GUT MICROBIAL CHANGES AND THEIR CONTRIBUTION TO POST-BURN PATHOLOGY

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Abstract

Burn injuries are a common form of traumatic injury that leads to significant morbidity and mortality worldwide. Burn injuries are characterized by inflammatory processes and alterations in numerous organ systems and functions. Recently, it has become apparent that the gastrointestinal bacterial microbiome is a key component of regulating the immune response and recovery from burn and can also contribute to significant detrimental sequelae after injury, such as sepsis and multiple organ failure. Microbial dysbiosis has been linked to multiple disease states, however, its role in exacerbating acute traumatic injuries, such as burn, are poorly understood. In this article, we review studies that document changes in the intestinal microbiome after burn injury, assess the implications in post-burn pathogenesis, and the potential for further discovery and research.

1. INTRODUCTION

Burn injuries are a subset of traumatic injuries to the skin that can be caused by a number of different insults. This includes thermal, chemical, radiation, electrical, friction, and cold burns (frostbite). The majority of burns are caused by thermal insults, including fire, hot

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objects, or hot liquids/scalding. As of 2016, an estimated 486,000 people in the United States received medical care related to a burn injury, with 40,000 people hospitalized due to their injury(1). Furthermore, incidents of burn injury are significantly higher in lower income countries with the WHO attributing up to 90% of the 11 million burn injuries world-wide in low income countries, and are a major cause of morbidity in the world(2).

Burn injury severity is categorized by the depth and size of the wound as demonstrated in Figure 1. First degree burns have the injury limited to the epidermis, or outermost and superficial layer of the skin. Second degree or partial thickness burns are defined by the injury extending past the outermost layer into the dermis and often form blisters that are extremely painful. Depending on the depth of the partial thickness burn, surgery may be required. Third- and fourth-degree burns penetrate the full thickness of the dermis, with fourth degree burns causing deeper damage to underlying muscle or bone. Third- and fourth-degree burns are not painful due to destroyed nerve endings and require surgery and careful management of the burn area to prevent infection. Fourth-degree burns will often lead to the loss of the injured area. Along with the depth of the injury, the total surface area is an important component of determining burn injury severity. If the burn covers less than 10% of the total body surface area (TBSA), it is categorized as a minor burn. The classification for major burns is less well-defined, however, the following guidelines are often used to indicate a major burn: greater than 20% TBSA in adults, greater than 30% TBSA in children, and greater than 10% TBSA in elderly patients(3).

2. RESPONSE TO BURN INJURY

The initial burn injury causes numerous detrimental effects not only to the immediate burn area but leads to a cascade of responses in the entire body that can lead to severe consequences such as shock and multiple organ failure. These systemic responses can cause serious detriment to the patient and their recovery. Immediately following injury, catecholamines, cortisone, and inflammatory cytokines like IL-6 and tumor necrosis factor (TNF) are released into systemic circulation. Burn injury usually leads to distributive shock, in which capillaries become leaky and fluid is lost to the extravascular space. This loss of fluid from the circulatory system results in edema, fluid accumulation in tissues, reduced cardiac output, and compromised delivery of oxygen to numerous bodily organs, including the gastrointestinal tract(4, 5). Furthermore, burns can be complicated by other injuries, such as inhalation injury which can compromise the airway. A small subset of burn injuries are also associated with other traumatic injuries, such as traumatic brain injury, soft tissue injury and/or fracture, or injuries to the thorax and/or abdomen(6, 7). Like with any trauma patient, airway, breathing, and circulation must be stabilized upon immediate arrival to the hospital.

Current standards in burn treatment require immediate fluid resuscitation to account for this hypovolemic state. Fluid requirements are calculated based on several different formulas; however, initial rates are dependent on the size of the burn area, the patient's body weight, and eventual fluid output by the patient (urine volume)(8-10). The purpose of fluid resuscitation is to adequately perfuse and oxygenate organs to avoid complications such as renal failure(11, 12). A delicate balance must be maintained while resuscitating burn patients, as over-resuscitation combined with endothelial leakage observed can lead to "fluid

creep" and severe consequences such as abdominal compartment syndrome, pulmonary edema, and decreased perfusion of the burn wound itself (9, 13).

The type of fluid used in resuscitation must also be considered: isotonic crystalloids, hypertonic solutions, colloids, and increasingly plasma is being used. Crystalloids, including Ringer's lactate (RL) and normal saline, are readily available products and commonly used. However, over-resuscitation with RL has been associated with increased neutrophil activation after hemorrhage, and high volume administration of saline can lead to hyperchloremic acidosis(14, 15). Due to problems associated with over-resuscitation and edema, hypertonic solutions have also been used but require close monitoring due to risks of hypernatremia and subsequent renal failure(16). Recently, there has been an increased shift to using blood products, including plasma, as it is a physiologic fluid that may help prevent excess vascular leakage after burn injury(17). A rat model of burn injury demonstrated that addition of fresh frozen plasma (FFP) to resuscitation fluid helped diminish endothelial leakage(18). However, there are limited studies on the effect of the type of resuscitation fluids on the burn microbiome. One study using a swine burn model demonstrated that resuscitation volumes could influence the gut microbiota, such as a dose-dependent increase in *Bacteroidetes* and ameliorating growth of harmful *Proteobacteria* populations(19). A similar study used different resuscitation fluid paradigms in a swine model and found that while all groups experienced intestinal microbial dysbiosis following burn injury, limited-volume crystalloid (LV-Cr) resuscitation led to the most drastic dysbiosis and hepatocellular damage(20). However, further research needs to be done to fully understand the impact of burn resuscitation protocols on the microbiome.

While fluid resuscitation is paramount to burn treatment, it does not fully restore organ function. Burn patients still have numerous systemic abnormalities that must be closely monitored and treated. Inflammatory cytokine levels are consistently elevated in mouse models of burn injury for several days and a similar trajectory was observed in pediatric burn patients(21, 22). Finally, patients with severe burns enter a hypermetabolic state, and combined with systemic inflammation, are at risk for secondary infection, sepsis, and multiple organ failure.

Even after sufficient fluid resuscitation, patients still have experienced significant organ ischemia, and secondly, aggressive infusion of fluids leads to rapid re-introduction of oxygen to ischemic tissues, production of reactive oxygen species (ROS), and formation of free radicals. A major source of free radicals are neutrophils activated by damage associated molecular patterns (DAMPs) derived from injured tissue, leading to oxidative stress and injury of organs(23-26). Free radical-mediated injury after burn injury has been documented in numerous organs, including the lungs, liver, and the gastrointestinal tract(27-30). A number of studies have investigated antioxidants such as Vitamin C as a supplemental therapy to combat the severe oxidative stress encountered after a burn injury(31, 32).

Furthermore, this systemic inflammatory phase is characterized by release of inflammatory cytokines such as IL-1, IL-6, IL-18, and tumor necrosis factor (TNF), leading to further detrimental effects in numerous organ systems(33). The uncontrolled inflammatory response and release of cytokines can lead to systemic inflammatory response syndrome (SIRS),

characterized by overactivation of the immune response(34). This uncontrolled activation of the inflammatory response leads to organ tissue damage, which exacerbates the injury itself. Under these circumstances, the inflammatory mediators produced by the host are causing more damage to organ systems rather than eliminating subsequent infections. Due to this, patients with severe burn injuries are also at risk to develop secondary infections, which can lead to sepsis and multiple organ failure. Several studies have implicated the gastrointestinal tract as a source of bacterial endotoxin products and infection due to its large reservoir of bacteria(35-38).

2.1 Gastrointestinal abnormalities following burn injury

Following a severe cutaneous burn injury, the gastrointestinal system is adversely impacted by the initial hypoxia, the subsequent free radical injury and inflammation, leading to deficits in gastrointestinal (GI) barrier integrity and immune function(39). The GI tract is host to the body's largest reservoir of bacteria and maintaining homeostasis between the host and the microbiome is crucial. Numerous abnormalities in the GI system have been documented in burn patients, including increased gut permeability, decreased gut motility/transit, and increased gut bacteria translocation(40-46). Bacterial products and bacteria themselves have been detected in the mesenteric lymph node, liver, and lungs following injury(35, 38, 47). This infiltration of bacteria can exacerbate the tissue damage by recruiting more neutrophils who continue to release ROS and free radicals(28). Burn injury has been reported not only to harm the gastrointestinal organs but alters the microbial populations themselves. Animal models of burn injury and reports from patients have shown that bacterial diversity is diminished and beneficial species, such as Bifidobacterium, are decreased compared to healthy controls(36, 48, 49). Trauma patients also exhibit these changes within 72hrs, showing a reduction in *Bacteroidales, Fusobacteriales* and *Verrucomicrobiales*, accompanied by an increase in *Clostridiales* and *Enterococcus* bacteria(50). GI functional deficiencies can be complicated by treatments used for the burn injury, including opioid analgesics, which may inhibit gut transit and reduce motility(51). This slowed transit of digestion products can lead to increased bacterial overgrowth in the intestines and overgrowth of pathogenic bacteria, in particular *Enterobacteriaceae*(47, 52). Similar to analgesics, antibiotic usage is ubiquitous due to high rates of mortality associated with secondary infections in burn patients. However, its usage leads to disruption of the gastrointestinal microbiome and introduces another confounding factor in the management of burn patients(53-56). As expected, antibiotic usage is linked to a decrease in the diversity of the gut flora and can allow multi-drug resistant strains to flourish(57). Others have demonstrated that the microbiome plays a crucial role in numerous disease states, including in unexpected areas, such as the regulation of cardiorespiratory control and the strength of the immune response derived from vaccines(58, 59). Some studies have used manipulation of the microbiome to their advantage. In trauma patients, selective decontamination of the digestive tract (SDD), a prophylactic regimen of non-absorbable antibiotics with the aim to prevent nosocomial infections in critically ill patients, has shown to have some benefit in morbidity and mortality(60-62). However, there is still limited research in how the microbiome can affect the disease course of burn patients, as well as their potential as a therapeutic target or therapeutic that may aid the recovery of burn patients.

3. CONFOUNDING VARIABLES IN BURN INJURY

3.1 Alcohol Use

Most instances of burn injury do not occur in isolation and are complicated by factors such as the patient alcohol and/or drug usage. In nearly half of all burn injuries, patients are intoxicated at the time of admission following burn injury(63). Alcohol leads to lowered inhibition, loss of dexterity and balance, and leads to increased accidental injuries, including burn injury(64). It is well-established that alcohol intoxication at the time of burn injury leads to worse outcomes, including increased hospital stay, increased infection rates, and a higher rate of surgical procedures(65). Alcohol alone is a risk factor and one of the leading causes of morbidity and mortality worldwide(66). Furthermore, alcohol usage can disrupt the microbiome, leading to dysbiosis, as well as alter permeability of the intestinal barrier. Studies have shown increased levels of endotoxin in alcoholic patients compared to controls, as well as increased small intestinal bacterial overgrowth(67-69). Ethanol exposure also alters immune responses, such as decreasing NLRP3 activation and cytokine production(70-72). Taken together, alcohol exposure at the time of burn injury potentiates end organ damage, including the lungs, liver, and gastrointestinal tract(73-77).

3.2 Advanced Age

Adults over the age of 65 are a growing percentage of the population(78). Within individuals of advanced age, there is increased incidence of chronic illnesses and a phenomenon known as "inflamm-aging," which is a constant low-grade inflammatory state(79). Aging individuals have higher levels of TNFα and IL-6, but have diminished immune responses and are more susceptible to infections such as pneumonia(80, 81). Advanced age also leads to poor immune responses after burn, including delayed wound-healing, delayed inflammatory responses, and dysregulation of immune cells such as monocytes(82-85). While the elderly experience immune senescence with advanced age, the composition of their microbiome also changes, including greater proportion of *Bacteroides* and *Clostridium* groups(86-88). In a study using germfree mice, FMT were performed from young or old donor mice and transferred into young germfree mice. The mice inoculated with aged gut microbiota demonstrated increased intestinal inflammation and increased translocation of bacterial products from the gut lumen into systemic circulation, as well as immune cell activation(89). As for burn injuries, elderly patients consistently have poorer outcomes compared to younger patients, including increased morbidity, mortality, a delayed initial inflammatory response, followed by a prolonged hyperinflammation long after burn(84, 90). All of these aberrations in the aged population can compound in increased morbidity and mortality observed in elderly patients following burn injury, but the contribution of the microbiome is still poorly understood in the context of the elderly and acute injuries such as burn.

3.3 Sex

It is well-documented that there are differences in immune responses, leading to sexual disparities in numerous areas including the rate of autoimmune diseases and mortality after infection. Sex hormones are known to regulate immunity, with a general trend of females having a stronger immune response during reproductive age, including a more robust

humoral response(91-93). Differences have also been observed in traumatic injuries(94-96). In a rat study of trauma/hemorrhagic shock (T/HS) and burn injury, females showed less lung and gut injury following both injury models compared to male mice. When the female rates were ovariectomized, this protection was lost(97). Neutrophil response to a T/HS or burn injury rat model also demonstrated sexual dimorphism. CD11b surface expression and respiratory burst activity was increased in male rats after injury compared to female rats. This effect was reduced with castration of male rats rats(98). In a study of trauma patients, there was a slight increase in survival for women under the age of 40 with an Injury Severity Score between 16 and 24. Women also had less infectious complications, however, they did have a higher rate of death with infection(99). Conversely, in an analysis of patients from the National Burn Repository, female patients had a higher incidence of death as a result of burn injury across all age groups compared to men(100). More studies are needed to elucidate the effect sex has on burn patient survival and recovery.

4 THE MICROBIOME

The microbiome constitutes a diverse and complex population of bacteria, fungi, viruses, and other microorganisms that colonizes a specific location of the body. Although there are several different microbiomes within the human body, including the skin and oral mucosa, this review will focus on the gastrointestinal microbiome. The GI or gut microbiota is the largest reservoir of bacterial species in the human body, with upwards of 10^{12} bacteria per gram of luminal contents(101, 102). In addition to large quantities of total bacteria, the human gut microbiota also exhibits sizable numbers of individual bacterial species. Although it was previously believed that around 1,000 bacterial species are present in the gut, recent studies indicate there may in fact be over 15,000 individual species of bacteria in the human gut microbiome(103-105).

The earliest studies into the microbiome were hampered by the inability to culture the majority of bacteria present in the gut. Scientists have since harnessed the power of high through-put sequencing to make advances in understanding the composition and function of the microbiome. A commonly used method to detect and assess bacterial species is 16S ribosomal RNA (rRNA) sequencing. The 16S rRNA gene is an evolutionarily conserved component of the ribosome consisting of both conserved and a hypervariable region. Researchers can differentiate between bacterial species by sequencing the hypervariable region of this gene. With the advent of next generation sequencing, newer studies are beginning to utilize whole-genome sequencing. These approaches allow scientists to detect strains of individual bacterial species and analyze pathogenic bacterial genes. Overall, these methods have expanded the studies able to be performed and shaped our understanding of the human microbiome in health and disease.

4.1 Normal Bacterial Composition

The human gut microbiome is a diverse community of bacterial species that varies widely between individuals. Bacterial community structure is thought to be influenced by individual genetics, diet, environmental factors and exposure to microbes(106). In addition, differences in both bacterial abundance and composition are apparent throughout the length of the

gastrointestinal tract. However, the microbiome of the distal small intestine, cecum and large intestine share many commonalities(107). Throughout the years, this complexity has hampered the characterization of how a normal and healthy gut microbiome is structured. Nevertheless, scientists have identified several bacterial phyla and associated genera that are present across a number of intestinal microbiome studies. In general, the human intestinal microbiome is predominately composed of bacteria belonging to the *Firmicutes, Actinobacteria* and *Bacteroidetes* phylum(102). Core microbial genera belonging to the Firmicutes phyla include Ruminococcus, Faecalibacterium, Eubacterium, Clostridium and Roseburia(108). Pathogenic bacterial species, such as *Enterobacteriaceae*, are generally present at a very low abundance of <0.1% or less of the bacterial population(102). Recent studies looking at gut bacteria compositions across individuals have identified three general bacterial profiles, or enterotypes, that normal human gut microbiome samples cluster into(109, 110). However, studies are ongoing to determine what impact a person's enterotype may have on disease outcomes and their diagnostic potential. In addition to the presence or absence of specific bacterial species, overall diversity of the gut microbiome is an important indicator of health. In the field of microbiome research, the diversity of bacteria found within a single sample is termed alpha diversity. This is determined by assessing the evenness (abundance of a species) and richness (number of different species present) of the microbiota. A healthy gut microbiome exhibits high diversity and studies indicate that individuals with lowered microbiome diversity are more associated with inflammatory phenotypes, obesity, and insulin resistance(111).

4.2 Functions of the Gut Microbiota and Microbial Metabolites

Studies involving germ-free mice, which lack a resident microbiome, reveal several crucial functions for the microbiota in regulating intestinal homeostasis. Compared to mice raised in normal conditions, germ-free mice exhibit severe defects in mucosal immunity. Additionally, they have been shown to have reduced intestinal motility and vascularity, deficient cytokine production and reduced epithelial cell turnover(112). Historically, the gut microbiome has been studied for its vital role in host metabolism. The resident bacteria of the gut are responsible for the fermentation of otherwise non-digestible complex carbohydrates and other xenobiotic compounds. The resulting bacterial metabolites provide a critical source of energy for cells throughout the body. Furthermore, these metabolites can enter the bloodstream where they have been shown to impact overall host metabolism, weight, and insulin sensitivity(113-115). Short-chain fatty acids are widely studied bacterial metabolites that include acetate, propionate, and butyrate. Acetate and propionate are primarily used by the liver and other peripheral tissues, where they are often converted into glucose or lipids. Butyrate is a major source of energy for the intestinal epithelium, with some studies indicating it may provide up to 80% of the energy source(116-118). A wide variety of bacteria belonging to the Bacteroidetes and Firmicutes phyla can produce different SCFAs. *Bifidobacterium* species are widely studied for their ability to produce the SCFA butyrate. Additional butyrate producing bacteria include *Ruminococcaceae* family members, such as *Faecalibacterium prausnitzii*, and various species of *Roseburia* within the family of *Lachnospiraceae*(119-121). In addition to regulating energy balance, SCFAs have been shown to promote intestinal barrier function via several mechanisms. Treatment of intestinal epithelial cells with SCFAs increases proliferation and tight junction expression

and reduces epithelial permeability(122-124). Furthermore, SCFAs can modulate mucosal immune responses to suppress intestinal inflammation. For example, acetate has been shown to promote intestinal IgA production(125). In general, butyrate is considered an anti-inflammatory molecule that maintains immune homeostasis in the gut by promoting anti-inflammatory cytokines like IL-10 and maintenance of regulatory T cell function(126-128). Apart from regulating host metabolism and providing sources of energy, the bacteria that comprise the gut microbiota also regulate GI tract structure and integrity, modulate immune functions, and provide antimicrobial protection(102, 108). Therefore, maintaining a healthy gut microbiome is essential for the proper function of the gastrointestinal tract.

4.3 Health Implications of Microbial Dysbiosis

Although the bacterial populations of an individual vary over time, an important aspect of the human microbiome is its resilience and ability to recover from disturbances, such as antibiotic use(129). The inability of the gut microbiome to return to a healthy state is associated with dysfunctional gut health and a variety of diseases. Bacterial dysbiosis is described as a significant change in the intestinal microbiome, such as reduced diversity and increased abundance of pathogenic bacteria, that can result in adverse health effects(122). Many studies over the years have focused on the connection between bacterial dysbiosis and chronic inflammatory disorders of the gut. For example, gut bacterial dysbiosis is a common feature of patients with colorectal cancer and has been associated with chronic inflammation and colorectal cancer progression(130-132). The dysregulated immune responses central to inflammatory bowel disease (IBD) have been linked to dysbiosis of gut bacteria. Patients suffering from IBD display gut microbiomes that are distinct from healthy controls, with decreases in several beneficial bacteria and increases in *Enterobacteriaceae*, such as *Proteobacteria*, correlating with disease state(103, 133). Far less is understood about the impact of gut bacterial dysbiosis on more acute disorders, such as trauma and burn injury. Therefore, more research is needed to understand the contributions bacterial dysbiosis may have on the pathophysiology of severe burns.

5 IMPACT OF BURN INJURY ON GUT MICROBIOTA

It is clear from studies of inflammatory GI disorders, like IBD, that changes in the microbiome can significantly influence disease progression and outcomes. Although there are distinct differences between chronic disorders and acute trauma, like severe burn injury, both are impacted by intestinal inflammation and gut barrier disruption. Consequently, it is likely that bacterial dysbiosis would be prominent after severe burn injury and contribute to post-burn pathologies. This section will discuss changes in gut microbiota composition that occur after severe burn injury.

To begin to assess the microbiome's impact on post-burn pathogenesis, early studies focused on detailing how the composition of the microbiome is altered after severe burn. Utilizing fecal samples from severe burn patients, Earley *et al.* showed clear evidence for bacterial dysbiosis following burn injury. The microbiome of control patients was dominated by *Bacteroidaceae* and *Rimunococcaceae* families, indicating healthy microbiome compositions. Both of these dominant bacterial groups were decreased in severe

burn patient samples, which exhibited overall significantly different compositions compared to control samples by non-metric multidimensional analysis(47). Additionally, they observed a significant increase in the abundance of Enterobacteriaceae, a bacterial family that includes several pathogenic species. Indeed, the most abundant *Enterobacteriaceae* taxon (OTU) found in severe burn patients was attributed to *E. coli* O83:H1, a pathogenic and invasive strain of *Escherichia coli*(47). Other studies investigating compositional changes in the microbiome of severe burn patients reiterate reduced bacterial diversity accompanied by a decrease in beneficial bacteria like *Bacteroidaceae* and an increased presence of pathogenic *Escherichia* bacteria belonging to the *Enterobactericeae* family(48, 134). *Escherichia coli* is commonly associated with bacterial translocation and sepsis, which are known complications of severe burn injury(135). Therefore, increased abundance of *Enterbactericeae*, particularly strains of *Escherichia coli*, could provide a therapeutic target for reducing mortality associated with severe burns. As the abundance of bacterial species in the gut change following burn injury, there are likely accompanied by changes in the concentration of different bacterial metabolites. As discussed previously, SCFAs are widely studied bacterial metabolites that provide energy, modulate immune responses, and regulate gut barrier integrity. These beneficial metabolites are produced by several species among the *Bacteroidaceae* and *Rimunococcaceae* families, which have been shown to be reduced after severe burn injury(47, 48). In particular, studies show that after burn, patients exhibit decreases in the abundance of fecal *Bifidobacterium*, which is a major producer of butyrate(48). Additionally, investigators have assessed the concentrations of SCFAs in the stool samples of severe burn patients. One study following five patients with severe burns noted that the levels of SCFAs, including acetate, propionate, and butyrate, were reduced relative to normal levels. Although SCFA levels generally recovered as the patient did, levels of propionate and butyrate remained undetectable in the lone non-surviving patient(48). It is important to note that the few human studies described here utilize small patients sample sizes. Further research using patient samples is required to advance our understanding of how severe burn injury impacts the gut microbiome.

To reduce the impact of confounding factors present in patient studies, such as the complex diversity of the human microbiome and therapeutic interventions like antibiotics, several studies have examined the effect of severe burn injury on the microbiome in murine models. It should be noted that although both the mouse and human gut microbiomes are dominated by *Bacteroidetes* and *Firmicutes* phyla and share similarities in composition at higher taxonomic levels, including similar enterotype clustering, there are significant differences in composition at the bacterial species level(136, 137). In addition, human microbiome studies utilize stool samples while murine studies generally examine the bacterial content of the cecum. While there is significant overlap in the bacteria present throughout the lower GI tract (distal small intestines, cecum, and large intestine), the differences in bacterial composition based on location of sample collection can complicate analysis and is an important consideration. Although species differences may be present between humans and mice, similarities in bacterial families and dominant microbes indicate some conservation of the influence the microbiome has on intestinal function. Therefore, murine models represent a valuable tool that allow scientists to map broad shifts in microbial composition and examine the functional outcomes that may contribute to post-burn pathophysiology.

Several studies utilizing murine models of severe burn injury have shown changes in the gut microbiome after burn that mirror the bacterial dysbiosis found in human studies, including decreased abundance of dominant beneficial bacteria accompanied by an increase in the *Enterobacteriaceae* family, such as *E. coli* species(47, 49, 138). In addition, murine studies have shown that severe burn results in decreased SCFA levels, particularly butyrate, in cecal contents immediately following injury and for up to 7 days after injury(36, 139, 140). Accordingly, it has been found that the abundance of SCFA producing bacteria are reduced in murine models of severe burn injury. A study utilizing Sprague-Dawley rats and a 30% TBSA burn model showed a significant decrease in *Clostridium IV* and *Clostridium XIV*, which are both known butyrate producers(138). Furthermore, they found reduced levels of *Lactobacillus*, a commonly used probiotic, after burn injury(138). Studies show that the metabolite lactate, produced by *Lactobacillus*, is readily utilized by other bacteria to produce butyrate, and is therefore an important contributor to SCFA levels(141). These changes found in rats are consistent with mouse models of severe burn injury. A study published by Beckmann et al. revealed a reduction in *Lactobacillaceae* and *Clostridiaceae* bacterial families in mouse cecal contents after burn injury(49). Likewise, Feng et al. showed that Lactobacillus was a principal species, reduced after burn injury, that significantly correlated with SCFA levels(36). Interestingly, they also found that butyrate levels negatively correlated with the abundance of a prominent enteric pathogen, *Escherichia-Shigella*(36). This indicates that changes in the levels of SCFAs and the bacteria that produce them could potentially influence the abundance of pathogenic bacteria, thereby contributing to post-burn pathogenesis via multiple pathways.

6   THE GUT BARRIER, BACTERIAL TRANSLOCATION, AND BURN

The gastrointestinal tract is the largest mucosal surface in the body, responsible for regulating nutrient absorption while maintaining a barrier against environmental toxins and pathogens (Figure 2). The proximity of resident microbiota ensures that the barrier formed by intestinal epithelial cells is crucial for maintaining a homeostatic relationship between the host immune response and resident microbes. This intestinal epithelial barrier includes tight junction proteins that maintain close association of epithelial cells with each other, in addition to antimicrobial peptides (AMPs) and mucus which prevent bacterial overgrowth and invasion(142, 143). These components make intestinal epithelial cells the primary physical barrier that prevents enteric infections. Consequently, disruption of the intestinal barrier can dramatically impact health and is associated with a wide variety of gastrointestinal disorders(144). Nearly every aspect of the intestinal epithelial barrier is affected by severe burn. Studies show that burn injury reduces epithelial cell tight junction protein expression, disrupts tight junction localization, reduces mucus production, and inhibits the expression of AMPs(145, 146). Overall, these changes result in increased intestinal permeability(145, 147). Bacterial translocation resulting from intestinal barrier disruption is thought to contribute to severe consequences after burn injury, including sepsis and multiple organ failure(135). Indeed, bacteria and their products have been detected in the lung, liver, and mesenteric lymph nodes after severe burn injury(47, 148, 149). Further research is required to investigate the mechanisms by which bacterial translocation after

severe burn contributes to mortality and the pathophysiology of systemic inflammation and organ dysfunction.

Due to the close interactions between intestinal epithelial cells and the gut microbiome, the impact of gut bacteria on intestinal barrier function has been of recent interest. In particular, the contributions of both commensal and pathogenic bacteria to chronic disorders, like IBD and celiac disease, have been widely studied. Beneficial commensal bacteria have been shown to promote epithelial cell barrier function by reducing epithelial permeability and increasing tight junction protein expression(150-152). Additionally, bacterial metabolites including butyrate, acetate, and indole, can enhance the intestinal barrier(153-155). On the other hand, several pathogenic bacteria have been linked to gut barrier disruption and intestinal permeability(150). Certain invasive strains of *Escherichia coli* have been shown to target components of epithelial cell tight junctions to promote permeability(156). Other pathogenic bacteria that are known to disrupt epithelial tight junctions include *Clostridium perfringens* and *Clostridium difficile*(157, 158). Although the connection between the gut microbiome and intestinal barrier integrity has been established in other models of gastrointestinal inflammation, few studies have been conducted to discern the impact that changes in the microbiome have on gut barrier integrity following severe burn injury. It is likely that the increased abundance of pathogenic bacteria, like *Enterobacteriaceae*, could exacerbate intestinal barrier disruption after burn injury and promote the translocation of bacteria and bacterial endotoxins from the gut into the bloodstream. Further research in this area could provide new therapeutic targets to reduce the severity of sepsis, multiple organ failure and other critical complications of severe burn.

7 SYSTEMIC IMMUNE RESPONSE TO BURN

The initial immune response to burn injury is characterized by rapid activation of the innate immune system, including neutrophils, monocytes, and macrophages (Figure 3). These cells are able to recognize damage associated molecular patterns (DAMPs) and pathogen associated molecular patterns (PAMPs) through Toll-like receptors and NOD-like receptors(159-161). This activates the transcription factor NFκB, which regulates numerous pro-inflammatory downstream mediators, including TNF, IL-6, and IL-1β(162). When looking at genome-wide changes in leukocytes following burn injury, changes in gene expression were similar to those observed under severe trauma or endotoxemia(163). Furthermore, these changes in transcripts were observed as early as 4h after injury and persisted for weeks, indicating that traumatic injuries, including burn, lead to a rapid alteration in immune response followed by a chronic disruption in their activity

7.1 Long-term Disruptions in Immunity

While the initial response to burn injury is characterized by rapid onset of inflammation and a hypermetabolic state, the long-term consequences of burn injury are characterized by chronic dysfunction of the immune system. Compared to non-burn trauma patients, burn-injured patients displayed higher levels of inflammatory markers IL-6 and IL-18 in the plasma(164). A study following pediatric patients showed that markers of hypermetabolism (resting energy expenditure, body composition, metabolic markers) were elevated for up

to three years post burn injury. Similarly, levels of catecholamines, cortisol, and acute phase proteins were elevated(165). A study of different types of skin trauma found that 84 days post-injury, burn injured mice had significantly elevated levels of IL-10 in the sera, accompanied by reduced numbers of lymphocytes, neutrophils, and eosinophils compared to animals receiving an excision injury(166) . In a study of sepsis patients, it was determined that higher IL-10 concentration in the sera correlated with increased mortality, likely due to immunosuppression(167). A similar elevation in IL-10 was observed in a prospective study of patients with systemic inflammatory response syndrome (SIRS)(168). Multiple studies have observed changes in serum cytokines between surviving and non-surviving burn patients and proposed prognostic indicators, including combinations of cytokines IL-6/IL-7/IL-10, and IL-1RA/IL-6/MCP-1(169, 170). Other cytokines, such as IL-27, have also been proposed as a diagnostic biomarker for sepsis or infection in critically ill patients(171-174).The cytokine milieu in burn patients is complex, and while numerous biomarkers have been identified for these critically ill patients, the profile of each patient is different and likely requires individualized care.

7.2 Neutrophils

There are numerous deficits in the pro-inflammatory response observed in several cell types following burn injury. For example, neutrophils are the first responders in many instances of injury, including burns. Studies have shown increased neutrophil populations in the blood, intestine, and lungs after burn injury(28, 29, 175). While key responders to the initial injury, neutrophils in burn patients have been reported to have reduced phagocytic and bacterial killing ability up to 28 days after burn injury(176). Furthermore, in an animal model of burn injury and secondary infection, neutrophils have been reported to exhibit reduced recruitment to the lungs after intranasal administration of lipopolysaccharide (LPS), as well as reduced NETosis or formation of neutrophil extracellular traps (NETs). NETs are a key component of neutrophil bactericidal activity to contain bacterial infection and infiltration(177). Similarly, directional migration speed of neutrophils was significantly reduced in neutrophils isolated from burn patients and correlated with the size and severity of the burn(178). Following the initial burn injury, neutrophil recruitment into the GI tract due to hypoxia and cellular damage compounds the injury through production of ROS, and studies have shown that neutrophils have delayed apoptosis after burn injury(179). This prolonged lifespan is problematic, as inflammation persists in organs and prevents resolution of the inflammatory process. Studies observing intestinal damage in Graft versus Host Disease (GVHD) have also identified neutrophils as a key player in intestinal damage once recruited and observed that eliminating their ability to produce ROS significantly improved tissue damage and reduced effector T cell recruitment(180). Paradoxically, while neutrophils have prolonged lifespans after burn injury, they are worse at eliminating repetitive infections from pathogens. A mouse model of cutaneous burn injury found that animals infected with *Pseudomonas aeruginosa* pneumonia were worse at clearing the bacteria when given two repeat infections. While they mounted a hyper-inflammatory response to the first infection, the repeated infection merely increased recruitment of neutrophils and macrophages to the lungs, without clearing the infection itself(181). Poor neutrophil bactericidal activity in response injury contributes to poor outcomes in burn patients and is likely exacerbated by documented shifts in the microbiome to overgrowth of pathogenic bacteria. However,

the effect of bacterial changes on neutrophil function in the context of burn injury is not sufficiently understood or explored.

7.3 Macrophages

Similar to neutrophils, macrophages are activated by DAMPs and PAMPs in response to burn injury. After burn injury, macrophages produce copious pro-inflammatory cytokines, prostaglandins, and reactive nitrogen species(181-183). This hyperactive response by macrophages is thought to contribute to increased susceptibility of burn patients to sepsis due to the "two-hit" theory, in which an initial insult primes the patient's immune response to be hyper-inflammatory, followed by a second insult (infection or sepsis). Following the second insult, the patient is unable to mount an appropriate immune response due to the initial hyperinflammatory state and succumbs to multiple organ failure(184). Again, dysbiosis in the burn microbiome to preferentially allow overgrowth of harmful species, such as the *Enterobacteriaceae* family, are likely to exacerbate the two-hit theory of burn injury and subsequent sepsis. Macrophage hyperactivity results in elevated TNF, IL-6, and nitric oxide (NO) levels following burn injury. In particular, excess NO production by macrophages isolated from burn injured mice suppressed proliferation of splenic T cells(185). While macrophages are hyperinflammatory in the short term, long-term effects of burn injury on hematopoiesis in the bone marrow lead to perturbed development of monocytes. As early as 48h after injury, monocyte progenitors isolated from bone marrow of burn and burn sepsis mice show impaired TNFα production following LPS stimulation(186). These unresponsive progenitors eventually replace the initial pool of hyperreactive macrophages, leading to the immunosuppression observed later in burn injury recovery.

7.4 Antigen Presenting Cells

Macrophages are only one type of antigen presenting cell (APC) that are crucial in initiating adaptive immune responses, including activation of T cells. APCs isolated from burn injured mice have a demonstrated weakness in activating the proliferation of naïve T cells(187). Dendritic cells (DCs) are another subset of professional antigen presenting cells and are an essential bridge between the innate and adaptive immune response. Dendritic cells isolated from burn-injured mice demonstrated a dampened response to TLR9 stimulation and had impaired T cell activation activity(188, 189). Burn injury also led to significant reduction of CD11c^{+} DCs in lymph nodes, along with upregulation of programmed death ligand 1 (PD-L1) expression on splenic DCs, which is a potent suppressor of T cell activation(190). PBMCs isolated from burn patients demonstrated that overexpression of transcription factor MafB correlated with the dendritic cell depletion observed in burn patients, and silencing MafB in an in vitro culture system restored myeloid dendritic cell differentiation(191). This long-term perturbance in APC development and differentiation contributes to post-burn pathogenesis. While trauma and burn injury is often described by its initial hyperinflammatory state, the long-term consequences of burn injury is characterized by immunosuppression and increased susceptibility to infection.

7.5   T Cells

In concert with deficiencies in the innate immune system, there are well-documented effects of burn injury on T cells and their ability to mount a response to infection. T cells maintain a balance of pro and anti-inflammatory activity in response to various stimuli. Naive T cells are activated by DCs through antigen presentation and antigen recognition by the T-cell receptor (TCR), along with co-stimulatory molecules such as CD28 and cytokines. Both CD4 and CD8 positive T cells have demonstrated perturbations following burn injury(192, 193). In a mouse model of burn injury, there was a significant decrease in the number of splenocytes following a 20% TBSA burn. This decrease in cellularity was persistent for 5 days after injury and a diminished proliferative response was observed in splenocytes(194, 195). Similarly, T cells isolated from burn injured mice demonstrated reduced IL-2 production and augmented interferon gamma (IFNγ) production(196). IL-2 is particularly important to stimulate proliferation of T cells, and a study of burn patients found that while all burn patients demonstrated a decrease in IL-2 production by peripheral blood mononuclear cells (PBMCs), surviving patients had a gradual recovery of IL-2 whereas non-survivors did not recover IL-2 production(197). In addition to decreased proliferation, T cell survival is reduced upon burn injury complicated with sepsis. Findings from a combined burn and sepsis model show significant reduction in T cell population of the mesenteric lymph node by apoptosis(198). T cells isolated from peripheral blood of burn injured patients did not show a significant change in the ratio of CD4 to CD8 T cells, however, there was a demonstrated increase in the release of cytokine IL-4 by day 5. This increase in IL-4 production was mainly by CD8 T cells(199). Regulatory T cells (Tregs) are another important subset of T-cells altered after traumatic injuries such as burn. Under normal conditions, Tregs maintain a careful balance by preventing overactivation of inflammatory mediators through suppression of T cell proliferation/activation and production of anti-inflammatory cytokines such as IL-10 and TGF-β. In a study of peripheral blood cells taken from patients, Tregs isolated from burn patients showed higher cell surface expression of key suppressive mediators such as CTLA-4. IL-10 and TGF-β production by Tregs was also increased in burn patients compared to healthy controls(200). Progressive immunosuppression is observed after trauma, and it is well-established that Tregs play a key role in depressing immune function, including reducing production of IFNγ by Th1 cells following injury(201). Tregs isolated lymph nodes draining the injured areas from burn injured animals are found to show markers of T cell receptor (TCR) activation after injury, including phosphorylation of ZAP70 and nuclear factor of activated T cells (NFAT)(202, 203). Increased Treg activity has also been noted in trauma patients and correlates with poor clinical outcomes(200, 201). Similarly, studies have shown that depletion of Tregs prior to injury restores the proliferative capacity of CD4 T cells following burn injury in mice(204). In addition, Th17 cells have been identified as crucial players after burn injury. At the injury site itself, Th17 cytokines such as IL-17 and IL-22 are elevated 3h post-injury(205). While IL-17 is considered inflammatory and can aid in the recruitment of neutrophils, IL-22 promotes integrity of mucosal barriers, such as in the lungs or GI tract. IL-22 acts to promote tight junction formation and proliferation of intestinal epithelial cells, which is integral to maintaining proper barrier function after burn injury and preventing multiple organ failure or sepsis from bacteria originating from the gut(205).

8 INFLAMMATORY RESPONSE IN THE GI TRACT

Some of the most dire consequences of burn injury are the result of remote organ injury, such as inflammation in the lungs, kidney, gastrointestinal system, and bone marrow(47, 186, 206-210). While systemic inflammatory responses account for some of the organ dysfunction observed, it is suggested that one of the main drivers of inflammation and perturbed immune responses following burn injury is the translocation of gut bacteria and/or bacterial products from the GI lumen to systemic circulation. The gut-origin hypothesis of sepsis, SIRS, and multiple organ dysfunction after burn injury states that breakdown of the gut barrier and hyperpermeability leads to the transport of toxic agents derived from gut bacteria into the portal circulation and mesenteric lymph(211). Toxic agents can include bacterial products like peptidoglycan or endotoxin, which can travel to distant organs to cause inflammation and organ failure. In support of this hypothesis, animal models of trauma have found that ligation of the mesenteric lymph duct can prevent acute lung and renal injury following intraperitoneal injection of LPS and models of hemorrhagic shock in rodents(212-214). Similarly, mice given prophylactic antibiotics prior to burn injury reduced hepatosteatosis and liver injury markers(215). However, further studies must be done on burn injury to connect microbial dysbiosis in the gut to end organ dysfunction.

The gut microbiome is a crucial regulator of the immune system under homeostatic conditions, and imbalances, such as during burn injury, can have serious consequences on the function of the immune system. As stated previously, it is well-established that a healthy gut microbiome is crucial to the development of a healthy and robust immune response. Studies on germ-free mice have shown that the microbiome is necessary for appropriate development of mucosal immunity. Similarly, peripheral lymphoid organs, such as mesenteric lymph nodes and Peyer's patches are underdeveloped in germfree mice. Abnormal IgA and T cell responses are also observed(216, 217). A study of healthy volunteers found that the composition of their microbiome is directly correlated to their cytokine response as measured by stimulation of their peripheral blood mononuclear cells (PBMCs), indicating that differences in alpha or beta diversity correlate directly with their immune response(218). Other studies have indicated that dysbiosis can lead to altered response in specific immune cells. For example, mononuclear cells isolated from patients following antibiotic usage and diminished microbial diversity produced significantly decreased levels of TNFα following LPS stimulation(219). In a model of acute kidney injury following ischemia and reperfusion injury, supplementation of bacteria metabolites acetate, propionate, and butyrate reduced organ damage and inhibited the maturation of DCs, as well as inhibiting proliferation of T cells(220). In mouse models of sepsis, fecal microbial transplantation (FMT) from healthy littermates improved survival by 70%(221). In a similar study, mice receiving FMT from septic patients followed by cecal ligation and puncture (CLP) to induce sepsis had more severe liver damage compared to those receiving FMT from healthy donors(222). However, the contribution of the microbiome to immunological abnormalities following burn injury is not well-understood and lacking in research.

9 THERAPEUTIC POTENTIAL OF MANIPULATING THE GUT MICROBIOME

Over the years, manipulation of the microbiome has proven a promising therapeutic for trauma patients. The earliest studies of critically ill patients found that complications including pneumonia, urinary tract infections and sepsis were highly associated with enteric pathogens(223). Following this, studies were conducted to identify the benefit of antibiotic administration in critically ill patients. The resulting regiment of systemic and enteric antibiotics was proposed as selective digestive decontamination (SDD), which would go on to be studied in a variety of critically ill patient populations(223). A systematic review of studies involving the use of SDD and non-absorbable enteral antibiotics in severe burn patients showed reduced incidence of bloodstream *Enterobacteriaceae*, reduced incidence of pneumonia and overall improved survival of severe burn patients(224). However, widespread and long-term use of antibiotics can contribute to the propagation of antibiotic resistance and chronic disruption of the gut microbiota(55, 56). Further investigation into the complications associated with antibiotic use are required to delineate the impact their use may have on patients suffering from traumatic injuries.

More recent studies attempt to manipulate the microbiome using more targeted methods than general antibiotic use. As an alternative to removing bacterial populations from the gut, several researchers are studying the impact of fecal microbiota transplant and probiotic administration. FMT involves transfer of fecal matter from a healthy individual into the intestinal tract of another. This process attempts to cultivate a healthy microbiota in the patient that supports microbiome stabilization and gut function. After gaining increased prominence for its successful treatment of *Clostridium difficile* infections in hospitals, FMT is being assessed experimentally as a treatment for a variety of other gastrointestinal disorders(225). Probiotics utilize the specific administration of a few beneficial bacterial species to bolster commensal populations and promote healthy gut functions. Although many preliminary studies have been limited to mouse models, several indicate that FMT and probiotics are promising therapeutic options for severe burn patients(226). A recent study by Kuethe et al. utilized a mouse model of severe burn injury in which intestinal permeability and bacterial dysbiosis are evident at six days post burn. FMT was prepared from the cecal contents of control mice and administered via oral gavage twice daily on post-burn day two and three. They found that FMT restored mucosal integrity and reduced bacterial dysbiosis evaluated on post-burn day six(140). Their study indicates that FMT may be a viable therapeutic for severe burn injury, although further studies are required to assess its relevance in human patients.

Bifidobacterium are important butyrate producing bacteria that have been shown to promote gastrointestinal health in a variety of human studies, including alleviating symptoms of IBS and antibiotic associated diarrhea(227). As mentioned previously, Bifidobacterium are among the SCFA producing bacterial species that are significantly reduced in severe burn patients(48). Although there are currently no comprehensive human studies in the use of *Bifidobacterium* in severe burn patients, a mouse study by Wang et al. reveals that administration of Bifidobacteria after burn injury reduced intestinal mucosa damage and bacterial translocation(228). Utilizing a mouse model, Zhang et al. identified significantly reduced abundance of the butyrate producing bacteria *Clostridium butyricum* (*C. butyricum*)

following burn injury, which correlated with reduced fecal butyrate and increased intestinal permeability. Oral administration of *C. butyricum* 24 hours after burn injury enhanced butyrate levels and reduced intestinal damage and permeability(229). In addition to the administration of butyrate producing bacteria, treatment of mice with sodium butyrate also protects against severe burn induced intestinal permeability and promotes the expression of intestinal epithelial cell tight junction proteins(146). Additionally, sodium butyrate treatment reduced acute lung injury and systemic inflammation after severe burn in a rat model(230). Therefore, the therapeutic targeting of butyrate production via probiotic administration appears to be a promising area of research in severe burn injury. However, it is important to note that not all bacterial strains and treatment regimens have been found to provide positive effects. In a human study of severe burn patients, prophylactic administration of *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* was not associated with improved patient outcomes(231). Consequently, additional research is required to fully understand the potential of targeting the microbiome in therapeutics for burn injury and the mechanisms underlying the impact individual bacteria have on gut barrier function.

10   FUTURE DIRECTIONS AND PERSPECTIVES

Altogether, there is much that is still unknown about the contribution of the microbiome and microbial changes in the context of burn injury. Recent studies are elucidating the changes observed in the gastrointestinal microbial population in acute traumatic injuries, however, their effect on the subsequent immune response and recovery from burn injury are still understudied. Although dysbiosis, overgrowth of potentially harmful bacteria, breakdown in the intestinal barrier, and aberrant immune responses have been documented following burn injury, the causal relationship between these components requires further investigation and study. Current studies indicate that the reservoir of gastrointestinal microbes is a contributor to end organ damage across the body, including the lungs, liver, and kidneys. Recent work in inflammatory disease states have suggested that pre- and probiotics may be an avenue for further exploration in treating acute trauma patients. Similarly, beneficial bacterial SCFAs may be another area of potential therapeutic development. Studies of the microbiome in the context of acute trauma, such as burn injury, are likely to provide a rich source of potential therapeutic targets or agents. In addition, more studies should be conducted to understand mechanistically how the microbiome contributes to immune responses and dysfunction after burn injury.

Acknowledgments

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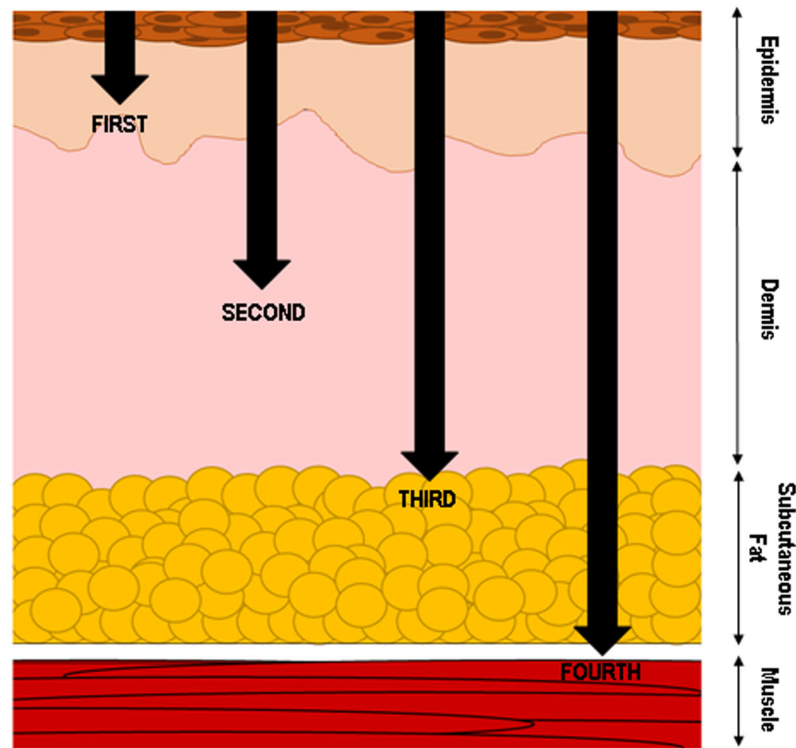
Figure 1. Categorization of burn wound injury.
In addition to wound size, the depth of tissue affected by burn injury contributes to the wound categorization, treatment and patient outcomes. Burn that are restricted to the epidermis are considered superficial and categorized as first-degree burns. Second-degree burns are partial thickness injuries that penetrate varying depths below the epidermis and into the dermis. Once the burn injury penetrates the entire dermal layer and begins to effect the subcutaneous fat layer, the injury is classified as a third-degree burn. These are considered full-thickness burns and result in the destruction of nerve endings which make the wound pain-free. However, burns of this depth require careful management and surgery to support healing and prevent wound infection. A burn that penetrates further and damages the underlying muscle, and even bone, are classified as fourth-degree burn. An injury of this severity often results in permanent damage to the tissue and possible amputation of the injured area.
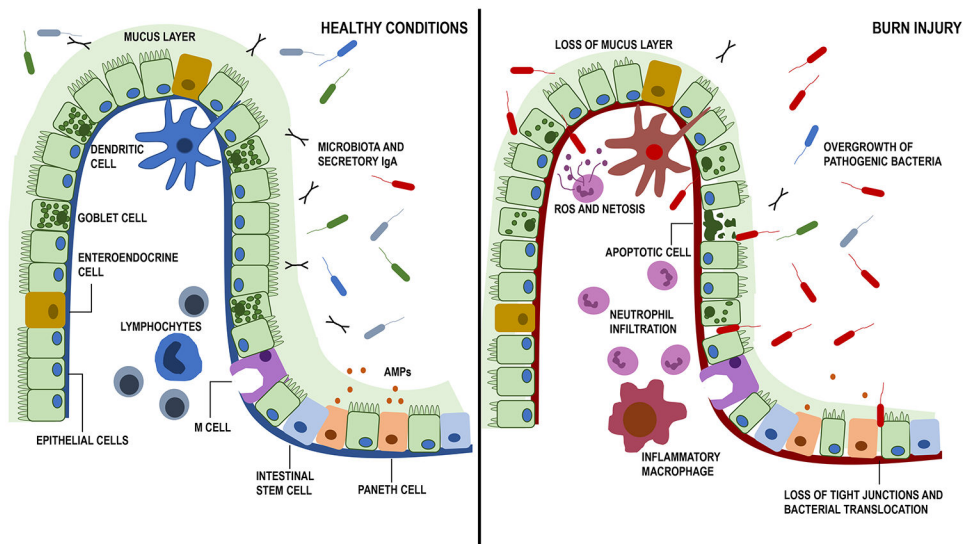
Figure 2. Representation of the intestinal barrier and gut microbiome under healthy and burn-injured conditions.

Under healthy conditions, the intestinal barrier is intact and composed of intestinal epithelial cells (IEC) that are tightly bound together by tight junction proteins. Goblet cells that produce mucus (light green) and enteroendocrine cells (yellow) are interspersed between IECs. In the intestinal crypt, there are intestinal stem cells (blue) that continuously replicate and regenerate IECs that are shed in the lumen. Paneth cells (orange) producing anti-microbial peptides (AMPs). M-cells (purple) are continuously testing luminal contents for uptake by any resident antigen presenting cells. Under the IEC barrier is the lamina propria, which contains a variety of immune cells, including dendritic cells, monocytes, and T/B cells (blue). Secretory IgA is continuously passed into the lumen to maintain homeostatic conditions with the microbiome. Under healthy conditions, there is a large diversity of intestinal microbiota with few pathogenic species. With burn injury (right panel), there is a loss of tight junction proteins and the mucus barrier, along with increased IEC apoptosis, leading to a leaky gut. Increased inflammatory cell infiltration by neutrophils and macrophages leads to production of reactive oxygen species (ROS) and further damage of the barrier, allowing for intestinal bacterial and bacterial products to translocate into systemic circulation. Furthermore, there is overgrowth of pathogenic bacterial species and loss of bacterial diversity in the lumen. This image was adapted from *Hammer et. al, Alcohol Res. 2015;37(2):209-22*.
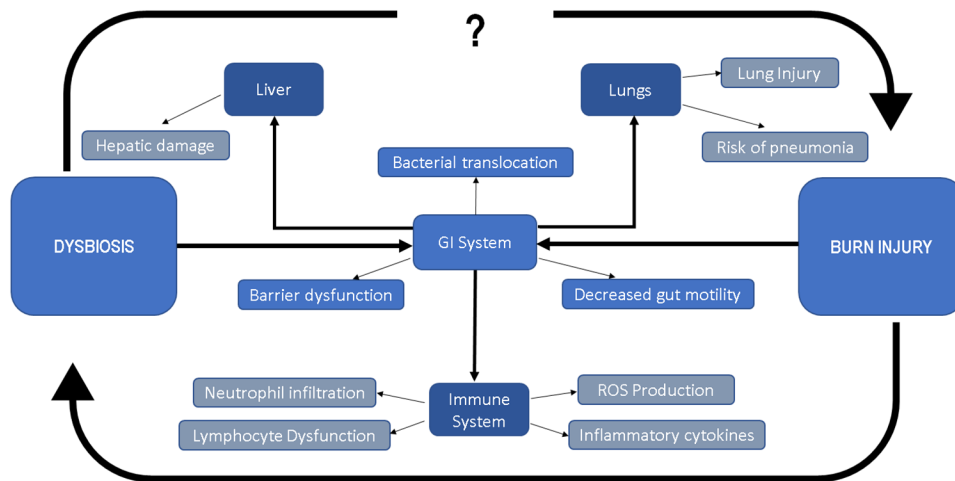
Figure 3. Representation of end organ injury and dysfunction following burn injury and microbial dysbiosis.

Both burn and dysbiosis are known to perturb the normal function of the gastrointestinal system. Factors altered in burn injury include barrier dysfunction, gut motility, and bacterial translocation into systemic circulation. This contributes to end organ dysfunction, including hepatic damage, lung injury, and pneumonia. Finally, burn injury and bacterial dysbiosis/translocation is linked to aberrant immune function, including neutrophil infiltration into various tissues, reactive oxygen species (ROS) and inflammatory cytokine production, and lymphocyte dysfunction. Burn injury itself is linked to dysbiosis, however, further work is needed to understand the contribution of microbial dysbiosis and its impact on deleterious outcomes for burn patients, as well as its impact on their recovery.