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Role of Heat Shock Proteins in Maintenance of the Gut Barrier Following Burn Injury

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ROLE OF HEAT SHOCK PROTEINS IN MAINTENANCE OF THE GUT BARRIER FOLLOWING BURN INJURY

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

ALS	Amyotrophic lateral sclerosis
BBB	Blood Brain Barrier
CAMs	Cell adhesion molecules
CARS	Counter anti-inflammatory response syndrome
CD	Crohn's disease
DC	Dendritic cells
GALT	Gut associated lymphoid tissue
HSF1	Heat shock transcription factor 1
HSPs	Heat shock proteins
IBD	Inflammatory Bowel Disease
IECs	Intestinal Epithelial Cells
IgA	Immunoglobulin A
IL	Interleukin
LP	Lamina propria
MLN	Mesenteric lymph nodes

MODS	Multiple Organ Dysfunction Syndrome
NF-kB	Nuclear-Factor-kappa-B
PPARγ	Peroxisome proliferator-activated receptor-γ
PP	Peyer's patches
SIRS	Systemic Inflammatory Response Syndrome
TBSA	Total body surface area
TJ	Tight junction
TNF-α	Tumor necrosis factor-α
UC	Ulcerative colitis
ZO	Zonal-occludin
5-ASA	5-aminosalysylic acid

CHAPTER ONE

INTRODUCTION

The two leading causes of increased morbidity and mortality after burn injury are sepsis and multiple organ dysfunction syndrome (MODs). Over 75% of all burn related deaths occur as a result of sepsis or infectious complications[1]. Immediately following injury patients experience a systemic inflammatory response, which presents as a surge of pro-inflammatory cytokine release[2]. This shift toward a pro-inflammatory environment from one of a healthy balance of pro- and anti-inflammatory cytokines manifests not only at the site of burn injury, but in extraneous sites of the lung, liver, and intestinal tract[3]. Coupled with the suppression of the immune system seen after serious burn injury, this inflammation increases patient susceptibility to sepsis and subsequent multiple organ failure[2-4]. Intestinal bacteria are the main source of bacterial infection following burn injury[3,5-7].

The intestinal tract contains over 100 trillion microbes, and under normal conditions maintains a symbiotic relationship benefiting the host by aiding in metabolism and nutrients, protection from invading pathogens, and immune system development and function[8-11]. In a healthy individual, intestinal epithelial cells maintain a physiological and immunological barrier sequestering both commensal and pathogenic bacteria to the luminal space[9,12,13]. After burn trauma intestinal epithelial cells undergo intense cellular

stress, which contributes to the barrier breakdown following injury[14,15]. This perturbation in gut barrier integrity could result in bacterial translocation out of the luminal space into extraintestinal sites ending in SIRS, sepsis, and multiple organ dysfunction[3-5,7,14-16]. However, the exact mechanism of gut barrier breakdown and subsequent burn related pathophysiologies remain largely unknown.

The intestinal barrier can be understood as both an immune barrier and a physical barrier. With T and B cells, macrophages, and dendritic cells of the Payer's patches (PP), mesenteric lymph nodes (MLN), and lamina propria (LP) of the gut associated lymphoid tissue (GALT) making up the intestinal immune barrier, and then tight junctional complexes, adherens junctions, and desmosomes between intestinal epithelial cells (IECs), contributing to the physical intestinal barrier. In particular tight junctional complexes of the small and large intestine are made up of the proteins: claudin, occludin, and zonal-occludin. These proteins are imperative to the maintenance of the physical intestinal barrier prohibiting translocation of bacteria out of the lumen while allowing the selective absorption of critical nutrients required by the host. Tight junctional complexes are not limited to the make-up of intestinal barrier, but exist in many other natural barriers of the body such as the lungs, kidney, and blood brain barrier, etc. Many studies on tight junction proteins at other barrier sites have implicated the role of heat shock proteins (HSPs) as support for tight junction protein integrity.

HSPs are classified as small cytoprotective proteins, which are induced after stresses such as heat, cytotoxic drugs, and bacterial endotoxins. Cell stress is alleviated by HSPs, which function to chaperone denatured proteins back to the endoplasmic

reticulum allowing for correct re-folding[17,18]. Burn injury can result in intense cellular stresses, which can consequently lead to an accumulation of denatured proteins. However, there exists a gap in the knowledge of the potential role HSPs could play in upholding the integrity of tight junction proteins in intestinal epithelial cells and, therefore, proper maintenance of the physical barrier of intestine following burn injury.

Understanding the interplay between burn related intestinal inflammation and the consequential intestinal barrier breakdown opens the doors for novel therapies in the treatment of burn patients. As Inflammatory Bowel Disease (IBD) mimics many of the inflammatory symptoms seen in the intestine after burn injury, applying common therapeutics currently used in IBD treatment and prevention, such as mesalamine, for the treatment of burn injury could yield promising results. Mesalamine inhibits inflammation through the inhibition of NF-κB and is theorized to upregulate the HSP response, resulting in alleviation of stress induced inflammation[19-21]. Although it's benefits in the treatment of ulcerative colitis and Crohn's disease have been extensively studied, mesalamine's potential for therapeutic intervention has yet to be applied in the context of burn injury. The studies performed herein profile the alterations in the heat shock response following burn trauma, which could contribute to changes tight junction proteins and gut barrier integrity. Additionally, studies were also carried out to examine whether treatment with mesalamine modulate HSPs and protect the barrier integrity after bun injury.

Hypothesis

Burn injury suppresses the heat shock protein response of intestinal epithelial cells altering tight junction proteins, which results in increased intestinal permeability.

Specific Aim 1

Characterize the expression of HSPs in the intestine and correlate with barrier integrity after burn injury.

Rationale

Our recent findings suggest that burn results in gut barrier disruption including increases in intestinal permeability and bacterial translocation to the mesenteric lymph nodes (MLNs). We now propose to study the effect of burn injury on HSP25, HSP72, and HSP90. These HSPs are implicated in the maintenance of gut homeostasis in response to cellular stresses such as changes in the intestinal microbiome or microbial products, hypoxia, and ischemia, all of which have been implicated as symptoms of burn injury[17,18,22-25]. Proper response to cell stressors, such as burn injury, by HSPs is essential due to their role in cell survival by facilitating the proper folding of denatured proteins. Thus, changes in expression of these cyto-protective HSPs post burn injury could adversely affect gut homeostasis resulting in barrier breakdown and increased leakiness and bacterial translocation. Therefore, it is imperative to assess the expression of HSPs in the intestine and correlate with barrier integrity after burn injury.

Specific Aim 2

Determine whether treatment with anti-inflammatory therapeutic, Mesalamine, restores gut barrier integrity via upregulation of HSPs after burn injury.

Rationale

Mesalamine or 5-ASA is currently used as treatment for patients with Inflammatory Bowel Disease (IBD) [11,21]. IBD is a chronic inflammatory disease characterized by gross elevations in pro-inflammatory cytokines and an over-active Th1 response in the intestinal tract. This pro-inflammatory environment results in severe intestinal epithelial stress resulting in intestinal tissue damage and severe pain in patients with the disease[26]. As of yet, there is no cure for IBD, but one therapeutic option is treatment with 5-ASA. The mechanism by which 5-ASA reduces symptoms of inflammation in IBD patients remains a matter of debate in the scientific community, but it is proposed to act through an inhibition of NF-κB and/or inhibition in the release of TNF-α[21]. However, recent studies *in vitro* have shown 5-ASA to up-regulate the heat shock protein response in intestinal epithelial cells in reaction to cellular stress. As burn injury induces intense intestinal epithelial cellular stresses as a result of increased inflammation and leakiness in the gastrointestinal tract. These downstream consequences of burn injury can mimic intestinal epithelial cellular stresses seen in IBD[13,26-28]. Experiments in this aim will determine whether treatment with 5-ASA after burn injury successfully up-regulates HSPs in IECs and consequently restores proper tight junctional complex integrity and intestinal barrier function.

CHAPTER TWO

REVIEW OF LITERATURE

Burn Injury

As estimated by the American Burn Association 450,00 individuals succumb to burn injuries every year, with approximately 4,000 of those individuals requiring subsequent hospitalization[29]. Burn injury remains a prominent medical issue to be resolved, not only due to the sheer number of injuries each year, but also due to the fact that burn trauma results in patients with increased risk of sepsis, progressing to septic shock, and ending in multiple organ dysfunction[5]. Sepsis and multiple organ dysfunction syndrome (MODS) continue to be the leading causes of burn related mortality[30].

The spiral towards increased mortality as a result of sepsis and MODS in burn patients begins with the onset of a global immune dysregulation[31]. Immediately following burn trauma patients suffer from an overwhelming inflammatory response. This initial injury response phase, termed systemic inflammatory response syndrome (SIRS), is dependent on cells of the innate immune system such as macrophages, dendritic cells, and neutrophils producing vast amount of pro-inflammatory cytokines of Il-1, IL-6, IL-10, and TNF[4,5,31-35]. Additionally, SIRS produces chemo-attractant proteins needed for recruitment of pro-inflammatory cells [31]. Conversely, the adaptive arm of the immune system generates a compensatory anti-inflammatory response syndrome

(CARS). CARS is defined by a host of immuno-suppressive actions. The disruption to the normally tightly controlled immune homeostasis after burn injury ultimately increases the patient's susceptibility to whole body bacterial infection[31]. The source of infection can be a consequence of externally acquired pathogens, or through internal commensal bacteria acquiring pathogenicity as a result of changes in their microenvironment post burn injury. However, it is hypothesized that the source of post burn infection is the gastrointestinal tract[36].

Burn Injury and Gut Barrier Maintenance

The lumen of the gastrointestinal tract is the major reservoir of bacteria in the human body harboring upwards of 100 trillion organisms[10]. This highlights the importance of the integrity of the gut barrier, which functions to sequester those high numbers of bacteria to the intraluminal space preventing bacterial translocation to extraintestinal sites. As septic shock is a major clinical problem after burn injury, any breakdown in gut barrier after burn trauma remains of particular interest[4,5,32]. Increases in burn related pro-inflammatory cytokines and inflammatory mediators in the gut after injury give rise to gut barrier dysfunction resulting in bacteria or bacterial endotoxins translocation across the intestinal barrier. Translocating bacteria after burn injury have been detected not only in the MLNs, but also in the bloodstream and other more distant organs to the intestines including the lungs, liver, and spleen[1,6,16,36]. The invasion of bacteria or their products to systemic organs post burn injury gives evidence to the hypothesis for the gastrointestinal tract as the origin of the major burn related complication: multiple organ dysfunction syndrome (MODS) [36].

Intestinal Epithelial Cell Barrier

As the largest mucosal surface in the human body, the gastrointestinal tract comes into contact with the most dietary antigens and largest diversity of microbial organisms[9]. However, it is the barrier created by the GI tract between the diverse community of microorganisms residing in a healthy gut and extraintestinal sites that is of critical importance. The intestinal barrier can be understood in two main parts: the immune barrier and the physical barrier.

The immune barrier consists of cells such as dendritic cells (DCs), macrophages, T cells, and B cells. DCs will constantly sample the intestinal lumen and present antigen to cells of both the adaptive and innate immune system[37]. This, in turn, prompts the proper immune responses of restricting commensal microbes to the lumen and effectively eliminating potential pathogenic bacteria via secretion of cytokines by activated epithelial cells or T cells and/or release of opsonizing antibodies such as IgA[37-39].

The physical barrier of the gastrointestinal tract mainly consists of intestinal columnar epithelial cells, which adjacently associate creating an epithelial cell lining of the intestinal lumen. Covering the epithelial cell lining is layer of mucus secreted by specialized epithelial cells called goblet cells[40]. The mucus layer prevents most bacteria residing in the lumen of the intestine from direct contact with the epithelial cells, and therefore, restricting improper immune responses to resident intestinal bacteria[9].

Critical to the maintenance of intestinal homeostasis and the integrity of the physical intestinal barrier are a class of proteins known as tight junction (TJ) proteins. Even in an intact intestinal epithelial cell layer, there still exits a paracellular pathway

allowing for transepithelial transport between adjacent cells, but, however, needs to be sealed against bacterial transport outside of the intestinal lumen[41,42]. Tight junctional proteins create tight junctional complexes, which aid in adjacent intestinal epithelial cell sealing. The claudins and occludins are two types of transmembrane TJ proteins, which associate with zonal-occludin-1,2 (ZO1, ZO2) proteins. ZO1 and ZO2 anchor the claudins and occludins into the intestinal epithelial cells cytoskeletal component F-actin[43,44]. Proper formation and integrity of tight junction complexes is required for a healthy gut.

Any breakdown in gut barrier integrity, such as after injury or inflammation, could allow for the invasion of resident bacteria into the epithelium or to extraintestinal sites. Bacteria translocating out of the intestinal lumen can exploit their newfound environments turning into opportunistic pathogens leading to infection and disease[3,7,45].

Heat Shock Proteins (HSPs)

Heat shock proteins or HSPs are a family of highly conserved stress proteins expressed ubiquitously across all organisms from humans to bacteria to yeast[17,24,25,46-50]. First discovered in 1962, HSPs are characterized by and subsequently named by their molecular weights, which range from approximately 15 to 110 kDa[47,51]. The distribution of HSPs in different cellular compartments is widespread as it includes the cytoplasm, endoplasmic reticulum, and nucleus[52]. Yet, the precise mechanistic function of HSPs has yet to be determined, but it is well known they are essential for survival at normal or elevated temperatures and in response to ischemia, cytokines, and energy depletion[38-42]. Although exact mechanisms are still not understood, researchers have determined that

HSPs have strong cytoprotective effects, are critical to many regulatory pathways, and act as molecular chaperones for other proteins[23,24,46,47,50,53,54].

Three of the most extensively studied HSPs are HSP72, HSP25, and HSP90, or HSP70, 27, and 90 in humans respectively. HSP70 has at least two regulatory sequences that interact with the major heat shock protein transcription factor, HSF1. The two sequences of HSP70, HSPA1A and HSPA1B, will code for almost identical amino acid sequences generating nearly indistinguishable proteins, which results in some redundancy[55,56]. In particular, HSP72 was shown to be the most temperature sensitive and highly conserved out of all the HSPs. In contrast to others in the HSP family, HSP72 is highly inducible in response to a variety of stressors, such as hypoxia, ischemia with over-expression of HSP70 protecting from ischemic heart injury by enhancing post-ischemic contractile function[57] , reactive oxygen species, and pro-inflammatory cytokines like TNF-α.

Unlike the ATP-dependent HSP72, HSP25 acts completely independently of ATP, as do many of the other small molecular weight HSPs. Mammalian HSP25 proteins have the ability to dimerize under conditions of stress. It theorized that a unique cysteine residue on HSP25 gives it its ability to act as an anti-apoptotic protein under threats of apoptosis due to injury[58,59]. Lastly, HSP90 comprises approximately 1-2% of all cellular proteins in a cell, as it is so ubiquitously expressed. Like HSP25, HSP90 requires dimerization to properly function, but it differs in its dependency on ATP, similar to that of HSP72. Unique to HSP90 is its ability to bind more than one naïve or stress-induced mis-folded protein in order to aid its proper folding/re-folding[60-62].

In the context of the gastrointestinal tract, there exists a fine-tuned relationship between the resident microflora, intestinal epithelial cells, and HSP induction[17,26,45,63-65]. It is of interest to note that HSP25 and 72 are present only at low basal amounts in the healthy distal small intestine and large intestine[66,67]. Kojima et. al found that this distribution of HSP25 and 72 was due to the differential amounts of bacteria as one descends down the GI tract, with greater number and diversity of bacteria from small to large intestine[66]. A healthy human gut requires the presence of the resident microflora, but under normal physiologic conditions microbial overgrowth is restricted, in part, by innate immune responses from intestinal epithelial cells. Their study, and others, have provided evidence for the fact that potential pathogenic bacteria or bacterial products upregulate the HSP25 and 72 response[65-68]. As both HSPs are known to be cytoprotective, their induction would allow for intestinal epithelial cell protection of critical cellular functions and viability. Compromised expression of either HSP25 or 72 in the small or large intestine could potentially increase susceptibility to invading pathogens and subsequent systemic complications.

HSPs and Disease

As HSPs are renowned for their cytoprotective roles, it follows that they have been implicated in the protection from various diseases, including Amyotrophic lateral sclerosis (ALS), cardiovascular disease, and Inflammatory Bowel Disease (IBD). ALS is a progressive paralysis disease characterized by the death of motoneurons in spinal cord and motor cortex. Researchers found that treatment with a broad-spectrum inducer of HSPs could drastically slow the progression of ALS[69].

In the context of cardiovascular disease, induction of HSPs by thermal stress can significantly improve the outcome of ischemic heart disease. Ischemic heart disease results in an accumulation of circulating leukocytes, leading to increased T cell and macrophage presence in the arterial way releasing pro-inflammatory cytokines of TNF, IL-6, and IFN-γ[70]. As a consequence of increased inflammation, endothelial cells undergo severe cellular stresses, which lead to tissue damage and necrosis. Currie et. al found that hearts with HSP over-expression had improved contractile functioning in response to ischemic conditions. Additionally, the reperfusion damage was significantly lower than in hearts with basal levels of HSPs[71].

IBD is described as a chronic inflammatory state of the gastrointestinal tract[26-28,72]. This pro-inflammatory state in IBD can be characterized not only by elevated production of the pro-inflammatory cytokines TNF-α, IL-6, and IL-1β, but also by elevated levels of cell adhesion molecules (CAMs) are crucial to the infiltration of leukocytes into the bowel. Leukocyte infiltration and chronic intestinal inflammation results in severe intestinal epithelial cell damage, which can proceed to colonic bleeding and intense discomfort in patients[26,27]. However, recently, Tanaka et. al have shown that the presence of HSPs can significantly reduce IBD symptoms of intestinal epithelial cell damage and leukocyte infiltration compared to that of an HSF1 null mouse. However, transgenic mice expressing the human HSP70 were found to have lower clinical scores of IBD symptoms, less intestinal epithelial damage, and reduced levels of the pro-inflammatory cytokines TNF-α, IL-6, and Il-1β. From this they concluded that HSP70 was essential in protection from symptoms of IBD[73,74].

The above studies show strong evidence for HSPs as targets for protection from various diseases. Therefore, it is of upmost importance to understand how pharmacological upregulation of HSPs could be a new, viable, therapeutic option is disease treatment.

Mesalamine Treatment

Crohn's disease (CD) and Ulcerative Colitis (UC) are two diseases encompassed by the classification of IBD. While CD can affect the entirety of the gastrointestinal tract, UC is restricted to the colon. Under normal homeostatic conditions, tolerance exists between the resident microbes of the intestinal tract[14,38-40]. However, in diseases states such as CD and UC that tolerance is broken leading to a hyper-active immune response characterized by dramatic increases in pro-inflammatory cells such as CD8+ T cells, pro-inflammatory cytokines of INF-λ, TNF, IL-12, and IL-13, and other pro-inflammatory chemokines[75,76]. No cure currently exists for patients with IBD, but several promising treatment options are available. One such option is treatment with drug mesalamine.

Mesalamine, or 5- aminosalysylic acid (5-ASA), is presently being used as first-line therapy for patients with either CD or UC[77,78]. The mechanism by which 5-ASA acts as an anti-inflammatory agent remains a matter of debate, but it is theorized to act in several ways. The first of which is a PPARγ agonist. In other studies, PPARγ agonists have been shown to inhibit the production of the pro-inflammatory cytokines of TNF-α, IL-6, and IL-1β, which are commonly elevated in IBD[20,79,80]. The second way in 5-ASA has been shown to act is through the inhibition of NF-κB, which would halt the transcriptional messages required for

the increased inflammation in IBD[19,20,77-79]. Lastly, 5-ASA has been shown to up-regulate HSPs, particularly HSP72, *in vitro* in rat intestinal epithelial cells[81]. Induction of HSPs in a pro-inflammatory environment, such as after injury, could potentially assuage the detrimental effects of the inflammation, i.e. intestinal barrier breakdown, by aiding in the re-folding of denatured or damaged proteins as a consequence of inflammatory cell stress[7,9,82]. Additionally, increases in HSPs could function to more efficiently fold naïve anti-inflammatory proteins required for alleviating inflammation[17,18,22,24,50,83-85]

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 as describes by Walker and Mason[86]. The mice divided into two treatment groups, those receiving burn injuries or sham injuries. The burn group was 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 normal saline. This procedure models a severe ~20% TBSA full thickness third degree burn. The animals were sacrificed 4 hours, day 1 and day 3 following injury. Small and large intestine were harvested and processed for the isolation of intestinal epithelial cells for downstream experiments.

Intestinal Epithelial Cell Isolation

Four hours, 1 or 3 days following the injury procedure, mice were humanley euthanized by isoflurane asphyxiation. Isolation of intestinal epithelial cell was performed as described previously by Weigmann *et al*[87]. Small and large intestines were removed from the peritoneal cavity. For small intestine ileum studies, the distal 10cm of the small intestine was separated from the remainder of the small intestine for analysis. The entirety of the colon was harvested for analysis. The tissues were cut longitudinally and placed in ice cold PBS + 1% penicillin/streptomycin (pen/strep) cocktail. Following two washes in PBS + pen/strep, tissues were placed in a digestion solution containing 5% heat-inactivated fetal bovine serum (FBS), 1% HEPES, 1% pen/strep, 0.5% gentamicin, 5mM EDTA, and 1mM dithiothreitol (D.T.T.) in Hank's Balanced Salt Solution (HBSS) at 37°C. Tissues were placed in a 37°C incubator and shaken on a rotator at 250rpm for 20 minutes. Tissues were vortexed to separate the epithelial cells from the tissue and passed through a 100μm filter. Cells were counted on a hemocytometer to determine epithelial cell purity (≥90%). Intestinal epithelial cells were then processed for downstream applications.

RNA Isolation and cDNA synthesis

RNA isolation was performed using a RNeasy Mini Kit (Qiagen, Valencia, CA) as described by the manufacturer. Genomic DNA was removed by DNase digestion using an RNase-free-DNase Set (Qiagen). Isolated RNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Bannockburn, IL). Only samples with a 260/280 ratio of ≥2.0 were used for cDNA synthesis. cDNA synthesis was

performed using a High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA) and reactions were run on a Veriti 96-well Fast Thermocycler (Life Technologies) per the manufacturer's instructions.

Real-Time PCR

Expression of claudin-4, claudin-8, occludin, HSP25, HSP72, HSP90, and HSF1 mRNA levels were analyzed by qPCR using TaqMan primer probes and TaqMan Fast Advanced Master Mix (Life Technologies). Target gene Ct cycle values were normalized to housekeeping control GAPDH or β-actin Ct values. Data were calculated using the ΔΔCt method, and all groups were expressed relative to the sham group.

Cytokine quantification

IECs were isolated either from the distal 10cm of the small intestine or the colon, allowed to incubate in 500uL of 1X cell lysis buffer (Cell Signaling Technology) containing 1mM PMSF, 1X Protease inhibitor, 1X Phosphatase Inhibitor added (Cell Signaling Technology). The homogenates were centrifuged at 10,000 RPM for 5min and the supernatant was removed, aliquoted, and stored in -80°C for HSP25 (Enzo Life Sciences), HSP72 (R&D), IL-18 (eBioscience), IL-6 (BD), KC (R&D), or MCP-1 (R&D) ELISAs. Protein measurements of the same samples were done from Bio-rad protein assay kit. Data were normalized as amount of cytokine/mg protein.

Immunofluorescence

Sections of the distal ileum (1cm) and proximal colon (1cm) were fixed in the cryoprotective embedding medium, OCT, and frozen on dry ice. Tissue sections were

prepared by the Loyola University Health and Sciences Division Processing Core. Briefly, tissues were semi-permeabilized with 100% ice cold ethanol for 30 min, fixed in 100% ice cold acetone for 3 min, allowed to rehydrate in PBS for 1 min, and blocked with Superblock (Scytek Laboratories) in humidity chamber for 5 min. Sections were washed in appropriate amount of primary antibody to either claudin-4 (abcam) or claudin-8 (Invitrogen) diluted at a concentration of 1:100 in 1%BSA in PBS, allowed to incubate for 2 hours in humidity chamber, and washed 3X with PBS. Appropriate amounts of secondary antibody conjugated to Alexa 488 diluted to a concentration of 1:1000 in 1% BSA in PBS were added and allowed to incubate in humidity chamber for 1 hour. Sections were washed in 3X PBS and phalliodin dye (Life Technologies) was added to stain F-actin for 30 min in humidity chamber. Tissues were washed 3X PBS, mounted with Prolong Gold antifade reagent with DAPI (Life Technologies), and sealed after 24 hours. 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. Stomach content, small intestine luminal content divided into three equal parts (with section #1 being proximal and section #3 being distal), and large intestine feces were collected. The blood was centrifuged at 8000rpm for 10min at 4°C, plasma isolated, and read spectrophotometrically at 480nm excitation and 520nm emission wavelengths for

intestinal permeability. Stomach content, small intestinal luminal contents #1, #2, and #3, and large intestine feces were weighed, normalized by amount of PBS added, sonicated (XL-2000 Misonix) until the solution was homogenous. Homogenates were centrifuged at 8000rpm for 10min at 4°C, supernatants were collected, and read spectrophotometrically at 480nm excitation and 520nm emission wavelengths for intestinal transit.

Statistics

The data, wherever applicable, are presented as means + SEM and were analyzed using analysis of variance (ANOVA) with Tukey's post-hoc test or Student t test (GraphPad Prism6). Unless otherwise noted, significance is reported as follows:

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

CHAPTER FOUR

RESULTS

Expression of Tight Junction Proteins Following Injury

As we had previously observed an increase in intestinal permeability and subsequent bacterial translocation to extraintestinal sites following burn injury, we first examined whether burn injury altered the expression of tight junction proteins. Tight junction proteins uphold the physical intestinal barrier by joining two adjacent intestinal epithelial cells allowing for the passive flux of nutrients from the lumen, but restricting the large number of bacteria our GI tract harbors to the luminal space[6,13,26,36,88]. Any change in expression of tight junction (TJ) proteins after burn injury could potentially break this selective barrier allowing an increase in bacterial translocation[7,82]. Therefore, we profiled the mRNA expression of several key TJ proteins, claudin-4, claudin-8, and occludin, in both small and large IECS one and three days following either burn or sham injury. In small intestine IECS, we observed a 54% decrease in claudin-4 expression and 49% decrease in claudin-8 expression relative to sham levels one day post burn on the same day. The expression of these proteins was normalized to that of sham levels three days after injury (Fig. 1). This is likely due to the extremely fast turnover rate of intestinal epithelial cells, which is about 2-3 days. However, the downstream effect of this early breakdown in tight junction complex integrity one day post burn injury could

allow for the consequential translocation of bacteria out of the intestinal lumen ending in one of the most common burn related complications: septic shock. There were no significant changes in occludin expression in small intestine IECS one or three days post burn injury (Fig. 1).

Next, we examined the same tight junction proteins in large intestine IECs one and three days following burn injury. Similar to small intestine, IECs from large intestine trend toward the same decreases in claudin-4 and claudin-8 but this was not found to be significantly different from sham animals. Furthermore, this trend remained visible on day three after burn (Fig. 2).

SMALL INTESTINE IECs

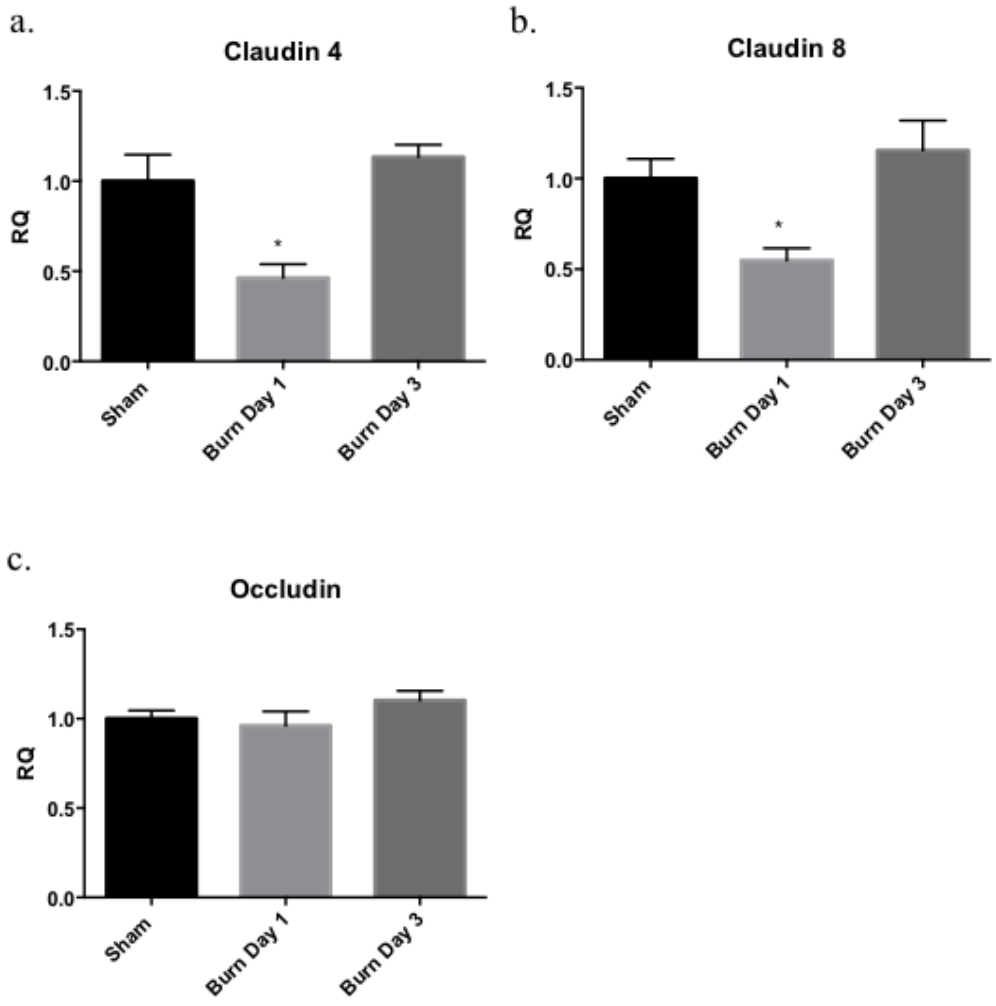


Figure 1: *Burn injury results in decreased expression of tight junction proteins in small intestine IECs one day following injury.* q-RTPCR Claudin-4, claudin-8, and occludin mRNA expression relative to GAPDH. Values are mean ± SEM of 6-8 animals per group expressed relative to sham. *, $p<0.05$ burn day one compared to sham by ANOVA and Tukey post hoc test.

LARGE INTESTINE IECs

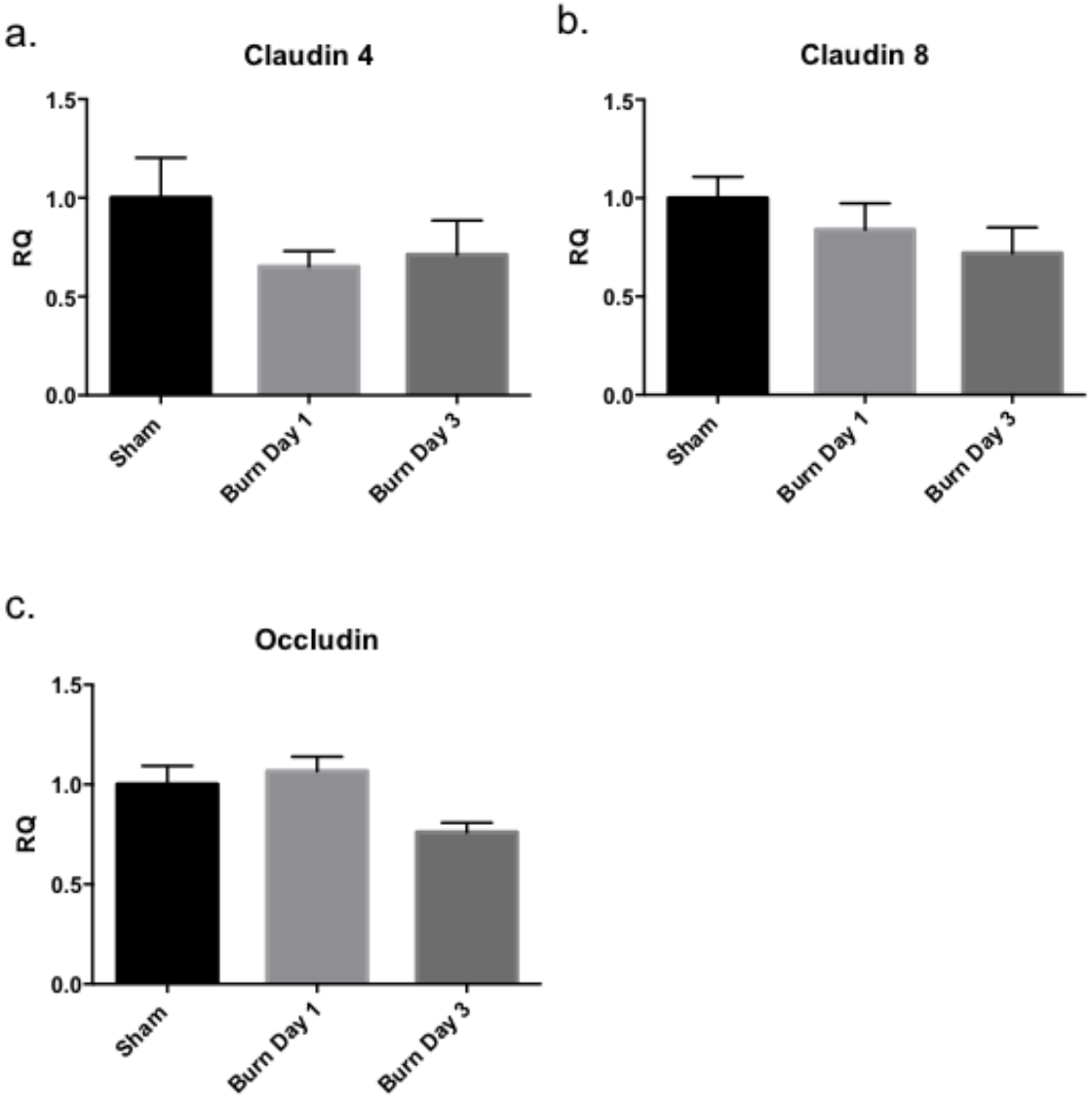
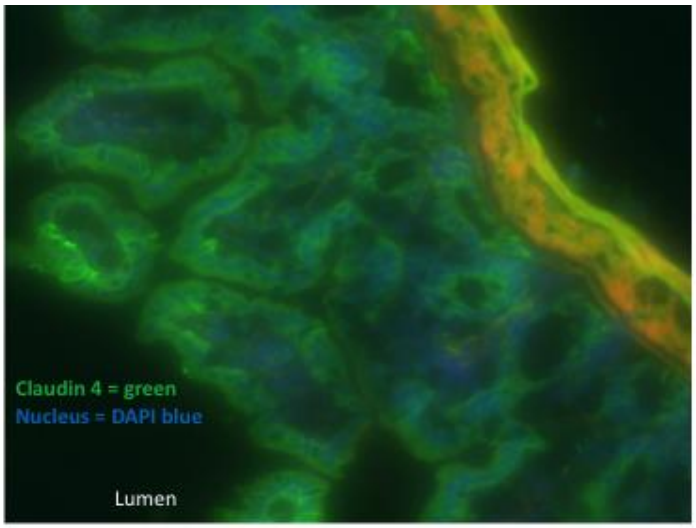


Figure 2: *Burn injury results in no significant changes in claudin4, claudin-8, or occludin in large intestine IECs.* q-RTPCR of Claudin-4, claudin-8, and occludin mRNA expression relative to GAPDH. Values are mean ± SEM of 6-8 animals per group expressed relative to sham. *, $p<0.05$ burn day one compared to sham by ANOVA and Tukey post hoc test.

Immunofluorescent Staining of Tight Junction Proteins

Additionally, we used immunofluorescence to visualize the distribution of the tight junction proteins claudin-4 and claudin-8 in small intestine tissue sections. It is imperative to understand how burn injury affects not only mRNA expression of tight junction proteins, but also how tight junctional complexes at the protein level are altered after burn injury. Using specific antibodies to claudin-4 or claudin-8, we observed decreases in claudin-4 protein levels one day after burn injury compared to sham. No significant changes in claudin-8 were observed in the small intestine.

Sham



Burn Day 1

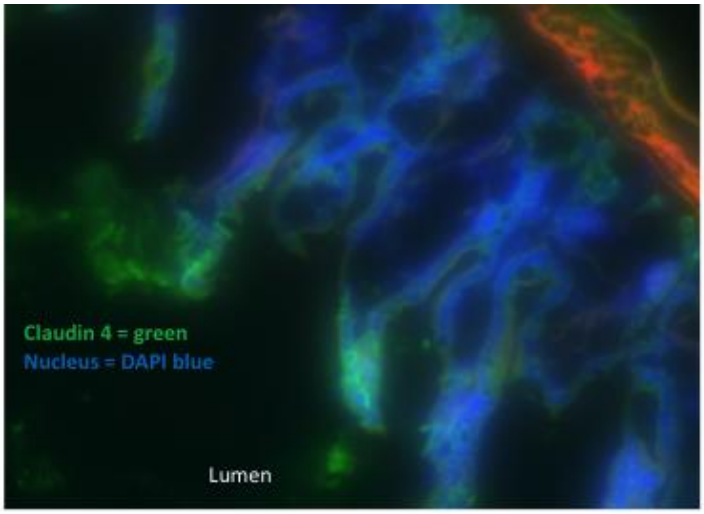
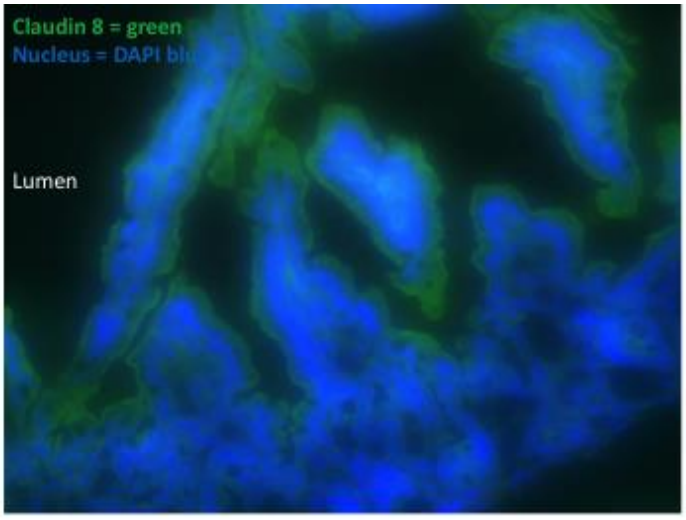


Figure 3: *Burn injury leads to a decrease in claudin-4 protein levels in small intestine tissue one day after burn injury.* Green color represents ALEXA 488 conjugated secondary antibody to that of primary antibody towards claudin-4. Blue is a DAPI stain for nuclei. The above image is 20X magnification.

Sham



Burn Day 1

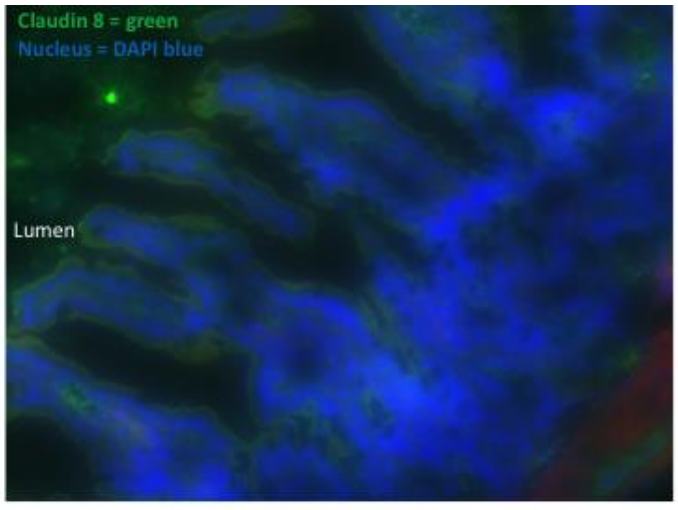


Figure 4: *No significant difference in claudin-8 protein levels one day after burn injury.* Green color represents ALEXA 488 conjugated secondary antibody to that of primary antibody towards claudin-8. Blue is a DAPI stain for nuclei. The above image is 20X magnification.

Burn Injury and Heat Shock Proteins (HSPs)

We next examined whether burn injury alters expression of HSPs in intestinal epithelial cells (IECs). Burn related decreases in tight junction proteins, as we observed in our studies, could result from alterations in HSPs. Other studies have implicated HSPs as a source of stabilization of tight junction proteins in other barriers of the body, including the blood brain barrier (BBB). The BBB is made up primarily of endothelial cells, while the intestinal physical barrier is made of epithelial cells[81]. Yet, the way in which both endothelial cells in the BBB and epithelial cells of intestinal tract structure their tight junctional complexes is almost identical[89]. They are both made up of the class of tight junction proteins, the claudins and occludins, and both allow for selective permeability[42,43,82,89]. If HSPs are stabilizing the tight junction proteins of the intestinal epithelial barrier in a manner similar to that of the BBB, then we would expect a decrease in HSPs correlating with the decrease in TJ proteins we observe one day after burn injury resulting in gut barrier breakdown. Therefore, we examined the HSPs 25, 72, and 90 in both small and large IECs four hours, one, and three days after burn injury as they all have been found to play a role in the maintenance of intestinal homeostasis[17,18,24,31,83]. More specifically, HSP72 was found to be critical in the stabilization of tight junction proteins in the BBB after injury[24,83]. At the mRNA level, we found significant decreases in HSP25, 72, and 90 expression (25%, 85%, and 51% respectively) in IECs harvested from the small intestine one day post burn injury compared to sham controls (Fig. 5). Understanding how the mRNA message for HSPs can be disrupted after burn is important, but it is critical to determine whether burn injury alters levels of the HSP

protein response. Hence, at the protein level, burn injury resulted in a decrease of 85% in HSP25 four hours after burn injury and significant decreases in HSP72 four (51%) and three days (46%) in small intestine IECs compared to sham controls (Fig 6).

Upon examination of the HSPs 25, 72, and 90 in large intestine IECs four hours, one day, and three days following burn injury, we observed a significant decrease, in all three HSPs, 4 hours after injury of 44%, 79%, and 33% respectively compared to sham. This significant decrease in HSP72 expression (81%) persisted to one day post burn injury (Fig. 7). Protein levels of HSP25 mimicked mRNA expression with significant decreases in HSP25 (71%) in large intestine IECs four hours after burn injury. Yet, HSP72 levels post burn injury were significantly down in large intestine IECs on both one (63%) and three days (46%) compared to sham controls (Fig. 8).

The suppression in HSPs in both small and large intestine post burn injury are correlating with the significant decreases we observe in the tight junction proteins. This gives evidence to our hypothesis that decreased expression of HSPs alters tight junction proteins, which could drive the breakdown in gut barrier integrity after burn injury potentiating the risk of sepsis and subsequent MODs in burn patients.

SMALL INTESTINE IECs

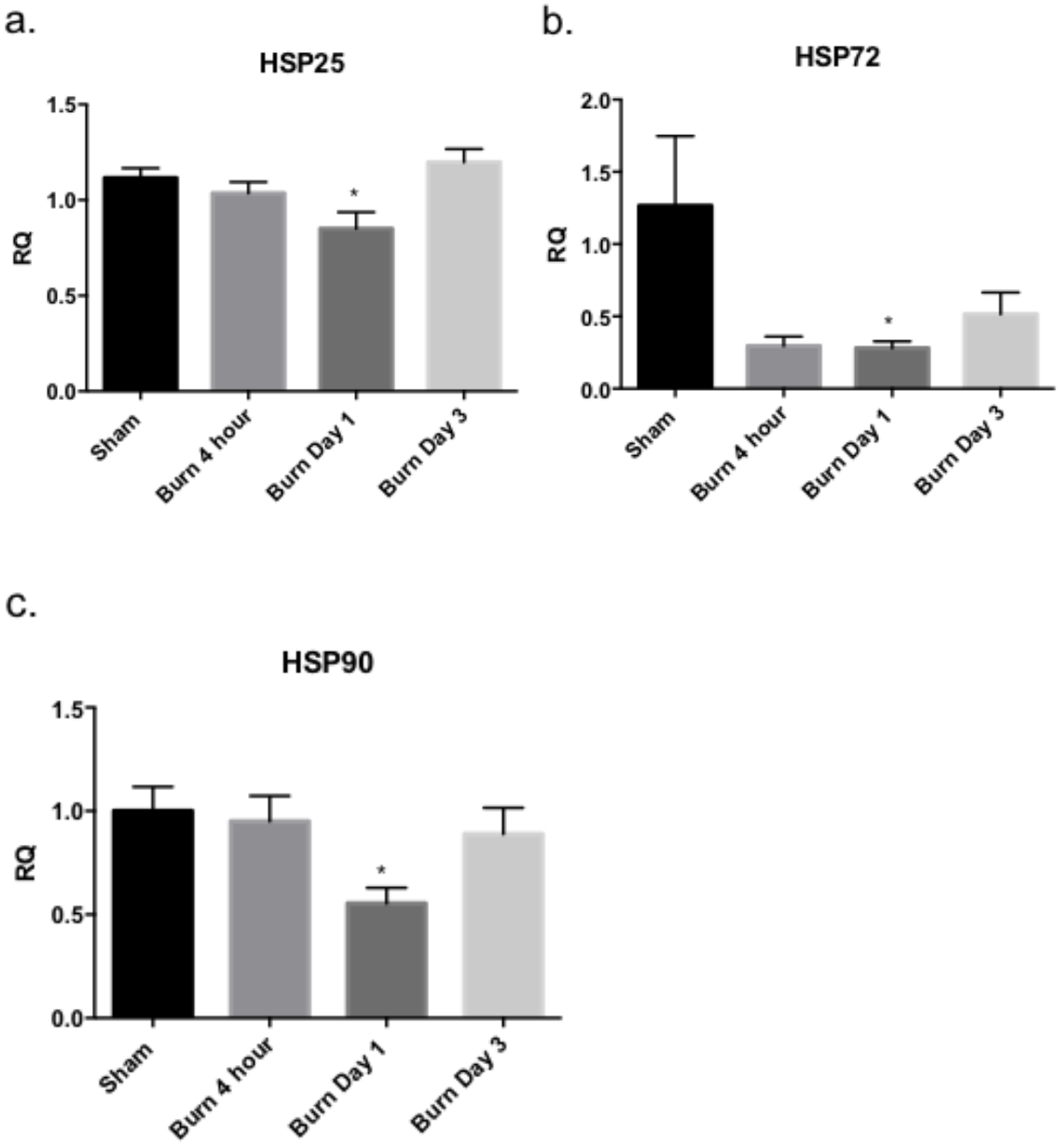


Figure 5: *Burn injury results in decreased expression of HSP25, 72, and 90 in small intestine IECs one day after burn injury.* q-RTPCR of HSP25, HSP72, and HSP90 mRNA expression relative to GAPDH. Values are mean ± SEM of 6-8 animals per group expressed relative to sham. *, $p<0.05$ burn day one compared to sham by ANOVA and Tukey post hoc test.

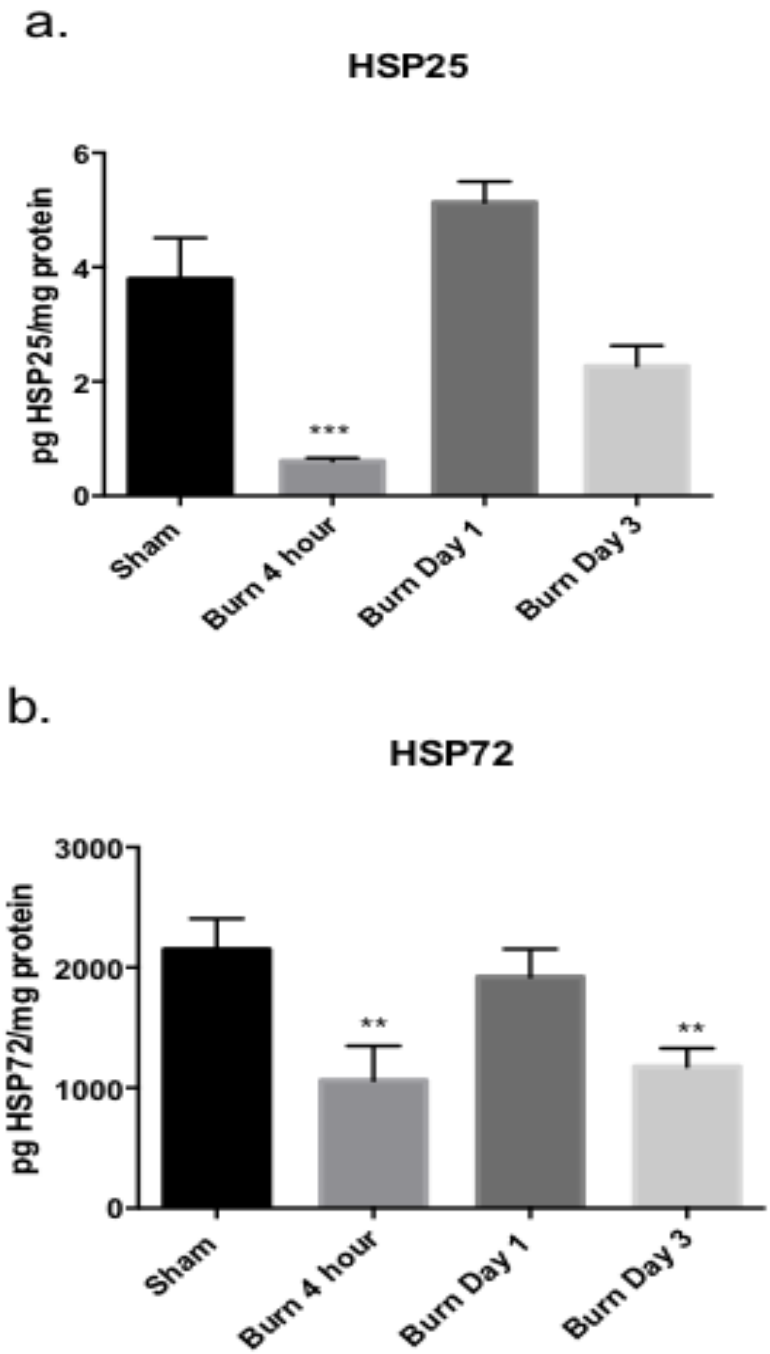


Figure 6: *Burn injury results in a suppressed HSP response in small intestine IECs in HSP25 four hours after injury and HSP72 four hours and three days after injury.* IECs of the small intestine were harvested and cells were lysed for protein extraction. ELISAs on HSP25 and HSP72 were performed on the protein homogenate four hours, one day, or three days after injury and expressed as pgHSP/mg protein. **, $p<0.001$, ***, $p<0.0001$ burn four hour or one day compared to sham, ANOVA and Tukey multiple comparison tests. Values are mean ± SEM of 6-8 animals per group.

LARGE INTESTINE IECs

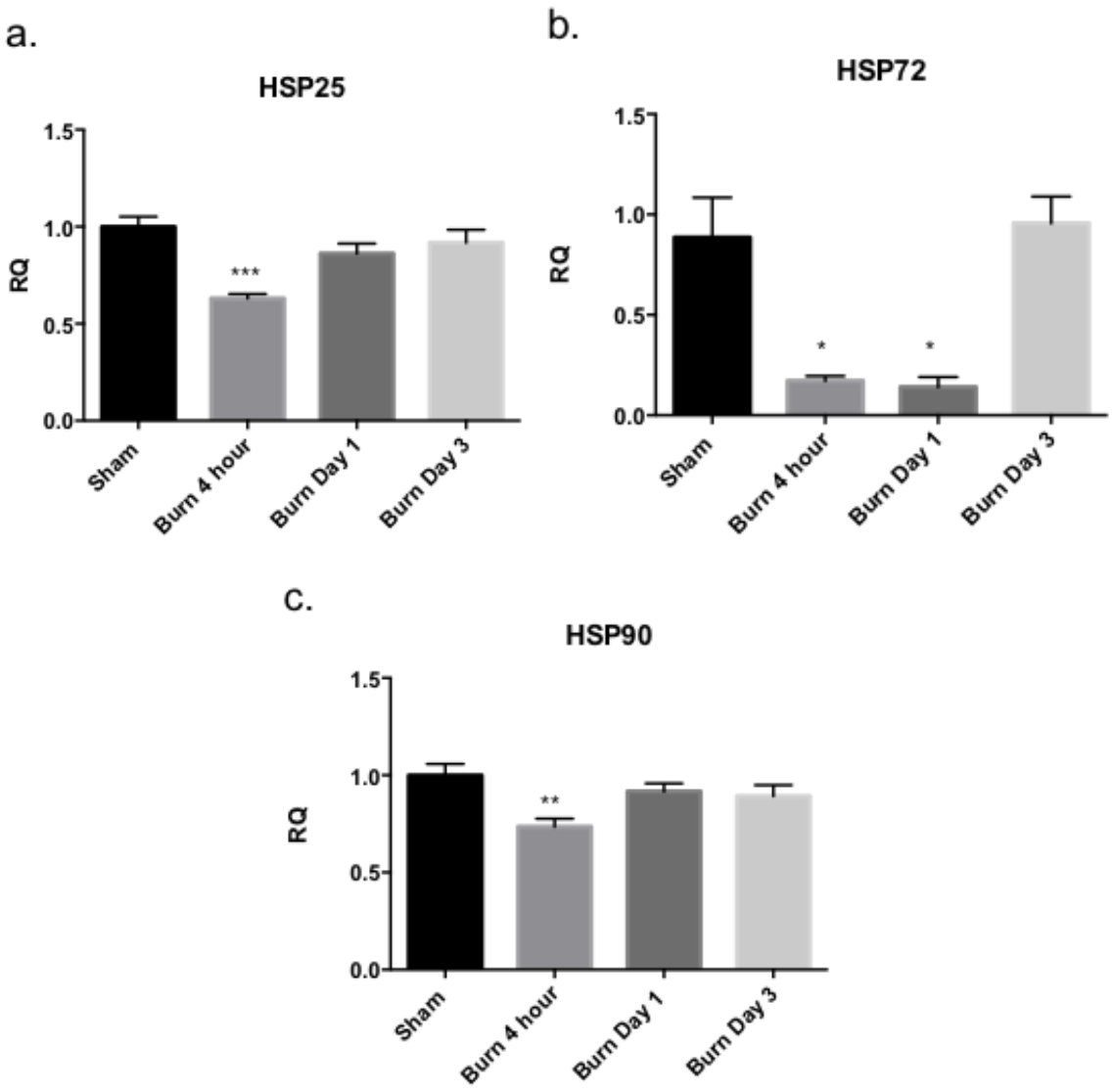


Figure 7: *Burn injury results in decreased expression of HSP25 four hours after injury, HSP72 four hours and one day after injury, and HSP90 four hours after injury in large intestine IECs.* q-RTPCR of HSP25, HSP72, and HSP90 mRNA expression relative to GAPDH. Values are mean ± SEM of 6-8 animals per group expressed relative to sham. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$ all burn time points relative to sham by ANOVA and Tukey post hoc test.

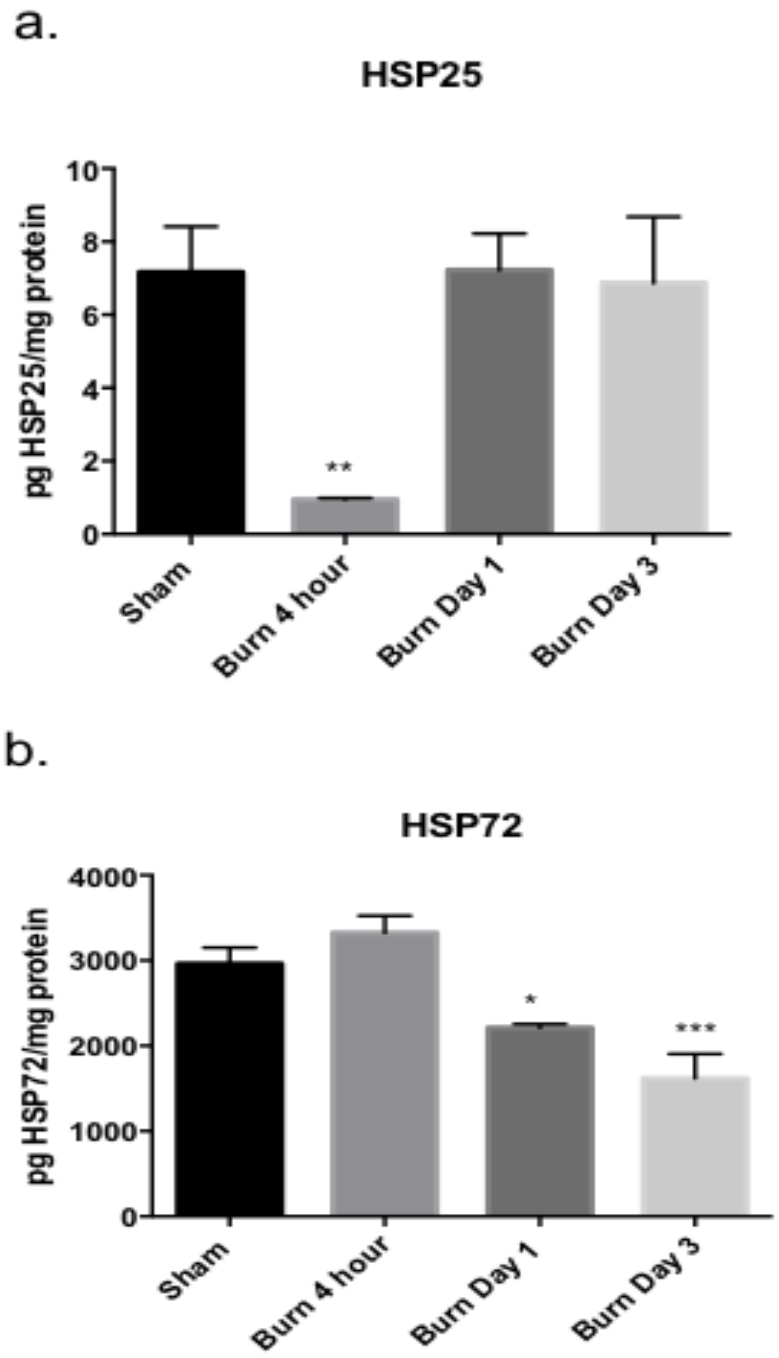


Figure 8: *Burn injury results in a suppressed HSP response in large intestine IECs in HSP25 four hours after injury and HSP72 one and three days after injury.* IECs of the large intestine were harvested and cells were lysed for protein extraction. ELISAs on HSP25 and HSP72 were performed on the protein homogenate four hours, one day, or three days after injury and expressed as pgHSP/mg protein. *, $p<0.05$, **, $p<0.001$, ***, $p<0.0001$ burn four hour or one day compared to sham, ANOVA and Tukey multiple comparison tests. Values are mean ± SEM of 6-8 animals per group.

Mesalamine Treatment

Mesalamine, or 5-aminosalysylic acid (5-ASA), is currently used as an anti-inflammatory treatment for patients with Inflammatory Bowel Disease (IBD), a disease characterized by severe elevations of pro-inflammatory cytokines in the gastrointestinal tract causing severe discomfort in affected patients[21,81]. Most commonly, 5-ASA is used in the treatment of ulcerative colitis or Crohn's disease where it is theorized to work through an inhibition of the major transcription factor of inflammatory mediators, NF-kB, or by inhibiting the synthesis of TNF-α[21,32,81]. However, another study looking at the mechanism by which 5-ASA acts as an anti-inflammatory agent found that 5-ASA up-regulates the heat shock protein HSP72 in rat intestinal epithelial cells[81]. Induction of HSP72 would allow for an alleviation of inflammation and stress to the cell via proper folding of naïve proteins needed to combat increased inflammation and proper re-folding of damaged proteins as a result of cell stress[24].

We attempted to induce HSPs in our murine model of burn injury with the hypothesis that if HSPs stabilize TJ proteins, up-regulating HSPs after burn injury could potentially restore the decrease in TJ proteins we observe and bring back normal barrier function. To perform this experiment, mice were divided into four groups: sham plus saline, sham plus 5-ASA, burn plus saline, and burn plus 5-ASA. Immediately after burn injury, mice were given an intraperitoneal (i.p.) injection of 100mg/kg of 5-ASA dissolved in 1 mL of saline used for the normal resuscitation. The mice were then sacrificed one day after burn injury, as it is the time point where we see the most significant changes. We found that mice treated with 100mg/kg 5-ASA at time of resuscitation, did not up-regulate HSP25 or 72 in either small or large intestine IECs as seen by mRNA expression in Figure 9.

Examination of HSP72 at the protein level confirmed that 5-ASA did not increase the HSP72 response after burn injury (Fig. 10). To further elucidate whether 5-ASA could upregulate HSPs in response to burn injury, we looked further upstream at the master transcriptional regulator of HSPs, HSF1[18]. Quantification of mRNA expression of HSF1 after treatment with 5-ASA in the context of burn injury showed no significant changes in either small or large intestine IECs compared to the burn injury alone group (Fig. 11a and 11b). It is of interest to note, that burn injury alone does produce a significant decrease in HSF1 in both small and large intestine IECs one day after burn compared to sham controls, which gives evidence to our observations of decreased levels of HSPs following burn injury.

However, 5-ASA treatment did significantly restore claudin-4 and trends toward restoration of occludin expression in small intestine IECs one day after burn injury (Fig. 12). No changes in claudin-8 expression were observed in small intestine IECs following 5-ASA treatment after burn injury as seen in Figure 12. There was no restoration of the large intestine IEC tight junction proteins, claudin-4, claudin-8, or occludin, one day after burn injury with 5-ASA treatment (Fig. 13).

Burn injury is associated with high levels of inflammation in the gastrointestinal tract, which can potentiate increases in intestinal permeability[82,90]. We broadened our analysis of 5-ASA treatment to determine whether 5-ASA could be deemed beneficial in reducing the high levels of pro-inflammatory cytokines and chemokines. We, and others, have previously seen elevated post burn injury, such as IL-18, IL-6, KC, and MCP-1[7,82,91]. 5-ASA significantly reduced the small intestine IEC pro-inflammatory cytokines IL-18 (62% increase one day after burn and back to sham levels with 5-ASA) and IL-6

(34% increase back to sham levels one day post burn) in IECs after burn injury (Fig. 14). No significant changes were observed in the pro-inflammatory chemokine MCP-1 in small intestine IECs with 5-ASA treatment (Fig. 14).

In large intestine IECs there was a trend towards a reduction in IL-6 and the pro-inflammatory cytokine KC with 5-ASA treatment one day following burn injury. Interestingly, there was a significant decrease (63%) in the pro-inflammatory chemokine MCP-1 following 5-ASA treatment one day after burn injury in large intestine IECs (Fig. 15).

Increases in intestinal inflammation have been shown to potentiate increases in intestinal permeability[82], both of which have been theorized to inhibit intestinal peristalsis[11,92]. The inability to effectively move luminal content down the GI tract could dramatically change the luminal microenvironment creating more favorable environments for opportunistic pathogens[11,36,42,45,92,93]. Our lab has previously reported drastic alterations in microbial communities of the intestinal tract after burn injury[94] (submitted manuscript). This compounded with stalled intestinal peristalsis and increases in intestinal permeability could give evidence to our reports of bacterial translocation to extraintestinal sites resulting in some of the most common burn related complications of sepsis and MODs[95]. Therefore, it is critical to determine whether burn injury not only results in increased intestinal permeability, but also whether burn inhibits intestinal peristalsis. Hence, we performed a FITC-dextran permeability and transit assay. Mice were gavaged with FITC-dextran one day after burn injury. Three hours later plasma, stomach content, small intestine luminal content divided into three equal sections, and large intestine feces were collected and analyzed for the presence of FITC spectrophotometrically.

We have previously reported increases in intestinal permeability one day following burn injury using the aforementioned FITC-dextran assay[94] (submitted manuscript). After applying the FITC-dextran assay to assess intestinal transit, we found that burn injury significantly inhibits normal intestinal transit in burn injured animals as evidenced by an accumulation of FITC-dextran in the stomach content and small intestine #1 (Fig. 16). To our knowledge, however, there has been no attempt to reduce intestinal permeability and restore intestinal peristalsis following burn injury via treatment with 5-ASA. Therefore, we performed the FITC-dextran assay with the addition of 5-ASA treatment to determine whether 5-ASA following burn injury would decrease intestinal permeability and restore intestinal peristalsis.

As we've seen previously, the concentration of FITC-dextran in the plasma was significantly increased in burn injury alone relative to sham control giving evidence to increased gut leakiness post burn injury. With 5-ASA treatment following burn, the increase in intestinal permeability was completely restored to that of sham levels as there is no statistically significant difference in FITC-dextran concentrations in the plasma between sham controls and 5-ASA treated burn animals (Fig. 17). Additionally, treatment with 5-ASA following burn injury helps restore normal functioning intestinal peristalsis. This can be seen in Figure 18 with sham animals having the largest concentration of FITC-dextran transiting all the way to the colon. On the other hand, the FITC-dextran transit is halted in burned animals evidenced by zero to very little FITC-dextran in the colon with the vast majority residing in the stomach and small intestinal content #1. Treatment with 5-ASA post burn

allows for more efficient transit of FITC-dextran as more FITC-dextran can be detected in large feces with treatment (Fig. 18). It is not, however, back to sham control levels.

SMALL INTESTINE IECs LARGE INTESTINE IECs

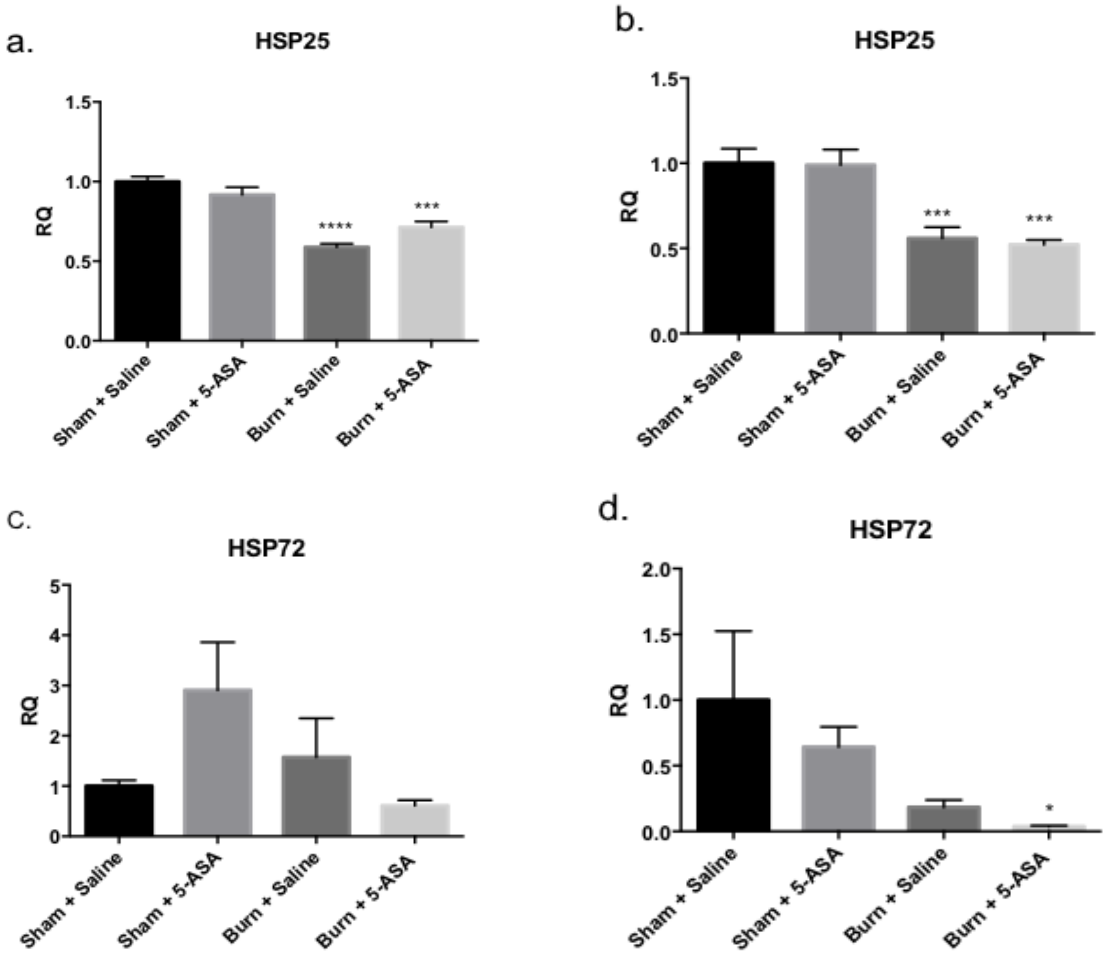


Figure 9: *5-ASA treatment after burn injury does not significantly induce mRNA expression of HSP25 or HSP72 following injury.* q-RTPCR of HSP25 and HSP72 mRNA expression in large and small intestine IECs relative to β-actin. Values are mean ± SEM of 6-8 animals per group expressed relative to sham. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, ****, p,0.0001 all groups relative to sham by ANOVA and Tukey post hoc test.

SMALL INTESTINE IECs LARGE INTESTINE IECs

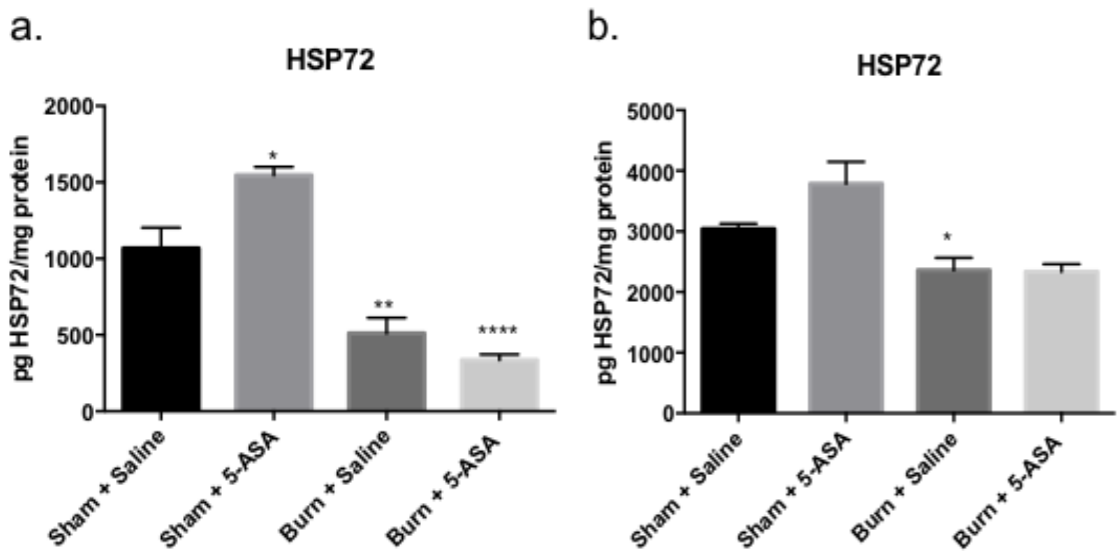


Figure 10: *5-ASA treatment after burn injury does not significantly induce protein levels of HSP72 in either small or large intestine IECs.* IECs of the small and large intestine were harvested and cells were lysed for protein extraction. ELISA on HSP72 was performed on the protein homogenate one day after injury and expressed as pg HSP/mg protein. *, $p<0.05$, **, $p<0.001$, ****, $p<0.0001$ all groups compared to sham, ANOVA and Tukey multiple comparison tests. Values are mean ± SEM of 6-8 animals per group.

SMALL INTESTINE IECs LARGE INTESTINE IECs

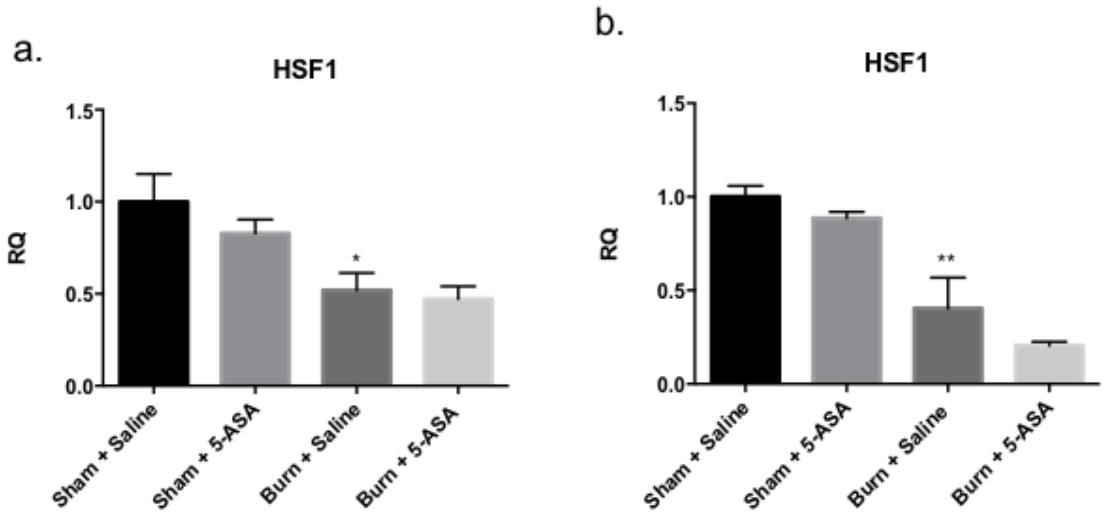


Figure 11: *5-ASA treatment after burn injury does not significantly induce mRNA expression of HSF1 in either small or large intestine IECs.* q-RTPCR of HSF1 mRNA expression in large and small intestine IECs relative to β-actin. Values are mean ± SEM of 6-8 animals per group expressed relative to sham. *, $p<0.001$, **, $p<0.0001$ all groups relative to sham by ANOVA and Tukey post hoc test.

SMALL INTESTINE IECs

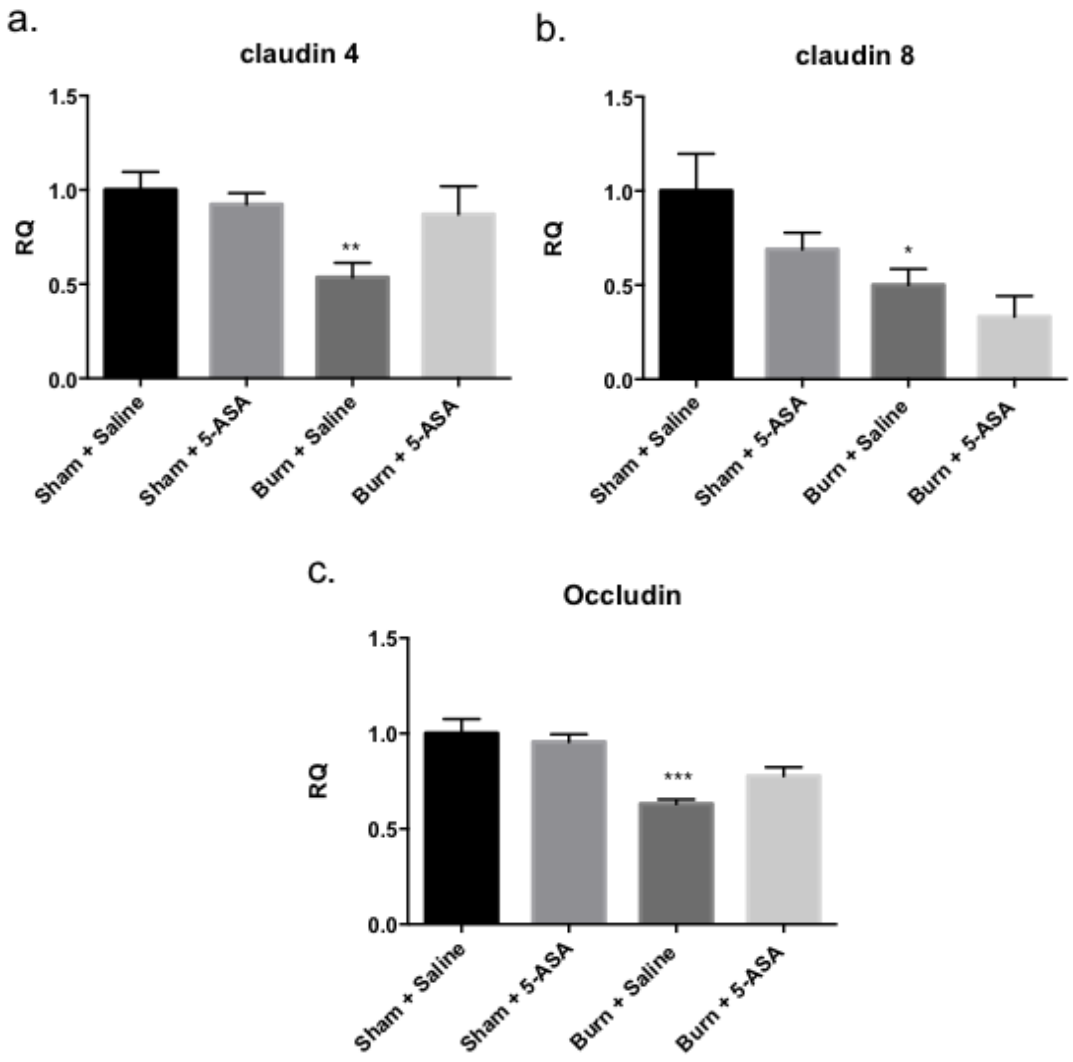


Figure 12: *Treatment with 5-ASA at time of burn injury significantly restores small intestine IEC claudin-4 expression to that of sham levels one day after injury, while a trend toward restoration exists of occludin expression in small intestine IECs one day post injury.* q-RTPCR of claudin-4, claudin-8, and occludin mRNA expression relative to β-actin. Values are mean ± SEM of 6-8 animals per group expressed relative to sham. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$ burn day one compared to sham by ANOVA and Tukey post hoc test.

LARGE INTESTINE IECs

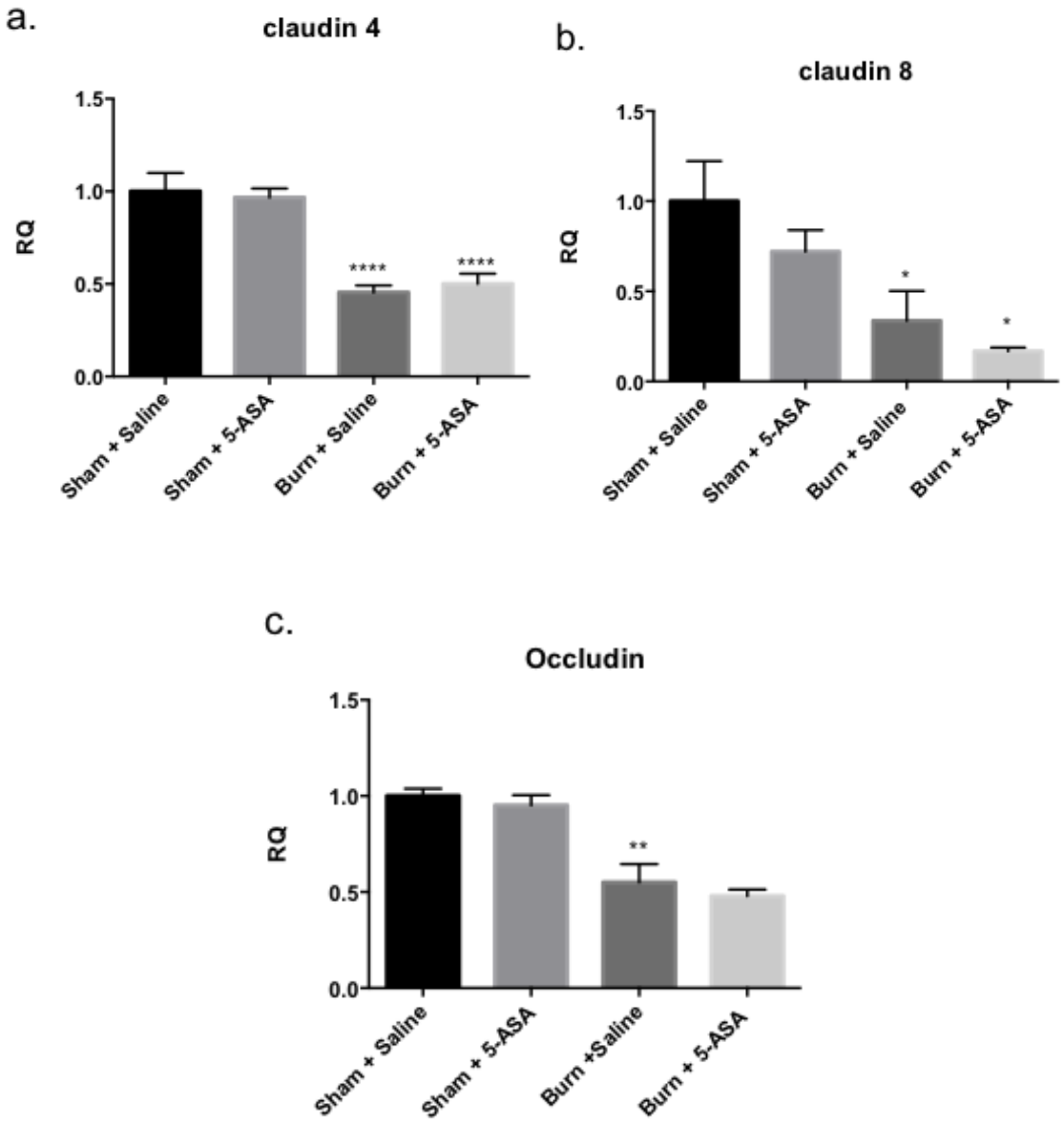


Figure 13: *Treatment with 5-ASA at time of burn injury does not affect expression of claudin4, claudin-8, or occludin in large intestine IECs after injury.* q-RTPCR of claudin-4, claudin-8, and occludin mRNA expression relative to β-actin. Values are mean ± SEM of 6-8 animals per group expressed relative to sham. *, $p<0.05$, **, $p<0.01$, ****, $p<0.001$ burn day one compared to sham by ANOVA and Tukey post hoc test.

SMALL INTESTINE IECs

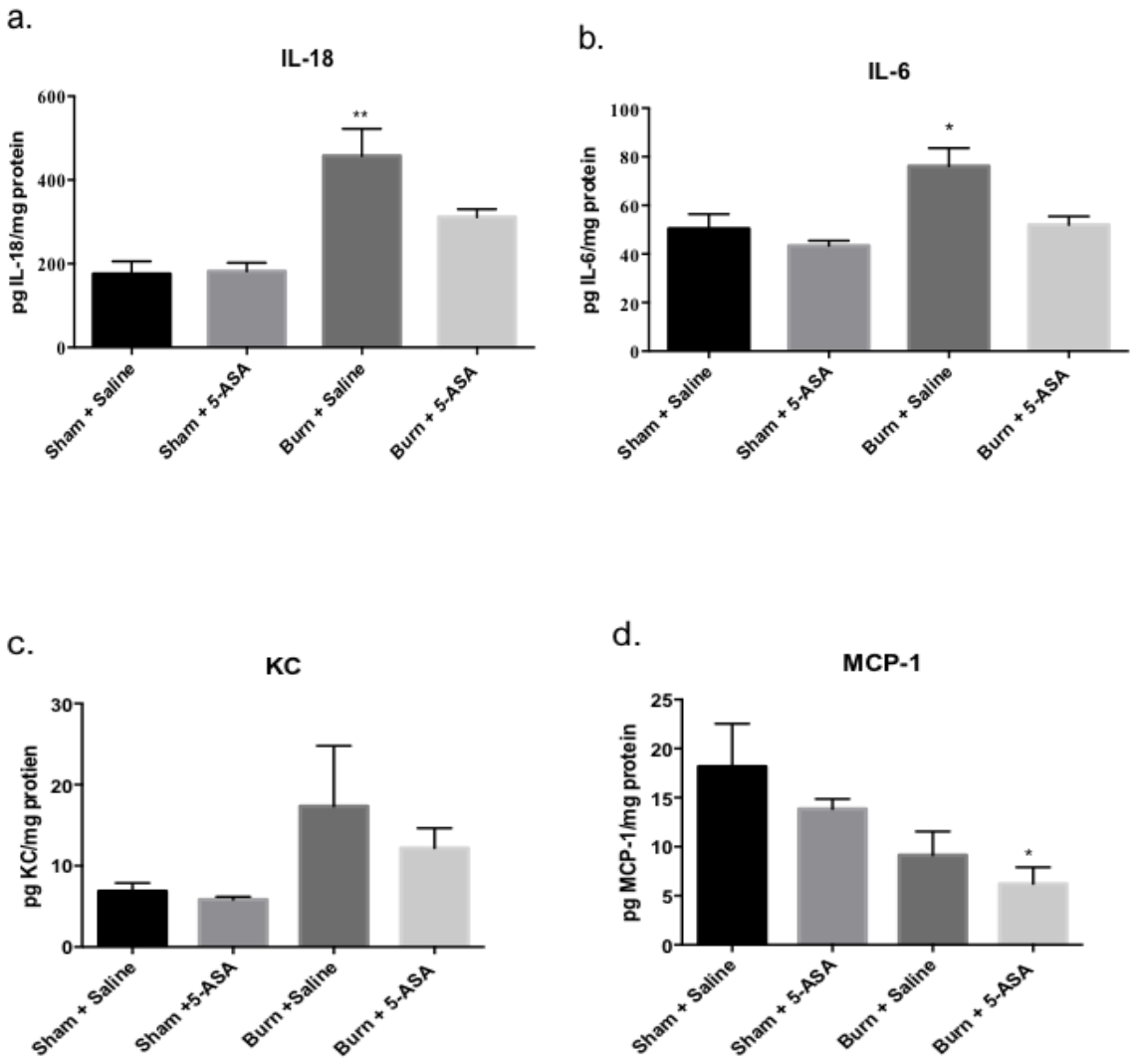


Figure 14: *Treatment with 5-ASA after burn injury significantly reduces the observed increase in the pro-inflammatory cytokines IL-18 and IL-6 back to that of sham levels one day after injury in small intestine IECs, with a trend toward reduction in the pro-inflammatory chemokine, KC, one day after injury.* IECs of the small intestine were harvested and cells were lysed for protein extraction. ELISAs on IL-18, IL-6, KC, and MCP-1 were performed on the protein homogenate one day after injury and expressed as pg/mg protein. *, $p<0.05$, **, $p<0.001$, all groups compared to sham, ANOVA and Tukey multiple comparison tests. Values are mean ± SEM of 6-8 animals per group.

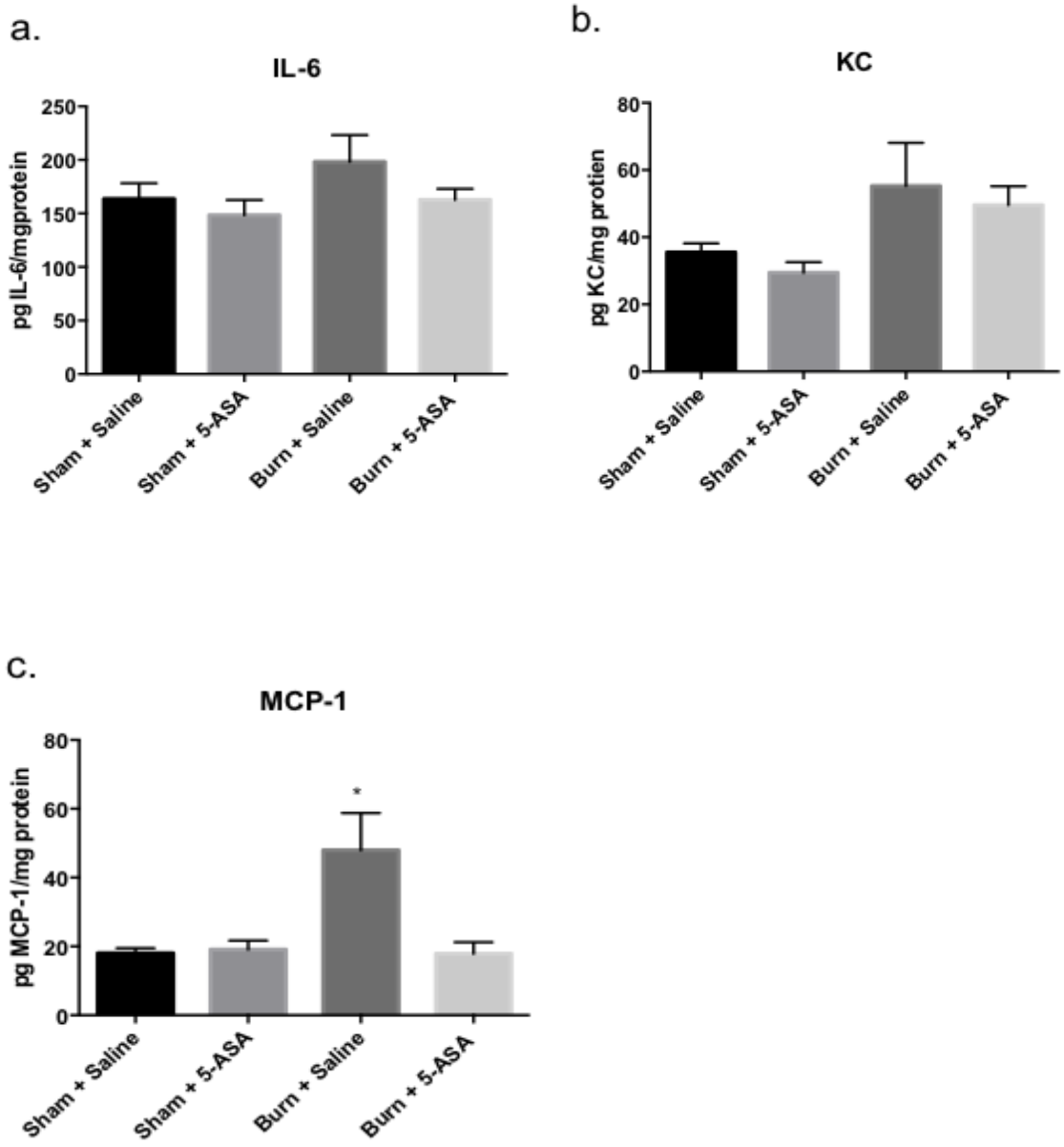


Figure 15: *Treatment with 5-ASA after burn injury significantly reduced the pro-inflammatory chemokine, MCP-1, back to that of sham levels in large intestine IECs one day after injury. 5-ASA treatment produced a trend towards a reduction in the pro-inflammatory cytokine/chemokines of IL-6 and KC one day after injury in large intestine IECs.* IECs of the large intestine were harvested and cells were lysed for protein extraction. ELISAs on IL-6, KC, and MCP-1 were performed on the protein homogenate one day after injury and expressed as pg/mg protein. *, $p<0.05$, all groups compared to sham, ANOVA and Tukey multiple comparison tests. Values are mean ± SEM of 6-8 animals per group.

INTESTINAL TRANSIT

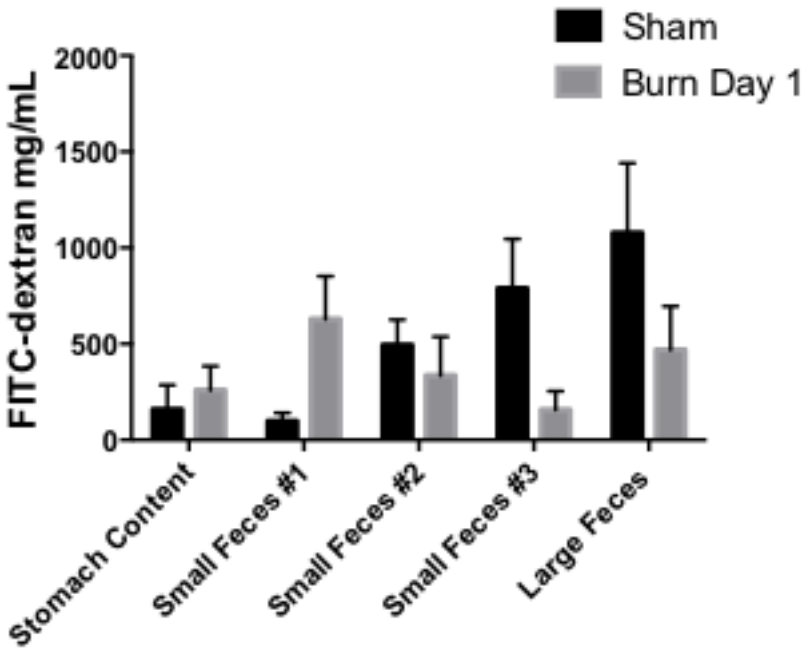


Figure 16: *Decreases in intestinal transit one day after burn injury.* . Mice were gavaged with FITC-dextran one day after burn or sham injury. Three hours after FITC-dextran gavage stomach content, small intestine luminal content divided into three equal lengths (#1 being proximal to stomach and #3 being distal), and large intestine feces were collected and visualized spectrophotometrically for presence of FITC-dextran. Values are mean ± SEM of 6-8 animals per group.

INTESTINAL PERMEABILITY AFTER 5-ASA

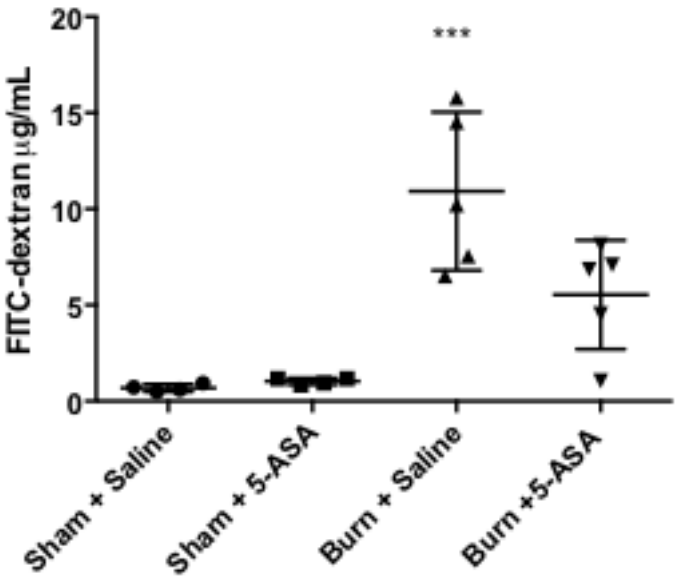


Figure 17: *Treatment with 5-ASA following burn injury significantly reduces intestinal permeability and restores intestinal peristalsis to that of sham levels one day after injury.* Mice were gavaged with one day after either burn or sham injury. Three hours later FITC-dextran was measured in plasma spectrophotometrically. Values are mean ± SEM of 4-6 animals per group. ***, $p<0.0001$ burn saline compared to sham control , ANOVA and Tukey multiple comparison tests.

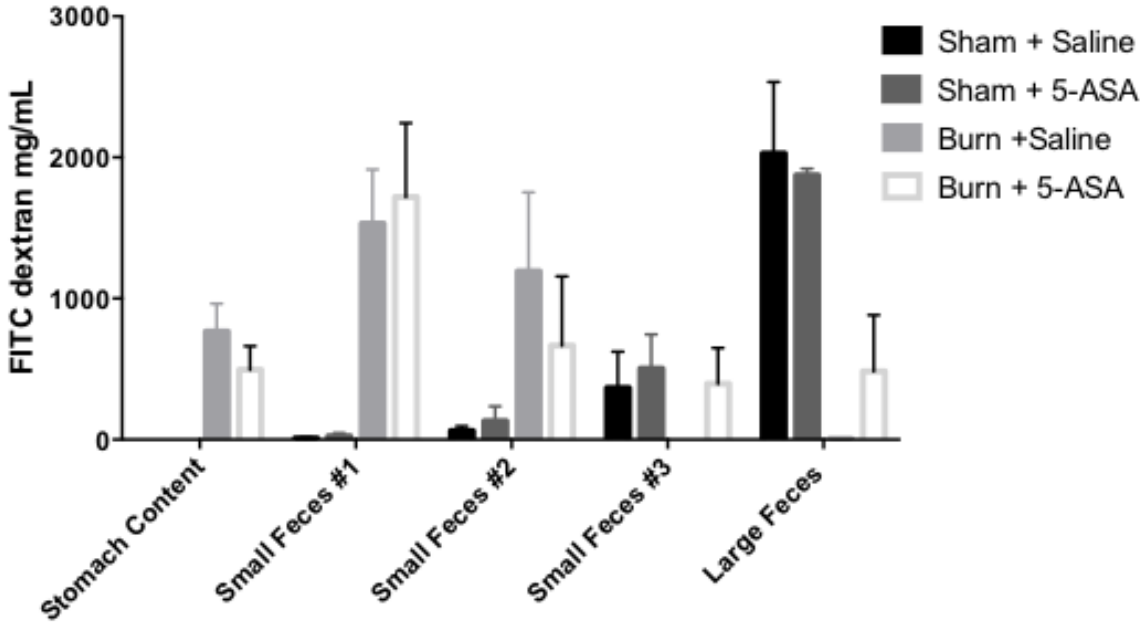


Figure 18: *5-ASA treatment partially restores normal intestinal transit one day after burn injury.* Mice were gavaged with FITC-dextran one day after burn or sham injury. Three hours after FITC-dextran gavage stomach content, small intestine luminal content divided into three equal lengths (#1 being proximal to stomach and #3 being distal), and large intestine feces were collected and visualized spectrophotometrically for presence of FITC-dextran. Values are mean ± SEM of 4-6 animals per group.

CHAPTER FIVE
SUMMARY AND DISSCUSSION

Sepsis and Multiple Organ Dysfunction syndrome remain the leading causes of post-burn morbidity and mortality [3-5,29]. Understanding the contributing factors and mechanisms behind the dysregulated immune response after burn trauma, which significantly predisposes patient's risk for secondary infections, is necessary for development of novel therapeutic intervention. As the gastrointestinal tract harbors trillions of resident bacteria, any breakdown in the intestinal epithelial barrier sequestering those bacteria to the luminal space can result in bacterial translocation to extraintestinal sites giving rise to patient's risk of sepsis [10,88]. IECs are sealed together via tight junctional complexes [43]. HSPs are known stabilizers of TJ proteins [24,89]. Therefore, it is critical to not only understand the role of TJ proteins in gut barrier integrity following burn, but also the role of HSPs. We hypothesized that decreased expression in the HSP response in IECs following burn injury potentiates decreases in TJ protein expression increasing intestinal permeability. The studies performed herein identify post burn changes in TJ proteins in IECs correlating with suppression of the HSP response, which suggests a potential role for HSPs in the maintenance of gut barrier integrity. This breakdown in HSPs and TJ proteins in IECs following burn injury could allow for bacterial translocation out of the lumen and into circulation, which could drive

the systemic inflammatory response and subsequent septicemia common in burn related trauma.

Under normal homeostatic conditions, IECs maintain the physical barrier of the intestinal tract by joining adjacent epithelial cells via formation of tight junctional complexes. This interaction is imperative to the integrity of a healthy gut, and any perturbation in tight junction protein expression of IECs could increase gut leakiness and bacterial translocation [7,15,16,43,82,95,96]. Therefore, we measured the expression of the tight junction proteins claudin-4, claudin-8, and occludin following burn injury. Using q-RTPCR we observed decreases in claudin-4 and claudin-8 expression one day after burn injury in small intestine IECs and a trend towards the same decreases on day one in large intestine IECs in both claudin-4 and claudin-8. Upon immunofluorescent staining of claudin-4 and claudin-8 in small intestinal tissue, we saw decreases in claudin-4 levels one day after injury. These alterations in TJ protein expression give evidence to our previously observed increases in intestinal permeability and bacterial translocation to extraintestinal sites, such as the MLNs, one day after burn injury [94]. As tight junctional complexes are comprised of many tight junction proteins such as claudin-1, claudin-2, claudin-3, zonal-occludin-3 and many others, expanding our analysis to include these other tight junction proteins would allow for better understanding of the breakdown in structure of tight junctional complexes following burn trauma. Understanding the contributing factors leading to TJ protein alterations after burn injury would allow for the intervention of new therapies directed at their specific upregulation or their upregulation via secondary targets.

We hypothesized that decreases in TJ protein levels following burn trauma was the result of a suppressed HSP response. At the cellular level, the host responds to acute environmental stress, such as disruptions in gut homeostasis after burn injury, by inducing the heat shock protein response. As cyto-protective proteins, the presence of HSPs would allow for alleviation of aforementioned stress by correctly re-folding denatured proteins as a consequence of stress and also aiding in the folding of naïve proteins needed to combat cellular stress [17,18,46,49,54,55,61,68]. Since it is not known how the HSP response is affected after burn injury, we measured the expression of the three most studied HSPs, HSP25, HSP72, and HSP90, in small and large intestine IECs four hours, one day, and three days post burn injury [47,56,57]. We found that burn injury decreased mRNA expression of HSP25, 72, and 90 in small intestine IECs one day after injury. In large intestine IECs, burn decreases expression of HSP25 four hours after injury, HSP72 four hours and one day after injury, and HSP90 four hours after injury. It is imperative to understand how burn injuries affect not only mRNA expression of HSPs, but also how HSPs at the protein level are altered after burn injury. Hence, at the proteins level, we found a suppressed HSP response in small intestine IECs in HSP25 four hours after injury and HSP72 four hours and three days after injury. In large intestine IECs, burn injury resulted in decreases in HSP25 four hours after injury and HSP72 one and three days after injury.

Although the exact mechanisms behind the cyto-protective functions of HSPs are yet to be fully understood, their expression has been linked to protection in various diseases such as ALS, ischemic heart disease, and IBD [14,50,54,57,61,68,70,74]. Transgenic mice have been created to overexpress the human isoform of HSP72, HSP70, and it would be interesting to

assess whether HSP70 overexpressing mice allow for protection from burn induced complications of intestinal permeability, inflammation, and bacterial translocation compared to wild type mice [57,74].

Mesalamine (5-ASA) has been shown to significantly reduce symptoms of intestinal inflammation in patients with two forms of IBD: Crohn's disease and ulcerative colitis [20,21,27,77,78]. 5-ASA has been shown to induce HSP72 *in vitro* in rat intestinal epithelial cells in response to cellular stress, providing a mechanism of protection to rat IECs [81]. As the intestinal inflammation and subsequent damage to intestinal epithelial cells in IBD mimics what we observe in burn intestines, we attempted to induce the HSP response *in vivo* with 5-ASA after burn injury. Measurement of HSP25, HSP72, and HSP90 after 5-ASA treatment yielded no significant induction in any of the three HSPs in both small and large intestine IECs. However, 5-ASA treatment did significantly restore claudin-4 and trends toward restoration of occludin expression in small intestine IECs one day after burn injury. As burn injury results in high levels of inflammation in the gastrointestinal tract, which can potentiate increases in intestinal permeability, we measured levels of IL-18, IL-6, KC, and MCP-1 in small and large IECs one day after burn with 5-ASA treatment [82,90]. We've previously reported increase in all four pro-inflammatory cytokines/chemokines in intestinal tissues, and found that 5-ASA significantly reduced the small intestine IEC pro-inflammatory cytokines IL-18 and IL-6 in IECs after burn injury [7,82,91]. In large intestine IECs there was a trend towards a reduction in IL-6 and the pro-inflammatory cytokine KC with 5-ASA treatment one day following burn injury. Interestingly, a drastic decrease in the pro-inflammatory chemokine MCP-1 was observed following 5-ASA treatment one day after burn injury in large intestine IECs.

Intestinal inflammation can significantly increase intestinal permeability, both of which can halt normal intestinal peristalsis [11,82,94]. Without the ability to move luminal content down the GI tract, drastic changes in the microenvironment of the gut can ensue, producing environments more suited to opportunistic pathogens [11,16,36,44,92,94,95]. We have previously reported severe dysbiosis of the intestinal tract after burn injury, and compounded with inhibition of intestinal peristalsis and increases in intestinal permeability, would give rise to our reports of bacterial translocation to extraintestinal sites [15,94,95]. Treatment with 5-ASA reduced intestinal permeability to that of sham levels and partially restored the intestinal peristalsis we observe one day following burn injury.

5-ASA treatment after burn injury has its limitations as the highest dosage able to be dissolved in normal saline is 100mg/kg. Future studies will be critical to determine dosages and delivery systems for optimal efficacy.

Treatment with 5-ASA does not act as an agonist of HSPs after burn. Yet, it could potentially be used as treatment to decrease intestinal permeability by restoring tight junctional complexes and decreasing the inflammation associated with burn injury.

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 yielding two groups. For the 5-ASA treatment, animals were divided into four groups sham + saline , sham + 5-ASA, burn + saline, and burn + 5-ASA. The mice were anesthetized with a 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=kW2/3, given k=10, and weight in grams[86]. 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. For the 5-ASA treatment, 5-ASA treatment groups received 100mg/kg 5-ASA (Santa Cruz) dissolved in normal saline. Animals were allowed food and water ad libitum. Mice were sacrificed and organs or tissue were collected four hours, 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 50.l 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/.l and 10.l of each sample was added to a 96 well plate
3. 10.l of RT master mix was added to each sample which contained, the reverse transcription enzyme, dNTPs, random primers, and H20 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/.l for qPCR

qPCR

1. The qPCR master mix was created using 10.l/rxn TaqMan Fast Advanced qPCR supermix, 1.l/rxn TaqMan primer and probe (FAM), and 1.l/rxn TaqMan GAPDH or β-actin endogenous control (VIC).
2. 12.l was pipetted into each well of a 96 well plate
3. 8.l of cDNA sample at 30/8 .l 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

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, phosphatase inhibitor, and PMSF to manufacturers instructions
2. Add 500.l lysis buffer per sample (small intestine IECs or entire large intestine IECs from cecum)
3. Keep samples on ice 45 min.
4. Centrifuge for 5 min at 10000rpm at 4°C.
5. Collect and aliquot.
6. Store homogenates at -80°C.

ELISA Supplies:

• HSP25 ELISA kit (Enzo Life Sciences)
• HSP72 ELISA kit (R&D)
• IL-18 ELISA kit (eBioscience)
• IL-6 ELISA kit (BD)
• KC ELISA kit (R&D)
• MCP-1 ELISA kit (R&D)

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 manufacturer's protocol, and suggested antibody dilutions
3. Read plate with a spectrophotometer at wavelengths suggested by manufacturer.

Immunofluorescence

Supplies

- Humidity Chamber
- Pap Pen
- 100% ice cold acetone
- 100% ice cold ethanol
- 1X PBS
- 1% BSA in 1X PBS
- Primary antibody to protein of interest
- Secondary antibody conjugated to fluorophore
- Prolong Gold antifade reagent with DAPI

Procedure

1. Mark area with pap pen
2. Immerse slides in 100% ice cold ethanol for 30 min in freezer.
3. Remove slide from ethanol and immerse in 100% ice cold acetone for 3 min in freezer.
4. Remove from acetone and allow to air dry for 3 min
5. Immerse in 1X PBS for 1 min
6. Put slide in humidity chamber. Add 200μL Superblock over tissue area. Incubate for 5 min.
7. Rinse 3X in PBS for 2 min
8. Add appropriate amount of primary antibody ~200μL in 1% BSA in PBS over tissue area in humidity chamber. Incubate for 2 hours.
9. Rinse 3X in PBS for 2 min.
10. Turn lights off.
11. Add appropriate amount of secondary antibody ~200μL in 1% BSA in PBS over tissue area in humidity chamber. Incubate for 1 hour.
12. Rinse 3X in PBS for 2 min.
13. Add ~200μL phalloidin dye to tissue area in humidity chamber. Incubate for 30 min.
14. Rinse 3X in PBS for 2 min.
15. Dry slides.
16. Mount with Prolong Gold antifade reagent with DAPI.
17. Allow to dry overnight.
18. Image with Zeiss Axiovert 200m fluorescent microscope

FITC-dextran Permeability and Transit Assay

1. On the day of sacrifice, gavage mice with .4ml of 22mg/ml FITC-dextran (Sigma-Aldrich) in PBS.
2. After 3 hours draw blood and sacrifice the mice.
3. Collect:
    -stomach content
    -small intestine luminal content divided into 3 equal parts: #1, #2, #3
        -#1 being proximal to stomach and #3 being most distal
4. The blood was centrifuged for 8000rpm for 5min at 4°C, plasma isolated.
5. Each animals stomach and luminal contents from each section were weighed and normalized to 5X PBS addition.
6. Sonicate for 30 seconds using XL-2000 Misonix.
7. Centrifuge samples for 10 min at 8000rpm at 4°C.
8. Remove supernatant.
9. Standards of FITC-dextran was prepared using 2 fold dilutions of pure FITC-dextran in PBS, the high standard being 3 mg/ml for luminal content and 100μg/ml for plasma.
10. Equal volumes of standard, plasma, and luminal content were pipetted into the corresponding well, 96 well plate.
11. The plate was read spectrophotometrically at 480nm excitation and 520nm.

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VITA

Abigail Cannon was born in Missoula, Montana and raised in Ottawa, Illinois. Before attending Loyola University Chicago Biomedical Sciences for her Master's of Science, she attended Loyola University Chicago for her undergraduate education. At Loyola she acquired a Bachelor's degree with a major in Biology and minor in Psychology in the winter of 2010.

She became interested in Microbiology and Immunology after completing a course in Microbiology during her undergraduate work. It was there that her love for Science and teaching flourished. Therefore, she has chosen to continue with the Ph.D. program at Loyola University Chicago Biomedical Sciences.

Once she has completed her Ph.D., she hopes to become a university professor while continuing research with her students.