flow cytometry analysis of small intestine epithelial cell IL-22R1 expression in sham vehicle and burn ethanol mice (A-B). Serum levels of IL-22 (C) and IL-22BP (D) in wildtype (black/white) and STAT3$^{-/-}$ knockout (gray bars) mice following IL-22 treatment and burn ethanol injury. n = 3-14 animals per group from one representative experiment, **p < 0.01, **** p < 0.0001 by One-Way ANOVA with Tukey post-hoc compared to untreated sham vehicle.

IL-22BP in expression (**Figure 16D**). We also assessed the serum IL-22 biological activity using young adult mouse colon (YAMC) epithelial cells. YAMCs were cultured in serum isolated from sham and burn alcohol groups and STAT3 phosphorylation was assessed. Our findings revealed a significant increase in phosphorylation of STAT3 in alcohol bur injured mice receiving IL-22 compared to all other groups (**Figure 26**), suggesting that IL-22 in circulation is biologically active.

Summary.

Here, we demonstrate that burn injury in the presence of alcohol results in decreased

intestinal epithelial cell proliferation and results in a significant elevation in Enterobacteriaceae. Administration of recombinant IL-22 restored epithelial cell proliferation, AMP production, and drastically diminished microbial dysbiosis in the small intestine. Our findings further demonstrate that IL-22-mediated protection requires STAT3 signaling in intestinal epithelial cells, as $STAT3^{-/-}$ knockout mice did not benefit from IL-22 treatment following injury. Together, our data highlight the importance of both IL-22 and STAT3 signaling for protection against gut dysbiosis, as well as in maintenance of gut barrier integrity following alcohol and burn injury.

CHAPTER FIVE

DISTINCT AND INDEPENDENT ROLES FOR INTERLEUKIN-22 AND INTERLEUKIN-18 IN INTESTNAL BARRIER MAINTENANCE FOLLOWING ALCOHOL AND BURN INJURY

Abstract.

Excessive consumption of alcohol (ethanol) has been shown to increase the incidence of traumatic injury, including burn injury. Previous studies in hospitalized patients have found those who have consumed alcohol prior to burn injury have higher incidence of infection, multiple organ failure, and mortality. We have also observed gut barrier leakiness and substantial increases in inflammatory markers IL-6, KC, and IL-18 in the small intestine of mice following alcohol and burn injury. However, the mechanisms of how alcohol exacerbates post-burn pathogenesis remain elusive. Previous studies from our laboratory have shown many benefits of independent recombinant IL-22 or αIL-18 antibody treatment to intestinal barrier maintenance following alcohol and burn injury. Here, we wanted to determine if a combination of IL-22 and αIL-18 antibody administration helps protect the intestinal barrier following the combined insult. Male C57BL/6 mice were gavaged with ~2.9g/kg alcohol or water, and then given ~12.5% body surface area burn. Mice were given resuscitation with or without 1mg/kg recombinant mouse IL-22, 1mg/kg αIL-18 antibody, or both. We found IL-22 alone does not result in a reduction of IL-6 or KC levels compared with untreated animal receiving alcohol and burn injury. Interestingly, αIL-18 antibodies completely reduce both cytokines to sham levels. *In vivo* results show both αIL-18 and IL-22 alone partially significantly decreased intestinal barrier leakiness compared

to animals receiving the combined injury alone. Together, these data suggest that IL-18 and IL-22 appear to have distinct roles following alcohol and burn injury, and that combining the treatment regimens appears to act cooperatively to protect the intestinal barrier following traumatic injury.

Introduction.

Alcohol Use Disorders (AUDs) remain one of the leading preventable causes of disease and death worldwide [1, 116]. While chronic alcohol intoxication is associated with a host of behavioral and mental disorders, most of the alcohol in the United States is consumed in a binge-like fashion [1, 116]. Not surprisingly, binge alcohol intoxication is correlated with high incidence of accidents and injuries, including burn injury [82]. Previous studies by our lab and others have shown that when intoxication precedes a burn injury, post-burn recovery is worsened in both pre-clinical and clinical settings. These studies further add that patients coming to burn units for treatment and have measurable levels of alcohol in circulation demonstrate higher rates of infection, sepsis, and mortality [2, 6, 72]. One potential cause of infection and sepsis could be the disruption of the intestinal barrier. As the largest reservoir of bacteria in the human host, intestinal barrier disruption may allow bacteria or bacterial endotoxins to gain access to extra-intestinal sites resulting in the sequelae associated with alcohol and burn injury. Thus it is important to understand the mechanisms by which alcohol combine with burn injury causes gut barrier disruption.

Interleukin-18 (IL-18) is a pro-inflammatory cytokine that is produced by both immune and non-immune cells, and is a key driver of the inflammatory response and neutrophil recruitment [117, 118]. Mature IL-18 is produced by the cleavage of its precursor, proIL-18, by caspase-1 [117]. Our previous studies, as well as studies from others have shown that the

presence of IL-18 contributes to delayed neutrophil apoptosis [119], and exacerbates resident tissue cell death in several experimental models [68, 120, 121]. We have shown neutralizing anti-IL-18 antibodies significantly restore occludin and claudin-1 expression in intestinal epithelial cells of rats following alcohol and burn injury [68]. In addition, caspase-1 inhibitors have been used in both animal models and clinical trials with promising results to limit inflammation in many conditions [122-124]. In spite of the promising improvements observed in our pre-clinical rodent model of alcohol and burn injury following anti-IL-18 antibody or caspase-1 inhibitor treatment, we do not observe complete recovery of animals following the combined alcohol and burn injury with these therapies alone.

Parallel studies from our laboratory have examined the role of interleukin-22 (IL-22) in the intestine following alcohol and burn injury [73]. IL-22 is a member of the IL-10 family, and has been shown to be protective in many models of intestinal injury and disease [77, 109, 125]. However, little work has been done examining the effects of IL-22 in an extremely acute setting of injury. Our laboratory has shown promising protective effects of treatment with recombinant IL-22 in mitigating bacterial overgrowth and restoring intestinal barrier function following alcohol and burn injury [73]. However, like inhibition of IL-18, recombinant IL-22 treatment alone does not result in complete recovery of animals following alcohol and burn injury.

As many recent studies have begun to illuminate an intimate relationship between IL-18 and IL-22 signaling, we wanted to investigate this relationship in our acute model of alcohol and burn injury [80, 126, 127]. In the present study, we utilized a two-pronged therapeutic approach using anti-IL-18 antibodies and recombinant IL-22 in the resuscitation fluid following combined alcohol and burn injury. We found that inhibition of IL-18 appears to have a more significant effect on restoring tight junction complexes and preventing apoptosis in intestinal epithelial cells.

Conversely, IL-22 administration does not appear to have protective effects on tight junctions and apoptosis. Rather, IL-22 promotes intestinal epithelial cell proliferation and anti-microbial peptide production. In addition, the combined IL-22 and αIL-18 antibody treatment completely restored intestinal barrier function following alcohol and burn injury. Together our results illuminate distinct and independent roles for IL-18 and IL-22 in the intestine following acute alcohol and burn injury, and suggest that the combination of these therapies may be beneficial due to their distinct roles in the intestine following trauma.

Materials and Methods.

Animals. Male 10-12 week old C57BL/6 mice (~23-25g body weight) were obtained from Charles River Laboratories (Wilmington, MA). Animals were housed at Loyola University Chicago Health Science Division, Maywood, IL, USA, in accordance with IACUC regulations.

Alcohol/Burn Injury, IL-22, and αIL-18 Treatment. Mice were subjected to alcohol and burn injury as previously described [28]. Mice were randomly separated into eight different experimental groups: sham vehicle, sham + IL-22, sham + αIL-18 , sham + IL-22 + αIL-18, burn alcohol, burn alcohol + IL-22, burn alcohol + αIL-18, burn alcohol + IL-22 + αIL-18. Mice received and oral gavage of 400 μl of 25% alcohol (~2.9g/kg) or vehicle (water). Four hours later, when blood alcohol was approximately ~100mg/dL, mice were anesthetized by intraperitoneal injection with ~80 mg/kg ketamine hydrochloride and ~1.2 mg/kg xylazine. Mice then had their dorsum shaved and were then placed in a pre-fabricated template to expose ~12.5% of their total body surface area. Burn injury was administered by submersing mice in 85-90°C water for 7 seconds, resulting in a full-thickness burn. Sham animals were immersed in 37°C water for 7 seconds. Mice were immediately given 1mL intraperitoneal normal saline resuscitation injection with or without 1mg/kg recombinant mouse IL-22 (GenScript, Piscataway

Township, NJ), 1mg/kg αIL-18 antibodies (MBL International, Woburn, MA), or both. Mice were returned to their cages and allowed food and water, and were humanely euthanized one day following the alcohol gavage.

Intestine Epithelial Cell Isolation. Intestinal epithelial cells were isolated from the small intestine as described previously [96]. Briefly, the distal 10cm of the small intestine was removed, opened longitudinally, and placed in ice cold PBS. Epithelial cells were then disrupted from the tissue by vigorous shaking in a digestion solution containing 5% heat-inactivated fetal bovine serum (FBS), 1% HEPES, 1% pen/strep, 0.5% gentamicin, 5mM EDTA, and 1mM dithiothreitol (D.T.T.) in 1X Hank's Balanced Salt Solution (HBSS) at 37°C. Cells were counted on a hemacytometer to determine purity (>90%), and were used in downstream experiments.

Immunofluorescent Tissue Staining. Clauin-8 staining was performed on tissues frozen in optimal cutting temperature (O.C.T.) medium. 5μm sections were cut and mounted on glass slides. Antibodies for the staining included a mouse claudin-8 primary antibody (Invitrogen, Carlsbad, CA) and an Alexa-488 conjugated secondary antibody (Invitrogen). Phycoerythrin-conjugated phalloidin was used to visualize actin filaments (Invitrogen). All tissues were counterstained using DAPI to visualize nuclei (Invitrogen). Images were obtained on a Zeiss Axiovert 200m at 200X total magnification. Brightness and contrast of images were adjusted for images using Photoshop CS3.

Quantitative Analyses of Fecal Microbiome. Quantitative real-time PCR was used to quantify bacterial ribosomal 16S rRNA gene abundance [100]. Primers targeting rRNA genes of microbes at the domain level (Bacteria) and family level (Enterobacteriaceae) were used. Primers included 340F: (ACTCCTACGGGAGGCAGCAGT) and
514R: (ATTACCGCGGCTGCTGGC) for domain-level analyses and

515F: (GTGCCAGCMGCCGCGGTAA) and

826R: (GCCTCAAGGGCACAACCTCCAAG) for Enterobacteriaceae analyses (Thermo Fisher Scientific). Standards were made by preparing 10-fold dilutions from purified genomic DNA of reference bacteria. PCR reactions were run at 95°C for 3', followed by 40 cycles of 95°C for 15" and a 63°C (Bacteria) or 67°C (Enterobacteriaceae) for 60" using a Step One Plus Real-Time PCR instrument (Applied Biosystems, Foster City, CA).

Enzyme-Linked Immunosorbent Assay. To determine circulating levels of IL-22 and IL-18, blood was collected by cardiac puncture and allowed to clot at room temperature for 30 minutes. Serum was separated from red blood cells by centrifugation. IL-22 ELISA (eBioscience, San Diego, CA), and IL-18 Platinum ELISA (eBioscience) were performed per the manufacturer's instructions. For intestinal tissue levels of inflammatory cytokines, IL-6, KC, (R&D Systems, Minneapolis, MN) ELISA kits were used on protein isolated from small intestine total tissue per the manufacturer's instructions. For cell death ELISA, Isolated epithelial cells were lysed using cell lysis buffer (Cell Signaling) with protease inhibitor (Cell signaling) and phosphatase inhibitor (Invitrogen). Relative cell death (apoptosis) was determined using a Cell Death ELISA Kit (Roche, Basel, Switzerland) per the manufacturer's instructions. Levels of cell death are expressed as relative absorbance measurement at 405nm and normalized to amount of protein loaded per well.

Real-time PCR Microarray and Analysis. Cell growth and apoptosis gene expression were analyzed using a Qiagen RT^2 PCR-Profiler Array Mouse Jak/STAT Signaling Pathway Assay. Briefly, RNA was isolated from intestinal epithelial cells using a Qigaen RNEasy Kit per the manufacturer's instructions. Reverse transcription and real-time PCR were performed per

manufacturer's instructions for RT^2 PCR Array. Heat maps were generated using Qiagen's software accessible at www.pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis

Statistics. Data from sham vehicle and alcohol + burn animals from independent experiments were combined and presented as the averages of those experiments. Comparisons within sham or injured groups were analyzed using a One-Way ANOVA with a Tukey post-hoc test. Comparisons between sham + vehicle and alcohol + burn mice was performed using a student's t test. All analysis was done using GraphPad Prism software (GraphPad, La Jolla, CA). A confidence level of $p < 0.05$ was considered statistically significant. Unless noted otherwise, significance is represented as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Results.

Previous studies from our laboratory have shown many benefits of IL-22 or αIL-18 antibody treatment to intestinal barrier maintenance following alcohol and burn injury [68, 73]. We have also observed substantial increases in inflammatory markers IL-6, KC, and IL-18 in the small intestine following alcohol and burn injury. In order to determine if IL-22 administration also helps prevent intestinal inflammation following the combined insult, we measured IL-6 and KC levels from small intestinal tissue after treatment with αIL-18 antibodies, recombinant IL-22 administration, or a combination of both (**Figure 17**). Results showed that IL-22 alone does not result in a reduction of IL-6 or KC levels compared with untreated animals receiving alcohol and burn injury. Interestingly, αIL-18 antibodies completely restored both cytokines to sham + vehicle levels (**Figures 17A-B**). Additionally, we quantified IL-22 and IL-18 levels in serum and found that αIL-18 antibodies or the combined IL-22 and αIL-18 treatment significantly decreased IL-18 levels in circulation. IL-22 was increased in circulation following IL-22 treatment, but

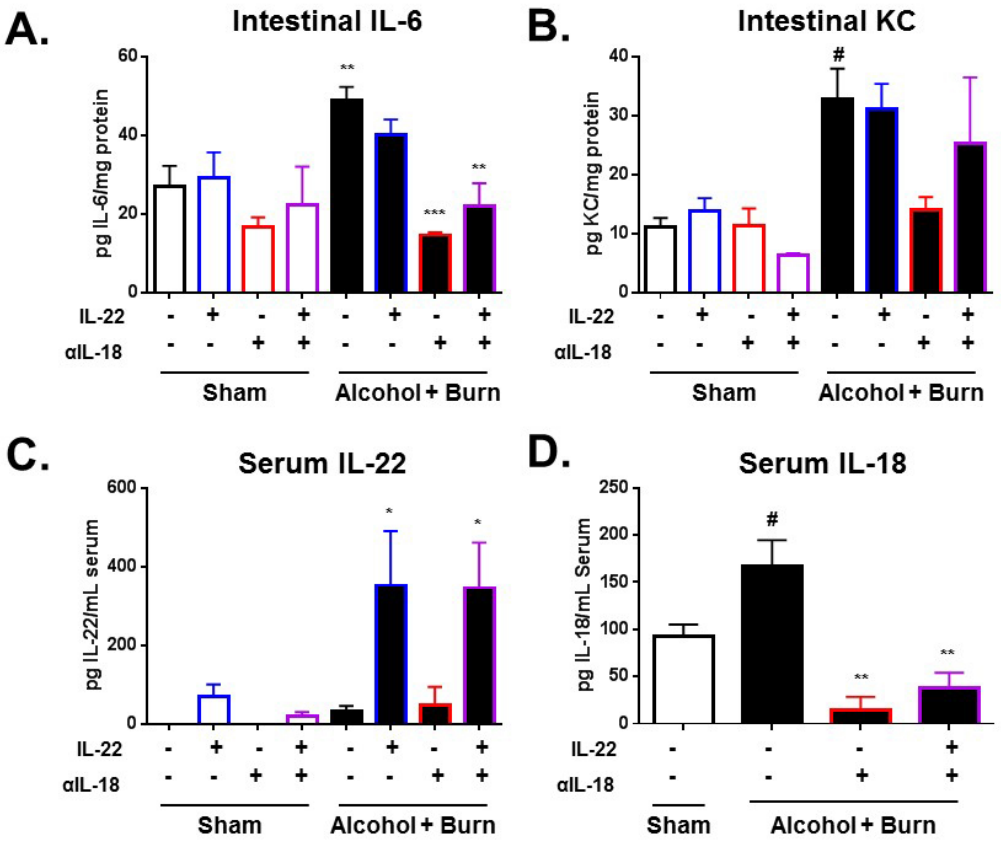

Figure 17. Inflammatory markers in intestine and serum following alcohol and burn injury. Total intestinal (distal 10cm) tissue homogenates were analyzed for the inflammatory markers **A)** IL-6 and **B)** KC by ELISA. Serum levels of **C)** IL-22, and **D)** IL-18 measured by ELISA (n = 4-18, *p < 0.05, **p < 0.01, ***p < 0.001 by ANOVA compared to untreated alcohol burn, #p < 0.05 by student t test compared to sham vehicle).

αIL-18 antibody administration did not affect circulating IL-22 levels (**Figures 17C-D**). These data suggest that IL-18 is a major driver of inflammation within the first 24 hours.

Previous studies from our laboratory have shown IL-22 or αIL-18 antibody administration significantly improves, but does not fully restore gut barrier leakiness one day following the combined injury. Therefore, we sought to determine if the combined treatment of IL-22 and αIL-18 antibodies could act cooperatively to completely prevent gut barrier leakiness following alcohol and burn. To determine this, mice were given a gavage of FITC-dextran 90 minutes before euthanasia, and blood was collected at the time of tissue harvest to measure levels

of FITC-dextran in circulation. Results showed that mice receiving alcohol and burn injury had significantly higher levels of FITC-dextran in circulation, which was partially reduced by IL-22 or αIL-18 antibody administration alone. However, the combined treatment showed very near complete reduction of leakiness in mice receiving alcohol and burn injury (**Figure 18**), suggesting these therapies can work in cooperatively to better prevent intestinal leakiness than either therapy individually.

In order to determine the underlying cause of gut leakiness following injury, we examined the effect of IL-22 and/or αIL-18 antibodies on tight junction expression and apoptosis levels in small intestine epithelial cells (**Figures 19-20**). Immunofluorescent staining of claudin-8, a major tight junction protein in rodent intestine, and F-actin revealed that both burn injury alone and the combined insult result in significant disruption of claudin-8 and actin co-localization (**Figure 19A**). Upon treatment with αIL-18 antibodies, we observed a significant restoration of claudin-8 and F-actin co-localization in small intestine epithelial cells. IL-22 administration did not improve claudin-8 co-localization in injured mice (**Figure 19B**). To determine if intestinal barrier leakiness following injury could also be due to cell undergoing apoptosis, we analyzed epithelial cell death using a cell death ELISA. Data demonstrated that mice receiving alcohol and burn injury have significantly elevated numbers of epithelial cells undergoing apoptosis (**Figure 20A**). Similar to tight junctions, αIL-18 treatment significantly decreased epithelial cell apoptosis, while IL-22 administration did not appear to have any effect in injured mice following the combined injury (**Figure 20B**).

Due to the efficacy of αIL-18 antibody treatment in restoring epithelial cell tight junctions and apoptosis, we sought to determine if αIL-18 antibodies also helped regulate the intestinal microbiome following alcohol and burn injury. Previous studies from our lab have

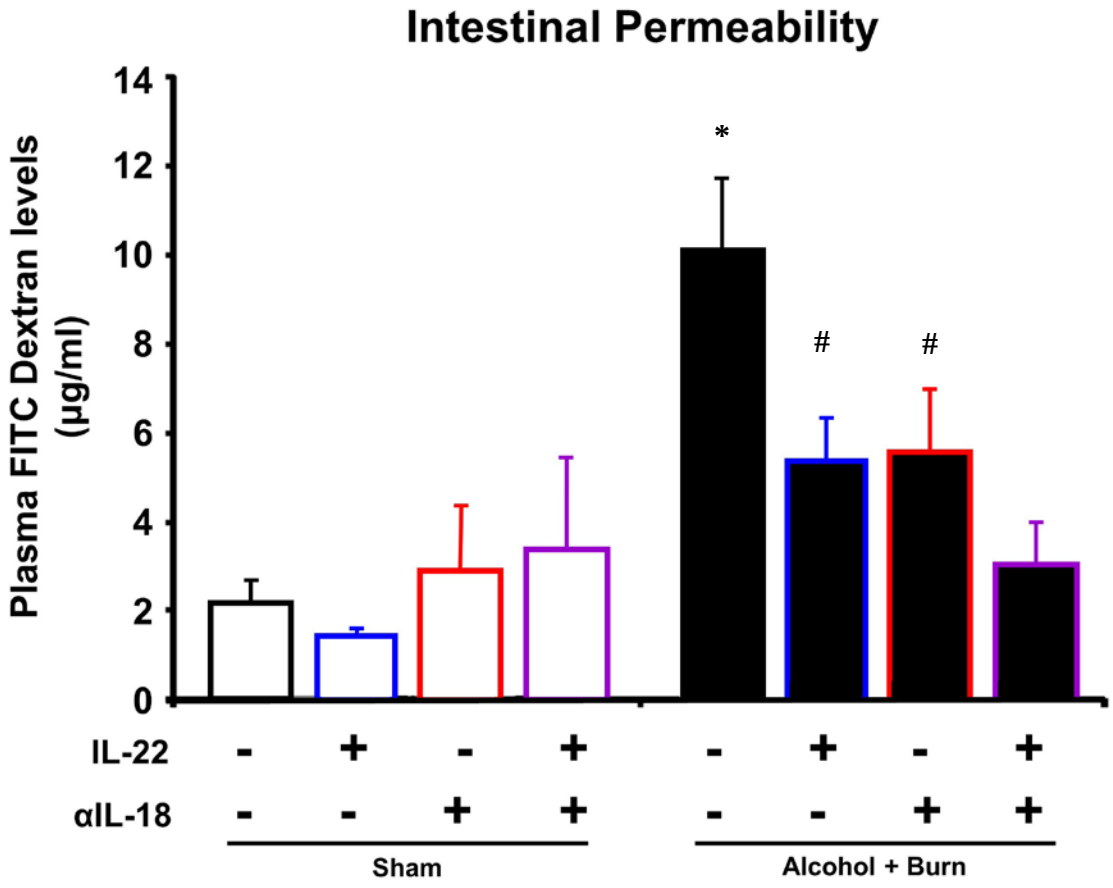

Figure 18. Intestinal permeability following alcohol and burn injury plus αIL-18 antibody and IL-22 treatment. Animals were gavaged with 4 kDa FITC-dextran 90 minutes before euthanasia, at which time blood was collected. FITC-dextran levels in serum were quantified by measuring absorbance (n = 3-6, *$p < 0.05$ compared to other groups by ANOVA, #$p < 0.05$ versus sham vehicle).

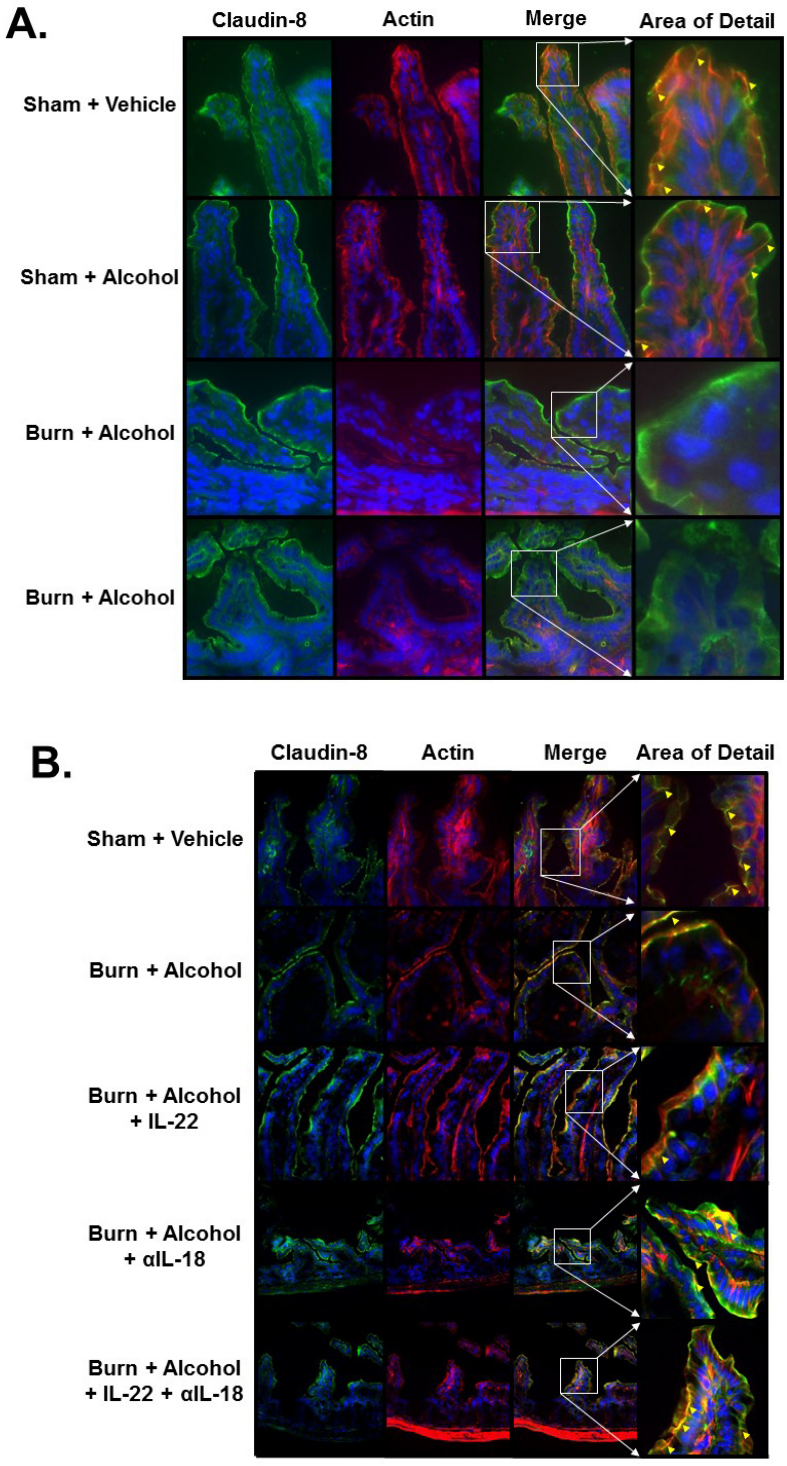

Figure 19. Decreases in claudin-8 and actin co-localization following alcohol and burn injury. Frozen sections of distal ileum were stained for claudin-8 (green) and F-actin (red) co-localization to analyze epithelial cell tight junction integrity. A) Effects of alcohol alone, burn alone, or alcohol and burn injury on epithelial cell tight junctions. B) Representative claudin-8 co-localization with actin following treatment with IL-22, αIL-18 antibodies, or both following the combined injury. (n = 4-5 per group).

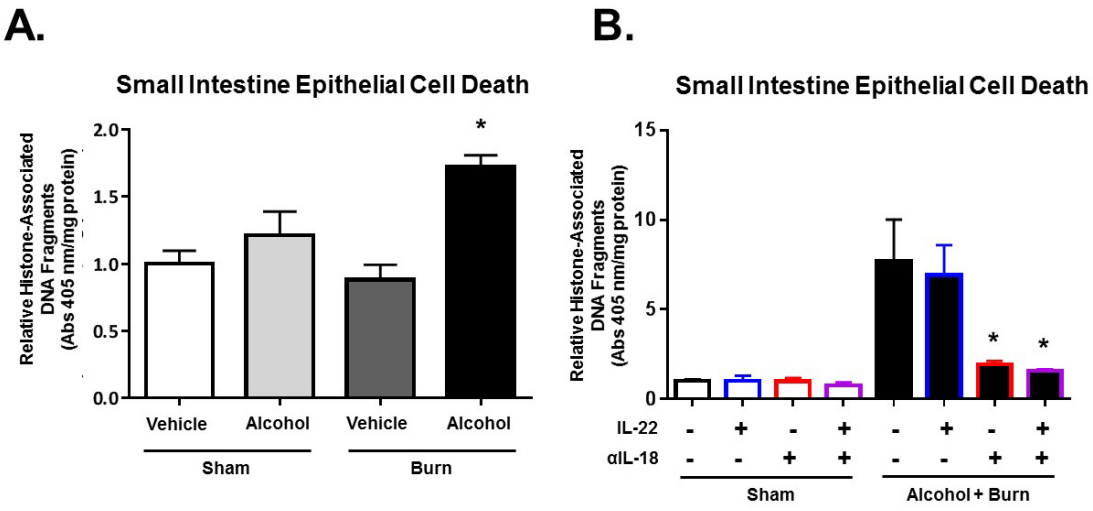

Figure 20. Small intestine epithelial cell apoptosis following alcohol and burn injury and combined IL-22 and αIL-18 treatment. Protein isolated from small intestine epithelial cells was used to quantify apoptosis by cell death ELISA. **A)** Relative apoptosis levels in intestinal epithelial cells following alcohol, burn injury, or the combined insult. **B)** Effects of IL-22 and αIL-18 treatment on small intestine epithelial cell apoptosis following alcohol and burn injury (n = 3-8, *p < 0.05 compared to **(A)** sham vehicle, or **(B)** untreated alcohol burn injured mice).

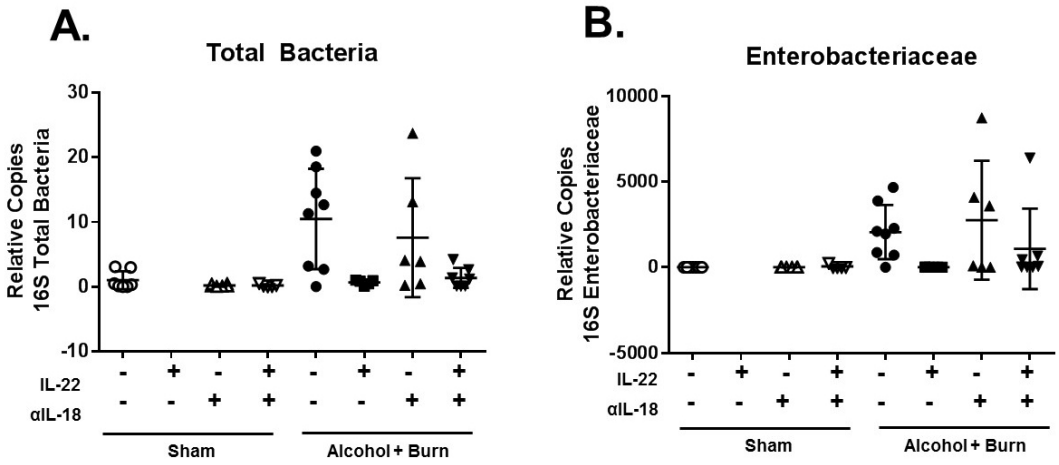

Figure 21. Total bacteria and Enterobacteriaceae 16S rRNA real-time PCR expression. DNA was isolated from small intestine luminal content and A) total bacterial and B) Enterobacteriaceae populations were analyzed using 16S rRNA qRT-PCR amplification. Because no changes were observed in sham + IL-22 in previous experiments, this group was omitted from this experiment (n = 4-8 mice per group).

shown that alcohol and burn injury results in a large overgrowth of total bacteria and

Enterobacteriaceae in the small intestine of mice [73, 113].

We performed 16S ribosomal RNA amplification by real-time PCR to quantify the number of total bacteria and Enterobacteriaceae present in the luminal content of small intestines following alcohol and burn injury and different treatments (**Figure 21**). Our findings demonstrate that αIL-18 antibody treatment does not significantly mitigate total bacteria (**Figure 21A**) or Enterobacteriaceae (**Figure 21B**) dysbiosis in the small intestines of mice receiving the combined injury. However, IL-22 administration paired with αIL-18 antibodies nearly completely restored both total bacteria and Enterobacteriaceae populations in alcohol and burn-injured mice. This data suggests that one of the primary protective roles of IL-22 treatment is preventing significant microbial dysbiosis following alcohol and burn injury.

Summary.

The results presented here suggest distinct and independent roles for IL-22 and IL-18 in the small intestine following alcohol and burn injury. We demonstrated that IL-18 inhibition appears to be important in reducing the inflammatory cytokines IL-6 and KC, and is sufficient to restore epithelial cell tight junction complexes and drastically reduce apoptosis. IL-22 on the other hand, prevents microbial dysbiosis 24 hours following the combined injury. Finally, when αIL-18 antibodies and recombinant IL-22 are administered in tandem, gut barrier leakiness is restored to very near sham vehicle levels. Collectively, our findings indicate that while both these treatments benefit the intestine independently following acute alcohol and burn injury, the combination of both IL-22 and αIL-18 antibodies act cooperatively through different mechanisms to provide broader protection to the intestinal barrier.

CHAPTER SIX

DISCUSSION

New Contributions to the Alcohol and Burn Injury Field.

At the outset, the overall aim of this work was to expand our understanding of how the intestinal barrier is disrupted following alcohol (ethanol) and burn injury, and to identify a potential therapy to improve post-burn outcomes. While a good amount of work has been done to understand the intestinal immune response and ways to suppress detrimental inflammatory responses, a major gap in the field before this work was the minimal understanding of what aspects (e.g. microbiome, epithelial barrier etc.) of the small intestine were negatively impacted by alcohol and burn injury. The results presented here demonstrate that there are significant changes to the intestinal microbiome, disruptions in epithelial tight junction complexes, and substantial dysregulation of the epithelial barrier regeneration within 24 hours following alcohol and burn injury. Administration of recombinant IL-22 was effective at restoring the intestinal microbiome and enhancing epithelial cell proliferation in a STAT3-dependent fashion. As a whole, these findings provide a new insight into cellular and molecular mechanisms that may contribute to intestine barrier disruption following burn injury in the presence of intoxication, and this may have implication in other forms of traumatic injury and disease conditions which acutely affect intestinal physiology.

Acute Gut Barrier Disruption.

The idea of bacteria leaking out of the gut was first published in a study from Harvard in

1948 examining the peritoneal lavage fluid from uremic dogs [128]. This idea was then largely forgotten until the 1980's when studies from the laboratories of Dr. Rodney Berg of Louisiana State and Dr. Edwin Deitch of Rutgers began to describe in great detail the process of bacterial translocation from the intestinal tract in mice [129, 130]. Since then, the idea of bacterial translocation out of the intestines contributing to systemic inflammation, sepsis, and other diseases has been examined by many different groups describing this process in varying disease and injury animal models and in patients [131-135].

In the context of alcohol and burn injury, previous work from our laboratory has demonstrated that the intestine becomes more leaky following the combined insult in mice, and this is paired with large increases in the populations of culturable Gram-negative bacteria in intestinal tissue and luminal content [73]. Our present work shows significantly diminished tight junction co-localization and intestinal epithelial barrier regeneration following the combined injury, and has led us to question whether disruptions to the intestinal barrier occur as a result of injury, or are a secondary consequence of Gram-negative bacterial overgrowth that causes epithelial barrier leakiness. It is well documented that fluid shifts that occur in the burn-injured patient result in shock and ischemic states in peripheral tissues, especially in non-vital organs such as the intestines [14]. This host-response to injury alone could certainly be one cause of direct epithelial barrier disruption in the intestines. On the other hand, changes in the intestinal microenvironment due to alcohol and burn injury may create a niche where Gram-negative Enterobacteriaceae species are able to outcompete commensal microbes. It is not unreasonable to hypothesize that the combination of intestinal ischemia and microbial dysbiosis both contribute to intestinal barrier leakiness following alcohol and burn injury. In order to fully illuminate

whether changes in the microbiome are the cause or merely a correlative observation of intestinal barrier disruption, further work will need to be done in gnotobiotic, or ideally, germ-free mice.

Few studies in the literature have examined the effects of alcohol or burn injury alone on the intestinal barrier in cell culture models and in mice [66, 88, 136, 137]. Ma *et al.* have shown in cultured Caco-2 cells that even low concentrations of alcohol (≤10%) can induce rapid disruptions of tight junction complexes including on ZO-1, actin, and myosin filaments, which was correlated with significantly increased myosin light-chain kinase (MLCK) activity [66]. MLCK activation results in the phosphorylation of myosin light chain (MLC), causing cytoskeletal shifting, and disruption of epithelial cell junctions. Supporting mouse *in vivo* studies in an alcohol burn injury model performed by Zahs *et al.* have also shown increased MLCK activity in the ileum of mice after alcohol and burn injury, which was rescued by using the membrane permeant inhibitor of MCLK (PIK) [67]. In addition, our laboratory has shown substantial decreases in phosphorylation of occludin and claudin-1 in rat ileum following alcohol and burn injury [68]. Our current findings not only add supporting data to these previous studies, but expand the analysis from the ileum to the colon. Furthermore, we profiled several additional tight junction genes (i.e. claudin-4 and claudin-8) integral to maintaining barrier integrity in addition to the ones already investigated [67, 68, 90]. The variance in gene expression observed between the small and large intestine in our studies highlight the significance of different junction proteins and mucins that may contribute to the increase in leakiness in these two segments of the intestine. Many claudins are expressed throughout the intestines of mammals, but most are only expressed within specific regions. While claudin-4 is highly expressed in both the small and large intestines, claudin-8 protein is only observed in the ileum and colon of rodents [92]. It is plausible that these regional variations in tight junction protein expression play

a significant role in gut leakiness following injury. In addition, enzyme activity modulating cytoskeletal rearrangements (e.g. MLCK) can certainly play a significant role in barrier integrity following alcohol and burn injury. Cytoskeletal rearrangements may also lead to limited tight junction gene expression in epithelial cells following a traumatic insult, but further studies will be needed to address these questions.

To our knowledge, very few studies have examined mucin expression in the intestines following alcohol exposure. Hartmann *et al.* examined the extent of alcoholic liver damage and found that Muc2-/- mice were protected from intestinal bacterial overgrowth and dysbiosis following chronic alcohol feeding [40]. They found that antimicrobial peptides were significantly elevated in Muc2-/- mice, which likely account for the decreased bacterial translocation observed. These studies suggest that it takes very high concentrations of chronic exposure to alcohol to perturb the mucus layer of the intestines. It is known that mucin levels increase in the intestine of chronic alcoholic feeding in rodents [59], however, in our model of a single binge of alcohol and burn injury, it is not known whether the combined insult influences the mucin expression/levels. In the current study, we observed significant reductions (20-60% decrease) in mucin gene expression in both the small and large intestines one day following this combined insult compared to mice in the sham vehicle group. Perhaps one reason for this observation is the acute nature of our intoxication and injury model, but this seems unlikely due to the measurable changes of mucus secretion in response to various acute stimuli reported in mice by others [138]. Regardless, such decreases in mucin protein expression could contribute to increased translocation of bacteria and other pathogens as mucin is known to contain bacteriophages and IgA antibodies to prevent such occurrences [139].

While the mechanisms underlying alterations in tight junction proteins and mucin expression remain to be explored, our findings suggest a protective role for IL-22 in intestinal barrier function after alcohol and burn injury in mice [73]. IL-22 has been shown to play a critical role in the maintenance of intestine homeostasis, and is known to modulate intestinal epithelial cell proliferation and apoptosis, and promote mucin and antimicrobial peptide secretion [76]. Previous work from our laboratory has shown that IL-22 levels in small intestine are significantly reduced following combined injury compared to sham mice, and this is paired with increased intestinal permeability [73]. In addition, we observe large increases in the amount of IL-18 present in the intestines following alcohol and burn injury compared to shams, which may also contribute to altered tight junction proteins and increases in gut leakiness [28, 68].

A surprising observation from these results is the lack of significant changes in tight junction and mucin expression following burn injury alone. A different rat model of burn injury without alcohol intoxication demonstrated significant changes in the intestinal barrier tight junctions and mucin expression [140]. These models of burn injury are much more extensive (20-60% total body surface area) than the burn injury we administer in our combined alcohol and burn model. We believe that the extent of our burn injury alone (~12.5% total body surface area) is not sufficient to induce intestinal damage similar to that observed in larger burn area models. Thus, our results highlight that alcohol intoxication at the time of burn injury exacerbates post-burn pathogenesis, even in small surface area burns. Our data further suggest that both ileum and colon respond differently to the combined insult of alcohol and burn injury, and that these changes are visible as early as one day following the combined injury. Although, the mechanism underlying the differential response remains to be established, previous studies suggest that the intestines are highly dynamic, and the microenvironments of the small and large intestines vary

drastically. As such, intestinal homeostasis is differentially regulated in the small and large intestines to account for the different environments that exist in each.

In a recent study, we observed that large intestine exhibits a higher inflammatory response to a combined insult of alcohol and burn injury compared to the small intestine in mice, which supports this notion [86]. The results as presented in Aim 1 clearly suggest that the combined alcohol and burn injury results in similar downregulation of tight junction genes in both small and large intestine epithelial cells one day following injury compared to sham vehicle. In contrast, more dramatic decreases in mucin gene expression were observed in colon epithelial cells. Together, our findings suggest that there are differential responses between the small and large intestines following alcohol and burn injury, which may then contribute to the development of gut leakiness following injury.

Our studies also demonstrate changes in the local microbiome may be a likely cause of the barrier disruption in the intestines following the combined injury, as we observed significant differences in the bacterial populations between the ileum and colon. While large increases in both total bacteria and Enterobacteriaceae populations were observed in the ileum, only Enterobacteriaceae increased in the colon following alcohol and burn injury compared to all other groups. Due to the substantially larger number of bacteria present in the large intestine compare to the small intestine, this was not a surprising observation. It is plausible that the environment in the ileum becomes permissive to Gram-negative populations to expand following the combined injury, accounting for the increase in both total and Enterobacteriaceae following the combined injury. In contrast, data from the large intestine are more suggestive of a shift in the bacterial populations from commensal species to pathogenic Gram-negative bacteria. Studies in mouse models of colitis and nonalcoholic fatty liver disease have demonstrated roles for the

intestinal barrier regulation by the microbiome, including tight junction and mucin expression [141, 142]. While many more studies will be needed to further understand the details of how alcohol and burn injury affects the intestinal microbiome, these preliminary experiments give some insight into the possible mechanism by which intestinal leakiness and inflammation may be occurring. We acknowledge that a lack of direct mechanistic support of these differential observations between the small and large intestines is a limitation of this study. IL-22 and STAT3 signaling in intestinal epithelial cells also appear to impact the diversity of intestinal microbial populations following alcohol and burn injury, which will be discussed in the next section.

Taken together, our findings from Aim 1 highlight the differential regulation that occurs in both tight junction and mucin genes in the small and large intestines following alcohol and burn injury. Regardless of the underlying mechanism, disruption in barrier integrity can allow gut bacteria to gain access to extra intestine sites. While the intestinal barrier is restored three days following the combined injury, some bacteria have already infiltrated and may contribute to infection, sepsis, and multiple organ failure observed following injury. These findings suggest that although acute administration of a single dose of alcohol alone is not likely to cause infection and/or sepsis in healthy individuals, the presence of alcohol in the context of a second insult such as burn injury becomes a serious confounding factor that significantly impacts the recovery of the host. Therefore, presence of alcohol at the time of injury should be considered as one factor in the overall outcome of burn patients.

Interleukin-22 Treatment and STAT3 Signaling.

IL-22 remains one of the most intriguing cytokines due to its ability to elicit completely different responses based on the microenvironment. In the context of the intestines, the presence

of IL-22 appears to be beneficial under most circumstances including inflammatory bowel disease, graft-versus-host disease, and many types of bacterial infection [73, 75-79]. Interestingly, certain bacterial infections, such as in mouse models using *T. gondii*, cause IL-22 to be pro-inflammatory [80, 81]. While the reasons behind these differential responses of IL-22 remain unknown, it illuminates the importance of understanding the role of IL-22 under different conditions. In addition, current clinical trials using Fc-fusion IL-22 administration for treatment of patients with graft-versus-host disease have shown promising preliminary results, indicating that IL-22 treatment may be efficacious in a clinical setting [143].

We have previously shown Gram-negative bacteria are present in mesenteric lymph nodes in a 20% total body surface area mouse model of burn injury [100]. A corollary study in our combined alcohol and burn injury model, which uses a burn injury nearly half the size (12.5% body surface area), showed intestinal barrier leakiness and overgrowth of culturable Gram-negative bacteria within one day following injury. IL-22 treatment significantly reduced gut leakiness and the presence of aerobic Gram-negative bacteria in the small intestine of mice [73]. The present study provides a mechanistic role for IL-22 mediated epithelial barrier regeneration and promotion of epithelial AMP secretion through STAT3, which could account for the observed barrier restoration. A recent report supporting this mechanism from Hanash *et al.* showed that IL-22 specifically signals through STAT3 in stem cells within the crypts of both small and large intestines to promote barrier regeneration in a murine model of graft versus host disease [79]. Interestingly, we observe increased pSTAT3 in the absence of IL-22 treatment, which may be due to elevated levels of IL-6 and/or IL-10 following the injury [28] (**Figures 24-25**). This suggests that IL-22 signaling either further activates STAT3, or changes the downstream targets of STAT3 to elicit a protective response. While IL-22 has been shown in

other models to signal through other STATs or MAPK/ERK pathways [144-147], we found epithelial cell STAT3 to be necessary for IL-22 mediated protection in our acute model of alcohol and burn injury.

Though this study focused on the role of IL-22 on the epithelial cell barrier and microbiome, it is plausible that IL-22 could be promoting protection through other mediators as well. Several lines of evidence suggest that IL-22 can act as a bridge between the innate and adaptive immune responses following bacterial and viral infections in mice [148, 149]. A recent mouse study showed IL-22 mediated Reg3γ synthesis prevented colonization by antibiotic-resistant *Enterococcus faecium* via Toll-like receptor (TLR)-7 stimulation on dendritic cells [148], and others have demonstrated that IL-22 mediated *Clostridium difficile* clearance from peripheral organs by upregulating systemic C3 complement activity [149]. It appears that T cell TLR signaling may play a role in our model, as we demonstrated that ex-vivo stimulation with TLR ligands restored IL-2 and IFN-γ production [150]. While the effects of IL-22 administration on immune cells in our system is still unclear, it is plausible that IL-22 has indirect effects on immune cell signaling in addition to its direct impact on epithelial cells in order to promote AMP secretion and mitigate bacterial overgrowth.

A surprising observation was the extremely high levels of IL-22 in circulation in injured mice receiving IL-22 treatment compared to all other groups. This most likely suggests either clearance of IL-22 following injury is hampered, or alternatively, IL-22 administration results in a positive feedback loop resulting in further IL-22 production from immune cells. Though literature suggests IL-22 can signal in a positive feedback loop in conjunction with IL-20 [151], there are currently no studies to our knowledge that have addressed how IL-22 is cleared from the host. The notion that IL-22 not only helps locally within the intestine, but also aids in

prevention and clearance of systemic infections and sepsis has been shown as well. One study in mice demonstrated that IL-22 helps prevent systemic infection with orally administered *Citrobacter rodentium* and opportunistic pathogens such as *Enterococcus faecalis* [152]. Elevated IL-22 in infection is observed clinically as well, as patients with abdominal sepsis have elevated serum levels of IL-22 [153]. However, whether IL-22 is beneficial is still conflicted, as another study by Weber *et al.* showed that IL-22 inhibition was beneficial to protect from polymicrobial sepsis in mice [154].

While currently we do not know the source of circulating IL-22, we believe what is present in serum is biologically active (i.e. not sequestered by the soluble IL-22 binding protein), and preliminary data suggest IL-22 is free in circulation, not cell-associated (unpublished data). We do not believe the modest increase in serum levels of IL-22 is sufficient to mount a beneficial physiological response to the combined injury alone, and it appears that only serum levels above a certain threshold following IL-22 treatment have a positive physiological effect. However, caution should be used in this regard, as high/prolonged levels of IL-22 are associated with both autoimmune diseases and cancer in rodents and humans [155, 156].

The intestinal microbiome likely plays a role in post-burn pathogenesis due to close interactions with the epithelial barrier. In a recent study, our lab showed that large increases in Enterobacteriaceae in the intestinal microbiome of mice following burn injury is also observed in humans following injury [100]. Overgrowth of Enterobacteriaceae has been shown to promote intestinal inflammation, increase gut barrier leakiness, and result in spontaneous colitis in both *in vitro* and *in vivo* mouse models [157-159]. Additionally, Enterobacteriaceae are able to out-compete normal commensal microbial species, specifically within the Firmicutes and Bacteroidetes phyla, and can perpetuate the effects of trauma and inflammation [159-161].

Research examining the efficacy of pre- and probiotics following burn injury in patients has failed to show any significant benefits [162], which may be due to changes in the intestinal microenvironment following trauma that allow Gram-negative facultative bacteria to selectively outcompete beneficial commensal microbe colonization. The cause and consequence of Enterobacteriaceae overgrowth in our model remains to be established, and this will be the focus of future studies, as the relationship between the microbiome and intestine epithelial cells is highly important for homeostasis.

In sum, our results highlight the importance of IL-22 and STAT3 signaling for protection against intestinal damage in an acute setting. We propose that IL-22 is mainly protective by regulating the intestinal microbiome and preventing overgrowth and translocation of Enterobacteriaceae. Gut barrier disruption and leakiness is common to many different pathologies, and while the mechanism that causes barrier disruption amongst these different diseases may not be the same, our model of alcohol and burn injury provides insight into how IL-22 may help protect the host in these various other conditions.

Intestinal IL-22 and IL-18 Axis.

Our lab has previously shown that αIL-18 treatment restores occludin claudin-1 expression in rats following alcohol and burn injury [68]. Thus, we were not surprised to see restoration of claudin-8 co-localization with the actin cytoskeleton following αIL-18 antibody treatment. In addition, this study demonstrated that αIL-18 antibodies minimized mucosal apoptosis following the combined insult. IL-22 treatment did not have a significant effect on epithelial cell tight junctions, and showed a very minimal improvement of epithelial cell apoptosis. While the exact mechanism behind these findings remains unknown, our findings support the current literature suggesting that IL-22 is protective to epithelial cells not by

inhibiting apoptosis genes, but rather by upregulating pro-proliferative genes through STAT3 [115]. In addition, our data showing IL-18 antibody treatment lowers levels of KC in intestinal tissue (**Figure 17**) is also reflective of many previous studies that suggest the excessive neutrophil infiltration significantly contributes to epithelial cell death and damage in rodents [64, 118, 121, 124,].

Following alcohol and burn injury, we have observed large increases in both total bacteria and Enterobacteriaceae in small intestine luminal contents of mice [73, 113]. We have also observed that IL-22 treatment at the time of burn injury completely restores total and Enterobacteriaceae populations in the intestine. As others have shown IL-18 is necessary for clearance of many bacterial infections [80, 163], we were surprised to see that neutralizing IL-18 in the intestines following alcohol and burn injury did not worsen microbial dysbiosis (**Figure 21**). A recent study by Thinwa et al. demonstrated that human Caco-2 intestinal epithelial cells infected with the Enterobacteriaceae family member *Yersinia enterocolitica* used integrins as pathogen recognition receptors [164]. While the status of integrins in epithelial cells in our mouse model of alcohol and burn injury have not yet been explored, it is not unreasonable to hypothesize that this may be one mechanism by which IL-18 expression is induced.

There have been several recent studies examining the role of the IL-22/IL-18 axis in the context of intestinal infection in mice [80, 121, 127, 165]. Although our model of alcohol and burn injury explores the relationship between IL-22 and IL-18 in an acute setting (24 hours following injury), our data corroborate with several of these recent findings. Studies from Heimesaat *et al.* suggest that IL-22 and IL-18 mount differential responses to *Campylobacter jejuni* infection [121], and showed that IL-22 knockout mice were more susceptible to *E. coli* infection than their IL-18 knockout counterparts. A similar study by Munoz et al. explored the

relationship between IL-18 and IL-22 in different mouse models of infection including *Toxoplasma gondii* and *Citrobacter rodentium* [80]. Their results demonstrated that IL-22 augmented *Il18* mRNA and pro-IL-18 expression following infection with either of *T. gondii* or *C. rodentium*. Interestingly, the authors also demonstrated that IL-18 was responsible for the up-regulation of IL-22 in *T. gondii* infection, a pathogen that has been suggested by others to generate a pro-inflammatory response from IL-22 as opposed to a protective role. Together, the present findings and previous literature suggest that the relationship between IL-22 and IL-18 is complex and likely depends on the intestinal microenvironment.

Finally, to explore possible cooperative signaling pathways between IL-22 and IL-18, we utilized a Jak/STAT signaling pathway microarray (**Figure 27**). It has been established that the main downstream signaling of the IL-22 receptor occurs through STAT3, however, IL-18 signaling is more complex. One study found in a rodent model of acute graft-versus-host disease (aGVHD) that treatment with αIL-18 antibodies led to reduced epithelial cell apoptosis, which was correlated with lower levels of Fas and FasL, as well as lower p38MAPK [166]. In similar models, the role of IL-22 is more controversial [144, 167]. While some studies have shown that IL-22 exacerbates inflammation in aGVHD via hyperactivation of STAT1 and CXCL10 [144], others have shown that IL-22 protects the intestine by specifically signaling in Lgr5 stem cells to induce intestinal barrier regeneration in mice [167]. Interestingly, IL-18 and the inflammasome have been shown to directly regulate IL-22 activity by inhibiting IL-22BP expression in DCs. While we have not observed changes in IL-22BP in circulation, it is possible that local expression of IL-22BP in the intestines is affected by high levels of IL-18 following alcohol and burn injury. This may serve as an additional explanation for the cooperative role between αIL-18 antibodies and recombinant IL-22 treatment in our model.

Collectively, the current study demonstrates that IL-22 and IL-18 appear to have independent roles in the context of the intestinal barrier following acute alcohol and burn injury. In spite of these divergent roles, there does appear to be a connection between IL-22 and IL-18 as the combined treatment produces more efficacious protection to the intestine. While the exact mechanism(s) that IL-22 and αIL-18 antibodies use to promote intestinal barrier protection are yet to be fully illuminated, but it appears that the Jak/STAT signaling pathway plays at least a partial role. Additionally, changes in the microbiome following the combined injury remain a likely contributor to changes in these molecules and their responses following alcohol and burn injury, and future studies will aim to examine those relationships. Our findings illuminate the possible efficacy of a combined αIL-18/IL-22 dual treatment option, which may provide broader protection than αIL-18 or IL-22 treatment alone.

Limitations.

We see several major limitations to our current studies. First is the inability to obtain patient tissue samples. While we would benefit greatly from studying the effects of alcohol and burn injury on human intestine samples, there is no reason for physicians to take intestinal biopsies, or even perform non-invasive procedures such as colonoscopies following burn injury. The closest we are currently able to get to directly studying the effects of alcohol and/or burn injury on the intestines is through patient fecal samples. Fecal samples are not insignificant, and previous work from out laboratory provided translation validation to our model by demonstrating changes to the mouse microbiome mimic those in humans following burn injury [100]. Hopefully, future imaging technologies will allow us a direct glimpse into how the human gastrointestinal tract, and other organs, are affects following alcohol and burn injury.

In addition to the lack of patient samples, a major limitation to the progress of our understanding how the microbiome may influence the intestinal epithelial barrier and/or intestinal immune cells. The role of the microbiome, and the ability to understand the contributions of individual species of bacteria to disease has largely been feasible due to experiments carried out in germ-free facilities. Another potential experiment to study the interaction between microbiome and gut pathophysiology is to perform fecal transplant from mice following alcohol and burn injury into naïve germ free mice to see if intestinal pathology would manifest in the absence of burn injury (i.e. just due to changes in the microbiome alone). Additionally, colonizing mice with a single species of bacteria, or a labeled species of bacteria would allow us to not only tease apart the influence of specific bacterial species following acute trauma, but also monitor the translocation of microbes from the gut and how that may directly influence other organs. We hope to establish collaborations in the future that may make performing these types of experiments possible in our model.

We also would like to acknowledge that some of the experiments in this dissertation (specifically in chapter 5) have only been performed once. While we have carried out these experiments with an acceptable number of animals to run our statistical analyses, we are currently repeating these studies to verify our results before submitting these data to peer-reviewed journals.

Finally, we would like to address the use of male mice in our studies. We recognize that by only using male mice, we are missing how males and females may respond differently to alcohol and burn injury. The largest reason for the absence of female mice from our studies is due to the need to align the estrous cycles of all the female animals involved in the study. Estrogen levels likely influence the post-burn response, and this is a mechanism that should be

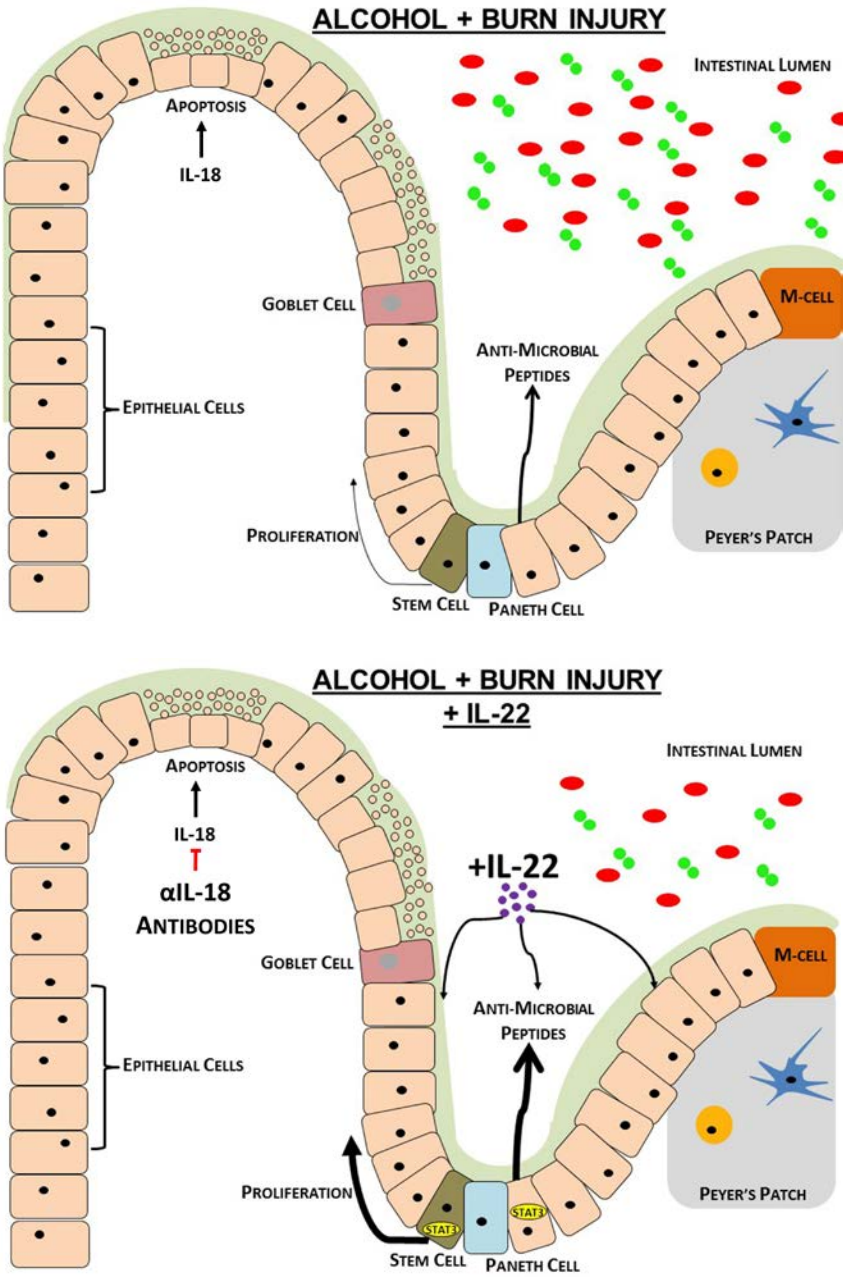

Figure 22. Schematic of major findings following alcohol and burn injury with and without IL-22 and αIL-18 antibody treatment. The combined injury leads to many changes in the small intestines including increased apoptosis and decreased proliferation of epithelial cells, elevated AMP gene expression, and bacterial overgrowth in the lumen (top panel). Treatment with IL-22 (purple), leads to downstream activation of STAT3 (yellow) in IECs, which results in intestinal barrier protection through further increasing AMP expression, reducing microbial dysbiosis, and promoting IEC proliferation to regenerate the epithelial barrier following injury (bottom panel).

studied in future work. Several studies have demonstrated that the post-burn response differs in men versus women [168-170]. New guidelines from the National Institutes of Health requiring both males and females to be used in experimental studies will allow for our laboratory to begin work on this mechanism.

Final Conclusions.

An exciting prospect of our studies is the new questions they have brought to light. First, we are still unclear on the exact cellular source of IL-22 observed in circulation following alcohol and burn injury. We believe that the source of IL-22 does not come from B or T cells, as data from preliminary studies performed in Rag1 knockout mice showed similar levels of circulating IL-22 as wildtype mice. Identifying this cell population will provide insight into a population of immune cells that are activated following the combined injury, and that may be specifically targeted to aid in intestinal barrier protection. Interestingly, only ~70% of mice receiving the combined injury display elevated levels of IL-22 in circulation. The reason for elevated IL-22 in circulation of some animals following combined injury also remains unknown.

Next, we have just begun to scratch the surface of studying the microbiome following alcohol and burn injury. While we observe massive increases in Enterobacteriaceae following the combined injury, the most pressing question is if these changes are physiologically relevant. While we predict that these Gram-negative microbes contribute to intestinal and perhaps systemic inflammation following injury, the direct relationship remains to be established. Finally, elucidating the precise effects of IL-22 treatment remains a pressing question in our model. While we have demonstrated that AMP transcript levels increase, we have not identified if AMPs are actually translated into protein, and furthermore, which of these AMPs actually regulate the microbiome following injury. Additionally, we would like to identify the effects of

alcohol and burn injury on Paneth cells and stem cells within the crypts, as both these cell populations have been shown to be influenced by IL-22. These questions, and many others, are being addressed in our ongoing studies.

Together, the current studies have bridged a significant gap in our current understanding of the effects of post-burn pathophysiology on the intestines (**Figure 22**). Our work has shed light onto the cellular and molecular alterations to the intestinal epithelial barrier following the combined injury, as well as begun to illuminate the significant dysbiosis that occurs within the microbiome. Additionally, our work has provided a potential therapeutic outlet using IL-22 and αIL-18 antibodies that showed has showed pre-clincial efficacy in our mouse model. Finally, we have explored the role of the Jak/STAT signaling pathway in intestinal epithelial cells following alcohol and burn injury using transgenic VillinCre STAT3 knockout mice to show the importance of this pathway in protection from gut barrier disruptions and dysbiosis. Future studies will certainly build on the foundation of this work, and will further contribute to our understanding of how alcohol exacerbates post-burn pathogenesis.

APPENDIX A

SUPPLEMENTAL FIGURES

To confirm knockout of STAT3 in intestine epithelial cell of transgenic mice, small intestine epithelial cells were isolated from wildtype and VillinCre STAT3$^{-/-}$ mice as described in Appendix B. Protein was extracted from IECs and analyzed by Western blot for STAT3 and β-actin expression. Our results showed that STAT3 expression is nearly absent in the IECs of our transgenic VillinCre STAT3-/- mice compared to our wildtype controls, confirming our transgenic mice did not express STAT3 in their IECs (**Figure 23**).

To examine the phosphorylation status of STAT3 following the combined injury, we isolated protein from small intestine IECs and analyzed phosphorylated STAT3 (pSTAT3) levels by Western blot (**Figure 24**). Our results show that pSTAT3 levels are increased following alcohol and burn injury compared to sham vehicle animals. We also wanted to confirm that pSTAT3 was actually activated and binding to its promoter. To address this, we performed a TransAM STAT3 activation assay, in which oligonucleotides containing binding motifs are bound to a 96-well plate and co-incubated with our IEC protein isolates containing pSTAT3 (**Figure 25**). Our results show that STAT3 has higher DNA-binding activity following alcohol and burn injury than in sham vehicle animals. Together, these data suggest higher STAT3 activation in small IECs following alcohol and burn injury.

We wanted to determine if the elevated levels of IL-22 in circulation were biologically active. To address this question, we performed an in vitro experiment using young adult mouse colon (YAMC) cells co-cultured with serum harvested from our experimental animals (**Figure 26**). YAMC cultures were incubated with serum or unsupplemented RMPI (negative control) for 30 minutes, at which time the cell were lysed an protein was isolated. Protein lystates were then analyzed by Western blot for pSTAT3 levels. We found that cells cultured with serum from animals receiving burn ethanol treatment had slightly elevated levels of pSTAT3 relative to sham

vehicle controls. However, mice that received both the combined injury and IL-22 treatment had significantly elevated levels of pSTAT3. These data suggest that IL-22 given to mice receiving alcohol and burn injury is biologically active. The next set of experiments will be performed using neutralizing IL-22 antibodies to confirm that phosphorylation of STAT3 is due to IL-22, and is not due other cytokines or signaling molecules in circulation.

We wanted to examine potential signaling pathways that IL-22, αIL-18, and the combined treatment may be using to protect the intestinal barrier following alcohol and burn injury. We ran a microarray of Jak/STAT pathway genes on small intestine epithelial cells as IL-22 and IL-18 have been shown to signal through the Jak/STAT pathway (**Figure 27**). Data generated from the array show that Myc and SOCS3 genes appear to be upregulated in animals receiving the combined treatment compared to those receiving injury alone, suggesting that these genes may be protective. Interestingly, more genes involved in apoptosis and cell growth were upregulated in mice receiving injury alone than mice receiving injury and treatment.

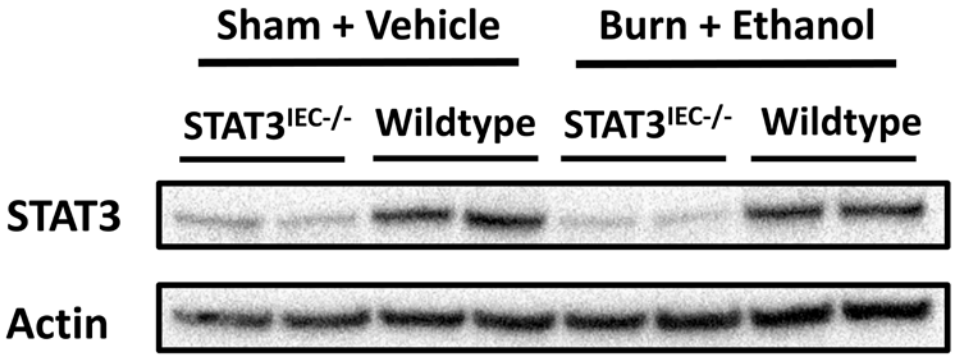

Figure 23. VillinCre STAT3$^{-/-}$ mice do not express STAT3 in small intestine epithelial cells. Intestinal epithelial cells isolated from the small intestine of sham vehicle and burn ethanol mice were probed for total STAT3 protein expression by Western blot.

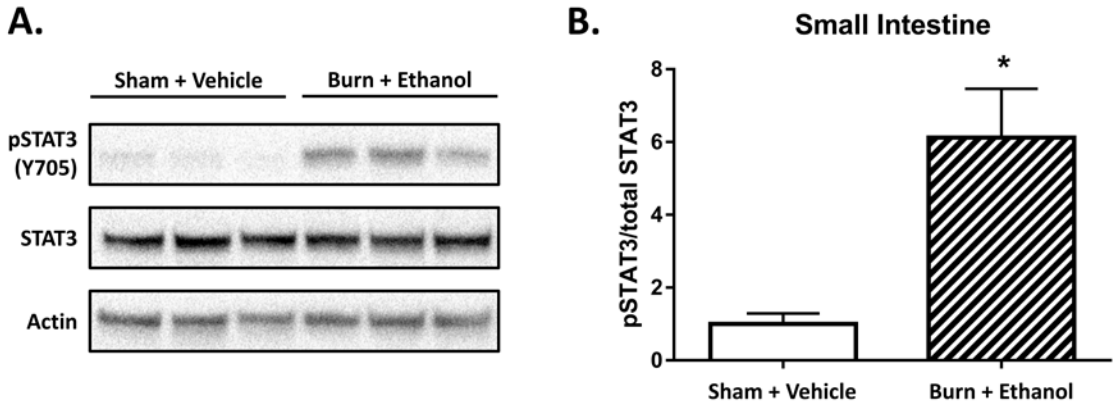

Figure 24. Small intestine pSTAT3 elevated following burn ethanol injury. Protein isolated total small intestine tissue was probed for STAT3 and pSTAT3 (Y705) by Western blot (A). Densiometric analysis was performed to express the ratio of pSTAT/STAT3 (B).

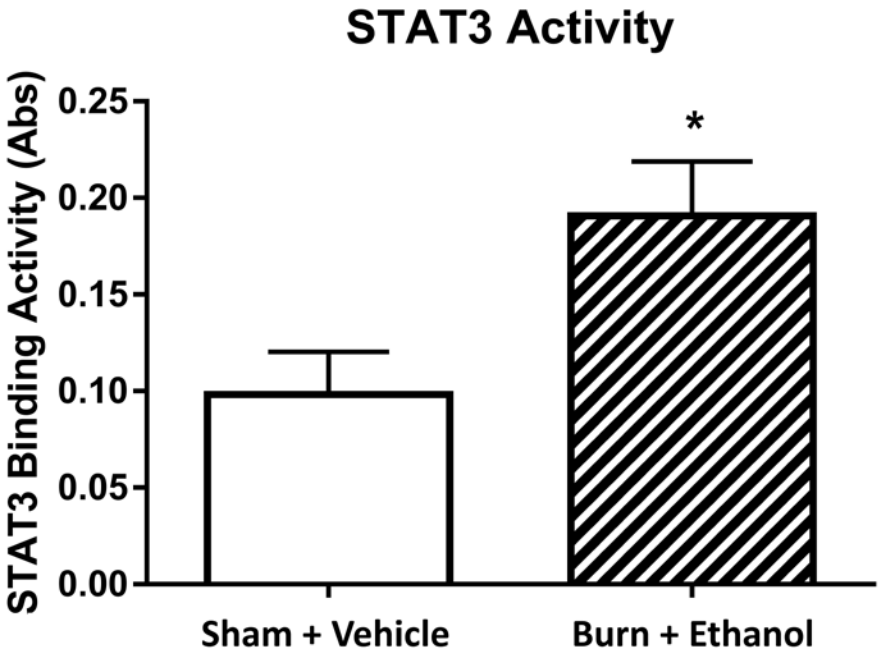

Figure 25. STAT3 from small intestine is biologically active following combined burn ethanol injury. Protein isolated from total small intestine tissue was analyzed by a TransAM STAT3 DNA-binding ELISA. Values are expressed as absorbance values normalized to the total amount of protein loaded.

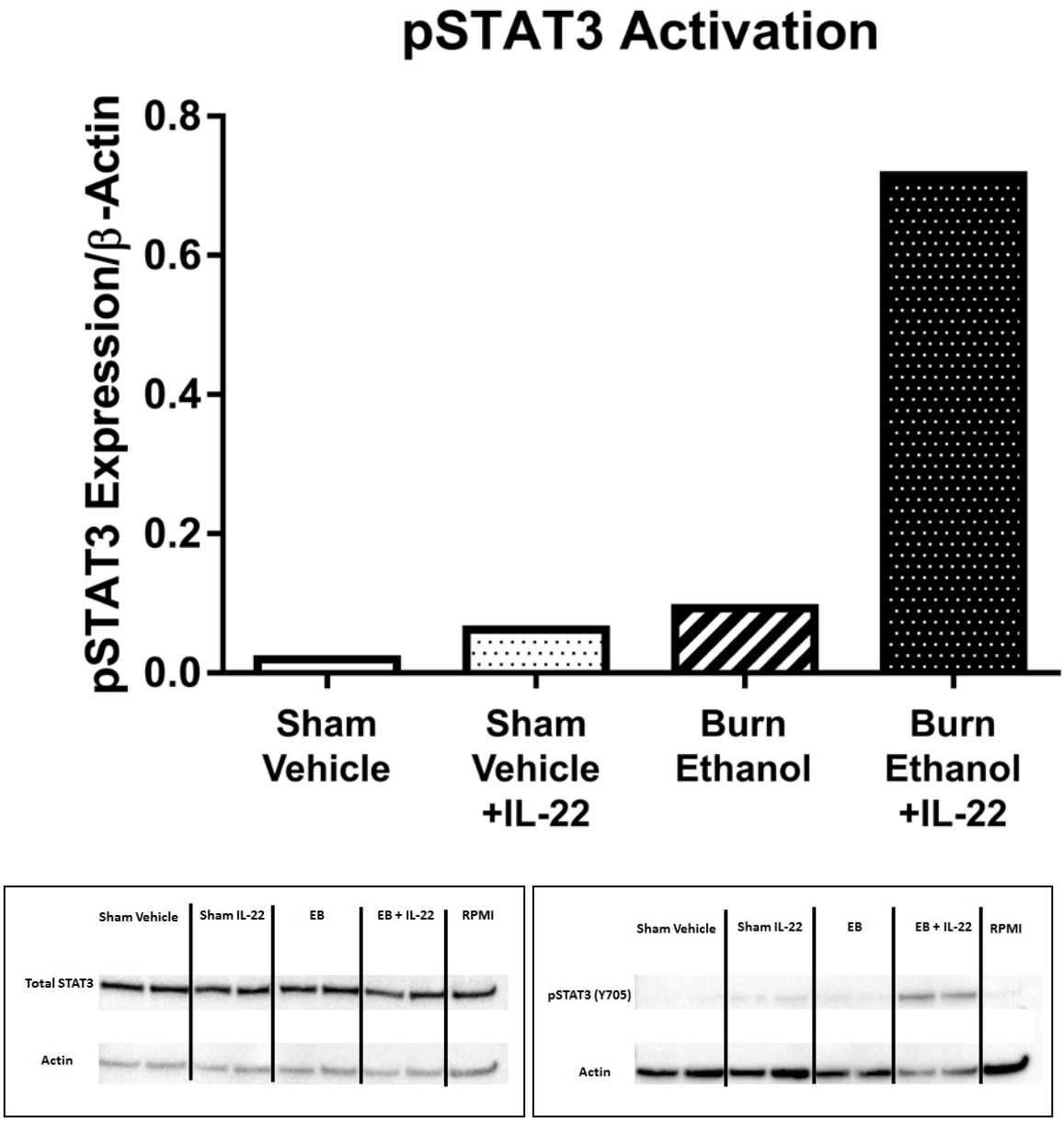

Figure 26. Elevated pSTAT3 in YAMC cells cultured with serum from mice receiving IL-22 and combined burn ethanol injury. Young adult mouse colon (YAMC) cells were cultured in serum from each experimental group. Protein was isolated and pSTAT3 levels were analyzed by Western blot.

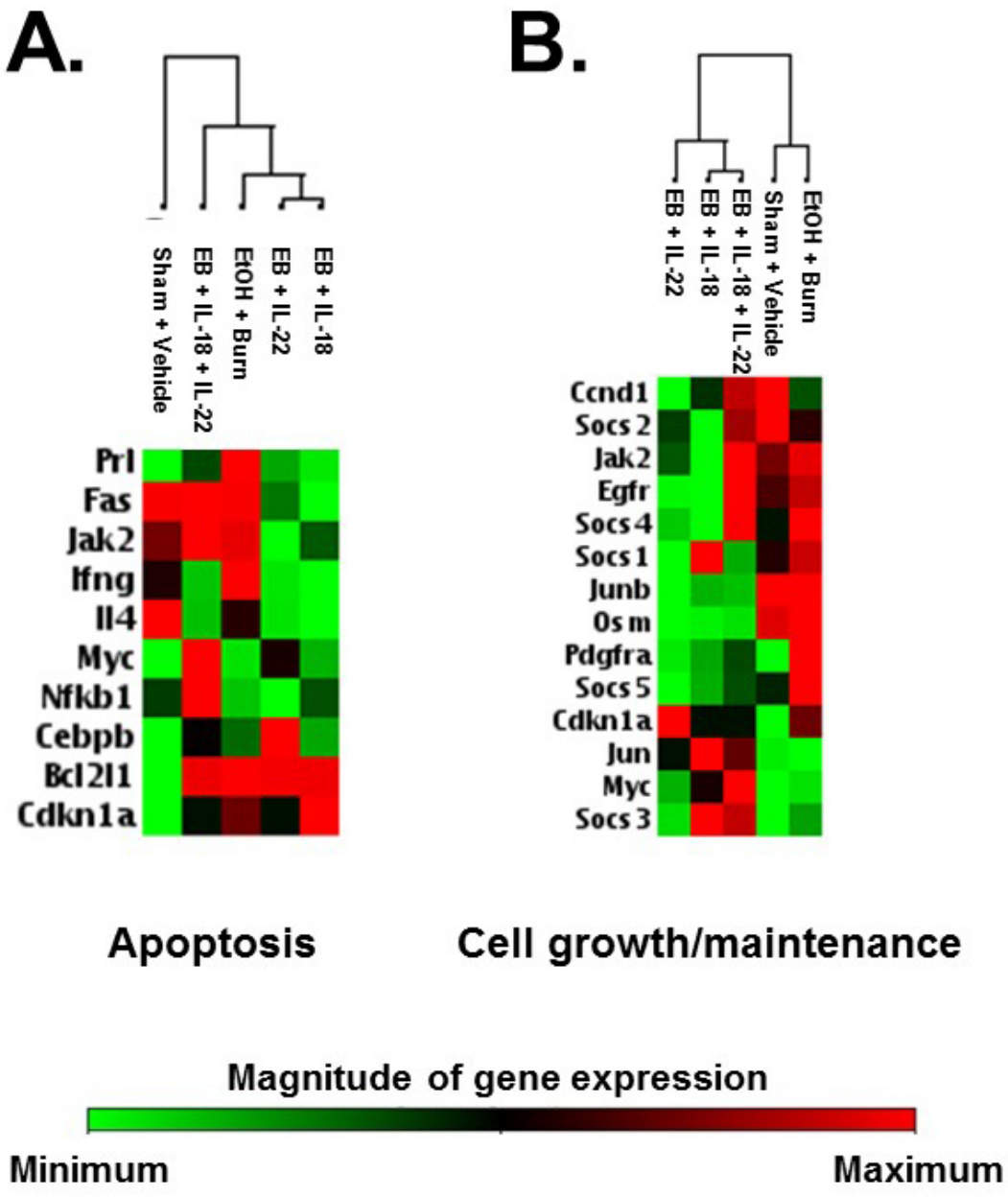

Figure 27. Jak/STAT microarray gene analysis of small intestinal epithelial cells. RNA was isolated from small intestine epithelial cells and analyzed for A) apoptosis and B) cell growth gene expression following alcohol and burn injury with or without treatment. (n = 3-6 per group.) EB = ethanol burn, EtOH = ethanol

APPENDIX B

DETAILED MATERIALS AND METHODS

Intoxication and Burn Injury Protocol.

Materials.

2 Water baths

Thermometer

Timer

Absorbent pads

Burn Injury Template (~12.5% surface area for 25g mouse)

1 mL syringes with 27 gauge needles

3 mL syringes with 27 gauge needles

1 mL syringe with 20 oral gavage needle

Ketamine

Xylazine

Ethanol

Sterile Water

Heating pads

Hair clippers

Scale

0.9% normal saline (sterile)

Protocol.

1. Fill water baths and heat one to 89C and the other to 37C

2. Prepare 25% ethanol solution in sterile water

3. Tail mark mice and gavage “ethanol” mice with 400ul of 25% ethanol solution. Gavage “vehicle” mice with 400ul of sterile water.

4. After four hours, weigh the animals and place cages on warmed heating pads.

5. Anesthetize mice by intraperitoneal injection with cocktail of 80 mg/kg ketamine hydrochloride and 1.2 mg/kg xylazine cocktail.

6. Once mice are asleep, shave the dorsal surface.

7. Place mice one at a time in burn template, ensuring dorsal surface is exposed through the template.

8. Administer burn injury by gentle lowering burn template with mouse into 87C water bath so that only the dorsal surface of the mouse is contacting the water.

9. After 7 seconds, remove mouse from water bath, gently remove mouse from template and dry dorsal surface on absorbent pad to prevent further scalding.

10. Immediately, give mouse 1mL pre-warmed normal saline resuscitation by intraperitoneal injection.

11. Return mouse to cage and monitor regularly for 3-4 hours. Cages can be returned to mouse room one mice have recovered fully from anesthesia and are ambulating.

12. Repeat steps 7-11 with sham animals but using the 37C water bath.

Intestinal Epithelial Cell Isolation.

Materials.

50 mL conical tubes

Vortexer

100um filters

37C incubator capable of rotation up to 250 RPM

Petri dishes

Forceps

Pre-chilled centrifuge to 4C

Pre-chilled sterile 1X PBS

Hemocytometer and microscope

PBS + antibiotics solution:

1X PBS (sterile), 500ml

Pen-Strp,5ml

Gentamycin (50mg/ml), 500µl

Pre-digestion solution:

1X HBSS (without $Ca2^{+}$ and Mg^{2+}), 500ml

FBS (Heat inactivated), 25ml

Gentamicin (50mg/ml), 500µl

HEPES (1M), 5ml

100X Penicillin/Streptomycin cocktail, 5ml

5mM EDTA

1 mM DTT

Protocol.

1. Make pre-digestion solution in sterile container and warm to 37C in water bath, grab a tub of ice.
2. Pre-chill centrifuge to 4C
3. Collect intestine without fat and Peyer's patches, open the intestine longitudinally and cut into 4-5 cm pieces.
4. Put intestine sections in 20ml ice cold PBS + antibiotics in 50ml tube on ice
5. Vortex for 10 seconds

6. Wash the intestinal pieces 2x in ice-cold PBS + antibiotics (by dumping into petri dish and washing with forceps by agitation).
7. Add 10 ml of prewarmed predigestion solution in 50 ml tube and incubate for 20 min at 37^0C under slow rotation (250 rpm)
8. Vortex for 10s. The epithelial cells are distrupted from the mucosa.
9. Draw up as much of the 10ml predigestion supernatant containing epithelial cells from the 50ml tube, leaving the tissue in the tube. Dispense epithelial cell supernatant through a 100um filter into new 50ml conical tube and store on ice. The flow-through contains epithelial cells and intraepithelial lymphocytes (IELs).
10. Repeat step 6-7 once with the same tissue and same filter.
11. Centrifuge tubes at 1500 rpm (300 x g) for 10 minutes at 4C
12. Wash cells with 10ml cold 1X PBS and repeat step 12
13. Resuspend cells in 1-5ml 1X PBS (depending upon pellet size)
14. Count cells on a hemocytometer using trypan blue
15. Epithelial cells are ready for use in downstream experiments

16S Bacterial qRT-PCR.

Materials.

96-well PCR Plate

Bacterial Standards

SYBR Green PCR Master Mix

Molecular grade PCR Water

Forward and Reverse Bacterial Primers

Nanodrop

Real-Time PCR Thermal Cycler

Centrifuge

Protocol.

1. Prepare standards, the first gDNA standard has to be at a concentration of 4.17 ng/µl, and then do a 1:10 serial dilution to get a total of 7 standards.
2. Prepare forward and reverse primers to a final concentration of 3 µM in RNase/DNase-free water.
3. Prepare MasterMix. Per reaction, you need 10 µl of SybrGreen and 2 µl of both forward and reverse primers.
4. Pippette 14 µl of MasterMix per well.
5. Add 6 µl of DNA or H_2O (water for the negative control). Standards and the negative control are done in triplicates, and samples are done in duplicates.
6. Spin the plate down for 1min at 2000 rpm, and setup the machine.
7. Make sure that the experiment properties are correct (Quantitation standard curve, SybrGreen, and is set for the standard run—2 hours). Under “Plate setup,” and “Assign Targets and Samples,” set your standards. To change the standards, click “Define and Set up standards,” and fill out the pop-up window. Enter the starting quantity number. Under “Run Method,” depending on which standard you are using, you will have a different annealing temperature. Change this temperature accordingly. Data collection should be on in the last step of the “Cycling stage,” and the last step of the “Melt Curve stage.”
8. Analyze your data using your standard curve.

Bacterial Fluorescent in situ Hybridization (FISH).

Materials.

10% Formalin Buffered Saline

Tissue cassette

Universal bacterial probe EUB338: Alexa 555 5'-GCTGCCTCCCGTAGGAGT -3'

Enterobacteriaceae probe ENTBAC 183: Alexa 488 5'-CTCTTTGGTCTTGCGACG -3'

Incubator capable of maintaining stable 50°C temperature

Coplin jar

Humidity chamber

Hybridization Buffer (.9M NaCl, 20mMTris-HCL, pH 7.5, .1% SDS)

Xylenes

Ethanol (100%)

Prolong Gold Anti-fade DAPI mounting reagent

Slide cover slips

Protocol.

1. Save a 1cm section of intestine in a tissue cassette immersed in 10% Formalin-buffered saline from animal experiment, obtain 5μm paraffin-embedded tissue sections from Loyola Tissue Processing Core or other tissue processing facility.
2. Deparaffinze slides by running them through 4x 3min incubations in xylene and 4x 3min incubations in absolute ethanol.
3. Dry slides for 25min at 50°C.
4. Dilute probes of interest to a final concentration of 1ng/μl in hybridization buffer (.9M NaCl, 20mMTris-HCL, pH 7.5, .1% SDS).
5. Pipette 500μl of probe in hybridization buffer on slides and incubate at 16hr or overnight at 50°C

in the dark inside a Tupperware container with moist paper towels.

6. Wash slides 3x for 15min in prewarmed wash buffer (.9M NaCl, 20mMTris-HCL, pH 7.5, 0.1% SDS) at 50°C.
7. Air-dry the slides and mount slides using ProLong Gold Antifade Reagent with DAPI. 7.) Image using a Zeiss Axiovert 200m fluorescent microscope at 200X total magnification.

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VITA

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