Role of Myosin Light Chain Kinase in Binge Ethanol and Burn Injury-Induced Intestinal Barrier Dysfunction

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Recommended Citation
Zahs, Anita, "Role of Myosin Light Chain Kinase in Binge Ethanol and Burn Injury-Induced Intestinal Barrier Dysfunction" (2012). Dissertations. 421.
https://ecommons.luc.edu/luc_diss/421
LOYOLA UNIVERSITY CHICAGO

ROLE OF MYOSIN LIGHT CHAIN KINASE IN BINGE ETHANOL AND BURN INJURY-INDUCED INTESTINAL BARRIER DYSFUNCTION

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTORATE OF PHILOSOPHY

PROGRAM IN MOLECULAR AND CELLULAR BIOLOGY

BY
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CHICAGO, ILLINOIS
DECEMBER 2012
ACKNOWLEDGEMENTS

I would like to thank my family, friends, and lab for their help with everything I do, for making me laugh, for distracting me, and for always being there no matter the circumstances. I would like to thank my mentor, Liz, for her guidance, support, and letting me raid her refrigerator for the past 4 years. Finally, I would also like to thank my committee for their suggestions, patience, and all they’ve done to aid in the development and completion of this project.
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LIST OF ABBREVIATIONS

AMP antimicrobial peptide
BAC Blood alcohol content
BMP bone morphogenic protein
Ca$^{2+}$ calcium
CFU colony forming unit
CPI-17 C-potentiated protein phosphatase-1 inhibitor
E-cadherin epithelial cadherin
EGF epithelial growth factor
ERK extracellular signal-related kinase
GPCR G protein coupled receptor
Grb2 growth factor receptor-bound protein 2
GDP guanosine diphosphate
GTP guanosine triphosphate
IFN$\gamma$ interferon gamma
IκB inhibitor of NF-κB
IKK IκB kinase
IL interleukin
IP$_3$ inositol triphosphate
SHP-2    SH-2 containing protein phosphatase
Socs3    suppressor of cytokine signaling 3
SOS      son of sevenless
Src      Src kinase
Stat3    signal transducer and activator of transcription 3
TBSA     total body surface area
Th17 cell T helper 17 cell
TNFα     tumor necrosis factor alpha
Tyr      tyrosine
Y        tyrosine
ZO-1     zonula occludens protein
CHAPTER 1
INTRODUCTION

Ethanol is a common factor in traumatic injury, including burn injury. Previous studies from our laboratory indicate that ethanol increases both pulmonary and gastrointestinal inflammation as well as susceptibility to infection in burn-injured mice; however, the mechanisms of these responses are not entirely known. Moreover, after burn injury, bacteria and their products leak out of the intestinal lumen and into the bloodstream. These bacteria can disperse throughout the body leading to pulmonary damage, acute lung injury, sepsis, and death. Ethanol in combination with burn injury has been shown to decrease intestinal barrier function greater than either insult alone. Long (210 kDa) smooth muscle myosin light chain kinase (MLCK), an enzyme important for epithelial tight junction maintenance, has been implicated in barrier alterations after burn injury or ethanol exposure alone. With this knowledge, we hypothesize that the combination of binge ethanol exposure and burn injury causes an increase in intestinal barrier dysfunction due to inflammation-mediated activation of myosin light chain kinase. To test this hypothesis, three aims are proposed: 1) to determine whether elevated myosin light chain kinase activation mediates the intestinal permeability alterations in mice receiving binge ethanol and burn injury treatment, 2) if interleukin-6 (IL-6) activates myosin light chain kinase and, 3) the signaling mechanism leading to MLCK activation after ethanol exposure and burn injury. Bacterial translocation along with
immunofluorescent staining for tight junction protein localization will be performed to investigate intestinal barrier function and integrity. MLCK activation will be determined by Western blot analysis. Inhibition and knock out of MLCK will be employed as well to examine the role of this molecule on intestinal permeability after acute ethanol and burn injury exposure. We will investigate MLCK activation and tight junction integrity in IL-6 knockout mice and wild type mice given an IL-6 neutralizing antibody. Finally, we will examine the signaling mechanism leading to MLCK activation in our model of ethanol and burn. To do this, activation of other mediators of the MLCK pathway, including Rho kinase (ROCK), myosin light chain phosphatase (MLCP) and Src kinase, will be determined. These studies will help gain an understanding for how even binge ethanol in combination with burn injury can result in decreased barrier function that has been clinically observed and can result in increased morbidity and mortality.
CHAPTER 2

REVIEW OF RELATED LITERATURE

**The intestinal barrier – homeostasis is key**

Enterocytes and junction complexes

Intestinal epithelial cells (IECs) form a single layer barrier separating the lumen of the intestine from the intestinal tissue, known as the lamina propria. On the luminal side of the intestinal barrier lie trillions of bacteria which have colonized the gut and aid in digestion of food. Commensal bacteria are not harmful to the host, and their presence in the intestine prevents harmful bacteria from colonizing that niche. Besides enterocytes, goblet cells, another type of intestinal epithelial cell, produces mucin to form a mucus layer to protect the barrier as well (1). In addition to protection, the intestinal barrier is also responsible for absorption of nutrients and some water balance though most of water reabsorption takes place in the large intestine. To form the epithelial layer, polarized IECs are connected by several types of junction complexes including tight junctions, adherens junctions, and desmosomes (Figure 1). The most apical junction is the tight junction and represents the first line of defense from luminal pathogens. Tight junctions are composed of various proteins including occludin, claudins, and zonula occludens proteins (ZOs) (2). Zonula occludens protein 1 (ZO-1) can bind and cross-link actin (3). Other ZO isoforms (ZO-2 and ZO-3) are also capable of binding but not cross-linking actin. ZO-1 is also known to bind occludin and claudins thus linking them to the actin
Figure 1: A variety of junction complexes adheres intestinal epithelial cells together to form a barrier. Intestinal epithelial cells are held together by tight junctions, adherens junctions, and desmosomes. Tight junctions are the most apical junction and are composed of occludin, claudins, and ZOs. These junctions are anchored to the actin cytoskeleton. Just basolateral to tight junctions are adherens junctions, which like tight junctions are attached to actin. Cadherins, E-cadherin in epithelial layers, are Ca^{2+}-dependent, transmembrane proteins that use α and β-catenins to anchor the complex to actin. Finally, desmosomes, composed of desmoplakin, desmoglein, and desmocollin, are anchored to keratin and are the most basolateral of the junction complexes.
cytoskeleton (4). While ZO-1 acts as a scaffolding and cross-linking protein, it is cytoplasmic and does not interact with other cells. Occludin and claudins are transmembrane proteins that bind occludin or claudins from neighboring cells thus forming a tight junction. As tight junctions are anchored to actin, they are partially under cytoskeletal control. Signals that mediate cytoskeletal contraction, such as histamine, thrombin, and sodium absorption also cause tight junction disruption and permeability (5-7). While this permeability is essential for absorption of some nutrients, extensive or prolonged openings in the barrier can allow bacteria to move into the intestinal tissue causing inflammation and tissue damage. Adherens junctions and desmosomes also connect IECs. Adherens junctions are just basolateral to tight junctions and contain cadherins, E-cadherin in epithelial barriers, as well as β and α-catenin (8). Cadherins are Ca²⁺-dependent, transmembrane proteins and using the catenin proteins, also anchor to the actin cytoskeleton (8). Unlike the other more apical junction complexes, desmosomes are anchored to keratin. Desmoglein and desmocollin are transmembrane proteins that bind to desmoplakin, which then anchors the entire complex to keratin (9).

Immune component of the intestinal epithelial barrier and lamina propria

Besides enterocytes, several other types of cells, such as intestinal epithelial lymphocytes (IEL) and specialized epithelial cells compose the intestinal epithelial barrier. (Figure 2). These cells fit predominantly into 2 classes, immune cells and other types of intestinal epithelial cells. IELs are T cells scattered throughout the epithelial barrier and are essential for fighting and killing bacteria and other attacks, such as viruses, on the barrier (10). These T cells also produce antimicrobial peptides which
Figure 2: The intestinal barrier contains a diverse population of cells. The intestinal epithelial barrier consists of many different cell types besides enterocytes. Goblet cells secrete mucin, which helps create the protective, mucus barrier. Paneth cells reside in the crypt and contain granules containing AMPs and other bacteria-killing compounds. Both goblet and Paneth cells prevent bacteria from adhering from the epithelial barrier. M cells reside in the epithelial lining of the ileum adjacent to Peyer’s patches. Bacteria translocate through these cells into the lamina propria where they are phagocyted by antigen presenting cells and presented to resident lymphocytes for killing or to develop tolerance. IELs are important for bacterial killing in cases of infection, but also for maintenance of tolerance to commensal bacteria. Finally, dendritic cells can intercalate themselves into the epithelial layer to sample the bacterial products in the lumen to generate tolerance.
prevents bacterial overgrowth (11). Furthermore, dendritic cells can intercalate into intestinal epithelial barrier in order to detect bacterial products and initiate subsequent activation of the immune response which involves activation of T cells and cytokine and chemokine production recruiting more lymphocytes to fight the insult (12). Dendritic cells also randomly sample the luminal bacterial products in order to promote tolerance to tolerance of commensal bacterial (12). Without tolerance, a constant immune response to the commensal bacteria would happen and lead to malabsorption and tissue damage. In addition to dendritic cells in the lamina propria and Peyer’s patches, macrophages reside in these areas as well and act as phagocytes and antigen presenting cells as necessary. Together, IELs, dendritic cells, and macrophages (as well as lamina propria resident lymphocytes and occasional neutrophils) comprise a vast component of the immune system in charge of regulating intestinal epithelial immunity and tolerance.

Specialized intestinal epithelial cells and stem cells

Paneth cells, goblet cells, M cells, transit amplifying cells and intestinal stem cells constitute the rest of the intestinal barrier. Found only in the crypts, Paneth cells contain granules consisting of antimicrobial peptides (AMPs) and other compounds necessary for killing bacteria (13). Enterocytes are also capable of producing AMPs to aid in the defense of the intestinal epithelial barrier (14). As mentioned previously, goblet cells release mucin, the predominant component of the mucus layer. Goblet cells are found throughout both crypts and villi to maintain the protective mucus layer (15). Finally, microfold (M) cells are specialized epithelial cells found only in the ileum. M cells sit directly above Peyer’s patches, organized areas of immune cells necessary for quick
response to infection or injury (16). Pathogens may translocate through M cells and encounter immune cells within the Peyer’s patch. This encounter leads to bacterial killing under normal conditions; however, following injury or exposure to other detrimental factors, the immune response is suppressed allowing bacteria to survive and perhaps disseminate through the body (17-19).

When intestinal epithelial cells die, they are shed predominantly from the villus tip. The intestinal epithelium is replaced every 3-5 days indicating the need for an active stem cell population in the gut. Intestinal stem cells reside in the crypts of the intestine and, when needed, differentiate, proliferate, and move up the villus. Intestinal stem cells can become any of the different epithelial lineages including enterocyte, Paneth cell, or goblet cell (20). Maintenance of this stem cell population and subsequent differentiation is tightly controlled by gradients in the Wnt and bone morphogenic protein (BMP) signaling pathways. High Wnt signaling in the crypt maintains the stem cell population and prevents differentiation, except in Paneth cells where Wnt signaling is necessary for Paneth cell maturation (21). Transit amplifying cells are the immediate progeny of stem cells and as these cells move up the villus, Wnt agonists decrease and BMP agonist increase promoting differentiation into secretory (Goblet) or epithelial cells (22).

The intestinal epithelial barrier is a complex system balancing absorption of nutrients and protection from outside insults including commensal bacteria and ingested toxins or allergens. Although the intestinal barrier is constantly balancing these issues, in healthy individuals, no problems are apparent as IECs are replaced every 3-5 days to preserve the barrier. Unfortunately, most insults on the body perturb the intestinal barrier
itself or the immune component within the intestine. In some cases, both are altered causing even greater damage. Maintenance of the intestinal epithelial barrier is one of our bodies’ first lines of defense against ingested toxins and contaminants and determining mechanisms of how this barrier is affected after injury is important for preventing further damage and discovering treatments to aid in faster recovery.

**Myosin light chain kinase activation and intestinal permeability**

**MLCK isoforms and activation**

Activation of numerous signaling pathways, including cytokines, bacteria, traumatic injury, sodium transport, and ethanol (5, 23-26), can lead to cytoskeletal contraction, tight junction disruption, and ultimately, intestinal permeability. Ultimately, most of these pathways end up in the activation of myosin light chain kinase (MLCK) (5, 24, 25). When examining permeability in an epithelial barrier, it is the long (210 kDa) form of smooth muscle MLCK that is being discussed. There are two genes for MLCK in vertebrates, one for skeletal muscle MLCK and the other for smooth muscle MLCK (27). Smooth muscle MLCK isoforms (long, 210 kDa and short, 130 kDa) are produced from the same gene but different promoters. The short and long forms of MLCK are very similar with the C-terminus and catalytic cores being exactly the same (28). Long MLCK does have an N-terminal extension consisting of two actin binding domains and six Ig domains (29). These actin binding domains allow MLCK to anchor to actin and bring the catalytic core in proximity of myosin light chain (MLC) so MLCK can phosphorylate MLC. Smooth muscle MLCK is ubiquitously expressed throughout the body; however, the long form is predominantly expressed in barrier tissues, such as the intestine, lung,
and kidney (27). MLCK activation is necessary for normal intestinal barrier function as it permits nutrient absorption and IEC proliferation.

Encoded in the MLCK protein is its own autoinhibitory sequence that blocks the catalytic core and prevents MLCK from phosphorylating MLC. In the presence of calcium (Ca^{2+}), calmodulin is activated and can bind to MLCK causing the inhibitory domain to move and expose the catalytic core (30). Active caspase-3 can also cleave MLCK, removing the inhibitory domain thus producing a constitutively active MLCK (31). Although the autoinhibitory sequence blocks catalytic activity, it does not prevent ATP from binding. As soon as inhibition is released, MLCK can phosphorylate MLC (30). Once MLC has been phosphorylated (pMLC, Ser 19) it can interact with actin and initiates cytoskeletal contraction.

Regulation of MLCK activation

As MLCK is a Ca^{2+}-dependent enzyme, any pathway that generates calcium release has the potential to activate MLCK. G-protein coupled receptors (GPCR), particularly those with α_{q} and α_{12/13} subunits, such as protease-activated receptors (PAR), and growth factors are a few common pathways known to initiate calcium release (6) (Figure 3). Additional to MLCK activation, several other key enzymes are also activated downstream of GPCR or growth receptor signaling that can regulate MLCK activity. The small GTPase, Rho, is one of these enzymes. Rho, when bound to GTP, can activate Rho kinase (ROCK) (32). ROCK is also a serine/threonine kinase and has been demonstrated to phosphorylate MLC’s activation site, serine 19 (33), but this is one of its minor targets. Recent work suggests that the role of ROCK in MLCK activation is through inactivation
Figure 3: MLCK activation can be regulated by different pathways. MLCK activation can be controlled by a variety of different pathways with the PAR-1 (GPCR) and EGFR pathway demonstrated. Following stimulation of PAR-1, $\alpha_{12/13}$ subunits can lead to the activation of Rho guanine exchange factors (Rho-GEF) which promote exchange of GDP for GTP thus initiating Rho function. Active Rho (Rho-GTP) can then bind ROCK, which phosphorylates myosin light chain phosphatase and inhibits its function. Other $\alpha$ subunits ($\alpha_q$) activate phospholipase C (PLC), ultimately triggering release of calcium. Calmodulin binds Ca$^{2+}$ which when bound to the autoinhibitory domain of MLCK, released the inhibition and allows MLCK to phosphorylate MLC. In addition to calcium regulation, tyrosine phosphorylation of MLCK enhances its activity. Src kinase, known to be activated following interleukin-6 (IL-6) stimulation, can phosphorylate MLCK and augment its ability to phosphorylate MLC.
of MLCP more so than direct phosphorylation of MLC (34). Phosphorylation at threonine 696 inhibits MLCP (35). When active, MLCP de-phosphorylates MLC consequently preventing its interaction with actin and cytoskeletal contraction. A balance between MLCK and MLCP activity (or inactivity) aids in the maintenance of the intestinal barrier and prevents intestinal permeability. ROCK also phosphorylates MLCP at threonine 850, which is a regulatory site and its significance is not known (36, 37). Finally, as previously mentioned, GPCR and PAR-1 signaling can also regulate MLCP activation. In addition to ROCK phosphorylation, CPI-17 (C-kinase potentiated protein phosphatase-1 inhibitor) binding also inhibits MLCP. CPI-17 must be phosphorylated to bind to MLCP and PKC is one known kinase for this modification of CPI-17 (38). Although CPI-17 has been revealed as a MLCP inhibitor, it is not known to be expressed in the intestine. In addition to the aforementioned proteins, MLCK activity is also regulated by phosphorylation of itself. Protein kinase C (PKC), a serine/threonine kinase is also calcium activated. PKC has many phosphorylation targets, but in relation to MLCK, it acts as an inhibitor. Phosphorylation within MLCK’s catalytic core or calmodulin binding site inhibits MLCK function, and both PKC (α and β) and PKA can phosphorylate this site (28). No other isoforms of PKC are known to phosphorylate MLCK; however, both PKCd and PKCe are linked with TNFα-induced intestinal barrier dysfunction (250, 251). Furthermore, PKC and PKA also phosphorylate MLC thus decreasing MLCK’s affinity for MLC, and if MLC has already been phosphorylated at serine 19, phosphorylation at serines 1 and 2 or threonine 9 (PKC consensus sites) it has a decreased ability to bind to actin (39, 40).
While PKC and PKA activity inhibit MLCK, Src kinase, can elevate MLCK activity. Within the N terminal extension of long MLCK are several Src consensus sites that when phosphorylated enhance MLCK activity (41). Furthermore, pro-inflammatory cytokines, particularly TNFα, IL-1β, and IL-6 have all been associated with augmented MLCK activation (24, 25, 42, 43). With numerous players involved in the regulation of MLCK activation (Figure 3), it becomes apparent that imbalance in either direction (too much MLCK activity or too little) may cause dire consequences for the intestine and the whole organism.

Consequences of an elevation in MLCK activity are observed in diseases, such as inflammatory bowel disease (IBD), as well as following infection, trauma, alcohol (ethanol) exposure (24, 25, 43, 44). Cytokines and MLCK activity have long been studied in the intestine, particularly TNFα and MLCK. Along with IBD, intestinal ischemia, and graft versus host disease are all linked with TNFα-induced MLCK activation (45-47). Interestingly, TNF-induced MLKC activity elevates paracellular permeability and anti-TNFα antibody treatment has become prominent in management of IBD (48-50). Both injury and disease affects the entire body, but is worsened in part by the “gut-lymph hypothesis”. This hypothesis states that bacteria (or bacterial components) translocating from the intestinal lumen (through a damaged intestinal epithelium) move into the mesenteric lymph nodes where, if not killed, eventually end up being moved back into the bloodstream (125). Bacteria-laden blood is then free to circulate throughout the body promoting further systemic problems. With this knowledge, understanding the role of MLCK following insult becomes important in the treatment of a patient.
**IL-6, intestinal homeostasis, and intestinal damage**

**IL-6 signaling**

IL-6 and IL-11, along with oncostatin M, leukemia inhibitor factor, and several others, make up a family of cytokines known primarily for their roles in inflammation. Using the IL-6 receptor (IL-6R) and gp130, IL-6 stimulates JAK3 activation which phosphorylates the transcription factor Stat3 (signal transducer and activator of transcription 3). Phosphorylated Stat3 then homodimerizes, and moves into the nucleus where it leads to the transcription of other pro-inflammatory cytokines, transcription factors, and an inhibitor to IL-6 signaling, SOCS3 (suppressor of cytokine signaling 3). SOCS3 inhibits JAK3 thereby limiting IL-6 signaling activity (51) (Figure 4).

Many cell types express IL-6R (52), such as hepatocytes, immune cells, and fibroblasts although its expression on enterocytes has been debated. (52, 53). Recently, a soluble form of IL-6R was reported (54). Soluble IL-6R could bind IL-6 and bind to any cell expressing gp130. This vastly enhances the number of IL-6 responsive cells as gp130 is ubiquitously expressed (55).

**IL-6 is necessary for homeostatic functions**

Although IL-6 is predominantly associated with inflammation, it is required for some normal cellular functions. Under healthy conditions, a nominal level of NF-κB signaling occurs in intestinal epithelial cells (56). IL-6, along with many other cytokines, induces NF-κB activation. This low level of NFκB activation does not cause an immune response, but instead, promotes cell survival and expression of anti-apoptotic genes (56). Inhibition of NF-κB causes intestinal damage and inflammation in the epithelial barrier.
Figure 4: IL-6 signaling activates NF-κB, ERK, and Src kinase. IL-6 signaling through IL-6R and gp130 activates Stat3 leading to transcription of other cytokines, transcription factors and its own inhibitor, Socs3. IL-6 binds to IL-6R, recruits gp130 and initiates the signaling cascade, ultimately ending in the activation of NF-κB, pStat3 and other transcription factors. These factors induce expression of genes including other cytokines and SOCS3, an inhibitor to IL-6 signaling. Activation of the IL-6 signaling pathways also activates Src kinase, a kinase known to enhance MLCK activity.
Loss of both IKK1 and IKK2 in intestinal epithelial cells led to development of colitis; however, knockout of only one IKK did not cause any epithelial damage (57). This would suggest that loss of one IKK is compensated for by the other enzyme. When NF-κB signaling is enhanced (in cases of infection, trauma, and chronic diseases) above this negligible level, it induces an inflammatory response.

**IL-6 and B cell and Th17 cell activation**

Known by many names including interferon β2, B-cell stimulatory factor-2 and later T-cell-replacing factor, IL-6 was first discovered as a T cell-secreted protein that induced B cells to secrete immunoglobulins (58). Since then, IL-6 has been described as an anti-apoptotic signal to multiple myeloma cells as IL-6 up-regulates Stat3 leading to production of bcl-xL and mcl-1. This enhancement of anti-apoptotic proteins contributes to the chemotherapy resistance of myeloma cells (59).

Along with transforming growth factor β, IL-6 induces the differentiation of Th17 cells (60, 61). Th17 cells are important for maintenance of mucosal immunity as they protect against pathogens such as *Klebsiella pneumonia* (61), and alteration of Th17 cytokines, IL-17 and IL-22, is associated with inflammatory bowel disease (61). Moreover, IL-17 induces ERK signaling in intestinal epithelial cells which enhances expression of the tight junction protein, claudin-1 (62).

**IL-6 and immunosuppression**

Increased IL-6 levels correlate with worse prognosis following injury (65, 66). In a model of ethanol exposure and burn injury, a rise in systemic IL-6 is associated with T cell suppression (67). Treatment with an IL-6 neutralizing antibody in the combined
injury model restored splenocyte proliferation and the DTH response (68). A similar immunosuppression was also observed in burn injury and sepsis patients (65, 66). IL-6 induced immunosuppression contributes to a rise in susceptibility to infection following injury and during chronic diseases.

**IL-6, inflammation, and chronic diseases**

Rheumatoid arthritis, obesity, IBD, and cancer are linked with elevated IL-6 levels (69-72). In IBD, IL-6 provides an anti-apoptotic signal to T cells that accumulate in the intestine (73). This loss of T cell apoptosis enhances the continuous immune response which is characteristic of the disease. Furthermore, IL-6 regulates claudin-2 expression (74). Elevations of claudin-2 (a “leaky” tight junction protein) inclusion in tight junctions increase the permeability of the junction which can promote further inflammation. Anti-IL-6 therapy represents another viable option for patients with chronic diseases and phase trials have been done in IBD patients as well as those suffering from arthritis (244, 245).

**Systemic effects of alcohol consumption**

**Alcohol-induced health burden and drinking patterns**

Despite high public awareness, alcohol (ethanol) continues to be one of the most abused drugs worldwide. Alcohol is a risk factor for infection, disease, and injury and contributes to nearly 4% of global deaths (75). Furthermore, both ethanol-induced mortality and ethanol-associated diseases were vastly higher in people under the age of 60, with the 15-29 and 30-44 age groups displaying the biggest ethanol effects (75). Less than 10% of drinkers in the United States are considered chronic or dependent, indicating
that most consumers are acute (occasional), moderate or binge drinkers. (76). A drink is defined as 12 oz. of beer, 5 oz. of wine, or 1.5 oz of liquor (77), and several different patterns of ethanol consumption exist. As defined by the National Institute on Alcohol and Alcoholism moderate or acute (“low risk”) drinking consists of no more than 14 drinks per week (2-3 per day) for men or no greater than 7 drinks per week (1-2 per day) for women. Binge drinking is classified as consuming enough ethanol to bring the blood alcohol content (BAC) above the legal limit of 0.08 mg/dL in 2 hours. Men can usually attain this BAC with 5 drinks while only 4 drinks will bring the BAC to this level in women (77). Chronic drinking is defined as greater than 8 drinks per day for many days (77).

Acute ethanol and cellular immune defects

Even short term ethanol exposures (acute and binge) can affect both the innate and adaptive immune responses. Acute ethanol has been demonstrated to decrease the pro-inflammatory cytokine response to microbial compounds like lipopolysaccharide (LPS), peptidoglycan, and polynosinic-polycytidylic acid (poly I:C) (17, 78, 79). Suppression of NF-κB activity also occurs following ethanol exposure and LPS challenge (80). Decreased ethanol-induced inflammatory responses lead to a rise in susceptibility to infection. Besides reduced production of cytokines, acute ethanol also dampens the phagocytic ability of macrophages and other antigen-presenting cells (81, 82). Inability to phagocytose pathogens not only permits these damaging organisms to survive, but it disrupts the communication between the innate and adaptive immune systems. Without phagocytosis, antigen presenting cells cannot present bacterial components to T cells,
further suppressing the immune response. Surprisingly, human studies indicate decreased levels of monocyte antigen presentation after only one alcohol exposure (82). In addition to less phagocytosis, acute ethanol has also been shown to reduce dendritic cell activation of T cells (82, 83).

**Chronic ethanol effects on the cellular immune response**

Interestingly, chronic ethanol exposure has an opposite effect on the innate immune response following lipopolysaccharide (LPS) challenge. Elevated production of inflammatory cytokines and NF-κB activation are associated with chronic ethanol exposure (84). Furthermore, fewer bone marrow-derived dendritic cells were observed in a chronic ethanol mouse model (85) and similar to acute ethanol, chronic levels of ethanol cause reduced phagocytosis (86, 87).

**Ethanol-induced alterations to the adaptive immune system also occur.** Elevated immunoglobulin (Ig) levels are commonly found in alcoholics (88). Despite a rise in Ig’s, a decrease in B cells was found in alcoholics although mouse models suggest that there is no effect of ethanol on B cell function (89). This elevation in Ig’s is actually associated with greater immunodeficiency as many of the Ig’s produced are against self-targets including lymphocytes, DNA, serum lipoproteins, and several liver proteins (90). Both acute and chronic ethanol exposure cause modifications in T cell function and activation. As previously mentioned, acute ethanol decreases antigen presentation, thereby reducing T cell activation. Moreover, T cell proliferation diminishes as is expression of co-stimulatory molecules following exposure to ethanol (82). Overall, these studies suggest severe alterations in immune cell action after both acute and chronic ethanol exposure.
Review of ethanol’s effects on liver and brain

In addition to immune system defects, many if not all tissues of the body experience changes due to ethanol exposure. This subject has been extensively described in the literature, particularly ethanol-induced alterations to the brain and liver (91).

Briefly, Kupffer cells (resident liver macrophages) produce elevated tumor necrosis factor-α (TNFα) and reactive oxygen species (ROS) in response to chronic ethanol (92, 93). Circulating LPS, commonly found in alcoholics, causes Kupffer cells to release IL-1β, IL-6 and chemokines (92, 94). All of these mediators promote inflammation, cell death, and fibrosis in the liver and represent characteristics of alcoholic liver disease (92, 93). Similarly, the inflammatory cytokines TNFα, IL-1β, monocyte chemoattractant protein-1 (MCP-1), and inducible nitric oxide synthase (iNOS) are up-regulated in the brain following ethanol exposure (95). Interestingly, an elevation in cytokines was actually also found in the brains of alcoholics and as were cell death biomarkers (96).

Alcohol-induced brain damage is diffuse and widespread and known to cause social withdrawal, and attention and impulse-control deficits (97, 98).

Increased susceptibility for lung damage following ethanol exposure

Despite ethanol’s primary association with neurological and liver damage, both the lung and intestine are also greatly influenced by ethanol exposure. Surprisingly, more patients in the United States die from alcohol-related lung dysfunction than liver cirrhosis (91) and chronic ethanol abuse is a risk factor for development of acute respiratory distress syndrome (99). The resident immune cells of the lung, primarily alveolar macrophages, display reduced phagocytosis and superoxide production following ethanol
exposure and bacterial challenge (100, 101). Lungs are highly vascularized which is necessary for oxygen exchange, but this characteristic also exposes this tissue to increased inflammatory mediators, ROS, and pathogens in the bloodstream. As already discussed, ethanol exposure causes an increased risk for infection and interestingly, chronic ethanol also reduces glutathione (102, 103). Glutathione is the major scavenger of excess ROS in the body. Metabolism of ethanol produces ROS, suggesting that excess ROS in the bloodstream combined with reductions in glutathione may also contribute to lung tissue injury (104).

Intestinal permeability, inflammation, and damage following ethanol

Finally, the first organ system to encounter ethanol after exposure is the gastrointestinal system. Both acute and chronic ethanol exposure trigger alterations to the structure of the entire tract (105, 106). Even acute ethanol exposure can cause intestinal damage, which is characterized by loss of the intestinal epithelium, particularly at the tips of villi, and infiltration of inflammatory cells (106). Chronic ethanol exposure produces more severe changes in intestinal morphological, as villi are often blunted following long term ethanol (105). Furthermore, intestinal permeability, MLCK activation, and intestinal pro-inflammatory levels rise with chronic ethanol exposure (24, 107, 108). Elevations in permeability are further associated with reduced localization of tight junction proteins, ZO-1 and occludin, to the junction (109, 110). Bacterial overgrowth and dysbiosis (abnormal microbiota composition) are also both induced with exposure to ethanol (111). Considering how extensively ethanol is abused, studies presented in this section indicate that ethanol has the potential to cause serious whole body damage.
Burn injury, inflammation, and intestinal permeability

A clinical perspective on burn injury

There are more than 1 million burn-related injuries in the United States each year, resulting in approximately 50,000 hospitalizations and 3,500 deaths (112). Burn injury, like many traumatic injuries, results in increased circulating levels of pro-inflammatory cytokines (113, 114), known as systemic inflammatory response (SIRS) (18, 115). Some trauma patients also develop compensatory anti-inflammatory response syndrome (CARS) increasing their risk for infection. Nearly 50% of burn patients suffer infectious complications during their time in the hospital (116). Deaths due to sepsis remain unchanged despite improvements in resuscitation and wound treatment. These observations indicate that burn patients are highly susceptible to infection and that the source of said infection may come from within the patient. Although the consequences of burn injury seems like they should be restricted to the skin, the opposite is actually closer to reality. Burn injures cause systemic complications, helping explain why multiple organ failure and multiple organ dysfunction syndrome are the two most common causes of death following burn (117, 118).

Lungs and multiple organ dysfunction following burn injury

Similar to many tissues, a rise in inflammatory mediators is evident in the lung (119) after burn injury. Inhalation combined with burn injury further amplifies these mediators, in particular IL-6 and monocyte chemotactic protein 1 (MCP-1) (120). SIRS causes lung tissue damage soon after insult partially due to their high vascularization and delicate architecture. Augmented levels of chemokines in the lungs recruit neutrophils,
which can kill invading pathogens, but can also damage delicate epithelial cells as well (121, 122). The lung epithelia constitute the walls of the alveoli and if damaged, reduce gas exchange and lung function. This pulmonary damage induces lung failure and the lungs are the first organ to fail in cases of multiple organ dysfunction syndrome (MODS) and multiple organ failure (MOF), the two most common causes of death in burn patients.

Burn injury induces intestinal inflammation and permeability

Edema formation at the burn site is evident early after insult in both humans and rodent models (122, 123). This edema transpires at the expense of other organs, such as the intestine, where water loss is evident. Animal models have demonstrated that burn injury causes mesenteric vasoconstriction and splanchnic vasoconstriction (123, 124). Both types of blood/lymph constriction trigger gut mucosal damage and bacterial translocation (125). Although controversial, bacterial translocation to the mesenteric lymph nodes (MLN) does occur in humans following some intestinal insults (125, 126). Intestinal permeability rises following burn injury in both lab models and in burn patients (127, 128) and numerous cytokines and chemokines, including IL-6 and KC (a neutrophil chemokine) increase in the ileum following burn injury (113). Further enhancing intestinal damage, chemokines, like KC and macrophage inflammatory protein 2 (MIP-2), recruit neutrophils to the tissue. Once in the intestine, neutrophils release various enzymes which aid in the killing of pathogens, but too many neutrophils can lead to tissue damage (129-131). IL-6, TNFα, IL-8 (human homolog of mouse KC and MIP-2), and LPS have all been demonstrated to inhibit neutrophil apoptosis (132-134). Elevated inflammatory cytokines and tissue damage are strong activators of MLCK, and not
surprisingly, MLCK activation increases in IECs in animal models of burn injury (135). Augmented MLCK activation is associated with intestinal morphological damage, decreased ZO-1, occludin, and claudin-1 protein levels, and intestinal permeability (135, 146). Inhibition of MLCK or inflammatory cytokine expression reduces burn-induced intestinal damage and permeability and restores tight junction protein levels in the intestinal epithelial barrier (135, 146). Using a rat model, Shao and colleagues discovered amplified occludin expression following burn injury although it was not localized to tight junctions (136). As occludin must be phosphorylated for inclusion in the junction complex, this suggests that burn injury may alter either kinases responsible for phosphorylating occludin or the phosphatases removing the phosphate mark. Additionally, exposure to burn serum reduced vascular endothelial cadherin and β-catenin at adherens junctions in endothelial cells (137). Burn injury also stimulates bacterial growth (138) which, when combined with burn-induced alterations in junction complexes, creates an ideal situation for bacterial translocation and possible systemic dissemination of these bacteria.

**Ethanol exposure and burn injury, a “second hit” yields additional problems**

Effects of ethanol and burn injury in the clinic

As already mentioned, ethanol is a risk factor for injury, infection, and surgical complications (76, 139). Nearly 50% of the adult burn patient population has a positive blood alcohol content at the time of admission (140-142). Clinical data from the Burn Intensive Care Unit at Loyola University Chicago indicate that despite the fact that ethanol-exposed burn patients have smaller burn sizes than their non-intoxicated
counterparts, they spent just as many days in the hospital and accrued the same amount of hospital costs (143). Ethanol exposure prior to burn injury also increases both morbidity and mortality (76, 140). Further clinical studies indicate that intoxicated burn patients had a rise in intensive care unit admissions, days on the ventilator, and fluid resuscitation requirements as compared to burn patients who were not intoxicated. Additionally, pneumonia and hypotension occurred more often in patients who were intoxicated at the time of burn when compared to burn patients who had no alcohol in their blood stream (139, 142).

Ethanol, burn injury, and the systemic immune response

The combined insult of ethanol exposure and burn injury generates an immunosuppressed cellular immune response which is characterized by a reduced delayed type hypersensitivity (DTH) response and decreased splenocyte activation and proliferation (144, 145). Enhanced systemic IL-6 levels contribute to this immunosuppression as anti-IL-6 antibody treatment restores the DTH response and splenocyte proliferation (68). In addition, the combined insult produces an elevated inflammatory state in tissues like the lung and intestine (147, 148).

Effect of the combined insult on the lung

The “gut lymph hypothesis”, which suggests that bacterial translocation into the lymphatic system can harm other tissues, is especially important when discussing the lung. The contents of the lymphatic system move back into the bloodstream at the thoracic duct, so the first capillary bed and tissue encountered by bacteria-laden blood is the lung. With its delicate architecture and extensive vascularization, the lungs are highly
susceptible to tissue damage and inflammation. This susceptibility is observed following ethanol exposure and burn injury. After the combined insult, intracellular adhesion molecule 1 (ICAM-1), and KC are elevated in the lung and this is associated with increased neutrophil infiltration (148). Despite this rise in neutrophils, reduced bacterial clearance is observed following the combined insult and intratracheal bacteria challenge (149). Furthermore, ethanol-exposed and burn-injured mice display thicker alveolar walls and greater tissue damage than mice exposed to either insult alone (148, 150). Reduced alveolar space and thicker alveolar walls will decrease lung function and oxygen exchange leading to lung dysfunction and failure.

Intestinal response to ethanol exposure and burn injury

A similar state of inflammation occurs in the ileum of mice exposed to ethanol and burn injury. IL-6, IL-18 and chemokines CINC 1/2 (rat homologs of KC and MIP-2) are elevated in the ileum following the combined insult. This rise in neutrophil chemoattractants trigger elevated neutrophil infiltration and ROS leading to tissue damage (151, 152). Ethanol exposure and burn injury reduce occludin and claudin-1 in the intestinal epithelial layer and a rise in apoptosis was also observed in the ileum following the combined insult. Interestingly, inhibition of IL-18 attenuates neutrophil infiltration, intestinal permeability, and apoptosis and restores occludin and claudin-1 levels in a mouse model of the combined insult (152-154). Additionally, T cells in the MLN and Peyer’s patches exhibit diminished activation, proliferation, and IL-2 and interferon γ (IFNγ) in mice exposed to ethanol and burn injury (155). This suggests that any bacteria that do get into the MLN or lamina propria may survive. As observed
following either injury alone, the combined insult also generates bacterial overgrowth (156) further demonstrating the importance of an intact epithelial barrier.

Other animal models demonstrate similar intestinal responses following combined insults. Using a model of intestinal ischemia/reperfusion and intestinal infection, Kubiak and colleagues discovered severe intestinal edema, epithelial sloughing and flattened villi following these insults (246). As infection and sepsis are common after burn injury, a model of burn and Enterococcus faecalis infection was employed to investigate intestinal permeability, a possible second source of infection. This second hit of infection led to greater intestinal permeability and decreased occludin expression than either insult alone (247). These two models indicate the importance of an intact intestinal barrier following not just one insult, but also in cases of combined injury.

Absorption of some nutrients requires intestinal permeability, but this is a highly controlled and short-lived occurrence. When intestinal permeability is prolonged, as observed following disease, injury, or ethanol exposure, intestinal tissue damage and inflammation occurs. The combination of ethanol exposure and burn injury elevates intestinal permeability greater than either insult alone (156). Bacterial translocation takes place when the IEC layer is damaged; however, with a rise in intestinal permeability, bacteria and other luminal contents can enter the lamina propria through areas of damaged epithelium as well as gaps in the epithelial barrier. Once in the lamina propria, these bacteria are normally killed by macrophages; however, in cases of immunosuppression or overwhelming numbers of bacteria, some microbes may survive and accumulate in the lymphatics, specifically the mesenteric lymph nodes. This allows
for the potential of systemic bacterial infection and inflammatory responses. While knowledge of the immune parameters occurring in the gut following ethanol exposure and burn injury exists, the mechanisms leading to intestinal permeability are not well understood.
CHAPTER 3

INHIBITION OF LONG MYOSIN LIGHT CHAIN KINASE ACTIVATION ALLEVIATES INTESTINAL DAMAGE AFTER BINGE ETHANOL EXPOSURE AND BURN INJURY

Abstract

Laboratory evidence suggests that intestinal permeability is elevated following either binge ethanol exposure or burn injury alone and this barrier dysfunction is further perturbed when these insults are combined. We and others have previously reported a rise in both systemic and local pro-inflammatory cytokine production in mice after the combined insult. Knowing that long myosin light chain kinase (MLCK) is important for epithelial barrier maintenance and can be activated by pro-inflammatory cytokines, we examined whether inhibition of MLCK alleviated detrimental intestinal responses seen after ethanol exposure and burn injury. To accomplish this, mice were given vehicle or a single binge ethanol exposure followed by a sham or dorsal scald burn injury. Following injury, one group of mice received PIK (membrane permeant inhibitor of MLCK). At 6 and 24 hours post-injury, bacterial translocation and intestinal levels of pro-inflammatory cytokines were measured and changes in tight junction protein localization and total intestinal morphology were analyzed. Elevated morphological damage, ileal IL-1β and IL-6 levels, and bacterial translocation were seen in mice exposed to ethanol and burn injury relative to either insult alone. This increase was not seen in mice receiving PIK.
after injury. Ethanol-exposed and burn-injured mice had reduced zonula occludens protein-1 (ZO-1) and occludin localization to the tight junction relative to sham-injured mice. However, the observed changes in junction complexes were not seen in our PIK-treated mice following the combined insult. These data suggest that MLCK activity may promote morphological and inflammatory responses in the ileum following ethanol exposure and burn injury.

**Introduction**

Nearly 1 million burn injuries occur annually in the United States with approximately 4,000 patients succumbing to their injuries (112). Both clinical and laboratory studies indicate that intestinal permeability and tissue injury increase after burn injury permitting bacteria to translocate into the lymphatic system and ultimately the bloodstream (125, 135). In a rodent model of binge ethanol exposure and burn injury, Kavanaugh et al. demonstrated that the combined insult promotes greater bacterial translocation to the mesenteric lymph nodes (MLN) and intestinal permeability than in mice receiving either treatment alone (63, 156). The distal small intestine and colon contains enormous volumes of bacteria ($10^5$-$10^8$ bacteria per gram of tissue) and endotoxin; therefore, changes in intestinal permeability could produce systemic complications. Intestinal barrier dysfunction/failure allowing gut flora and its components to invade the intestinal mucosa is the basis for the gut-lymph hypothesis of multiple organ dysfunction syndrome (MODS) (64), which states that traumatic injury promotes a rise in intestinal permeability leading to a release of bacteria and endotoxin.
into the lymphatic system and ultimately the bloodstream. As the bacteria-laden blood encounters the lung vascular bed first, pulmonary injury is possible thus promoting the early stages of MODS (64, 157) and ultimately multiple organ failure (MOF), which are the two most common causes of death after burn injury (158, 159).

Intestinal epithelial cells, physically connected by multiple types of junction complexes, create a semi-permeable mucosal barrier, which permits absorption of nutrients but prevents other contents of the lumen from entering the lamina propria (160, 161). Regulation of this barrier can be affected by various stimuli, including bacteria, cytokines, traumatic injury, and ethanol (23-25, 125). Junction proteins, such as the tight junction proteins zonula occludens protein-1 (ZO-1) and occludin, as well as enzymes important for the maintenance of this barrier can be affected by these stimuli. One such enzyme, long (210 kDa) myosin light chain kinase, phosphorylates myosin regulatory light chain (MLC) at serine 19 allowing it to interact with actin. Myosin-actin interaction causes cytoskeletal sliding, which induces tight junction disruption and a gap in the epithelial barrier. Actin reorganization, elevated permeability, and ZO-1 and occludin redistribution away from tight junctions are associated with MLC phosphorylation (109, 110).

Inflammatory diseases and traumatic injury amplify pro-inflammatory cytokines in the gut (158, 162). A rise in pro-inflammatory cytokines is associated with MLCK activation and it has been shown that TNF-α, IL-1β, and lymphotoxin-like inducible protein that competes with glycoprotein D for herpesvirus entry mediator on T cells (LIGHT) can up-regulate MLCK transcription (25, 42, 49, 163). Furthermore, increased
MLCK activation has been linked to intestinal permeability after burn injury or chronic ethanol exposure alone (24, 164). Traumatic injury, like burn, causes an over-exuberant systemic inflammatory response characterized by a rise in systemic and tissue levels of IL-6, TNF-α, and IL-1β (19, 68, 145, 165). When injury is combined with ethanol exposure, these immune responses are often further increased. Recently, a specific MLCK inhibitor, PIK, was found to reduce occludin reorganization away from tight junctions and restore water absorption in a model of T cell-mediated diarrhea (166). Moreover, TNFα and interferon-γ–induced barrier dysfunction was reversed with PIK treatment (167).

Ethanol is a common risk factor in traumatic injury (76, 168), and approximately 50% of the adult burn patient population has a positive blood alcohol content at the time of admission (140, 141). Further evidence suggests that the combination of ethanol and burn injury leads to enhanced immunosuppression promoting greater susceptibility to bacterial infection at both the wound site as well as remote organs, such as the lung and ileum (169-171).

The combination of insult-induced immunosuppression and inflammation along with amplified bacterial translocation in the gut contribute to a physiological state that is ideal for tissue destruction and dysfunction. We hypothesized that inhibition of MLCK would alleviate the intestinal damage and inflammatory responses seen after binge ethanol exposure and burn injury. Mice had greater villus blunting and edema as well as a redistribution of ZO-1 and occludin following ethanol and burn injury compared to sham treated mice. These changes coincided with elevated phosphorylated myosin light
chain (pMLC), bacterial translocation and ileum IL-6 levels. In contrast, PIK-treated mice exposed to ethanol and burn injury had significantly reduced intestinal damage, normal tight junction protein localization, and decreased bacterial translocation and IL-6. This indicates a crucial role for MLCK in intestinal barrier leakiness and/or inflammation in our model of ethanol and burn injury.

**Materials & Methods**

**Mice**

Eight to ten week old male (C57BL/6, 23-25 grams,) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed in sterile microisolator cages in the Loyola University Medical Center Comparative Medicine facility. All experiments were conducted in accordance with the Loyola Institutional Animal Care and Use Committee.

**Murine Model of Ethanol and Burn Injury**

A murine model of a single binge ethanol exposure and burn injury was performed as described previously (172) with minor modifications (149). Briefly, mice were given a single binge dose of 1.11 g/kg of 20% (v/v) ethanol solution intraperitoneally that resulted in a blood ethanol level of 150-180 mg/dl at 30 minutes. The mice were then anesthetized with 100 mg/kg of Ketamine and 10 mg/kg of Xylazine (Webster Veterinary, Sterling, MA), their dorsum shaved, and placed in a plastic template exposing 15% of the total body surface area (TBSA) and subjected to a scald injury in 90-92° C water bath or a sham injury in room-temperature water. The scald injury
resulted in an insensate, full-thickness burn injury of approximately 15% total body surface area (173). Mice received 1 mL of saline resuscitation and their cages were placed on warming pads until mice recovered from anesthesia. At 30 minutes after burn injury, mice were given the specific long MLCK inhibitor PIK (i.p, 50 uM) (174). Mice were sacrificed by CO$_2$ narcosis followed by cervical dislocation at 2, 3, 6, or 24 hours following injury.

Histopathologic examination of the ileum

At 6 and 24 hours post-injury, mice were euthanized by CO$_2$ narcosis and the ileum was harvested and fixed overnight in 10% formalin. Samples were then embedded in paraffin, sectioned at 5 microns, and stained with hematoxylin and eosin (H&E). Images were taken at 200x.

Immunofluorescent staining of ZO-1 and occludin in the ileum

Immunofluorescent staining was done as previously described (167) with minor modifications. Briefly, a small section of ileum (5 mm) was embedded in OCT and frozen for immunofluorescent staining. The ileum was sectioned (5 microns) and stained with either rabbit anti-ZO-1 or rabbit anti-occludin (Invitrogen, Carlsbad, CA) followed by goat anti-rabbit Alexa 488 (Invitrogen, Carlsbad, CA). Sections were further stained with fluorescent-conjugated phalloidin (actin) and Hoechst nuclear stain (Invitrogen, Carlsbad, CA). Using Zeiss software (Zeiss LSM 510 Version 4.2 SP1), a 20 epithelial cell section (crypt or villus) was outlined and only within this outlined section were the number of co-localized (both red and green fluorescence) pixels determined. The number
of co-localized pixels was divided by the total number of pixels in that section and expressed as a percentage. This process was repeated an additional 4 epithelial cell sections per animal leading to 100 total epithelial cells examined for each animal. Results were averaged for each animal and this average was then used to determine the group average.

Bacterial translocation

Bacterial translocation was assessed as previously described with minor modifications (156). Briefly, 5-6 mesenteric lymph nodes (MLN) per mouse were removed at 6 or 24 hours, placed in cold RPMI and kept on ice. Nodes were separated from connective tissue and homogenized in RPMI using frosted glass slides. Homogenates were plated in triplicate on tryptic soy agar (TSA) plates and placed in a 37°C incubator overnight. Colonies were counted the following day, averaged, and divided by the total number of lymph nodes harvested.

Intestinal epithelial cell isolation and western blotting

Intestinal epithelial cells were isolated as previously described (167) with minor modifications. Briefly, ileum sections were opened lengthwise, washed with calcium and magnesium free Hanks Basic Salt solution (HBSS), and placed in tubes containing 10 mM DTT and phosphatase inhibitor cocktail in HBSS. Samples were placed at 4°C for 30 minutes, after which tubes were shaken briefly and ileum sections moved to tubes containing 1 mM EDTA and phosphatase inhibitor cocktail in HBSS for 1 hour at 4°C. After incubation, tubes were shaken robustly and large pieces of tissue removed.
Samples were centrifuged at 1000 g for 10 minutes and the supernatant discarded. Intestinal epithelial cells were confirmed through microscopy and villin 1 staining. Pelleted cells were lysed in 100 uL of Cell Lysis Buffer according to manufacturer’s protocol (BioRad, Hercules, CA). Twenty milligrams of whole cell lysate protein was boiled for 5 minutes, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes and blotted with primary antibodies specific for phosphorylated myosin light chain (pMLC Ser19, Cell Signaling Technology, Danvers, MA), total MLCK (Abcam, Cambridge, MA), and villin-1 (Cell Signaling Technology).

Detection of Circulating Cytokine Levels

Blood was collected from cardiac puncture and serum was obtained by centrifugation after clotting. IL-6 and TNFα levels were then determined using ELISA according to manufacturer’s instructions (BD Biosciences, San Diego, CA).

Cytokine determination and ileum

Two one-inch sections of ileum were homogenized in 1 mL of Cell Lysis Buffer according to manufacturer’s protocol (BioRad, Hercules, CA). Homogenates were then filtered and analyzed for IL-6 levels using ELISA (BD Biosciences, San Diego CA). The results were normalized to total protein present in the homogenate using the BioRad protein assay based on the methods of Bradford (175) (BioRad, Hercules, CA).
Statistical Analysis

Statistical comparisons (GraphPad Instat and Prism) were made between the sham vehicle, sham ethanol, burn vehicle, and burn ethanol treatment groups, resulting in 4 total groups analyzed. One-way analysis of variance was used to determine differences between treatment responses, and Tukey’s post-hoc test once significance was achieved (p < 0.05). Statistical comparisons made between the burn ethanol and burn ethanol plus PIK treatment groups were done using student’s t-test and Tukey’s post-hoc test (p<0.05).

Results

Following exposure to binge ethanol and burn injury a rise in IL-6 and intestinal permeability as well as a shortening of villus heights has been observed in the ileum (147). These changes can promote further tissue damage and may contribute to systemic complications. We sought to determine whether inhibition of MLCK after insult alleviates these detrimental responses.

MLCK activated early after insult

TNFα often peaks early systemically after injury (176) and we see an increase in serum levels at 2 hours post ethanol exposure and burn injury (Figure 5A). Interestingly, IL-6 levels, thought to peak at later time points, were also significantly elevated in the serum at 2 hours post insult (Figure 5B). Neither TNFα nor IL-6 is significantly elevated in ileum tissue (Figure 5C, D); however, we do see an increase in total MLCK in intestinal epithelial cells isolated from ethanol-exposed and burn-injured mice (Figure 6A) at 3 hours post combined insult. This elevation was not significant, but dually
Figure 5: TNFα and IL-6 elevated in serum at 2 hours post insult. Levels of TNFα and IL-6 in the serum (A, B) and ileum (C, D) were quantified by ELISA. Cytokine concentrations were normalized to total protein in ileum samples (C, D) as determined by BioRad protein assay and all data is presented as concentration ± SEM. *p<0.05 versus both sham groups. n = 3-6 per group.
Figure 6: Ethanol exposure and burn injury elevates total MLCK and pMLC (Ser19) in intestinal epithelial cells. Isolated intestinal epithelial cells were lysed and analyzed by western blot for levels of MLCK (A) and pMLC (B, C) 3 hours after exposure to treatment. Quantification of MLCK and pMLC levels were normalized to villin-1 levels and done using BioRad Image Lab software. Images are representative of 3 experiments, *p<0.05 versus all other groups, # p<0.05 versus Burn ethanol + PIK group. Quantification is of n = 6-8 per group.
exposed mice did have a significant increase in pMLC as compared to all other groups (Figure 6B) and PIK treatment significantly reduced pMLC following ethanol exposure and burn injury (Figure 6C). These data suggest that within a few hours of the combined insult the MLCK pathway has been activated, possibly due to elevated serum levels of TNFα and IL-6.

Morphological damage observed by 6 hours post ethanol exposure and burn injury

Intestinal damage, characterized by villus blunting and edema as well as intestinal inflammation, commonly occurs after traumatic injury (43, 177, 178). Six hours after exposure to ethanol and burn injury, ileum morphology begins to change. Mice in both burn groups had blunted villi (Figure 7C, D) as compared to mice in either sham group (Figure 7A, B). The intestinal epithelial layer is also altered in mice exposed to the combined insult with gaps appearing between epithelial cells, although no changes in ZO-1 or occludin localization were observed at this time (data not shown). Inhibition of MLCK using PIK yields to little or no change in the intestinal epithelial barrier morphology following exposure to ethanol and burn injury (Figure 7E).

After either traumatic injury or chronic ethanol exposure, bacterial translocation was reported to be elevated as a result of epithelial cell damage, bacterial overgrowth, epithelial barrier permeability and MLN T-cell suppression (155, 156). This translocation occurs in small amounts in healthy individuals and under normal conditions MLN resident T cells clear the bacteria (155). Six hours after ethanol exposure and burn injury mice had significantly greater bacterial accumulation in the MLN when compared to all other groups (Figure 7F). This elevation in bacterial accumulation was associated
Sham vehicle  Sham ethanol  Burn vehicle

Burn ethanol  Burn ethanol + PIK

Figure 7 continued on next page.
Figure 7: PIK treatment reduces morphological damage and inflammation at 6 hours post combined insult. Ileum sections were stained with hematoxylin and eosin (A-E) and examined for degree of inflammation and damage. n= 6-8 per group. Mesenteric lymph nodes were isolated from mice sacrificed at 6 hours following insult (F), homogenized and plated on tryptic soy agar (TSA) plates. Colonies were counted the next day and normalized on the total number of lymph nodes removed from each mouse. Levels presented as concentration ± SEM. n = 6-10 per group. Ileum levels of IL-1β and IL-6 were quantified by ELISA (G, H) and normalized to total protein. Data presented as concentration ± SEM. *p<0.05 versus all groups except the sham ethanol group. &p<0.05 versus sham vehicle and burn vehicle. n = 3-6 per group. @p<0.05 versus sham vehicle.
with a rise in ileum levels of IL-1β and IL-6 (Figure 7G, H). PIK treatment significantly reduced bacterial accumulation in the MLN of mice given ethanol and burn injury and also decreased IL-1β in the ileum (Figure 7G). These data indicate the continued inhibition of MLCK preserves intestinal morphology and reduces inflammation following ethanol exposure and burn injury.

**PIK treatment alleviates intestinal epithelial barrier alterations**

Previous work in this model has revealed that the heights of villi are lower at 24 hours following the combined insult (147). This response was verified in these experiments as villi in mice exposed to burn alone or the combined insult (Figure 8C, D) had shorter (115 μm ± 9) and wider villi than sham treated animals (200 μm ± 14, p<0.05, Figure 8A, B). Furthermore, there was a decrease in intact villi and the epithelial cell layer in mice given ethanol and burn injury as compared to all other groups. As seen at 6 hours, PIK treatment was coupled with reduced intestinal morphological damage (Figure 8E) as mice given PIK after ethanol and burn injury had tall (170 μm ± 9) and narrow villi similar to villi seen in sham treated mice. Since greater gross morphological changes/damage were observed at 24 hours following insult, we examined the effect of ethanol and burn injury on the localization of two of the major tight junction proteins in the ileal epithelial cell layer, ZO-1 and occludin (2). Intact tight junction complexes in the intestinal epithelial layer prevent bacteria and their products from moving into the lamina propria and initiating an immune response. Representative images from wild type sham vehicle and sham ethanol mice (Figure 8F, G) show ZO-1 in its characteristic chicken wire pattern and its co-localization with actin indicating an intact tight junction.
Figure 8 continued on next page.
Figure 8: MLCK inhibition preserves intestinal morphology and tight junction protein localization following ethanol exposure and burn injury. Ileum sections from mice were stained with hematoxylin and eosin (A-E) and then examined for degree of inflammation and damage. Representative H&E images taken at 200x. Frozen ileum sections were stained with antibodies against ZO-1 (green, F-J) or occludin (green, K-O) as well as phalloidin (red) and nuclei (blue). Representative immunofluorescent images taken at 400x. n = 6-8 per group. Immunofluorescent images were analyzed for co-localization of ZO-1 or occludin with actin (P, Q). Co-localization is presented as % colocalized pixels/100 epithelial cells. *p<0.05 versus all groups except burn vehicle. #p<0.05 versus sham groups. n = 4-6 per group.
Animals exposed to burn injury alone had a slight, but insignificant, decrease in ZO-1 localizing with actin (Figure 8H), while almost no ZO-1 co-localizes with actin in animals exposed to the combined insult (Figure 8I). Quantification of ZO-1 and actin co-localization (Figure 8P) indicated that significantly less ZO-1 co-localized with actin in wild type mice exposed to ethanol and burn injury than in sham treated animals. PIK treatment of wild type mice exposed to the combined insult preserved ZO-1 localization at the periphery of cells and its interaction with actin (Figure 8J, P). Occludin position can be affected by a variety of stimuli including burn injury alone, acetaldehyde (the predominant ethanol metabolite), TNFα, and LIGHT (49, 110, 154, 179). Occludin localization alterations were also observed by visual examination, but this decrease in co-localization was not as obvious as found with ZO-1 (Figure 8K-O). Quantification, however, of occludin and actin co-localization indicated that association of occludin and actin was significantly reduced in mice exposed to the burn injury alone or the combined insult, and this localization was re-established in mice receiving PIK after ethanol exposure and burn injury as compared to mice not receiving PIK treatment (Figure 8O, Q). As seen at 6 hours post insult, MLCK inhibition continues to decrease intestinal damage and intestinal epithelial cell barrier alterations induced following ethanol exposure and burn injury.

Intestinal damage and inflammation reduced following PIK treatment

In conjunction with a reduction in intestinal morphological damage as observed in Figure 8, PIK treatment also led to a 33% reduction in bacterial translocation at 24 hours post insult; however, this difference was not significant (Figure 9A). This reduction is
Figure 9: Decreased IL-6 following PIK treatment in mice exposed to ethanol and burn injury. Mesenteric lymph nodes were isolated from mice sacrificed at 24 hours following insult (A). Lymph nodes were homogenized and plated on tryptic soy agar (TSA) plates. Colonies were counted the next day and normalized on the total number of lymph nodes removed from each mouse. Levels presented as concentration ± SEM. *p<0.05 versus all other groups except burn ethanol + PIK. n = 6-8 per group. IL-6 (B) and IL-1β (C) levels in the ileum were measured by ELISA and normalized to total protein per sample. Data presented as concentration ± SEM. #p<0.05 versus all other groups. n = 4-6 per group.
likely due to the decrease seen at 6 hours, but to maintain this reduction another dose of PIK may be needed. Although bacterial translocation was not reduced following PIK treatment, IL-6 levels in the ileum were significantly less in PIK-treated mice exposed to ethanol and burn injury as compared to mice not receiving PIK (Figure 9B). By 24 hours following insult, no differences in IL-1β levels were observed between groups (Figure 9C). As seen in Figure 8, early inhibition of MLCK alleviates intestinal damage and inflammation and preserves tight junctions in the intestinal epithelial barrier at 24 hours post combined insult.

These data confirm previous studies that gut inflammation is greater after ethanol exposure and burn injury than burn injury alone. Furthermore, the loss or inhibition of MLCK promotes maintenance of intestinal epithelial tight junctions after ethanol and burn, thereby preventing bacterial translocation and the subsequent immune response and leading to less intestinal damage and inflammation.

**Summary**

The combined insult of binge ethanol and burn injury causes systemic increases in TNFα and IL-6 as well as elevated inflammation, bacterial translocation, and intestinal permeability in the ileum. Bacterial translocation and intestinal permeability occur as a result of intestinal epithelial barrier damage or gaps between epithelial cells. One mechanism for a rise in permeability is enhanced MLCK activity. Either burn injury or ethanol exposure alone augments MLCK activity in intestinal epithelial cells leading to permeability. We hypothesized that increased MLCK activity causes intestinal epithelial damage and permeability in a murine model of binge ethanol and burn injury.
As early as 2 hours post combined insult, both TNFα and IL-6 are elevated in the serum of mice exposed to ethanol and burn. By 3 hours, pMLC, a measurement of MLCK activity, is elevated in intestinal epithelial cell isolated from mice exposed to the combined insult. To investigate the role of MLCK in this model, mice were administered PIK, a specific inhibitor to MLCK, after ethanol and burn injury. Ethanol-exposed and burn-injured mice given PIK demonstrate reduced pMLC following the combined insult as compared similarly treated mice not receiving PIK. Shorter villi and increased intestinal epithelial layer damage was observed at both 6 and 24 hours following burn or the combined insult; however, PIK-treated animals displayed little to no alterations in villus morphology and barrier damage at either time point. A rise in morphological damage was associated with less ZO-1 and occludin localization with actin. Interestingly, PIK-treated mice do not demonstrate reduced villus heights or decreased ZO-1 and occludin localizing with actin following ethanol exposure and burn injury. Augmented intestinal damage after the combined insult leads to significantly enhanced bacterial translocation and IL-6 levels in the ileum. Animals given PIK following binge ethanol and burn exhibit a significant decrease in IL-6, but only a 33% reduction in bacterial translocation, which is not a significant change. These studies were also conducted in MLCK knockout mice and are described in Appendix A.

The combined insult of binge ethanol and burn injury amplifies MLCK activity, intestinal damage, and inflammation. Inhibition of MLCK alleviates these responses suggesting that this enzyme plays a role in ethanol and burn-induced epithelial permeability.
CHAPTER 4

ANTI-IL-6 ANTIBODY TREATMENT, BUT NOT IL-6 KNOCKOUT, IMPROVES INTESTINAL BARRIER FUNCTION AND REDUCES INFLAMMATION FOLLOWING BINGE ETHANOL EXPOSURE AND BURN INJURY

Abstract

IL-6 is an inflammatory cytokine known to be elevated in chronic diseases and following insults such as trauma and infection. While necessary for the development of B cells and Th17 cells, IL-6, at elevated levels, can also cause tissue damage and lead to a rise in inflammation. Previous work in our laboratory has shown that IL-6 is increased both systemically as well as in multiple organ systems including the ileum following ethanol exposure and burn injury. As this combined insult causes elevated intestinal morphological damage, tight junction protein localization alterations, and phospho myosin light chain (pMLC) levels, we sought to determine the role of IL-6 in these intestinal responses using a model of binge ethanol exposure and burn injury. IL-6 antibody treatment after the combined insult reduced morphological changes in the ileum, bacterial translocation, and pMLC levels relative to either injury alone. ZO-1 and occludin localization was also re-established in wild type mice given IL-6 antibody after ethanol and burn. IL-6 knockout mice given ethanol and burn injury also had reduced intestinal damage; however, no changes in bacterial translocation or tight junction protein localization were observed as compared to similarly treated wild type mice. These data suggest that IL-6 may have a role in intestinal tissue damage observed following the
combined insult of binge ethanol exposure and burn injury although complete loss of IL-6 does not appear to be beneficial in this model. Modulation of IL-6 may present a new option for preventing intestinal damage and associated inflammation following a combined insult of ethanol exposure and burn injury.

**Introduction**

Dysfunction of the intestinal epithelial barrier occurs following numerous insults including infection, trauma, and disease (43, 167, 180, 181). Our laboratory and others have demonstrated that the combined insult of ethanol exposure and burn injury causes elevated intestinal inflammation, neutrophil influx, and immunosuppression of mesenteric lymph node T-cells (151, 182). Furthermore, this combined insult is also associated with elevations in intestinal permeability and bacterial translocation, decreased ZO-1 and occludin localization to tight junctions, and increased phospho myosin light chain (pMLC) (156, 183). A common molecule found in the serum as well as many tissues of mice exposed to ethanol and burn injury is the inflammatory cytokine, interleukin (IL) 6 (182, 183).

Important for a variety of cellular responses, IL-6 has a predominant role in the inflammatory response. Signaling through its receptor, IL-6R and gp130, IL-6 helps mediate the transition from acute to sustained inflammation (184), induces fever and acute phase responses following infection (185), and may contribute to tissue damage in states of elevated inflammation (72). Tumor necrosis factor-α (TNFα), IL-1β, lipopolysaccharide (LPS), and viral infections can all induce IL-6 (186-188) indicating its importance in the immune response. Not all cells are known to actually express IL-6Rα
(52); however, with the discovery of a soluble form of the IL-6 receptor (54), all cells expressing gp130 are able to respond to IL-6. As most cells express gp130 on their surfaces, the effect of elevated IL-6 becomes global and allows for the possibility of tissue injury or damage in various organs of the body.

IL-6 has long been known as an important component of the immune response. Interestingly, recent work also indicates that IL-6 can also act as a causative or prolonging agent in disease and other cellular processes. Obesity and insulin resistance (69), rheumatoid arthritis (70), aging (189) and cancer (71) all have symptoms or outcomes associated with elevated systemic or local levels of IL-6. With relation to the gut, IL-6 provides an anti-apoptotic signal to CD4+ T cells that aggregate in inflammatory bowel disease allowing for further inflammation and tissue damage (73). Along with transforming growth factor β (TGF-β), IL-6 aids in the induction of Th17 cell differentiation (190). Th17 cells produce IL-17, a known pro-inflammatory cytokine, which, along with IL-6, is elevated in IBD patients (72, 73).

Following acute insults, such as injury or infectious challenge, serum levels of IL-6 rise (181, 191). In particular, burn injury-induced mortality often correlates with increased IL-6 levels (192). Mouse models of burn injury indicate elevated levels of IL-6 in the ileum (43) and when mice are exposed to a combined insult of binge ethanol and burn injury, IL-6 levels in the ileum are further increased (150, 182). Knockout or inhibition of IL-6 had previously been described as effective in the prevention of intestinal morphological damage and permeability in animal models of splanchnic arterial occlusion and reperfusion, sepsis, and hemorrhagic shock and resuscitation (193-195).
These data suggest that IL-6 has a role in causing or perpetuating intestinal responses following injury. With the knowledge that IL-6 is elevated both systemically and at the tissue level following exposure to either ethanol or burn injury alone as well as the combined insult (19, 91, 147), we sought to determine if IL-6 promotes the intestinal inflammation and barrier dysfunction observed after the combined insult of ethanol exposure and burn injury.

**Materials & Methods**

**Mice**

Wild type (C57BL/6) and IL-6 knockout (B6.129S2-7 IL6(tm1Kopf)/J) mice (6-7 week old,) were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed in sterile microisolator cages in the Loyola University Health Sciences Division Comparative Medicine facility until 8-10 weeks of age (23-25 grams). All experiments were conducted in accordance to National Institutes of Health guidelines and were approved by the Loyola Institutional Animal Care and Use Committee.

**Murine Model of Ethanol and Burn Injury**

A murine model of a binge ethanol exposure and burn injury was performed as described previously (172) with minor modifications (149). Briefly, mice were given a single dose of 1.11 g/kg of 20% (v/v) ethanol solution intraperitoneally that resulted in a blood ethanol level of 150-180 mg/dl at 30 minutes. The mice were then anesthetized with 100 mg/kg of Ketamine and 10 mg/kg of Xylazine (Webster Veterinary, Sterling,
MA), their dorsum shaved, and placed in a plastic template exposing 15% of the total body surface area (TBSA) and subjected to a scald injury in 90-92°C water bath or a sham injury in room-temperature water. The scald injury resulted in an insensate, full-thickness burn injury (173). Mice received 1 mL of saline resuscitation and their cages were placed on warming pads until they recovered from anesthesia. At 30 minutes after burn injury, mice were given either rat IgG or rat anti mouse IL-6 antibody (i.p.) at a dose (eBioscience, 5 ug/mouse) previous determined in our lab (68). This dose has previously been shown to restore DTH responses in our model of ethanol exposure and burn injury. As no differences were found between IgG and anti-IL-6 treatment in the sham groups and burn vehicle group, only mice given ethanol and burn injury plus anti-IL-6 are shown in the results section. All other groups are treatment plus IgG. Mice were sacrificed by CO₂ narcosis followed by cervical dislocation at 3 and 24 hours following injury.

Histopathologic and immunofluorescent examination of the ileum

At the time of sacrifice, the ileum was harvested and fixed overnight in 10% formalin. Samples were then embedded in paraffin, sectioned at 5 microns, and stained with hematoxylin and eosin (H&E). Images were taken at 200x. Immunofluorescent staining was done as previously described (167) with minor modifications. Briefly, a small section of ileum (5 mm) was embedded in OCT and frozen for immunofluorescent staining. The ileum was sectioned (8 microns) and stained with either rabbit anti-ZO-1 or rabbit anti-occludin (Invitrogen, Carlsbad, CA) followed by goat anti-rabbit Alexa 488 (Invitrogen, Carlsbad, CA). Sections were further stained with fluorescent-conjugated phalloidin (actin) and Hoechst nuclear stain (Invitrogen, Carlsbad, CA). Using Zeiss
software (Zeiss LSM 510 Version 4.2 SP1), a 20 epithelial cell section (crypt or villus) was outlined and only within this outlined section were the number of co-localized (both red and green fluorescence) pixels determined. The number of co-localized pixels was divided by the total number of pixels in that section and expressed as a percentage. This process was repeated an additional 4 epithelial cell sections per animal leading to 100 total epithelial cells examined for each animal. Results were averaged for each animal and this average was then used to determine the group average.

Bacterial translocation

Bacterial translocation was assessed as previously described with minor modifications (156). Briefly, 5-6 mesenteric lymph nodes (MLN) per mouse were removed, placed in cold RPMI (with 5% fetal bovine serum) and kept on ice. Nodes were separated from connective tissue and homogenized in RPMI using frosted glass slides. Homogenates were plated in triplicate on tryptic soy agar (TSA) plates and placed in a 37°C incubator overnight. Colonies were counted the following day, averaged, and divided by the total number of lymph nodes harvested.

Intestinal epithelial cell isolation and western blotting

Intestinal epithelial cells were isolated as previously described (167) with minor modifications. Briefly, ileum sections were opened lengthwise, washed with calcium and magnesium free Hanks Basic Salt solution (HBSS), and placed in tubes containing 10 mM DTT and phosphatase inhibitor cocktail in HBSS. Samples were placed at 4°C for 30 minutes, after which tubes were shaken briefly and ileum sections moved to tubes containing 1 mM EDTA and protease inhibitor cocktail in HBSS for 1 hour at 4°C. After
incubation, tubes were shaken robustly and large pieces of tissue removed. Samples were centrifuged at 1000 g for 10 minutes and the supernatant discarded. Pelleted cells were lysed in 100 uL of Cell Lysis Buffer according to manufacturer’s protocol (BioRad, Hercules, CA). Twenty micrograms of whole cell lysate protein was boiled for 5 minutes, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes and blotted with primary antibodies specific for phosphorylated myosin light chain (pMLC Ser19, Cell Signaling Technology, Danvers, MA), total MLCK (Abcam, Cambridge, MA), and villin-1 (Cell Signaling Technology).

Cytokine determination in the ileum

Two one-inch sections of ileum were removed, luminal contents removed, and homogenized in 1 mL of Cell Lysis Buffer according to manufacturer’s protocol (BioRad, Hercules, CA). Homogenates were then filtered and analyzed for IL-6 levels using ELISA (BD Biosciences, San Diego CA). The results were normalized to total protein present in the homogenate using the BioRad protein assay based on the methods of Bradford (175) (BioRad, Hercules, CA).

Statistical Analysis

Statistical comparisons (GraphPad Instat and Prism) were made between the sham vehicle, sham ethanol, burn vehicle, and burn ethanol treatment groups, resulting in 4 total groups analyzed. One-way analysis of variance was used to determine
differences between treatment responses, and Tukey’s post-hoc test once significance was achieved (p < 0.05). Statistical comparisons made between the burn ethanol and burn ethanol plus anti-IL-6 treatment groups were done using student’s t-test and Tukey’s post-hoc test (p<0.05). Comparisons made between wild type and knockout in the same treatment group (i.e. burn ethanol) were done using two-way ANOVA and Bonferroni’s post-hoc test (p<0.05).

Results

Lower IL-6 levels following anti-IL-6 antibody treatment

Previous work from our laboratory indicates that IL-6 is increased both systemically and in the lung and ileum following ethanol exposure and burn injury (144, 147, 150). Consistent with these observations, we found higher IL-6 levels in both the serum and ileum of mice exposed to the combined insult and IgG (650 ± 150 pg/mL, 35 ± 5 pg/mg protein, Figure 10A, B). However, 24 hours following IL-6 neutralizing antibody treatment, IL-6 was not significantly decreased in the serum or ileum (400 ± 20 pg/mL, 20 ± 7 pg/mg protein), but this was not expected as antibody treatment was only administered once following insult.

IL-6 antibody treatment reduces pMLC levels in intestinal epithelial cells

Our laboratory has recently described a role for myosin light chain kinase activity in intestinal barrier damage following ethanol exposure and burn injury (183). To determine if inhibition of IL-6 affects MLCK activity, we measured phosphorylation (Ser 19) of myosin light chain (pMLC) in isolated intestinal epithelial cells. Treatment with an IL-6 neutralizing antibody reduced pMLC levels by nearly 70% 3 hours after exposure to
Figure 10: IL-6 antibody treatment reduces IL-6 in serum (A) and ileum (B) following ethanol and burn. Levels of IL-6 were quantified by ELISA. Cytokine concentrations in ileum were normalized to total protein in the sample as determined by BioRad protein assay and all levels are presented as concentration ± SEM. *p<0.05 versus all groups except antibody treated. n = 4-6 per group.
the combined insult as compared to mice not receiving antibody treatment after exposure to ethanol and burn injury (Figure 11A). At 24 hours post antibody treatment, pMLC was still reduced in mice exposed to the combined insult as compared to mice not receiving antibody; however, this difference was no longer significant (data no shown). Interestingly, at 24 hours post insult mice deficient in IL-6 had no change in pMLC following any of the treatments (Figure 11D, F). As these samples were not run on the same gels, no statistical analyses between wild type and IL-6 knockout mice were done. Due to this result and subsequent outcomes to be described in upcoming sections, westerns were not completed at the 3 hour time point in IL-6 deficient mice.

Antibody treatment prevents tissue and tight junction protein alterations

As found previously (183), mice given ethanol and burn injury had visually shorter and wider villi along with an elevation in intestinal epithelial cell damage as compared to sham treated animals (Figure 12, only burn ethanol exposed animals shown). IL-6 knockout or wild type mice given an IL-6 neutralizing antibody had little to no intestinal morphological damage following exposure to the combined insult. Villi were taller and narrower in ethanol-exposed and burn-injured IL-6 knockout or antibody-treated wild type mice in contrast to burn ethanol wild type mice not treated with anti-IL-6 antibody (Figure 12 B, C). Greater damage to intestinal epithelial cells correlated with a reduction in tight junction protein co-localization with actin. Specifically, we found that mice given ethanol and burn injury had a significantly less zonula occludens protein-1 (ZO-1) (88%, p<0.05) and occludin (83%, p<0.05) at tight junctions when compared to mice
Figure 11: Anti-IL-6 antibody treatment reduces pMLC (Ser19) in intestinal epithelial cells following ethanol exposure and burn injury. Isolated intestinal epithelial cells were lysed and analyzed by western blot for levels of pMLC 3 hours (A) and 24 hours (C, D) in IL-6 antibody-treated (A) and IL-6 knockout mice (C). Quantification of pMLC levels was normalized to villin-1 levels and done using BioRad Image Lab software. Images are representative of 3 experiments, *p<0.05 versus antibody treated group. #p<0.05 versus sham groups. Quantification is of n = 4-6 per group.
Figure 12: Treatment with anti-IL-6 preserves morphology after ethanol exposure and burn injury. Ileum sections from wild type, antibody-treated, and IL-6 knockout mice were stained with hematoxylin and eosin (A-C) and then examined for degree of inflammation and damage. Representative H&E images taken at 200x. n = 4-6 per group.
exposed to sham injury (Figure 13G). Despite the resolution of intestinal morphological
damage observed, IL-6 knockout mice exposed to ethanol and burn injury had similar
localization patterns of ZO-1 as wild type mice given the combined insult (Figure 13C,
G). Interestingly, occludin localization was restored in ethanol-exposed and burn-injured
knockout mice (Figure 13 C, H), but this reestablishment was not nearly as effective as
antibody treatment. Treatment of wild type mice given ethanol and burn injury with an
IL-6 neutralizing antibody promotes maintenance of ZO-1 and occludin localization with
actin (Figure 13 B, E, G, H). These data suggest that inhibition, but not life-long
deficiency, of IL-6 is protective of intact tight junctions within the intestinal epithelial
cell layer of mice exposed to ethanol and burn injury.

Reduced bacterial translocation following antibody treatment

Alterations in tight junction protein localization affect the integrity and function
of the intestinal epithelial cell barrier. As observed previously in our model (183), mice
exposed to ethanol and burn injury have significantly greater bacterial translocation to the
mesenteric lymph nodes (MLN) as compared to mice receiving either insult alone (Figure
14A). Anti-IL-6 antibody treatment decreased bacterial translocation by 50% (p<0.05)
when compared to mice not receiving antibody after the combined insult (Figure 14A). In
contrast to antibody treated mice, knockout of IL-6 had no effect on bacteria
accumulating in the MLN and ethanol-exposed and burn-injured knockout mice actually
demonstrated an increase in bacterial translocation (Figure 14B). This increase in bacteria
in the MLN of knockout mice exposed to the combined insult is associated with a
decrease in ZO-1 localization at tight junctions as seen in Figure 13. These data indicate
Figure 13 continued on next page.
Figure 13: Tight junction protein localization maintained following anti-IL-6 treatment in combined injury animals. Frozen ileum sections were stained with antibodies against ZO-1 (green, A-C) or occludin (green, D-F) as well as phalloidin (red) and nuclei (blue). Representative immunofluorescent images taken at 400x. Immunofluorescent images were analyzed for co-localization of ZO-1 or occludin with actin (G, H). Co-localization is presented as % colocalized pixels/100 epithelial cells. *p<0.05 versus wild type sham vehicle, sham ethanol and antibody treated groups. #p<0.05 versus wild type sham vehicle and sham ethanol. @p<0.05 versus knockout burn ethanol and antibody treated groups. n = 4-6 per group.
that IL-6 may have a role in the intestinal epithelial damage, loss of tight junction integrity, and rise in bacterial translocation following ethanol exposure and burn injury; however, complete loss of IL-6 is perhaps more harmful than beneficial.

**Summary**

IL-6, along with TNFα and IL-1β, are pro-inflammatory cytokines that are commonly up-regulated after injury, infection, and other insults. In addition to its role in the inflammatory response, IL-6 also aids in differentiation of some cell types and induces mucin production. Recent work also suggests that IL-6 can activate MLCK.

Following binge ethanol and burn injury, MLCK activity and IL-6 were increased in the ileum. With this knowledge, we hypothesized that IL-6 activates MLCK and promotes intestinal damage, tight junction protein localization changes, and bacterial translocation in a mouse model of ethanol and burn injury.

For these studies IL-6 was inhibited with an IL-6 neutralizing antibody or IL-6 knockout mice were used. IL-6 antibody treatment significantly decreased pMLC in intestinal epithelial cells at 3 hours post binge ethanol and burn injury; however, knockout of IL-6 had no effect on pMLC at 24 hours. (Earlier time points for western blots were not analyzed in IL-6 knockout mice as analyses done at the 24 hour time point suggest that deficiency of IL-6 may be more detrimental than beneficial to the animals in our model of ethanol and burn.) At 24 hours post combined insult, both inhibition and life-long deficiency of IL-6 lessened intestinal morphological damage when compared to wild type animals not receiving anti-IL-6 treatment. Only antibody treated animals demonstrated restored ZO-1 and occludin localization with actin following the 2 insults.
Figure 14: Decreased bacterial translocation to mesenteric lymph following antibody treatment in mice exposed to ethanol and burn injury. Mesenteric lymph nodes were isolated from antibody-treated mice (A) and knockout mice (B) at 24 hours following insult. Lymph nodes were homogenized and plated on tryptic soy agar plates. Colonies were counted the next day and normalized on the total number of lymph nodes removed from each mouse. Levels presented as CFU/MLN ± SEM. *p<0.05 versus all other groups. #p<0.05 versus all other wild type groups. @p<0.05 versus all other wild type groups and knockout burn ethanol. n= 6-8 per group.
IL-6 knockout mice given ethanol and burn did have greater occludin localization to tight junctions than wild type mice exposed to the combined insult; however, this recovery was not nearly as efficient as observed with antibody treatment. In conjunction with the tight junction protein results, animals treated with IL-6 antibody displayed dampened bacterial translocation following binge ethanol and burn injury. Interestingly, ethanol-exposed and burn-injured IL-6 deficient mice had comparable amounts of bacterial translocation as similarly treated wild type mice.

These data suggest that anti-IL-6 antibody treatment following binge ethanol and burn injury reduces MLCK activity. Subsequent intestinal morphological damage and bacterial translocation are also diminished after the combined insult. IL-6 knockout mice exposed to ethanol and burn also had less intestinal damage; however, life-long deficiency in IL-6 does not improve intestinal barrier junctions or intestinal function after the dual insult. Responses observed in IL-6 knockout mice are likely due to the role of IL-6 in homeostatic functions or other cytokines compensating for the lack of IL-6. While complete loss of IL-6 does not decrease intestinal damage and inflammation following the combined insult, anti-IL-6 antibody treatment could possibly be used as a therapeutic for intestinal barrier disorders.
CHAPTER 5

ROCK AND MLCP ACTIVATION ARE NOT ALTERED FOLLOWING BINGE ETHANOL EXPOSURE AND BURN INJURY

Abstract

Binge ethanol exposure or burn injury alone are known to cause intestinal tissue damage, permeability, and inflammation. When these insults are combined detrimental intestinal responses are even greater. Previous work in our laboratory indicates that myosin light chain kinase (MLCK) activity plays a role in intestinal damage, bacterial translocation, and inflammation. We wanted to determine the signaling pathway leading to elevated MLCK activity. One pathway involves Rho-kinase (ROCK), which is known to inactivate myosin phosphatase consequently leading to a rise in phospho-myosin light chain (pMLC). ROCK levels were not changed between groups at 90 minutes or 3 hours following ethanol exposure or burn injury. No alterations in ROCK corresponded with no changes in the phosphorylation sites (inactivation and regulatory) of myosin phosphatase. Furthermore, Src kinase, one activator of ROCK, activation was also not affected by the combined insult. These data suggest that the increase in MLCK activity observed following exposure to ethanol and burn injury is not due to elevated ROCK activity although the possibility remains that we are not examining the correct time point for this analysis.
Introduction

Actin reorganization is a necessary occurrence for cellular migration, proliferation, and general cellular function. One pathway involved in actin reorganization is the Rho and Rho kinase (ROCK) pathway (196). Agonists of G-coupled protein receptors (specifically Gq or G12/13), such as thrombin (197), lead to the activation of the small GTPase Rho. Once active, Rho (Rho-GTP) can then bind to the Rho-binding domain of ROCK thus increasing ROCK’s activity (32). Besides Rho binding, cleavage of ROCK’s C-terminal domain by caspase-3 leads to a constitutively active ROCK (198), and binding of arachidonic acid can also lead to ROCK activation (199). ROCK is a serine/threonine kinase (32) that can phosphorylate numerous targets including the myosin binding subunit of myosin phosphatase, myosin light chain (MLC), and CPI-17 (33, 200, 201). Phosphorylation of myosin phosphatase inhibits its activity and tips the scale toward elevated phospho-MLC (pMLC) and actin contraction (35). A rise in pMLC is associated with barrier dysfunction (161), increased inflammation (202), and chemokine expression and leukocyte recruitment (203).

Following traumatic injury, like burn injury, an elevation in intestinal epithelial and endothelial permeability has been observed (135, 204). Increases in intestinal permeability are associated with greater inflammation, bacterial translocation, and tissue damage (205). Previous work from our laboratory and others indicates that when ethanol exposure is combined with burn injury, these detrimental intestinal responses are exacerbated (156, 183). Interestingly, elevations in intestinal epithelial cell levels of pMLC were also found following the combined insult (183) but no elevation in myosin
light chain kinase (MLCK) was observed. This suggested that the increase in pMLC may be due to a rise in ROCK activity and greater myosin phosphatase inactivation.

Activation of the ROCK pathway has been attributed to alterations in intestinal barrier function due to various insults. ROCK activity was recently shown to mediate radiation-induced intestinal barrier function (206), and endotoxin exposure led to decreased epithelial restitution as a result of elevated ROCK activity (207). Furthermore, *H. pylori* infection induces ROCK activity ultimately leading to disruptions in tight junction proteins (208). ROCK activation also contributes to leukocyte recruitment following intestinal ischemia/reperfusion injury (203). These studies suggest that the Rho/ROCK pathway plays a role in intestinal epithelial barrier homeostasis as well as inflammation following burn injury. Using a model of ethanol exposure and burn injury, we hypothesized that intestinal barrier alterations observed following the combined insult was due to elevated ROCK activity.

**Materials and Methods**

**Mice**

Wild type (C57BL/6) and IL-6 knockout male (8-10 week old, 23-25 grams) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed in sterile microisolator cages in the Loyola University Medical Center Comparative Medicine facility. All experiments were conducted in accordance with the Loyola Institutional Animal Care and Use Committee.
Murine Model of Ethanol and Burn Injury

A murine model of a single binge ethanol exposure and burn injury was performed as described previously (172) with minor modifications (149). Briefly, mice were given one dose of 1.11 g/kg of 20% (v/v) ethanol solution intraperitoneally that resulted in a blood ethanol level of 150-180 mg/dL at 30 minutes. The mice were then anesthetized with 100 mg/kg of Ketamine and 10 mg/kg of Xylazine (Webster Veterinary, Sterling, MA), their dorsum shaved, and placed in a plastic template exposing 15% of the total body surface area (TBSA) and subjected to a scald injury in 90-92° C water bath or a sham injury in room-temperature water. The scald injury resulted in an insensate, full-thickness burn injury of approximately 15% total body surface area (173). Mice received 1 mL of saline resuscitation and their cages were placed on warming pads until mice recovered from anesthesia. Animals were sacrificed by CO₂ narcosis followed by cervical dislocation three hours following injury.

Intestinal epithelial cell isolation and western blotting

Intestinal epithelial cells were isolated as previously described (167) with minor modifications. Briefly, ileum sections were opened lengthwise, washed with calcium and magnesium free Hanks Basic Salt solution (HBSS), and placed in tubes containing 10 mM DTT and phosphatase inhibitor cocktail in HBSS. Samples were placed at 4°C for 30 minutes, after which tubes were shaken briefly and ileum sections moved to tubes containing 1 mM EDTA and PIC in HBSS for 1 hour at 4°C. After incubation, tubes were shaken robustly and large pieces of tissue removed. Samples were centrifuged at 1000 g for 10 minutes and the supernatant discarded. Pelleted cells were lysed in 100 uL
of Cell Lysis Buffer according to manufacturer’s protocol (BioRad, Hercules, CA). Twenty milligrams of whole cell lysate protein was boiled for 5 minutes, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes and blotted with primary antibodies specific for phosphorylated myosin light chain (pMLC Ser19, Cell Signaling Technology, Danvers, MA), ROCK1 (Cell Signaling Technology), phosphorylated myosin phosphatase (Thr696 and Thr850, Millipore, Danvers, MA) and villin-1 (Cell Signaling Technology).

Statistics

Statistical comparisons (GraphPad Instat and Prism) were made between the sham vehicle, sham ethanol, burn vehicle, and burn ethanol treatment groups, resulting in 4 total groups analyzed. One-way analysis of variance was used to determine differences between treatment responses, and Tukey’s post-hoc test once significance was achieved (p < 0.05).

Results

Recent work from our laboratory indicates that the dual insult of binge ethanol and burn injury leads to a significant elevation in pMLC in intestinal epithelial cells (183); however, no elevation in MLCK was observed. We sought to determine whether this rise in pMLC was due to ROCK-induced phospho-myosin phosphatase (pMLCP) inactivation.
No changes in ROCK levels following ethanol and burn

As the earliest increase in pMLC was observed at 3 hours following the combined insult, we examined intestinal epithelial cell levels of ROCK at both 90 minutes and 3 hours after ethanol and burn injury. Surprisingly, no changes in ROCK were observed between groups at either time point (Figure 15A, B). As no differences between groups were found at the 3 hour time point, only the sham vehicle and burn ethanol groups were employed at the 90 minute time point.

Inhibitory and regulatory MLCP phosphorylation sites not altered by combined insult

Although ROCK did not appear to be altered following exposure to ethanol and burn injury, we analyzed the phosphorylation status of the inactivation site (Thr696) and regulatory site (Thr850) on MLCP. ROCK can phosphorylate both sites (36, 37). Neither Thr696 nor Thr850 phosphorylation were changed by ethanol exposure, burn injury, or the combined insult (Figure 16A, C).

Activators of ROCK not altered following combined insult

Besides the binding of Rho, other factors are known to enhance ROCK activity. Both IL-1β and IL-6 have been shown to active the Rho signaling pathway ultimately leading to ROCK activation (208, 209). While IL-1β and IL-6 are both elevated in the ileum at varying time points, 6 and 24 hours respectively, serum levels of IL-6 are increased by 2 hours post insult (183). The work of Lin and colleagues indicates that IL-6 induces ROCK via Src (209); however, we found no differences in levels of active Src at 3 hours post insult (Figure 17A). Interestingly, it appears that mice exposed to burn injury
Figure 15: ROCK levels not altered following ethanol exposure and burn injury. Isolated intestinal epithelial cells were lysed and analyzed by western blot for levels of ROCK either 90 minutes (A) or 3 hours (B) after exposure to treatment. Quantification of ROCK levels was normalized to villin-1 levels and done using BioRad Image Lab software. Images are representative of 3 experiments. Quantification is of n = 4-6 per group.
or the combined insult had less active Src than sham-injured mice, but this change was not significant.

Overall, these data reveal that there is no significant increase in ROCK pathway activity following ethanol exposure and burn injury. Although we observed an increase in pMLC in IECs, neither inhibition of MLCP or direct phosphorylation by ROCK appear to be responsible for this effect.

**Summary**

We previously described a role for MLCK in intestinal damage, permeability, and tight junction protein localization in a murine model of binge ethanol and burn injury. Furthermore, IL-6 signaling also contributes to MLCK activity as inhibition of IL-6 reduced pMLC in animals exposed to the combined insult. Neither of these observations, however, detail a mechanism leading to MLCK activation. Here, we examined the activation of ROCK, MLCP, and Src kinase in intestinal epithelial cells isolated from mice exposed to binge ethanol and burn injury.

At 90 minutes and 3 hours post combined insult, no differences in ROCK were discovered between groups. ROCK phosphorylates (and inactivates) MLCP at both threonine 696 and threonine 850, but pMLCP (Thr696 and Thr850) were not altered at 3 hours following binge ethanol and burn. Finally, Src kinase can phosphorylate MLCK causing enhanced MLCK activity. Despite an observed increase in pMLC at 3 hours post combined insult, Src kinase (pTyr416) levels were not different between groups.

These data suggest that the combined insult of binge ethanol and burn injury do not cause a significant elevation in the activity of the ROCK pathway. This does not
Figure 16: Combined insult does not change regulatory of inhibitory phosphorylation levels of myosin phosphatase at 3 hours after ethanol and burn. Isolated intestinal epithelial cells were lysed and analyzed by western blot for levels of pMLCP at either Thr696 (A) or Thr850 (C) after exposure to treatment. Quantification (B, D) of pMLCP levels was normalized to villin-1 levels and done using BioRad Image Lab software. Images are representative of 3 experiments. Quantification is of n = 4-6 per group.
Figure 17: Src kinase activation not increased at 3 hours following ethanol and burn injury. Isolated intestinal epithelial cells were lysed and analyzed by western blot for levels of pSrc kinase (A) after exposure to treatment. Quantification (B) of pMLCP levels was normalized to villin-1 levels and done using BioRad Image Lab software. Images are representative of 2 experiments. Quantification is of n = 4-6 per group.
insinuate that ROCK has no influence on MLCK activation. MLCP is phosphorylated indicating that ROCK is active. It is possible that a significant rise in ROCK and pMLCP (inactive) is not necessary for the elevation in MLCK activity observed after binge ethanol and burn injury.
CHAPTER 6
DISCUSSION

In review, we used a mouse model of ethanol exposure and burn injury to investigate the role of MLCK in intestinal permeability and inflammation. Previous studies in a rodent model have shown that binge ethanol exposure and burn injury can cause intestinal permeability and bacterial translocation (63, 155); however, the possible mechanism of these changes has not been determined. The results described in the present study indicate that after the combined insult of ethanol exposure and burn injury MLCK contributes to alterations in tight junction localization, bacterial translocation, along with intestinal inflammation and damage (Figures 8, 9, Table 1). PIK-treated mice had a reduction in these parameters including less villus blunting, edema, destruction of the intestinal epithelial layer, and tight junction protein localization alterations as compared to similarly treated wild type mice not receiving PIK (Figure 8). Although MLCK is an enzyme necessary for epithelial barrier maintenance, inhibition of MLCK could be beneficial for decreasing intestinal morphological damage and inflammation following ethanol and burn injury. This decline in morphological damage corresponded with reduced ileum IL-6 levels in PIK-treated mice (Figure 9). While PIK-treated mice did not have significantly lower bacterial translocation 24 hours after the combined insult, it was 33% less than ethanol-exposed and burn-injured mice not given PIK. It is likely that more than one PIK administration would be necessary to significantly reduce
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Table 1: Summary of ethanol exposure and burn injury-induced responses in ileum in first 72 hours post insult. The combined insult of binge ethanol exposure and burn injury causes intestinal damage, inflammation, and barrier dysfunction. This table summarizes the events occurring in ileum at the designated time points, starting at 2 hours and ending at 72 hours.
bacterial translocation at later time points, which is an ongoing study in our laboratory.

Studies examining immune cell function suggest that bacterial accumulation in the MLN occurs in a model of ethanol exposure and burn injury, likely due to decreased T cell proliferation and reduced production of IL-2 and interferon-γ (63, 155). Moreover, previous work in our model indicates an increase in ileum levels of IL-10 at 24 hours post combined insult (147). Overall these data suggest that the combination of ethanol exposure and burn injury not only affect barrier permeability but also delay resolution of bacteria in the MLN allowing for bacterial accumulation and the possibility of bacterial dissemination.

Alterations in intestinal permeability have long been studied as the cause or outcome of numerous diseases and injuries. Common in chronic inflammatory disorders, such as inflammatory bowel syndrome, elevated intestinal barrier dysfunction may contribute to progression of the disease due to a continuous immune response (210). Pro-inflammatory mediators, particularly TNFα, LIGHT (a member of the TNFα superfamily), IL-1β, and IL-6 have all been shown to increase intestinal permeability in animal models of hemorrhagic shock and resuscitation, diarrhea and epithelial barrier dysfunction (49, 193, 211). Of these cytokines, most studies have focused on TNFα in barrier dysfunction. TNFα was shown to directly up-regulate MLCK transcription as well as protein levels (48, 163) and the cytokine also promotes occludin internalization (49, 212). In models of inflammatory bowel disease, antagonism of TNFα resulted in partial restoration of T-cell mediated barrier dysfunction (167); however, in our model of binge ethanol exposure and burn injury, we only see an early rise in serum levels of
TNFα. This initial rise in TNFα suggests that it could still be an activator of MLCK as our elevation in MLCK and pMLC are observed early after the insult as well. Furthermore, TNFα is also known to increase levels of other pro-inflammatory cytokines, such as IL-1β and IL-6, which may be important regulators of the alterations seen in the intestine after exposure to the combined insult. In line with this thought, we observed an increase in ileum levels of IL-1β at 6 hours and IL-6 at 24 hours following the combined insult (Figures 7 and 9). Interestingly, IL-6 was also elevated in the serum at 2 hours post combined insult and previous work indicates that it remains elevated until at least 24 hours (68). Taken together, these data indicate that both TNFα and IL-6 may have a role in activation of the MLCK pathway and later tissue damage, inflammation, and alterations in the intestinal barrier.

Our studies support previous work that ethanol exposure combined with burn injury causes additive damage on intestinal barrier dysfunction than burn injury or ethanol exposure alone. Choudhry and colleagues have shown greater bacterial translocation to the mesenteric lymph nodes in a rat model of ethanol exposure and burn injury (155, 156). This correlated with increased in vivo gut permeability and bacterial overgrowth, but not with changes in intestinal morphology. Furthermore, intestinal edema and MPO were higher in mice exposed to the combined insult than in mice given either injury alone (151). Neutrophil depletion or anti-IL-18 antibody treatment reduced ileum neutrophil infiltration and MPO levels in the same model (151, 152). The findings presented in this study are similar to those previously published by our group and others despite using a slightly different model. We do, however, find greater intestinal
morphological damage than was observed seen in other models of ethanol exposure and burn injury. This difference in intestinal damage might be attributed to subtle differences in ethanol administration, timing of administration relative to injury, peak blood alcohol level or small variations in burn injury protocol; however, both models produce elevations in inflammation and barrier dysfunction.

Unlike previous studies, data presented here show, for the first time, a possible mechanism, which could explain how the previously reported marked elevation in systemic and local levels of cytokine might trigger the observed increase in intestinal damage after exposure to ethanol and burn injury (Figure 5-9, 18-20). While MLCK has been extensively studied in chronic inflammatory diseases, the role of this enzyme after a combined insult or other acute insults has not been as well defined. Using a MLCK inhibitor, we have demonstrated that MLCK functions in tight junction protein localization and subsequent bacterial translocation and inflammation in a model of ethanol exposure and burn injury. The mechanism of this restoration is currently being studied with several possible pathways being involved. MLCK activation is highly correlated with TNFα and while we do not see changes in TNFα in the ileum after insult, it may very well be the predominant signal for MLCK activation. TNFα signaling causes increases in both expression and protein levels of IL-6 (213) and MLCK (163) and can also activate numerous kinase cascades, which could easily lead to MLCK activation. Multiple pathways may converge leading to the damage and alterations seen in the ileum after the combined insult.
Figure 18 continued on next page.
Figure 18: Serum levels of IL-6 and TNFα elevated 2 hours after combined insult. The intestinal epithelial layer is composed of many cell types held together by a variety of junction complexes (A). If we look closer at this barrier (B), we see that these complexes consist of tight junctions (TJ, purple boxes), adherens junctions (AJ, green boxes), and desmosomes (D, grey boxes). Early (2 hours) after binge ethanol and burn injury, an elevation in pro-inflammatory cytokines can be observed in the blood of exposed animals.
Figure 19: MLCK activation is associated with an elevation in inflammation, tissue damage, and tight junction protein localization changes at 24 hours post combined insult. MLCK activity promotes intestinal barrier damage, inflammation, and alterations in tight junction protein localization. By 3 hours post combined insult, an elevation in pMLC is observed. This is followed by decreased tight junction integrity and a rise in epithelial cell damage, bacterial translocation and ileum levels of IL-6.
Figure 20: Inhibition of MLCK or IL-6 reduces detrimental intestinal responses following binge ethanol and burn injury. Inhibition of MLCK or IL-6 reduces pMLC in intestinal epithelial cells and is associated with decreased intestinal epithelial cell damage, bacterial translocation, inflammation, and tight junction protein localization alterations.
In summary, the results of Chapter 3 indicate that inhibition of MLCK is beneficial for the reduction of intestinal damage, barrier leakiness, and inflammation after exposure to ethanol and burn injury. As previously stated, MODS and MOF are the most common outcomes of burn injury (158, 159). Recent clinical studies indicate that trauma-induced sepsis patients with a history of alcohol abuse have an increased risk of acute respiratory distress syndrome (ARDS), the first stage of MODS. (214). Interestingly, preliminary work in our laboratory suggests that PIK treatment also reduces the neutrophil chemokine, KC, in the lung as well as the total number of neutrophils infiltrating into the lung following ethanol exposure and burn injury. Furthermore, studies conducted in Loyola’s Burn ICU demonstrate that intoxicated patients who suffer burn injuries have smaller burns than non-drinking burn patients, but spend the same number of days in the hospital, have as many days on the ventilator and accrue the same amount of hospital costs as non-drinking patients with much larger burns (143). Thus, intoxicated patients with minor burn injuries suffer more complications than their not drinking counterparts. Data presented here implicate that patients who suffer a burn injury with ethanol in their system may have impaired intestinal barrier function thus contributing to an elevated state of inflammation and perhaps severe downstream complications. Overall, these data associate MLCK in the pathogenesis of binge ethanol and burn-induced intestinal barrier dysfunction and elevated intestinal inflammation and suggest a possible role for MLCK in other acute gastrointestinal injuries.

MLCK activity is regulated by various factors, including inflammatory cytokines. IL-6 is a multi-functional cytokine capable of promoting both beneficial and detrimental
outcomes depending on the level of IL-6. At elevated levels, as seen in inflammatory bowel disease and rheumatoid arthritis, IL-6 is known to perpetuate the inflammatory state and tissue destruction of these diseases partially through the induction of Th17 cells (215, 216). Previous work indicates that increased systemic IL-6 observed following sepsis and burn injury leads to cellular immunosuppression (65, 66) and our laboratory has demonstrated that ethanol exposure combined with burn injury causes even further cellular immunosuppression thus allowing for increased susceptibility to infection (68, 144). A minimal amount of IL-6 is required for normal cellular functions like the development of B and Th17 cells (58, 190); however, it is the sustained elevation in systemic and tissue levels of IL-6 that can cause tissue injury, increased inflammation, and delayed resolution of the immune response following insult (43, 72, 181, 192). In the gut, intestinal epithelial cells have been shown to produce IL-6 in response to cytokine and TLR4 signaling induced by infection, disease, or injury (186-188). As seen in Figure 10, the combined insult of ethanol exposure and burn injury leads to significant increase in both serum and ileum levels of IL-6. IL-6 has been associated with permeability in models of ethanol exposure or burn injury alone (43, 217). Other studies indicate that elevations in IL-6 are linked to mucosal permeability in both rodents and humans (218, 219). Additionally, using Caco-2 cells as a model of the intestinal epithelial cell barrier, work by Suzuki and colleagues suggests that IL-6 increases tight junction permeability via an increase in expression of the leaky tight junction protein, claudin-2 (74). Combined these studies indicate the importance of controlled IL-6 signaling in the intestine both in a healthy individual and following insult.
The role of IL-6 in tissue injury is dependent on the type of injury, the specific tissue, and the level of IL-6. IL-6 treatment was found to be protective of ischemia/reperfusion injury in a fatty liver (220) and in ethanol-induced hepatocyte oxidative stress and mitochondrial dysfunction (221). Interestingly, IL-6 has also been described to up-regulate keratins in the intestinal epithelial cell barrier in a dodecyl sodium sulfate (DSS)-induced barrier dysfunction model (222). This up-regulation of keratins protected barrier function suggesting that in situations of intestinal barrier compromise, IL-6 may be necessary for maintenance of the intestinal epithelial cells (222). The work of Wang et al. may provide a possible explanation for why our IL-6 knockout mice exposed to ethanol and burn had patterns of localization similar to comparably treated wild type mice (Figure 13).

In contrast to the above studies, IL-6 has also been shown to be necessary for intestinal barrier dysfunction in a model of hemorrhagic shock and resuscitation (193). Knockout of IL-6 led to decreased intestinal permeability following induction of sepsis (194) and reduced ileal morphological injury, neutrophil infiltration into the intestine and serum levels of pro-inflammatory cytokines in a model of splanchnic artery occlusion (195). Interestingly, data presented in Chapter 4 suggest that knockout of IL-6 is not protective of intestinal barrier function but does prevent morphological damage induced by the combined insult of ethanol exposure and burn injury (Figures 12 and 14). IL-6 knockout mice subjected to hind limb ischemia or hemorrhage also did not have a reduction in bacterial translocation (223), further indicative that IL-6 may play different roles in tissue inflammation and injury depending on the nature of the insult. Moreover,
as is true in many knockout strains, a compensatory response may have been activated
thus producing responses observed following ethanol exposure and burn injury. Whether
that compensatory response occurred in our model is not known, but increased activity of
other members of the IL-6 family or other pro-inflammatory pathways may have
produced the responses we discovered in the IL-6 knockout mice.

IL-6 signaling causes numerous responses depending on the cell type. Known to
signal through signal transducer and activator of transcription 3 (Stat3) and induce
transcription of genes (224), stimulation through IL-6 also leads to activation of the
phosphatidyl-inositol 3 kinase (PI3K)/Akt pathway (225). Signaling through the
PI3K/Akt pathway is usually associated with being anti-apoptotic (226). Furthermore, IL-
6 activation induces the mitogen activated protein kinase (MAPK) cascade (227),
specifically ERK1/2 (extracellular signal-related kinase), a pathway associated with cell
survival. While numerous stimuli activate the MAPK and PI3K/Akt pathways (228, 229)
IL-6 deficiency may be a compounding factor in detrimental intestinal responses
observed following ethanol exposure and burn injury. Grivennikov and colleagues found
decreased intestinal epithelial cell survival and crypt cell proliferation in dextran sodium
sulfate (DSS)-treated IL-6 knockout mice as compared to similarly treated wild type mice
(230). This further supports the idea that IL-6 is important for IEC survival and
maintenance in time of injury.

Tight junction proteins are often disrupted in the intestinal barrier following many
insults including trauma, disease, and infection (180, 231, 232). These insults also lead to
elevated IL-6 levels (72, 147, 233); however, the majority of studies have examined other
cytokines, such as TNFα and IL-1β, in tight junction protein regulation. IL-6 treatment of HUVECs (human umbilical vein endothelial cells) led to an elevation in monolayer permeability and alterations in ZO-1 localization. Using a PKCα (protein kinase C) and PKCβ inhibitor in conjunction with an IL-6 antibody partially restored barrier function of the HUVECs (234). This result is similar to our ethanol-exposed and burn-injured mice given an IL-6 antibody (Figures 13 and 14); however, when no IL-6 was present the therapeutic effect was lost suggesting that some IL-6 is necessary for proper tight junctions in the IEC barrier. In a model of reflux esophagitis, Li and colleagues found that IL-6 was elevated in infiltrating inflammatory cells (235). This elevation in IL-6 corresponded with a rise in claudin-1, occludin and ZO-1 in surrounding epithelial cells; however, newly synthesized tight junction proteins failed to organize at the plasma membrane. Furthermore, production of junction proteins was not enough to repair lesions induced in the model. Whether IL-6 affects tight junction protein localization directly or indirectly is not clear from these studies, but it does insinuate that IL-6 has an effect on tight junction protein production and possibly localization.

Pro-inflammatory cytokines, in particular TNFα, have been shown to induce barrier dysfunction in models of IBD as well as traumatic injury (43, 210, 236). TNFα, as well as IL-1β and LIGHT, are known enhancers of MLCK transcription and activation (42, 49, 163). Interestingly, we found that anti-IL-6 antibody treatment led to less pMLC (Figure 11) following ethanol exposure and burn injury. Few other studies have examined IL-6’s role in MLCK signaling, but Suzuki et al. discovered that IL-6 treatment had no effect on pMLC in a Caco-2 cell culture model (74) although barrier function of this
monolayer was decreased. While this would advocate for IL-6-induced barrier function not being MLCK-dependent, this work was done in cell culture indicating that other cells may be necessary for an effect. Current research in our laboratory is examining whether IL-6 does have a direct effect on MLCK activity. Preliminary experiments will examine changes in Src kinase activation, an enzyme downstream of IL-6 signaling and known to enhance MLCK activity (41).

Our data indicate that an elevation in serum and ileum levels of IL-6 may have a role in intestinal epithelial cell barrier alterations following ethanol exposure and burn injury. When IL-6 was inhibited with a neutralizing antibody bacterial translocation was reduced and greater ZO-1 and occludin localized to the tight junction. These results were accompanied by reduced pMLC in intestinal epithelial cells (Figures 11-14, 18-20). While complete knockout of IL-6 did not appear to be beneficial for intestinal responses following the combined insult, some reduction in IL-6 may present a new option for aid in models of intestinal barrier dysfunction.

In addition to inflammatory stimulation, MLCK’s activity is affected by proteins such as ROCK, MLCP, and Src kinase. Activation of the ROCK pathway is known to cause numerous effects including cellular contraction, proliferation, mobility, and apoptosis (237). ROCK has also been shown to play a role in inflammation and tissue damage following infection (208), radiation (206), endotoxin exposure (207), and ischemia-reperfusion injury (203). In our model of ethanol exposure and burn injury, we hypothesized that ROCK may be inducing the up-regulation of pMLC in intestinal epithelial cells thus causing downstream barrier dysfunction and inflammation. Despite
ROCK’s involvement in the MLCK pathway, its levels are not greater in IECs of mice exposed to ethanol and burn injury (Figure 15). This is associated with no alterations in MLCP phosphorylation (Figure 16) and no changes in the ROCK activator, Src (Figure 17). These data suggest that ROCK activity is not elevated following exposure to ethanol and burn injury; however, it does not mean that ROCK is not functioning in responses observed in the intestine after the combined insult. We examined ROCK at 90 minutes post insult, but the possibility remains that we need to analyze an even earlier time point such as 30 minutes. Moreover, little to no information exists on the effect of ethanol or burn injury on ROCK. Boe and colleagues have described a role for a single dose of ethanol on an increase in ROCK activity, but the ethanol dose used in these studies was 3 times higher than used in our work and done entirely in vitro (238). Comparing in vivo and in vitro studies is challenging, but the 50-100 mM ethanol doses used in this paper are equivalent to approximately 400 mg/dL and higher in vivo. This level is 5 times the legal limit and is not physiologic so direct comparisons between our studies should be limited. As our model does not cause as severe an inflammatory response as previous work (209, 239), this may explain why we do not observe an elevation in ROCK activity.

Other proteins are capable of inhibiting the ROCK/MLCP pathway that may be up-regulated following the combined insult of ethanol exposure and burn injury. While RhoA, B, and C are activators when bound to ROCK, RhoE inhibits ROCK activity when bound (196, 240). RhoE levels are not known following exposure to ethanol and burn injury. Besides ROCK phosphorylation, MLCP can also be inhibited by the binding of CPI-17. CPI-17, when phosphorylated by PKC, binds to MLCP and prevents it from de-
phosphorylating MLC (38). CPI-17 is currently not known to be expressed in intestinal epithelial cells, but it is expressed in smooth muscle (38). Although smooth muscle is a large component of the intestine, it is not near the epithelium and CPI-17 is not known to be excreted. This would suggest that CPI-17 does not have a role in MLCP inhibition in isolated intestinal epithelial cells. Recent studies indicate that ROCK activates IκB kinase, which activates the NF-κB pathway (202). NF-κB is the predominant transcription factor for many inflammatory proteins including TNFα and IL-6 (241). The work of Segain and colleagues indicates that ROCK’s role in our model of ethanol exposure and burn injury may be in the early induction of inflammation although with no measureable increase in ROCK, this does not appear to be a likely occurrence. Data presented here suggest that the combined insult of ethanol exposure and burn injury does not elevate ROCK pathway activity, but it does not rule out the chance of ROCK having a role in intestinal responses following the dual insult. Tissue damage, neutrophil infiltration, or cytokine levels are not increased to the extent as others in the literature (202, 203, 206, 208) which could explain why no significant increases in ROCK were found.

Two other enzymes are known to phosphorylate MLC in addition to MLCK and ROCK, integrin-linked kinase (ILK) and zipper-interacting protein kinase (ZPK) (248, 249). Little information exists about these kinases in intestinal epithelial cells; however, a role for ILK was discovered in intestinal epithelial restitution (249). Currently, it is not known whether ZPK is expressed in intestinal epithelial cells, but both ILK and ZPK represent other possible pathways to investigate as an explanation of our pMLC results.
Overall, data described here indicate that following ethanol exposure and burn injury, MLCK activity rises in intestinal epithelial cells. Elevated MLCK activity, as measured by pMLC levels, is linked with enhanced bacterial translocation and ileum IL-6 amounts (Figure 18-21, Table 1). Furthermore, ZO-1 and occludin localization to tight junctions is decreased following the combined insult. Inhibition of MLCK using the specific inhibitor PIK, or inhibition of IL-6 using a neutralizing antibody diminishes MLCK activity, intestinal inflammation, and bacterial translocation (Figure 18). Both inhibition of MLCK or IL-6 also restored tight junction protein localization. Surprisingly, none of the other proteins involved in the MLCK pathway (ROCK, MLCP) are altered in levels or activity after ethanol exposure and burn injury.

Clinical studies suggest that burn patients and alcoholics suffer from gastrointestinal problems such as permeability and inflammation. Moreover, these patients also have an increased susceptibility to infection. The intestine is a large reservoir of bacteria that in healthy individuals causes no troubles, but if the intestinal barrier is compromised, these bacteria can enter the intestinal tissue and perhaps lead to a systemic infection. Although medical treatment has gotten better at preventing infections in hospitalized patients, the source of bacteria may also come from within the patient. In other words, if the intestinal barrier breaks down, a patient’s own bacteria can cause harm. All of these clinical data indicate that prevention of intestinal barrier damage and permeability could thwart further systemic complications, such as lung infection (Figure 19). As MLCK’s predominant function is maintenance of the epithelial barrier and its
Figure 21: Proposed model of binge ethanol and burn-induced intestinal damage, inflammation, and possible systemic consequences. The combined insult causes elevated serum and ileum levels of pro-inflammatory cytokines leading to MLCK activation and phosphorylation of MLC. Increased MLCK activity triggers intestinal tissue damage and bacterial translocation, which can further augment inflammation in the ileum and other organs, such as the lung.
activation is commonly augmented following injury and ethanol exposure, it represents a possible target to better treat ethanol-exposed and burn-injured patients.
APPENDIX A

MLCK KNOCKOUT MICE DISPLAY REDUCED INTESTINAL EPITHELIAL BARRIER DYSFUNCTION AND INFLAMMATION FOLLOWING BINGE ETHANOL AND BURN INJURY
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MLCK KNOCKOUT MICE DISPLAY REDUCED INTESTINAL EPITHELIAL BARRIER DYSFUNCTION AND INFLAMMATION FOLLOWING BINGE ETHANOL AND BURN INJURY

Results

Previous data described in Chapter 3 indicate that inhibition of MLCK reduces intestinal damage, tight junction protein position alterations, bacterial translocation, and ileum inflammation. Similar studies were conducted in MLCK knockout mice, which lack only the long form of the enzyme.

Elevation in pMLC despite knockout of MLCK following binge ethanol and burn injury (Figure 6). An increase in pMLC was previously described at 3 hours after binge ethanol and burn injury (Figure 6). Inhibition of MLCK using PIK significantly reduced pMLC following the combined insult (Figure 6). Surprisingly, MLCK knockout mice do not display less pMLC following ethanol exposure and burn injury (Figure 22). In fact, pMLC levels are significantly higher in MLCK knockout mice exposed to the combined insult as compared to comparably exposed wild type mice. A similar finding was observed in the burn injury alone group as well (only densitometry shown, Figure 22). Further studies need to be conducted to determine if other kinases, such as ROCK, are responsible for the elevated phosphorylation of MLC in the absence of MLCK.

Knockout of the MLCK gene alleviates morphological damage after combined insult

Twenty-four hours following the combined insult, villi of MLCK knockout mice (Figure 23) exposed to burn injury alone (data not shown) or ethanol plus burn injury
Figure 22: pMLC not decreased in MLCK knockout mice following burn injury or binge ethanol and burn injury. Isolated intestinal epithelial cells were lysed and analyzed by western blot for levels of pMLC at serine 19 in wild type and MLCK knockout mice exposed to binge ethanol and burn injury. Quantification of pMLC levels was normalized to villin-1 levels and done using BioRad Image Lab software. Images are representative of 2 experiments. *p<0.05 versus both sham vehicle groups and comparably treated wild type group. Quantification is of n = 3-6 per group.
were comparable to sham treated animals (Figure 8) with tall (179 µm ± 16), narrow villi. Furthermore, no indications of intestinal epithelial barrier damage are observed in histological samples from MLCK knockout mice exposed to binge ethanol and burn.

Unlike wild type mice, neither burn alone nor the combined insult affected ZO-1 localization in MLCK knockout mice. All MLCK knockout treatment groups (only burn ethanol shown) had high levels of ZO-1 and actin co-localization (Figure 23 D, G). As described previously, quantification of ZO-1 and actin co-localization (Figure 8) indicated that significantly less ZO-1 co-localized with actin in wild type mice exposed to ethanol and burn injury than in sham treated animals. MLCK knockout mice given the combined insult had greater ZO-1 co-localization than their wild type counterparts and all MLCK knockout treatment groups had statistically greater co-localization than ethanol-exposed and burn-injured wild type mice (p<0.05). While MLCK knockout mice exposed to ethanol and burn injury did not have occludin co-localization that were restored to sham levels, it was significantly elevated (p<0.05) as compared to similarly treated wild type mice (p<0.05, Figure 23 F, H).

Decreased MLCK activity reduces epithelial damage and inflammation after combined insult

Using bacterial translocation as a measure of epithelial damage, we again saw that wild type mice exposed to the combined insult had greater bacteria accumulation in the mesenteric lymph nodes than in mice exposed to either injury alone. In contrast, MLCK knockout mice did not have elevated levels of bacteria in the MLN after ethanol and burn injury (Figure 24). These results correspond with findings from PIK treatment and
Figure 23: Reduced intestinal damage and tight junction protein localization change in MLCK knockout mice 24 hours after ethanol and burn. Ileum sections from wild type mice were stained with hematoxylin and eosin (A, B) and then examined for degree of inflammation and damage. Representative H&E images taken at 200x. Frozen ileum sections were stained with antibodies against ZO-1 (green, C, D) or occludin (green, E, F) as well as phalloidin (red) and nuclei (blue). Representative immunofluorescent images taken at 400x. Co-localization is presented as % colocalized pixels/100 epithelial cells. *p<0.05 versus wild type sham vehicle and sham ethanol, #p<0.05 versus comparably treated knockout group. n = 6-8 per group.
Figure 24: Decreased bacterial translocation and ileum levels of IL-6 in MLCK knockout mice exposed to ethanol and burn injury. Mesenteric lymph nodes were homogenized and plated on tryptic soy agar (TSA) plates from MLCK knockout and wild type mice (A) 24 hours after insult. Colonies were counted the next day and normalized on the total number of lymph nodes removed from each mouse. Levels presented as concentration ± SEM. Levels of IL-6 were quantified in MLCK knockout and wild type mice by ELISA (B). Cytokine concentrations were normalized to total protein in the sample as determined by BioRad protein assay and presented as concentration ± SEM. *p<0.05 versus all other wild type groups, #p<0.05 versus comparably treated knockout group. n = 3-6 per group.
indicate that MLCK promotes barrier damage and bacterial translocation in a model of binge ethanol and burn injury.

With the knowledge that ethanol and burn injury incur greater morphological damage and bacterial translocation than either insult alone, we examined the effect of MLCK deficiency on inflammatory cytokines. As observed previously, there was a 33% increase in ileal IL-6 as compared to burn injury alone (Figure 9). Unlike wild type mice, the ileum IL-6 levels in all MLCK knockout groups were significantly lower than wild type mice given ethanol and burn injury (Figure 24). IL-1β and TNFα levels remained constant in both wild type and MLCK knockout mice in all treatment groups (data not shown).

These data support the results observed following PIK treatment. Both inhibition and knockout of MLCK decrease intestinal morphological damage and inflammation following binge ethanol and burn injury. Furthermore, ZO-1 and occludin localization with actin is maintained in intestinal epithelial cells of MLCK knockout or PIK-treated wild type mice exposed to the combined insult. Overall, ethanol and burn-induced MLCK activation contributes to subsequent intestinal epithelial damage, bacterial translocation, and inflammation. Although knockout of MLCK did not reduce pMLC after the combined insult, other results suggest that it does have a role in intestinal barrier dysfunction and inflammation after binge ethanol and burn injury.
APPENDIX B

DETAILED METHODS DESCRIPTION
APPENDIX B

DETAILED METHODS

Burn Injury (+ethanol) Protocol:

Materials:

Hot plate
Metal pan with lid
Metal or plastic pan for control room temp water
diH₂O
Animal clippers with #40 surgical clip comb
Thermometer
1cc syringes with 27g needle
3cc syringes with 25g needle
0.9% normal saline (sterile)
Burn injury template (check for proper size according to weight of mice)
Paper towels
Ketamine/Xylazine anesthesia (see solutions)
20% ethanol (see solutions)
Timer

Procedure:

Fill the pans with diH₂O and turn the dial on the hotplate up to get the water heated while you administer alcohol, anesthetize the mice, etc. The water needs to be heated and maintained at 92-95°C. At this point, the water will have some small bubbles in it on the
bottom of the pan, but will not be actually boiling. It is important to use the thermometer and maintain this temperature for all of the burn-injured animals.

1) Weigh mice in grams

2) Inject mice (i.p.) with 150ul (or chosen dose volume) of either 0.9% sterile saline vehicle or 20% v/v ethanol solution (preparation indicated in Solutions), using a 1cc syringe with a 27g needle. Allow 2-3 minutes between cages so there is time to shave and burn the mice at their scheduled time.

3) Thirty (30) min. after saline/ethanol injection, anesthetize the mice by injecting the appropriate volume of Ketamine/Xylazine i.p. (preparation indicated in Solutions, refer to “Ketamine Xylazine” protocol for proper dosing of animals)

4) As soon as they are asleep (approx. 5 min. after anesthesia injection), thoroughly shave the backs of the mice (Oster animal clippers with #40 comb).

5) One at a time, place animals in appropriate burning template to give a 14-16% total body surface area scald injury according to their weight (see chart in procedure room), and proceed with the burn injury (or sham – dunking mice in room temp water). Make sure the shaved part of the back is “sealed” on the burning template by gently pressing the mouse down into the template.

6) Hold the animal+template in the water for 7 seconds. Quickly blot the mouse’s back on paper towels. Removing as much hot water as possible from the animal prevents further scalding, and helps keep their body temp. up after burn. Put the mouse back in a clean cage. As soon as possible after the injury, resuscitate each
mouse with 1.0 ml of 0.9% sterile saline (i.p.), pre-warmed in 37°C water bath, or on warming pads.

7) Keep cages on heating pads for the next 3-4 hrs. to help the mice recover from procedure. Mice can be returned to the mouse room once they are walking and fairly alert.

8) Place some food pellets on the bottom of each cage; the mice can’t stretch their necks as easily to reach for food after burn injury. Replace water bottles.

Solutions:
Ketamine/Xylazine: Diluted with 0.9% saline, 100 mg/kg ketamine, 10 mg/kg xylazine, 70.0 uL/mouse i.p. (see Ketamine/Xylazine protocol for how to make)

Ethanol:

- 20% solution is the final concentration, made from the 95% stock ethanol (marked for use in mice only). Dilute with 0.9% sterile saline.
- Inject i.p., 150 uL for “low-dose” which will give a final BAC between 150-180 g/dL.

Notes:
We perform our burn injury procedures between 7-10 am

Anesthesia: Initially, this protocol used Nembutal for anesthesia (through 2008).

However, it is no longer available and JR modified the protocol in 2009 to switch to a ketamine/xylazine mixture, as previously used in the Faunce lab, but giving less than a burn only experiment or other procedure (70.0 uL instead of 100.0 uL for the average 20-25g mouse) due to the combined effects of the anesthesia and ethanol. JR ran several
pilot experiments to work out the optimal dosage. Animals wake up faster and more alert from ketamine/xylazine than they did previously with Nembutal (i.e. wake up within an hour instead of several hours). The different anesthesia protocol does not appear to affect parameters measured this far compared to historical data with Nembutal.
Intestinal epithelial cell isolation protocol

(Keep samples on ice at all times, centrifuge runs done at 4C)

- Open intestine lengthwise and wash with 4C CMF-HBSS (calcium and magnesium free), scrape away excess luminal contents/fecal matter
- Transfer to 1.5 mL epi tube containing 10 mM DTT in CMF-HBSS [plus phosphatase inhibitor cocktail (PIC) tablet] and put on ice (samples should be in DTT solution for a minimum of 30 minutes either on ice or at 4C but can be in it longer if needed
- Shake tube briefly and transfer tissue to 1.5 mL epi tube containing 1 mM EDTA in CMF-HBSS (plus PIC tablet) and leave at 4C or on ice for 1 hour.
- After incubation, shake tube vigorously, remove and discard any large pieces of tissue
- Run isolated cells (what’s left in the tube after removing large pieces of tissue) through a 25g syringe several times to break up large chunks of cells. Vortexing immediately prior to running through syringe helps prevent tissue clogging syringe needle.
- Centrifuge samples at 1000g for 10 minutes
- Discard supernate, add 100 uL cell lysis buffer to pelleted cells
- Vortex samples for 30 seconds and then put on ice for 4 minutes
- Centrifuge samples at 16000g for 15 minutes
- Remove supernate (cell lysates) and place into another tube and aliquot as needed

If desire to count cells before lysis, resuspend pellet in 100 uL cold PBS and count cells. Centrifuge again and remove supernate and then add cell lysis buffer to lyse cells.
Immunohistochemistry for Frozen Ileum

Translated from Christian Gomez’s notes – 8-1-08, Jerry Turner (U Chicago) (AZ)

Materials

- Coplin jars
- PAP pen
- Rectangular Tupperware container
- Paper towels
- 1X PBS (1 L)
- Normal Goat Serum (serum should be of same species as secondary Ab)
- Primary Ab’s of choice
- Secondary Ab’s (Fluorochrome or other of choice)
- Mounting media
- coverslips

Protocol

1. Defrost frozen slides for 20 minutes
2. Mark with PAP pen
3. Fix 30’ in 1% paraformaldehyde, 1mM CaCl₂, PBS
4. Wash 3 x 5’ in PBS
5. Block 1 hr. in 5% Normal Goat Serum, Triton (0.3%), in PBS
6. Tap off NGS, add primary Ab [diluted in Triton (0.3%) in PBS], leave ON at 4C
7. Wash 3 x 5’ in PBS
8. Incubate with secondary Ab (Alexa Fluor Ab’s are in 4C deli fridge, red box) [diluted in Triton (0.3%) in PBS)] for 1 hr.

9. Wash 3 x 5’ in PBS (keep slides in dark during washes)

10. Incubate with Phalloidin [1:100 for 30 min. (-20C) and Hoechst 1:20,000 (-20C) for 2 min] if desired, wash 3 x 5 minutes between phalloidin and Hoechst

11. Wash with tap water

12. Air dry and coverslip
   - Never let slides dry except when defrosting and before coverslipping
   - Write down Lot, date opened of all Ab’s
   - Keep slides in dark place while doing PBS washes once add secondary Ab
Generic Western Blot Protocol (JK)

Solutions to make (recipes at end of protocol):

1L 5x Running Buffer (stock) 10% SDS (in dH2O)
1L 5x Transfer buffer (stock) 5M NaCl
TEN, TBST, or PBST solution 0.5M EDTA
3% milk in 0.05% Tween/PBS 1M Tris (pH 7.5)
1L 1x Running buffer 1L 1x Transfer buffer

Other solutions needed:

β-mercaptoethanol or DTT
Sample Buffer (4x sample buffer Fisher Cat. #CB31010)
Methanol

Supplies needed:

Electropheresis and transfer apparatus
Gels (usually use BioRad Tris-HCl 4-20%, Cat # 456-1093)
Filter Paper (BioRad Cat. #1703932)
Foam/thin sponges
Membrane (PVDF membrane: Millipore Cat #IPVH07850)
Razor blade
Gel loading tips (Fisher Cat# 02-707-138)
1.5mL conical Eppendorf tubes (for samples, ladders)
Western blot ladders (unstained: Biorad Cat # 161-0363, stained: Biorad Cat # 161-0324)
Electrophoresis

- Protein assay on samples to determine amount of sample to use that equals 15 ug protein. (Use sample Western Blot 15 ug Excel file in protocols. You can load higher amounts of protein if you choose.)

- Turn on heating block (Rm 4228) and set temperature for 100F. (allow ~30 minutes for heating to set temperature)

- Make 1x running buffer (from 5x) and prepare samples. Remove protein ladders from freezer (-20) and place in ice bucket to defrost. Add ddH2O, 2x (dilute 4X in dH2O) sample buffer, and β-mercaptoethanol to tubes according to amounts calculated in Western blot sample calculator. Keep samples on ice at all times! Add sample last and right before boiling!

- Ladders prepared at following amounts – 10uL ddH2O, 25 uL Sample buffer, 5uL β-mercaptoethanol, 10uL ladder.

- Once plate heater warms to temperature, place samples in heater and boil for 3-5 minutes. Remove samples from heater and place back on ice immediately.

- Gels are stored in 4C fridge. To open, remove green tape along bottom. Remove comb to expose wells. Load gel(s) ensuring that green gasket ridge matches up with the gel. If running only one gel, use buffer dam on other side. Lock gels in place and load into electrophoresis dish – creates 2 outer and 1 inner reservoir.

- Add 1x running buffer to inner reservoir first and check for leaks. If none apparent, continue to add buffer to inner reservoir, allowing it to run over into outer reservoirs. Fill
until wells are covered entirely by buffer, but do not go over top of gel/plastic.

(Combining reservoirs will abolish the potential difference and nothing will happen)

- Load wells with 20uL (depending on gel can load up to 30 uL) of sample using gel-loading tips. Be careful to not dispense any air bubbles into wells.

- Put cap on dish – black to black, red to red. Run gel at 103V for ~1 hour. Check gel to make sure it is running. Sample buffer (blue line) should be almost to bottom before stopping electrophoresis.

Transferring to Membrane

- Mark top of membrane with “Top” and initials, date, expt. # on bottom with pencil. DO NOT TOUCH MEMBRANE!

- Pour methanol into one small plastic dish. Prepare 1L 1x Transfer buffer (recipe at end) and pour some (not all) into small plastic dish. (one dish per transfer done, and one for removal of gel). Place black portion of transfer holder into dish w/ buffer. Place sponge against pegs of holder, and place filter paper on top of sponge. Remove air bubbles (roll test tube or pipette piece over filter paper).

- To remove gel, crack open case (in dish with transfer buffer) with razor blade or spatula, gel will stick to one of the sides of the case. Keep gel in transfer buffer and cut off wells using razor blade. Slide gel onto filter paper; straighten and remove air bubbles. Remove membrane from methanol dish and rinse in ddH2O and then in transfer buffer. Using forceps, lay membrane (written side to gel) onto gel. Move membrane around so as to cover entire gel and remove air bubbles. Place another piece of filter paper over
membrane and remove air bubbles; place another sponge over filter paper and remove air bubbles. Closer transfer holder and keep in transfer buffer until ready to load into transfer apparatus.

- Wash electrophoresis equipment within minutes of finishing to prevent salt build-up which can affect equipment! (Wash with tap water, rinse with dH20)

- Place transfer dish into rectangular ice container and surround dish with ice. Place a stir bar at bottom of dish and put transfer holder (red and black) into dish. Add ice container and your “transfer sandwich” (black part of transfer holder to black side of transfer apparatus) to dish. Fill dish with transfer buffer (can use buffer used to make transfer sandwich). Buffer should cover entire “sandwich”, but not overflow dish.

- Place ice container on stir plate and put lid on dish - red to red, black to black.

1 membrane – 200 mA for 1 hr.

2 membranes – 350 mA for 1 hr.

Blocking and Primary Ab

- Carefully remove membrane from “transfer sandwich” and put into plastic dish containing TEN solution.

- Wash transfer equipment within minutes of finishing to prevent build-up of salt affecting equipment!

- Pour off TEN solution and add ~10-12 mL of 3% milk in 0.05% Tween/PBS. Place on shaker at RT for 30 minutes - 1 hour.
- Add primary antibody to 10-12 mL milk solution. Pour off milk solution and add primary antibody. Cover with parafilm and place on shaker at 4C overnight.
- (If primary antibody is phospho-specific dilute antibody in 5% BSA in TBST solution)

- Primary Ab’s usually used at 1:1000-1:5000 dilution, but check your individual antibody product sheet.

**Washing and Secondary Antibody**

- Pour off primary antibody into conical tube (Ab dilutions can be saved for ~1 week) and add 10-12 mL of TEN solution onto membrane. Place on shaker for 10 minutes. Repeat 2x for total of 3 washes.
- Dilute secondary Ab in milk solution. (Secondary Ab usually used at 1:2000-1:10,000 dilution. Secondary Ab’s conjugated to HRP or other detection agent. Unstained ladder requires StreptActin addition to secondary Ab dilution. (1uL StreptActin/5mL milk solution)
- Pour off TEN solution; add secondary Ab. Place on shaker at RT for 1 hour. Wash 3x in TEN solution. (Secondary Ab dilutions can also be saved!)

**Detection (HRP)**

- Place piece of saran wrap on counter. In 15 mL conical tube mix 1mL brown bottle, 1mL clear bottle (Supersignal West Dura extended Duration Substrate- upstairs in common room) per membrane. Place membrane (remove excess moisture by wiping
membrane against dish edges) on saran wrap. Pour 2 mL detection reagent on
membrane. Using forceps pick up edges of membrane to ensure detection reagent gets to
all portions of membrane. Leave detection reagent on membrane for 2 minutes. Pick up
membrane and blot edges on paper towels to remove excess buffer. Blot excess reagent
from saran wrap as well. Place membrane back on saran wrap and cover with saran
wrap. Keep saran wrap as flat as possible to prevent detection reagent sitting on one part
of gel. Fold edges of saran wrap to seal.

- Use chapel to detect bands. (Chemi-Hi light)

- After detection in chapel, remove membrane from saran wrap and put back into plastic
dish containing TEN solution. Wash 3x for 10 minutes each.

- If wish to blot membrane with another primary antibody, add 10-12 mL of Stripping
buffer to membrane and place in 37C incubator for 10-30 minutes.

-Wash 3x for 10 minutes each in TEN solution. After washes repeat procedure from
blocking step.

Recipes:

<table>
<thead>
<tr>
<th>5x Running Buffer (1L)</th>
<th>5x Transfer Buffer (1L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50mL 10% SDS</td>
<td>25 mL 10% SDS</td>
</tr>
<tr>
<td>72g glycine</td>
<td>72g glycine</td>
</tr>
<tr>
<td>15.15g Trizma Base</td>
<td>15.15g Trizma Base</td>
</tr>
<tr>
<td>ddH20 to 1L</td>
<td>ddH20 to 1L</td>
</tr>
<tr>
<td>10% SDS (1L)</td>
<td>5M NaCl (250mL)</td>
</tr>
<tr>
<td>100g SDS</td>
<td>73.05g NaCl</td>
</tr>
<tr>
<td>Solution</td>
<td>Volume and Composition</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>----------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>1L ddH20</td>
<td>250 mL ddH20</td>
</tr>
<tr>
<td>1x Transfer Buffer (1L)</td>
<td>3% milk/0.05% Tween-PBS</td>
</tr>
<tr>
<td>600mL ddH20</td>
<td>3 g dry milk</td>
</tr>
<tr>
<td>200mL 5x Transfer buffer</td>
<td>100 mL 0.05% Tween-PBS</td>
</tr>
<tr>
<td>200mL Methanol</td>
<td></td>
</tr>
<tr>
<td>Stripping Buffer (100mL)</td>
<td>TEN solution</td>
</tr>
<tr>
<td>20 mL 10% SDS</td>
<td>1M Tris pH 7.5 (25ml)</td>
</tr>
<tr>
<td>6.25 mL 1M Tris-HCl (pH 6.8)</td>
<td>0.5M EDTA pH 8.0 (2ml)</td>
</tr>
<tr>
<td>72.95 mL ddH2O</td>
<td>5M NaCl (28ml or 56ml of 2.5M NaCl)</td>
</tr>
<tr>
<td>0.8 mL β-mercaptoethanol</td>
<td>ddH2O to 1L</td>
</tr>
<tr>
<td>Other solutions possibly needed:</td>
<td></td>
</tr>
<tr>
<td>10x TBS (1 L)</td>
<td>TBS-tween (TBST, 1L)</td>
</tr>
<tr>
<td>24.2 g Trizma base</td>
<td>100 mL 10x TBS</td>
</tr>
<tr>
<td>80 g NaCL</td>
<td>900 mL ddH2O</td>
</tr>
<tr>
<td>pH to 7.6 (~20-30 ml HCL)</td>
<td>1 mL Tween 20</td>
</tr>
<tr>
<td>fill to 1L with ddH2O</td>
<td></td>
</tr>
</tbody>
</table>
Glass slide homogenization for Lymph Nodes

Supplies needed:

- 1.5 mL Epi tubes
- RPMI with 5% FBS
- Frosted glass slides
- Small petri dishes
- Tryptic soy agar plates (large petri dishes)
- Cell spreaders (sterile or autoclaved)

1. Collect lymph nodes in a 1.5 mL tube with 0.5 mL RPMI (without antibiotic and with 5% FBS), add RPMI steriley to tubes on day of sac, keep on ice.

2. Separate lymph nodes from connective tissue and transfer to a new 1.5 mL tube containing 0.5 mL RPMI. (Lymph nodes should sink in RPMI.)

3. Transfer nodes (cut tip from a 1 mL pipette tip to allow transfer of nodes and RPMI) to the frosted side of one glass slide. Homogenize by crushing lymph nodes between the frosted sides of 2 glass slides over a small petri dish to collect the homogenate. Wash slides with 200 uL of RPMI and transfer collected homogenate from small petri dish to 1.5 mL epi tube.


5. Leave plates overnight in 37C incubator.

6. Count colonies at 24 hour.
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VITA

Anita Marie Zahs was born on April 4, 1983 to Donald and Nannette Zahs in Washington, Iowa. Anita received her secondary education at Highland High School in Riverside, IA. In August 2001, Anita entered Simpson College, where she majored in Biochemistry. After graduation, Anita spent 2 years working as a chemistry technician at Red Star Yeast, LLC in Cedar Rapids, Iowa.

In July 2005, Anita began her graduate education at Loyola University Chicago in the Molecular and Cellular Biochemistry program. During her time as a graduate student at Loyola, Anita served as the biochemistry program representative on the Graduate Student Council and also served on the Graduate Student Community Service Organization. In May 2006, Anita entered the laboratory of Dr. Elizabeth Kovacs studying the effect of ethanol on intestinal epithelial barrier function following burn injury.

Anita held a predoctoral position on the Alcohol Research Program training grant from 2007 to 2009. In 2010, Anita received research support through a predoctoral Ruth L. Kirchstein National Research Service Award from the National Institute of Health. Upon completion of her graduate studies, Anita will begin post-doctoral research in the laboratory of Dr. Gail Hecht at University of Illinois Chicago.