Role of the Intestinal Microbiota in Gut Barrier Dysfunction Following Burn Injury

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LOYOLA UNIVERSITY CHICAGO

ROLE OF THE INTESTINAL MICROBIOTA IN GUT BARRIER DYSFUNCTION FOLLOWING BURN INJURY

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

PROGRAM IN INTEGRATIVE CELL BIOLOGY

BY
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CHICAGO, IL
AUGUST 2014
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<tr>
<td>AMP</td>
<td>Antimicrobial peptide</td>
</tr>
<tr>
<td>MLN</td>
<td>Mesenteric lymph nodes</td>
</tr>
<tr>
<td>IEC</td>
<td>Intestinal epithelial cell</td>
</tr>
<tr>
<td>MODS</td>
<td>Multiple organ dysfunction syndrome</td>
</tr>
<tr>
<td>MOF</td>
<td>Multiple organ failure</td>
</tr>
<tr>
<td>SIRS</td>
<td>Systemic inflammatory response syndrome</td>
</tr>
<tr>
<td>CARS</td>
<td>Counter anti-inflammatory response syndrome</td>
</tr>
<tr>
<td>TJ</td>
<td>Tight Junctions</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>REG</td>
<td>Regenerating</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>UC</td>
<td>Ulcerative colitis</td>
</tr>
<tr>
<td>CRS</td>
<td>Cryptdin related sequence</td>
</tr>
<tr>
<td>CRD</td>
<td>Carbohydrate recognition domain</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide oligomerization domain</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>-----------</td>
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<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>SCFA</td>
<td>Short chain fatty acid</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone Deacetylase</td>
</tr>
<tr>
<td>TSA</td>
<td>Tryptic Soy Agar</td>
</tr>
<tr>
<td>LGG</td>
<td><em>Lactobacillus rhamnosus GG</em></td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>C. leptum</td>
<td><em>Clostridium leptum</em></td>
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<td>C. coccoides</td>
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CHAPTER ONE

INTRODUCTION

Sepsis and multiple organ failure are the leading complications in patients who sustain serious burn injuries. 75% of all deaths are related to sepsis or infectious complications arising from the injury\(^1\). Following insult, there is an immediate systemic inflammatory response that spreads throughout the body and affects secondary organs\(^2\). In addition to the skin, there is reported inflammation in the lungs, liver, and intestines after burn injury\(^3\). This inflammation, coupled with a suppressed adaptive immune response, increases the risk of organ failure and septic infections. Bacterial infections may arise externally from the environment or internally from opportunistic pathogens that are members of the commensal microbial flora.

The gastrointestinal tract contains over 100 trillion microbes, termed the microbiota, that live symbiotically and provide numerous benefits for the host such as metabolism and *de novo* synthesis of nutrients, protection against pathogenic microbes, and immune development and function\(^4\). Under healthy homeostatic conditions, the gut functions as an epithelial barrier that segregates the commensal bacteria from host tissue and prevents inadvertent activation of an immune response. However, after trauma, this barrier may break down allowing for translocation of bacteria or endotoxin from the gut to extraintestinal sites, which can then lead to sepsis and organ failure\(^5\)\(^-\)\(^7\). The precise
mechanisms of post burn induced gut barrier breakdown and its role in the pathology of
infections and organ failure remain to be elucidated.

Since the immune system’s principal role is the recognition of self from non-self, the innate and adaptive immune system function in parallel to establish tolerance to the commensal microbes in the gut. This tolerance is maintained by certain protective bacterial species and broken by imbalances in bacterial communities and overgrowth of pathogenic microbes. The innate immune system directly regulates the intestinal microbiota and protects against pathogenic infections through the production of antimicrobial peptides (AMPs). AMPs are small endogenously expressed proteins produced largely by epithelial and immune cells to kill microbes. A few studies have suggested a protective function of AMPs following burn injury but none have investigated the role these proteins play in regulating the intestinal microbiota and gut barrier following burn injury. Furthermore, the bacterial diversity of the gut and its potential role in inducing intestinal inflammation and permeability following burn injury remain largely unexplored.

Understanding the mechanisms exploited by intestinal bacteria to cause post burn pathogenic complications may yield novel therapeutics for patients struggling with these conditions. Probiotic therapy, or introduction of protective gut bacteria, is revealing promising results in restoring the microbiome, reducing gut leakiness, and alleviating intestinal inflammation in colitis and other conditions, but has not yet been studied nor implemented as a post burn treatment. The studies performed herein identify burn-
induced alterations of the intestinal microbiota and increases of gut permeability and inflammation.

**Hypothesis**

Burn injury decreases the expression of antimicrobial peptides, which leads to alterations in the intestinal microbiota and contributes to an increase of intestinal permeability and inflammation.

**Specific Aim 1**

Determine whether burn injury decreases expression of AMPs and alters the intestinal microbiome of the small and large intestine.

**Rationale**

The antimicrobial peptides α-defensins and C-type lectins have been implicated in the establishment and regulation of the intestinal microbiota. Recent studies have shown that a reduction in α-defensins promote shifts in microbial communities and the overgrowth of pathogenic bacteria, which can lead to intestinal inflammation in Crohn’s disease \(^{12-14}\). C-type lectins are another class of antimicrobials which protect against intestinal inflammation and colitis by segregating the commensal bacteria from the intestinal epithelium. However, it is not known how burn injury changes the expression of these two antimicrobials in the small intestine. Therefore we will determine whether burn injury leads to a change in the expression of α-defensins and C-type lectins in the gut.
Next, we will determine whether changes in these classes of antimicrobials accompany shifts in the relative diversity of the intestinal microbiota. Previous research from our laboratory and others have shown increases in total and Gram-negative bacteria in the intestines and mesenteric lymph nodes (MLN) by culture following burn injury\textsuperscript{15}. This suggests that Gram-negative bacteria are able to outcompete the rest of the commensal bacteria and lead to potential phylogenetic shifts in the microbial flora. However, it is not known how the diversity of the microbiome is altered following burn injury. The healthy microbiota has been shown to possess immunomodulatory and immunostimulatory functions and expansion of opportunistic pathogenic bacteria may skew the immune response to promote increases of intestinal inflammation and permeability as is seen in chronic inflammatory diseases such as inflammatory bowel disease (IBD)\textsuperscript{16}. Thus, experiments will further determine how burn injury alters the diversity of the intestinal microbiota, with a focus on abundant bacterial phyla and specific groups that have anti-inflammatory or proinflammatory potential.

**Specific Aim 2**

Determine whether burn injury increases gut permeability and inflammation.

**Rationale**

Previous data has identified the gut as a cytokine-generating organ in response to burn injury but no studies have established a link between alterations in the microbial flora inducing intestinal inflammation following burn\textsuperscript{17}. Therefore experiments will determine which specific cytokines and chemokines change in the intestines and when
these changes occur following injury. In addition, we will determine whether these changes in inflammatory mediators accompany changes in specific groups of bacteria. Furthermore, the intestine is physiologically impermeable to the luminal bacteria, however, a breakdown in the epithelial barrier may allow for bacterial translocation to the MLN. Published data from our laboratory show increases of culturable bacteria in the MLN following burn injury, but these bacteria were not identified nor measured in the intestines. Thus, we will first determine whether burn increases gut leakiness and bacterial translocation and whether these bacteria that are present in the MLN are also increased in the small or large intestines. Hence, experiments in this aim will determine whether intestinal inflammation and permeability may be due to increases in pathogenic or pro-inflammatory groups of bacteria.

**Specific Aim 3**

Determine whether probiotic treatment of *Lactobacillus* *gg* restores the gut barrier following burn injury.

**Rationale**

Probiotic treatment has shown to reduce intestinal inflammation in various mouse models of colitis and may show some benefit in patient cohorts of inflammatory bowel disease. *Lactobacillus rhamnosus* GG is bacterial strain with anti-inflammatory properties that attaches to the intestinal epithelial cells and secretes proteins, p40 and p75, which reduce inflammatory cytokines, and increase expression of tight junction
proteins\textsuperscript{11,18,19}. Therefore experiments in this aim will determine whether in vivo treatment of \textit{Lactobacillus gg} probiotic reduces intestinal inflammation and leakiness following burn injury.
CHAPTER TWO

REVIEW OF LITERATURE

Burn Injury

Burn injury accounts for approximately half a million cases requiring medical treatment and 4,000 deaths reported annually each year\textsuperscript{20}. Of these deaths, sepsis and multiple organ dysfunction syndrome (MODS) remain the leading causes of mortality in burn related trauma\textsuperscript{5,21}. Following burn injury, patients suffer a global immune dysregulation characterized by a heightened innate proinflammatory response and subsequently followed by a compromised adaptive immune response\textsuperscript{5}. This innate response, termed systemic inflammatory response syndrome (SIRS), is marked by an increase of cytokines, chemokines, and acute phase proteins. The host response to injury induces a “cytokine” storm of TNF, IL-1, -6, and -8 as well as elevated levels of acute phase proteins such as serum amyloid A, and C-reactive protein\textsuperscript{22,23}. Ensuing, this initial response is a counter anti-inflammatory response (CARS) marked by decreased T cell proliferation, Th1 and Th17 cell responses, and antigen presentation\textsuperscript{2,3,6,7,23}. It is hypothesized that this disruption in immune homeostasis from injury predisposes susceptible individuals to opportunistic infections\textsuperscript{2}. These infections may be derived externally from the environment or internally from the indigenous commensal bacteria.

Burn Injury and Gut Barrier Dysfunction
Since the gut is a reservoir of an enormous amount of bacteria in the body, it has the potential to become a major clinical problem following burn related trauma increasing the susceptibility of patients to developing septic infections. In the context of the gut, burn injury may lead to a breakdown in the epithelial barrier characterized by increased apoptotic cell death and enhanced production of proinflammatory mediators allowing for the translocation of viable bacteria or endotoxin to extraintestinal sites. This barrier dysfunction may allow gut derived factors to travel into the lymphatic circulation to the mesenteric lymph nodes (MLN), liver, and lungs to produce systemic tissue damage and increase the risk for septic complications. Studies have shown that viable bacteria may be cultured from the MLN days after the injury suggesting prolonged alterations in intestinal permeability after burn. These observations led to the gut origin hypothesis of multiple organ dysfunction syndrome, which describe the gut or gut-derived factors as possible sources of sepsis or MODS.

Ischemia, reperfusion injury, and gut bacteria are all burn induced factors which can potentiate this barrier breakdown leading to increases of inflammation and permeability. Immediately following injury, there is a severe mesenteric vasoconstriction as blood is diverted from the intestines to the site of injury to aid in wound healing. This vasoconstriction, produces an ischemic environment for the gut, and this lack of oxygen, may lead to cell death and inflammation in the intestinal bed. On the other hand, the vigorous reperfusion and diversion of blood back to the intestines may augment this inflammation. Increases of inflammatory cytokines and chemokines have been reported
in the feces of burn patients. This inflammatory environment allows for the overgrowth of luminal intestinal bacteria. Although it not known how the diversity of the intestinal flora is altered following burn injury and the relative impact this has on the host, there are data suggesting the overgrowth of Gram-negative bacilli. Gram-negative infection and LPS are potent inducers of TNFα signaling and neutrophil recruitment, which can potentially lead to tissue damage, increased permeability in the intestines after burn, and septic shock.

The Intestinal Epithelial Barrier

The gut is an organ specialized for the digestion and absorption of nutrients. These processes are due in large part to the commensal bacteria which facilitate the digestion of complex plant polysaccharides and the synthesis of essential vitamins that humans are incapable of. In turn, our digestive tract provides a nutrient rich ecosystem that aids the development of complex bacterial communities. This coevolution of microbial mutualism accompanied the development of elaborate mechanisms to promote tolerance and to prevent our immune system from generating inflammatory responses against these bacteria. At the host microbial interface is a mucosal barrier which physically separates bacteria from the intestinal tissue. Specialized secretory epithelial cells, goblet cells, secrete mucus which contains hydrated glycoproteins, immunoglobulins, and antimicrobial peptides. The mucus layer of both the small and large intestine is composed of an outer and inner layer. Since mucin glycoproteins sterically hinder bacteria from attaching to the intestinal epithelial cells, bacteria are
usually found in the outer mucus layer and the inner layer which is relatively devoid of bacteria\textsuperscript{37}. There are some species that do however attach to the intestinal epithelial cells, and interaction of these bacteria with pattern recognition receptors, Toll-like receptors and Nod-like receptors, is necessary for homeostasis in the intestine\textsuperscript{38,39}. B cells in the lamina propria produce IgA which is transported and secreted into the intestinal lumen. This immunoglobulin binds antigens on commensal bacteria and prevents invasion from pathogenic microbes\textsuperscript{40}. Antimicrobial peptides are secreted from specialized epithelial cells, Paneth cells, located in the intestinal crypts. These proteins aid in spatial segregation, regulate the commensal flora, and protect against pathogenic infection.

The intestine is also a selectively permeable organ allowing for the bidirectional flow of nutrients and wastes but impermeable to the translocation of bacteria from the lumen to the lamina propria\textsuperscript{41}. This seal is maintained by tight junction proteins (TJ), which are transmembrane proteins composed of claudin and occludin, that prevent the paracellular transport of molecules. Furthermore, adherens junctions and desmosomes further assist in maintaining this cell-to-cell connectivity in the intestine.

Following injury or inflammation, this barrier may break down allowing for the invasion and translocation of commensal microbes into the epithelium. Certain members of the commensal flora can exploit this environment and become opportunistic pathogens and lead to infection and disease\textsuperscript{42}.

\textbf{Antimicrobial Peptides}
The innate immune system is directly involved in maintaining the tolerogenic mucosal and commensal bacterial interface in the gut through physical and chemical regulatory networks, both of which maintain gut permeability and prevent chronic inflammation by restricting contact of resident bacteria with intestinal epithelial cells. Antimicrobial peptides (AMPs) are proteins that are constitutively produced at mucosal surfaces by epithelial and lymphoid cells to regulate commensal bacteria and to protect against pathogenic microbial infections. These peptides are small proteins (12-50 amino acids) that possess an amphipathic structure and a net cationic charge. In addition to participating in direct bacterial killing, AMPs have other diverse functions such as chemotactic activity, and wound repair.

Defensins are a major family of AMPs, and the α-defensins are the most highly expressed antimicrobial in the human gastrointestinal tract. These proteins are produced mainly by Paneth cells, specialized secretory intestinal epithelial cells, which are found at the base of the crypts of Lieberkühn. In humans, two Paneth cell specific α-defensins have been identified, HD-5, and 6. Interestingly, mice express ~19 evolutionarily related proteins that are homologous to human α-defensins termed cryptdins or cryptdin-related sequence (CRS) peptides. α-defensins are constitutively expressed as inactive pro-peptides and proteolytically processed to the mature form by trypsin in humans and MMP-7 in mice, whereby they are secreted into the intestinal lumen. These AMPs possess a cationic charge and are electrostatically attracted to negatively-charged microbial membranes forming pores which lyse bacterial cells. This
class of AMPs has broad range microbicidal activity against both Gram-positive and Gram-negative bacteria as well as fungi such as: *Listeria monocytogenes*, *Escherichia coli*, *S. typhimurium* and *Candida albicans*.

Another key class of antimicrobials in the intestine is the regenerating (REG) family of C-type lectins, Reg3α or HIP/PAP in humans and Reg3γ in mice. These antimicrobials possess a carbohydrate recognition domain (CRD), which facilitates the binding of Reg3 to peptidoglycan present on Gram-positive bacteria. Once bound, Reg3 is able to form hexameric membrane-permeabilizing oligomeric pores which selectively lyse only Gram-positive bacteria. Similar to the α-defensins, these peptides are primarily produced by the Paneth cells of the small intestine, but in contrast, their production is induced by Toll-like receptor stimuli from the resident microbiota and inflammatory stimuli.

In the literature, both α-defensins and Reg3γ have been implicated in the establishment, regulation, and segregation of the healthy intestinal microbiota. Reduction or overexpression of these proteins can alter bacterial communities and intestinal homeostasis allowing for an increase in inflammation. It is hypothesized that Paneth cell dysfunction of antimicrobial release can lead to disease development such as inflammatory bowel diseases (IBD), Crohn’s disease and Ulcerative Colitis (UC), which are chronic inflammatory conditions of the gut. Furthermore, Crohn’s disease patients show a reduction of α-defensins in the ileum. This attenuation of α-defensin expression is linked to aberrant inflammasome signaling in Crohn’s patients with NOD2.
mutations\textsuperscript{54}. Since knockouts of $\alpha$-defensin genes are embryonic lethal, mouse models have been developed to study the role of $\alpha$-defensins in intestinal homeostasis by utilizing MMP-7\textsuperscript{+/−}, the protein involved in processing the mature secreted form of $\alpha$-defensins, and transgenic mice overexpressing human HD-5\textsuperscript{55}. Results from these experiments show that $\alpha$-defensins regulate the composition of the intestinal microbiota and reductions of these proteins have the potential to skew the microbiota toward a proinflammatory phenotype\textsuperscript{12}. Similar to $\alpha$-defensins, Reg3$\gamma$ is also involved in maintaining the microbiota\textsuperscript{56}. Hooper’s laboratory showed that the spatial segregation of the commensal bacteria from the intestine tissue is dependent on the expression of this antimicrobial. Reg3$\gamma$\textsuperscript{−/−} showed an altered microbiota composition and increased bacterial colonization of the intestinal epithelial surface\textsuperscript{50}.

The Intestinal Microbiota

All mammalian epithelial surfaces are colonized by a diverse consortium of microbes collectively known as the microbiota, and their genetic composition is termed the microbiome. In the human body, there are approximately 100 trillion microbial cells, which outnumber human cells 10 to 1 and contain 100 times as many genes as our own genome\textsuperscript{57}. Establishment of this microbiota occurs immediately following birth, and it is the temporal and spatial colonization of these microbes, which help shape our developing immune system. The vast majority of these symbiotic microbes, upwards of $\sim$1000 different bacterial species and 100 trillion organisms, inhabit the gastrointestinal tract and provide numerous physiological benefits for the host including metabolism of nutrients,
vitamin synthesis, colonization resistance, and immune development and function. Abnormal development or ecological disturbances of these microbial communities play critical roles in determining health and disease.

**Diversity of the Healthy Gut Microbiome**

As infants, the phylogenetic diversity and functional capacity of our intestinal microbiota drastically increases in the first three years of life. Succession of bacterial communities abruptly changes with the introduction of various life events such as breast-feeding, solid foods, and antibiotic use. After this three-year time point, the matured infant microbiome becomes more stable and resilient resembling the adult microbiome.

The healthy adult intestinal microbiota is dominated primarily by the Bacteroidetes and Firmicutes phyla. The Bacteroidetes are Gram-negative anaerobic bacteria, which play critical roles in the metabolism of complex plant polysaccharides and are divided primarily into the *Bacteroides* and *Prevotella* genera. The Firmicutes are the most diverse phylum in the gut and are a group of Gram-positive, low G+C content bacteria, which participate in the production of short chain fatty acids (SCFA) and lactic acid fermentation. This heterogeneous phylum is further divided into the Clostridia, Bacilli, and Mollicutes classes, with the anaerobic Clostridia comprising 95% of the total Firmicutes. The Clostridia are primarily of the Lachnospiraceae family, clusters IV (*Clostridium* genera) and XIVa (*Eubacterium* and *Ruminococcus* genera) and most species are producers of the SCFA butyrate. The remaining 5% of the Firmicutes are divided into the Mollicutes, and Bacilli classes (*Streptococcus*, and
Lactococcus genera). Lactobacillus is a genus of bacteria in the Bacilli class, which has potent anti-inflammatory properties and is used as a common probiotic. Collectively the Bacteroidetes and the Firmicutes comprise more than 90% of the bacteria in the gut. The rest of the intestinal microbiota is comprised of the Proteobacteria, Actinobacteria, Verrucomicrobia, Fusobacteria, and TM7 phyla along with a predominant archael phylotype, Methanobrevibacter smithii. The Proteobacteria are Gram-negative aerobic bacteria with a lipopolysaccharide containing outer membrane. Many members of this phylum are opportunistic pathogens and infectious organisms, which are seen in the γ-Proteobacteria class and the Enterobacteriaceae family (Escherichia, Klebsiella, Enterobacter, Proteus, Citrobacter, Salmonella, and Serratia genera). The Actinobacteria are Gram-positive bacteria containing the Bifidobacterium genera, another lactic acid producing taxa which is also used in probiotic supplementation. There is not much known about the composition, presence, and function of the other rare bacterial phyla in the gut. Many of these bacteria are unculturable and sequences of these phylotypes were discovered during 16s rDNA sequencing experiments. Collectively, the Verrucomicrobia, Fusobacteria, TM7, and other unidentified phyla comprise less than 1% of the healthy microbial flora.

Even though the presence of these bacteria has been identified in the healthy human gut, there is considerable variation in genera of the predominant phyla, Firmicutes and Bacteroidetes, between individuals. It is reported that among different persons, there are common core functional capacities but significant interindividual variations of
bacterial compositions allowing for the classification of three different enterotype
dominated genera: *Bacteroides*, *Prevotella*, or *Ruminococcus*\(^{64}\). However, it remains to
be shown the long-term impact these different enterotype dominated microbiotas impart
on the host’s health and immune function. Classification of the healthy intestinal
microbiome is clinically necessary for determining normal or abnormal states to elucidate
how altered microbiota composition may play a role in disease development and
progression.

**Microbiota and Immune Function**

The intestinal immune system encounters more antigens than any other part of the
body. Therefore, the recognition of “self” and “non-self” is critical to discriminate the
harmless commensal microbiota and food antigens from harmful pathogenic microbes.
This equilibrium in part is established by the balance of effector T cells—the amount and
function of anti-inflammatory regulatory T cells (Tregs) versus proinflammatory Th17
cells\(^{65}\). Tregs are a subset of CD4+, CD25+ helper T cells, which express the
transcription factor FOXP3, secrete IL-10, and maintain tolerance to self-antigens
protecting against autoimmune diseases\(^{66}\). Th17 cells are another subset of CD4+ cells
which secrete IL-17A, F, and IL-22 and protect against bacterial infections at mucosal
surfaces\(^{67}\). These cells cause inflammation and autoreactive Th17 cells have been
implicated in various autoimmune diseases: inflammatory bowel disease, arthritis, and
multiple sclerosis. Research has shown that these cells can differentiate and expand
locally in the gut and then spread systemically to other organs. Antigenic stimulation
from the intestinal microbiota shapes this balance of Treg/Th17 cells and determines homeostasis or inflammation. In the gut, M cells and dendritic cells constantly sample microbial antigens from the lumen. The dendritic cells traffic from lymphoid follicles in the lamina propria and Peyers patches to the mesenteric lymph nodes to present these antigens to naïve CD4+ Th0 cells. The presentation of specific antigens and production cytokines skews and directs the differentiation of Th17, and Tregs.

The composition of the intestinal microbiota facilitates the development of lymphoid organs and directs immune cell responses—the differentiation of adaptive immune cells and the production of effector cytokines. Studies using germ free mice, mice devoid of any microbes, reveal that these mice are more susceptible to colonization by pathogenic microbes, have small and undeveloped lymphoid organs, and show reductions in: CD4+ and CD8+ T cells, IgA secretion, and production of AMPs. Therefore the commensal microbes are essential for regulating immune physiology and the innate and adaptive immune systems. Current research is focused on identifying these mechanisms and microbial signals that direct immune development and function.

One specific commensal, Bacteroides fragilis produces an immunomodulatory molecule polysaccharide A (PSA) which directs lymphoid organogenesis, modulates the Th1 and Th2 balance, directs regulatory T cell development (Treg), and protects against models of intestinal inflammation. Mazmanian et al. showed that therapeutic treatment of PSA led to the production of anti-inflammatory IL-10 and alleviated intestinal inflammation in various models of IBD.
Segmented Filamentous Bacteria, or *Candidatus Arthromitus* a group of Gram-positive bacteria related to Clostridia that have not yet been cultured, have been shown to attach to small intestine epithelial cells and lead to the production of serum amyloid A (SAA). SAA then stimulates dendritic cells in the lamina propria to secrete IL-6 and IL-23 which promotes Th17 cell differentiation and maturation\(^74\). Littman’s laboratory showed that germ free mice have reductions of Th17 cells in the small intestine but could be restored when colonizing mice with feces taken from mono-colonized SFB mice\(^74\). Therefore colonization of SFB is necessary and sufficient to induce Th17 cell differentiation in the gut. Furthermore, they determined the specific membrane bound antigenic proteins of the SFB that direct Th17 production\(^75\). This bacterial group is also necessary for the secretion of IgA and Reg3\(\gamma\)\(^47\). Nevertheless, overgrowth of this bacteria may upset the Th17/Treg balance in favor of overactive Th17 cells that can potentially lead to autoimmune diseases: inflammatory bowel disease, arthritis, and multiple sclerosis\(^76,77\).

Indigenous *Clostridium* species particularly the spore forming bacteria of clusters IV and XIVa, *Clostridium leptum* and *coccoides* respectively, are sufficient to induce the development of Tregs in the colon\(^78\). Colonization of mice with a mix of *Clostridium* strains produced a TGF-\(\beta\) enriched environment, the cytokine necessary for FOXP3 activation and Treg cell differentiation, and led to Treg cell development in the colon but not the small intestine lamina propria. Probiotic therapy of 17 *Clostridium* strains protected against various models of intestinal inflammation and allergies\(^79\). These
Commensal bacteria ferment sugars to produce the SCFAs butyrate and propionate which act directly on T cells and dendritic cells to inhibit histone deacetylase (HDAC). Acetylation of the FOXP3 promoter CNS1 allows for the binding of FOXP3 and transcription of Treg dependent genes \(^{80}\).

**Microbiota and Disease**

A person’s intestinal microbiota is largely determined by diet, antibiotic use, hygiene, lifestyle, and genetics. Genetic or environment factors may lead to disturbances in bacterial communities, or a microbial dysbiosis. These microbial imbalances of the gut are seen in numerous disease states: obesity, inflammatory bowel disease, rheumatoid arthritis, multiple sclerosis, autism, allergies, diabetes, and others \(^{81}\). Furthermore, it is hypothesized that dysbiosis of the intestinal microbiota can lead to disease development and progression \(^{4}\). Different diets can leads to variations in intestinal bacteria. A “western” diet high in animal fat and protein has been seen to promote a *Bacteroides* dominated enterotype whereas a carbohydrate based diet facilitates a *Prevotella* dominated microbiome \(^{82}\). These diet induced changes in gut bacteria are suggested to play a role in obesity, which is seen by a reduction in the Bacteroidetes and an increase in the Firmicutes phyla allowing the individual to extract more energy from their food \(^{83}\). Antibiotic use is another example, which can alter the intestinal microbiome. A study that examined the changes of the intestinal microbiome in patients following ciprofloxacin treatment revealed that antibiotic use changed the diversity and species richness of every
patient, and although many communities of bacteria recovered by 4 weeks, there were some populations that did not recover up to 6 months after treatment. Therefore certain environmental factors can potentially cause permanent damage to the intestinal microbiome. This dysbiosis has the potential to change the antigenic variation of the intestinal microbiota leading to an overactive inflammatory response. It is hypothesized that when these disturbances occur in genetically susceptible individuals it has the potential to lead to inflammatory bowel disease. IBD patients have an altered microbial flora seen by a reduction in the Bacteroidetes and Firmicutes and overgrowth of the Proteobacteria and Actinobacteria phyla. More specifically, there are reductions in the Lachnospiraceae family, which contain Clostridium clusters IV and XIVa, Clostridium leptum and coccoides respectively relative to healthy controls. These protective bacteria produce the SCFA butyrate, a key nutrient for colonocytes and an important source for energy production. Butyrate and other SCFAs may enhance the epithelial barrier integrity. Furthermore, a reduction in Faecalibacterium prausnitzii a specific species in Clostridium cluster IV, leads to a higher risk of postoperative surgical recurrence in IBD patients. F. Prausnitzii secretes specific factors, which block NF-κB activation and increase the secretion of anti-inflammatory IL-10 in the colon. In addition to reductions of protective bacteria there are overgrowths of potentially harmful bacteria termed pathobionts. Increases of the γ-Proteobacteria specifically the Enterobacteriaceae family are seen concurrently and in relapsing IBD. Specific species of the Enterobacteriaceae are sufficient to induce colitis in animal models such as, adherent
E. coli, Klebsiella pneumonia, and Proteus mirabilis. Furthermore, there are also increases of minor phyla of bacteria such as Actinobacteria and TM7, which are also suggested to be colitogenic.

**Probiotic Treatment**

Restoring the healthy microbiota through probiotic treatment is a potential therapeutic intervention that may protect against disease development and progression by modulating the immune system. Probiotics are live beneficial bacteria that colonize the gut and exert immunological protections through the production of cell surface antigens or secreted proteins. Lactobacilli, Bifidobacteria, E.coli Nissle 1917, Faecalibacterium prausnitzii, Bacteroides fragilis, and Bacteroides thetaiotaomicron have all been shown to be beneficial groups or strains of bacteria. Recent research is focused on identifying the protective mechanisms of these bacteria so that they can be combined as cocktails and used as treatments to restore the intestinal microbiota and immune function.

Various probiotic strains or combinations have shown to be protective in models of inflammation, infection, and disease. Bacteroides fragilis has been shown to alleviate intestinal inflammation and restore gut permeability protecting against inflammation in various models of colitis and autism. E. coli Nissle 1917 has been shown to increase tight junction protein levels, human beta-defensin 2 production (hBD-2), and maintain remission in ulcerative colitis. VSL #3 is a high concentration prescription formulation of 8 bacterial strains consisting of Lactobacilli, Bifidobacteria, and Streptococci which is FDA approved for ulcerative colitis, ileal pouch, and irritable
bowel syndrome. This combination of bacteria has been shown to induce IL-10 production and TGFβ expressing T cells and is effective in achieving remission in ulcerative colitis patients.

Lactobacilli are potent protective bacteria that are necessary for immune homeostasis in the gut. One specific strain, *Lactobacillus rhamnosus GG* (LGG), has been shown to be protective against intestinal inflammation in models of colitis. This bacteria encodes a SpaC pillin which facilitates its colonization by adhering to mucin glycoproteins. In addition, these pilli interact with TLR2 and play a role in balancing IL-8 secretion by intestinal epithelial cells. It has also been shown that LGG secretes soluble proteins, p40 and p75, which act on intestinal epithelial cells to inhibit TNF induced apoptosis. Furthermore, research suggests that LGG may protect against pathogenic infections such as *E.coli O157:H7* and *C. difficile*. 
CHAPTER THREE
MATERIALS AND METHODS

Animals

Male C57BL/6 mice, 8-9 week old, weighing 22-25g, were obtained from Charles River Laboratories are used in all experiments. Animals were allowed to acclimate to the facility for 7-10 days before being used for the experiments. All experiments were conducted in accordance with the guidelines set forth by the Animal Welfare Act and were approved by the Institution Animal Care and Use Committee at the Loyola University Chicago Health Sciences Division.

Burn Injury Procedure

Mice were anesthetized with xylazine and ketamine, their dorsal surface shaved, and placed in a template exposing ~20% total body surface area (TBSA) as calculated by the Meeh formula \(^{107}\). The mice divided into two treatment groups, those receiving burn injuries or sham injuries. The burn group were then submerged in a water bath set to 85-95°C for 7-9 seconds while the sham group were submerged in a water bath set to 37°C. Following burn or sham burn, all animals were resuscitated with 1ml of saline. This procedure models a severe ~20% TBSA full thickness third degree burn. The animals were sacrificed on days 1, 3, and 5 following injury, and intestine tissue and luminal contents as well as mesenteric lymph nodes were harvested for downstream experiments.
Histology

Small, 3-5mm sections of tissue were taken from the ileocecal wall and placed immediately in cold Carnoy solution and left to incubate overnight. Paraffin blacks were prepared by Loyola University Medical Center Tissue Processing Core, 5μm sections were cut, and 1 slide from each animal was H&E stained for tissue pathology. The procedure for fluorescent in-situ hybridization staining was performed as described previously with minor adjustments\textsuperscript{108}. Slides were deparaffinized by running them through 4x 3min incubations in xylene and 4x 3min incubations in absolute ethanol. Next, the slides were dried for 25min at 50°C. The indicated probes were diluted to a final concentration of 1ng/μl in hybridization buffer (.9M NaCl, 20mMTris-HCL, pH 7.5, .1% SDS)\textsuperscript{109}. 500μl of probe in hybridization buffer was placed on the slides and left to incubate at 16hr or overnight at 50°C in the dark inside a Tupperware container with moist paper towels. The probe sequences were as follows and purchased from Invitrogen\textsuperscript{12,53,93,109,110}:

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

Enterobacteriaceae probe ENTBAC 183:
Alexa 488 5’-CTCTTGTGTCCTGGACG -3’

SFB probe, SFB1008:
Alexa 488 5’-GCGAGCTTTCCCTATTACAAGG-3’
Following the incubation, the slides were washed 3x for 15min in prewarmed wash buffer (.9M NaCl, 20mMTris-HCL, pH 7.5, .1% SDS) at 50°C. The slides were air dried, mounted, and counterstained using ProLong Gold Antifade Reagent with DAPI (Molecular Probes). The sections were imaged using a Zeiss Axiovert 200m fluorescent microscope and images were processed using Axiovision software.

**FITC-dextran assay**

One day after the aforementioned burn or sham injury procedure the mice were gavaged with .4ml of 22mg/ml FITC-dextran in PBS. After 3 hours blood was drawn and the mice were sacrificed. The blood was centrifuged for 8000rpm for 5min at 4°C, plasma isolated, and read spectrophotometrically at 480nm excitation and 520nm emission wavelengths.

**Cytokine quantification**

The distal 5cm of the small intestine and the whole large intestine from the cecum were collected and sonicated (XL-2000 Misonix) until the solution was homogenous in 700μl of 1X cell lysis buffer (Cell Signaling Technology) with 1mM PMSF and 1X Protease inhibitor added (Cell Signaling Technology). The homogenates were centrifuged at 12,000 RPM for 30min and the supernatant was removed, aliquoted, and stored in -80°C for Inflammation Multiplex (Bio-rad), IL-6 (BD), KC (R&D), and Cell death ELISAs (Roche). Protein measurements of the same samples were done from Bio-rad protein assay kit. Data were normalized as amount of cytokine/mg protein.

**RNA and DNA purification**
Tissue for RNA purification was immediately placed in RNA later (Ambion) and left at 4°C overnight before being stored at -80°C. RNA was purified from the tissue using Qiagen RNeasy mini-kit with the optional DNase digestion step (Qiagen). cDNA was prepared using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). For purification of genomic bacterial DNA, the feces were collected from the distal 5cm in small intestine and the whole large intestine from cecum and purified using Qiagen DNA stool mini kit with an initial sonication step in lysis buffer ASL and the optional high temperature 95°C incubation step to achieve sufficient lysis of both Gram-positive and Gram-negative bacteria.

**Real-time PCR**

Real-time PCR was preformed on mRNA and gDNA to quantify gene expression levels and copy numbers of various bacterial groups with the Step One Plus real-time pcr machine (Applied Biosystems). Copy numbers do not equal actual bacteria numbers or CFU but both are directly related and correlate well \(^{53}\). For gene expression, TaqMan Fast Advanced Master Mix (Applied Biosystems) was used along with TaqMan gene expression primers and probe for the target gene, and the endogenous control, GAPDH (Applied Biosystems). dCT was calculated for each sample of target gene normalized to GAPDH and expressed relative to sham as follows:

\[
\text{Fold} = 2^{(Ct \, \text{Target} - Ct \, \text{Control})}
\]

Relative Quantification = (1/fold)\(\times\)100.
For bacterial quantification, purified gDNA was diluted down to 4ng/μl for small intestine feces or .4ng/μl for large intestine feces to fall within the range of the standard curve. The final qPCR master mix contained 1X iTaq Universal SYBR Green supermix (Bio-rad), 3μM forward and reverse primer for the indicated bacterial group see table 1, and 24ng (small intestine feces DNA) or 2.4ng (large intestine feces DNA). The reaction was run at 95°C for 3min and 40 cycles of 95°C for 15sec and the indicated annealing temperature of the primer sets, table 1, for 60 seconds. The data was read at the final step. Samples were done in duplicates and standards in triplicate. For standards, purified genomic DNA from reference bacteria or plasmids were used. Copies of DNA were calculated from the following formula:

\[
\text{Copies} = (g \text{ DNA}) \times \frac{(6.022 \times 10^{23} \text{ copies/mole})/(650 \text{ g/mol bp}) \times \#\text{bp}}
\]

A standard curve was created from plotting the threshold cycle (Ct) against the Copy # of 10 fold dilutions ranging from 10^6 copies to 1 copy for genomic DNA or 10^7 to 10 copies for plasmid DNA. A semilog linear regression line was made and copy # of unknowns was calculated from the following formula relating the Ct value for each sample to the slope and the y-intercept:

\[
\text{Copy#} = 10^{(\text{Ct-b})/m}
\]

A melt curve was performed for quality assurance and efficiencies ranged under the accepted values, 90-110%. Data were normalized as copies of bacterial 16s per gram feces, taking into account DNA extraction efficiencies per g feces.
## Bacterial Primers

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward Primer 5'-3'</th>
<th>Reverse Primer 5'-3'</th>
<th>Annealing Temp (°C)</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td>Total Bacteria</td>
<td>ACTCTACGGGAGGCAGCAGT</td>
<td>ATTACCAGCGGTGCTGCG</td>
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<td>Barman¹⁵³</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>CGAACAGGATTAGATACCCCT</td>
<td>GGTAAGGTTCCTCGCTAT</td>
<td>61.5</td>
<td>Bacchetti¹¹¹</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>TCGTCAGCTCGTGYGTGA</td>
<td>AGCTGACGACAAACCAGAC</td>
<td>60</td>
<td>Hooper¹⁰⁹</td>
</tr>
<tr>
<td>γ-Proteobacteria</td>
<td>GGAGYATGTGGTTATTCGAA</td>
<td>CGTAAAGGCGATGATG</td>
<td>61.5</td>
<td>Bacchetti¹¹¹</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
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<td>GCCTCAAGGGCAACCTCAA</td>
<td>67</td>
<td>Barman¹⁵³</td>
</tr>
<tr>
<td>Escherichia coli</td>
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<td>CGGGTCAACGTCAGAAA</td>
<td>60</td>
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<tr>
<td>Clostridium leptum</td>
<td>CTTCCGTGCGCGAGTTA</td>
<td>GAATTCACCAGACACATCCACT</td>
<td>60</td>
<td>Corthier¹¹³</td>
</tr>
<tr>
<td>Clostridium Coccoides</td>
<td>ACTCTACGAGGAGGCAGC</td>
<td>GCTTCTAATCGAGGTCTCAT</td>
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</tr>
<tr>
<td>Bacteroides</td>
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<td>GCTGCTCCGCTGAGATTAGG</td>
<td>61</td>
<td>Barman¹⁵³</td>
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<tr>
<td>SFB</td>
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<td>GACGGAGAGATTATGTTTCA</td>
<td>58</td>
<td>Barman¹⁵³</td>
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<td>Lactobacillus</td>
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<td>CACCGCTACACATGGAG</td>
<td>56</td>
<td>Barman¹⁵³</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>CGAAGCCACGAGGCTATA</td>
<td>TCRTCCACACTTCCCTCCG</td>
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<td>Bacchetti¹¹¹</td>
</tr>
<tr>
<td>MIB</td>
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<td>CGCATTTCCGATCTTCCT</td>
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<td>Barman¹⁵³</td>
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<tr>
<td>Prevotellaceae</td>
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<tr>
<td>TM7</td>
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<td>α-Proteobacteria</td>
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</tr>
<tr>
<td>Faecalibacterium prausnitzii</td>
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<td>GAGCCTACGCGTCGTGGTTG</td>
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<tr>
<td>Bifidobacteria</td>
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<td>TGGATAGGACCGACACCCA</td>
<td>60</td>
<td>Corthier¹¹³</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>AGCACAGAGAGCTTG</td>
<td>ACTTTGCTTTTGCGAC</td>
<td>60</td>
<td>Kurupati¹¹⁵</td>
</tr>
</tbody>
</table>

**Table 1.** List of bacterial primer sequences, annealing temperature, and reference
**Bacterial Culture**

To culture bacteria from the lymph nodes, the mesenteric lymph nodes were aseptically removed, weighed, and homogenized in PBS to achieve a 50mg/ml concentration. 50μl of the homogenate was plated on Tryptic soy agar plates with 5% sheep blood, and MacConkey agar to grow total and Gram-negative bacteria respectively. The plates were cultured aerobically in a 37°C incubator with 5% CO₂ for 24 hours.

**Statistical Analysis**

Data are expressed as mean ± standard error of the mean (SEM) unless otherwise noted. Differences between groups were determined by ANOVA with tukey’s post hoc test or student’s t-test using GraphPab InStat. P<.05 was considered statistically significant.
CHAPTER FOUR

RESULTS

Gross Intestine Pathology

We first examined the gross intestine pathology, and the histology by H&E staining to observe physical and structural changes to the organ and intestinal villi following burn injury. The intestine, specifically the cecum, appeared desiccated, hard, and necrotic by gross examination (Fig.1a). H&E staining revealed some blunting of the small intestine villi indicative of mild inflammation (Fig.1b).
Gross Pathology

Figure 1 *Necrotic intestine tissue and mild blunting of the small intestine villi one day post burn injury.* a. depicts gross intestine pathology and b. reveals histology of the distal small intestine by H&E staining in a representative sham and burn animal (20X magnification).
Expression of AMPs following burn injury

We next determined the expression of AMPs in the gut after burn injury. The role of α-defensins and C-type have been previously described in other studies to play a significant role in epithelial barrier defense and regulating the intestinal microbiota in the distal small intestine, however it is not known how burn injury alters the expression of these proteins. Therefore we measured the levels of mRNA expression of five various α-defensin genes, Defcr1, CR2, Defcr5, Defcr21, and Defa-rs1 and two C-type lectins, Reg3γ and Reg3β by reverse transcription qPCR in the distal small intestine one and three days after burn or sham injury. We observed a 40-50% reduction of all the α-defensins in the small intestine one day after injury relative to the sham group, p<.05 ANOVA (Fig. 2). These reductions normalized to sham levels three days after injury. The α-defensin mRNA levels in the large intestine tissue were undetectable (data not shown). This is most likely due to the fact that Paneth cells are the primary producers of these proteins and these specialized cells are found specifically in the small intestine.

Concerning the C-type lectins, Reg3γ expression displayed a 50% reduction three days after injury in the tissue relative to sham, p<.05 ANOVA (Fig. 2). Reg3β levels were not significantly different when analyzed by ANOVA but showed a significant decrease when comparing days three post burn with sham by an unpaired student t-test, p<.05 (Fig2).
Figure 2. *Burn injury suppresses α-defensin and C-type lectin expression one and three days after injury respectively.* RNA was purified from the distal small intestine, reverse transcribed to cDNA, and quantitated by qPCR using primers and probes specific for the target gene and an endogenous control. AMP expression was normalized to GAPDH by the ΔCt method and expressed relative to sham. Values are mean ± SEM from 7-15 animals/group. *, p <0.05 ANOVA and Tukey’s post hoc test burn day 1 or 3 compared to sham. #, p<.05 unpaired t-test burn day 3 compared to sham.

**Burn Injury and the Intestinal Microbiota**

Reductions of AMPs in the intestine have been shown to accompany disturbances in bacterial communities, thus we asked whether the intestinal microbiota was changing on days one and three following injury to mirror the changes we observed in the α-defensins and C-type lectins. To quantify changes in the predominant bacterial groups of the gut we used primers for 16s, the gene for the small bacterial subunit, which have been previously designed and validated (see table 1). 16s is a gene contained by all prokaryotes that is highly evolutionarily conserved but contains enough nucleotide variability necessary to determine different phylogenetic bacterial lineages. We first
asked if burn injury led to changes in total bacterial load in the feces of the small and large intestine. The data revealed a 23-fold increase in the small intestine one day after burn relative to sham but no significant changes in the large intestine (Fig. 3).

**Figure 3.** Burn injury leads to increases in total bacteria content in the small intestine but not the large intestine one day after injury. Genomic bacterial DNA was purified from the feces of the small (a) and large (b) intestine and quantified by qPCR using universal bacterial primers. Copies of 16s were calculated based on a standard curve normalized to gram feces and expressed relative to sham. Values are mean ± SEM from 5-9 animals/group. *, p <0.05 burn day 1 compared to sham ANOVA and Tukey post hoc test.
In addition, we examined the bacterial diversity of the intestinal microbiome with focus on the most abundant bacterial groups in the healthy intestine and key bacterial groups that have pro-inflammatory or anti-inflammatory potential in burn patients or other inflammatory diseases. In the small intestine, we observed no change in the most abundant bacterial phyla, the Gram-positive Firmicutes and Gram-negative Bacteroidetes, but saw an enormous increase, ~1300 fold in the Gram-negative aerobic γ-Proteobacteria class, one day after burn relative to sham animals (Fig. 4a). Furthermore within the γ-Proteobacteria class, we observed an ~4000 fold increase of Enterobacteriaceae and ~3000 fold increase in *E. coli* on the same day post injury (Fig. 4a). Segmented filamentous bacteria and Lactobacillus were significantly decreased in the small intestine feces three days after burn relative to sham (Fig. 4a). Astonishingly, every bacterial group we measured in the large intestine showed a significant change one day after burn relative to sham. These changes were more diverse but less intense than the changes of bacteria in the small intestine. Concerning the major taxa, there was a significant increase in the Bacteroidetes and a reciprocal decrease of the Firmicutes (Fig. 4b). Of the prominent genera and species of bacteria within these phyla, the Bacteroides a genus within the Bacteroidetes, was similarly elevated and *Clostridium leptum* and *coccoides*, prominent species within the Firmicutes, were significantly reduced (Fig. 4b). Furthermore similar to the small intestine, the γ-Proteobacteria, Enterobacteriaceae, and *E. coli* were all significantly elevated and were some of the most pronounced changes of any bacterial groups (Fig. 4b). In addition, the Enterococcus genus and *Enterococcus faecalis* species
were increased ~12 and 76 fold respectively (Fig. 4b). Another minor phyla, the Gram-positive Actinobacteria, were significantly elevated in the large intestine (Fig. 4b). These bacterial changes are summarized in table 2.
Figure 4. *Burn injury alters the intestinal microbiome one or three days after injury relative to sham.* Genomic bacterial DNA was purified from the feces of the small (a) and large (b) intestine and quantified by qPCR using bacterial primers for the indicated group. Copies of 16s were calculated based on a standard curve normalized to gram feces and expressed relative to sham. Values are mean ± SEM from 5-9 animals/group. *p <0.05
burn day 1 compared to sham ANOVA and Tukey post hoc test. #, p<.05 Burn day 3 compared to sham student t-test.

### Small Intestine

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Burn Day 1</th>
<th>Burn Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteroidetes</td>
<td>13.43156523</td>
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<tr>
<td>Firmicutes</td>
<td>4.509607998</td>
<td>2.76553264</td>
</tr>
<tr>
<td>γ-Proteobacteria</td>
<td>1311.641382</td>
<td>9.617735076</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>4373.958842</td>
<td>3.470150519</td>
</tr>
<tr>
<td>E coli</td>
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<td>1.009265903</td>
</tr>
<tr>
<td>SFB</td>
<td>4.050059795</td>
<td>0.269216568</td>
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<tr>
<td>Lactobacillus</td>
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<td>Bacteroides</td>
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### Large Intestine

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Burn Day 1</th>
<th>Burn Day 3</th>
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</thead>
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<tr>
<td>Bacteroidetes</td>
<td>1.560458447</td>
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<tr>
<td>Firmicutes</td>
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</tr>
<tr>
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<td>C. leptum</td>
<td>0.591921874</td>
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<td>C. coccoides</td>
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<tr>
<td>Enterococcus faecalis</td>
<td>76.77106235</td>
<td>1.013609503</td>
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</table>

**Table 2**: Fold change of copies 16s/g feces relative to sham levels of the indicated bacterial group in the small or large intestine feces one and three days after injury.
**Fluorescent in-situ hybridization**

Using qPCR for 16s we identified an ~4000 fold increase in Enterobacteriaceae in the small intestine feces and an ~400 fold increase in the large intestine feces (Fig. 4a,b). Other studies have reported that the Enterobacteriaceae is a potentially proinflammatory group of bacteria that contains many opportunistic pathogens which may overgrow in response to inflammation and induce colitis\(^93,122\). This family of bacteria comprises a minor part of the healthy intestinal microbiota and is rarely found adhering to the intestinal villi\(^123\). Adherence of these bacteria to intestinal epithelial cells is necessary for these bacteria to induce intestinal inflammation or translocate to extraintestinal organs. Therefore, to visualize the proximity of these bacteria to the intestinal villi we stained the 16s rRNA of this bacterial group by fluorescent in-situ hybridization (FISH). Using a universal bacterial probe in combination with an Enterobacteriaceae specific probe, we detected overgrowth of Enterobacteriaceae adjacent to the epithelium in the small intestine one day after injury in the burn animals but not the sham (Fig. 5). This suggests that the Enterobacteriaceae may have invasive potential and the ability to induce inflammation by activating pattern recognition receptors present on the intestinal epithelial cells.
Figure 5. *Burn injury leads to an increase of Enterobacteriaceae adjacent to the small intestinal villi one day after insult.* Red represents Alexa 555 EUB probe for total bacteria. Green represents Alexa 488 Entbac probe for Enterobacteriaceae. Orange depicts colocalization of both probes and the Enterobacteriaceae. Blue is a DAPI stain for nuclei. The above image is 100x magnification.
**Intestinal Inflammation**

Dysbiosis of the microbiota has been previously shown to potentiate intestinal inflammation in IBD. Therefore we asked whether burn-induced changes in the intestinal microbiome that we observe one or three days after injury accompanies increases of inflammatory cytokines and apoptosis. To test this hypothesis we ran a multiplex assay on inflammatory cytokines in both the small and large intestines. Numerous innate cytokines and chemokines were increased in both the small and large intestine one day after injury. To confirm these results, we next ran ELISA’s on two key inflammatory markers of burn injury IL-6 and KC. Both IL-6 and KC were significantly elevated in the small intestine one day after burn but only KC was increased in the colon (Fig. 6 a-d). Inflammation has the potential to be detrimental to the host and lead to increases of cell death. Thus, we ran a cell death apoptosis ELISA and noted increases in apoptosis both one and three days after injury in the small intestine (Fig. 7). Therefore, we noticed that burn injury is leading to increases in intestinal inflammation in both the small and large intestines one day post burn and increases of apoptosis in the small intestine one and three days post burn.
Figure 6. Burn injury leads to an increase of cytokines in the small and large intestine. Total small and large intestine tissues were homogenized. IL-6, and KC ELISAs were performed on sham, burn day one, and burn day three on the small intestine (a,c) or the large intestine (b,d).* p<.05, ** p<.01, *** p<.001 burn day 1 compared to sham, ANOVA and Tukey multiple comparison tests. Values are all expressed as mean ± SEM 8-11 animals per group.
**Figure 7.** *Burn injury leads to an increase of cell death in the small intestine.* Total small intestine tissue was homogenized. A cell death ELISA was performed on sham, burn day one, and burn day three on the small intestine. Data is expressed as OD value at 450nm per mg protein for each sample. *, p<.05, ** p<.01, burn day 1 or 3 compared to sham, ANOVA and Tukey multiple comparison tests. Values are all expressed as mean ± SEM 8-11 animals per group.
**Intestinal Permeability**

In order to determine whether an increase of intestinal inflammation accompanied an increase of gut leakiness following burn injury, we ran a FITC-dextran permeability assay. Mice were gavaged with FITC-dextran one and three days after burn. Three hours later, blood was drawn and the concentration of this fluorescent conjugated sugar moiety was determined spectrophotometrically. In normal physiologic conditions, the size of this molecule prevents its translocation from the intestinal lumen into the circulation. The concentration of FITC-dextran increased in the plasma one day after burn relative to the sham animals (Fig. 8a). In addition to the FITC-dextran assay, we quantified the gene expression of two tight junction proteins, claudin 4,8 in the small and large intestine. We saw a 50% reduction in claudin 4, and 8 in the small intestine but no change in the large intestine (Fig. 8b,c).
**Figure 8.** Increases of intestinal permeability one day after burn injury. a. represents the FITC-dextran permeability. Mice were gavaged with FITC-dextran one and three days after burn or sham injury. Three hours later the concentration of this dye was measured spectrophotometrically in the plasma of the mice. b. and c. represent RT-qPCR from the distal small intestine or large intestine, using primers and probes specific for the target gene and an endogenous control. TJ expression was normalized to GAPDH by the ΔCt method and expressed relative to sham. Values are mean ± SEM from 5-10 animals/group. *, p <0.05 ANOVA and Tukey’s post hoc test burn day 1 to sham.
Increased gut leakiness has been shown to lead to bacterial translocation to the lamina propria and the mesenteric lymph nodes (MLN)\textsuperscript{28}. Therefore we asked whether increases in gut bacteria could be leading to increases of bacterial translocation. We first stained for total bacteria by FISH and noticed bacteria in the lamina propria of the small intestine after burn injury (Fig. 9). Furthermore, we measured total bacteria and Enterobacteriaceae in the MLN by qPCR and culture methods. We found that burn injury resulted in a significant increase in total bacteria and Enterobacteriaceae in the MLN by qPCR one day after injury (Fig. 10). In addition, bacteria were cultured aerobically on tryptic soy agar (TSA) with 5% sheep blood, to identify total aerobic bacteria, and MacConkey agar, to identify Gram-negative aerobic bacteria or the Enterobacteriaceae for 24hrs. We noted a significant increase of colonies present on the TSA and MacConkey plates from burn day one animals and no colonies were observed on the plates from the sham animals (Fig. 11b). Some colonies were also detected on the TSA plates three days after burn (Fig 11c). Collectively this data shows that the intestinal permeability increases one day after injury, and this permeability may be due to alterations in tight junction proteins. This increased gut leakiness leads to bacterial translocation to the lamina propria and the MLN seen one or three days after burn. Furthermore, we observed bacteria present in the MLN and most of these bacteria were Enterobacteriaceae. Therefore, burn injury leads to an overgrowth of Enterobacteriaceae in the small and large intestines that occurs one day after burn and has the potential to
spread systemically to other organs. Systemic Gram-negative bacteremia has the potential to lead to sepsis and multiple organ failure for burn patients.

**Figure 9.** *Bacteria present in the small intestine lamina propria one day after burn.* Red represents Alexa 555 EUB probe for total bacteria. Blue is a DAPI stain for nuclei.
Figure 10. *Total bacteria and Enterobacteriaceae in the MLN one and three days after injury.* MLN were removed from sham burn day 1, and burn day three animals. The tissue was homogenized, total DNA was purified from the tissue, and qPCR was performed on the DNA using universal bacterial primers and primers specific for Enterobacteriaceae (table1). Data is expressed as qPCR copies bacteria/ng total DNA relative to sham. Values are mean ± SEM n=6 animals per group. ANOVA and Tukey post hoc test *,p<.05 burn day 1 or burn day 3 compared to sham.
**Figure 11.** *Bacteria in the MLN one or three days after burn.* The lymph nodes were aseptically removed from sham (a) burn day 1 (b) or burn day 3 (c) animals and homogenized in PBS to achieve a 50mg/ml concentration. Equal volume for each sample was plated on tryptic soy agar plates with sheep blood or MacConkey agar plates and cultured aerobically for 24 hours to grow total bacteria or Enterobacteriaceae respectively. The above plates are a representative image taken from one sham animal, one burn day one, and one burn day three.
Probiotic Treatment

Probiotic treatment with live beneficial bacteria has shown promise in restoring the epithelial barrier and preventing intestinal inflammation in various models of disease. Lactobacillus rhamnosus GG (LGG) is a probiotic strain which adheres to intestinal mucins and secretes various factors which increase epithelial cell proliferation, regulate tight junction expression, and modulate cytokine secretion. Previous research has shown that LGG is an effective therapy for reducing intestinal inflammation in models of colitis. This efficacy was achieved by oral inoculation of $10^{10}$ colonies/ml of LGG. Therefore we decided to treat mice with $10^{10}$ colonies/ml of LGG by gavage at the time of injury and measure various parameters associated with gut barrier dysfunction—antimicrobial peptide production, tight junction expression, and inflammatory cytokine levels. To perform this experiment, mice were divided into four groups, sham vehicle, burn vehicle, sham plus lactobacillus, and burn plus lactobacillus. Immediately before injury mice were given a gavage of 0.2ml of PBS or LGG. The mice were then sacrificed one day after injury; the time point where we see the most pronounced changes.

We used real-time qPCR to measure α-defensin expression one day after injury in the small intestine and chose to focus on a single defensin gene, defα1. Relative to sham vehicle, we saw a 40% reduction in burn vehicle, which indicates consistent observations to what was earlier reported (Fig. 12). There was no change in gene expression of defα1 in sham lacto. relative to sham vehicle suggesting that LGG does not modulate the α-
defensin gene expression in the wild type sham animals. However the burn lacto group showed 57% reduction in *defa1* relative to sham vehicle a similar result to what was reported in the burn vehicle (Fig. 12). Therefore, this experiment showed us that at this concentration and time point LGG did not restore α-defensin expression.

We next measured whether LGG restored the expression of a key tight junction protein *cldn4*, which we previously observed down in the small intestine one day after burn. This experiment revealed similar results to what we observed in *defa1*, there was a decrease in burn vehicle, no change in sham lacto, and a decrease in burn lacto suggesting that this experiment did not restore expression of *cldn4*.

Previously we observed significant increases of KC in the small intestine following burn injury, and there is data suggesting LGG may decrease expression of this cytokine in models of colitis. However, it has not been determined whether this therapeutic may be protective in burn injury. Therefore, we measured KC production in the small intestine following LGG treatment to investigate whether this probiotic may reduce intestinal inflammation following burn (Fig. 13). We saw no reduction of KC in the lactobacillus treated burn group in the small intestine one day after injury. Therefore the $10^{10}$ CFU/ml dose of LGG did not show any therapeutic benefit with regard to α-defensin or tight junction expression, and KC levels in the small intestine one day after injury.
Figure 12. LGG treatment does not restore α-defensin 1 or claudin 4 expression in the small intestine one day after burn injury. RNA was purified from the distal small intestine, reverse transcribed to cDNA, and quantitated by qPCR using primers and probes specific for α-defensin 1 or claudin 4 and an endogenous control. Gene expression was normalized to GAPDH by the ΔCt method and expressed relative to sham. Values are mean ± SEM from 7-10 animals/group. *, p <0.05 ANOVA and Tukey’s post hoc test burn vehicle or burn lacto. compared to sham vehicle.
Figure 13. LGG treatment does not restore KC levels one day after burn injury in the small intestine. Total small intestine tissue was homogenized and KC concentration was measured by ELISA on sham vehicle, burn vehicle, sham lacto, and burn lacto groups. Values are all expressed as mean ± SEM 7-10 animals per group. *, p<.05 burn vehicle or burn lacto compared to sham vehicle, ANOVA and Tukey multiple comparison tests.
CHAPTER FIVE
SUMMARY AND DISCUSSION

Multiple organ failure and sepsis are the leading complications contributing to post burn morbidity and mortality in patients who sustain thermal injuries. Identifying the contributing factors and mechanisms leading to a dysregulated immune response, which predisposes patients to second hit infections, is necessary to develop novel therapeutics to prevent sepsis and organ failure. Overgrowth of specific gut bacteria and gut barrier disruption may play a role in generating a systemic inflammatory response—the principal etiological factor contributing to sepsis. Therefore, it is necessary to determine whether burn injury influences the intestinal microbiota and whether the change in the microbiome is related to gut barrier dysfunction following burn injury. We hypothesized that burn injury leads to a decrease in AMPs which allow for changes in the intestinal microbiota that contribute to increases of inflammation and permeability in the intestines. Our findings reported herein suggest that burn-mediated increase in gut leakiness could result from a potential change in the microbiota. We further observed an increase in intestinal inflammation, gut leakiness, and bacterial translocation to extraintestinal sites. This translocation of bacteria into the circulation may potentiate a systemic inflammatory response that may then lead to sepsis.
The host regulates various species and abundances of microbes in the intestine through the production of antimicrobial peptides. Antimicrobials are constitutively secreted primarily by the Paneth cells of the small intestine, and two classes of AMPs, α-defensins and C-type lectins, have been shown to be critical in maintaining normal proportions of bacteria and homeostasis in the intestine. Since, it is not known how these AMPs respond following burn injury we measured the expression of these proteins in the small intestine and found that α-defensins, Reg3γ, and Reg3β decreased one and three days after injury respectively. Unlike humans who express two different isoforms of Paneth cell α-defensins, HD-5 and -6, mice express highly polymorphic structurally similar proteins termed cryptdins. To date, there have been 17 different cryptdins that have been identified and only six of which have been purified. Cryptdins 1-4 possess the most microbicidal activity but the specific role each of these peptides have in vivo is unclear. Thus further research is needed to determine the various immunological functions of the α-defensins in the intestine following burn injury.

Although, the mechanisms leading to the release of α-defensins are largely unknown, α-defensins have been shown to be downstream of toll-like receptor or NOD-like receptor pathways. Further research is needed to determine why expression of these proteins decreases following burn injury and whether burn induces the production of unknown ligands which interact through these pattern-recognition receptors to inhibit AMP production. On the other hand, it is known that Reg3γ and Reg3β secretion is regulated through SFB and the IL-22 pathway. SFB interact with intestinal epithelial
cells to lead to the production of serum amyloid a (SAA). SAA then acts on dendritic cells to promote Th17 cell differentiation. Th17 cells secrete IL-22 which acts on the IL-22R expressed exclusively on intestinal epithelial cells which leads to the production of AMPs such as Reg3γ and Reg3β. We see decreases of IL-17 and SFB three days after burn which may explain why Reg3γ and Reg3β are also down three days after injury. Another potential mechanism leading to antimicrobial decrease is that burn induces a hypoxic environment in the intestine and this lack of oxygen may lead to apoptosis. We observed an increase of apoptosis in the small intestine one and three days after injury, and this cell death may explain why we noticed decreases of α-defensins and C-type lectins on these days respectively.

Mouse cryptdins and C-type lectins may be differentially transcribed or possess varying functions than their human homologous counterparts. Therefore, the levels and functions of human α-defensins and C-type lectins need to be investigated as well. To study the in vivo roles of these AMPs, α-defensin and Reg3γ knockout mice have been created as well as transgenic mice overexpressing human HD-5 in intestinal epithelial cells. It would be interesting to determine whether the AMP specific knockouts have increased mortality, morbidity, intestinal inflammation, gut leakiness, and microbiota changes than the wild type mice have after burn injury. In addition, it is crucial to determine whether the α-defensin overexpressing mice are protected from burn induced complications as compared to the wild type mice. If so, these experiments would
provide support that AMPs could be used as a novel potential therapeutic to prevent infections for burn patients.

We hypothesized that it is this decrease of AMPs which allows for changes in the intestinal microbiota. However, there are many other factors which could be contributing to these changes. For instance, nutrient abundance is a key factor that contributes to niche specific bacterial communities. Disruption of a continual nutrient supply may allow for colonization by foreign pathogens or expansion of underrepresented bacterial species which can outcompete the other commensals. After the burn injury, the mice become less active and are less likely to eat, and this change in diet, may allow for changes in the bacterial flora. In addition, burn injury may change the physiologic oxygen levels of the gut which could potentiate expansions of aerobic and reductions of anaerobic bacteria. We observed increases of aerobic \(\gamma\)-Proteobacteria and Enterobacteriaceae, but decreases in the anaerobic Firmicutes and Clostridia one day after injury. Research shows that following burn there is a mesenteric vasoconstriction which reduces blood flow and creates an ischemic environment in the intestines. After some elapsed time, the blood flow vigorously returns and this reperfusion creates an oxygen rich environment. The earliest time point we studied was one day after injury, and we do not know the levels of oxygen in the intestine at this time point. It would be interesting to determine whether the increases of Gram-negative aerobic bacteria also occur at earlier time points. In addition, if we reduce blood flow through ligation of the carotid artery, do we still observe similar changes in the microbiota? Any factor, which alters the normal
physiological habitat of the bacteria, may play a role in inducing changes in the microbiota. *In vivo*, numerous factors in addition to changes in AMPs most likely impart a certain magnitude in altering the intestinal microbiota, but further research is needed to identify these variables.

To explore the changes in the intestinal microbiota, we used qPCR of the 16s gene for the bacterial ribosome with various primer sets that have been previously designed and validated (Table 1) which amplify select bacterial taxa. Concerning the major taxa, we observed increases in only the γ-Proteobacteria in the small intestine feces. However the large intestine showed more diverse but less intense changes—a decrease in the Firmicutes and an increase of the Bacteroidetes and γ-Proteobacteria one day after burn. Next, we explored various families, genera, and species within these taxonomic groups that are either in high abundance in the gut, or that have been shown to be potentially protective or harmful. In both the small and large intestine the bacterial group that showed the most exaggerated change was the Enterobacteriaceae family. This family of bacteria is under the γ-Proteobacteria class and contains many opportunistic pathogenic bacteria such as the Escherichia, Proteus, Salmonella, and Klebsiella genera. Furthermore, we noticed increases in *E. coli* in both the small and large intestines. Overgrowth of Enterobacteriaceae has been shown to cause intestinal inflammation and increased gut permeability in models of colitis. Furthermore, the proportions of these bacteria are increased in the intestines of inflammatory bowel disease patients and in
patients that present relapsing recurrent Crohn’s disease\textsuperscript{139}. Therefore this bacterial group may also be harmful for burn patients.

Concerning the protective bacteria, we noticed decreases in Clostridia, \textit{Clostridium leptum} or \textit{Clostridium coccoides}, in the large intestine one day after burn. These bacteria have been shown to induce Treg cell differentiation and decrease intestinal inflammation in models of colitis\textsuperscript{78,79,140}. A decrease in Treg cell numbers or function may explain the intestinal inflammation that we see one day after burn. These bacteria produce the short chain fatty acids butyrate and propionate which acts on dendritic cells and CD4+ T cells to increase the differentiation of Tregs\textsuperscript{80}. These SCFA’s also act on colonic epithelial cells to increase cell proliferation. A decrease in these bacteria along with their SCFA metabolites may offer additional therapeutic interventions to reduce intestinal inflammation in burn injured patients.

We also detected decreases in segmented filamentous bacteria three days after injury in the small intestine feces. These bacteria produce various cell surface antigens which selectively lead to Th17 cell maturation in the small intestine lamina propria\textsuperscript{75,141}. Previously our lab has shown that Th17 cells have a protective function regulating the gut barrier in burn injury by inducing AMP production and preventing pathogenic bacterial infections\textsuperscript{7,142}. Therefore SFB bacteria may serve a protective role in burn injury by increasing Th17 cell counts and effector cytokines. It remains to be determined whether reconstituting the gut with these bacteria could boost the adaptive immune response and prevent immunosuppression and infectious complications in burn patients.
Another potentially protective bacteria that we observed decrease in the small intestine three days after injury were the Lactobacilli. There are numerous protective strains of these bacteria which modulate the innate immune system through various mechanisms\(^\text{11}\). This genera of bacteria adheres to the inner mucous layer and interacts with TLR2 with their pilli\(^\text{18}\). In addition, these bacteria secrete factors, p40 and p75, which increase tight junction protein expression and inhibit apoptosis of the intestinal epithelial cells\(^\text{104}\). Various strains of lactobacillus have been shown to reduce intestinal inflammation and be protective in models of IBD, however more research is needed to determine which strains may confer protection in burn patients.

Microbial dysbiosis has been suggested to lead to intestinal inflammation and permeability in various diseases: IBD, obesity, diabetes, rheumatoid arthritis, and autism\(^\text{143}\). This led us to question whether microbial dysbiosis was also involved in burn injury and may be contributing to similar increases of intestinal inflammation and permeability. We therefore measured intestinal inflammation and permeability one and three days after injury—the same days we observed changes in the bacterial flora of the small or large intestines. We saw an increase in innate cytokines, IL-6 and KC, in the small intestine one day after injury. In addition we ran an \textit{in vivo} FITC-dextran gut permeability assay and observed increases in intestinal permeability also one day after injury. Furthermore we wanted to determine whether the increased intestinal permeability could be leading to bacterial translocation to the lamina propria or extraintestinal sites\(^\text{144}\). Since we noted the highest increases in Enterobacteriaceae in both the small and large intestine feces, we
decided to stain this family of bacteria with FISH to visualize their location in relation to the intestinal epithelial cells—a place where these bacteria are not usually found in the healthy microbiome\textsuperscript{93}. We showed that Enterobacteriaceae were found adjacent to intestinal villi which suggests that these bacteria may possess invasive potential. We next measured this bacteria in the MLN by culture and qPCR and noted increases of viable bacteria in the lymph nodes both one and three days after injury. These data suggest that Enterobacteriaceae are able to overgrow in the intestines after burn and translocate to extraintestinal sites.

Enterobacteriaceae are Gram-negative bacteria containing LPS on their bacterial cell wall. LPS binds to TLR4 and activates downstream transcription factors which upregulates the production of inflammatory cytokines\textsuperscript{145}. Therefore the increase of Enterobacteriaceae that we detected may be causing inflammation through this signaling pathway. Previous work has shown that signaling through the TLR4 pathway is harmful but TLR2 is protective after burn injury\textsuperscript{146}. Changes in the intestinal microbiota may help to explain this phenomenon. Gram-positive bacteria contain a peptidoglycan cell wall which is a ligand for TLR2. We noted decreases of Clostridia the predominant Gram-positive bacterial group in the gut. Therefore a combination of a loss of protective Clostridia and pathogenic Enterobacteriaceae may explain the increases of inflammation in the intestines following burn. An increase of intestinal inflammation can lead to cell death and an apparent breakdown of the epithelial barrier allowing for bacterial translocation. In addition to inflammation and permeability we also noted increases of
apoptosis in the small intestine both one and three days after injury. Further research is still needed to determine whether the Enterobacteriaceae are indeed causing inflammation in the intestines. Knocking down the Enterobacteriaceae group through selective antibiotic treatment with polymyxin B and Clostridia through a vancomycin and metronidazole combination will determine the role these bacteria play in the intestines following burn injury.

Probiotic therapy has shown promise in reducing intestinal inflammation in models of colitis. Therefore we tested whether a probiotic supplement of *Lactobacillus rhamnosus* GG could restore the epithelial barrier by increasing AMP production, increasing tight junction protein expression, and reducing intestinal inflammation one day after burn injury. We gavaged mice with $10^{10}$ CFU/ml of bacteria at the time of injury and measured these parameters. Previous research has shown that this bacteria secretes factors, p40 and p75, which increase tight junction protein expression and reduce intestinal inflammation in models of colitis but it is not yet determined whether this bacteria is protective in burn injury. However, we did not see a change in α-defensin 1 or cldn 4 expression nor a reduction in KC or IL-6 production. These results could be due to numerous factors. First, it is possible that 24 hours is not enough time for this bacteria to adhere to the inner mucus layer, colonize the intestine, and exert its protective effects. It’s also possible that $10^{10}$ CFU/ml may not be a high enough dose for protection in burn injury as opposed to colitis models. Another possibility may be that this bacterial strain is simply not protective in burn injury and that other probiotic alternatives need to be
explored. Probiotic therapy still remains a novel therapeutic avenue which may modulate the immune system, fix the intestinal epithelial barrier, and prevent gut bacteria from becoming a source of septic infections or organ failure.

Sepsis and multiple organ failure remain the leading problems associated with post burn complications. Therefore it is crucial to elucidate the scientific mechanisms leading to these disease pathologies. Previous burn research has suggested that translocating gut derived factors may potentiate a systemic inflammatory response. Nevertheless, the mechanisms leading to this phenomenon remain largely unexplored. This is the first study which suggests that burn injury induces a microbial dysbiosis which may play a role in propagating intestinal inflammation, and gut leakiness. We showed that specific gut bacteria, Enterobacteriaceae, overgrow in response to injury, and can translocate to extraintestinal sites such as the MLN. Previous research has suggested a link between Gram-negative infection and sepsis. Therefore elevations of this family of bacteria may be potentially problematic for burn patients and increase the risk for sepsis. More research is necessary to elucidate the mechanisms of Enterobacteriaceae translocation to the MLN and activation of inflammatory pathways. In addition, this study suggests that reducing the Enterobacteriaceae to normal physiological levels through the use of probiotic cocktails or antimicrobial therapy may be potentially beneficial to burn patients.
APPENDIX

SPECIFIC METHODS
Mouse Model of Thermal Injury

Adult C57BL/6 mall mice (7-8 weeks old, 22-25g body weight, Charles River Laboratories) were chosen randomly for all experiments. Animals received sham or burn injury yield two groups. For the LGG treatment, animals were divided into four groups sham PBS, burn PBS, sham LGG, and burn LGG. The mice were anesthetized with an intraperitoneal injection of ketamine hydrochloride/ xylazine cocktail (~80mg/kg and 1.2 mg/kg, respectively). The dorsal surface was shaved and the mice were transferred to a template calculated to expose ~20% TBSA as calculated by the Meeh formula, $A=kW^{2/3}$, given $k=10$, and weight in grams$^{107}$. The mice were submerged in a water bath set to 85°C for 7-9 seconds to emulate burn injury or a water bath set to 37°C to emulate sham injury. Following the burn the mice were resuscitated with an intraperitoneal injection of 1ml of saline. Animals were allowed food and water ad libitum. Mice were sacrificed and organs or tissue were collected one or three days after injury.
qPCR

For quantification of gene expression, RT-qPCR

RNA Purification

1. RNA was purified using RNeasy mini kit by Qiagen.
2. Tissue stored in RNA later was excised, 20 mg, and homogenized in lysis buffer with a rotor fixed tissue shredder for 30 seconds.
3. Sample was added to Qiagen spin columns and DNA was digested using the Qiagen DNase digest to remove any contaminating genomic DNA following the manufacturers.
4. Inhibitors were washed off the columns using buffers AW1 and AW2
5. Sample was eluted using 50μl of TE

Reverse Transcription

1. Reverse transcription reaction was performed using Applied Biosystems high capacity cDNA reverse transcription kit and following the manufacturers instructions.
2. Each sample was diluted to 33.75ng/μl and 10μl of each sample was added to a 96 well plate
3. 10μl of RT master mix was added to each sample which contained, the reverse transcription enzyme, dNTPs, random primers, and H2O at a 1X concentration
4. The reaction was run using Applied Biosystems Veriti thermal cycler using the manufacturers recommendations.
5. The cDNA was diluted down to 30/8 ng/μl for qPCR

qPCR

1. The qPCR master mix was created using 10μl/rxn TaqMan Fast Advanced qPCR supermix, 1μl/rxn TaqMan primer and probe (FAM), and 1μl/rxn TaqMan GAPDH endogenous control (VIC).
2. 12μl was pipetted into each well of a 96 well plate
3. 8μl of cDNA sample at 30/8 μl was pipetted into the corresponding well
4. The reaction was run using the FAST Applied Biosystems protocol on Step One Plus qPCR machine, Applied Biosystems
qPCR

For quantification of bacterial copy numbers

DNA purification

1. Feces were gently scraped from the intestine tissue one and three days after burn, weighed, and gDNA was purified using Qiagen DNA Stool mini kit with the procedures for optimizing prokaryotic to eukaryotic DNA.
2. Feces were lysed in ASL lysis buffer, sonicated for 1 min, and placed in a 95°C water bath to lyse Gram-positive bacteria.
3. Protease K digestion was performed with the manufacturers instructions to digest histone proteins.
4. DNA was precipitated with 100% ETOH and placed on spin columns.
5. Proteins and other inhibitors were washed using buffer AW1 and AW2.
6. DNA was eluted in 200μl of buffer TE.

Bacterial Standards

1. Purified bacterial gDNA or cloned bacterial plasmid DNA were used as standards. Using 25ng of genomic bacterial DNA with approx. 6 Mbp and 0.1ng of plasmids with approx. 2500 bp gives 10^6 or 10^7 copies per standard respectively using the formula: Copies = (g DNA)(6.022E10^22 copies/mol)/(bp)(650g/mol/bp)
2. 10 fold dilutions of standards were made for qPCR.

qPCR

1. Master mix was prepared using SYBR Green Universal qPCR reaction mix (Bio-Rad). 12μl/rxn of SYBR Green supermix, 2μl/rxn of forward primer (3μM), 2μl/rxn of reverse primer (3μM). (Table 1 gives primer sequences)
2. 14μl of master mix were pipetted into a 96 well plate
3. 6μl of purified gDNA from feces, bacterial standard, or H2O were pipetted into the corresponding well
4. The reaction was run at 95°C for 3 min and 40 cycles of 95°C for 15 sec and the corresponding annealing temperature for the primers (Table 1) for 60 sec. on a Step One Plus Real Time PCR Machine
5. Bacterial copy numbers are calculated relative to the standard curve.
FISH

Supplies

- 5M NaCl
- 1M Tris-HCL PH, 7.5
- 10% SDS
- Pap Pen
- Hybridization Container
- Fluorescent probes
- Xylene
- 100% Ethanol
- 50°C Oven
- Prolong Gold Antifade with Dapi (Molecular probes)

Procedures

1. Deparaffinize slides by running them through 4X 3min incubations in Xylene and 4X 3min incubations in ethanol
2. Heat the slides at 50°C for approx. 20 min to dry the slides
3. Prepare hybridization/wash buffer (.9M NaCl, 20mMTris-HCL, pH 7.5, .1% SDS), and hybridization container
4. Mark area of hybridization with Pap pen
5. Place 500μl of probe diluted to 1ng/ul in hybridization buffer on tissue section
6. Incubate for 16 hours overnight in dark oven at 50°C
7. Wash slides in pre-warmed washing buffer (.9M NaCl, 20mMTris-HCL, pH 7.5, .1% SDS) for 3X 15minute incubations.
8. Dry the slides
9. Counterstain and mount with Prolong Gold Antifade with Dapi
10. Image using Zeiss 500m fluorescent microscope with AxioVision software
ELISA

Tissue Homogenization:

Supplies:

- Lysis buffer, Cell Signaling Technologies
- Protease Inhibitor Cocktail
- PMSF
- Homogenizer Qiagen
- Sonicator

Procedure:

1. Prepare lysis buffer: Cell signaling technologies, protease inhibitor cocktail, and PMSF to manufacturers instructions
2. Add 700μl lysis buffer per sample (5cm small intestine tissue or entire large intestine tissue from cecum)
3. Homogenize for 30 seconds with a rotor fixed homogenizer
4. Sonicate for 10 seconds using a sonicator at setting 3.5
5. Centrifuge for 30min at 12,000 rpm at 4°C
6. Remove supernatant and aliquot

ELISA

Supplies:

- IL-6 ELISA kit (BD)
- KC ELISA kit (R&D)
- Cell Death ELISA kit (Roche)

Procedure:

1. Samples were diluted depending on the specific ELISA kit used so that the unknowns were within the standard curve
2. ELISA procedure was performed exactly as according to the manufacturers protocol, and suggested antibody dilutions
3. Read plate with a spectrophotometer at 450nm
FITC-dextran Permeability Assay

1. On the day of sacrifice, gavage mice 4ml of 22mg/ml FITC-dextran (Sigma-Aldrich) in PBS.
2. After 3 hours draw blood and sacrifice the mice.
3. The blood was centrifuged for 8000rpm for 5min at 4°C, plasma isolated.
4. Standards of FITC-dextran was prepared using 10 fold dilutions of pure FITC-dextran in PBS, the high standard being 1mg/ml
5. Equal volumes of plasma and standard were pipetted into the corresponding well, 96 well plate
6. The plate was read spectrophotometrically at 480nm excitation and 520nm
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VITA

The author, Zachary Earley was born in Chicago, IL on February 22, 1990 to Michael and Cathryn Earley. He graduated *summa cum laude* with a Bachelor of Science in Biology from Loyola University Chicago in May of 2012.

In August of 2012, Zachary joined the Program of Integrative Cell Biology at Loyola University Medical Center (Maywood, IL). Shortly thereafter, he joined the laboratory of Dr. Mashkoor A. Choudhry, where he studied burn injury and the role of the intestinal microbiota in gut barrier dysfunction.

His research showed that, following injury, the composition of the intestinal microbiota is altered increasing the load of Gram-negative aerobic bacteria, which may translocate to extraintestinal sites and lead to sepsis or multiple organ failure.

Based on his findings, Zachary gave a podium presentation titled, “*Burn injury Alters the Intestinal Microbiota and Increases Gut Inflammation and Permeability*” at the American Burn Association annual meeting in Boston MA, (March 2014). He also presented his work at the Burn & Shock Trauma Research Institute Seminar Series at Loyola University Stritch School of Medicine in May, 2014. Zachary is a co-author in a review titled, The First Line of defense: The Effects of Alcohol on Post-Burn Intestinal Barrier, Immune Cells, and Microbiome.