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Muc2 Mucin Alters Intestinal Epithelial Barrier Function

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Muc2 Mucin Alters Intestinal Epithelial Barrier Function

by

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ABSTRACT

Intestinal epithelial barrier function is critical to host protection against pathogens, and it is one of the first defence mechanisms affected in gastrointestinal disease. Barrier function is maintained by a three part system involving bacteria and host proteins in the intestinal lumen, the mucus layer and peptides embedded in it, and intestinal epithelial cells and tight junctions between them. Together these components act synergistically to maintain barrier function, and a change in any of these may result in increased permeability to pathogens and antigenic stimuli, and aberrant activation of immune cells. The intestinal mucus layer, at the centre of this system is critical to the proper functioning of all components. These studies illustrate that in the absence of an intact mucus layer, intestinal barrier function is compromised, disease susceptibility is increased and recovery following injury delayed. These studies also investigated whether or not susceptibility to dextran sulfate sodium induced colitis could be modulated with probiotics. In addition, the functional roles of an intact mucus layer in *Entamoeba histolytica*-induced acute inflammatory and permeability responses in the colon were examined. Probiotic therapy using VSL#3 was ineffective at limiting injury or accelerating colonic healing. The data presented here illustrate that *Muc2*$^{-/-}$ animals were highly susceptible to *E. histolytica* induced secretory responses through altered expression of various tight junction proteins. These findings unravel a novel role for MUC2 mucin in conferring both luminal and epithelial barrier functions critical in innate host defence against intestinal infection and wound healing.

PREFACE

This thesis consists of two book chapters and three manuscripts co-authored with my supervisor, Dr. Kris Chadee. Dr. Chadee provided financial support for the laboratory work, advice regarding experimental design and correction of the thesis and manuscripts.

SECTION II: LITERATURE REVIEW

CHAPTER 1: This chapter contains excerpts and figures from a book chapter, The Effect of *E. histolytica* on Muc2 Mucin and Intestinal Permeability, in revision for publication in *Amebiasis: Biology and Pathogenesis of Entamoeba*, 2013. As the first author, my contribution was to design the book chapter, research and write the chapter, create tables and figures, and make revisions. Elizabeth Trusevych is a co-author in this book chapter, and wrote the section on tight junctions with the accompanying figure, and created the figure on Muc2 structure.

CHAPTER 3: Includes excerpts from a book chapter *Entamoeba histolytica* Cathepsin-like Enzymes: Interaction with the Host Gut published in *Cysteine Proteases of Pathogenic Organisms*, 2011. My contribution was to provide a framework for the book chapter, research and write the chapter, create tables and figures, and make revisions. Leanne Mortimer is a co-author in this book chapter, and assisted with the editing and revisions.

SECTION IV: RESULTS

CHAPTER 4: This is a manuscript which is being prepared for submission. My contribution to this work involved initial design and conducting of experiments, acquisition and analysis of data and preparation and revision of the manuscript. Elizabeth Trusevych as second author in this work conducted the initial studies characterizing the *Muc2*$^{-/-}$ phenotype in the absence of disease, and Figures 4-1, 4-3 and 4-5 are from her studies. She also contributed to the data shown in Figures 4-4, 4-7 and 4-9. In the work involving dextran sulphate sodium, she assisted with experimental design, sample acquisition and revision of the manuscript. France Moreau also assisted with sample acquisition and provided technical advice in these studies.

CHAPTER 5: This is a manuscript which is being prepared for submission. In these studies, experimental design and conducting of experiments, sample acquisition, data analysis and preparation and revision of the manuscript were my responsibility. Elizabeth Trusevych assisted with experimental design, conducting of preliminary experiments, and contributed to Figures 5-1, and 5-4 to 5-6. France Moreau assisted with sample acquisition and provided technical advice in these studies.

CHAPTER 6: This is a complete manuscript published in the *American Journal of Pathology* (*Am J Pathol*. 182(3): 852-65, 2013). My contribution to these studies included experimental design, sample collection and analysis, preparation and revision of the manuscript. France Moreau, a co-author in this study maintained the *E. histolytica* collection in the lab, assisted with animal surgery and provided technical advice.

Elizabeth Trusevych assisted with the intestinal permeability studies involving fluorescein isothiocyanate (FITC) dextran.

This is a manuscript-based thesis, written as per the guidelines of the Faculty of Graduate Studies. The introduction provides a brief overview of the major studies presented in this thesis. The conclusion brings together the key findings of these studies, establishes the relationship between these and discusses further implications and future directions. All chapters are followed by a complete list of references for the content therein.

ACKNOWLEDGEMENTS

I am extremely grateful for the assistance of my supervisor Dr. K. Chadee. Thank you for accepting me into your laboratory, teaching me and providing continued advice and insight.

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You have all inspired me to strive for my personal best,

and for this I thank you!

DEDICATION

I would like to dedicate this to my parents and grandparents:

This degree would not have been possible without the constant encouragement and support of my parents Drs. Sandy & Jean Kissoon-Singh.

I could never repay you for all you've done!

To my grandparents, Mr. & Mrs. Ramdass-Singh and Mr. & Mrs. Kissoon-Singh,

I love you all, and you will be missed.

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LIST OF SYMBOLS, ABBREVIATIONS AND NOMENCLATURE

AJ	adherens junction
AJC	apical junctional complex
CD	Crohn's disease
CLDN	claudin
DAI	disease activity index
DSS	dextran sulphate sodium
ECM	extracellular matrix
EGF	epidermal growth factor
Eh	*Entamoeba histolytica*
*Eh*CP5	*Entamoeba histolytica* cysteine proteinase 5
FGF	fibroblast growth factor
FITC-dextran	fluorescein isothiocyanate-dextran
Gal	galactose
GalNAc	*N*-acetyl-*D*-galactosamine
GI	gastrointestinal
IBD	inflammatory bowel disease
IEC	intestinal epithelial cell
IFN	interferon
IL	interleukin
JAM	junctional adhesion molecule

KC	keratinocyte chemoattractant
LIX	lipopolysaccharide induced CXC chemokine
MCP	monocyte chemotactic protein
MIP	macrophage inflammatory protein
NLR	NOD-like receptor
OCLN	occludin
RETNL-β	resistin-like molecule beta
RT	room temperature
TER	transepithelial electrical resistance
TFF-3	intestinal trefoil peptide
TGF	transforming growth factor
TJ	tight junction
TLR	toll-like receptor
TNF	tumour necrosis factor
UC	ulcerative colitis
ZO-1	Zona occludens 1

SECTION I: INTRODUCTION

The gastrointestinal (GI) tract is an essential component of a person's overall health. Therefore, it is not surprising that many researchers continue to study and analyze the causes of disease and the defence mechanisms of the gastrointestinal system [1-4]. One primary reason is because thousands of preventable deaths occur annually, worldwide and many of these could be attributed to gastrointestinal infections. Indeed, in developing countries, millions of dollars are spent each year on treating preventable diseases of the GI tract[5], and diarrhea is one of the leading causes of childhood morbidity and mortality [6, 7]. In the developed world, other diseases of the GI are prevalent and in many of these, such as inflammatory bowel disease (IBD) the etiology is unclear.

This multifaceted study seeks to gain a clearer understanding of the defence mechanisms of the gastrointestinal system, in particular the role of the mucus layer. This thesis summarizes the findings of these studies in three major research articles. These articles have been peer-evaluated at laboratory research presentations, international gastrointestinal research conferences and also presented in research journals and textbook formats. In this thesis, the innate host defences of the GI tract, in particular intestinal epithelial barrier function will be examined with particular focus on the mucus layer. This study also examines factors that compromise and those that strengthen this barrier, with a view to altering disease onset, progression or recovery in infected individuals.

A single layer of columnar epithelial cells lines the GI tract and this has traditionally been viewed as the barrier that prevents translocation of bacteria and other luminal contents into sterile interstitial spaces. This view of epithelial barrier function however is only partially complete. In fact, barrier function is maintained by a multi-layered system of which intestinal epithelial cells (IEC) are but one small component. Luminal contents including the commensal microflora forms the first line of innate host defence against antigenic stimulation while the intestinal mucus layer and proteins embedded in it, the second. Finally the IEC and tight junctions (TJ) between them as well as the mucosal cells below provide the final line of protection. Therefore, for pathogenic organisms or other antigenic stimuli to gain access to the interstitial spaces, they must find a way to circumvent each of these natural host defences. In the colon in particular, intestinal epithelial barrier function is essential for overall health, and disease prevention since the host is exposed to a plethora of antigenic stimuli ranging from food antigens to commensal/pathogenic bacteria and bacterial products.

The colonic mucus layer lies at the centre of this barrier, and it is critical for proper functioning of all components of it. Commensal bacteria colonize the mucus layer and this prevents them from being washed through the colon. At the cellular level, proteins secreted by enteroendocrine cells must also be contained in the lumen, and this is accomplished through entanglement in the mucus layer. In addition, the mucus layer also serves other protective functions discussed in subsequent chapters. Therefore it is critical to intestinal epithelial barrier function, throughout the GI tract but particularly in the colon.

Although it is an essential component of normal GI function, little is known about the role of the mucus layer itself, or the role of MUC2 in innate host defence. This study examines the role of the mucus layer in maintaining epithelial barrier function as well as its role in the genesis, progression, and repair of chemically induced colitis using the dextran sulfate sodium (DSS) model of injury. In addition, this study evaluates whether or not probiotics which have been shown to enhance epithelial barrier function both *in vitro* and *in vivo*, are able to exert their protective effects in the absence of an intact mucin barrier. Finally, the role of the mucus layer in innate host defence against pathogenic organisms, in particular *E. histolytica* is examined.

In these studies, animals with and without an intact mucus layer were examined to tease apart the role of Muc2 in maintaining epithelial barrier function. Here we show that in the absence of an intact mucus layer, intestinal morphology is altered and colonic permeability is increased. Furthermore, these animals were more sensitive to chemically induced colitis, and damage was evident throughout the full length of the colon. Curiously, although these animals did improve in the recovery phase of disease, they did not fully recover to their pre-disease levels, suggesting that the mucus layer may also be involved in recovery following injury.

In as much as the mucus layer is important for maintaining epithelial barrier function, it was of interest to determine whether augmenting this host defence could alter the genesis, progression or recovery from colitis. Probiotic mixtures especially VSL#3 have shown efficacy in clinical trials, and one of the mechanisms by which this may be achieved is by

fortifying the integrity or function of the mucus layer. However, little is known about the mechanism of action of this probiotics mixture or whether an intact mucus layer is required for VSL#3 to exert its protective effects. In healthy controls, VSL#3 treatment alone did not alter intestinal permeability though increased mucin secretion was observed. Furthermore, VSL#3 did not alter the genesis, progression or recovery associated with DSS colitis either in the presence or absence of a mucus layer. High, medium or low dosages of VSL#3 did not alter disease progression in severe colitis either in WT mice or those deficient in Muc2, and VSL#3 did not alter disease progression in mild disease in either of these animal groups.

Since the mucus layer is essential for normal function in the colon, and loss of this layer resulted in increased disease susceptibility and impaired recovery *in vivo*, it was of interest to determine whether or not this layer could also provide protection against pathogenic organisms. Intestinal amebiasis causes significant morbidity and mortality through diarrheal disease in the developing world but many infections are asymptomatic. Since the parasite colonizes the colon by binding to the mucus layer, it is believed that this may hold the key to explain why this is the case. Using an *in vivo* colonic loop model, the interaction of the parasite with the host epithelium both in the presence and absence of a mucus layer was investigated. The results of this study revealed that in the absence of an intact mucus layer, *Muc2*$^{-/-}$ mice were more susceptible to *E. histolytica* challenge resulting in a robust pro-inflammatory response with altered TJ permeability. Furthermore, while the parasite did not bind to or destroy the epithelium directly, it

caused increased permeability to serum proteins, clearly demonstrating that in pathogenic infections, the mucus layer is also important for maintaining epithelial barrier function.

Taken together the results presented here clearly indicate that the mucus layer is an essential component in maintaining epithelial barrier function *in vivo*. In healthy animals, this layer is critical for maintaining barrier integrity. In disease conditions however, the mucus layer shields the epithelium thereby protecting the host from the full impact of the injurious agent, while at the same time facilitating recovery. In the disease models investigated here, augmenting epithelial barrier function through the addition of probiotic bacteria did not alter the outcome of disease either in the presence or absence of an intact mucus layer. Furthermore, in models of pathogenic injury, the mucus layer augmented barrier function to prevent increased permeability that could lead to further damage.

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SECTION II: LITERATURE REVIEW

This section includes excerpts from

The Effect of *E. histolytica* on Muc2 Mucin and Intestinal Permeability

Vanessa Kissoon-Singh, Elizabeth Trusevych, Kris Chadee

Book chapter in revision for publication in
Amebiasis: Biology and Pathogenesis of Entamoeba, 2013

CHAPTER 1: INTESTINAL EPITHELIAL BARRIER FUNCTION

The gastrointestinal tract (GIT) is lined by a single layer of columnar epithelial cells which act as a barrier between the intestinal lumen and host connective tissue[1]. This barrier is multi-layered and consists of host and microbial products found in the intestinal lumen, the mucus layer and peptides embedded in it, and intestinal epithelial cells (IEC) and tight junctions (TJ) between them[2, 3] (Fig. 1-1).

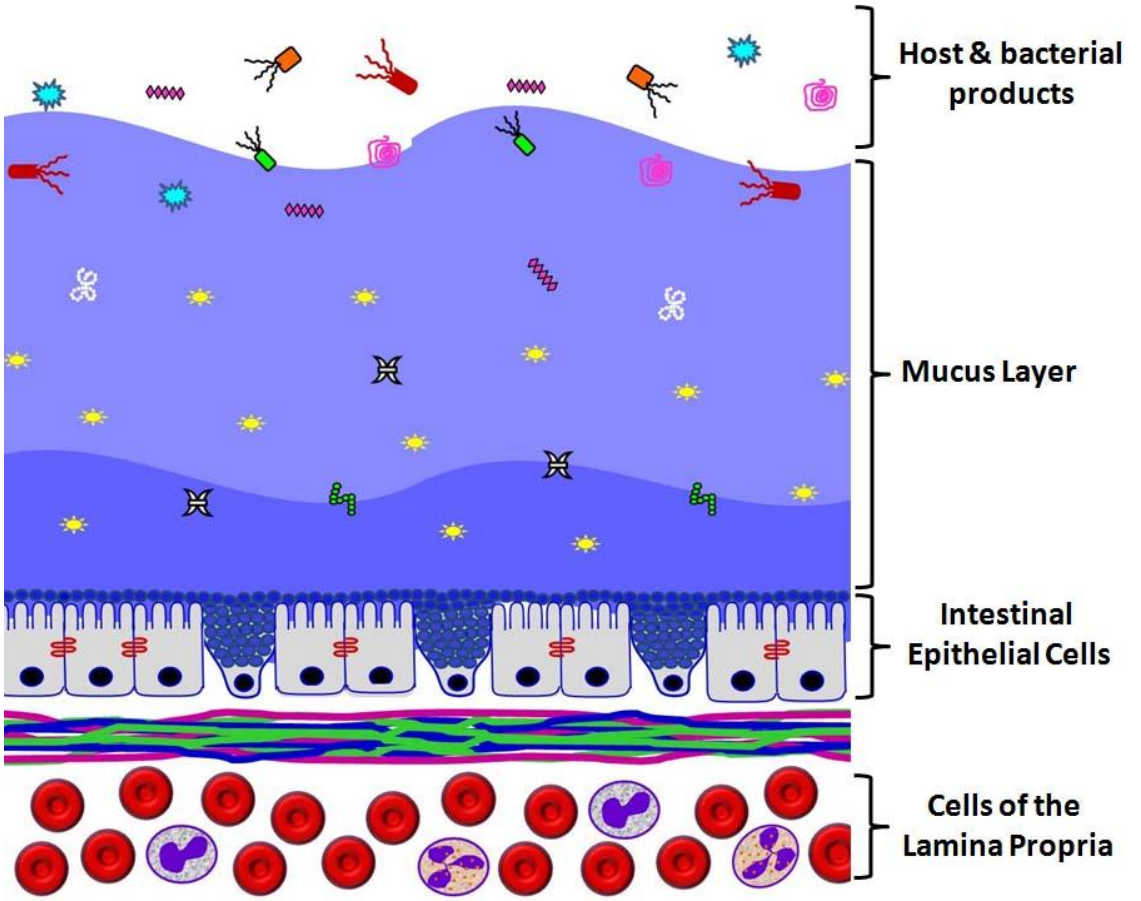

Figure 1-1: The intestinal epithelial barrier. This barrier is composed of three layers: the commensal bacteria and host and bacterial proteins present on the luminal surface, the mucus layer with antimicrobial peptides, secretory IgA, trefoil peptides and other secretions of IEC embedded in it and the intestinal epithelial cells. Below the IEC are cells of the lamina propria including red blood cells, macrophages, neutrophils and other cell types that sample luminal antigens and respond to injury. Interspersed among the IEC are the goblet cells which secrete MUC2.

The main function of this intestinal barrier is to protect the host from bacterial products and antigenic stimulation by luminal contents which could induce an excessive/aberrant inflammatory response. Disturbance in any component of the intestinal barrier could facilitate disruption in others leading to unrestrained movement from the lumen into the interstitial spaces and this may occur even before the development of overt disease[2]. Indeed, many GI infections can be traced to changes at any or all levels of barrier function[2] and dysregulation of the intestinal immune response can lead to a variety of inflammatory conditions including food allergies, IBD and intestinal cancers[4].

Strategically located at the centre of the epithelial barrier, the colonic mucus layer primarily composed of MUC2 mucin (Muc2 in mice) is critical to proper functioning of all aspects of this multi-layered system. The mucus layer acts as a lubricant in the intestine, is an essential food source for indigenous commensal bacteria found in the intestinal lumen, provides a substrate to facilitate colonization and prevents binding of potential microbes to the underlying IEC. This study shows that the MUC2 mucus layer plays a central role in maintaining intestinal epithelial barrier integrity both *in vitro* and *in vivo*.

1.1 Luminal Components and Commensal Bacteria

A critical component of the intestinal epithelial barrier is the luminal bacteria which make up the bulk of the host microbiota. The intestine is home to an estimated 10^{14} microorganisms which live in a symbiotic relationship with the host, and they out-number the total cells of the body by a factor of 10[1, 5-7]. The mammalian host provides

microbes with a stable temperature, in a hospitable environment, rich in nutrients while the commensal bacteria acquired shortly after birth, aid in normal physiological function of the intestine[1, 6, 8]. Commensal bacteria facilitate the digestion, absorption and storage of nutrients, function in the development of the intestinal epithelium, protect against tissue injury and promote angiogenesis[2, 7, 9]. In addition, commensal bacteria occupy many of the niches in the intestine and compete with pathogens for the nutrients present there, while some species also secrete antimicrobial peptides to prevent colonization by pathogenic organisms[5]. In this way, the commensal microflora forms a physical barrier to maintain intestinal barrier function.

It stands to reason therefore, that changes in the acquisition or composition of commensal bacteria may have a number of deleterious effects on the host. Indeed, changes in the microbial composition may trigger metabolic and inflammatory disease states or the susceptibility and progression of immune mediated diseases[1, 8]. Digestion of starch by commensal bacteria leads to the production of short chain fatty acids like butyrate, which inhibit the expression of pro-inflammatory cytokines while at the same time inducing the expression of regulatory cytokines. Interestingly, selective immune pressures can cause over-growth of commensal species rendering them potentially pathogenic, and this may have negative consequences for the host[1]. In fact, the immune status of the host is intimately linked with the composition of the commensal communities and these bacteria can modulate immune signalling in mammalian IECs[1]. In addition, the microflora has been shown to inhibit the NF-κB mediated pathway activated by toll-like receptors (TLR) and NOD-like receptors (NLR) responding to pathogenic bacteria. Activation of NF-κB

results from the phosphorylation of IκB, targeting it for ubiquitylation and proteosomal degradation, and commensal bacteria inhibit this, thereby preventing the activation of NF-κB, and induction of pro-inflammatory responses[10]. Thus microbial communities are essential for the normal physiological functions of the intestine.

In addition, the host and bacterial products present in the intestinal lumen are critical for proper functioning of other layers of intestinal barrier function. Indeed, animals grown under germ-free conditions, that lack a commensal microflora have a very thin adherent mucus layer, but when they are treated with bacterial products this is restored to the levels observed in conventionally housed animals[11]. In addition these animals are more susceptible to chemically induced injury[12], and epithelial restitution and wound repair functions may be compromised in them[13, 14]. Therefore the commensal microbiota and bacterial products present in the intestinal lumen clearly have an effect on the thickness and integrity of the mucus layer, and the level of protection it affords.

1.2 The Intestinal Mucus Layer

The full length of the GI tract is lined by a viscous, gel-like mucus layer[15], which varies in composition and thickness along the length, and reaches maximal thickness in the large intestine, the site of greatest bacterial load[16]. This layer serves a number of protective functions *in vivo*, and acts as a physical and chemical barrier to shield the epithelium[17-19].

In the stomach and duodenum, the mucus layer protects against acidic secretions and prevents tissue dehydration, while lubricating the GI tract for the passage of food[15]. In

the colon, it acts as a substrate to facilitate colonization of the colon and is the main source from which many species derive nutrients[17, 20, 21]. In addition, the mucus layer forms a diffusion barrier across which drugs and toxins must pass in order to directly contact the underlying IEC[20, 22]. Therefore, in the harsh environment of the colon, in order for commensal or pathogenic organisms to survive, they must be able to colonize the mucus layer and find the food and nutrients necessary to survive there.

The mucus layer is dynamic and always changing. Host and bacterial proteases cleave colonic mucins thereby degrading mucus, while continued production and secretion work to maintain a constant thickness[23]. This ensures that pathogenic and commensal species are able colonize the mucus layer for a time, but are eventually sloughed off during peristalsis or defecation[16]. Indeed mucins bind to whole bacteria and bacterial toxins and trap these in the mucus layer such that they are unable to traverse through and bind to, or cause damage of, the epithelial cells below[22]. Therefore as long as the rate of secretion exceeds that of degradation, the thickness of the mucus layer is maintained, and offending organisms are held at bay[16].

Small molecules and ions are able to pass through the mucus layer with relative ease, while bulk fluid flow is limited, resulting in the formation of an unstirred portion closest to the epithelial surface[24]. Therefore the colonic mucus layer appears to be composed of two distinct parts: a dense, inner and a more viscous outer one[25, 26]. The inner part which consists of a series of alternating layers[15] is firmly adherent to the underlying IEC and impermeable to bacterial species[26], while the outer portion composed of alternating

laminated, and loose curl-like structures[15] is colonized by bacteria and luminal contents[25-27] (Fig. 1-1). Although little is known about the formation of these layers, both consist of the secreted mucin Muc2, and differences in the fucosylation state may explain some of the differences observed. Indeed, distinct glycoforms have been identified, and these remain unmixed which may result in the striated appearance observed[15]. Furthermore, embedded in this layer are antimicrobial compounds and peptides including secretory IgA which may cross-react with membrane antigens to prevent the binding of microorganisms and harmful agents[28]. Therefore colonization of the mucus layer is facilitated in commensal species, and this establishes competitive inhibition where these are favoured over pathogenic organisms[28].

1.3 The Colonic Mucin MUC2

The intestinal mucus layer contains water, electrolytes, ions, sloughed-off epithelial cells, immunoglobulins and secreted proteins including trefoil peptides, resistin-like molecules and defensins[16, 17, 23]. However, the main components of this layer are the secretory mucins, in particular MUC2, which contribute to the heterogeneity of this layer, and give it many of its unique properties[29].

In the colon, mucins are synthesized and secreted by intestinal goblet cells[16, 30], though other cells types including mucosal epithelial cells and submucosal glands are important for mucin production in other organs[30]. GI mucins are very large glycoproteins (500 to 25,000 kDa) and they are either membrane bound, forming the glycocalyx of the IEC or

they may be secreted, forming a protective gel layer[16, 30]. This study focuses on the role of secreted mucins in host defense.

Much of the molecular weight of secreted mucins is due to the presence of sugar side chains, which account for up to 90% of the dry weight of the mucin, and give it its bottle-brush appearance[16, 20, 31]. The MUC2 monomer is a 5100 amino-acid protein backbone, with O-linked and N-linked oligosaccharides[23, 31, 32]. The N- and the C- terminal regions of the mucin monomer are poorly glycosylated, but contain N-linked oligosaccharide side chains that consist mainly of mannose[23] (Fig. 1-2). In addition, these termini contain von Willebrand factor-like D domains which are rich in cysteine residues[16]. These facilitate the linking of monomers by disulphide bonds to form mucin polymers (Fig. 2B), that are critical for the gel like properties of the MUC2 mucus layer[30, 33].

The structure of the MUC2 mucin is crucial in its role as a protective barrier in the colon. Mucin glycoproteins consist of a peptide core, rich in serine, proline and threonine residues (PTS domains), which are linked through the peptide hydroxyl group to oligosaccharide side-chains via O-glycosidic bonds[18, 23, 30, 32]. The length of these side chains varies in different mucins but is typically 4-12 sugars long, and rich in galactose (Gal) and N-acetyl-*D*-galactosamine (GalNAc) residues, N-acetyl-glucosamine (GluNAc), N-acetyl-neuraminic acid and fucose[20, 29]. Together, these sugar residues provide the necessary anchor by which commensal and pathogenic organisms are able to colonize the colon through lectin binding[22]. The terminal end of the side chains consist

of sialic acid and sulfate residues[22, 23, 30], which give the MUC2 monomer a negative charge under physiological conditions[20].

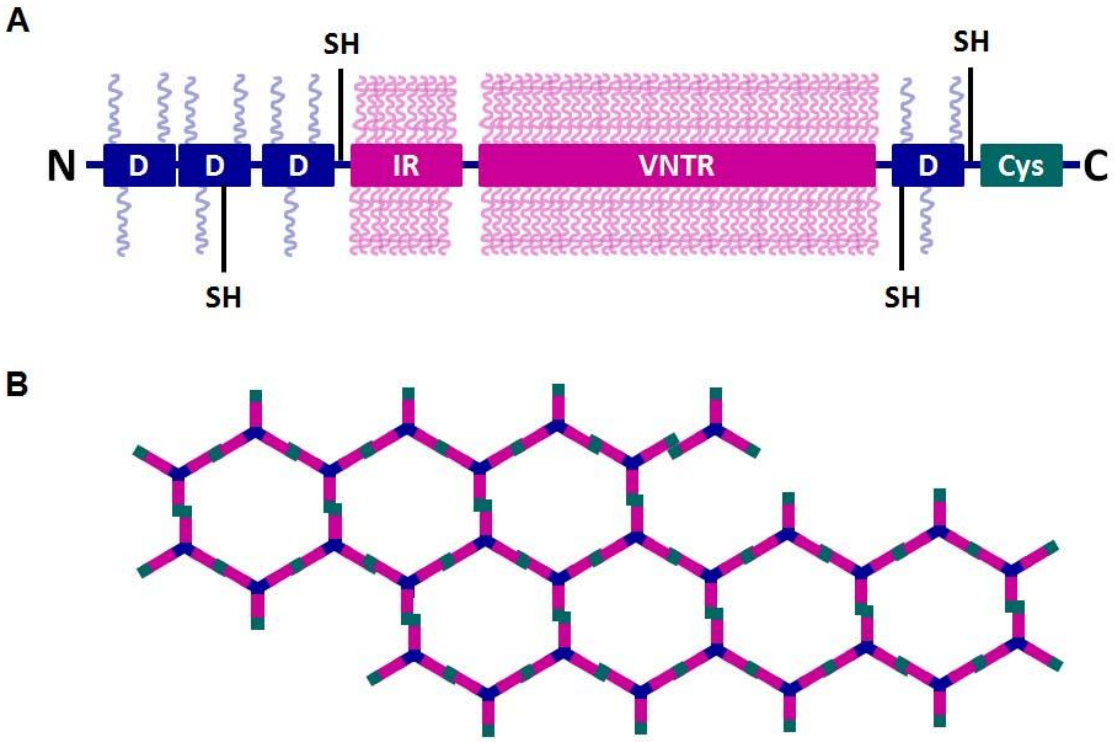

Figure 1-2: The Monomeric and Polymeric Structure of MUC2. A: The MUC2 monomer consists of a protein backbone with four von Willebrand D domains (D): three at the N-terminal end and one at the C-terminus. Much of the monomer is composed of serine, threonine and proline residues which from the irregular repeat (IR) and variable number tandem repeat region (VNTR) both of which are heavily glycosylated. In addition, there are multiple sites where the monomer can form disulphide bonds (SH) and at the C-terminal end, MUC2 contains a cysteine knot (Cys) structure. Panel B: MUC2 monomers join by "head to head" or "tail to tail" binding, to form polymers, which are rapidly hydrated in the intestinal lumen, to give rise to the mucus layer.

Many of the unique characteristics of colonic mucins are dependent on the O-glycosylation pattern[30]. In MUC2, glycosylation leads to a high charge density ratio due to the sialic acid and sulfate residues present, and this facilitates hydration and gives rise to the rigidity, viscosity and gel-like nature of the mucus layer[16]. In addition, the N- and C- termini, due to the lack of glycosylation, are susceptible to proteolytic degradation

while the core of the MUC2 monomer is not[16]. Therefore differences in the mucin monomer and it glycosylation pattern may have robust effects on the efficacy of this barrier *in vivo*[30], and in disease conditions where mucin structure is compromised, the integrity of the mucin barrier is lost[34].

Studies in Muc2$^{-/-}$ Mice

Early studies demonstrating a protective role for the colonic mucus layer were conducted *in vitro* using molecules designed to mimic mucin glycoproteins such as *D*-galactose[18], cancer cell lines including LS 174T cells which constitutively secrete mucins[22] or the addition of exogenous mucins[18]. While these studies provided valuable information regarding potential roles of mucins *in vivo*, it was not until the advent of mice deficient in Muc2 (*Muc2*$^{-/-}$), that it became possible to not only determine the specific role of Muc2 *in vivo*, but also to examine other mechanisms of innate host defences which were previously masked by the effects of the protective mucin barrier.

In 2002 a mouse model deficient in Muc2 was developed that showed a role for mucins in intestinal carcinogenesis[35]. These mice contained targeted inactivation of the Muc2 gene by replacing a genomic fragment with a phosphoglycerate kinase-neomycin (PKG-Neo) cassette in animals on a C57BL6/J x 129/SvOla background. *Muc2*$^{-/-}$ mice gained the same amount of weight as mice heterozygous for the Muc2 gene (*Muc2*$^{+/-}$) or WT mice up to 12 months of age, but developed gastrointestinal tumours leading to invasive adenocarcinomas and rectal tumors[35]. Furthermore, these authors noted increased cell proliferation but decreased apoptosis in these mice, as well as crypt elongation due to

increased cell numbers. In addition, altered cell maturation and migration was observed and IECs appeared to migrate faster toward the luminal surface in *Muc2*$^{-/-}$ mice[35]. Histologically, *Muc2*$^{-/-}$ mice lacked recognizable goblet cells in the colon[36] though these cells remained present as they could be detected by the presence of intestinal trefoil peptide (Tff3)[35, 36]. In addition, while no compensatory increase in the expression of other apomucins (Muc5ac, Muc3, Muc13) was observed in *Muc2*$^{-/-}$ mice, at 5 weeks transient *de-novo* Muc6 expression was observed in the distal colon of these animals, but this was lost at 16 weeks[36].

Since the advent of the *Muc2*$^{-/-}$ mice, these animals have been used to provide valuable insights into the role of secreted mucins in host-pathogen interactions[37, 38], gastric wound healing[39], and spontaneous and inducible models of colitis[36, 40]. Studies have clearly shown up-regulation of genes involving the immune response to antigen presentation and processing, pro-inflammatory cytokines, chemokines and antimicrobial peptides in *Muc2*$^{-/-}$ mice[40], suggesting that under baseline conditions, intestinal permeability and the immune response is altered in these animals which may manifest as basal low grade inflammation. Indeed, low-grade inflammation in the colon of *Muc2*$^{-/-}$ mice, may explain the predisposition to colitis[11] and colon cancer development[26]. In fact bacteria are intimately attached to epithelial cells in *Muc2*$^{-/-}$ mice, and they are able penetrate deep into the intestinal crypt and this occurs prior to the onset of disease[26, 41]. Furthermore, colitis was observed in *Muc2*$^{-/-}$ animals in the distal colon at 5 weeks of age and this developed to full-blown colitis and colorectal cancer (CRC), implying a critical role for Muc2 in intestinal protection and defence[35, 36]. In addition, during aging of *Muc2*$^{-/-}$ mice,

colitis is exacerbated and the risk of developing cancer is increased[40]. Furthermore, *Muc2*$^{-/-}$ mice are more susceptible to *Citrobacter rodentium* infections, and although WT mice are able to recover, *Muc2*$^{-/-}$ mice frequently succumb to their infections[37]. In addition, DSS induced an exacerbated response in *Muc2*$^{-/-}$ mice[36], suggesting that Muc2 is indeed crucial to maintaining epithelial barrier function *in vivo*. Indeed, in *Trichuris muris* infections, the number of PAS positive goblet cells was increased in WT mice resistant to infection and this correlated with worm expulsion[42]. In contrast, in *Muc2*$^{-/-}$ mice worm expulsion was delayed. Furthermore, energy status of the worms as determined by ATP levels was not diminished in *Muc2*$^{-/-}$ mice as compared to those from resistant animals suggesting that the mucin barrier may also impair parasite motility or survival in the host[42].

Taken together, these studies reveal that MUC2 is involved in maintaining intestinal homeostasis and the suppression of CRC[35]. Furthermore, in the absence of MUC2, increased cell proliferation and decreased apoptosis are observed, leading to increased cell migration[35]. In addition, aberrant inflammatory responses and increased susceptibility to infection are observed in *Muc2*$^{-/-}$ mice suggesting a role for this mucin in intestinal barrier function.

1.4 Intestinal Epithelial Cells and Tight Junctions

Intestinal Epithelial Cells

The IEC of the GI tract are crucial in maintaining intestinal homeostasis[1]. They are essential for nutrient absorption, electrolyte transport, production of mucus glycoproteins,

antimicrobial peptides and bacteriolytic enzymes[43-46]. In addition, they prevent luminal antigens, solutes and other microorganisms from making direct contact with the immune cells below, while at the same time allowing some non-pathogenic bacteria to communicate with the immune system to facilitate maturation, immune tolerance and intestinal homeostasis[43, 47, 48].

The mucosal surface of IECs is lined by a brush border composed of microvillar extensions, which prevent microbial attachment and invasion[49], and the glycocalyx, formed by the secreted and apically attached mucin layer prevents binding to the surface of these cells[1]. IECs themselves also secrete a host of antimicrobial peptides including defensins, cathelicidins, and calprotectins that further prevent microbial attachment[1, 50]. Therefore, in addition to providing a physical barrier IECs are a primary line of innate defense against pathogenic and commensal bacteria, and they can influence the function of antigen presenting cells and lymphocytes in the intestinal micro-environment[1].

Since IEC are surrounded by a phospholipid bilayer, they are readily permeable to lipid molecules or those dissolved in lipid solvents. Hydrophilic molecules however must be actively transported into or out of the intestinal lumen by trans- (through the cell) or paracellular (in-between cells) routes[51]. These routes are tightly regulated and allow the barrier to be extremely selective. In the colon, low molecular weight molecules (< 600 Da), as well as small ions and water are thought to move through the paracellular space while larger molecules require active transport through the IEC[52, 53], though this may not always be the case.

Tight Junctions

The paracellular space between adjacent cells is protected by the apical junctional complex (AJC) composed of the tight junction (TJ), adherens junction (AJ), and desmosome[24] (Fig. 1-3). The TJ is a dynamic protein complex responsible for determining the rate of paracellular passage, while AJ and desmosomes form strong adhesive bonds between cells which facilitates cell proximity, intercellular communication, contact with the extracellular matrix (ECM) and proper cell polarization[24]. Movement through the paracellular route is governed by the concentration gradient, epithelial surface area, intestinal transit time and the intrinsic leakiness of the TJ[51].

Intestinal TJ are the main determinants of epithelial, endothelial and paracellular barrier function, and permeability varies between different tissues and can be regulated in response to pharmacological, physiological, and pathophysiological stimuli[52]. TJ are composed of thin protein complexes that completely encircle the apex of the cell and make contact with TJ of adjacent cells[54], thus forming a continuous paracellular seal[52]. It is a multiprotein complex, composed of over 50 proteins, which connect the plasma membrane to the actomyosin cytoskeleton[51].

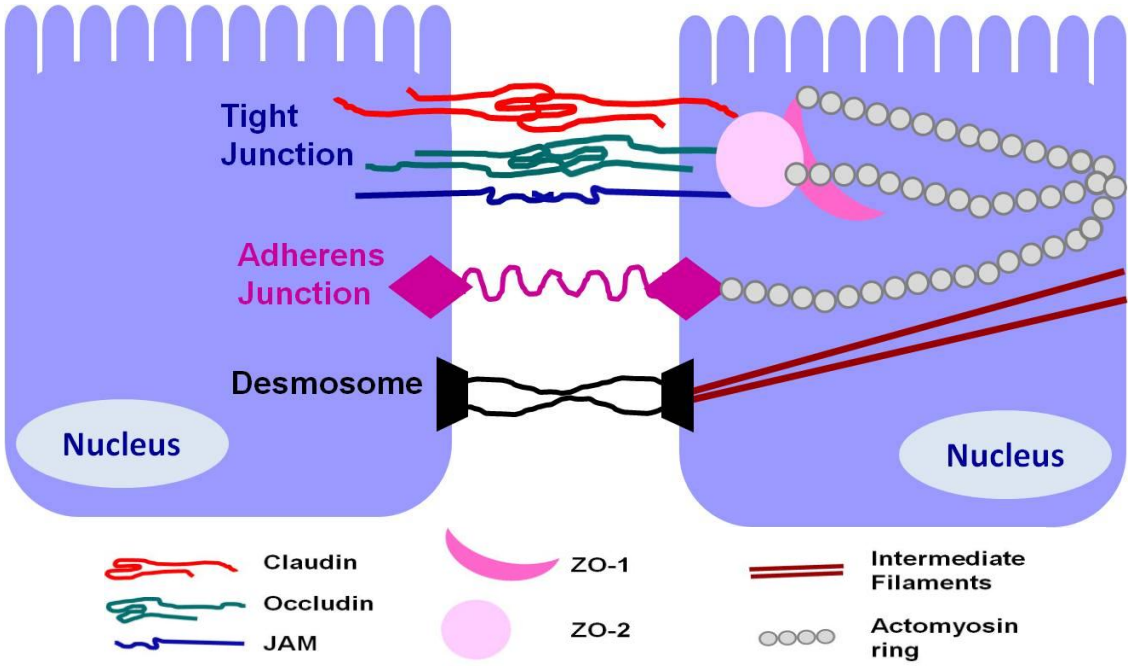

Figure 1-3: The tight junction. The tight junction of the apical junctional complex forms an effective barrier to regulate paracellular transport. The TJ consists of membrane bound proteins including members of the claudin family, occludin and junctional adhesion molecules (JAM), on adjacent cells which meet between cells to form a barrier. These proteins are connected to intracellular proteins notably the zona occludens (ZO-1 and ZO-2) which act as a conduit to join membrane bound proteins to the actomyosin ring.

While a complete review of these proteins is beyond the scope of this review, certain members bear discussion. The most abundant of the TJ proteins are the claudins, which regulate the movement of fluid and ions between cells[55]. To date over 24 different claudins have been identified, ranging in size from 20kDa – 27kDa[56], and they interact in a tissue specific manner to form charge- and size-selective barriers[24, 55]. The claudins can be subdivided based on their function into: those that increase epithelial resistance by reducing cation permeability (CLDN-1, 3-5, 7, 8, 14), others that create cation selective pores (CLDN-2, -10, -16) and some that increase or decrease epithelial resistance (CLDN-11, -15) depending on the presence of other claudins (reviewed in[57]). Occludin (OCLN), another transmembrane TJ protein interacts with the claudins present and the

actin cytoskeleton[24]. It is a 65kDa protein with 2 extracellular loops and N- and C-terminal cytoplasmic domains[58]. OCLN expression is significantly reduced in a number of permeability disorders including IBD and celiac disease[54]. In addition, studies with OCLN siRNA demonstrated that trans-epithelial flux of various probes was increased without a similar change in TER, and siRNA knockdown of Ocln in mice led to increased intestinal permeability to dextran[54]. This data clearly demonstrates that OCLN is important in maintaining epithelial barrier integrity by modulating the non-restrictive large channel pathway[54]. The zona occludens consisting of three peripheral membrane proteins (ZO-1, -2 and -3) is critical to the assembly and maintenance of the TJ[24]. They include multiple domains which interact with other proteins including the claudins and occludin and connect these to the cytoskeletal proteins most notably the actin microfilaments[24, 59]. Consequently these proteins are direct targets and effectors of the signalling pathways that alter TJ assembly, maintenance and barrier function[60]. Indeed ZO-1 acts a physical bridge between occludin and F-actin, and disruption of this leads to increases in paracellular permeability[61].

In cell lines, there appear to be two pathways that regulate movement from the apical to the basolateral surface[62]. A pore pathway which is permeable to molecules of approximately 4Å, and a second pathway which allows the movement of large-sized macromolecules and bacterial antigens[54]. A series of small pores exist in the spaces between IEC and their size and selectivity are defined by the claudins present [24, 52]. This 'pore' pathway is charge selective[63], and mediates transport of small molecules and ions[64], but prevents the movement of larger uncharged molecules[62]. In contrast, the low

capacity 'leak' pathway allows the movement of large ions, solutes and bacterial antigens and the limited flux of proteins and other macromolecules irrespective of the charge[52, 62]. This pathway appears to be under the control of ZO-1 and OCLN[62]. While an upper size limit has not been defined for this pathway, evidence has shown that whole bacteria are unable to pass through[24].

Therefore, under normal physiologic conditions, the 'pore' pathway is essential for the movement of small ions and uncharged molecules[52], but in disease conditions *in vivo* myosin light chain kinase (MLCK) activation leads to mucosal production of IL-13 and induction of CLDN-2, leading to ion-selective barrier dysfunction[65]. In healthy conditions, movement across the epithelial barrier allows the movement of small molecules and ions, but inhibits macromolecular flux[66]. In disease conditions however, movement of ZO-1 and OCLN away from the TJ leads to the opening of a non-restrictive pathway that allows paracellular flux of macromolecules which may induce an inflammatory response[52, 54]. The TJ is particularly sensitive to cytokines produced during inflammation[47, 67] and pro-inflammatory cytokines have been shown to induce redistribution/endocytosis of TJ proteins[52, 68] or disassembly of the AJC. IL-1β has been shown to increase intestinal TJ permeability[69-71], and TNF-α induces increased cell permeability leading to impairment of intestinal barrier function[72-74]. Furthermore, numerous reports have also shown that IFN-γ and TNF-α act synergistically to increase intestinal permeability by altering the expression and localization of various TJ proteins including CLDN-2, OCLN and JAM[75-78]. Indeed, flux through the leak pathway maybe increased by treatment with IFN-γ *in vitro* and TNF-α both *in vitro* and *in vivo*[66].

Furthermore, the exchange of OCLN and ZO-1 between the TJ and cytosolic pools has been linked to TNF-α induced changes[52, 68].

Clearly the intestinal epithelial barrier is a complex system involving the cooperation of many different parts. The luminal contents including the commensal bacteria present there forms the first line of defence while the mucus layer is the second and the IEC, the third. Although the mucus layer is often thought of as a distinct part of the barrier, it has effects on all levels and is therefore a crucial component of intestinal epithelial barrier function. In disease conditions, as discussed in Chapter 2, a disruption may occur at any level of this system that can have profound effects on barrier integrity, intestinal permeability and disease pathogenesis.

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This section contains excerpts from

Entamoeba histolytica cathepsin-like enzymes: Interactions with the host gut

Vanessa Kissoon-Singh, Leanne Mortimer, Kris Chadee

Book Chapter published in Cysteine Proteases of Pathogenic Organisms
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CHAPTER 2: FACTORS THAT COMPROMISE BARRIER INTEGRITY

A number of GI diseases are associated with changes in intestinal barrier function, including allergic responses and inflammatory conditions like IBD. In many of these the composition of the intestinal microflora or the thickness of the mucus layer is compromised, but whether this is a cause or a result of the disease remains unclear. In this section, two models in which the integrity of the mucin barrier has been linked to disease pathogenesis will be discussed.

2.1 Intestinal Barrier Function in IBD

In GI infections, mucin structure is modified or the integrity of the mucus layer compromised, and this is linked to a variety of inflammatory conditions, including IBD. While the etiology of Crohn's Disease (CD) and Ulcerative Colitis (UC) are unknown, they appear to involve environmental factors and host components in genetically susceptible individuals[1, 2]. In particular, the intestinal microbiota, barrier function and innate and adaptive immunity are implicated in disease pathogenesis[1-3], and a compositional shift in the microbiota has been observed in patients with IBD[4]. CD and UC are chronic recurring inflammatory conditions of the intestinal mucosa, often characterized by loss of epithelial barrier function[5]. CD may affect any site along the length of the GI tract though the terminal ileum is most commonly affected, and inflammation is transmural, but the distribution of the disease tends to be patchy[1]. UC however predominantly involves the distal colon and rectum, and inflammation though extensive is relegated to superficial mucosal ulcers[1]. Importantly, UC frequently

involves depletion of goblet cell mucin and loss of the integrity of the mucus layer, while this is not as well documented in CD.

In quiescent UC the mucus layer appears continuous and is similar to that in healthy individuals while in active disease, it is discontinuous and severely disrupted[6]. Indeed, a 60-70% reduction in the thickness of this layer and in the numbers of goblet cells and secreted mucins was observed during active disease[6, 7]. Additionally the mucin oligosaccharide side chains were shorter with a disruption in the sulphate and sialic acid residues[7] resulting in altered polymerization and gel-forming capacity of the mucins present. Furthermore, a 4-fold increase in proteolytic activity may also contribute to, or further degrade this protective barrier[7]. In CD however, while a continuous mucus layer is maintained, a decrease is observed in the mucus glycoproteins present, concomitant with an increase in the inflammatory cell infiltrate and the non-mucin components[6], and mucins from inflamed colons have a decreased functional capacity to bind to bacteria [8].

One of the major clinical manifestations of IBD is chronic and relapsing diarrhea which occurs by a leak-flux mechanism, and may promote mucosal inflammation and/or uptake of luminal antigens[5, 9]. Indeed, an abnormally leaky barrier results in aberrant tissue exposure to luminal antigens and pathogens and may induce or contribute to disease pathogenesis[5]. This is supported by data demonstrating enhanced barrier dysfunction in first degree relatives of patients with CD, and also suggests that there may be a genetic component to the loss of barrier function in this family of diseases[10]. While the specific causes of this loss of function are unclear, both clinical IBD cases and experimental

models of inflammation have suggested that ZO-1 and OCLN expression are altered in this family of diseases[11].

A number of different models have been used to examine the salient features of IBD *in vivo*, and these can be broadly categorized into:

- antigenic or microbial induced,
- induced by other forms including chemical, immunological or physical means,
- genetically induced,
- adoptive transfer models, or
- spontaneously induced[12].

As such, the choice of the model is largely dependent on the aspect of IBD under investigation and the resources available. A good animal model is one in which there is chronic or relapsing inflammation in the GI tract with features that resemble aspects of human disease[12].

2.1.1 The DSS Model of Colitis

A number of animal models have been used to study IBD *in vivo*, and one of the most widely used is the chemically induced dextran sulphate sodium (DSS) model of colitis. This model bears similarities to UC in terms of the etiology, pathogenesis and therapeutic response [13, 14], and it is a good model to examine innate immune responses in the colon[15]. The DSS model of colitis has been used to induce acute or chronic colitis in a number of animal species including mice and rats[13], since the dose can be varied both in terms of the amount of DSS and the duration of exposure[14]. Typically acute colitis is induced with

concentrations ranging from 1% to 7%[16, 17] DSS in drinking water for 5 to 7 days in mice depending on the animal strain used[18, 19].

The disease progression of DSS is especially well documented in mice. Within the first few days of exposure shortening of the colon occurs and this precedes histological evidence of inflammation or damage[14]. These initial changes are associated with crypt loss but the surface mucosal epithelial cells remain morphologically intact, and increased mucosal permeability occurs before the appearance of inflammatory processes[20]. In fact, erosion of the mucosal surface appears to be delayed, and does not actually occur until the fifth day of DSS in some animals[20]. In addition, DSS primarily affects the distal colon and rectum[21], and induces bloody diarrhea, ulceration and histological damage[22, 23]. While the specific method by which DSS induces disease pathogenesis remains unclear, it appears to alter intestinal barrier function by directly or indirectly attacking all levels of the barrier. Thus DSS alters the microbial composition of the intestinal lumen, compromises the integrity of the mucus layer and destroys IEC and the TJ between them.

2.1.2 DSS Alters the Composition of the Intestinal Microflora

DSS may exert damage by permitting bacterial invasion and preventing macrophages from ingesting microbes in the area[19]. Indeed, dramatic shifts in the bacterial composition and consequently the function of the intestinal microbiota are observed in WT mice treated with DSS[24], and several bacterial families are increased including the Bacteroidaceae and Enterobacteriaceae, while other families are lost[24]. Furthermore, the

intestinal microbiota is thought to play a role in modifying the disease susceptibility as well as the responsiveness to DSS induced damage of IEC[25].

2.1.3 DSS Changes the Mucus Layer

The mucus layer and the goblet cells responsible for the secretion of Muc2 are compromised in DSS colitis. Indeed, DSS induced a loss of goblet cells leading to increased levels of the precursors of Muc2 synthesis and total levels of Muc2[26]. In addition, DSS modified mucin structure, resulting in decreased serine and threonine residues on the Muc2 mucin which could result in a reduction in the potential O-glycosylation sites, leading to and compromised integrity of the mucus layer[27].

Indeed, a reduction in the thickness of the firmly adherent mucus layer was observed in mice treated with 3% DSS for 5d, and this paralleled the change in disease activity index (DAI)[28]. Recent studies have shown that a decrease in Muc2 and Muc3 gene expression occurs at 3 to 6 days post DSS treatment in rats, and this was accompanied by depletion in the number of goblet cells and adherent mucins[29]. Furthermore the severity of DSS colitis, and goblet cell and adherent mucins, was restored if animals were treated with anti-Tnf-α neutralizing antibody, indicating that Tnf-α is in part responsible for DSS severity and mucin depletion[29]. In addition, depletion of mucin containing goblet cells was observed in the vicinity of DSS lesions[27], and an overall reduction in the mucin content in the distal colon that was proportional to the duration of DSS was observed[21]. Interestingly however, a significant increase was observed in the mucins of the proximal colon in these animals[21] suggestive of a compensatory function of these mucins.

In addition to mucins, other goblet cell secretions may be important in mediating the response to DSS. Indeed, it has been suggested that goblet cells contribute to mucosal protection following DSS through the production of trefoil peptides[30], and Tff3 is up-regulated during the induction of colitis in BALB/c mice[16]. In addition, this protective response may be mediated by both Muc2 and Tff3 since the levels of both of these were elevated in the proximal colon of rats during DSS colitis[31, 32]. Furthermore, more severe colitis is observed in animals deficient in certain components of the innate immune system such as the intestinal mucus layer[18, 28] or trefoil peptides[33] suggesting a role for these in modulating disease pathogenesis.

2.1.4 DSS Disrupts Tight Junctions

DSS is thought to exert direct toxicity on epithelial cells *in vivo*[15, 33, 34] leading to cell destruction and increased permeability of the intestinal mucosa[20], or it may act directly on the TJ. Indeed in IEC-18 and CMT-93 cell lines DSS was toxic to epithelial cells, and this was concentration and time dependent[34].

DSS colitis also induced changes in intestinal barrier function by acting on the TJ. DSS increased trans-mucosal permeability, and this was lost when the DSS was removed[35]. In addition, permeation of Evan's blue was detected as early as 3 to 7 days post DSS treatment in mice[20]; and increased mucosal permeability was observed even before the induction of inflammatory processes[20]. Furthermore 2.5% DSS for 7 days induced a robust inflammatory response and DAI, which was driven by Ifn-γ since mice that lacked this cytokine were protected from DSS induced colitis[36]. In rats the early onset of colitis

is dependent on Tnf-α and the change in this cytokine occurred even before mucin depletion and epithelial damage[29]. This toxicity to IECs and disruption of TJ *in vivo* would in turn facilitate the movement of bacterial antigens and other noxious substances across the intestinal barrier leading to an exacerbated immune response[36].

A common form of intestinal injury is chemically induced through the ingestion of toxins. The DSS model of colitis in mice provides an excellent opportunity to study the effect of chemical injury one all layers of epithelial barrier function. DSS alters the intestinal microflora, depletes colonic mucins, and acts directly and indirectly on IEC and TJ. In addition, since the lesions induced by DSS bear similarity to those in UC, this model may serve to give some insight into the human condition.

2.2 Non-invasive disease: E. histolytica

Another common form of intestinal injury is derived from pathogenic organisms. When a pathogenic organism encounters the mucus layer one of three possible scenarios may occur[37]. It may become trapped in the mucus layer and is removed when this is sloughed off, or remain confined to this layer but unable to penetrate through to cause epithelial damage. Only pathogenic organisms with virulence factors to facilitate destruction of and movement through the mucus layer, can bind to and destroy IEC below to invade the colon[37, 38].

The intestinal parasite *Entamoeba histolytica* (*Eh*) is one such organism. It is transmitted via the fecal-oral route, and is the causative agent of intestinal amebiasis which infects an

estimated 500 million people each year, and claims 100,000 lives[39, 40]. While 90% of those infected are asymptomatic carriers, the remaining 10% get invasive disease leading to diarrhea, amoebic dysentery, and in acute cases amoebic liver abscesses. Diarrhea is one of the hallmark symptoms of amebiasis, and 12-25% of infections are associated with invasive diarrheal disease. *Eh* disrupts all levels of the intestinal epithelial barrier to cause invasive disease[41].

2.2.1 The Intestinal Microflora Modifies Eh Virulence

In areas of poor sanitation, where most people infected with *Eh* also harbour other GI infections, the balance between commensal and pathogenic bacteria is altered, and the bacterial flora is thought to be an important determinant of *Eh* virulence[42, 43]. *In vitro*, *Eh* trophozoites phagocytosed bacteria and this enhanced the cytopathic effect of *Eh* by increasing the expression of key virulence factors including the Gal/GalNAc lectin and *Eh* cysteine protease (*Eh*CP) activity[44](Fig. 2-1 B). In addition, *Eh* adhesion to and damage of epithelial cells was increased with prior exposure to bacteria, and this was augmented if *Eh* had first phagocytosed bacteria[44]. While further studies are necessary to determine if these relationships hold true *in vivo*, there is clear evidence that bacterial composition in the intestinal lumen may have an impact *Eh* virulence.

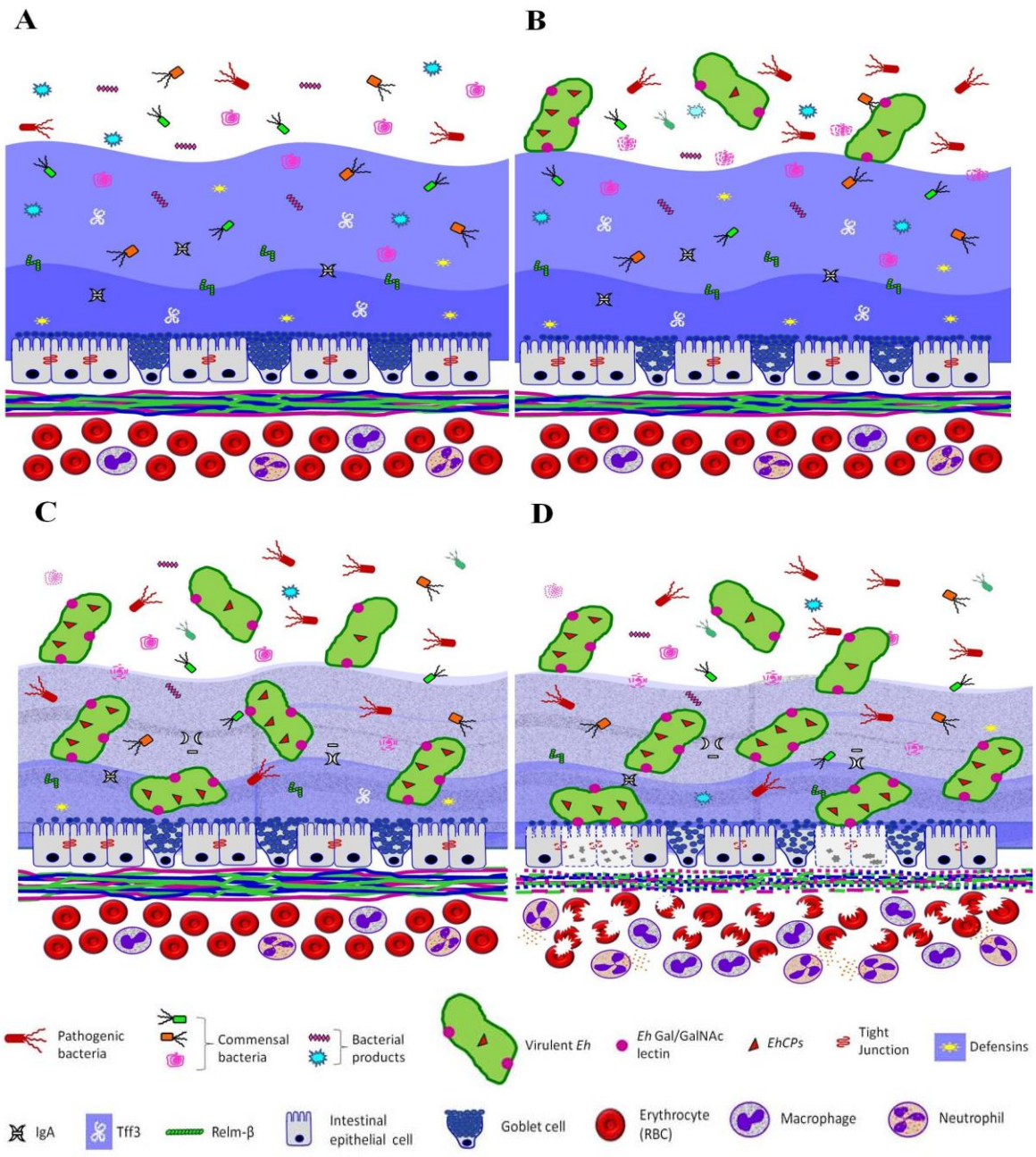

Figure 2-1: *Eh* overcomes the intestinal mucosal barrier to cause invasive disease. **A.** The intact intestinal barrier is formed by host proteins and commensal and pathogenic bacteria present in the intestinal lumen, a mucus layer with embedded host and bacterial proteins and the intestinal epithelial cells and the tight junctions between them. *Eh* compromises intestinal barrier integrity by disrupting every level of the barrier. **B.** *Eh* alters the luminal composition by changing the balance of commensal and pathogenic bacteria and parasite virulence is modified by phagocytosis of pathogenic bacterial species. **C.** *Eh* colonizes the mucus layer by binding via the Gal/GalNAc lectin, and *Eh*CP degrade colonic mucins. **D.** *Eh* binds to epithelial cells, disrupts the microvilli and the TJ between cells, destroys the extracellular matrix, erythrocytes and induces an inflammatory cell infiltrate.

2.2.2 Mucins are Protective in Intestinal Amebiasis

The interaction between *Eh* and colonic mucins has been extensively investigated in this laboratory and some of that research is presented here. In the absence of an intact mucin barrier, Chinese hamster ovary cells (CHO) were significantly disrupted by *Eh* trophozoites. In comparison, LS 174T cells which constitutively produce and secrete mucins, and were highly protected against *Eh*-induced damage[45]. Indeed *Eh* destroyed CHO cells in a contact dependent manner, and this could be inhibited by pre-incubating the trophozoites with *D*-galactose[46, 47] or purified rat or human colonic mucins[46], demonstrating a protective role for mucins *in vitro*. *In vivo*, the earliest response of the cecal mucosa to amoebic trophozoites in gerbils was the release of inter-glandular and goblet cell mucins[48] and in the colon of mice and rats, *Eh* induced a dose-dependent increase in mucin and non-mucin glycoprotein secretion[49, 50] demonstrating that *Eh* is a potent mucin secretagogue which depletes mature mucin stores to facilitate attachment to epithelial cells[51, 52]. Therefore, colonic mucins are the first line of mucosal defense against *Eh* and invasive disease only occurs when the parasite it able to overcome this barrier to bind to, and destroy epithelial cells in a contact-dependent manner[37, 45].

Eh colonizes the mucus layer by high affinity binding of the parasite Gal/GalNAc lectin to Gal and GalNAc residues of colonic mucins [45, 53, 54] (Fig. 2-1 B) and this prevents the parasite from making contact with the underlying colonic epithelia. The evidence for this is that enzymatic cleavage of rat mucin galactose and *N*-acetyl-galactosamine residues prevented this protective function as did inhibition of mucin O-linked glycosylation[45, 46], indicating that mucin sugar moieties were critical for protection. Indeed, the heavily

glycosylated regions of colonic mucins were essential for mediating protective activity, and only those mucin fragments which were rich in carbohydrates inhibited amoebic adherence to target cells[55].

In addition to the Gal/GalNAc lectin, *Eh* expresses other virulence factors that can compromise the integrity of the mucus layer, and the most important of these are *Eh*CP[56]. *Eh*CP cleave MUC2 monomers at the C-terminal end in two different places[57], which destroys the polymeric structure of mucin and compromises the integrity of the mucus layer[56, 57] (Fig. 2-1 C). This permits *Eh* to traverse the mucus layer and bind to and destroy epithelial cells in a contact-dependent manner. In addition, *Eh*CP cleave various components of the ECM leading to cell apoptosis[56], and this facilitates invasion into the lamina propria and submucosal tissue layers (Fig. 2-1 D). Curiously, the cleavage site for *Eh*CPs on human colonic MUC2 is very specific, and since murine Muc2 lacks this, it may explain why mice are not highly susceptible to invasive colonic disease[57].

2.2.3 Eh Modifies Tight Junctions

In addition to compromising the integrity of the mucus layer, amebae also disturb the organization of TJ proteins *in vitro* leading to increased paracellular permeability. Trophozoites placed in direct contact with T84 epithelial cells, increased paracellular permeability and [^{3}H] mannitol flux, reduced trans-epithelial resistance (TER), and this preceded lysis of the IECs[58]. *Eh* also induced loss of the scaffolding proteins ZO-1 and -2, with degradation of ZO-1 and dephosphorylation of ZO-2[58], clearly demonstrating that *Eh* modifies the TJ *in vitro*.

In both the chemically induced and pathogen induced models of injury discussed here, all components of the intestinal epithelial barrier are involved in disease pathogenesis either directly or indirectly. Since the epithelial barrier is involved in disease prevention and pathogenesis as discussed here, strengthening it may facilitate recovery following injury. While many therapies act on one or more components of this barrier, few act on all. Probiotics are one of the few, as discussed in the next chapter.

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CHAPTER 3: PROBIOTICS AND INTESTINAL BARRIER FUNCTION

The World Health Organization (WHO) defines probiotics as live organisms which provide a benefit to the host when administered in adequate quantities[1]. Probiotics are bacteria – a single species or a cocktail that are administered as spores or freeze-dried bacteria and may be ingested alone or with food. Once in the GI system, these bacteria travel through the digestive tract and spend most of the time in the intestine where they may colonize the mucus layer or remain suspended in the luminal contents. From here probiotics may act at the luminal, mucosal or submucosal levels, to maintain or improve epithelial barrier function[2]. Probiotics have been shown to modulate intestinal epithelial barrier function through a variety of mechanisms including preventing pathogenic contact with the cell surface, stabilization of the cytoskeleton and induction of mucin expression[3]. Furthermore since gram-positive and gram-negative bacteria appear to activate different mechanisms, it has been suggested that a probiotic cocktail may be more effective than a single strain or species[3].

Arguably, one of the most successful probiotic cocktails currently on the market is VSL#3. VSL#3 is composed of eight bacterial species all of which are normally found the in the commensal microflora, and these include: *Lactobacillus acidophilus, L. paracasei, L. plantarum, L. bulgaricus, Bifidobacterium longum, B. breve, B. infantis* and *Streptococcus thermophilus*. While the role of specific bacterial species in the VSL#3 cocktail is unclear, the rationale for using this cocktail is that high efficacy is expected through the synergistic action of the different bacterial strains present[4]. Indeed, the

Lactobacilli though they make a very small contribution to the bacteria present in the colon, are among the best studied species for their probiotic potential[5].

3.1 VSL#3 in IBD

VSL#3 is one of the few probiotics which has been shown to exert beneficial effects in human clinical trials, especially in IBD[6, 7]. Moreover, many of these species are reduced in disease conditions such as IBD, suggesting that they may mediate various protective functions and/or facilitate wound repair *in vivo*. Indeed, patients who were intolerant or allergic to 5-ASA (5-aminosalicylic acid) responded well to VSL#3 and daily treatment with this probiotic maintained remission in UC patients for 12 months. Curiously, the probiotic bacteria colonized the colon, but the total numbers of aerobic and anaerobic bacteria were not significantly altered, and no significant side-effects were reported[7]. However, VSL#3 therapy increased the fecal concentration of *S. thermophilus*, as well as various *Lactobacillus* and *Bifidobacterium* species[7]. VSL#3 was also found to maintain remission in 85% of UC patients with pouchitis, and again a number of the bacterial species composing VSL#3 were found in the feces suggesting colonization[6].

3.2 VSL#3 Modifies the Intestinal Microflora

It is widely believed that one of the key roles of probiotics is to restore the microbial imbalance following disease conditions. As observed in human IBD, probiotics may transiently colonize the colon and promote beneficial microbes and microbial products or they may be involved in maintaining host-microbe interactions and pathogen exclusion[8]. Indeed, some probiotics produce antibacterial factors including bacteriocins which inhibit

the growth and virulence of enteric pathogens[2] or lactic acid which reduces the pH in the local microenvironment making it harder for other organisms to thrive[2, 5]. In addition to antimicrobial peptides some species also promote defence by inducing the production of host antimicrobials including beta-defensin 2 *in vitro*[9].

Probiotics may act in a variety of ways to modify bacterial load *in vivo*. In clinical trials, VSL#3 induced remission in patients, and this effect may have been mediated by increased bacterial, but reduced fungal diversity[10]. Indeed, VSL#3 increased the total number of bacterial cells as well as the richness and diversity of the microbiota in these patients[10]. In mice, probiotics have been shown to survive in the colon and alter the metabolic activity of the cecal microflora in DSS treated animals[11], and they normalized the fecal microbiome which was altered following *C. rodentium* infection[12]. In addition, some probiotic strains may act on mucin secretion or the IEC and TJ to promote barrier function[14, 21].

3.3 VSL#3 Induces Mucin Secretion

Since the mucus layer is the first line of innate host defense against pathogens, it stands to reason that probiotics may induce goblet cell mucin secretion[13]. *In vivo*, the probiotic mixture VSL#3 increased the basal mucin content by up to 60%, and induced *Muc2* gene expression and secretion in rat colonic loops[14]. *In vitro* live VSL#3 bacterial species did not induce mucin secretion in cultured cells however bacterial products present in VSL#3 conditioned media did[14]. Moreover, of the bacterial species tested, *Lactobacillus* were the most effective at inducing mucin secretion[14].

Probiotics have also been shown to upregulate Muc2 expression and inhibit bacterial adherence. *Lactobacillus casei* GG significantly increased Muc2 expression in a dose-dependent manner and this prevented bacterial translocation across a Caco-2 monolayer[15]. In other studies *L. plantarum* 299v and *L. rhamnosus* GG inhibited enteropathogenic *E. coli* by inducing MUC2 gene expression in HT-29 cells[16]. *L. plantarum* 299v colonized the intestinal tract *in vivo* and induced epithelial cells to secrete Muc2 and Muc3 mucins which prevented the binding of pathogenic organisms like attaching and effacing bacteria (A/E) to IECs[16], and binding of *Campylobacter* species to the mucus layer was reduced when this was colonized by probiotic strains[17]. Curiously many probiotic bacteria do not behave like the normal commensal microflora. Bacteria in the intestinal lumen typically release proteases which cleave Muc2 mucin, and this is in part responsible for the formation of the outer less dense mucus layer. Interestingly, the probiotic bacteria: *L. brevis*, *L. plantarum*, *L. bulgaricus* and *B. lactis*, some of which are found in VSL#3 did not cleave the Muc2 core protein thereby maintaining barrier integrity, although all secreted active proteases[5].

3.4 VSL#3 Prevents Changes in Tight Junctions

In addition to inducing the expression and secretion of colonic mucins, probiotics may influence other aspects of the mucosa to protect the intestinal epithelium. At the level of the IECs, VSL#3 protected against apoptotic induced changes in intestinal permeability. Indeed, VSL#3 prevented epithelial apoptosis induced by acute colitis or infection with *Salmonella dublin*[3, 18]. VSL#3 also prevented the reduction in paracellular permeability observed in a number of disease models[3, 19-21], and much of this was mediated by changes

in the TJ proteins. VSL#3 and soluble products from these bacteria maintained TER, and prevented pathogen induced changes in TER[3].

VSL#3 also reduced the DAI in rats treated with 3.5%DSS for 7 days, from as early as 4 days post DSS and ameliorated the increased intestinal epithelial permeability associated with colitis[20]. In the same study, VSL#3 prevented the changes in TJ expression of Ocln, Zo-1 and Cldn-2 and their redistribution induced by DSS[20], and improved various parameters of disease in weanling animals, including IκBα, Il-1β and MPO[22]. VSL#3 conditioned media was also shown to decrease paracellular permeability in the ileum of SAMP mice by reducing Cldn-2 expression while at the same time inducing Ocln, and these changes appeared to be mediated by Tnf-α[19]. In the SAMP1/yit mouse model of IBD, VSL#3 improved intestinal barrier function and increased the production of Tnf-α and IκB-α mRNA[23]. In fact, *C. rodentium* infections induced transcription of *Tnf-α* and *Ifn-γ* and this was reduced with probiotic treatment[12]. Furthermore, VSL#3 treatments in *IL-10*$^{-/-}$ mice reduced the mucosal secretion of Tnf-α and Ifn-γ[18].

At the submucosal level, probiotics may also exert their beneficial effects in a number of different ways. Some probiotic species prevent the activation of the pro-inflammatory nuclear transcription factor NF-κB[24] while other species may activate NF-κB leading to an increase in the production of various pro-inflammatory cytokines including IL-6[25]. Furthermore, some probiotic species may alter the secretion of pro- and anti-inflammatory cytokines by immune cells[26] and modulate down-stream pro-inflammatory

signalling in IECs to maintain epithelial barrier function and regulate cytokine secretion [27].

3.5 Unresolved Issues with the Use of Probiotics

There is controversy in the literature regarding the mechanism of action of probiotic mixtures. Previous studies have shown that VSL#3 does not significantly alter the bacterial composition of the intestinal microflora or the relative numbers of aerobic and anaerobic bacteria following treatment[7]. However, subsequently VSL#3 was found to increase the relative numbers of anaerobic bacteria and reduce the number of fungal species in the intestine[10]. Furthermore, VSL#3 induced Muc2 gene expression and the thickness of the mucus layer under basal conditions[14], but did not induce Muc2 following DSS induction [11]. In another group of studies, probiotics reversed the signs of DSS induced inflammation in female BALB/c mice, but not in male animals[11, 21]. VSL#3 promoted epithelial barrier function by stimulating rather than suppressing innate immune responses, and this was driven by increased production of epithelial derived TNF-α and NF-κB activation[23]. In the *IL-10*$^{-/-}$ mouse model of colitis however, VSL#3 maintained barrier integrity by reducing Tnf-α and Ifn-γ levels[18]. Based on these studies, while it appears that VSL#3 may be beneficial under certain conditions, the mechanisms by which this is accomplished are unclear.

In addition, although this bacterial mixture has been shown to be beneficial in certain human infections, the paucity of appropriate controls in human studies involving VSL#3 is of great concern. A promising study conducted on a cohort of patients in Italy with

mild pouchitis reported that VSL#3 was effective in inducing and maintaining remission in 69% of patients when administered at high doses (3.6×10^{12} bacteria twice a day)[28]. This study however lacked either controls receiving no treatment (to determine the incidence of relapse) or those treated with broad-spectrum antibiotics (to facilitate comparison with the currently accepted method of therapy). To date, the lack of data obtained from randomized control trials with appropriate controls, makes it difficult to draw firm conclusions from these findings.

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SECTION III: HYPOTHESIS & SPECIFIC AIMS

The Muc2 mucus layer is a major component of the intestinal epithelial barrier, but its function remains unclear. Although valuable insight has been gained through the use of *in vitro* cells lines that lack or produce mucins, and *in vivo* with animals that have an intact mucus layer, much of the function of this layer remains unknown. Indeed, it was not until the advent of the *Muc2*$^{-/-}$ mouse that it became possible to investigate the role of the mucus layer along a continuum with animals that have (WT) or lack an intact mucin barrier (*Muc2*$^{-/-}$) or those in which this barrier though present, may be compromised (*Muc2*$^{+/-}$). *Muc2*$^{-/-}$ mice spontaneously develop adenomas and adenocarcinomas in the first year of life, and these animals are more susceptible to *C. rodentium* challenge and nematode infections, demonstrating the protective nature of mucins. However, little is known about the effect of Muc2 on intestinal epithelial barrier function, or how it mediates these protective effects *in vivo*.

In DSS, while a great deal is known about disease progression and pathogenesis *in vivo*, little is known about how this relates to the mucus layer. In the context of improved epithelial barrier function, studies using the probiotic cocktail VSL#3 have shown promising results, and it appears that VSL#3 may act at all levels of the barrier to improve function but it is unclear whether or not a mucin substrate is necessary for it to exert these protective effects. In studies with *Eh*, although much is known about the pathogenesis of disease through *in vitro* model systems, the lack of appropriate *in vivo*

models has significantly hampered further progress. This study aims to address these concerns by examining the role of Muc2 in epithelial barrier function in health and disease.

HYPOTHESIS

The Muc2 mucus layer plays a central role in intestinal epithelial barrier function in health, and in disease states.

SPECIFIC AIMS

1. To examine the role of an intact mucin barrier in disease susceptibility and wound repair following DSS induced colitis.

2. To investigate if probiotics which enhance intestinal epithelial barrier function can modulate disease genesis, progression, or recovery in the absence of an intact mucus layer.

3. To determine the role of this mucus layer in innate host defences against the parasite *E. histolytica*.

SECTION IV: RESULTS

CHAPTER 4: MANUSCRIPT I

Muc2$^{-/-}$ mice Exhibit Increased Intestinal Permeability and Susceptibility to DSS Colitis

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Manuscript submitted for publication

Abstract

In the intestine, the Muc2 mucus layer forms a protective barrier against pathogens and noxious agents. However, little is known about how Muc2 regulates intestinal permeability, disease pathogenesis or wound healing. In this study, the constitutive defects in *Muc2*$^{-/-}$ mice on colon histopathology, gut permeability and susceptibility to dextran sulphate sodium (DSS)-induced colitis and restitution were examined. In the absence of Muc2, the colonic muscle layer was thickened with marked crypt elongation and branching as compared to *Muc2*$^{+/-}$ and WT controls. *Muc2*$^{-/-}$ mice lacked acidic and neutral mucins and the microbial community was modified. Colonic transit time was significantly reduced (p<0.001) that was associated with increased intestinal permeability at 1 and 5 months of age (p<0.05). In the absence of Muc2, animals were hypersensitive to DSS-induced colitis even with dosages as low as 0.75% DSS, and this was associated with deep ulceration along the full length of the colon, loss of crypt architecture and thickening of the muscle layer. WT and *Muc2*$^{+/-}$ did not show similar pathology at these dosages. With low DSS dosages, *Muc2*$^{-/-}$ animals moderately recovered but robust expression of Ifn-γ and Il-8 homologs (KC, MCP-1 and LIX) at Day 15 (p<0.05) remained high. Curiously, no substantial increased was observed in any of the growth factors examined. Taken together, these data highlight an important role for Muc2 in intestinal epithelial barrier function and protection against injury and a novel role for Muc2 in repair and wound healing.

Introduction

The intestinal mucus layer acts as a barrier to protect the delicate epithelial cell surface from pathogens and noxious substances [1]. In the colon, the main component of this mucus layer is MUC2 mucin. MUC2 is a large glycoprotein produced and secreted by intestinal goblet cells, that is rapidly hydrated to form the thick gel-like mucus layer [2-5], which is colonized by a variety of commensal bacterial species[6]. This layer facilitates movement of particulate matter through the GI tract, lubricates cells, and acts as a diffusion barrier to prevent luminal stimuli from making direct contact with intestinal epithelial cells (IEC)[4, 7, 8]. Consequently the MUC2 mucus layer is intimately involved in the proper functioning of all layers of the epithelial barrier. Indeed, in the absence of this layer, animals spontaneously develop adenomas and adenocarcinomas within the first year of life[9], and they are highly susceptible to infections with the attaching and effacing bacteria *Citrobacter rodentium*[10], the murine whipworm *Trichuris muris*[11], and the intestinal parasite *Entamoeba histolytica*[12]. This suggests that intestinal barrier function that delays onset, reduces severity, or aids in recovery following infection is compromised in *Muc2*$^{-/-}$ animals. However, none of these have been directly examined to date. Furthermore, while some morphological differences have been observed between WT and *Muc2*$^{-/-}$ mice in the intestinal mucosa[13], little is known about how these translate to changes in epithelial barrier function in health or disease states.

The dextran sulfate sodium (DSS) model of colitis is versatile and can be used to induce mild, severe, or chronic disease, and is described to act on all layers of the epithelial barrier. This model has also been used to investigate disease onset (Day 0-3),

progression (Day 4-7) and recovery (Day 10-15) in a variety of rodent models[14, 15]. In this study, the DSS model of colitis was used to induce mild and severe colitis in WT and *Muc2*$^{-/-}$ mice to investigate the role of the mucus layer in disease. Our study shows that in the absence of Muc2 intestinal morphology was altered with crypt elongation and branching, and noticeable thickening of the muscle layers. In addition, *Muc2*$^{-/-}$ mice differed from their WT counterparts in their microbial composition as well as whole gut transit time and intestinal permeability. Notably, *Muc2*$^{-/-}$ animals were highly susceptible to DSS induced injury. In severe disease, the absence of Muc2 resulted in earlier disease onset and rapid progression and recovery was significantly impaired. This study highlights a role for the mucus layer in maintaining intestinal barrier function in healthy individuals. In addition, a novel role for Muc2 in delaying disease progression and facilitating recovery is described for the first time.

Materials & Methods

In these studies, male and female C57BL/6, *Muc2*$^{+/-}$ and *Muc2*$^{-/-}$ mice weighing approximately 20g were used. C57BL/6 mice were obtained from Charles River (Saint Constant, QC) while *Muc2*$^{+/-}$ and *Muc2*$^{-/-}$ animals were bred in house. All animals were housed under specific pathogen free conditions (SPF) in filter top cages and fed autoclaved food and water *ad libitum*. Throughout the study animals were closely monitored to ensure that they were treated humanely, and that all experiments adhered to the University of Calgary Animal Care Committee standards.

Intestinal Permeability Studies

Metabolic cages (Tecniplast, Philadelphia, PA) were used to assess temporal changes in intestinal permeability. Animals were given an acclimatization period of three weeks prior to commencing the study, during which they were gavaged with tap water or sugar probes and placed in metabolic cages overnight, once a week. During the study, animals received 0.2mL sugar solution containing sucrose (100 mg), mannitol (8 mg), lactulose (12 mg) and sucralose (6 mg) by oral gavage, and were placed in metabolic cages overnight. The following day, urine was collected in a 15mL falcon tube, and stool in eppendorf tubes (VWR International, Edmonton, AB) and samples were stored at -20^{o}C.

Fluorescein isothiocyanate (FITC) dextran was also used to examine intestinal permeability as previously described[16]. Briefly, mice were gavaged with 15mg FITC-dextran (3-5kDa, Sigma Aldrich), sacrificed by CO_2 asphyxiation 3h later, and blood collected by cardiac puncture. Whole blood was allowed to clot in the dark for 3h (RT) and centrifuged at 10,000 x *g* (10 min, RT). Serum was transferred to a clean eppendorf and diluted with an equal volume of phosphate buffered saline (PBS). 100µL of each sample was loaded onto a black bottom 96-well plate in duplicate, and fluorescence was determined with a plate reader (absorption 485nm, emission 535nm) and is expressed as relative fluorescence units.

Gut Transit Time

Whole gut transit time was determined as previously described[17]. Briefly, animals were gavaged with Evans blue, placed in clean bedding free cages, and the time to pass stool

that was blue in colour was recorded. To determine upper GI transit time, mice were gavaged with Evans blue and sacrificed by cervical dislocation after 15 min and the small intestine between the pyloric sphincter and the ileocecal junction removed. The distance which blue staining had travelled was measured and expressed as a percentage of the entire length of the small intestine.

Microbial profiling using quantitative PCR

The microbial composition was examined using qRT-PCR as previously described [18]. Briefly, the QIAamp DNA Stool Mini Kit (Qiagen, Mississauaga, ON) was used to extract total bacterial DNA from fecal samples, and validation of the stool extraction was performed using quantitative PCR. The concentration of DNA was determined using the Pico-Green DNA quantification kit (Invitrogen, Carlsbad, CA). Bacterial groups were quantified using real-time assays run in duplicate, with group-specific primers on the BioRad iCycler (BioRad Inc., Mississauga, ON). Standard curves for each primer were constructed using standard bacterial genomic DNA (American Type Culture Collection, Manassas, VA). Cell numbers of bacteria in fecal samples are expressed as mean pg bacterial DNA/ng total genomic DNA.

DSS-induced colitis

Colitis was induced using 1% - 4% dextran sulphate sodium (DSS; MP Biochemicals MW: 36,000-50,000) dissolved in tap water. Animals were treated with DSS for 5d after which DSS was removed and they were maintained on tap water for the duration of the experiment. Throughout this study, animals were weighed and examined daily to

determine the disease activity index (DAI) score, and where necessary, euthanized when the clinical endpoint was reached. The cumulative DAI score was obtained by scoring animals on the basis of weight loss, stool consistency, blood loss and appearance (Table 4-1). In studies to examine recovery and wound repair, animals were sacrificed on Day 7 and Day 15.

Histology & Staining Procedures

At the endpoint of the study, animals were sacrificed by cervical dislocation and the colon was excised. The colon was opened longitudinally along the mesenteric border and examined for signs of macroscopic lesions and mucosal thickening before it was rolled from the distal end to form a swiss roll. The swiss roll was sectioned into three parts with the middle portion being used for histological analysis while the outer portions were used for gene expression and protein isolation. For histology, colonic tissues were fixed in Carnoy's solution, and embedded in paraffin blocks. 7μm tissue sections were rehydrated through an ethanol gradient to water and stained with hematoxylin and eosin (H&E; EMD Chemicals Gibbstown, New Jersey) to examine overall tissue morphology or Periodic acid Schiff's reagent (PAS) and Alcian blue (AB; Diagnostic BioSystems Pleasanton, CA) to visualize neutral and acidic mucins respectively.

Table 4-1: Disease activity index in animals treated with DSS

	0	1	2	3
Weight loss	No loss	10-15%	15-20%	>25%
Stool consistency	Solid	Loose	Wet anal fur	Diarrhea
Blood loss	None	Little, dried on bum	Little, red on bum	Lots; fresh blood on bum & tail
Appearance	Lively/normal	Slower, not hunched	Hunched	Lethargic

Animals were given a disease activity index score daily, based on the parameters outlined here. A cumulative DAI score was calculated per animal per day, and mean scores ± standard error of the mean was plotted in DAI graphs.

Quantification of cytokine expression

Total RNA was isolated from snap-frozen tissue using the trizol reagent method (Invitrogen; Life Technologies, Burlington, ON) as per manufacturer's specifications, and the yield and purity determined by spectroscopic analysis. RNA was re-precipitated with lithium chloride precipitation solution (Ambion; Life Technologies, Burlington, ON) and random hexamer primers (Invitrogen) and Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen) were used to prepare cDNA from 1µg of RNA. qRT-PCR was performed using 1µg cDNA with 10µL SyBr Green master mix (Invitrogen) and 100µM primer concentration in a final volume of 20µL per reaction. A complete list of the primer sequences and conditions used are listed in Table 4.2. qRT-PCR was performed using a Rotor Gene 3000 (Corbett Research Kirkland, QC), and the results analyzed using the $2^{-\Delta\Delta CT}$ method[19].

Table 4-2: Primer Sequences used for Quantitative Real-Time PCR.

Name	Sequence 5` 3`	Annealing Temp.	Ref.
Il-1β	For: GCCTCGTGCTGTCGGACCCA Rev: CTGCAGGGTGGGTGTGCCGT	65^{o}	
Tnf-α	For: ATGAGCACAGAAAGCATGATC Rev: TACAGGCTTGTCACTCGAATT	56^{o}	[20]
Ifn-γ	For: TCAAGTGGCATAGATGTGGAAGAA Rev: TGGCTCTGCAGGATTTTCATG	54^{o}	[19]
Cox-2	For: ATCCTGCCAGCTCCACCG Rev: TGGTCAAATCCTGTGCTCATACAT	60^{o}	[21]
Tgf-β	For: TGACGTCACTGGAGTTGTACGG Rev: GGTTCATGTCATGGATGGTGC	60^{o}	
Egf	For: TTCTGGACGGACGTAGGGAT Rev: CGTGATTCCACTGGGTTCCA	65^{o}	
Fgf-1	For: ACCGAGAGGTTCAACCTGCC Rev: GCCATAGTGAGTCCGAGGACC	65^{o}	[22]
Vegf-A	For: GGAGATCCTTCGAGGAGCACTT Rev: GGCGATTTAGCAGCAGATATAAGAA	65^{o}	[23]
Ocln	For: AGAGGCTATGGGACAGGGCTCTTTGG Rev: CCAACAGGAAGCCTTTGGCTGCTCTTGG	60^{o}	[24]
Zo-1	For: GGAGCTACGCTTGCCACACT Rev: GGTCAATCAGGACAGAAACACAGT	60^{o}	[25]
Actin	For: CTACAATGAGCTGCGTGTG Rev: TGGGGTGTTGAAGGTCTC	54^{o}	

The primer sequences, and the annealing temperatures listed here were used in qRT-PCR studies in mice. Where applicable, references are also provided.

Protein Isolation and Estimation

Tissue samples were homogenized in phosphate buffered saline containing protease inhibitor cocktail (Sigma-Aldrich) and snap frozen. Thawed samples were centrifuged (10,000 x *g*, 5 min, 4^{o}C) and 70µL of the supernatant was passed through a pre-soaked 0.2µm filter (Millipore, Billerica, MA). Filtered samples were analyzed by Mouse 32-plex cytokine-chemokine panel (Eve Technologies, Calgary, AB), to examine the levels

of various Il-8 homologs (KC, MCP-1, MIP-2 and LIX), pro-inflammatory and immunomodulatory cytokines (Il-1α, Il-1β, Ifn-γ, Tnf-α).

Statistics

Data were analyzed using the student t-test and analysis of variance (ANOVA) with a Tukey or Kruskal Wallis post-test application where necessary, using the software GraphPad Prism version 4 (Graph-Pad Software, San Diego, CA). Data are reported as the mean ± standard error of the mean (SEM).

Results

The intestinal microflora is altered in Muc2$^{-/-}$ *mice*

In our hands, *Muc2*$^{-/-}$ mice on a C57BL/6 background do not spontaneously develop colitis or adenomas, but they are more susceptible to disease [1]. Since the mucus layer is necessary for bacterial colonization and nutrient acquisition, and it is effectively shaped by the intestinal microflora, it was of interest to determine if the composition of the fecal microflora was altered in *Muc2*$^{-/-}$ animals. Mice were fasted and placed in metabolic cages overnight and urine and stool samples collected. In WT mice, over 50% of the fecal microflora was composed of the *Firmicutes* phyla while *Bacteroides* comprised about 20% and various other bacteria constituted the remainder (Fig. 4-1). This was altered in *Muc2*$^{-/-}$ animals where *Bacteroides* and *Firmicutes* combined accounted < 50% of the total bacterial content. Indeed, most of the fecal microflora belonged to neither of these phyla. Notably, the *Bacteroides* and *Firmicutes* populations were reduced to one

half of that present in WT mice, clearly demonstrating that the intestinal microflora was altered in the absence of an intact mucus layer.

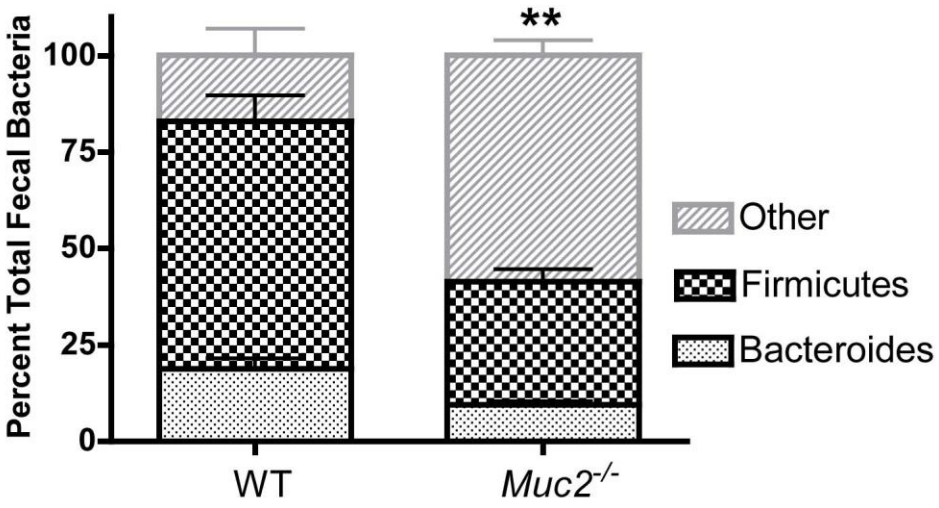

Figure 4-1: The fecal microbiota of WT and *Muc2*$^{-/-}$ mice. The composition of the fecal microflora was significantly altered in the absence of an intact mucus layer. In WT mice, this is largely composed of bacteria in the *Firmicutes* and *Bacteroides* phyla which account for over 75% of the microflora. In *Muc2*$^{-/-}$ animals however, these phyla accounted for less than 50% of the total bacterial content, and other species predominate. ** $p<0.01$.

Crypt architecture is altered in Muc2$^{-/-}$ *mice*

To determine if in the absence of Muc2 intestinal morphology was altered, the colon was excised, swiss rolled and fixed in Carnoy's solution. Paraffin embedded tissue sections were stained with PAS reagent to demonstrate secreted and goblet cell mucin. Colon morphology was altered in *Muc2*$^{-/-}$ mice as evidenced by longer crypts with increased crypt branching (Fig. 4-2). As expected, *Muc2*$^{-/-}$ mice lacked PAS positive goblet cells throughout the full thickness of the colon. Furthermore, the muscle layers were thicker in *Muc2*$^{-/-}$ suggestive of ongoing low grade inflammation as compared to *Muc2*$^{+/-}$ or WT animals. Histologically *Muc2*$^{+/-}$ mice were similar to their WT counterparts. PAS filled

goblet cells was most evident in the middle and proximal colon with constitutively secreted mucus forming a dense layer in the intestinal lumen. Furthermore, there was no evidence of muscle thickening or ongoing basal low grade inflammation in these animals (Fig 4-2).

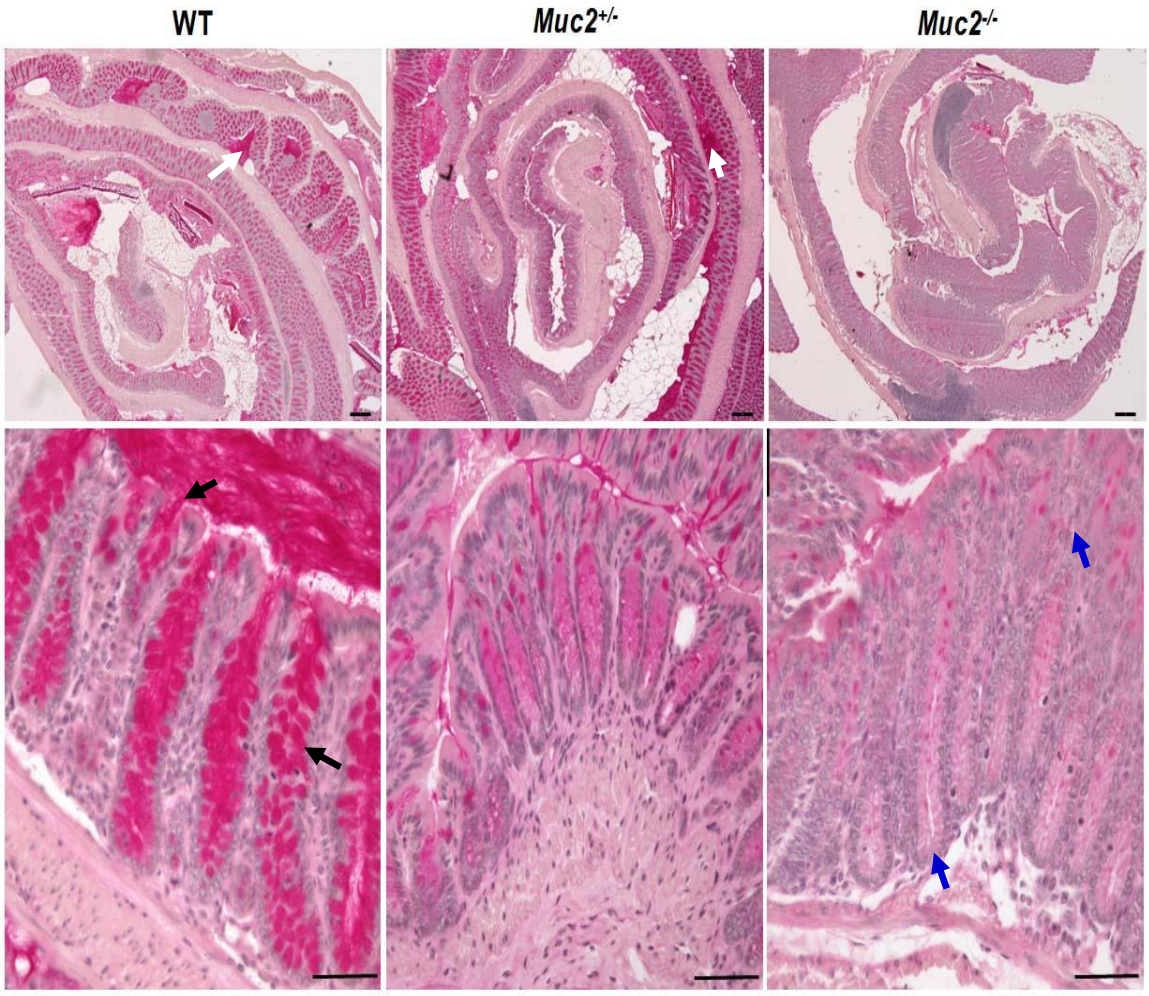

Figure 4-2: Intestinal morphology is altered in the absence of Muc2. Top panel: Swiss roll sections of WT, *Muc2*$^{+/-}$ and *Muc2*$^{-/-}$ stained with PAS reagent; the distal colon is at the center of the roll. Note, *Muc2*$^{-/-}$ mice are devoid of neutral mucins present throughout the colon as compared to WT and *Muc2*$^{+/-}$ animals. The white arrows point to mucins which are being secreted onto the epithelial surface forming a mucus plug. Scale bar 200µm. Bottom panels: Goblet cells full of mucins can be easily visualized as bright magenta (black arrow), and secreted mucins can be observed forming a protective layer over the epithelial surface of WT mice (black arrows). In *Muc2*$^{+/-}$ there was an intermediate magenta staining and the mucus plug was not as thick or organized as WT. In contrast, *Muc2*$^{-/-}$ lack the bright magenta staining goblet cells, and the crypts are elongated with branching (blue arrows). Scale bar 50µm.

Intestinal permeability is altered in Muc2*$^{-/-}$ *mice

To determine if the changes in colonic histopathology and bacterial composition in *Muc2*$^{-/-}$ animals affected other colonic functions, intestinal permeability was evaluated. One of the best ways to quantify this is to measure short circuit current and permeability in Ussing chambers. Unfortunately as the muscle layer was substantially thicker in *Muc2*$^{-/-}$ mice, our preliminary Ussing chamber studies (data not shown) were inconsistent. For this reason, whole gut transit time and permeability *in vivo* were assessed in WT, *Muc2*$^{+/-}$ and *Muc2*$^{-/-}$ mice (Fig. 4-3). Whole gut transit time was significantly decreased in *Muc2*$^{-/-}$ mice as compared to *Muc2*$^{+/-}$ or WT mice (Fig. 4-3A). These results suggest a possible role for luminal Muc2 in increased colonic secretion and/or gut motility. To determine whether this change was due to alterations in either the small or large intestine, upper GI transit time was examined. As shown in Fig. 4-3B, the presence or absence of an intact mucus layer did not alter upper GI transit time clearly illustrating that any differences in whole gut transit time were likely due to alterations in colon transit.

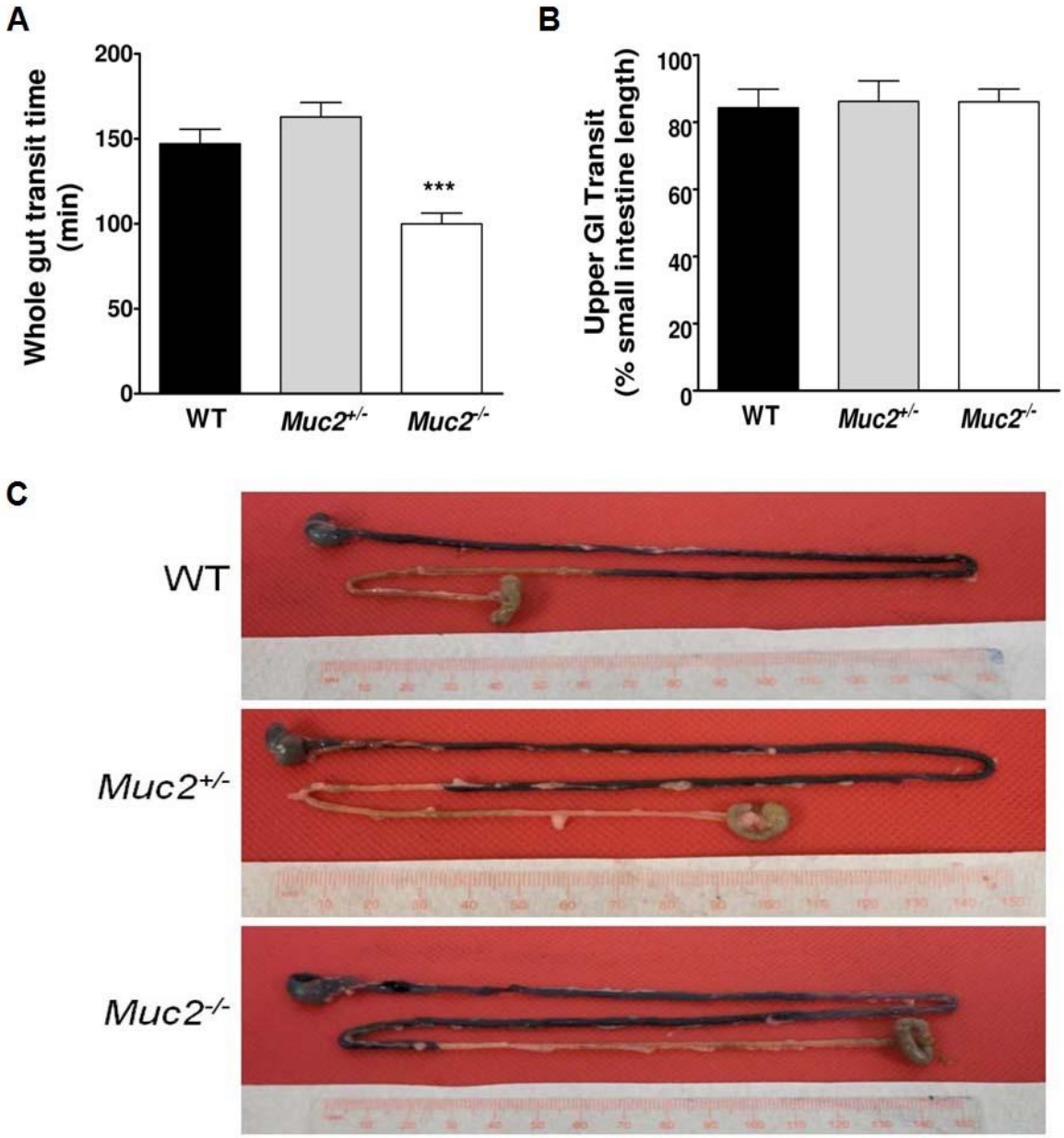

Figure 4-3: Whole gut transit time is decreased in *Muc2*$^{-/-}$ mice. As shown in Panel A, in the absence of an intact mucus layer (*Muc2*$^{-/-}$), whole gut transit time was significantly decreased as compared to WT or *Muc2*$^{+/-}$ mice. Panel B: This change in transit however was not due to alterations in the upper GI tract as upper GI transit time remained the same in all animals. Panel C: Animals were gavaged with Evans blue and sacrificed after 15 min, and the small intestine excised. Upper GI transit time was assessed by movement of blue dye through the small intestine, and expressed as percent of small intestine length. Data is shown as mean ± SEM. n=6 animals per group ***p<0.001.

Colonic permeability is increased in Muc2$^{-/-}$ *mice*

To determine if colonic permeability was specifically altered in *Muc2*$^{-/-}$ mice the movement of FITC dextran and fractional sucralose excretion rates were examined. As shown in Fig. 4-4, shortly after weaning at one month of age and later at five months, intestinal permeability to FITC was significantly higher in *Muc2*$^{-/-}$ indicative of increased intestinal permeability. In contrast, FITC levels were modestly though not significantly increased in *Muc2*$^{+/-}$ as compared to their WT counterparts.

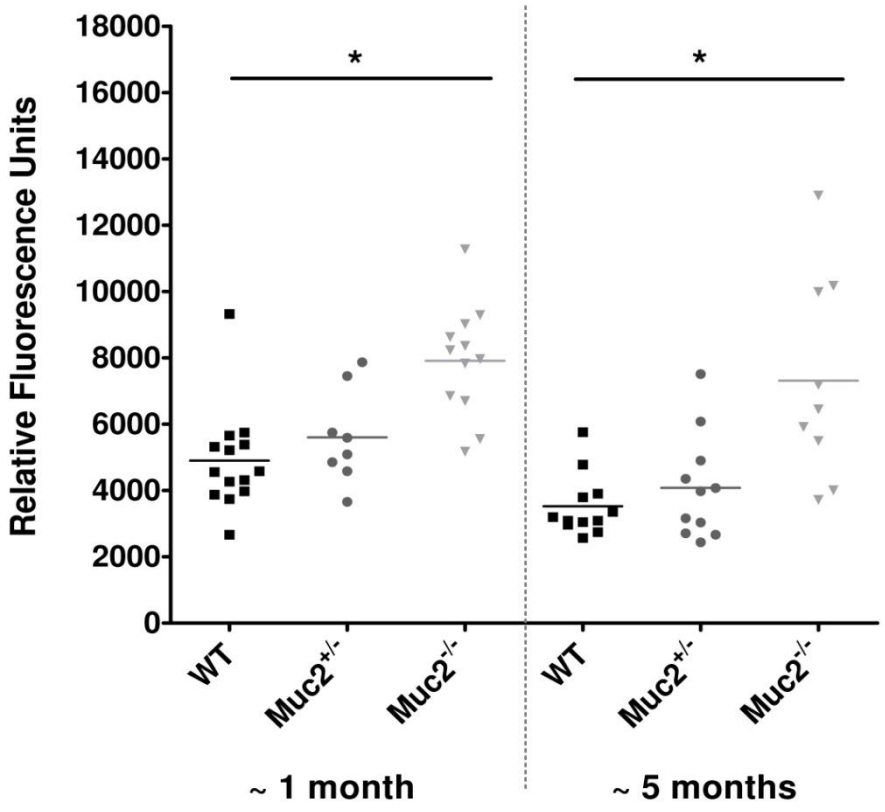

Figure 4-4: Intestinal permeability is significantly higher in *Muc2*$^{-/-}$ mice. Intestinal permeability to 3-5kDa FITC dextran was examined in WT, *Muc2*$^{+/-}$ and *Muc2*$^{-/-}$ animals shortly after weaning at 3 weeks to 1 month of age (left) and again at 5 months (right). In this dot plot, each animal is represented as: WT (■), *Muc2*$^{+/-}$ (●) and *Muc2*$^{-/-}$ (▼), and the mean of each group is shown. *$p<0.05$.

To further demonstrate that colonic permeability was involved in the changes observed in GI transit time, mice were gavaged with inert sugar probes containing sucralose, placed in metabolic cages and the fractional excretion rates in the urine were assessed. This technique was used since it has been shown in previous studies [26] to accurately reflect colonic permeability in human GI studies. As shown in Fig. 4-5, sucralose excretion was modestly increased in *Muc2*$^{-/-}$ mice from 14 weeks of age onwards, highlighting an age dependent increase in the colonic permeability phenotype.

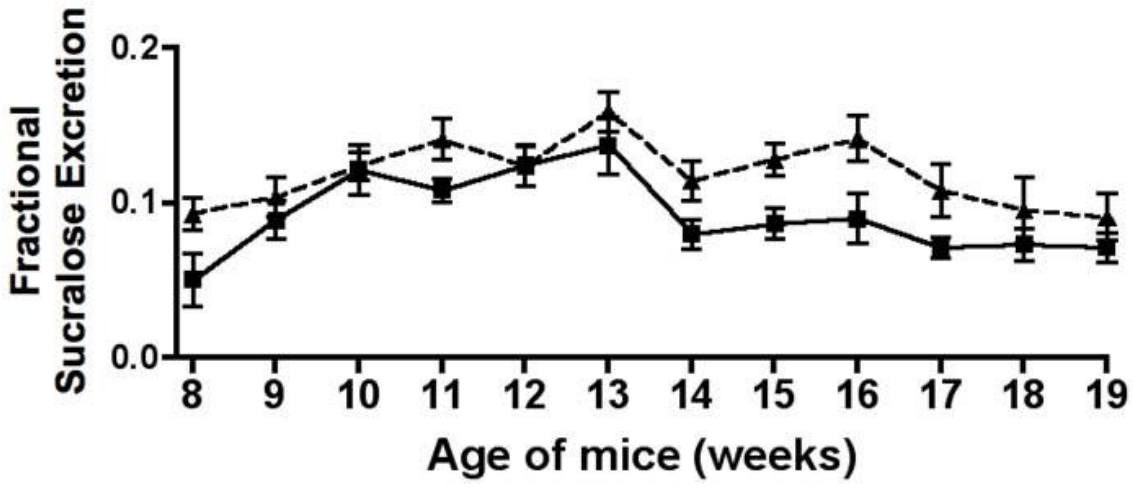

Figure 4-5: *Muc2*$^{-/-}$ mice have modestly higher colonic permeability. Colonic permeability was assessed by sucralose excretion. Fractional sucralose excretion rates were elevated in *Muc2*$^{-/-}$ mice (▲) as compared to WT animals (■) from 8 weeks onwards. This change was most evident between 14 and 17 weeks of age. Each data point represents the mean ± SEM for 10 animals per day.

Taken together, the data presented here demonstrate that in the absence of an intact mucus layer, intestinal morphology was altered and associated with increased GI motility and increased permeability of the colon. As intestinal permeability was increased in

Muc2$^{-/-}$ it was of interest to determine if these animals were more sensitive to intestinal injury.

Muc2$^{-/-}$ *mice are more susceptible to DSS induced colitis*

Numerous studies have shown that the mucus layer plays a protective role *in vivo*[12, 21, 27]. Therefore we sought to determine if in the absence of this protection, animals were more susceptible to DSS-induced injury. Previous studies have shown that *Muc2*$^{-/-}$ mice bred on a 129Sv background were susceptible to 2.5% DSS administered in the drinking water for 5d[13]. However as DSS susceptibility varies by strain it was necessary to test this on a C57BL/6 background using the same dosage of DSS. As shown in Fig. 4-6, *Muc2*$^{-/-}$ mice on a C57BL/6 background were highly susceptible to this dose, necessitating early termination of these studies. Moreover these animals were also extremely susceptible to dosages as low as 0.75% for 5d (Fig. 4-6B).

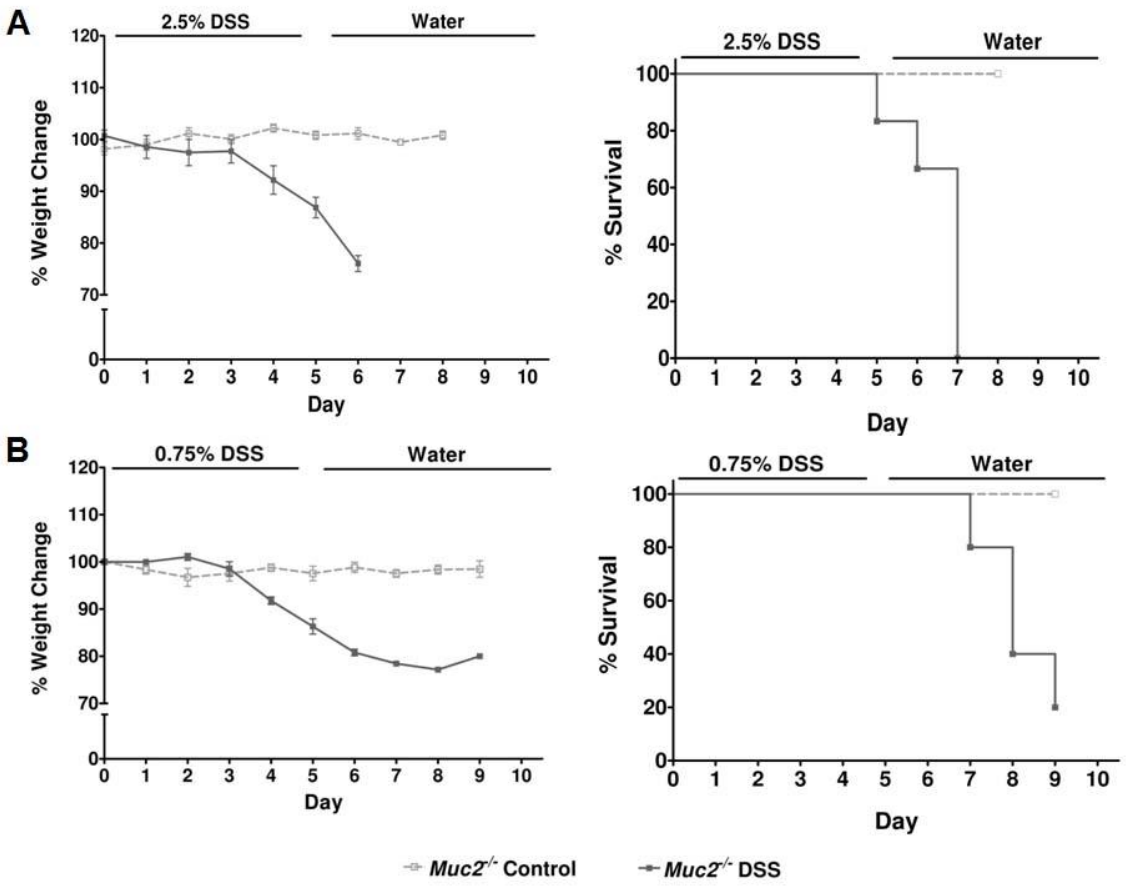

Figure 4-6: *Muc2*$^{-/-}$ mice are extremely sensitive to DSS. Mice were treated with 2.5% DSS (**A**) and 0.75% DSS (**B**) for 5 days, after which DSS was replaced with tap water. At both doses, significant weight loss (in excess of 20%) was observed (left) and this was accompanied by high mortality rates (right). Each data point represents the mean ± SEM per animal group per day. n = 4-6 animals per group.

In stark contrast, WT animals with an intact mucus layer tolerated dosages as high as 4% DSS for 5d (Fig 4-7). Curiously, even at these high doses, animals still only lost 10-15% of their body weight and significant mortality was not observed.

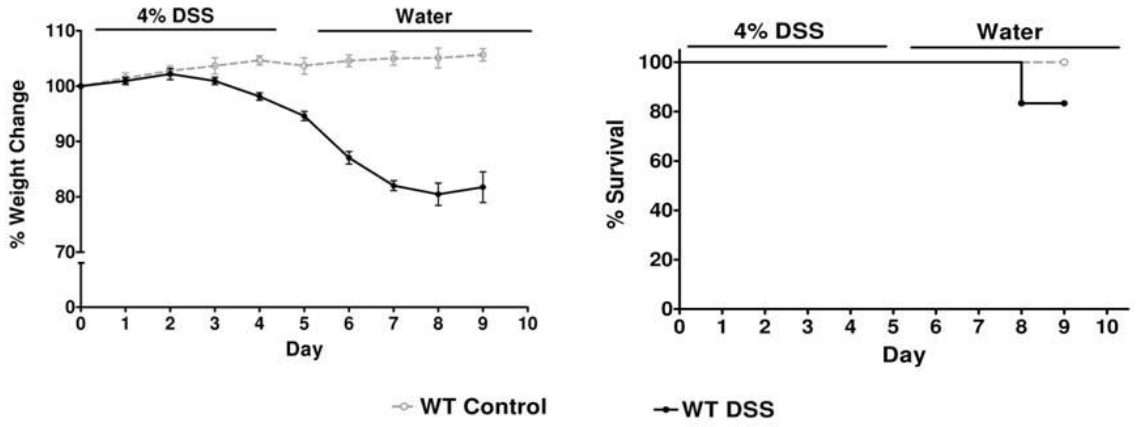

Figure 4-7: In the presence of an intact mucus layer animals are less sensitive to DSS. WT mice were treated with 4% DSS in the drinking water for 5d, after which this was replaced with tap water. On this dosage, animals lost approximately 20% body weight, and 80% survived to Day 8 of the study. n = 4-5 animals per group.

Since *Muc2*$^{-/-}$ mice were highly sensitive to DSS as compared to their WT counterparts, it was of interest to determine where the damage was localized along the colon. For this reason the colon was opened longitudinally along the mesenteric border, and rolled from the distal to the proximal end to form a swiss roll. As shown in Fig 4-8, the damage in WT mice was primarily localized to the distal portion of the colon as indicated by loss of goblet cell mucin, an intense inflammatory cell infiltrate and loss of crypt architecture. While some thickening of the muscle layers was observed much of the damage appeared to occur on the surface of the mucosa, and the middle and proximal regions of the colon remained largely unaffected by DSS, though mucin secretion was increased in these areas. In contrast, damage was significantly increased in *Muc2*$^{-/-}$ along the full length of the colon with complete denuding of the epithelium in the middle and distal colon, and inflammatory cell infiltration and loss of crypt architecture evident in the proximal portion. Moreover, the damage in *Muc2*$^{-/-}$ mice affected all levels of the mucosa and sub-mucosa with thickening of the muscle layer evident throughout much of the colon.

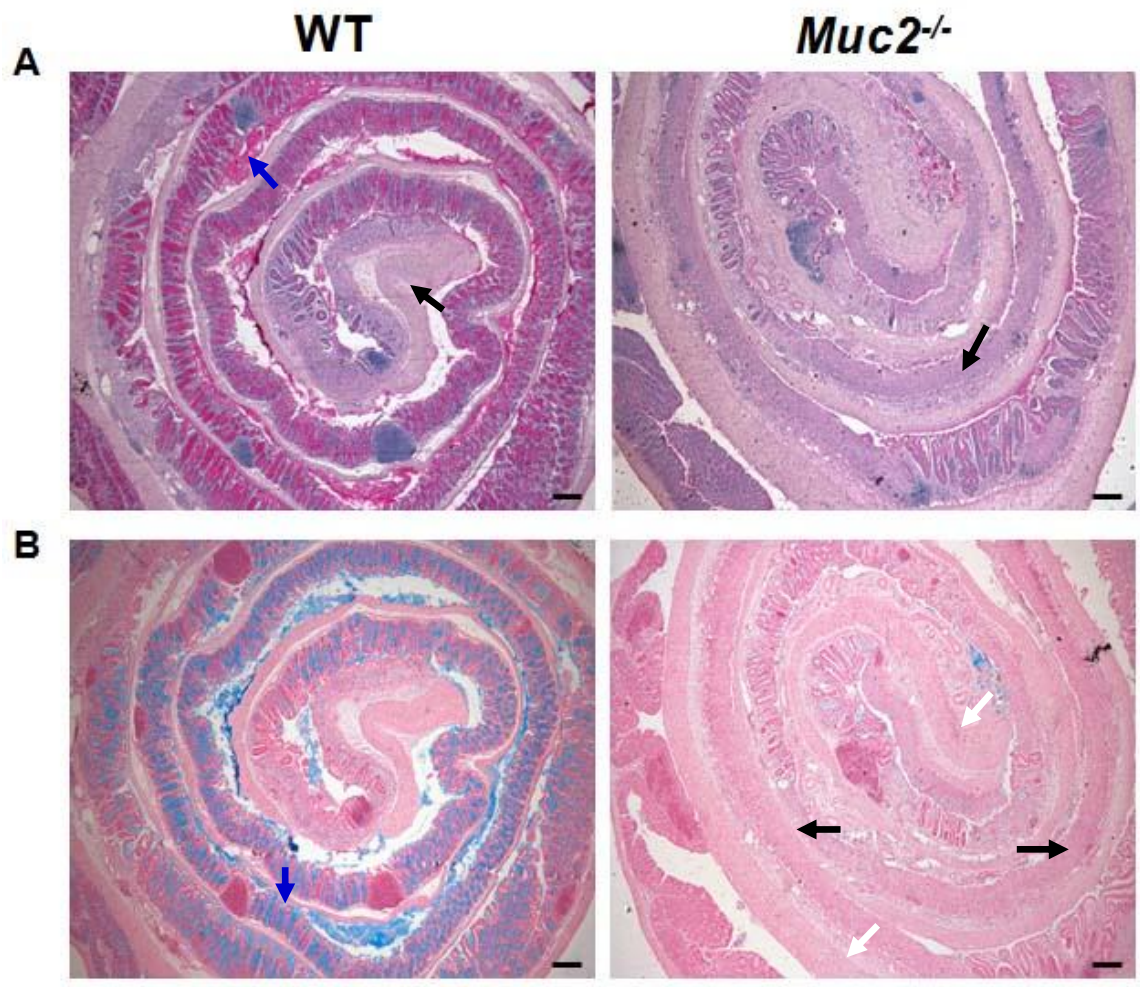

Figure 4-8: DSS induced damage along the full length of the colon in *Muc2*$^{-/-}$ mice. The full length of the colon was swiss-rolled from the distal (centre of picture) to the proximal end, and stained with PAS reagent (**A**) or Alcian blue (**B**). In WT mice (3.5% DSS, 5d) damage was localized to the distal colon, as indicated by loss of mucins, thickening of the muscle layer, edema, and loss of crypt architecture (black arrow). In the middle and proximal colon of these animals however, DSS induced neutral (A) and acidic (B) mucin secretion (blue arrows). In contrast, in *Muc2*$^{-/-}$ mice (1.0% DSS, 5d) damage was evident along the entire length of the colon, with extensive ulceration in the middle and distal colon (black arrows). This affected all levels of the mucosa, and muscle thickening (white arrows) was observed throughout the colon in these animals. Scale bar 200μm.

Since *Muc2*$^{-/-}$ animals were hypersensitive to DSS, even at very low dosages, wound repair and epithelial restitution were examined in these animals. For these experiments it was necessary to determine a dosage at which the epithelium could be injured in *Muc2*$^{-/-}$ without extensive damage and high mortality. After extensive studies, the only dosages

for which significant mortality was not observed were 1% DSS for 3d or 4d (Fig. 4-9). At these dosages however, changes in body weight loss did not correlate with a noticeable increase in DAI scores.

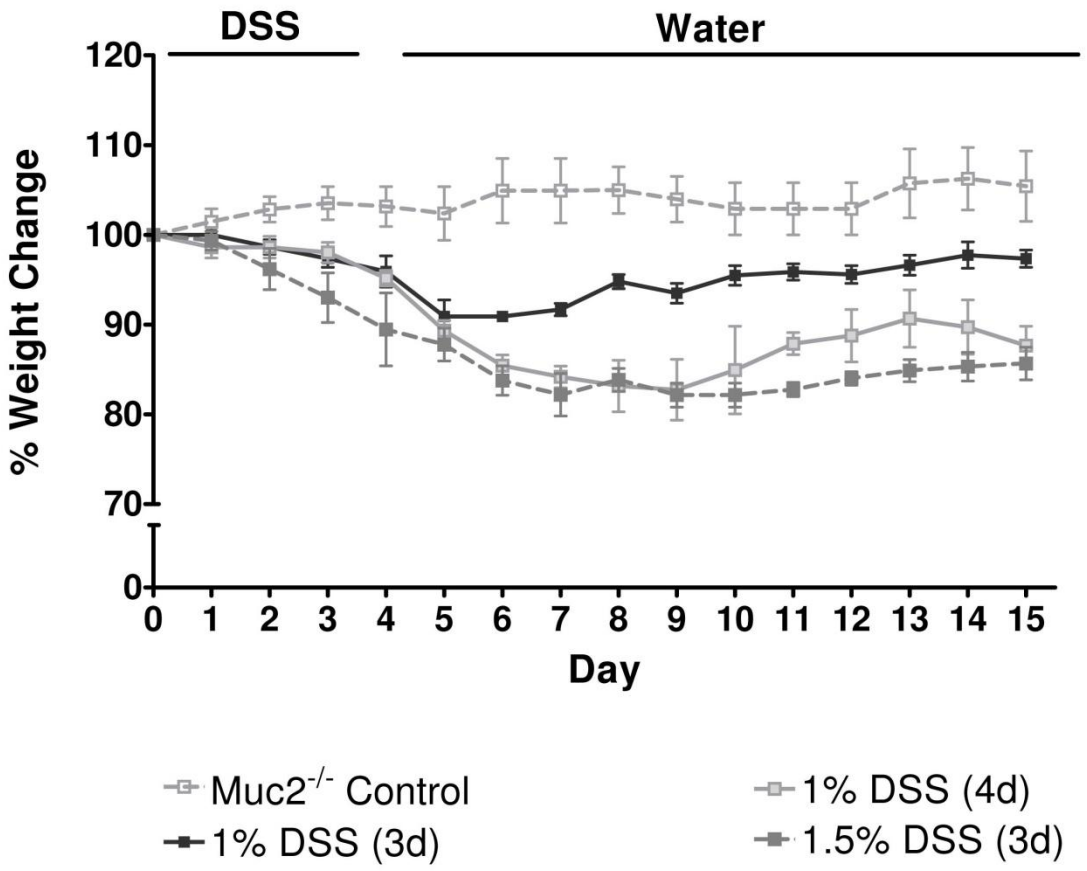

Figure 4-9: DSS induced weight loss in *Muc2*$^{-/-}$ mice. Animals were treated with different dosages of DSS and weight loss was examined. In all groups, approximately 15% weight loss was observed, but recovery as assessed by weight gain was only evident in animals given 1% DSS, and this was most evident in those treated for 3d. Each data point represents the mean ± SEM per animal group per day; n = 3-4 animals per group.

Previous studies have shown that the mucus layer is reduced but not completely lost in patients with UC[28]. While *Muc2*$^{-/-}$ mice provide a valuable tool in understanding the role of the mucus layer in disease progression and recovery, they do not adequately represent

the human condition, since patients do not completely lack MUC2. For this reason, it was also necessary to compare animals with a partially intact mucus layer as well as those that lacked Muc2. As previously shown (Fig. 4-2) *Muc2*$^{+/-}$ animals produce and secrete intermediate levels of PAS positive mucins into the lumen in a manner analogous to that observed in WT mice and modest, but not significant changes were observed in intestinal permeability. However, since these animals do not lack a complete mucus layer, it was necessary to determine if they were as sensitive to DSS as their *Muc2*$^{-/-}$ counterparts. Accordingly, animals were treated with 1% DSS (3d) and as shown in Fig. 4-10, no change in weight loss was observed in *Muc2*$^{+/-}$ mice demonstrating that even a partially intact mucus layer can protect against low levels of intestinal injury.

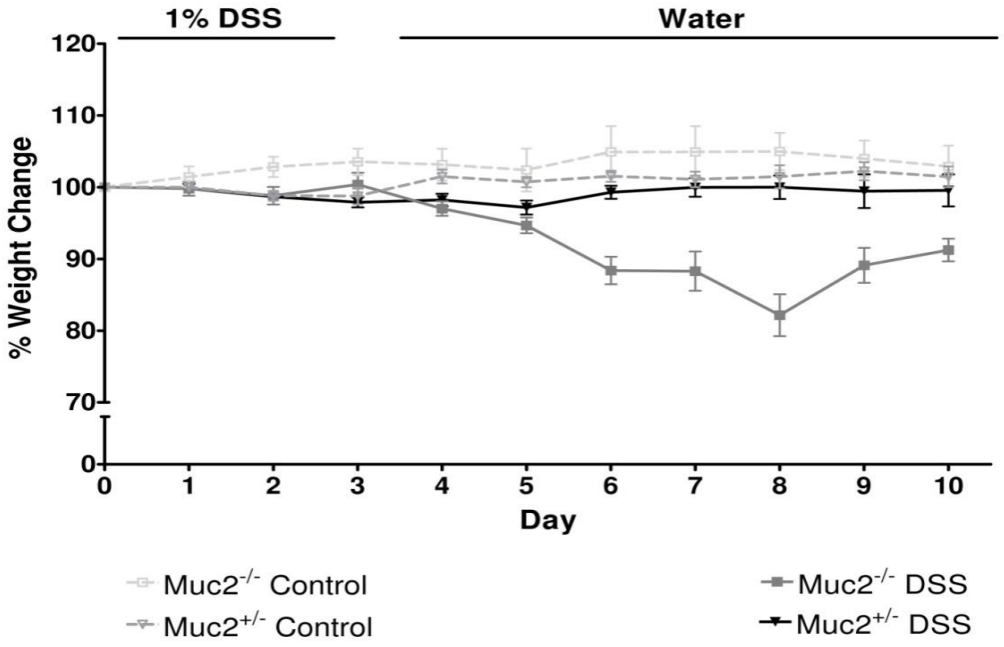

Figure 4-10: *Muc2*$^{+/-}$ mice are more resistant to DSS than *Muc2*$^{-/-}$ mice. *Muc2*$^{+/-}$ and *Muc2*$^{-/-}$ animals were treated with 1% DSS (3d) after which DSS was replaced with tap water. DSS induced a 15% weight loss in *Muc2*$^{-/-}$ mice between Day 6 and Day 9, though a similar trend was not observed in *Muc2*$^{+/-}$ mice. Each data point represents the mean ± SEM per group per day; controls n = 3, *Muc2*$^{-/-}$ DSS n = 5, *Muc2*$^{+/-}$ DSS n = 11.

An intact mucus layer is critical for wound repair in vivo

To further examine the role of the mucus layer in wound repair, WT and *Muc2*$^{+/-}$ were treated with 3% DSS (5d) while *Muc2*$^{-/-}$ mice were treated with 1% DSS (3d), after which all animals were given tap water (Fig. 4-11). Disease onset as indicated by weight loss occurred as early as Day 0-4, while maximal damage was between Day 6 and 9. Using weight loss as a surrogate marker of overall health, animals were sacrificed at Day 7 to denote maximal damage and Day 15 to evaluate mucosal repair. While all animals lost between 10% and 15% of their body weight at 7d, no significant changes were observed with one group losing significantly more weight than the others, suggesting that approximately equivalent damage occurred even with differing doses.

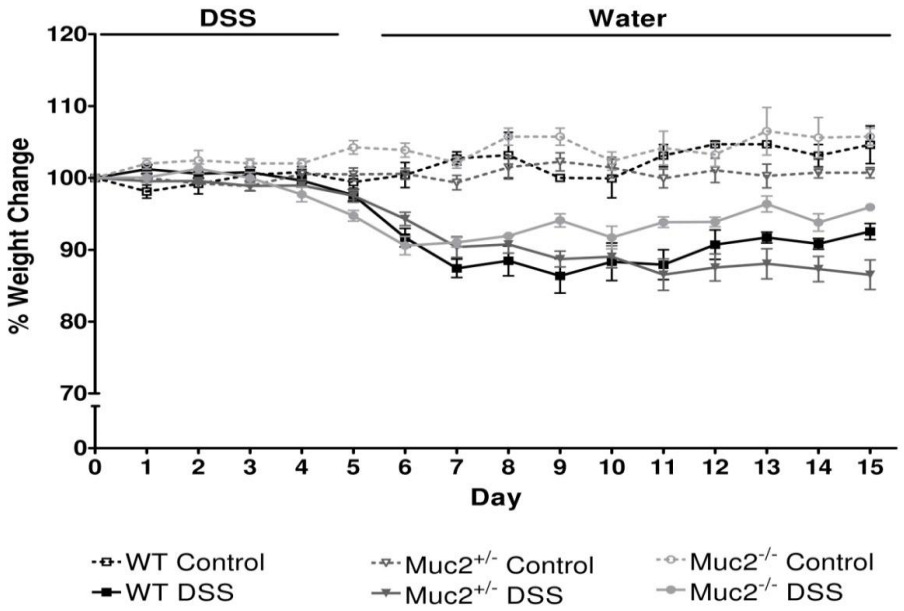

Figure 4-11: Differing doses of DSS were required to induce approximately equivalent levels of damage in WT, *Muc2*$^{+/-}$ and *Muc2*$^{-/-}$ mice. In WT and *Muc2*$^{+/-}$ mice, 3% DSS (5d) in the drinking water induced a 15% weight loss commencing at Day 4, with maximal loss between Day 6 and 9. In *Muc2*$^{-/-}$ animals however, 1% DSS for 3 days was required to induce this level of damage. For control groups n = 6 where 3 mice were sacrificed on Day 7 and the remainder on Day 15. For all DSS treated groups n = 9-10 mice where half were euthanized on Day 7 and the remainder on Day 15.

Interestingly, WT mice lost the most weight at Day 7 (12.58%), which may be indicative of slightly higher disease severity (Table 4-3), but some of that was regained at Day 15 suggesting that repair was indeed occurring. Although they were not as sensitive to DSS as their *Muc2*$^{-/-}$ counterparts, *Muc2*$^{+/-}$ animals were more susceptible than WT animals. At the same dosage of DSS as WT animals, *Muc2*$^{+/-}$ lost more weight and recovery was impaired. Indeed, maximal weight loss in WT mice occurred between Day 7 and 9 and recovery commenced by Day 10 in these animals. *Muc2*$^{+/-}$ animals however continued to lose weight up until Day 15. While a similar pattern was observed in *Muc2*$^{-/-}$ mice, although they lost less weight than their WT counterparts, they regained about the same amount by Day 15. These data indicate that WT mice are better able to recover from colonic injury at a faster rate than their Muc2 deficient counterparts, suggesting that recovery may be delayed in *Muc2*$^{-/-}$ animals. This was not observed in *Muc2*$^{+/-}$ animals however, since maximal damage occurred at Day 15.

Table 4-3: Percent weight change in WT, *Muc2*$^{+/-}$ and *Muc2*$^{-/-}$ animals over time.

Animal Group	% of initial weight at Day 7	Weight Change (Day 0 to Day 7)	Day 15	Weight Change (Day 7 to Day 15)
WT	87.42%	↓ 12.58%	92.54%	↑ 5.12%
***Muc2*$^{+/-}$**	90.38%	↓ 9.62%	86.53%	↓ 3.84%
***Muc2*$^{-/-}$**	91.03%	↓ 8.97%	95.94%	↑ 4.91%

The average percentage weight change per animal group is shown as well as increase or decrease during disease progression and recovery. DSS doses: WT and *Muc2*$^{+/-}$ mice 3% DSS 5d, *Muc2*$^{-/-}$ 1% DSS 3d. Control groups n = 6, DSS treated groups n = 9-10 animals per group.

In murine models of DSS, inflammation of the colon frequently manifests as thickening of the mucosa, with overall shortening of the colon and these signs are reversed during recovery. To determine whether colon length could be used as an indicator for restitution, it was measured at Day 7 and Day 15. As shown in Fig. 4-12, colon length was significantly shorter at 7d during acute injury, indicative of ongoing colitis. At Day 15 however colon length was significantly increased in WT mice suggestive of recovery, while this was absent in both *Muc2*$^{+/-}$ and *Muc2*$^{-/-}$ animals. In these animals colon length remained the same at Day 7 and Day 15, suggestive of similar levels of damage at these time points and delayed restitution. Furthermore, colitis as indicated by a shortened colon length was severe in acute disease in both WT and *Muc2*$^{+/-}$ mice, but where improvement was observed in WT mice, this was not the case in *Muc2*$^{+/-}$ animals.

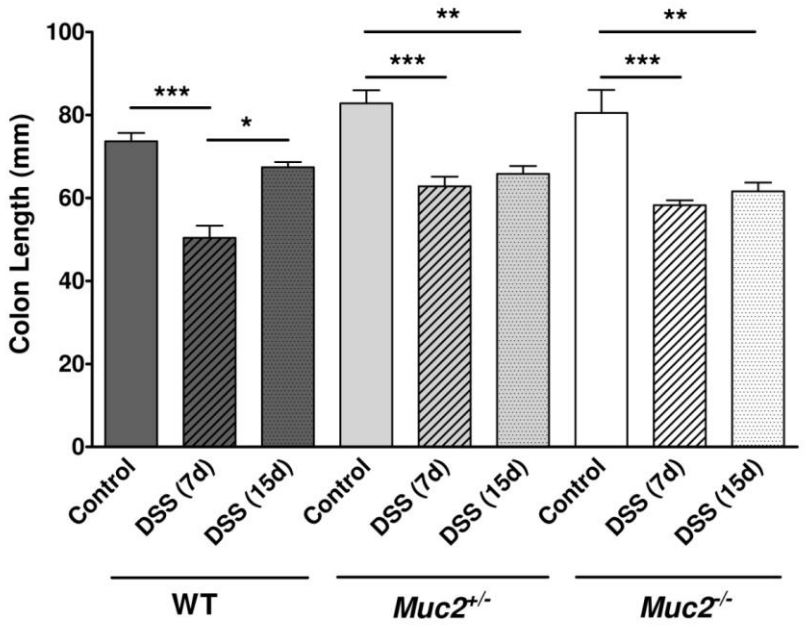

Figure 4-12: Colon length was significantly reduced in acute DSS injury. The colon length was measured at time of necropsy. During acute colitis (Day 7) the colon was significantly shortened in all animal groups. However an increase in colon length was observed in WT animals during the recovery phase (Day 15) while *Muc2*$^{+/-}$ and *Muc2*$^{-/-}$ animals maintained a shortened colon. DSS doses: WT and *Muc2*$^{+/-}$ mice 3% DSS 5d, *Muc2*$^{-/-}$ 1% DSS 3d. Each bar represents 4-6 animals. *p<0.05, **p<0.01, ***p<0.001.

Histological examination of these colons revealed that in WT mice, DSS induced damage was mainly localized to the distal colon (Fig. 4-13). At Day 7 thickening of the muscle layer, loss of goblet cells and luminal mucins and loss of crypt architecture was evident, while in areas less affected by DSS, most notably the proximal colon, extensive mucin production and secretion was observed in WT mice. At higher magnification, complete loss of crypt architecture was visible, along with an extensive inflammatory cell infiltrate. At Day 15 however, despite the presence of the cellular infiltrate and thickening of the muscle layer, granulation tissues were evident. Epithelial restitution had occurred as demonstrated by cell proliferation over the ulcer lesions and crypts with immature and mature goblet cells were evident. A similar pattern was observed in *Muc2*$^{+/-}$ with damage being localized to the distal colon, though some evidence of damage in patches in the mid colon was also observed. In addition, mucin production and secretion was reduced in *Muc2*$^{+/-}$ animals at Day 7. At Day 15, the epithelial surface of recovering *Muc2*$^{+/-}$ mice resembled that of WT animals with immature/mature goblet cells producing mucins. In stark contrast, *Muc2*$^{-/-}$ mice at all stages of disease showed lesions throughout the colon. Extensive thickening of the muscle layer and loss of crypt architecture was evident at Day 7 as was cellular infiltration. At Day 15, the epithelium appeared normal in parts, but thickening of the muscle layer and inflammatory cell infiltration persisted.

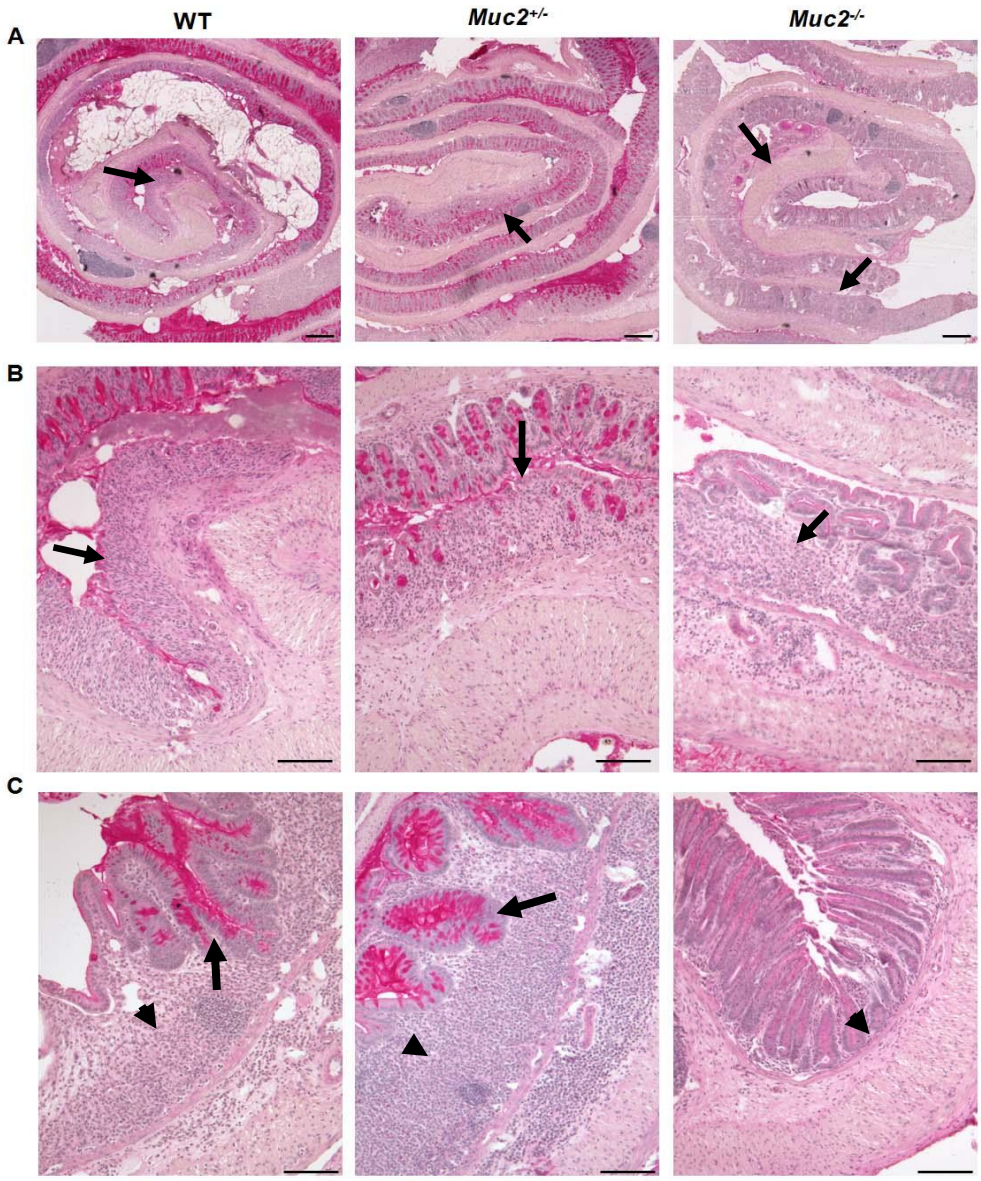

Figure 4-13: Epithelial repair was evident in all animal groups at Day 15 despite extensive damage at Day 7. The full length of the colon was swiss-rolled from the distal (centre of picture) to the proximal end, and sectioned and stained with PAS reagent. **A**: DSS induced damage in all animals at Day 7. In WT and *Muc2*$^{+/-}$ animals, damage was primarily localized to the distal colon as evident by loss of goblet cells, and crypt architecture in these areas as well as thickening of the muscle layer (arrows). In *Muc2*$^{-/-}$ animals in contrast, damage was evident throughout the colon with muscle thickening in the distal colon and loss of crypt architecture in the mid colon (arrows). Scale bar: 200µm. **B**: At higher magnification, at Day 7 loss of crypt architecture and inflammatory cell infiltration was evident in all animal groups (arrows) with noticeable thickening of the muscle layer. **C**: At Day 15, muscle thickening and cellular infiltration (arrowheads) was evident in all animals indicative of colitis. Signs of recovery were evident in WT and *Muc2*$^{+/-}$ animals with immature goblet cells secreting mucins (arrows). *Muc2*$^{-/-}$ mice however lacked mucin secretion despite well formed crypts. DSS doses: WT and *Muc2*$^{+/-}$ mice 3% DSS 5d, *Muc2*$^{-/-}$ 1% DSS 3d. In panels B and C, scale bar: 100µm.

Inflammatory chemokine expression is associated with recovery

In humans IL-8 is a potent neutrophil chemoattractant, leading to much of the cellular infiltration during inflammation. In mice, several Il-8 homologs serve a similar purpose[12]. Therefore the protein levels of the murine Il-8 homologs KC, MCP-1, MIP-2, and LIX were examined (Fig. 4-14) to correlate the severity of disease with restitution. As expected, in acute colitis at Day 7, the relative expression of KC, MCP-1 and MIP-2 were significantly increased. Furthermore, at Day 15 while restitution was occurring, the relative expression of these chemokines was reduced to approximately the same levels as control animals. While a similar pattern was observed in *Muc2*$^{+/-}$ and *Muc2*$^{-/-}$ animals with elevated expression at Day 7, this remained high at Day 15. This data may, in part, explain the lack of complete repair observed in these animals.

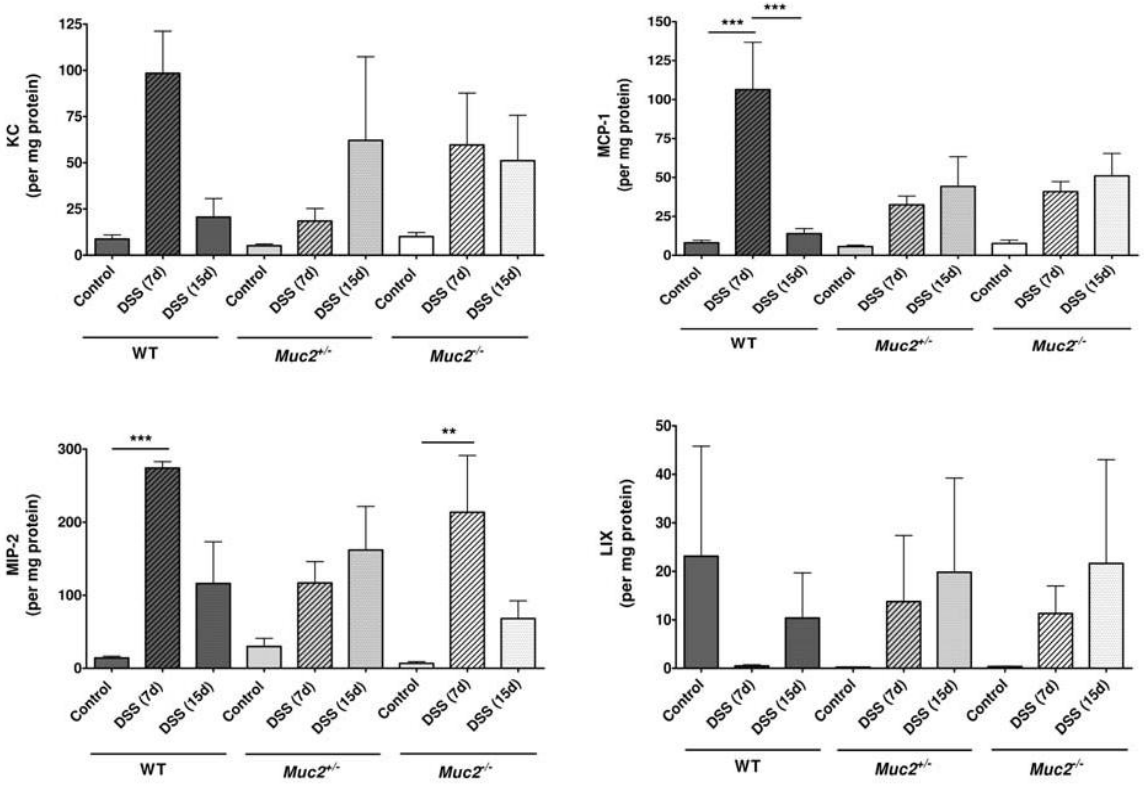

Figure 4-14: Acute colitis resulted in increased expression of murine IL-8 homologs. Animals were treated with DSS to induce acute colitis, and the murine Il-8 homologs KC, MCP-1,MIP-2 and LIX were examined in tissue homogenates obtained from the colon. Acute injury in WT mice resulted in a significant increase in KC, MCP-1 and MIP-2 levels at Day 7 and this was significantly reduced at Day 15. Similar patterns were not observed in *Muc2*$^{+/-}$ or *Muc2*$^{-/-}$ animals at Day 15 however. DSS doses: WT and *Muc2*$^{+/-}$ mice 3% DSS 5d, *Muc2*$^{-/-}$ 1% DSS 3d. n = 4-6 animals per group, $^{**}p<0.01$, $^{***}p<0.001$.

Biomarkers of inflammation at 7d and those of epithelial repair at 15d were examined by quantitative real-time PCR. DSS significantly induced *Il-1β* and *Ifn-γ* expression in all animal groups, with a modest increase in *Cox-2* and *Tnf-α*. In WT mice, although no change was observed in *Il-1β* or *Cox-2* expression, a marked increase in *Ifn-γ* was observed. In *Muc2*$^{-/-}$ mice, reduced *Il-1β*, *Cox-2* and *Tnf-α* expression was observed at Day 15 (Fig. 4-15), while Ifn-γ was increased. Since Ifn-γ levels were considerably

higher in all animal groups at Day 15 this cytokine when expressed within a narrow range, may facilitate wound healing *in vivo*.

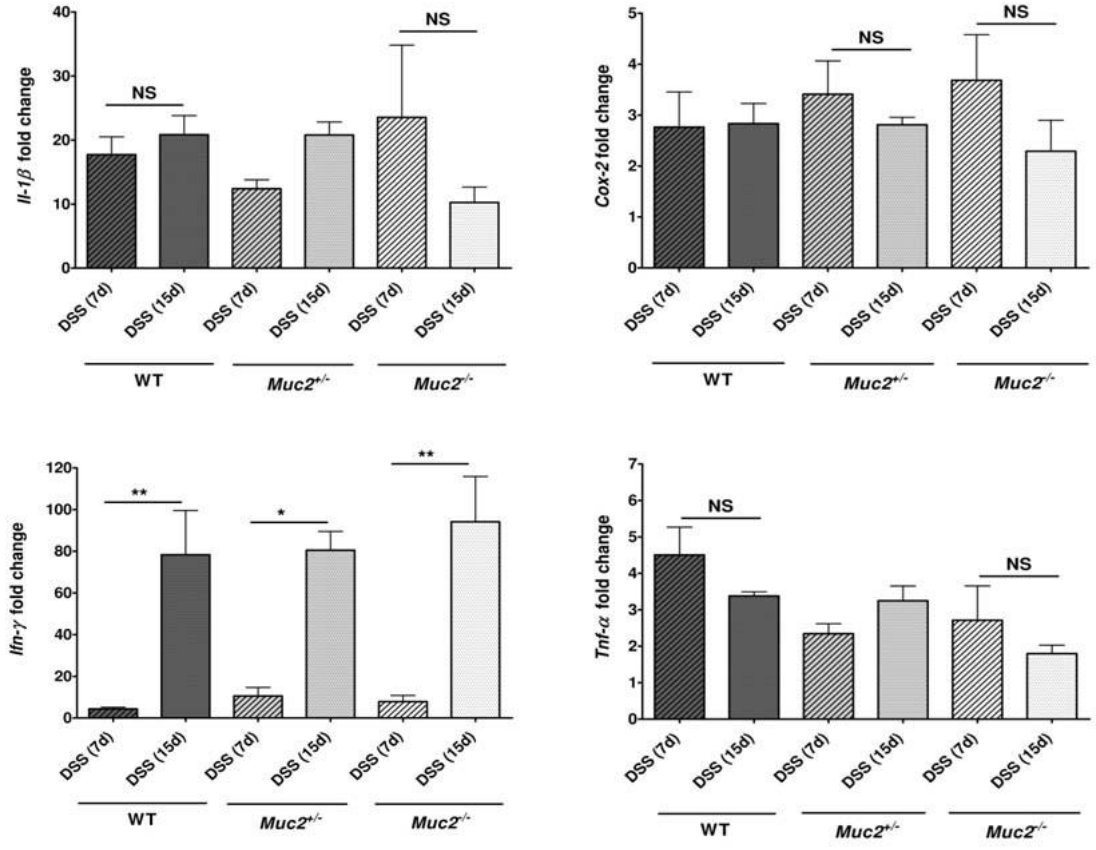

Figure 4-15: DSS induces changes in *Il-1β*, *Cox-2* and *Ifn-γ* expression. The relative gene expression of *Il-1β*, *Cox-2*, *Ifn-γ* and *Tnf-α* were assessed by qRT-PCR. DSS induced robust *Il-1β* and *Ifn-γ* expression in all animal groups at Day 7 and this remained high at Day 15. Although modest changes were observed in the levels of these cytokines in all animal groups during recovery only those with *Ifn-γ* were statistically significant. DSS doses: WT and *Muc2*$^{+/-}$ mice 3% DSS 5d, *Muc2*$^{-/-}$ 1% DSS 3d. n = 4-6 mice per group. Data expressed as fold change compared to control. NS = not significant, *p<0.05, **p<0.01, ***p<0.001.

At the protein level, Il-1α, Il-1β and Ifn-γ levels were elevated following DSS treatment, and these remained high even into the recovery phase while Tnf-α increased in acute disease then decreased by Day 15. A time dependent increase in both Il-1α and Il-1β was

observed in all animal groups following DSS, which suggests a possible role for these cytokines in disease progression or recovery.

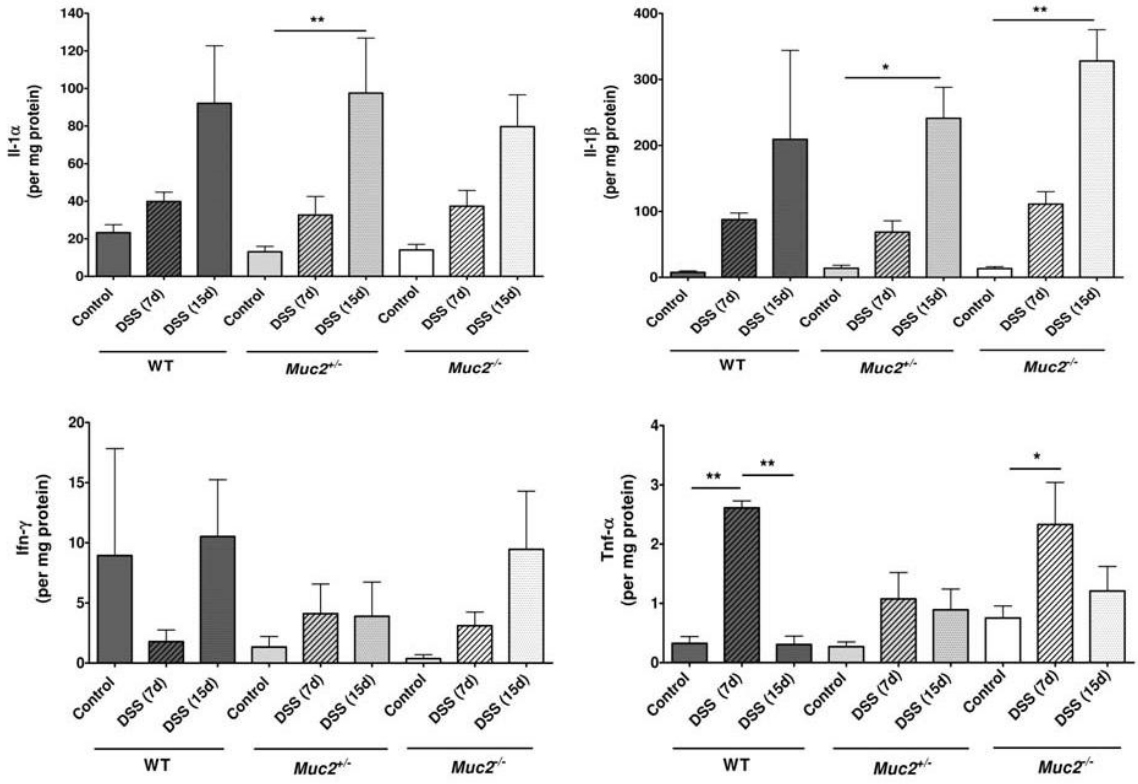

Figure 4-16: DSS induced a robust increase in Il-1α and Il-1β levels at Day 15. The expression of inflammatory cytokines was assessed in tissue lysates from control animals and those treated with DSS at Day 7 and Day 15. DSS injury induced an increase in Il-1α and Il-1β expression during acute disease (Day 7) and this remained elevated during recovery at Day 15. In addition, in WT mice and to a lesser extent *Muc2*$^{-/-}$, DSS induced a robust increase in Tnf-α expression at Day 7 which was significantly reduced at Day 15. DSS doses: WT and *Muc2*$^{+/-}$ mice 3% DSS 5d, *Muc2*$^{-/-}$ 1% DSS 3d. n = 4-6 mice per group, **p*<0.05, ***p*<0.01.

Egf and Fgf-1 may be involved in epithelial repair

Since DSS colitis altered the levels of pro-inflammatory mediators it was of interest to determine if the expression of growth factors important in restitution were increased at Day 15. As shown in Fig. 4-17, higher levels of *Egf* and *Fgf-1* was observed in the

recovery phase of disease as compared to the acute stage (Fig. 4-17), but these were only slightly elevated compared to control.

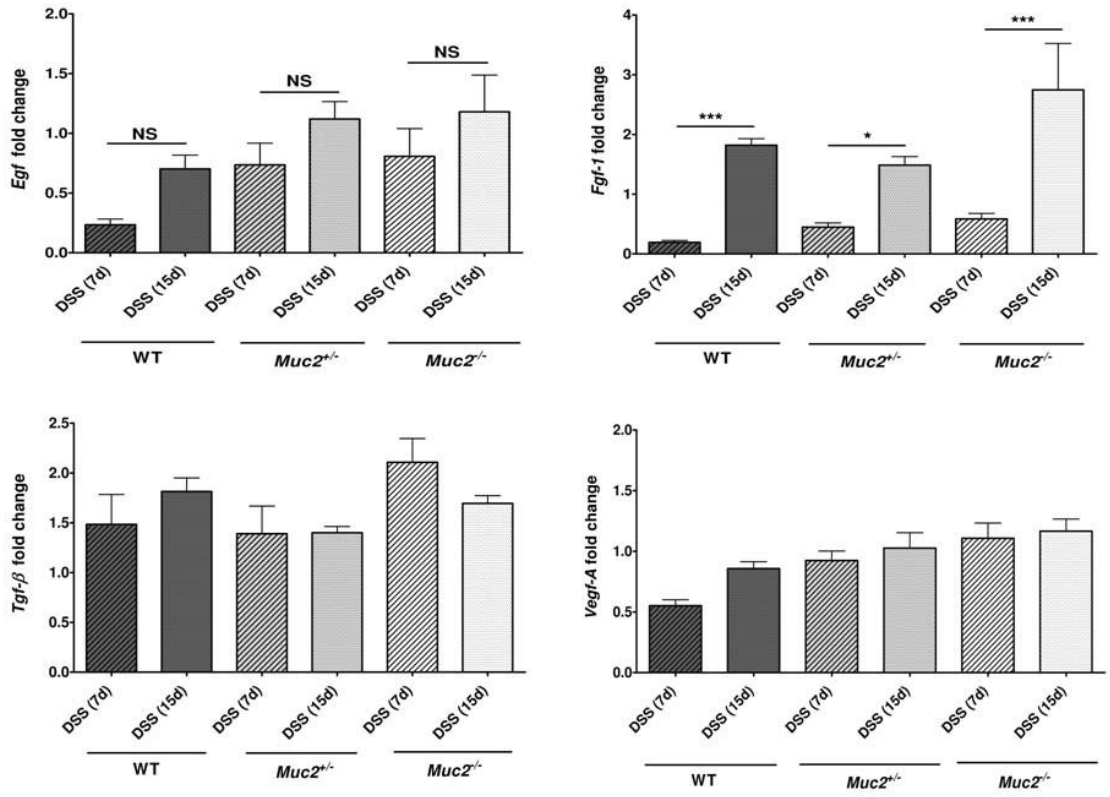

Figure 4-17: Increased expression of *Egf* and *Fgf-1* was observed at Day 15. To determine if wound healing was occurring, gene expression of *Egf*, *Fgf-1*, *Tgf-β* and *Vegf-A* was examined by qRT-PCR. A trend towards increased *Egf* and *Fgf-1* expression was observed in the recovery phase of disease. DSS doses: WT and *Muc2*$^{+/-}$ mice 3% DSS 5d, *Muc2*$^{-/-}$ 1% DSS 3d. n = 4-6 mice per group, NS = not significant, *p<0.05, ***p<0.001. Data expressed as fold change compared to control.

Discussion

The intestinal mucus layer plays a central role in innate host defense against pathogens, and in this study it was shown to play a critical role in maintaining epithelial barrier function both in the absence and presence of an injury. Intestinal morphology was severely altered in *Muc2*$^{-/-}$ mice evidenced by the absence of an intact mucus layer, and PAS positive stained goblet cells as well as crypt elongation and branching. In addition,

the composition of the fecal microbiota was altered with a reduction in the Bacteroides and Firmicutes phyla, and elevated levels of other bacteria. Furthermore whole gut transit time was significantly decreased in *Muc2*$^{-/-}$ mice, and this was not accompanied by changes in upper GI transit, suggesting that movement through the colon was increased. Indeed, intestinal permeability to FITC dextran was increased in *Muc2*$^{-/-}$ mice as was the fractional excretion of sucralose, further demonstrating that movement and intestinal function were altered in these animals. As expected, *Muc2*$^{-/-}$ were highly susceptible to intestinal injury and recovery was significantly impaired. Taken together this data clearly indicate that the Muc2 mucus layer plays an important role in intestinal epithelial barrier function and its response to acute injury and restitution.

The Muc2 monomer consists of a protein backbone surrounded by sugar-side chains that facilitate bacterial colonization[4, 5], while at the same time providing the nutrients required to sustain a microbial community. Therefore, the mucus layer effectively shapes the host microbiota, and changes in the integrity of this layer will result in changes to the bacterial community. The present study shows that the microbial community is altered in *Muc2*$^{-/-}$ mice, resulting in fewer species of the *Bacteroides* and *Firmicutes* phyla. Since these constitute the major components of the murine and human microbiota [29], changes in these may have significant implications in overall health. Indeed, bacteria in these phyla are involved in the digestion of complex molecules, energy resorption and obesity, and changes in this may explain why *Muc2*$^{-/-}$ mice rapidly gain weight later in life (unpublished observations). In addition, bacteria of the *Bacteroides* phyla exclude

potential pathogens from colonizing the colon, so a loss of these bacteria could result in increased disease susceptibility.

In addition to weight gain, *Muc2*$^{-/-}$ animals frequently pass loose or watery stools suggestive of changes in water absorption in the colon. In the absence of Muc2, whole gut transit time was decreased which may be indicative of increased gut secretion and/or motility. Thus, it was not surprising that intestinal permeability to FITC dextran was increased in these mice. These data suggest that epithelial barrier function was compromised in *Muc2*$^{-/-}$ mice which could facilitate increased exposure to antigenic stimuli and result in low grade colonic inflammation as has been previously described in these animals [13]. The DSS model of intestinal injury was used to determine sensitivity to a colitis inducing agent and to study the disease process from onset to complete recovery[15, 30]. Moreover, DSS has been used to induce acute or chronic colitis, so the severity of the disease can be modified and it can be used to examine the role of innate immune mechanisms such as the mucus layer in disease pathogenesis[31-33]. In fact, a treatment course of 3% DSS for 5d, where the animals are sacrificed 1-4 wks post DSS has been used as a model to investigate disease recovery[34], and termination on Day 8 or Day 10 resulted in high damage scores and epithelial repair activity [31]. The altered intestinal morphology and epithelial barrier function impairment in *Muc2*$^{-/-}$ mice resulted in hypersensitivity to DSS colitis even at dosages as low as 0.75% DSS (5d). In most cases the damage was so severe that many animals succumb to injury as compared to their WT counterparts. While the specific mechanism by which DSS induces injury is not clear, it appears to be toxic to IEC[32] and exerts its effects from the luminal surface of

the colonic mucosa[35], leading to changes in epithelial barrier function[31]. In addition to its numerous other functions, the mucus layer acts as a diffusion barrier effectively lowering the concentration of toxin to which the IEC are exposed. In *Muc2*$^{-/-}$ mice which lack an intact mucus layer, this dilution effect is lost and IEC are exposed to the full force of the toxin. Therefore, one reason why a much higher dose of DSS was required in WT mice to induce a comparable level of damage to that in *Muc2*$^{-/-}$ could be to compensate for the dilution effect of the mucus layer.

Mucosal permeability is increased towards DSS even before inflammation is observed in WT[36] and bacterial species have been found deep in the intestinal crypts of DSS-treated Muc2 deficient mice [37]. In addition, DSS has been shown to alter the composition of the intestinal microflora [38], though whether this is a cause or a consequence of disease remains unclear. DSS typically induces disease of the distal colon, leading to loss of goblet cell mucin, loss of crypt architecture and an inflammatory cell infiltrate (reviewed in [39]). Furthermore, although thickening of the muscle layer and edema are frequently observed in diseased animals, DSS lesions are typically superficial resulting in damage similar to that observed in human UC[39, 40]. In this study, WT and *Muc2*$^{+/-}$ mice treated with DSS induced superficial lesions with some thickening of the muscle layer, and damage was primarily localized to the distal colon, while the proximal colon appeared normal though mucin secretion was increased. A very different pattern was observed in *Muc2*$^{-/-}$ where the damage in these animals occurred along the full length of the colon and affected all layers of the mucosa, which may explain why at higher dosages these animals

succumbed to disease. The reason for this variation in distribution however remains unclear.

In addition to changes in the distribution of damage in WT and *Muc2*$^{-/-}$ mice, the intensity of the inflammatory response also varied. In rodents, DSS is associated with bloody diarrhea, ulcerations and granulocyte infiltration[31], and in acute models of colitis DSS has been shown to induce a massive inflammatory cell infiltrate consisting of T and B lymphocytes, neutrophils and macrophages at the site of the lesion[40]. In this study, histopathology was evident in all animals treated with DSS, and an inflammatory cell infiltrate was observed, along with increased expression of KC, MCP-1 and MIP-2, during acute injury in WT mice, and throughout the disease in *Muc2*$^{+/-}$ and *Muc2*$^{-/-}$ animals. Robust changes were observed in *Il-1β and Ifn-γ* gene expression, and Il-1α, Il-1β and Ifn-γ protein levels increased. Further increase in these cytokines at Day 15 suggests that they may be involved in epithelial restitution or wound repair functions.

The relationship between DSS and mucin synthesis and secretion has been well characterized and goblet cells are important for epithelial protection and wound repair in DSS[30]. As early as Day 1 of DSS, mucin content in the proximal colon was increased, and this was maintained for a five day treatment course[35] which corroborates the findings of this study. Furthermore, mucin content in the distal colon was significantly reduced during disease, and was thought to be proportional to the duration of DSS administration[35]. In addition, in rat models of DSS, mucin threonine and serine content was reduced in the gut which could result in a reduction of the number of potential O-

glycosylation sites, resulting in diminished protective abilities by these mucins[41]. This therefore raises the question whether or not diseases that affect the mucus layer in humans, could also result in compromised barrier integrity leading to reduced protective abilities by these mucins.

This data is of particular interest as it shows similarities in IBD patients. In acute DSS the expression profile of various cytokines as well as the histological changes observed are consistent with those observed in patients with UC[40]. In fact, the colonic lesions in DSS and UC are homogeneous and highly reproducible[42]. Furthermore, DSS colitis and human UC are similar in that both induce diarrhea, rectal bleeding, and they predominantly involve the distal colon where lesions encircle the mucosa[43]. This is of great significance since UC is also associated with changes in MUC2 structure as well as reduced mucin secretion and thinning of the mucus layer [28]. The data presented here show that in the absence of an intact mucin barrier, intestinal permeability was increased which could lead to invasion of potentially pathogenic species into the sub-mucosal layers. Furthermore, these animals were severely limited in their ability to heal colonic lesions. These findings suggest that in cases like UC where the integrity of the mucin barrier is compromised, treatment therapies should focus not only on managing the symptoms but also replenishing the mucus layer as a means to restoring epithelial barrier function.

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CHAPTER 5: MANUSCRIPT II

The Probiotic Mixture VSL#3 does not Alter DSS Induced Colitis Onset or Recovery

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Manuscript submitted for publication

Abstract

The intestinal epithelial barrier is compromised under disease conditions resulting in increased susceptibility to invasion with luminal bacteria and uptake of antigens. Probiotics including VSL#3 have been shown to enhance various components of barrier function through enhanced MUC2 expression and secretion, altered pro-inflammatory responses and enhanced expression of growth factors that alter the disease onset, progression or recovery. In this study, we examined whether the probiotic mixture VSL#3 could modulate DSS-induced colitis in WT and Muc2 deficient mice. Prolonged administration of VSL#3 to WT and *Muc2*$^{-/-}$ mice did not alter intestinal permeability in healthy animals. In DSS-induced colitis with different dosages, VSL#3 did not alter disease onset, progression or recovery in WT or *Muc2*$^{-/-}$ mice. In addition at low dosages of DSS weight loss and colon length were not altered with VSL#3 even though intestinal permeability was not increased in WT and *Muc2*$^{-/-}$ animals. Surprisingly, VSL#3 administration did not significantly alter pro-inflammatory or growth factors responses in WT and *Muc2*$^{-/-}$ mice during the acute phase of disease (Day 7) or following recovery (Day 15). This study indicates that colitis induced by DSS that directly affects epithelial barrier function and causes severe, acute, and deep ulcerations, was insensitive to probiotic treatments with VSL#3.

Introduction

The intestinal epithelial barrier is composed of host and bacterial proteins present in the lumen, the mucus layer and proteins embedded in it, and intestinal epithelial cells (IEC) and tight junctions (TJ) between them [1, 2]. Central to this barrier is the mucus layer which is critical for the proper functioning of the barrier and for maintaining a healthy gut. The inner portion of the mucus layer is impermeable to bacterial species and forms a protective coat on the surface of IEC while the outer layer is colonized by commensal bacteria to maintain homeostasis. In Muc2 deficient animals the epithelial cell surface is directly exposed to bacteria which can bind to and penetrate deep into the intestinal crypts, even prior to intestinal injury[3]. In addition, without the normal compliment of commensal bacteria which occupy the mucus layers, intestinal barrier integrity is compromised as discussed in Chapter 4, and animals are more susceptible to a variety of infections including attaching and effacing bacteria[4], and parasitic infections[5, 6].

The probiotic mixture VSL#3 modulates epithelial barrier function in various models[7]. VSL#3 as well as other probiotics can alter the composition of bacteria or bacterial proteins present in the lumen favouring beneficial commensals while others induce mucin secretion to prevent contact with IEC or alter the expression of pro-inflammatory cytokines expressed by these cells. Thus probiotics, either single or multi-species can act on a several parameters to increase barrier function. The probiotic mixture VSL#3 contains eight different bacterial species all of which are normally found in the intestinal microflora, and this mixture has shown efficacy in a variety of human clinical trials[8-10]. At present it is not known how these bacteria exert their beneficial effects and it is

unclear whether these probiotics require an intact mucin barrier from which to exert their protective functions. The aim of this study was to examine whether the probiotic mixture VSL#3 could alter the onset, progression or recovery of DSS-induced colitis in WT and Muc2 deficient mice.

Materials & Methods

Animals and Treatment Regimen

In these studies, 10-12 week old C57BL/6 mice (WT) were obtained from Charles River (Saint-Constant, QC), while *Muc2*$^{-/-}$ mice of the same genetic background were bred in house. Animals were housed in filter top cages and fed irradiated, autoclaved chow *ad libitum*. VSL#3 was re-suspended in 0.5mL tap water and administered by oral gavage once daily from three days prior to DSS to Day 15. Three different concentrations of VSL were used, referred to as low (2.25×10^9), medium (4.5×10^9) and high dose (9.0×10^9 cfu/ mouse/ day). Control animals received an equivalent volume of tap water by oral gavage. Control and VSL#3 treated animals received tap water in their bottles, while the DSS and DSS+VSL#3 mice were exposed to DSS dissolved in tap water. To induce mild colitis, WT mice were given 3% DSS for 5d, while *Muc2*$^{-/-}$ animals received 1% DSS for 3d. More severe colitis was induced in WT mice with 3.5% DSS for 5d while *Muc2*$^{-/-}$ received 1% DSS for 5d. All animals received water *ad libitum*. Weight loss and disease activity index (DAI) scores were assessed daily. DAI was determined based on weight loss, stool consistency, blood loss and overall appearance as previously described in Chapter 4. Where necessary, animals were euthanized prior to the terminal endpoint of the study in accordance with the animal handling protocols at the University of Calgary.

Intestinal permeability

Permeability of both the small and large intestine was assessed using specific sugar probes in metabolic cages. The sugar probes used were sucrose (100mg), lactulose (12mg), mannitol (8mg) and sucralose (6mg), per mouse dissolved in 200µL tap water. For three weeks prior to commencing the study, animals were gavaged with sugar probes and placed in metabolic cages for 16h (overnight) once per week, to become acclimatized to the new surroundings and reduce stress during the study. During their stay in the metabolic cages, the drinking water was supplemented with 1% sucrose to prevent the animals from becoming dehydrated. Animals were then treated with low dose VSL#3 from Days 0 to 35 and gavaged and placed in metabolic cages overnight on the 5^{th} day. Urine samples were collected and analyzed for the sugar probes by high-performance liquid chromatography (HPLC; Dr. J. Meddings, University of Calgary, AB).

In studies where animals were placed on DSS, intestinal permeability was assessed by movement of the inert marker fluorescein isothiocyanate (FITC) dextran (3-5kDa; Sigma Aldrich) from the lumen into the circulation as previously described in Chapter 4. Briefly, animals were gavaged with 15mg of FITC dextran dissolved in 100µL of tap water. After 3h, animals were euthanized by CO_2 asphyxiation and the blood collected by cardiac puncture using a $25^{5}/_{8}$-gauge needle (Becton Dickinson, Mississauga, ON). Blood was stored in the dark at room temperature for approximately 2h to facilitate clotting, and red blood cells were precipitated by centrifugation (10,000 x *g*, 10 min, RT). Serum was diluted with phosphate buffered saline (pH 7.3), and 100µL of each sample was loaded in duplicate on a black bottom 96-well plate and the fluorescence was

measured at an excitation wavelength of 485 nm with an emission wavelength of 530nm on a fluorescence plate reader.

Histopathology

The full length of the colon (cecum to rectum) was excised and the length measured in millimeters. The colon was opened longitudinally along the mesenteric border and the fecal contents removed. The colon was then rolled from the distal end to form a swiss roll and pinned using a $25^{5}/_{8}$-gauge needle. A sharp razor blade was used to section the swiss rolled colon into 3 parts, where one portion was snap frozen for protein isolation, another frozen in Qiazol reagent (Qiagen, Toronto, ON) for RNA isolation and the third fixed in Carnoy's solution for histology. Tissue samples were paraffin embedded, and microtome sections (7μm) were rehydrated through an ethanol gradient to water. Slides were stained with hematoxylin and eosin (H&E; EMD Chemicals, Gibbstown, New Jersey) to visualize overall morphology or Periodic acid Schiff`s reagent (PAS) to examine for neutral mucins.

Quantification of cytokine expression

Total RNA was isolated from snap-frozen tissue using the trizol reagent method (Invitrogen) as per manufacturer's specifications, and the yield and purity determined by spectroscopic analysis. To further purify the RNA, it was precipitated using lithium chloride precipitating solution (Ambion; Invitrogen) before random hexamer primers (Invitrogen) and Moloney murine leukemia virus (MMLV) reverse transcriptase

(Invitrogen) were used to prepare cDNA from 1µg of RNA. qRT-PCR was performed using 1µg cDNA with 10µL SyBr Green master mix (Invitrogen) and 100µM primer concentration in a final volume of 20µL per reaction. qRT-PCR was performed using a Rotor Gene 3000 (Corbett Research Kirkland, QC), and the results analyzed using the $2^{-\Delta\Delta CT}$ method[11].

Inflammatory mediators and growth factors

One third of the full length of the colon was snap frozen at -80^{o}C for protein isolation. Samples were thawed in 300µL phosphate buffered saline containing 30µL protease inhibitor cocktail (Sigma-Aldrich), homogenized and again frozen at -80^{o}C to facilitate further cell lysis. Samples were centrifuged at 14,000 x *g* for 5 min and 70µL of the clear supernatant filtered through a 0.2µm filter (Millipore, Billerica, MA). This filtered product was analyzed to determine the protein concentrations of various chemokines and cytokines by Mouse 32-plex assay (Eve Technologies, Calgary, AB), while the unfiltered product was stored for complete cell lysis and use in western blotting and ELISA.

Statistics

Data were analyzed using the student t-test and analysis of variance (ANOVA) with a Tukey or Kruskal Wallis post-test application where necessary, using the software GraphPad Prism version 4 (Graph-Pad Software, San Diego, CA). Data are reported as the mean ± standard error of the mean (SEM).

Results

The probiotic mixture VSL#3 is widely used in clinical trials and has shown efficacy in maintaining remission in mild and moderate ulcerative colitis (UC) in adults and children[12-14], and preventing pouchitis following ileal pouch-anal anastomosis (IPAA) surgery[9, 10]. However the mechanism by which VSL#3 exerts these protective effects is not well understood. In this study, the murine model of DSS colitis was used since it has been shown to induce colonic lesions that are similar to UC[15]. In addition, DSS colitis is an excellent model to investigate the entire disease process from onset, through progression, into the recovery phase of injury[16, 17].

VSL#3 does not alter intestinal permeability in healthy controls

Probiotics modulate intestinal epithelial barrier function both *in vitro* and *in vivo*, and strengthen barrier integrity. Therefore, it was of interest to determine the effect of probiotic treatment on epithelial barrier function in healthy controls. Mice were gavaged daily with a low dosage of VSL#3 and intestinal permeability was examined using the sugar probes lactulose and mannitol since the Lac:Man ratio measures small intestine permeability and sucralose measures colonic permeability. As shown in Fig. 5-1 continuous probiotic treatment over 35 days did not alter the small or large intestinal permeability in WT and Muc2 deficient animals, as compared to controls.

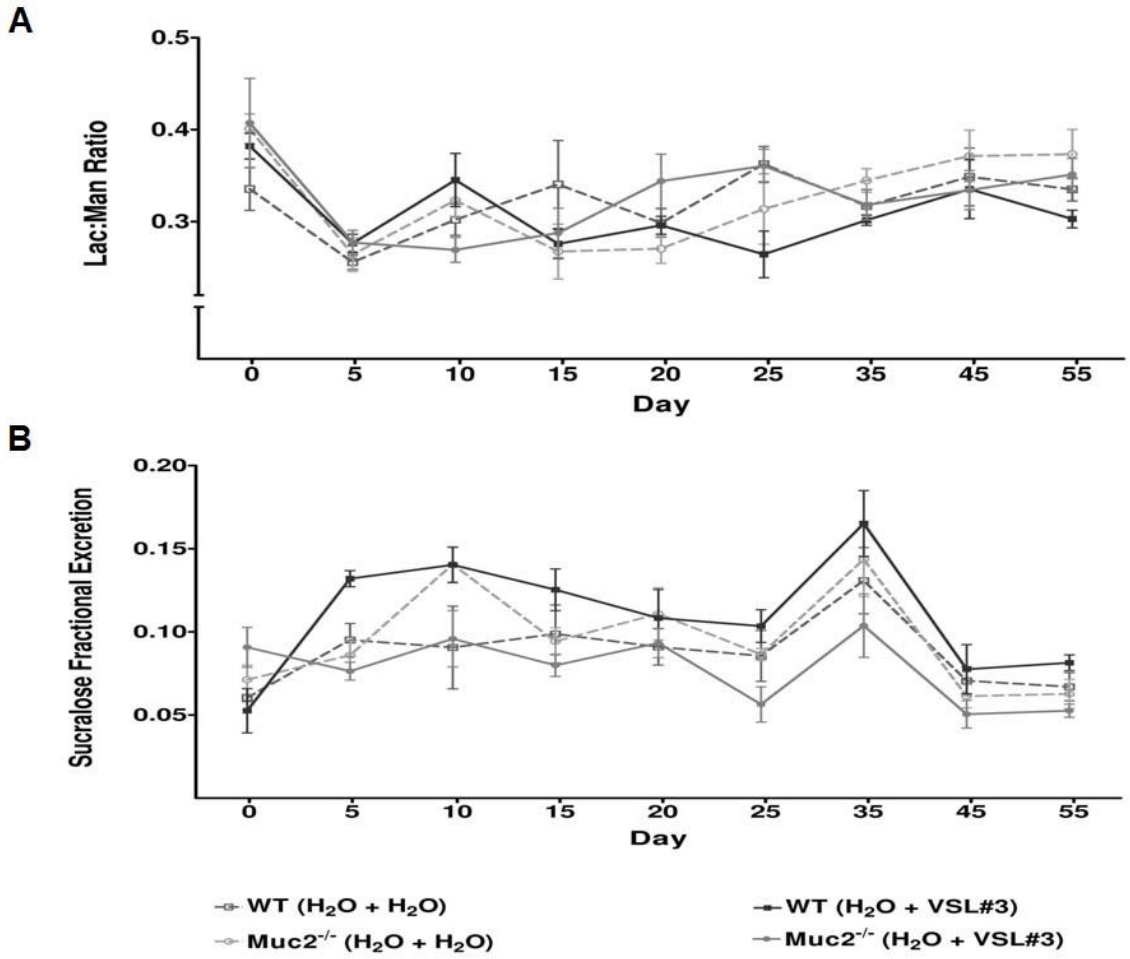

Figure 5-1: Prolonged exposure to VSL#3 does not alter intestinal permeability. Animals were gavaged with a low dose of VSL#3 (2.25 x 10^9 cfu/ mouse/ day) as well as a sugar solution containing sucrose, lactulose, mannitol and sucralose, and placed in a metabolic cage overnight. The urine sugar content was examined by HPLC. Prolonged treatment with VSL#3 did not alter small (**A**) or large (**B**) intestinal permeability in healthy WT or *Muc2*$^{-/-}$ mice. n = 5-6 animals per group

VSL#3 induced mucin secretion in WT but not Muc2$^{-/-}$ *mice*

Even though VSL#3 did not alter intestinal permeability in response to the sugar probes, it was of interest to determine if the probiotic altered mucin secretion or induced signs of histological healing. As shown in Fig. 5-2, VSL#3 treated WT animals showed intense mucin filled goblet cells and robust mucus secretion forming a mucus plug throughout

the gut similar to what has been observed in rats[18]. In Muc2 deficient animals however, VSL#3 did not alter crypt morphology or the thickness of the muscle layer.

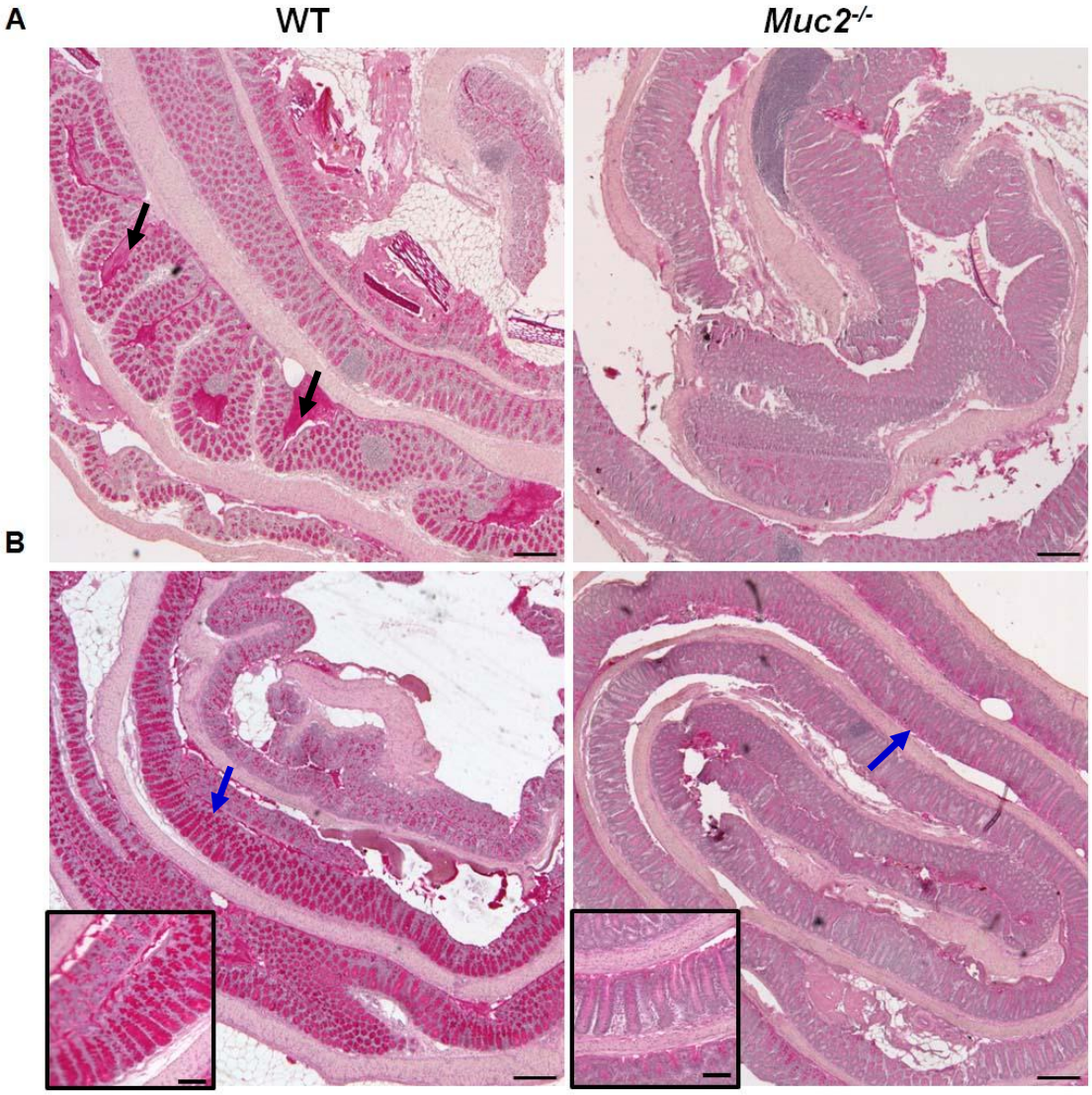

Figure 5-2: VSL#3 induced mucin secretion in WT mice but had no effect in *Muc2*$^{-/-}$ animals. The full length of the colon was swiss-rolled from the distal (centre of picture) to the proximal end. Tissue sections from control animals treated with water only (Panel A), and those gavaged with low dose VSL (Panel B) were stained with PAS reagent to visualize neutral mucins. **A.** WT mice constitutively produce and secrete mucins into the intestinal lumen and these form a protective coat on the surface of IEC (black arrows). *Muc2*$^{-/-}$ animals however lack these mucins. **B.** VSL#3 induced mucin secretion in WT mice as demonstrated by the magenta stained crypts throughout the colon, and these were absent in *Muc2*$^{-/-}$ animals (blue arrows). As shown in the insets, at higher magnification, goblet cells of WT mice were teeming with neutral mucin stained magenta, while this was not apparent in *Muc2*$^{-/-}$ animals. Scale bar represents 200μm, and 100μm in insets.

WT and Muc2$^{-/-}$ mice recover following DSS induced colitis

Since VSL#3 enhanced mucin secretion in WT animals, it was reasoned that it might be beneficial in disease conditions where the mucin layer is critical in disease pathogenesis. Furthermore, VSL#3 may require a mucin substrate to colonize and/or to exert its beneficial effects. Based on this premise, it was of interest to determine the effect of probiotic treatment in injured animals with and without an intact mucin barrier. In addition, since VSL#3 could exert its effects during disease onset, progression or recovery, it was necessary to utilize a disease model that facilitated examination of each of these distinct phases. For this reason the DSS model of colitis was used.

As was previously shown in Chapter 4, WT and *Muc2*$^{-/-}$ mice differ in their susceptibility to DSS, necessitating different dosages in these studies. WT mice were given a dosage of 3% DSS for 5d, and these animals lost approximately 15 - 20% body weight with no significant changes in disease activity index (DAI) scores, as assessed by stool consistency, rectal bleeding or overall appearance. Furthermore, when DSS was removed, these animals proceeded to gain weight until Day 15 where almost complete recovery (as assessed by weight gain) had occurred (Fig. 5-3). A comparable level of damage was induced in *Muc2*$^{-/-}$ mice treated with 1% DSS for 3d followed by a recovery phase until Day 15. *Muc2*$^{-/-}$ mice however, were slower to recover and weight gain did not reach that of control values by Day 15 (Fig 5-3).

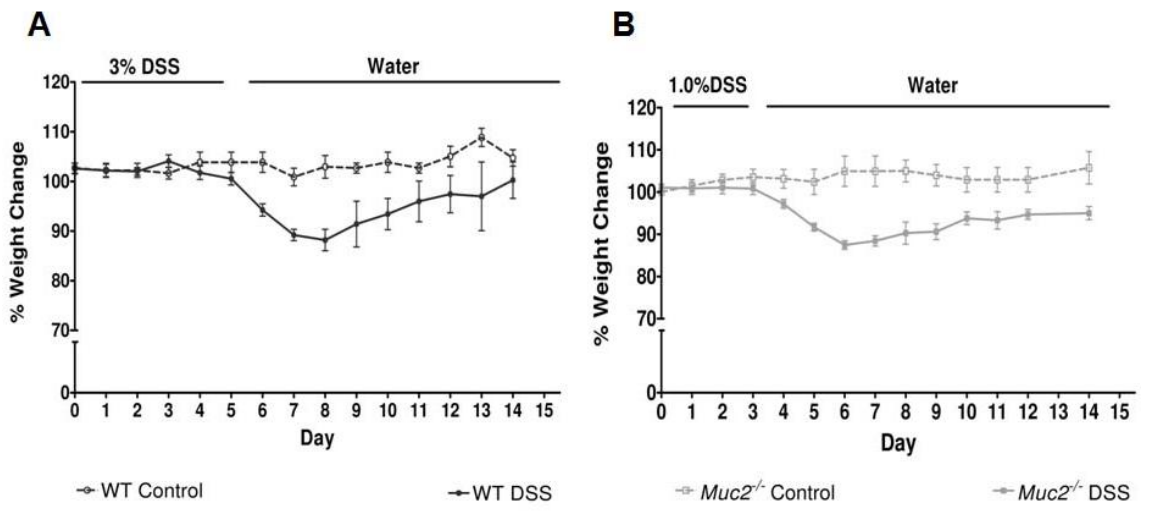

Figure 5-3: WT and *Muc2*$^{-/-}$ mice treated with DSS initially lose weight, but regain this shortly after DSS is removed. WT and *Muc2*$^{-/-}$ mice were treated with DSS in the drinking water and weight change and disease activity index scores measured. WT animals received 3% DSS for 5d (**A**) while *Muc2*$^{-/-}$ mice got 1% DSS for 3d (**B**). DSS was removed and replaced with tap water and animals were followed for 15 days. WT control n = 4, WT DSS n = 8, *Muc2*$^{-/-}$ control n = 3, *Muc2*$^{-/-}$ DSS n = 12.

VSL#3 does not alter disease progression in severe DSS colitis

While all animals lost weight at the dosages used, no changes were observed in any of the other DAI parameters assessed including stool consistency, blood loss or overall appearance. Therefore it was reasoned that a modest increase in DSS would result in a more severe colitis where other measures of DAI, could also be assessed. In addition, VSL#3 reduced the disease severity in WT mice treated with 3.5% DSS for 7d[19] suggesting that VSL#3 was beneficial in these animals even in severe disease. Therefore in this study, the dosage used in WT mice was increased to 3.5% DSS for 5d. Since *Muc2*$^{-/-}$ were severely susceptible to DSS however, rather than increasing the amount of DSS, the time of exposure was slightly increased to 5d instead of 3d. In these studies, animals were examined daily to assess weight change and DAI as markers of disease

progression, and sacrificed at Day 15 to assess the extent to which recovery had occurred. As was shown in Chapter 4 and also in Fig. 5-3, when DSS is removed, animals continue to lose weight and maximal weight loss occurs between Day 6 and Day 8. Both WT and *Muc2*$^{-/-}$ mice however gained weight after Day 9 indicating that animals are recovering. Therefore the question assessed here was whether probiotic treatment could reduce the severity or facilitate recovery following intestinal injury.

Currently, there exists no clear consensus on what dosage of probiotic is required to achieve clinical efficacy in rodent models or humans infections. Thus, it was necessary to determine which dosage of VSL#3 was most efficacious in this model. Three dosages of VSL#3 ranging from low (2.25 x 10^9 cfu), medium (4.5 x 10^9 cfu) to high (9.0 x 10^9cfu/mouse/day) were arbitrarily chosen. In addition, since the goal of this study was to determine whether or not VSL#3 could alter the severity of DSS induced injury animals were pre-treated with VSL#3 for 3 days prior to the induction of colitis, and this was continued during disease progression and for a 10-day recovery period afterwards. Disappointingly, VSL#3 did not alter disease onset, progression or recovery with either low (Fig. 5-4) or medium (Fig. 5-5) or high dosage (Fig. 5-6).

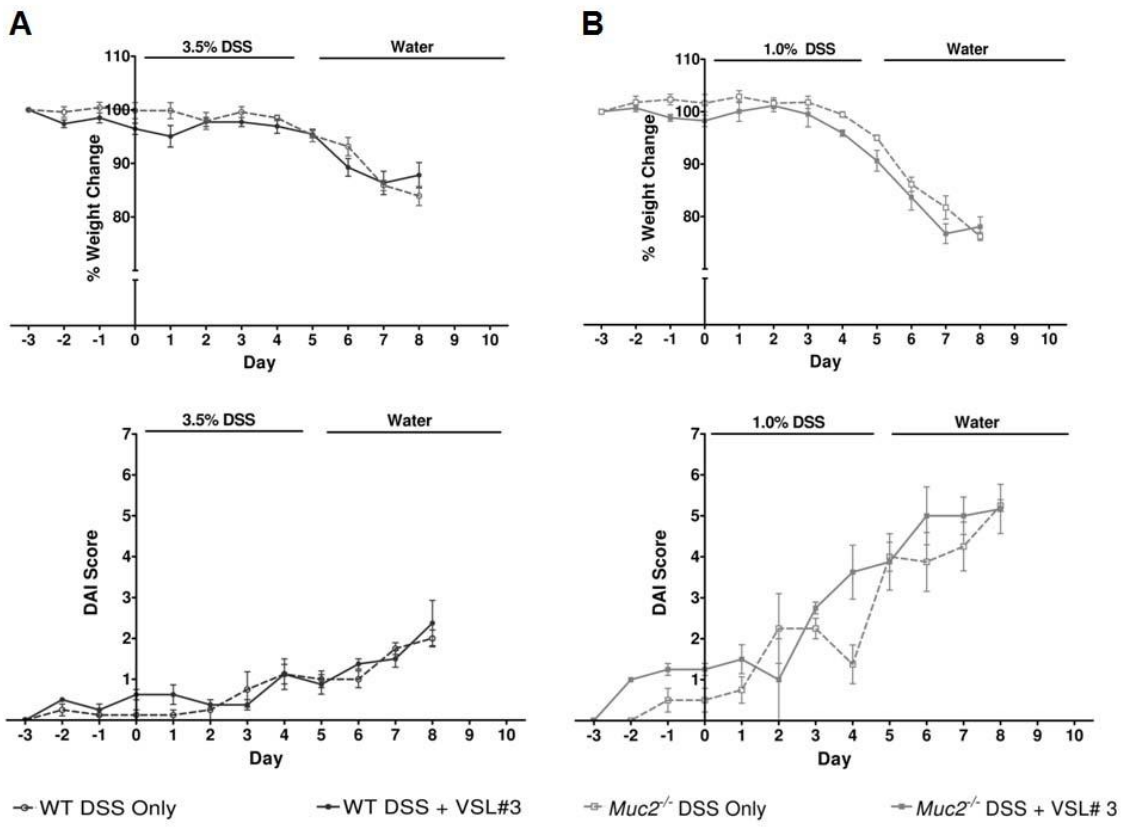

Figure 5-4: At low doses VSL#3 did not alter the severity of DSS induced colitis. Animals were treated with $2.25x10^9$ cfu/ mouse/ day for the duration of the study, and weight change and DAI scores assessed. Both DSS and DSS + VSL#3 induced similar levels of weight loss and DAI scores in WT (**A**) and *Muc2*$^{-/-}$ (**B**) mice commencing from as early as Day 4 of treatment. n= 4 animals per group.

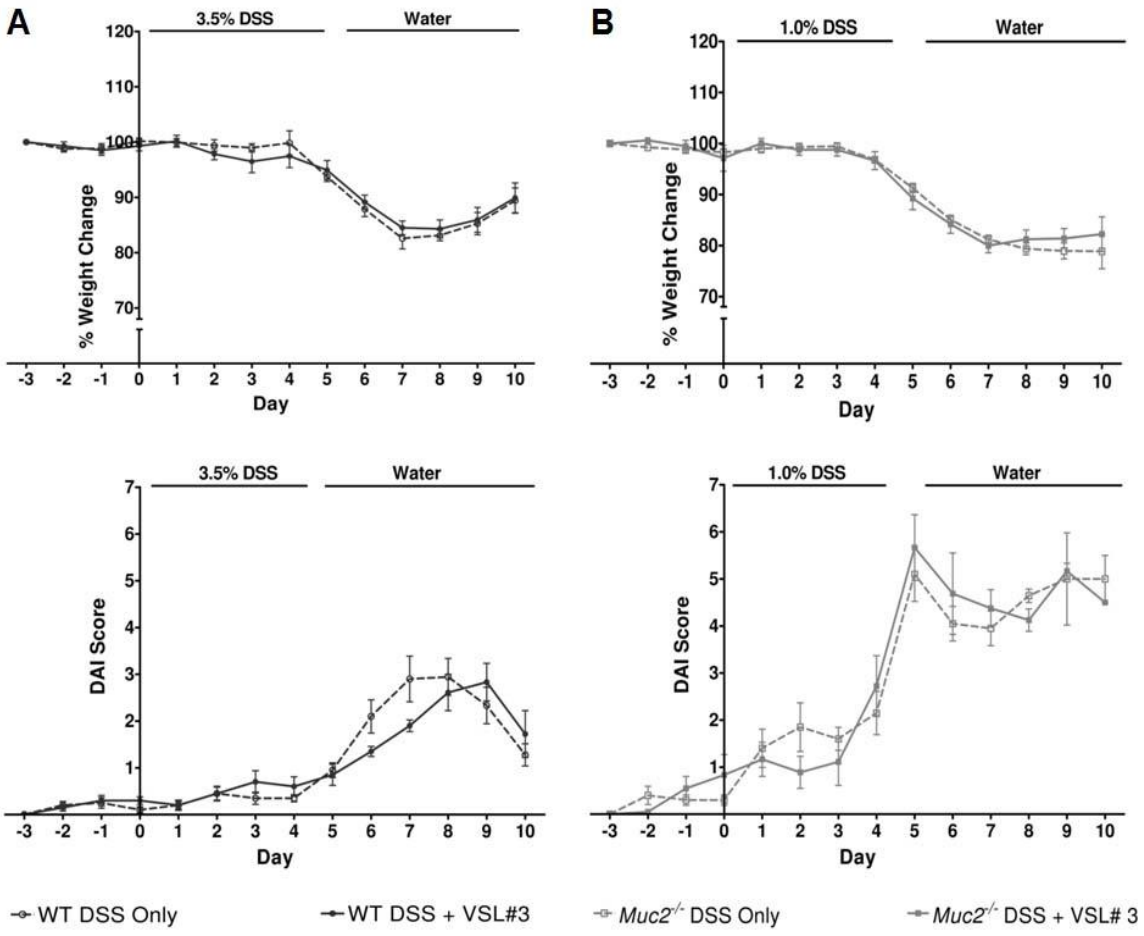

Figure 5-5: At medium doses VSL#3 did not change the disease profile in either animal group. Animals were treated with a medium dose of VSL#3 (4.5×10^9 cfu/ mouse/ day), and the effect on disease progression assessed by weight change and DAI. At medium doses VSL#3 did not improve DSS severity in the presence (**A**) or in the absence (**B**) of an intact mucin layer. n = 9-10 animals per group.

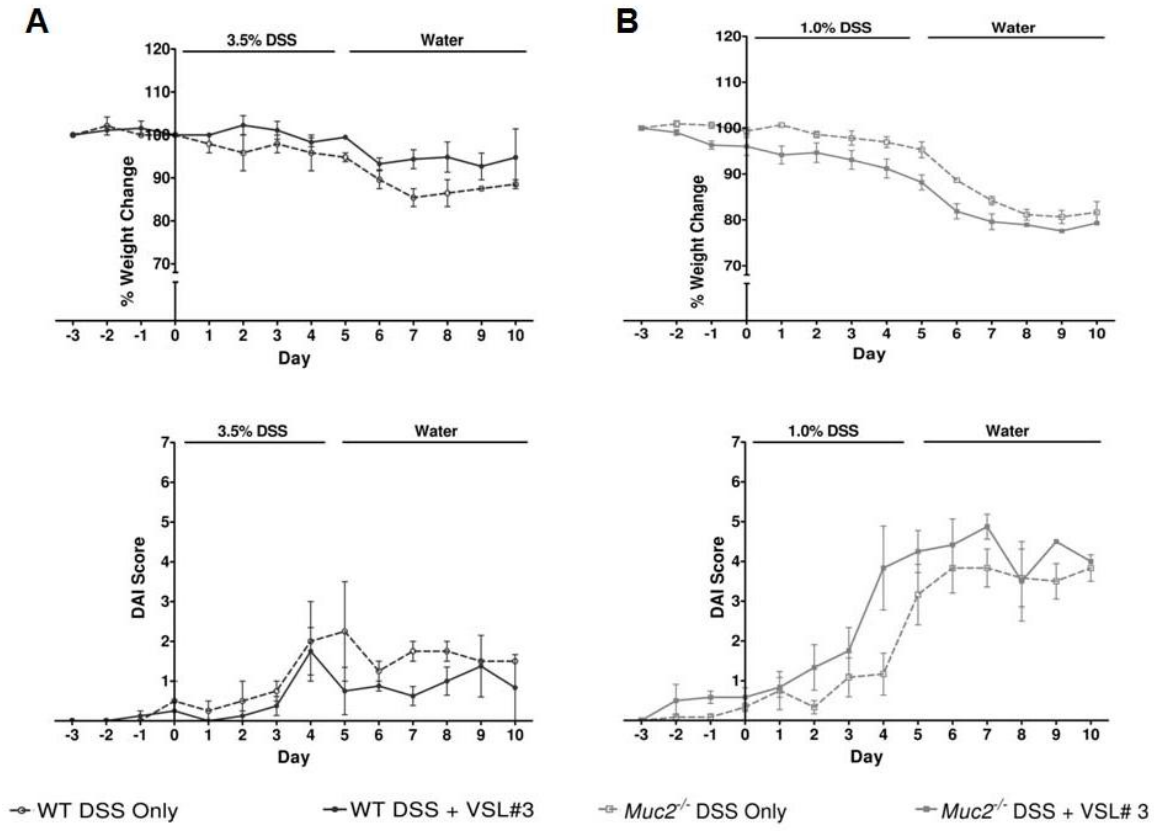

Figure 5-6: High doses of VSL#3 did not significantly alter DSS colitis. The effect of a high dose (9.0 x 10^9 cfu/ mouse/ day) of VSL#3 was assessed in WT (**A**) and *Muc2*$^{-/-}$ (**B**) mice with severe colitis. VSL appeared to improve weight loss in WT mice, but this was not significant and no change was observed in DAI scores in these animals. In contrast, at high doses, VSL#3 did not significantly alter disease progression in *Muc2*$^{-/-}$ mice. WT DSS and DSS + VSL#3 n = 2-4 animals per group, *Muc2*$^{-/-}$ DSS and DSS + VSL#3 n = 6.

VSL#3 did not alter disease progression in mild DSS colitis

At medium and high dosages VSL#3 treatment did not significantly improve disease progression in WT mice or *Muc2*$^{-/-}$ mice. However, WT mice treated with low dose VSL appeared to recover earlier than their DSS only treated counterparts, suggesting that a low dose may be more beneficial in this model. Previous studies have shown that low dosages of VSL# 3 ameliorated the severity of DSS induced injury in BALB/c mice[19]. Therefore it was reasoned that perhaps at the levels used here, the damage induced by

DSS was too severe to see any beneficial effect of the probiotic. Accordingly, the dosage of DSS given to WT or *Muc2*$^{-/-}$ animals was lowered to attain 100% survival rate and similar levels of damage as assessed by weight loss. Under these conditions, VSL#3 still did not alter disease onset, progression or recovery as measured by weight change (Fig. 5-7).

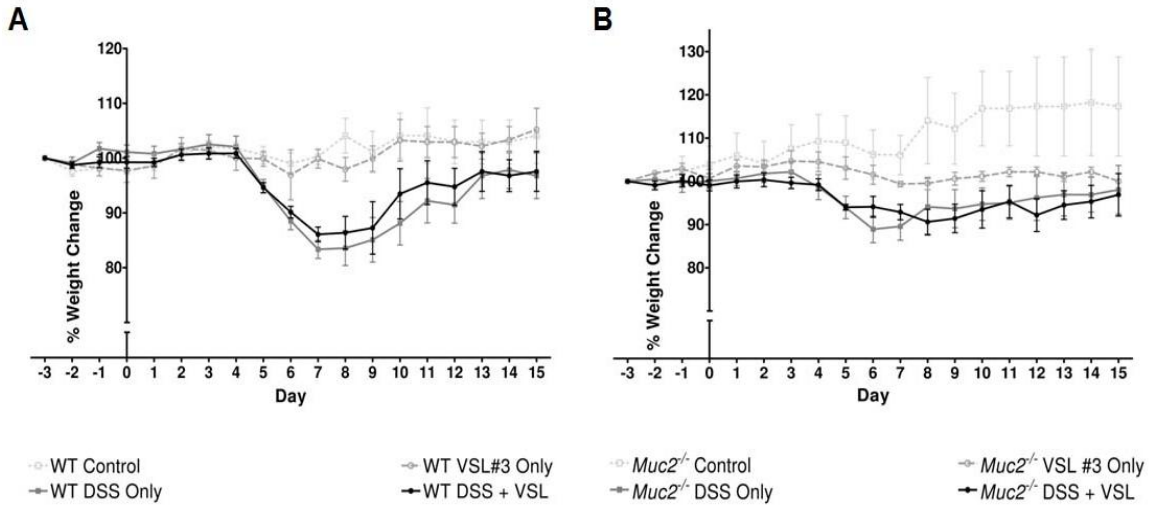

Figure 5-7: VSL#3 did not ameliorate disease severity in mild colitis. Since VSL#3 did not ameliorate the severity of colitis in earlier studies, it was reasoned that the colitis was too severe. Therefore milder colitis was induced (WT: 3% DSS, 5d; *Muc2*$^{-/-}$ 1% DSS, 3d). At low doses (2.25×10^9 cfu) VSL#3 did not ameliorate the severity of colitis in WT (**A**) or *Muc2*$^{-/-}$ (**B**) mice as compared to their non-VSL treated counterparts. n = 6-8 animals per group.

Since VSL#3 was administered by oral gavage while DSS was applied to the drinking water, it was possible that VSL#3 treatment may have been affecting water consumption. To determine if this was the case, water consumption of mice given DSS was monitored. As shown in Fig. 5-8, there was no significant change in water consumption regardless of the treatment regime indicating that water consumption was not a contributory factor in the response observed in VSL#3 treated animals.

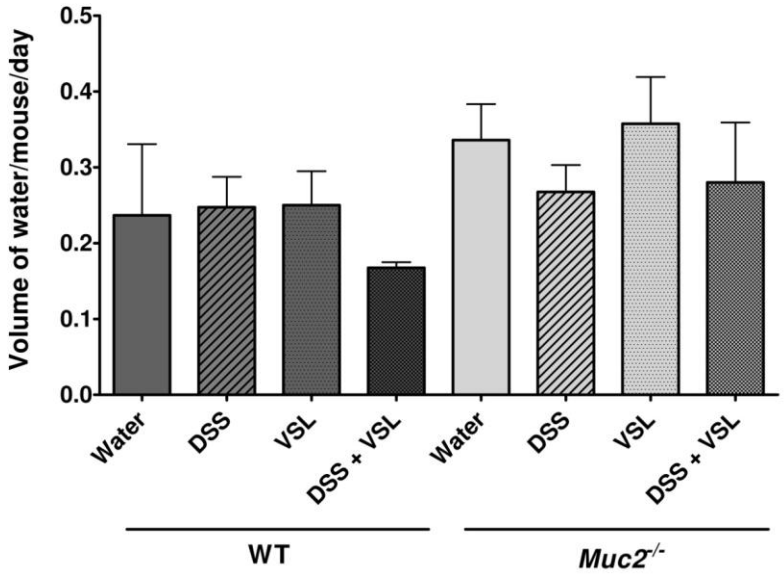

Figure 5-8: VSL#3 did not alter water consumption in WT or *Muc2*$^{-/-}$ colitic animals. The change in water level was assessed in each cage during the period over which animals were treated with DSS. VSL#3 gavage did not alter the amount of water consumed per animal. DSS doses: WT mice 3% DSS 5d, *Muc2*$^{-/-}$ 1% DSS 3d. n=4-6 animals per group.

Since colon shortening is associated with colitis, colon length following treatment was examined (Fig. 5-9). As expected, DSS treatment significantly shortened the colon in WT mice during the peak phase of disease (7d), but this returned to normal levels during the recovery phase (15d). In *Muc2*$^{-/-}$ mice however, recovery was not attained as assessed by colon length. VSL#3 treatment did not alter the length of the colon at either Day 7 or Day 15 as compared to the non-VSL treated controls, suggesting that VSL treatment was neither beneficial nor deleterious under these conditions.

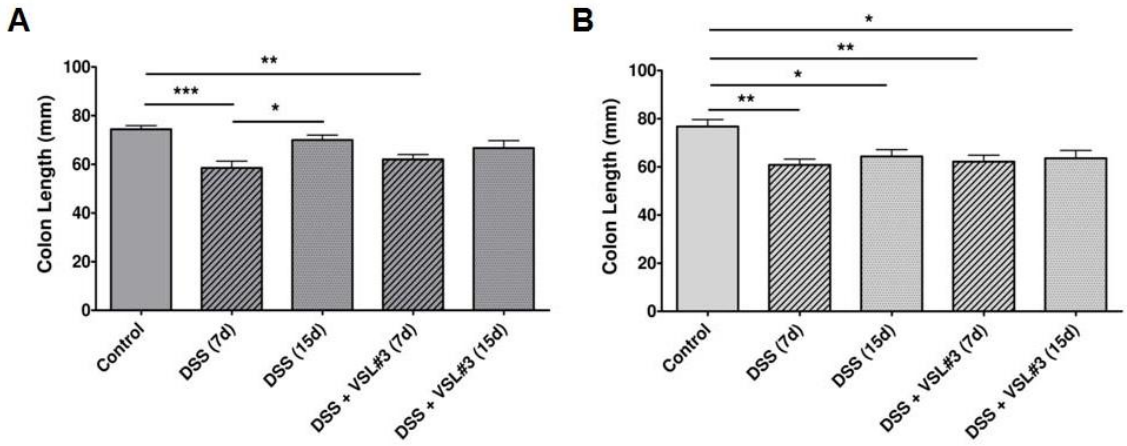

Figure 5-9: VSL#3 did not alter colon length in response to DSS in WT or *Muc2*$^{-/-}$ mice. Since acute colitis is associated with shortening of the colon, length was assessed in response to DSS and VSL#3. DSS alone induced colon shortening in WT (**A**) and *Muc2*$^{-/-}$ (**B**) animals during acute disease (7d), but in WT mice this increased to control levels at Day 15, suggestive of repair, while it did not in *Muc2*$^{-/-}$ mice. Treatment with VSL#3 did not alter colon length in either animal group at Day 7 or Day 15. DSS doses: WT mice 3% DSS 5d, *Muc2*$^{-/-}$ 1% DSS 3d. n = 4-6 animals per group, *p<0.05, **p<0.01, ***p<0.001.

Since no overt changes were observed in weight gain or colon length it was necessary to evaluate whether histological healing was present in recovering animals. As shown in Fig. 5-10, DSS induced an intense inflammatory cellular infiltrate in both WT and *Muc2*$^{-/-}$ mice at Day 7 which was attenuated in WT animals at Day 15. In VSL#3 treated animals, a noticeable change in the inflammatory cellular infiltrate was not observed despite increased mucin secretion in WT mice at Day 15 (Fig. 5-11).

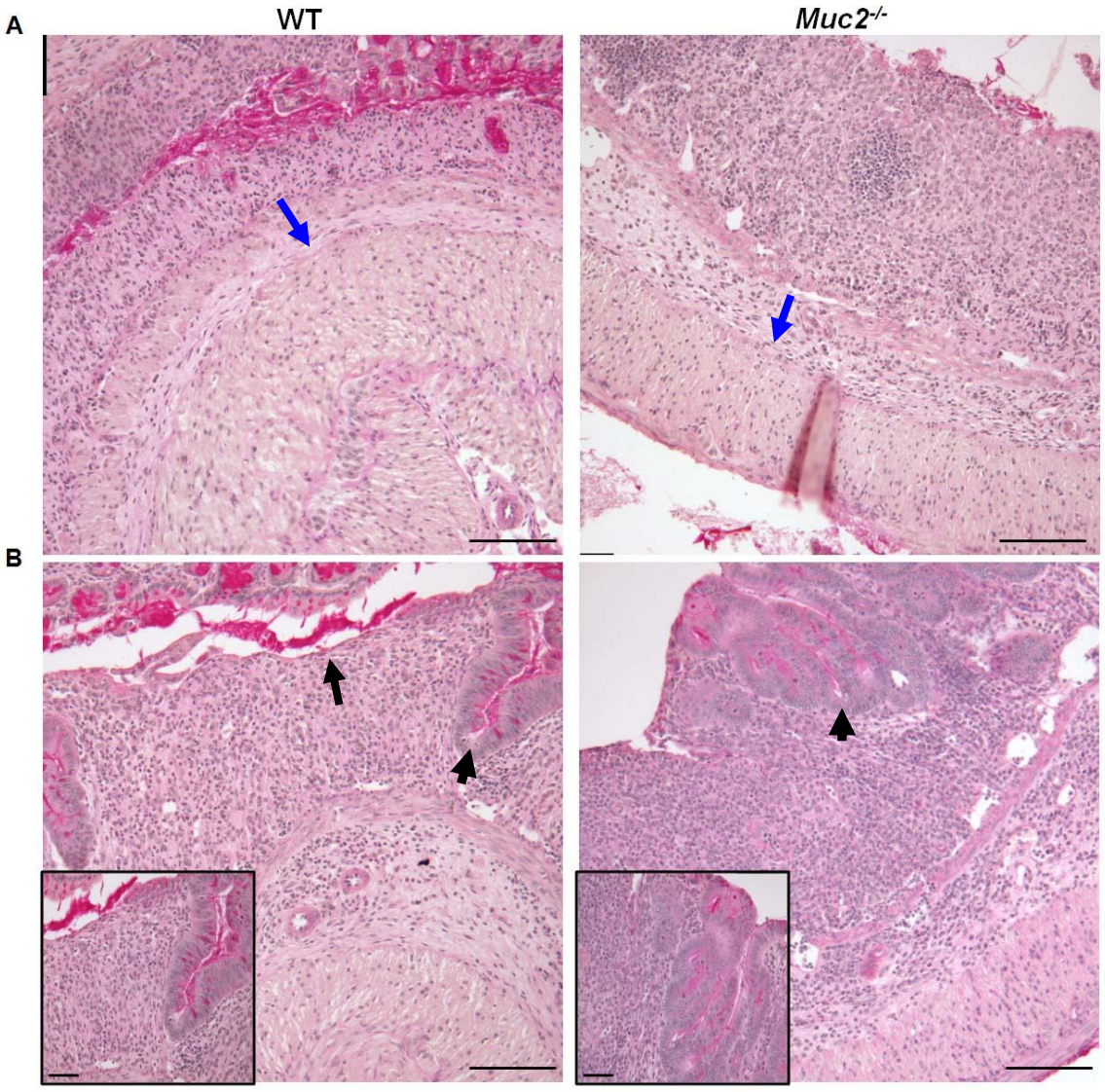

Figure 5-10: WT and *Muc2*$^{-/-}$ mice show signs of repair following DSS colitis. **A**: At Day 7, during the acute phase of injury, DSS induced lesions in WT and *Muc2*$^{-/-}$ mice as indicated by loss of goblet cell mucin, thickening of the muscle layer (blue arrows), and loss of crypt architecture. **B:** Signs of epithelial repair are evident at Day 15, as indicated by epithelial restitution (arrows) and the formation of crypts (arrow heads). Insets show the region of crypt formation at higher magnification. DSS doses: WT mice 3% DSS 5d, *Muc2*$^{-/-}$ 1% DSS 3d. All sections were stained with PAS reagent; scale bar 100μm in panels A and B, and 50μm in insets.

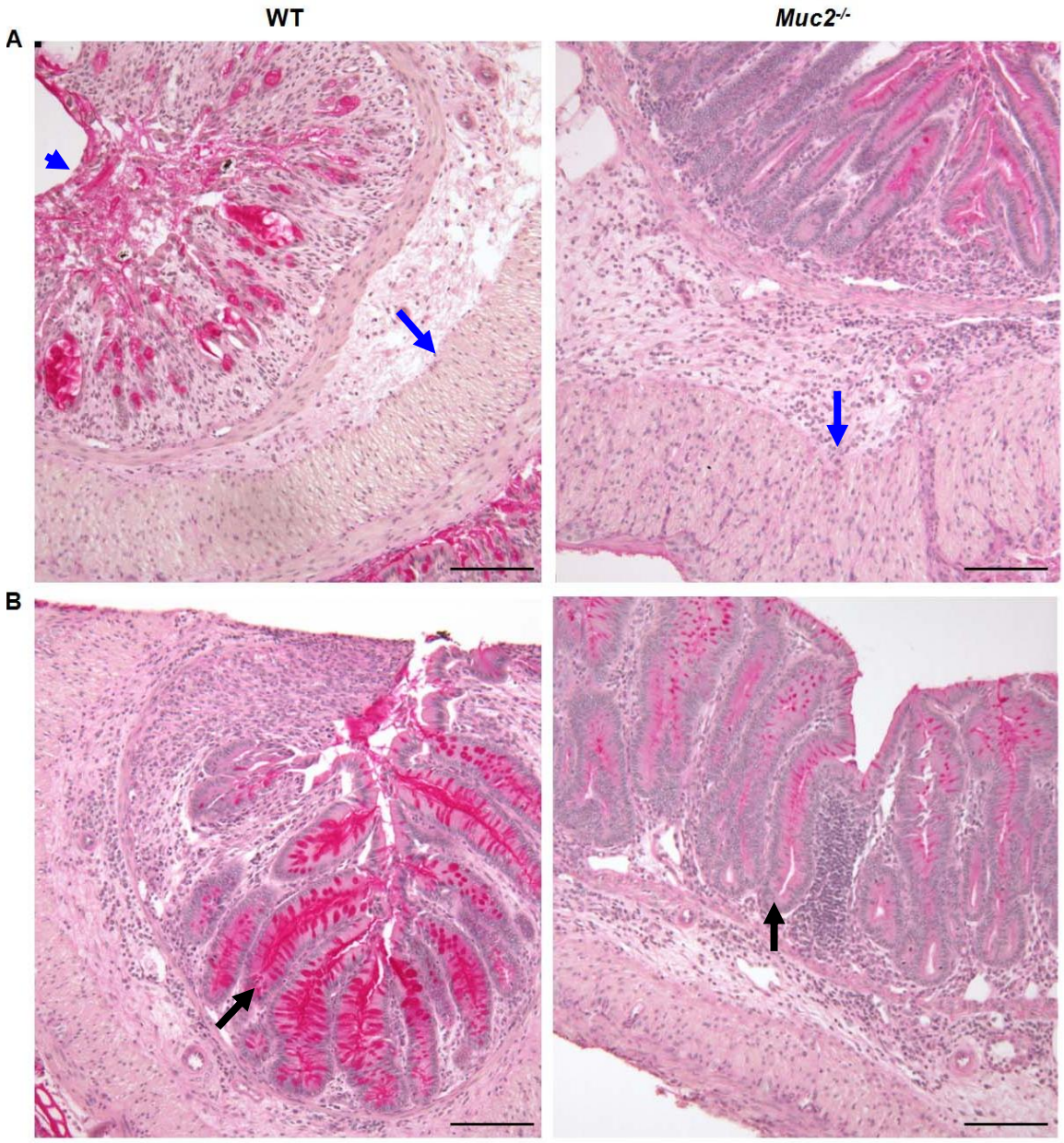

Figure 5-11: VSL#3 did not alter the histopathology associated with DSS colitis in WT or *Muc2*$^{-/-}$ mice. **A**: During acute injury, VSL#3 did not attenuate the damage induced by DSS as evidence by thickening of the muscle layer (blue arrows), cellular infiltrate and loss of crypt architecture (blue arrow head). **B**: When DSS was removed, animals recovered following injury as shown by epithelial restitution. VSL#3 treatment did not induce accelerated healing of the lesions in WT or *Muc2*$^{-/-}$ mice though increased mucin secretion was observed in WT mice at Day 15, and this was absent in *Muc2*$^{-/-}$ animals (black arrows). DSS doses: WT mice 3% DSS 5d, *Muc2*$^{-/-}$ 1% DSS 3d. All tissue was stained with PAS reagent; scale bar: 100μm.

VSL#3 did not alter inflammation induced by colitis

One of the hallmarks of intestinal injury is the movement of leukocytes and other inflammatory cells into the site, and this was evident in the histological sections in WT and *Muc2*$^{-/-}$ mice treated with DSS. Since human IL-8 is a key player in leukocyte trafficking, the murine homologs of this chemokine were examined.

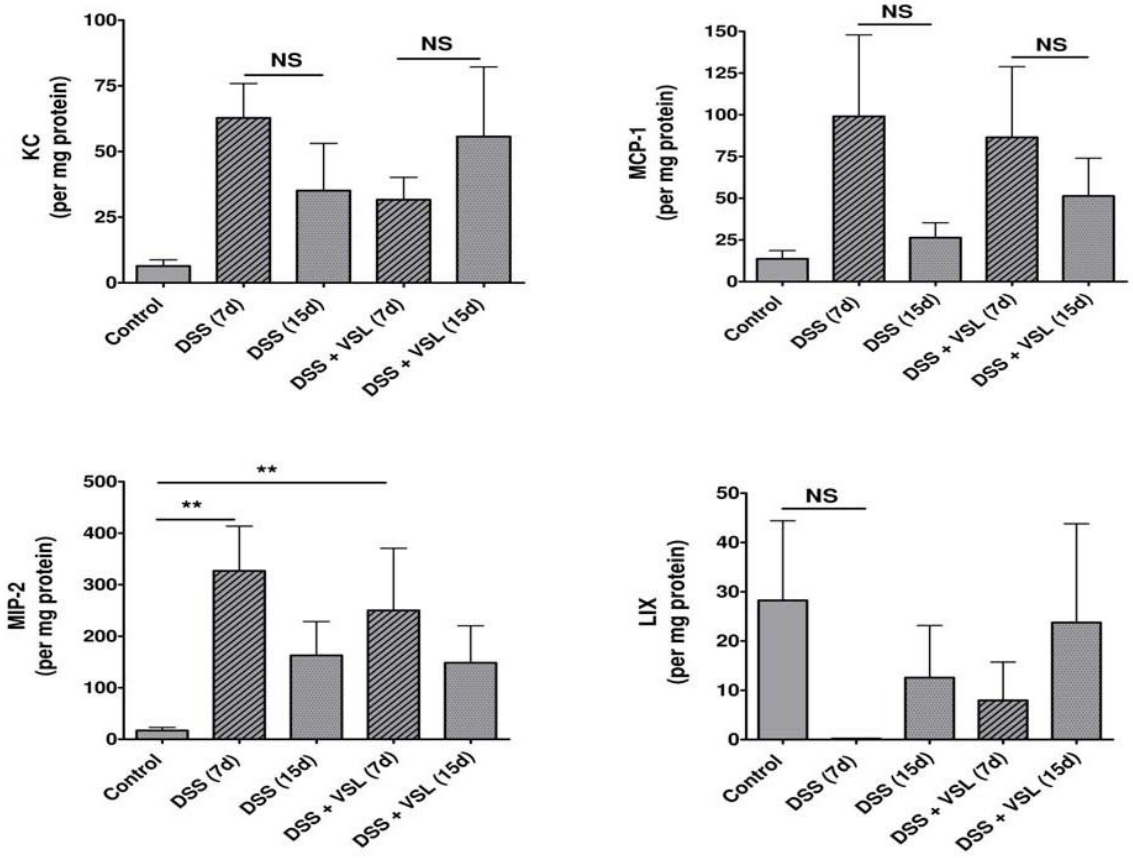

Figure 5-12: VSL#3 induced MIP-2 in WT mice at Day 7. During acute disease, DSS induced the expression of KC, MCP-1, and MIP-2 in WT and this was reduced during recovery. However, statistical significance was only attained in MIP-2 in WT mice. VSL#3 treatment induced MCP-1 and MIP-2 in WT mice at Day 7. DSS doses: WT mice 3% DSS 5d, *Muc2*$^{-/-}$ 1% DSS 3d. n= 4-6 animals per group, NS = not significant, ** $p < 0.01$.

As shown in Fig. 5-12, DSS treatment alone induced KC, MCP-1 and MIP-2 in WT at Day 7 during the acute phase of injury, and this was reduced at Day 15, though statistical

significance was only attained in MIP-2 in WT mice. VSL treatment induced both MCP-1 and MIP-2 at Day 7, and this was reduced at Day 15.

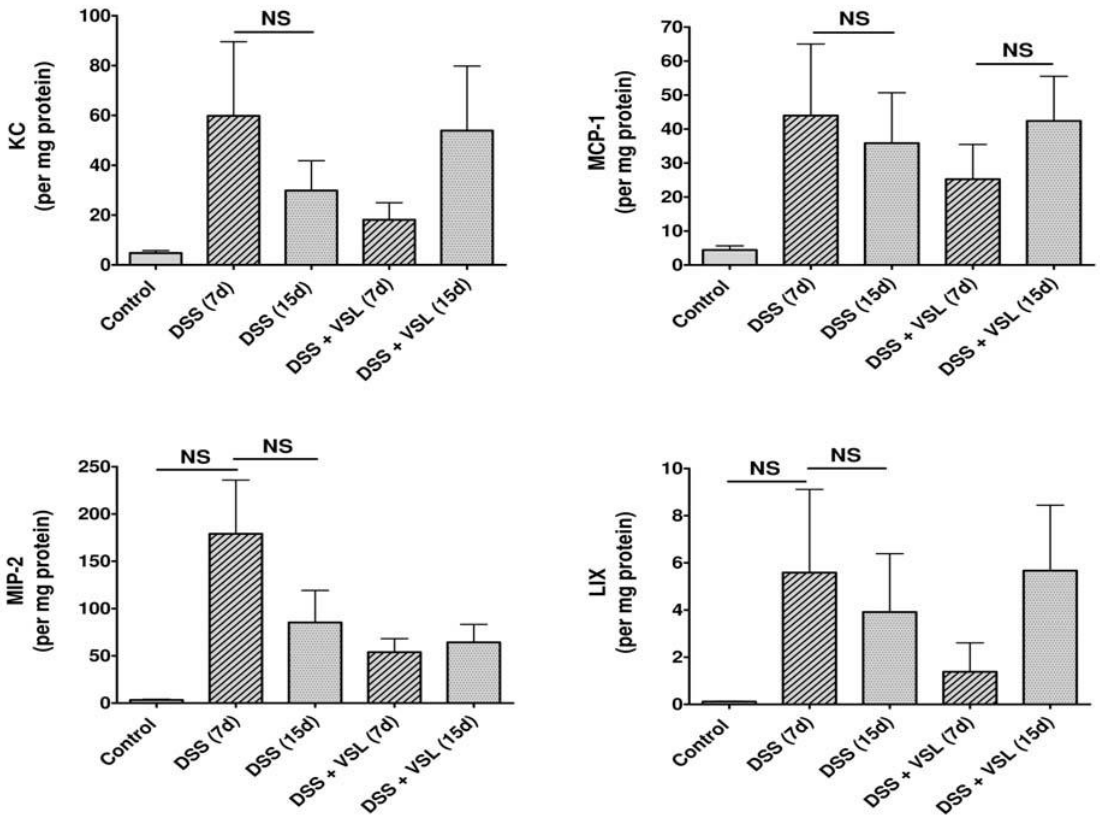

Figure 5-13: VSL#3 did not alter murine IL-8 homologs in *Muc2*$^{-/-}$ mice. During acute disease, DSS induced the expression of KC, MCP-1, MIP-2 and LIX in *Muc2*$^{-/-}$ animals and this was reduced during recovery, though statistical significance was not attained. VSL#3 treatment did not alter the expression of these chemokines at Day 7 or Day 15. DSS doses: WT mice 3% DSS 5d, *Muc2*$^{-/-}$ 1% DSS 3d. n= 4-6 animals per group, NS = not significant.

In *Muc2*$^{-/-}$ animals, while a trend to increased KC, MCP-1, MIP-2 and LIX levels was observed at Day 7 and reduced at day 15, significance was not observed (Fig. 5-13). While changes in colon length and histopathology provide meaningful data, neither of these provide information pertaining to the integrity of the epithelial cells or the tight junctions between them, and both are critical in maintaining epithelial barrier function.

For this reason, it was of interest to determine if VSL#3 altered intestinal permeability in the absence of overt histological changes. For these experiments intestinal permeability to FITC dextran was examined. As predicted, DSS treatment increased intestinal permeability to FITC at Day 7, and this was reduced at Day 15 in both WT and *Muc2*$^{-/-}$ mice (Fig. 5-14). No change in intestinal permeability was observed in animals treated with VSL at Day 7 and 15. Interestingly, intestinal permeability in *Muc2*$^{-/-}$ mice was almost twice that observed in WT animals (based on fluorescence units) indicating that colonic damage was more severe in these animals.

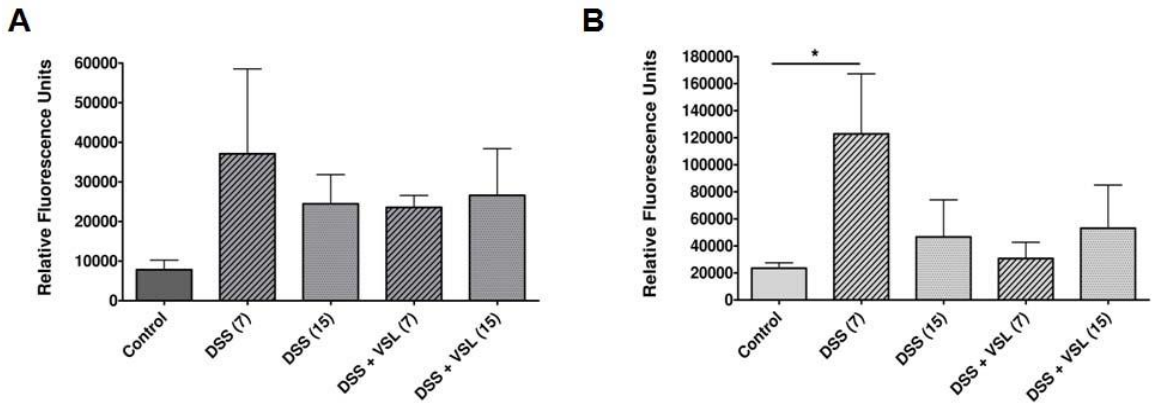

Figure 5-14: Intestinal permeability in DSS and VSL#3 treated animals. Intestinal permeability in WT (**A**) and *Muc2*$^{-/-}$ (**B**) mice was assessed by examining the levels of 3-5kDa FITC dextran in serum. WT and *Muc2*$^{-/-}$ mice showed increased permeability in response to DSS at Day 7, and this was reduced at Day 15. FITC dextran movement in *Muc2*$^{-/-}$ animals was almost twice that observed in WT animals. DSS doses: WT mice 3% DSS 5d, *Muc2*$^{-/-}$ 1% DSS 3d. n=4-6 animals per group, * $p < 0.05$.

VSL#3 did not alter the inflammatory response in WT or Muc2$^{-/-}$ *mice*

One of the ways by which VSL#3 exerts its protective effects in limiting colitis and maintaining epithelial barrier function is by suppressing acute and chronic inflammation and promoting growth factors[7, 20, 21]. Therefore it was of interest to determine if VSL#3 altered either the gene expression or the protein levels of the inflammatory mediators.

Four pro-inflammatory mediators were examined based on their role associated with gut injury and involvement in increased intestinal permeability. One of the earliest markers of inflammation is Il-1β, and IEC produce this cytokine in response to injury[22], necessitating its examination in this model. In addition, the inflammation induced by DSS is primarily driven by Tnf-α and Ifn-γ, and both of these have been shown to increase intestinal permeability by altering TJ proteins, so it was of interest to examine these as well. Furthermore, PGE_2 has been shown to alter TJ permeability leading to diarrhea[23], so it was of interest to examine this lipid mediator.

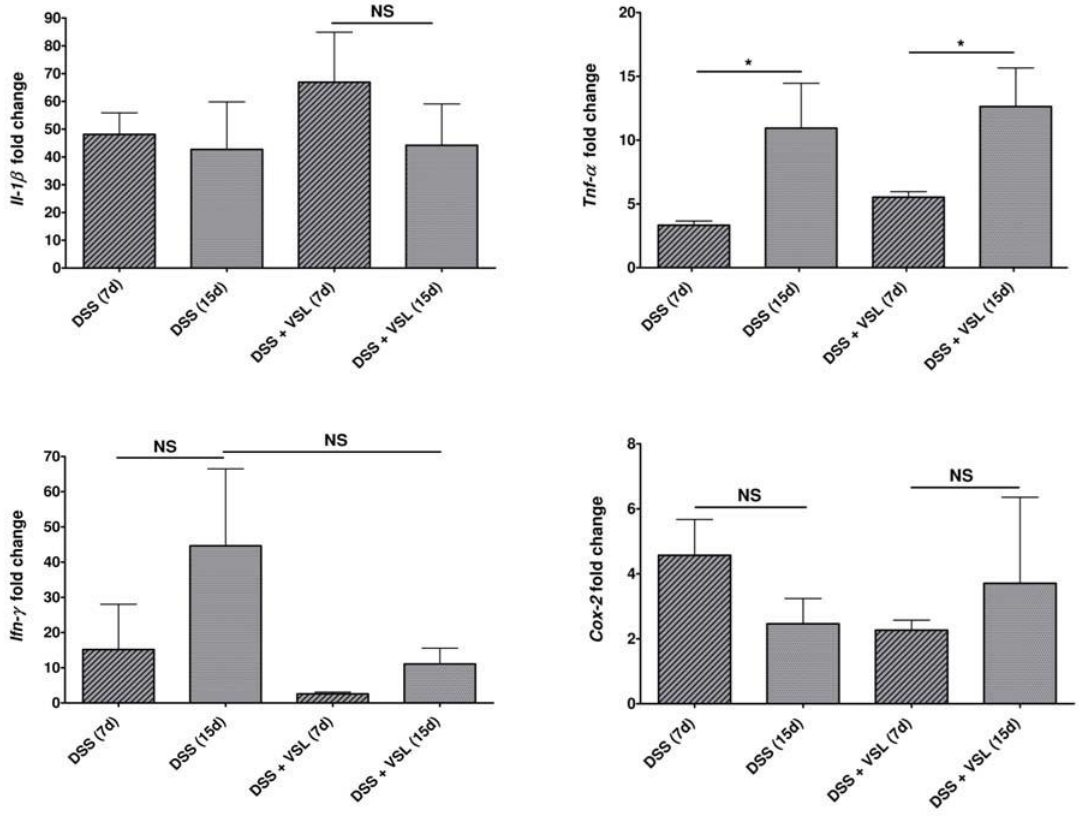

Figure 5-15: VSL#3 did not alter the inflammatory response in WT mice treated with DSS. qRT-PCR was used to assess the relative expression of the genes encoding *Il-1β*, *Tnf-α*, *Ifn-γ* and *Cox-2* in WT mice. DSS alone induced the expression of these cytokines at Day 7 and this remained high at Day 15. Surprisingly, *Tnf-α* levels were increased during recovery, as was *Ifn-γ* in these animals VSL#3 did not alter the expression in any of these cytokines at either Day 7 or Day 15. DSS doses: WT mice 3% DSS 5d, *Muc2*$^{-/-}$ 1% DSS 3d. n=4-6 animals per group. Data expressed as fold changed compared to control. NS = not significant, *p<0.05.

As shown in Fig. 5-15, DSS alone induced the expression *Il-1β*, *Tnf-α*, *Ifn-γ* and *Cox-2* in WT mice at Day 7, and in the case of *Tnf-α* and *Ifn-γ* this was further increased at Day 15. VSL#3 treatment did not significantly alter these at either Day 7 or Day 15. A similar trend was observed in *Muc2*$^{-/-}$ animals (Fig. 5-16) however the fold change was considerably less in these mice as compared to their WT counterparts.

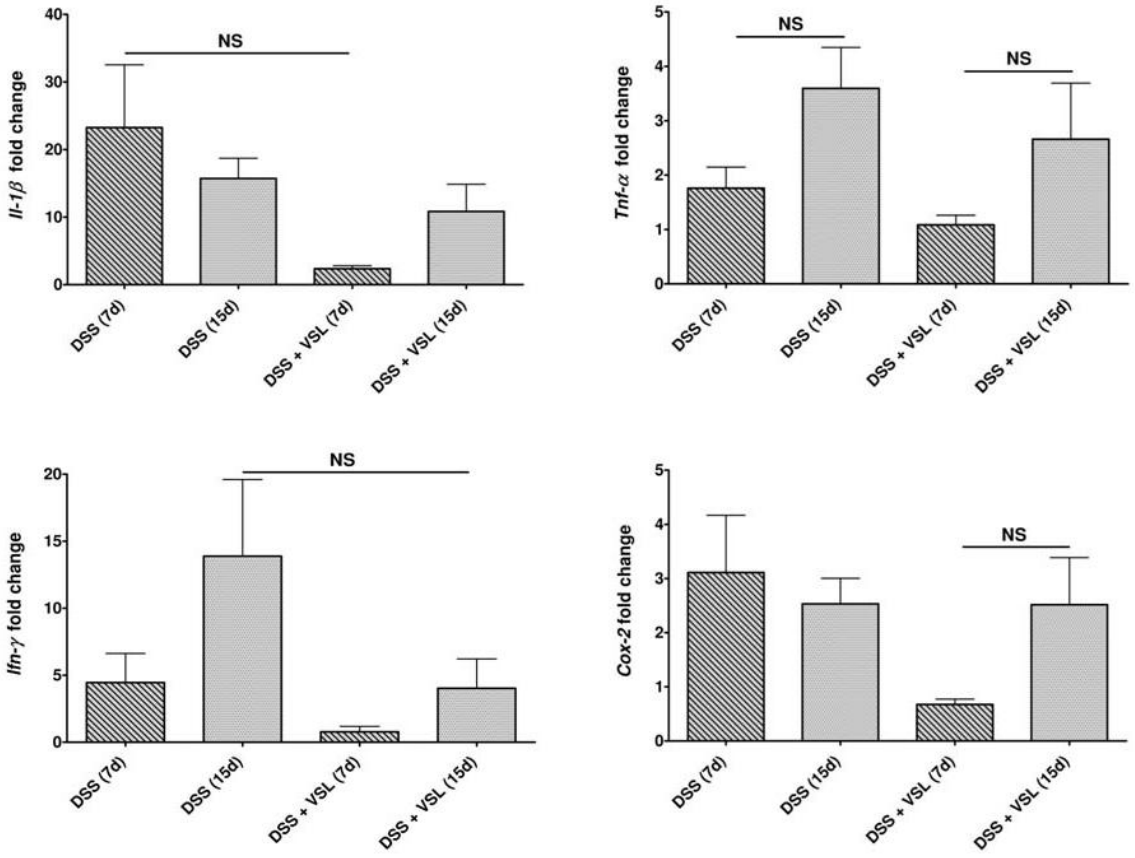

Figure 5-16: VSL#3 did not alter the inflammatory response in *Muc2*$^{-/-}$ mice treated with DSS. The relative expression of *Il-1β*, *Tnf-α*, *Ifn-γ* and *Cox2* was assessed by qRT-PCR in *Muc2*$^{-/-}$ mice. DSS alone substantially induced *Il-1β* and *Ifn-γ* at Day 7 and this was not significantly changed at Day 15. Curiously DSS did not significantly alter the expression of *Tnf-α* or *Cox-2* in these animals at Day 7 or Day 15. Furthermore, VSL#3 treatment did not alter the expression of these cytokines as compared to the DSS only treated animals. DSS doses: WT mice 3% DSS 5d, *Muc2*$^{-/-}$ 1% DSS 3d. n = 4-6 animals per group. Data expressed as fold change compared to control. NS = not significant.

It was also of interest to examine the protein levels of the pro-inflammatory cytokines in response to VSL#3. DSS induced the expression of Il-1α and Il-1β in WT (Fig 5-17) mice during acute injury and this was further increased in the recovery phase. VSL#3 treatment further induced Il-1α and Il-1β in WT mice at Day 15. DSS also induced Tnf-α in WT mice at both Day 7 and Day 15, though no significant change in Ifn-γ was observed. In the case of Ifn-γ and Tnf-α, VSL#3 did not alter the levels of these cytokines compared to DSS only levels.

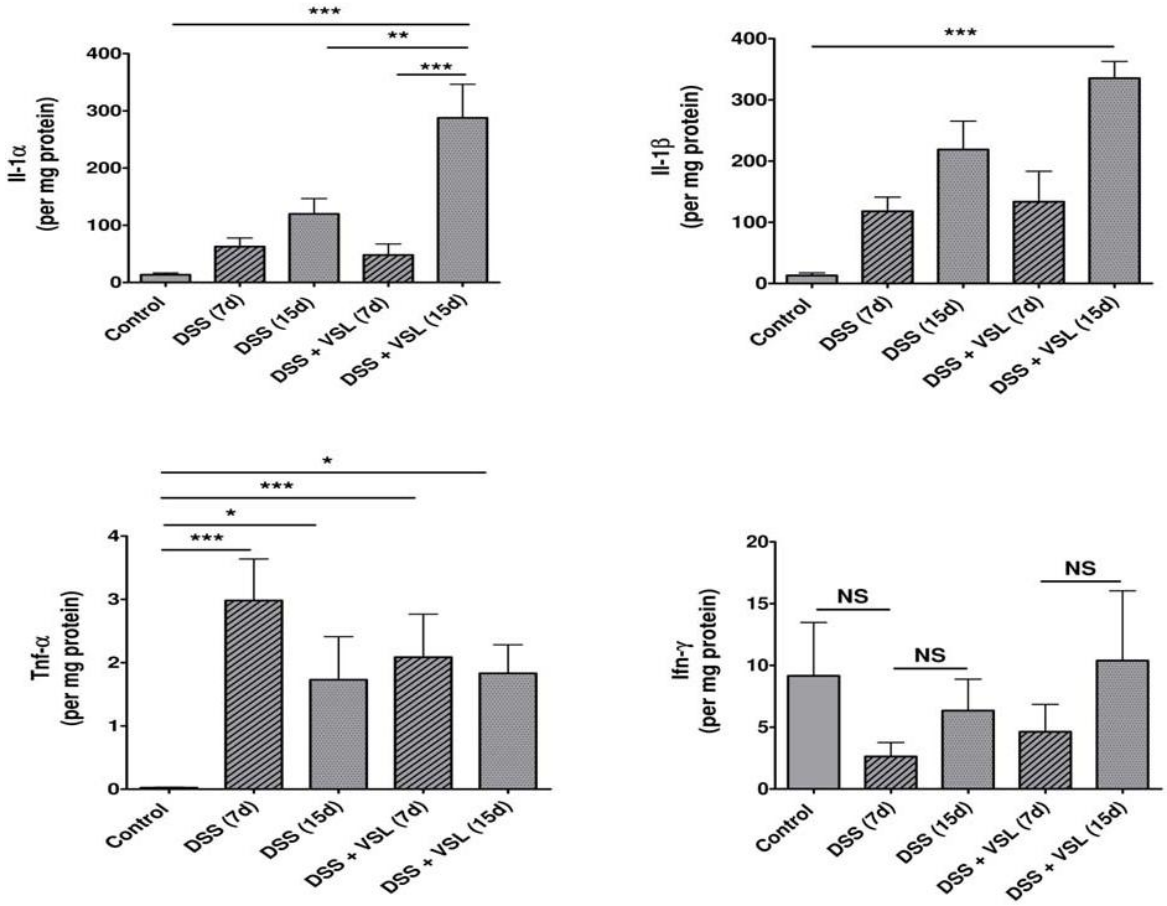

Figure 5-17: VSL#3 did not alter the protein release of the pro-inflammatory cytokines in WT mice. In WT mice, DSS induced the expression of Il-1α, Il-1β and Tnf-α at Day 7 and in the case of Il-1α, and Il-1β, this was further increased at Day 15, while a decrease was observed in Tnf-α. VSL#3 treatment also induced Il-1α and Il-1β at Day 15, but did not alter the levels of Ifn-γ and Tnf-α. DSS doses: WT mice 3% DSS 5d, *Muc2*$^{-/-}$ 1% DSS 3d. n=4-6 animals per group, NS = not significant, *p<0.05, **p<0.01, ***p<0.001.

In *Muc2*$^{-/-}$ mice (Fig 5-18), Il-1α, Il-1β and Ifn-γ were induced in response to DSS at Day 7 and this persisted at Day 15, however only Il-1β changes were significant at Day 15. Again, VSL#3 treatment significantly increased Il-1β at Day 15 compared to DSS only treated animals.

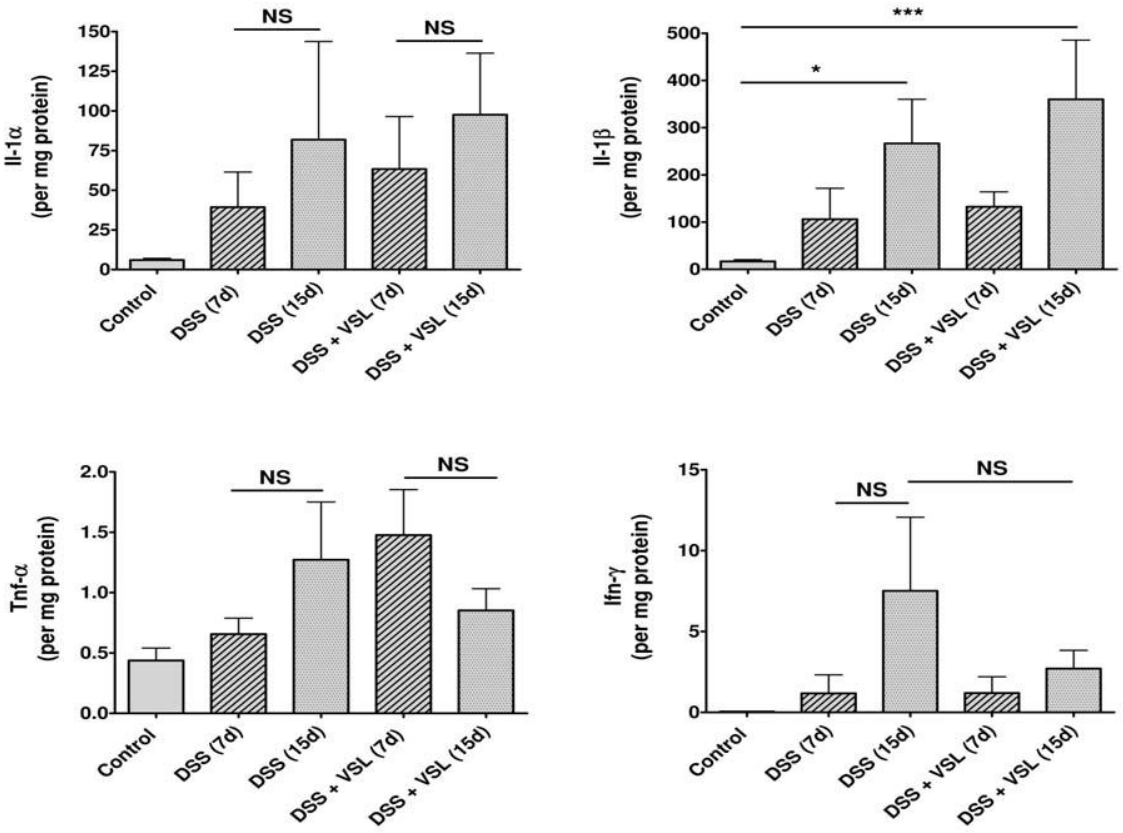

Figure 5-18: VSL did not alter the protein release of the pro-inflammatory cytokines in *Muc2*$^{-/-}$ mice. DSS induced Il-1α, Il-1β and Ifn-γ in *Muc2*$^{-/-}$ mice at Day 7 and these levels remained high at Day 15, however only Il-1β changes were significant, and this only at Day 15. VSL treatment did not significantly change the levels of these cytokines as compare to DSS only treated animals. DSS doses: WT mice 3% DSS 5d, *Muc2*$^{-/-}$ 1% DSS 3d. n=4-6 animals per group, NS = not significant, *p<0.05, ***p<0.001.

One of the key features of intestinal repair following injury is the expression of a variety of growth factors. Both Tgf-β and Vegf-A have been shown to be involved in gastric

ulcer wound healing[24]. Therefore, if was of interest to examine the gene expression levels of these cytokines. Neither DSS alone nor DSS + VSL significantly induced the expression of any of the growth factors examined in WT (Fig. 5-19) or *Muc2*$^{-/-}$ (Fig. 5-20) mice, suggesting that these are not involved in disease recovery at least in this model.

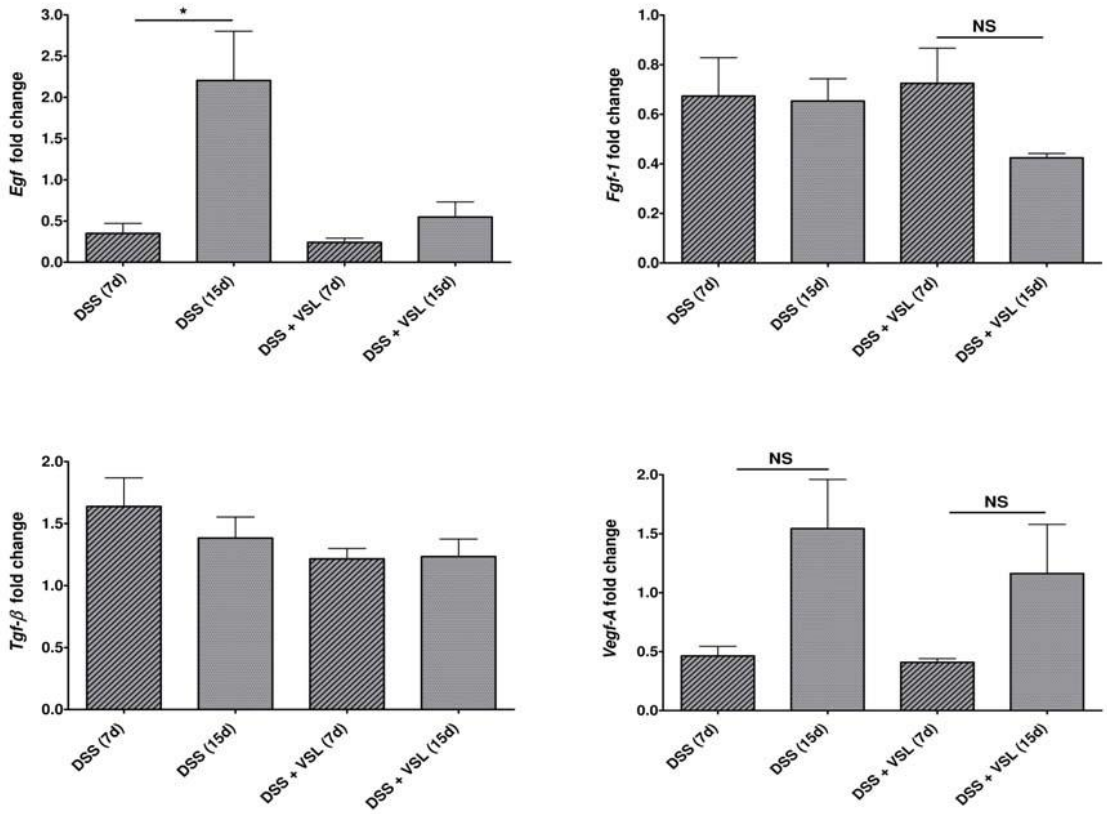

Figure 5-19: VSL#3 did not significantly enhance the expression of growth factors in WT mice. Relative gene expression of the growth factors *Egf*, *Fgf-1*, *Tgf-β* and *Vegf-A* were examined in response to DSS or DSS + VSL#3. *Egf* expression was induced at Day 15, and this was statistically significant, but this represented a 2 fold change compared to controls. Neither DSS nor VSL#3 altered the expression of any of these growth factors in WT mice at Day 7 or Day 15. DSS doses: WT mice 3% DSS 5d, *Muc2*$^{-/-}$ 1% DSS 3d. n=4-6 animals per group, NS = not significant, *$p<0.05$. Data expressed as fold change compared to control.

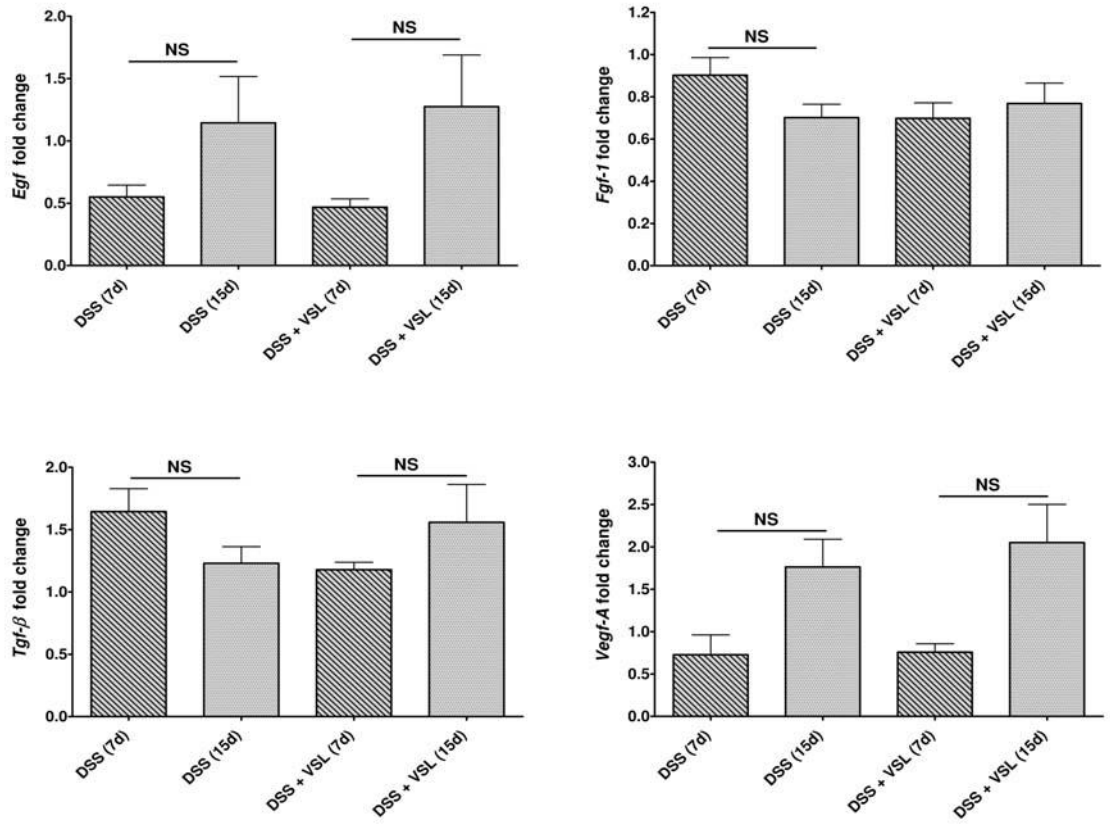

Figure 5-20: VSL#3 did not significantly enhance the expression of growth factors in *Muc2*$^{-/-}$ mice. Gene expression of *Egf*, *Fgf-1*, *Tgf-β* and *Vegf-A* were examined in response to DSS or DSS + VSL#3. None of these growth factors were significantly induced in recovery following DSS, or in response to VSL#3 treatment, at either Day 7 or Day 15. DSS doses: WT mice 3% DSS 5d, *Muc2*$^{-/-}$ 1% DSS 3d. Data expressed as fold change compared to control. NS = not significant.

Discussion

Probiotics have been shown to mediate protective functions *in vivo* to enhance epithelial barrier function. Based on these beneficial effects, and the observation that many bacterial species require the presence of other bacteria to exert their effects, use of probiotic mixtures has received much attention. Perhaps the most successful of these is the mixture VSL#3 consisting of four species of *Lactobacillus* (*L. acidophilus, L. bulgaricus, L. paracasei, L. plantarum*), three *Bifidobacteria* (*B. breve, B. infantum, B.*

longum), and one *Streptococcal* species (*S. thermophilus*). These bacteria species are normal constituents of the healthy, human intestinal microbiota and as such VSL#3 supplementation is simply adding to the already existing bacterial population. In addition, VSL#3 has been shown to mediate protective functions in disease models most notably human UC, but the specific mechanisms of action remain unclear.

The aim of this study was to determine whether or not a mucin substrate was necessary for VSL#3 to exert its protective effects, and secondly to assess if treatment with VSL#3 could compensate for the compromised barrier integrity observed in *Muc2*$^{-/-}$ mice, which rendered them more susceptible to gastrointestinal injury. The data presented here illustrates that in healthy individuals, prolonged exposure to VSL#3 did not alter intestinal permeability and the same was true of DSS colitis animals. In severe colitis low, medium, or high doses, of VSL did not attenuate the severity of disease in either WT or *Muc2*$^{-/-}$ mice as determined by weight loss and DAI scores, and the same was true at lower doses of DSS, which induced less severe disease. In mild colitis, VSL#3 treatment did not alter colon length nor did it significantly alter intestinal permeability as assessed by movement of FITC dextran, in WT animals. In fact, in the absence of an intact mucin barrier DSS increased intestinal permeability to FITC. In addition, VSL#3 treatment did not attenuate the inflammatory responses induced by DSS alone, nor did it significantly change the expression of any of the growth factors associated with wound healing and repair assessed.

When interpreted in light of other data these findings are somewhat controversial. Indeed, other studies using VSL#3 in the murine DSS model of colitis have met with mixed results. One study found that while VSL#3 altered the microflora, it did not induce healing nor did it reinforce the mucus layer [25]. In contrast, others have shown that VSL#3 reduced inflammation and intestinal permeability induced by DSS colitis and this was in part mediated by preventing loss of the various TJ proteins, notably occludin (Ocln) and zona occludens 1 (Zo-1)[19]. On closer examination, it was observed that while both studies were conducted in BALB/c mice, they differed in the dose of DSS and that of VSL#3 used. Gaudier *et al.* used 1% DSS (5d) and 4 x 10^9 cfu VSL#3 while Mennigen *et al.* used 3.5% DSS and 2.7 x 10^9 cfu. Based on these differing responses the dose of VSL#3 in our study was varied to determine if perhaps too much of a good thing was indeed bad. In this model, significant improvement at lower doses of VSL#3 was not observed. In addition, attempts were made to induce milder as well as more severe colitis. Approximately equivalent levels of damage were induced with 3% DSS (5d) in WT mice and 1% (3d) in *Muc2*$^{-/-}$. In either case no improvement occurred in animals treated with VSL#3.

There are several plausible reasons why VSL#3 did not improve disease pathogenesis in DSS colitis. It is possible that the model used is not the best to study the effects of VSL#3. While the exact mechanism of action is unclear, DSS appears to be directly toxic to IEC leading to cell destruction and apoptosis, thereby exposing cells of the lamina propria to luminal contents, commensal bacteria and VSL#3. Therefore, in this setting with a damaged epithelium exposure of the sterile mucosa to VSL#3 could result

in more harm than good. Another alternative is that the level of damage was too great to facilitate recovery. This is unlikely however since WT mice on DSS alone spontaneously recovered as evidenced by improved colon length and reduced intestinal permeability.

Some important recommendations can however be gleaned from these surprising results. Firstly, VSL#3 treatment alone did not alter intestinal permeability in WT or *Muc2*$^{-/-}$ animals, indicating that any effect the probiotic treatment may be having is independent of an intact mucus layer. Secondly, more damage and increased intestinal permeability was observed in *Muc2*$^{-/-}$ mice whether treated with DSS alone or DSS + VSL#3 as compared to their WT counterparts, further demonstrating a protective role for the mucus layer in disease conditions.

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CHAPTER 6: MANUSCRIPT III

Entamoeba histolytica Exacerbates Epithelial Tight Junction Permeability and Pro-inflammatory Responses in *Muc2*$^{-/-}$ Mice

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Abstract

Human MUC2 mucin is the first line of innate host-defence in preventing pathogen-induced epithelial injury. *Entamoeba histolytica* (*Eh*) colonizes the mucus layer by binding of the parasite surface Gal-lectin to Gal and GalNAc residues on colonic MUC2, preventing parasite contact-dependent cytolysis of epithelial cells. In this study, we quantified early innate responses toward *Eh* in WT and Muc2 deficient mice (*Muc2*$^{-/-}$) using closed colonic loops. *Eh* infection in WT but not *Muc2*$^{-/-}$ mice induced a time-dependent increase in ^{3}H-labeled mucin and non-mucin glycoprotein secretion. Immunohistochemical staining revealed intense Muc2 secretion that formed a thick, protective mucus plug overlying the surface epithelium, entrapping *Eh*. In *Muc2*$^{-/-}$ mice, *Eh* induced a pronounced time-dependent secretory exudate with increased gross pathology scores and serum albumin leakage. Colonic pathology, secretory responses and increased pro-inflammatory cytokine secretions of Tnf-α, Ifn-γ and Il-13 correlated with altered expression of the tight junction proteins claudin-2, occludin and Zo-1. The putative *Eh* virulence factor that elicited the pro-inflammatory responses and altered tight junction permeability was identified as *Eh* cysteine protease 5 (EhCP5). This study reveals that colonic mucins confer both luminal and epithelial barrier functions and in the absence of Muc2, animals are more susceptible to *Eh*-induced secretory and pro-inflammatory responses mediated by EhCP5.

Introduction

The surface of the gastrointestinal tract is lined by a viscous mucus layer which acts as a lubricant overlying the surface of intestinal epithelial cells (IECs)[1]. The mucus layer is composed of a dense inner portion, impermeable to enteric bacteria and a more viscous outer part colonized by a variety of commensal species[2]. Colonic Muc2 mucin is the main component of this mucus layer, and is secreted by intestinal goblet cells together with intestinal trefoil peptide (Tff3) and resistin-like molecule (Retnl-β), to mediate innate protective functions[3-5]. The thickness of the colonic mucus layer is reduced in inflammatory bowel diseases (IBD)[6], and *Muc2*$^{-/-}$ mice are highly susceptible to DSS-induced injury and are prone to spontaneously develop colitis and adenomas[7]. Thus, unravelling the myriad of functions of Muc2 in innate host defences is critical for our understanding of host-pathogen interactions and diseases like IBD where the etiology is unclear.

One of the best studied colonic pathogens that interacts with MUC2 is the enteric human protozoan parasite *Entamoeba histolytica* (*Eh*)[1, 8]. *Eh* is the causative agent of amebiasis, resulting in 100,000 deaths worldwide and is the second leading cause of death due to a parasitic infection[9]. In the majority of infections, *Eh* remains as a harmless commensal, but in ~10% of cases invades the colon causing amebic dysentery and/or liver abscesses[9]. *Eh* colonizes the mucus layer and can degrade MUC2 allowing the parasite to bind to and destroy the underlying epithelial cells[1, 5]. In genetically resistant mice, *Eh* does not invade, but colonizes the mucus layer and this is a useful model to investigate the early events in host-parasite interactions in the gut. In this study, we use a closed colonic loop

model of infection to investigate innate immune responses in wild-type (WT) and *Muc2*$^{-/-}$ mice to discern host-defences independent of Muc2 in epithelial barrier function. Our studies reveal that in the absence of Muc2, animals are highly susceptible to *Eh* induced acute pro-inflammatory responses, leading to aberrant protein secretions and altered tight junction (TJ) permeability. These studies highlight the novel role of Muc2 in both luminal and epithelial barrier functions.

Materials & Methods

Animals and colonic loop study

For experiments 10-12 week old male C57BL/6 and *Muc2*$^{-/-}$ mice on the same genetic background were used. WT mice were purchased from Charles River, while *Muc2*$^{-/-}$ animals were obtained from Dr. Velcich[7], and bred in house. Mice were kept in sterilized, filter-top cages, maintained under specific pathogen free (SPF) conditions and provided food and water *ad libitum*. All studies were approved under the University of Calgary Animal Care Committee.

Colonic loops were used as a model for short-term infection studies (1-6h p.i.) as previously described[10]. Briefly, WT and *Muc2*$^{-/-}$ mice weighing 23-25g were fasted for 12h prior to the study. Animals were anaesthetized with 4.25mg/kg sodium pentobarbital (Ceva Santé Animale), dissolved in sterile water, administered by intraperitoneal (i.p.) injection. Under anaesthetic, a laparotomy was performed and the colon exteriorized and ligated with 3.0 black silk sutures (Ethicon Inc., Peterborough, Ontario, Canada) at the proximal (distal to the cecum) and distal end (proximal to the rectum) and one more

ligation was made in the middle forming 2 colonic loops. Care was taken to keep the mesenteries, blood vessels and nerves intact. One million virulent log-phase *Eh* trophozoites in 100μL phosphate buffered saline (PBS, pH 7.3) were inoculated into the proximal loop and the distal loop received PBS. Another group of animals underwent a similar surgical procedure without the ligation of the colon to quantify the effects of surgical manipulations only (sham). Colonic tissues were fixed in Carnoy's solution (3h at 4^{o}C) and embedded in paraffin blocks. Microtome sections (7μm) were rehydrated through an ethanol gradient to water, and stained with hematoxylin and eosin (H&E; EMD Chemicals, Gibbstown, New Jersey), Periodic acid Schiff`s reagent (PAS), alcian blue (AB; Diagnostic BioSystems, Pleasanton, CA) or dual PAS and AB staining.

Assessment of gross pathology and myeloperoxidase activity

Gross pathology of colonic loops and inflammation was scored on a scale from 1-4 using the following criteria: 1: Normal colon (uniform thickness, no colon dilation or distension, no blood in luminal contents); 2: Minimal damage (visible mucosal thickening and colonic distension, visible mucosal exudates, expanded loop occupies less than 50% of abdominal cavity); 3: Extensive damage (thickening of the colonic mucosa, visible dilation of surface blood vessels, colon distension with visible luminal contents, mucosal exudates and expanded loop occupying 50% of the abdominal cavity); 4: Inflamed colon (extensive colon thickening, colon surface with extensive inflamed dilated blood vessels with or without haemorrhage, extensive colon distension with or without visible brown or bloody luminal contents, mucosal exudates under extreme pressure leading to ballooning of the colon, and expanded loop occupying most of the abdominal cavity).

Myeloperoxidase (MPO) activity were determined using a standard protocol[11], with some modifications. In brief, colonic tissues were homogenized and sonicated in hexadecyltrimethylammonium bromide buffer. Samples were centrifuged at 9,000 x *g* (10 min at 4°C), and the supernatant collected. O-dianisidine dihydrochloride (0.53mM) and hydrogen peroxide (1%) were added to 14µL of supernatant and the absorbance was determined at 420nm. Results are expressed as MPO units per milligram of tissue.

In vivo mucin secretion studies

To quantify mucin secretion *in vivo*, animals were fasted overnight and injected i.p., with 20µCi of ^{3}H-glucosamine (Perkin Elmer, Waltham, MA) in PBS for 3h to metabolically label the newly synthesized mucin pool as previously described[4, 5]. ^{3}H-glucosamine is converted into sialic acid, *N*-acetyl-glucosamine and *N*-acetyl-*D*-galactosamine on the mucin monomer and has been used previously to determine the secretion of mucin in response to various agonists[12]. Following laparotomy, a single colonic loop was made and injected with 2 x 10^{6} log-phase amebic trophozoites. Animals were sacrificed after 3h p.i., the colon excised and opened longitudinally and mucosal secretions obtained as previously described[13]. Mucosal secretions were vortexed (maximum speed, 15 min) and centrifuged (1,000 x *g*, 10 min, 4°C) to remove cellular debris. The ^{3}H-labelled secreted glycoproteins were precipitated overnight at 4°C by adding an equal volume of 10% trichloroacetic acid (TCA) and 1% phosphotungstic acid (PTA; Sigma-Aldrich)[13, 14]. The TCA/PTA precipitated proteins were isolated by centrifugation (400 x *g*, 10 min, 4°C), re-suspended in PBS and neutralized to pH 7.0 with 10M NaOH. The total ^{3}H-labelled glycoproteins were obtained by adding 3mL Scintiverse I (Fisher Scientific, Ottawa, ON)

scintillation fluid and quantified as counts per minute (CPMs) with a scintillation counter (Beckman Coulter, Fullerton, CA)[13, 14]. To enumerate the secretion of high molecular weight (V_o) mucin and non-mucin components, secreted ^{3}H-labelled glycoproteins were fractionated by Sepharose 4B column chromatography and the CPMs for each fraction determined. The column (Bio-Rad Laboratories, Mississauga, ON) was equilibrated with 0.01mol/L Tris HCl buffer containing 0.001% sodium azide (pH 8.0; Sigma Chemical Co). Prior to use, the column was calibrated with blue dextran (BD; M_r 2,000 kDa) to denote the void volume (V_o) and other gel filtration standards (Bio-Rad Laboratories, Mississauga, ON) including thyroglobulin (TG; M_r 670 kDa), chicken ovalbumin (Ova; M_r 44 kDa) and vitamin B12 (B12; M_r 1.35 kDa).

E. histolytica cultivation and harvesting

Eh trophozoites (HM1:IMSS) were grown axenically in TYI-S-33 media and regularly passaged through gerbil livers to maintain high virulence[14]. Trophozoites were harvested during the logarithmic growth phase (48-72 hours) by chilling on ice for 10 minutes and pelleted by centrifugation at 700 x *g* (5 min at 4^{o}C).

Quantification of pro-inflammatory cytokines and tight junction proteins in colonic tissues

Gene expression of pro-inflammatory (*Il-1β*, *Tnf-α*, *Ifn-γ*) and immune-regulatory cytokines (*Il-10* and *Tgf-β*), tight junction proteins (*Cldn 1-5*, *Cldn 8*, *Cldn 10*, *Ocln* and *Zo-1*) and secretory mucins (*Muc2* and *Muc6*) were examined by quantitative real-time

PCR (qRT-PCR). This method was also used to assess the expression of other goblet cell mediators (*Tff3* and *Retln-β*). Total RNA was prepared from full thickness tissue samples with Trizol reagent (Invitrogen) as per the manufacturer's specifications. Spectroscopic analysis was used to determine the purity and yield of the RNA. cDNA was prepared from 1μg mRNA using random hexamer primers and Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen). For qRT-PCR, 1μL cDNA was used with 10μL SyBr Green master mix (Invitrogen), and 100μM primer concentration in a final reaction volume of 20μL. Primer sequences and conditions are listed in Table 6-1. qRT-PCR were performed on a Rotor Gene 3000 (Corbett Research, Kirkland, QC), and results were analyzed using the $2^{-\Delta\Delta CT}$ method.

Table 6- 1: Murine Primers and Conditions.

Name	Sequence 5` 3`	Annealing Temp.	Ref
Il-1β	For: GCCTCGTGCTGTCGGACCCA Rev: CTGCAGGGTGGGTGTGCCGT	60^{o}	
Tnf-α	For: ATGAGCACAGAAAGCATGATC Rev: TACAGGCTTGTCACTCGAATT	56^{o}	14
Ifn-γ	For: TCAAGTGGCATAGATGTGGAAGAA Rev: TGGCTCTGCAGGATTTTCATG	54^{o}	15
Muc6	For: TGGACCACCCCCAAGGAATCCAC Rev: AGGAGGCCAAGGTGATGTCGGAG	60^{o}	
Cldn-1	For: TCTACGAGGGACTGTGGATG Rev: TCAGATTCAGCAAGGAGTCG	60^{o}	16
Cldn-2	For: CCTCGCTGGCTTGTATTATCTCTG Rev: GAGTAGAAGTCCCGAAGGA	60^{o}	17
Cldn-3	For: AAGCCGAATGGACAAAGAA Rev: CTGGCAAGTAGCTGCAGTG	60^{o}	16
Cldn-4	For: TGGATGAACTGCGTGGTG Rev: GGTTGTAGAAGTCGCGGATG	60^{o}	18
Cldn-5	For: GTGGAACGCTCAGATTTCAT Rev: TGGACATTAAGGCAGCATCT	60^{o}	16
Cldn-8	For: GCCGGAATCATCTTCTTCAT Rev: CATCCACCAGTGGGTTGTAG	60^{o}	
Cldn-10	For: CCCAGAATGGGCTACACATA Rev: CCTTCTCCGCCTTGATACTT	60^{o}	16
Ocln	For: AGAGGCTATGGGACAGGGCTCTTTGG Rev: CCAACAGGAAGCCTTTGGCTGCTCTTGG	60^{o}	17
Zo-1	For: GGAGCTACGCTTGCCACACT Rev: GGTCAATCAGGACAGAAACACAGT	60^{o}	19
Actin	For: CTACAATGAGCTGCGTGTG Rev: TGGGGTGTTGAAGGTCTC	54^{o}	

The primer sequences and conditions used in qRT-PCR are listed, with relevant references.

Enzyme-linked immunosorbent assay (ELISA) was used to quantify the secreted pro-inflammatory cytokines and serum albumin in the luminal exudates from control and *Eh* inoculated loops. Briefly, the colon was excised and the two loops separated. The distal suture was removed and the luminal contents in the loop collected in an eppendorf tube

that was stored on a bed of dry ice. Prior to use, luminal secretions were thawed, protein concentrations determined using the BCA protein kit (Pierce, Rockford, IL) as per manufacturer's specifications, and samples were equilibrated to 100μg protein per 100μL reaction. Briefly, 96-well ELISA plates were coated with 1μL primary antibody against murine serum albumin, secretory IgA or IgG (Bethyl Laboratories, Montgomery, TX) in 100μL coating buffer (0.05M carbonate-bicarbonate solution (pH 9.6) and incubated (RT, 1h)). Plates were rinsed and blocked for 30min (RT) in blocking solution (50mM Tris, 0.14M NaCl, and 1%BSA, pH8.0). One hundred micrograms of protein sample was added to each well, as per manufacturer's specifications and plates incubated at RT for 60 min. Horseradish peroxidise (HRP) detection antibody was added and the plate further incubated for 60 min. Finally, 3, 3', 5, 5'-Tetramethylbenzidine (TMB) substrate was added followed by the stop solution, and the protein concentration determined by colorimetric assay using H_2O_2. Luminal exudates were also analyzed by Mouse 32-plex Cytokine-Chemokine panel (Eve Technologies, Calgary, AB). This allowed us to examine the levels of the Il-8 homologs (KC, LIF, MCP-1 and MIP-2) and the pro-inflammatory (Il-1β, Ifn-γ, Tnf-α) and immunomodulatory cytokines (Il-10) among others. For western blotting, whole thickness colonic tissue preparations were homogenized and sonicated in cell lysis buffer to obtain protein extracts from lysed cells[15]. Cell lysis buffer contained 20mM Tris HCl, 1mM EDTA, 100mM NaCl, 0.1% SDS, 0.5% Triton X-100, and protease inhibitor cocktail (Sigma-Aldrich). Protein samples were analyzed by western blotting as previously described[15]. The protein content of each sample was estimated using the BCA protein assay kit and adjusted for a final concentration of 3.33μg/μL. Protein samples were mixed with 1x sample buffer and

boiled for 5 minutes at 100^{o}C. One hundred micrograms of protein was loaded per well of a 12.5% SDS polyacrylamide gel, and transferred to a nitrocellulose membrane (Bio-Rad, Mississauga, ON). Membranes were blocked with 5% skim milk in PBS-T (phosphate buffered saline with 0.1% Tween20) for 1h (RT) and incubated with primary antibodies against mouse claudin-2 and occludin (Invitrogen) in 5% bovine serum albumin (BSA) overnight at 4^{o}C. Blots were washed three times with PBS-T and incubated with secondary antibody in 5% skim milk-PBS-T for 1h at room temperature. Blots were washed with PBS-T and developed with Immobilon Western chemiluminescent HRP Substrate (Millipore, Billerica, MA) as per the manufacturer's instructions. Confocal immunofluorescence microscopy was used to determine the localization of TJ proteins in full thickness tissue preparations. Intestinal tissue was fixed in 1% paraformaldehyde for 15 min, after which it was passed through a 10% and 20% sucrose gradient for 30 min each followed by a 30% sucrose solution (overnight at 4^{o}C). Tissue was then embedded in Tissue-Tek Optimum Cutting Temperature (OCT) medium (VWR). Five-micron tissue sections were rinsed in PBS-T, and blocked in 3% normal goat serum. Sections were blotted with anti-mouse claudin 2 (Invitrogen Cat. # 325600), anti-mouse Occludin (Invitrogen Cat. # 331500), and anti-rabbit Zo-1 (Invitrogen Cat. # 617300) primary antibodies overnight in a humid chamber at 4^{o}C. Sections were rinsed with PBS-T and incubated with fluorescently labelled secondary antibodies (anti-mouse Alexa-488, anti-rabbit Dylight 594) at room temperature for 1h. Finally, nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) and sections were mounted with FluorSave Reagent (Calbiochem). Slides were examined using a FluoView FV1000 confocal immunofluorescence microscope (Olympus), where immunofluorescent signals

for the proteins of interest were determined in *en face* planes through the cellular *z* axis at 0.35μm intervals.

Intestinal permeability studies

Intestinal permeability was examined using FITC-dextran, as previously described[16]. Mice were anesthetized and colonic loops were inoculated with 10mg FITC-dextran (3-5kDa, Sigma Aldrich) and 1 x 10^6 *Eh* trophozoites suspended in 100uL PBS, while control animals received the same dose without *Eh*. At 3h p.i. mice were sacrificed by CO_2 asphyxiation and blood was collected by cardiac puncture. Whole blood was allowed to clot for 3h (in the dark) then centrifuged at 10,000 x g (10 min, RT). Serum was collected in a clean eppendorf and diluted with an equal volume of PBS. Serial dilutions of the stock FITC-dextran solution were used to generate a standard curve and 100uL of samples were loaded on a 96-well plate in duplicate. Fluorescence was determined with a plate reader (absorption 485nm, emission 535nm).

Statistics

Data were analyzed using the student t-test and analysis of variance (ANOVA) with a Tukey or Kruskal Wallis post-test application where necessary, using the software GraphPad Prism version 4 (Graph-Pad Software, San Diego, CA). Data are reported as the mean ± standard error of the mean (SEM).

Results

E. histolytica induces a robust secretory response in Muc2$^{-/-}$ mice

Although *Eh* colonizes the mucus layer which acts as an innate host defence mechanism to prevent parasite contact-dependent cytolysis of the underlying mucosa[23], the specific role of Muc2 in innate immunity against *Eh* remains unclear. *Muc2*$^{-/-}$ mice offer an excellent opportunity to examine luminal and epithelial barrier functions in the absence of an intact mucus layer. Here, we show that *Eh* inoculated into colonic loops induced a differential, temporal increase in both gross pathology and secretory responses in *Muc2*$^{-/-}$ mice as compared to WT animals (Fig. 6-1). Gross pathology scores were significantly increased in *Muc2*$^{-/-}$ as early as 1h p.i., and steadily increased up to 6h (p<.001). At 3h and 6h p.i., colons were severely dilated with luminal secretions under intense pressure and loops occupied >50% of the peritoneal cavity. Mucosal exudates from *Muc2*$^{-/-}$ were brownish (bloody) and watery whereas, those from WT animals were clear, thick and mucoid. Importantly, *Muc2*$^{-/-}$ animals exhibited a temporal progressive increase in pathology such that at 6h p.i., both the proximal and distal loops were severely dilated in some animals as compared to WT secretory responses which did not worsen significantly over time (Fig. 6-1).

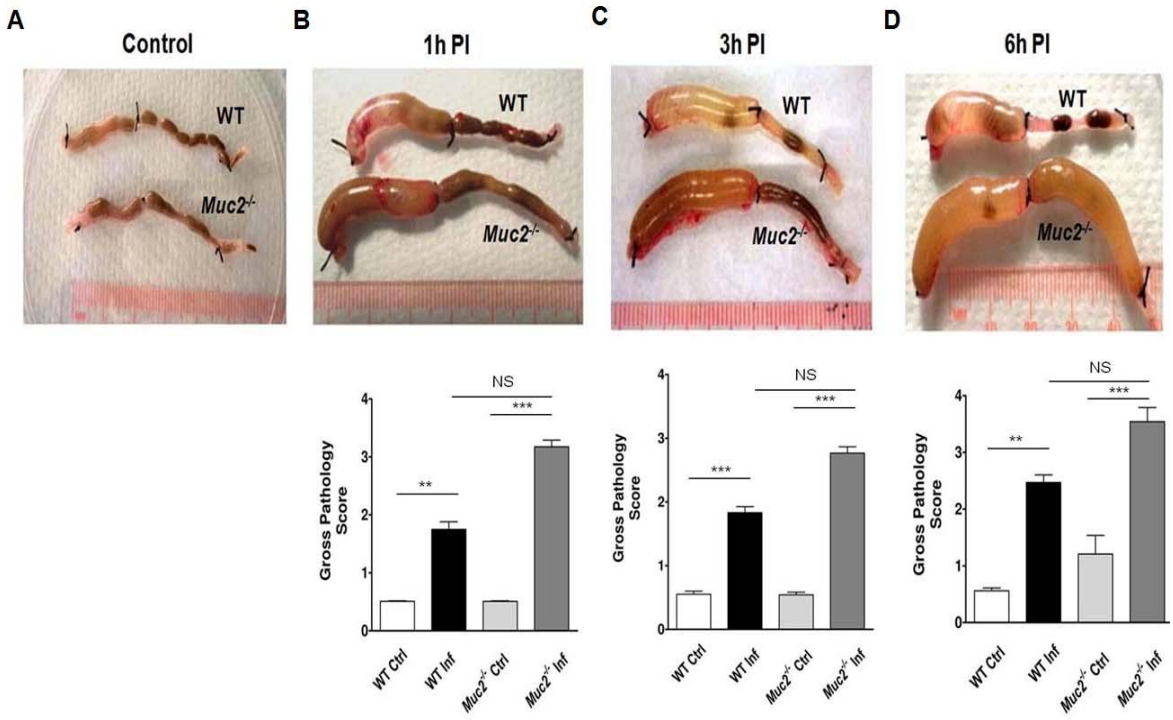


Figure 6-1: *E. histolytica* induces a rapid sustained secretory response with increased gross pathology in colonic loops of *Muc2*$^{-/-}$ animals. WT and *Muc2*$^{-/-}$ animals were anesthetized and closed colonic loops were infected with 1x10^6 virulent, log-phase trophozoites for 1, 3 or 6h p.i. Following infection, the colon was excised, and gross pathology scores determined (histograms). Panels A-D show the excised colons of representative WT and *Muc2*$^{-/-}$ animals, with the gross pathology scoring for each group. Controls in panel A, are sham animals with colon ligations shown for comparison purposes only. n=12 animals per group. $^{**}p<0.01$ and $^{***}p<0.001$. NS = not significant.

E. histolytica stimulates mucin and non-mucin glycoprotein secretion in WT mice

As *Eh* stimulates mucin secretion in animal models of amebiasis[24] and in human colonic goblet cells *in vitro*[14], it was of importance to determine the composition of the secretory responses in *Muc2*$^{-/-}$ mice. To do this, newly synthesized glycoproteins were metabolically labelled with ^{3}H-glucosamine and the ^{3}H-mucin and ^{3}H-non-mucin secretions quantified in response to *Eh* infection. Surprisingly, whereas *Eh* induced a 5-fold increase in total ^{3}H-glycoprotein secretions in WT this response was absent in *Muc2*$^{-/-}$ animals where the total ^{3}H-glycoprotein pool remained unchanged (Fig. 6-2A). To

determine if the ^{3}H-glycoproteins were high molecular weight V_o mucin and/or non-mucin components, proteins were fractionated by Sepharose 4B column chromatography (Fig. 6-2B), which separates the high molecular weight mucins from the non-mucin components[10, 22]. Predictably, the luminal secretions from WT infected mice showed a 1.8-fold and 1.7-fold increase in mucin and non-mucin ^{3}H-glycoproteins respectively as compared to those from *Muc2*$^{-/-}$ animals. These results demonstrate that glycoprotein secretions dominated the responses in WT mice whereas *Muc2*$^{-/-}$ secretory responses consisted primarily of water, ionic secretions and/or non-glycosylated proteins (Fig. 6-2B).

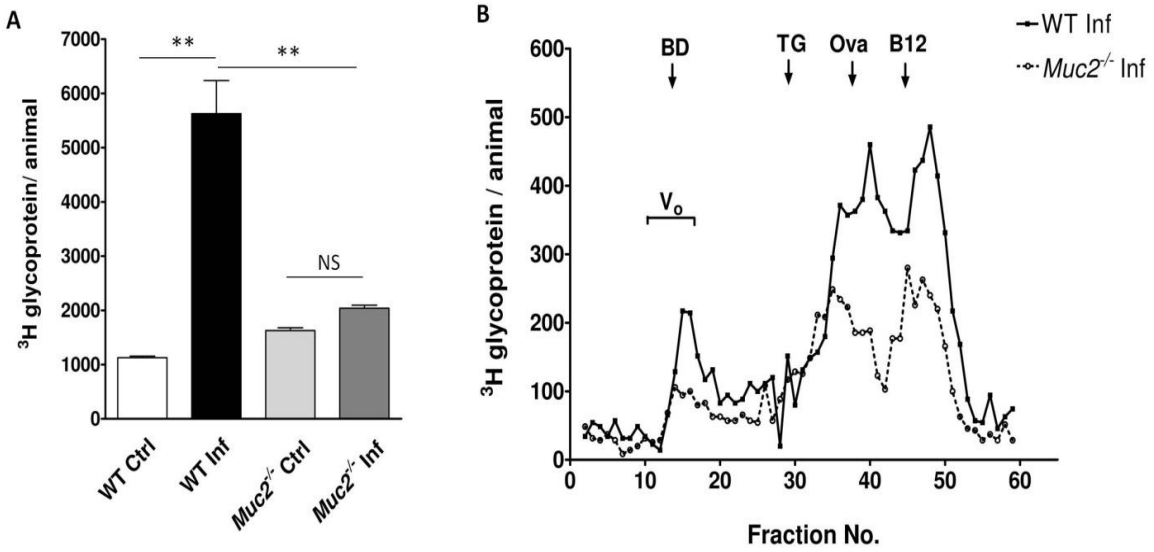


Figure 6-2: *E. histolytica* stimulates mucin and non-mucin glycoprotein secretion in WT and *Muc2*$^{-/-}$ mice. Mucosal secretions obtained from WT and *Muc2*$^{-/-}$ mice after 3h p.i. were pooled (n=6 for control and n = 7 for infected animals, repeated twice) and precipitated with a 10% TCA and 1% PTA solution. Total ^{3}H-labelled glycoprotein secretions are shown in Panel **A**. Note, significantly more glycoproteins were obtained from mucosal secretions of WT infected animals as compared to WT control or *Muc2*$^{-/-}$ infected animals (**p<0.01). NS = not significant. Panel **B** shows the Sepharose 4B column chromatography profiles obtained from the ^{3}H-labelled glycoprotein secretions in Panel A from WT and *Muc2*$^{-/-}$ infected animals. Note that the mucosal secretions of WT infected animals were rich in mucin (V_o fractions) and non-mucin glycoproteins. The column was calibrated with various molecular weight markers as shown.

Since Muc2 has been shown to protect against contact dependent cytolysis *in vitro*[23], we sought to determine if in the absence of Muc2, mice were more susceptible to *Eh* induced damage. Accordingly, colonic tissues were carefully excised, fixed in Carnoy's solution to preserve the mucus layer and stained to visualize cellular morphology (H&E) and to quantify neutral (PAS) and acidic (AB) mucin. Not surprising, tissues from WT mice showed filled PAS positive goblet cells throughout the length of the crypts and secreted mucus was visible overlying the surface IECs (Fig. 6-3). In contrast, control *Muc2*$^{-/-}$ colons showed crypt elongation with branching and only AB positive mucin in the deep crypts (data not shown). Moreover, no abnormal inflammatory cellular infiltrate was observed under basal conditions or following *Eh* infection after 6h p.i. Predictably, *Eh* induced a prominent mucus secretagogue effect in WT as early as 1h p.i., which formed a thick well organized mucus plug at 6h p.i. (Fig. 6-3). At 6h p.i., the colonic mucosa in *Muc2*$^{-/-}$ mice was severely compressed as a result of increased pressure inside the loop due to the intense secretory responses. Ameba did not bind to/or invade the epithelium in either WT or *Muc2*$^{-/-}$ animals at any time point examined. In WT animals, *Eh* trophozoites was found embedded in the thick mucus blanket away from the mucosal surface while in *Muc2*$^{-/-}$ mice, trophozoites were seen throughout the intestinal lumen engulfing bacteria and *Trichomonas* spp.

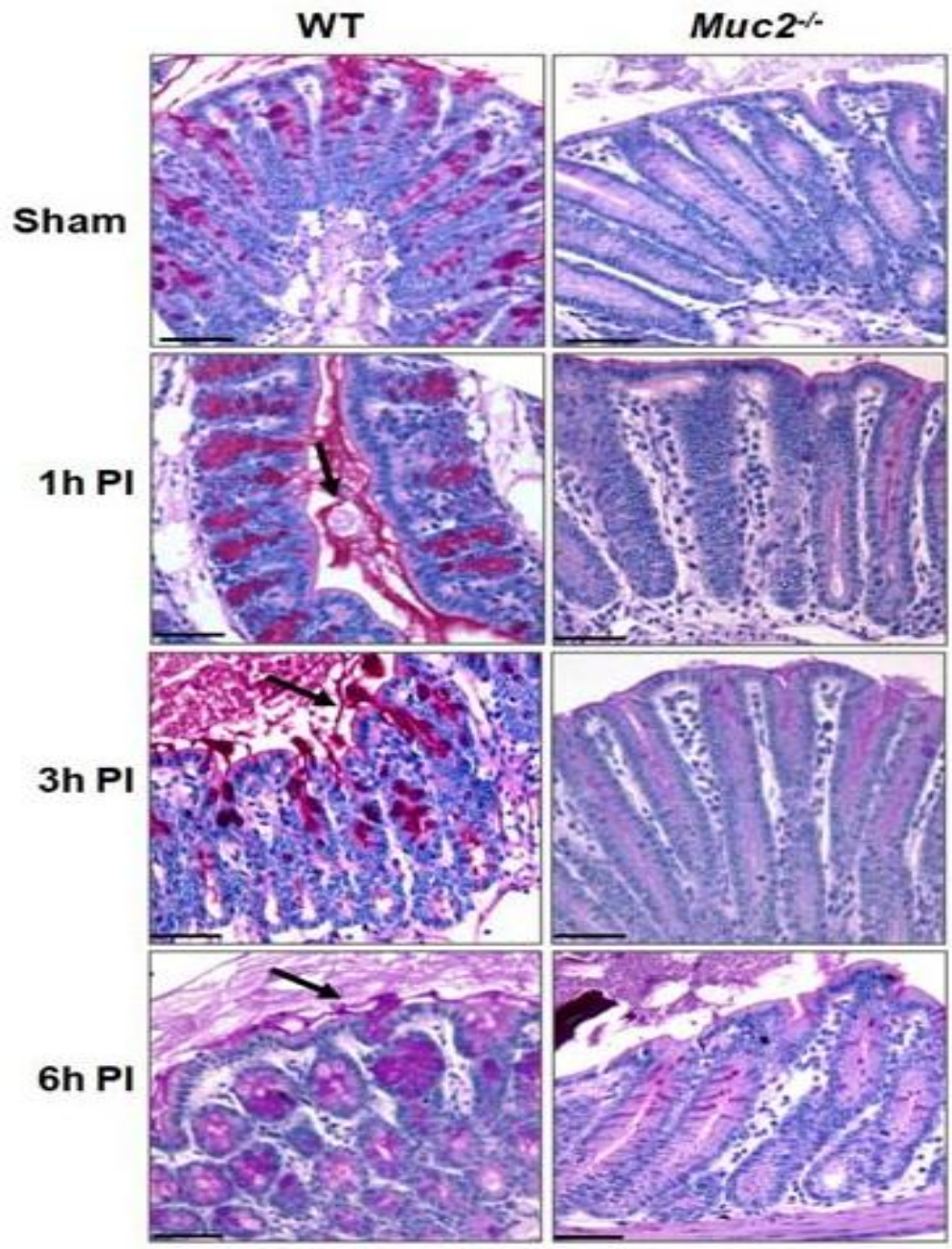


Figure 6-3: *E. histolytica* evokes a robust mucin secretagogue effect in WT animals. Colonic tissue sections from WT and *Muc2*$^{-/-}$ infected mice were fixed in Carnoy's solution and stained with PAS reagent to visualize neutral mucins. In WT sham animals, PAS filled goblet cells are visible throughout the length of the crypts and in the intestinal lumen (mucus). In contrast, *Muc2*$^{-/-}$ animals are devoid of PAS positive neutral mucins and crypts are elongated with branching. Note the PAS positive (magenta) mucin secretion in WT but not in *Muc2*$^{-/-}$ mice in response to *Eh*. In WT at 1h p.i., the arrow shows an ameba engulfed by neutral mucins. In WT at 3h p.i., the arrow shows mucin released from goblet cells forming a mucus plug at the opening of the crypt. At 6h p.i., arrow shows the inner (deep magenta color) and outer parts (lighter magenta color) of the mucus layer. Note the paucity of PAS positive staining in *Muc2*$^{-/-}$ tissues. Scale bar represents 50μm.

Effect of E. histolytica on other goblet cell mediators

Previous studies have shown that goblet cells synthesize and secrete Muc2, Tff3 and Retnl-β and in the absence of Muc2, lose their characteristic goblet cell phenotype[7, 25]. Since all three goblet cells mediators play critical roles in innate host-defence and wound repair, we sought to determine if there was a compensatory increase in these or other secretory mucins in *Muc2*$^{-/-}$ animals following *Eh* infection. Surprisingly, there was no increase in *Tff3* or *Retnl-β* gene expression in response to *Eh*. Curiously however, *Muc6* mRNA expression was notably increased in WT and *Muc2*$^{-/-}$ animals starting at 3h suggesting that it might play a protective role (Fig. 6-4).

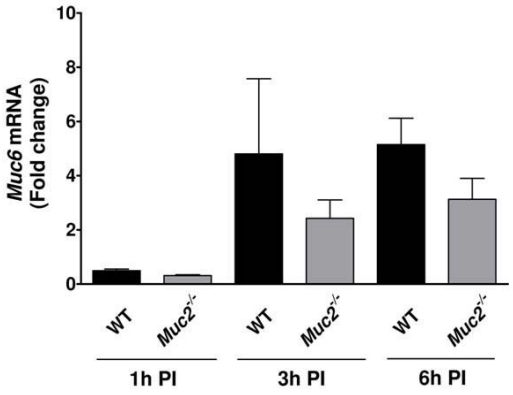


Figure 6-4: *Eh* induced a modest increase in *Muc6* expression. WT and *Muc2*$^{-/-}$ mice were infected with virulent *Eh* in a colonic loop, and *Muc6* gene expression was examined by qRT-PCR. *Eh* induced a subtle, though not statistically significant increase in *Muc6* in both WT and *Muc2*$^{-/-}$ animals at both 3 and 6h p.i, suggesting that this mucin may play a protective role in *Eh* infection. NS = not significant. Data expressed as fold change compared to control.

E. histolytica induces a differential pro-inflammatory response in WT and Muc2*$^{-/-}$ *animals

Goblet and columnar epithelial cells respond to inflammatory stimuli including *Eh* by releasing pre-formed and newly synthesized mucins[1, 5, 8] and increased expression and

secretion of pro-inflammatory mediators[26]. Thus, it was of interest to determine if *Eh* induced inflammatory responses were responsible for the enhanced secretory responses observed in *Muc2*$^{-/-}$ mice. Accordingly, inflammation was assessed by MPO activity as a measure of neutrophil influx/activation, qRT-PCR for cytokine gene expression and ELISA to detect the presence of secreted cytokines in colonic exudates. Surprisingly, even though there was no significant increase in MPO activity at 3 and 6h p.i. (Fig. 6-5), increase protein secretion of the Il-8 homologs KC, MIP-2 and MCP-1 in *Muc2*$^{-/-}$ animals were observed (Fig. 6-6). In addition, *Eh* infection increased mRNA expression and/or protein release of the pro-inflammatory cytokines Tnf-α, Ifn-γ and Il-13 indicative of a robust but differential inflammatory response in *Muc2*$^{-/-}$ as compared to WT animals (Fig. 6-7A). In particular, *Eh* induced a robust pro-inflammatory response characterized by elevated *Il-1β* expression and protein release in WT animals (Fig. 6-7B). In *Muc2*$^{-/-}$ mice however, this response was characterized by elevated levels of Tnf-α, Ifn-γ and Il-13 cytokine release.

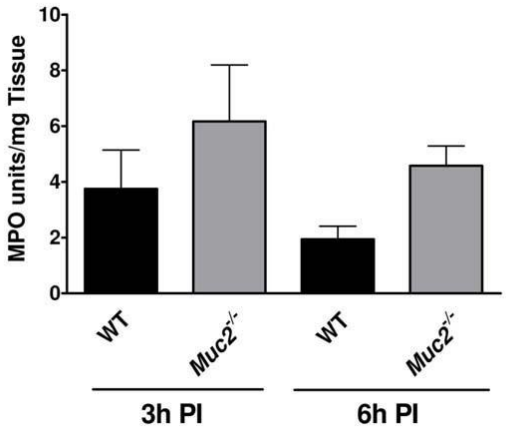


Figure 6-5: *Eh* infection did not induce robust changes in myeloperoxidase activity. MPO activity was assayed in tissue samples obtained from WT and *Muc2*$^{-/-}$ mice at 3h and 6h p.i. Although a modest increase in MPO was observed in *Muc2*$^{-/-}$ animals compared to their WT counterparts, this was not statistically significant (NS).

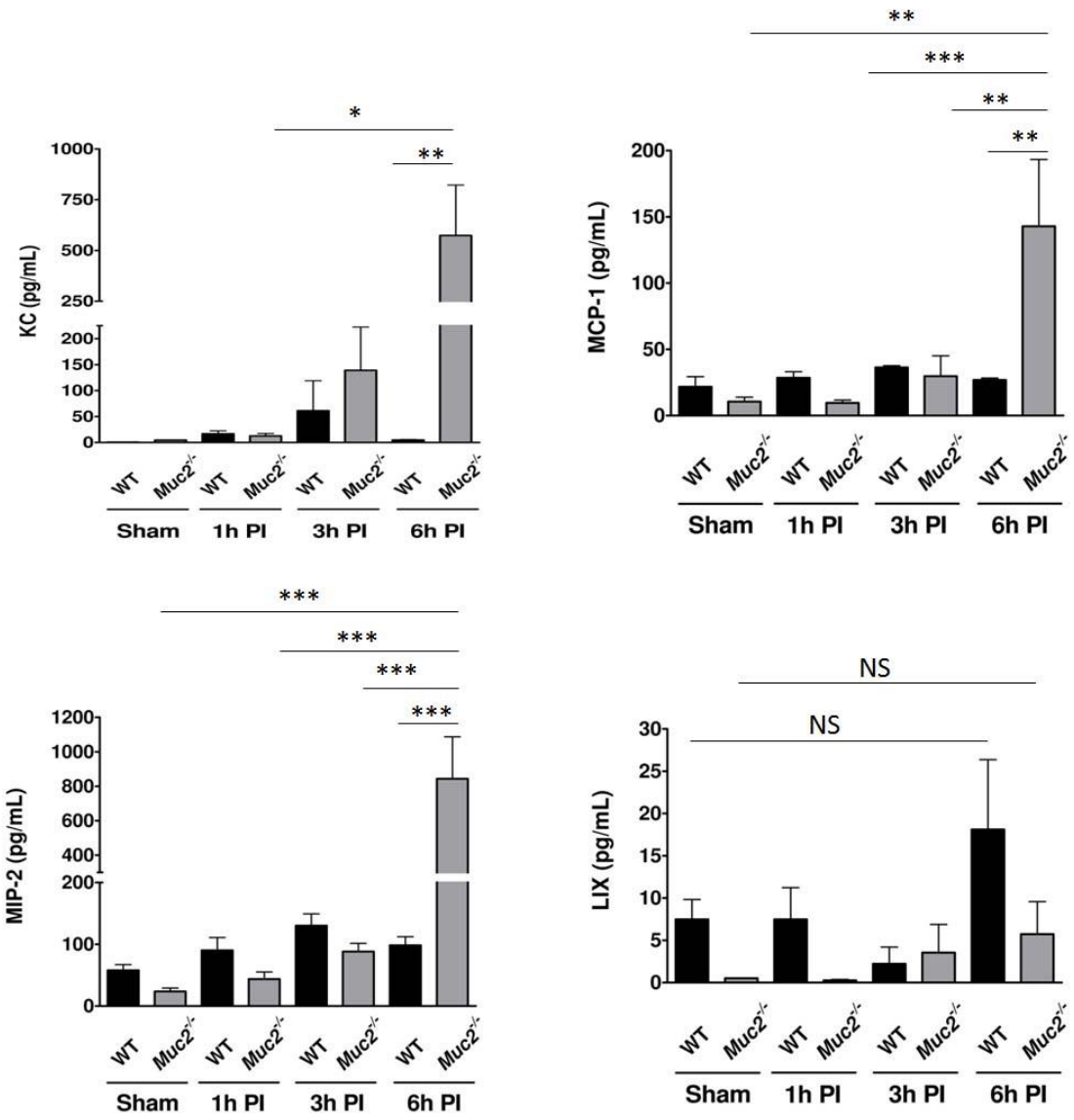
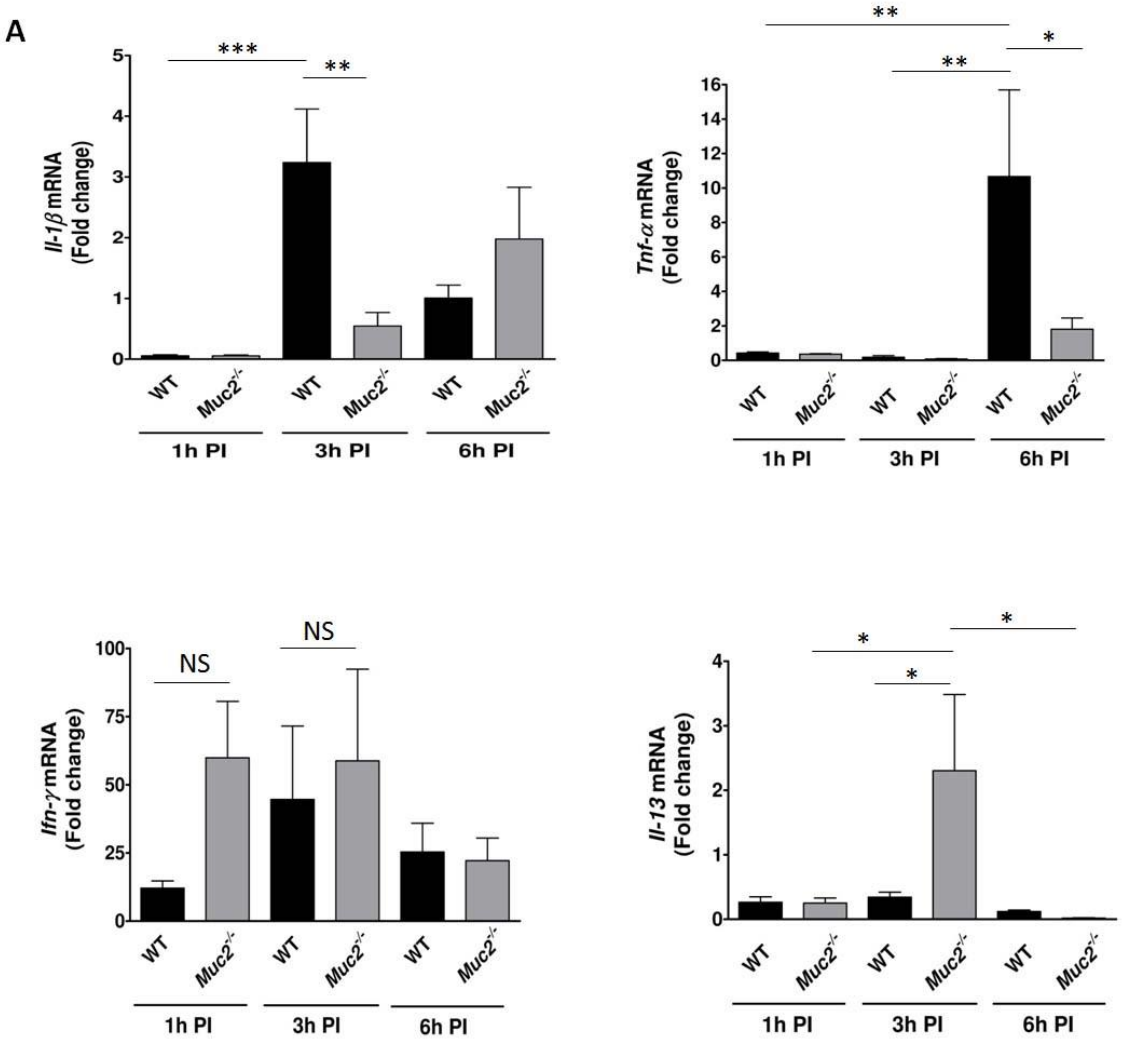


Figure 6-6: *Eh* induced an increase in cytokine production in colonic loops. Cytokine protein release of the murine homolog of IL-8 (KC, MCP-1, MIP-2 and LIX) was examined in the luminal exudates of WT and *Muc2*$^{-/-}$ mice by ELISA. Note *Eh* induced a robust increase in KC, MCP-1 and MIP-2 but not LIX in *Muc2*$^{-/-}$ but not WT animals. Curiously, LIX expression was elevated in WT mice at 1 and 6h p.i., but this was not statistically significant (NS). *p<0.05, **p<0.01, ***p<0.001. Sham animals: n=3, infected animals: n=6.


A
Il-1β mRNA (Fold change)
***
**
Tnf-α mRNA (Fold change)
**
**
*
Ifn-γ mRNA (Fold change)
NS
NS
Il-13 mRNA (Fold change)
*
*
*
WT
Muc2-/-
1h PI
3h PI
6h PI

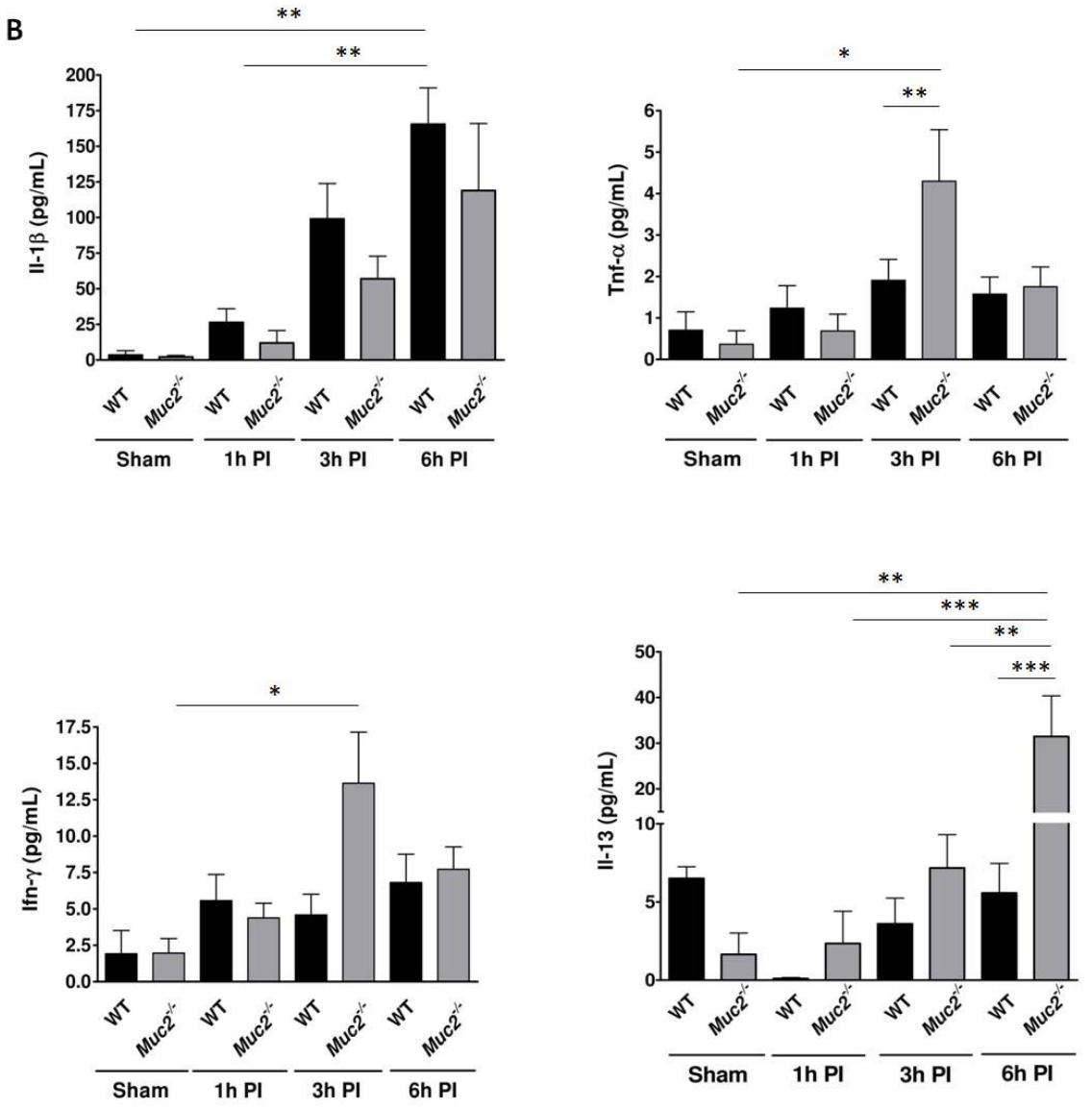


Figure 6-7: *E. histolytica* induces a differential pro-inflammatory response in WT and *Muc2*$^{-/-}$ mice. Full thickness colonic loop tissues were processed for pro-inflammatory gene expression (Panel **A**) and mucosal exudates for cytokine protein levels (Panel **B**) at various times following *Eh* infection. Gene expression data is shown as fold change compared to sham animals, *p<0.05, **p<0.01, ***p<0.001 NS = not significant. The experiment was repeated twice with similar results.

E. histolytica alters tight junction proteins in Muc2$^{-/-}$ mice

Recent studies have shown that pro-inflammatory cytokines including Tnf-α alter TJ permeability in IECs resulting in occludin (Ocln) endocytosis and increased paracellular permeability[27]. To investigate if the increased pro-inflammatory cytokines observed in *Muc2*$^{-/-}$ mice were associated with altered TJ permeability, and increased secretory responses, TJ mRNA expression was examined by qRT-PCR and protein levels by western blot and immunofluorescence microscopy. As shown in Figure 6-8A, *Eh* significantly increased the gene expression of the pore-forming claudin-2 (*Cldn-2)* at 1 and 3h p.i., in *Muc2*$^{-/-}$ as compared to WT animals, concomitant with no change or variable expression of the barrier-tightening *Cldn-1, 3*, *4, 5, 8* and *10* mRNA (Fig. 6-9). Correspondingly, *Ocln* mRNA expression was significantly decreased in *Muc2*$^{-/-}$ animals at 3 and 6h p.i. (Fig. 6-8). The mRNA expression data was consistent with protein levels where WT mice showed low basal expression of Cldn-2 and Ocln, however; only Ocln increased in response to *Eh*, and peaked at 6h p.i. (Fig. 6-8 top panel and relative protein intensity). In contrast, *Muc2*$^{-/-}$ basal levels of Cldn-2 and Ocln were consistently higher than WT and Cldn-2 protein increased steadily in response to *Eh* (Fig. 6-8).

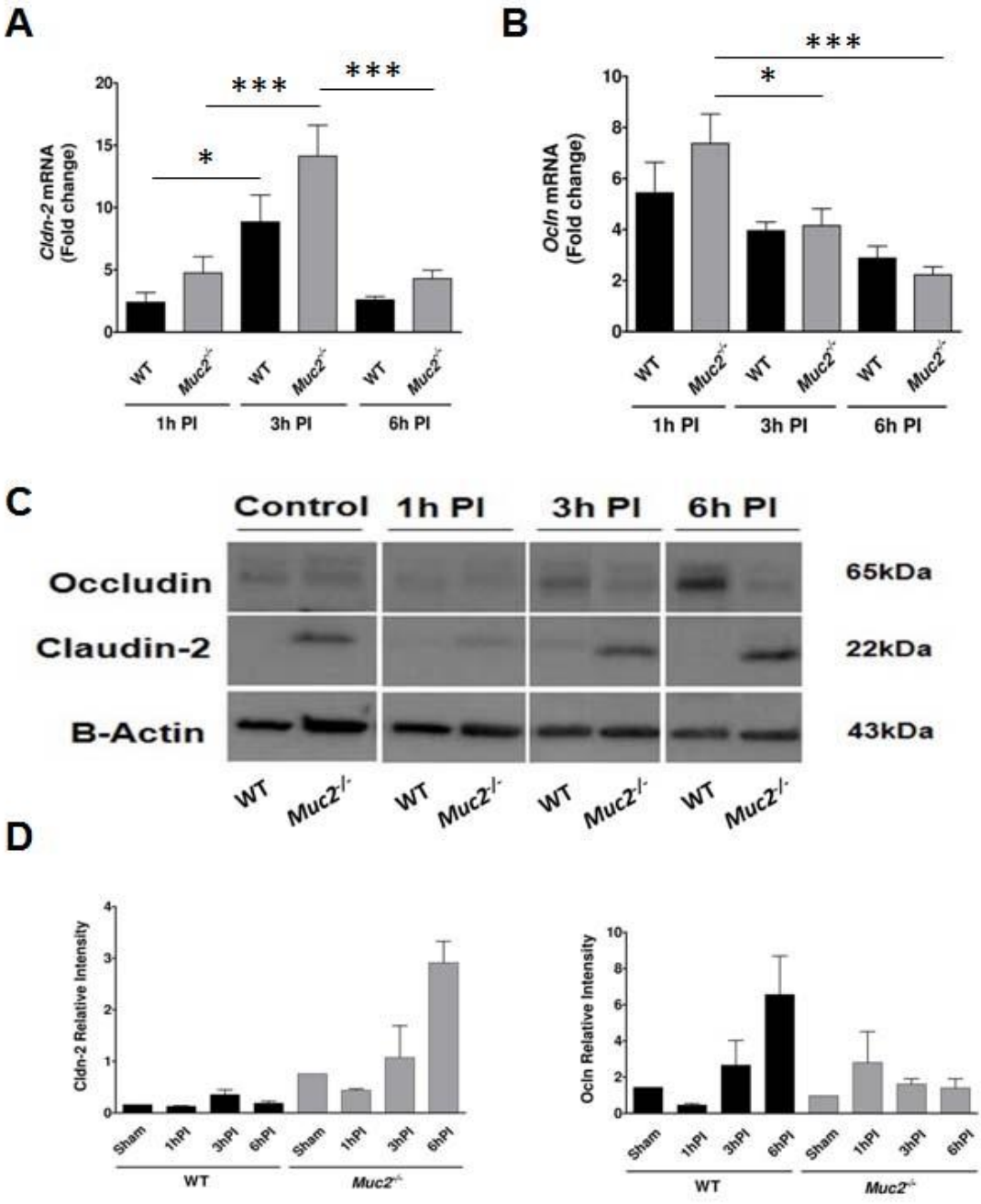


Figure 6-8: *E. histolytica* alters gene expression of tight junction proteins in *Muc2*$^{-/-}$ mice. Intestinal permeability in *Muc2*$^{-/-}$ animals was assessed for claudin-2 and occludin gene expression, and displayed as fold change compared to sham. *p<0.05, **p<0.01, ***p<0.001. *Cldn-2* gene expression levels were consistently higher in *Muc2*$^{-/-}$ mice at all time points investigated though this peaked at 3h p.i. (Panel A). Concomitantly *Eh* induced a time dependent decrease in Ocln levels in these animals (Panel B). Panel C shows a representative western blot for WT and *Muc2*$^{-/-}$ mice following *Eh* infection at 1h, 3h and 6h p.i. Panel D shows the relative intensity for claudin-2 (left) and occludin (right) protein for the western blot above.

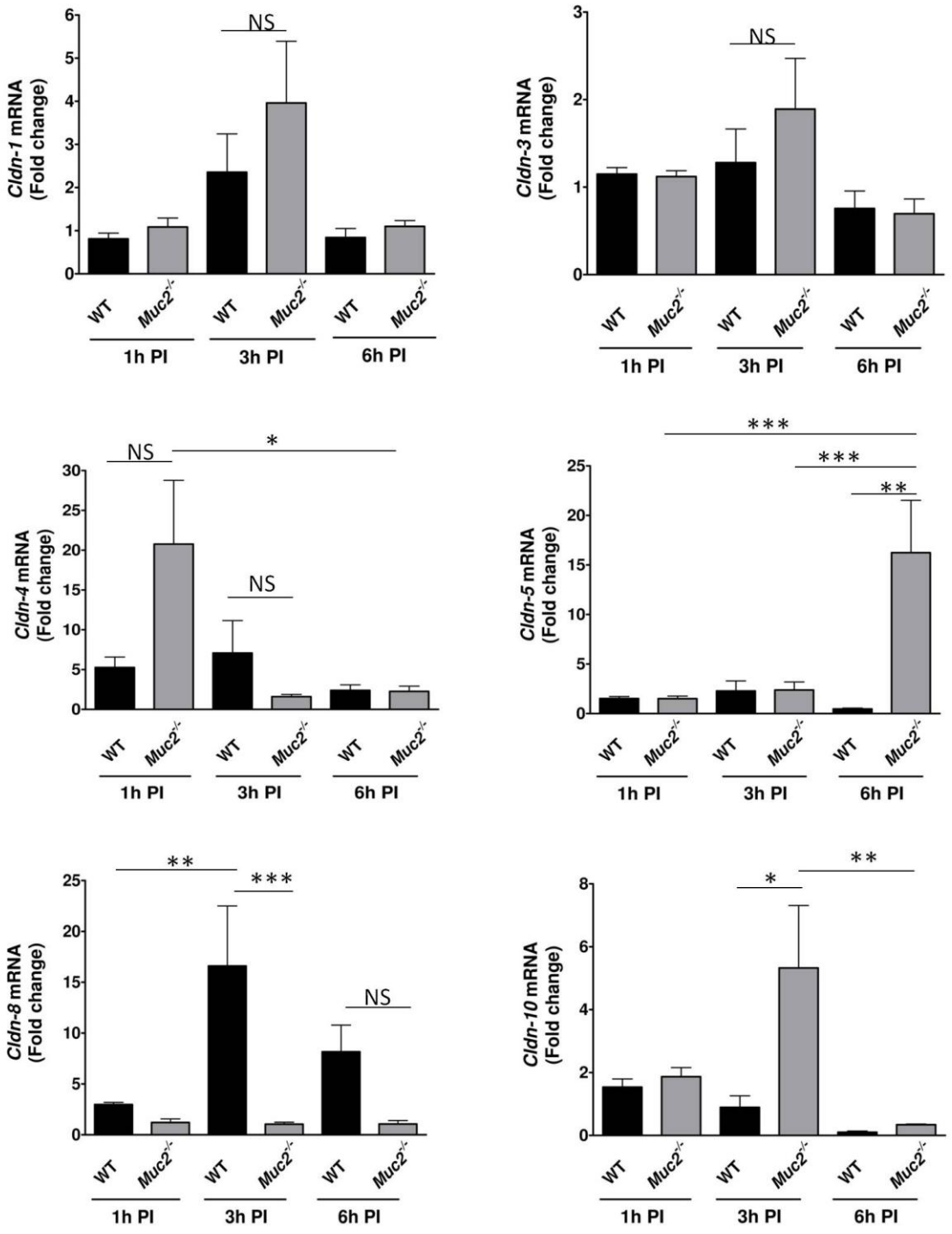
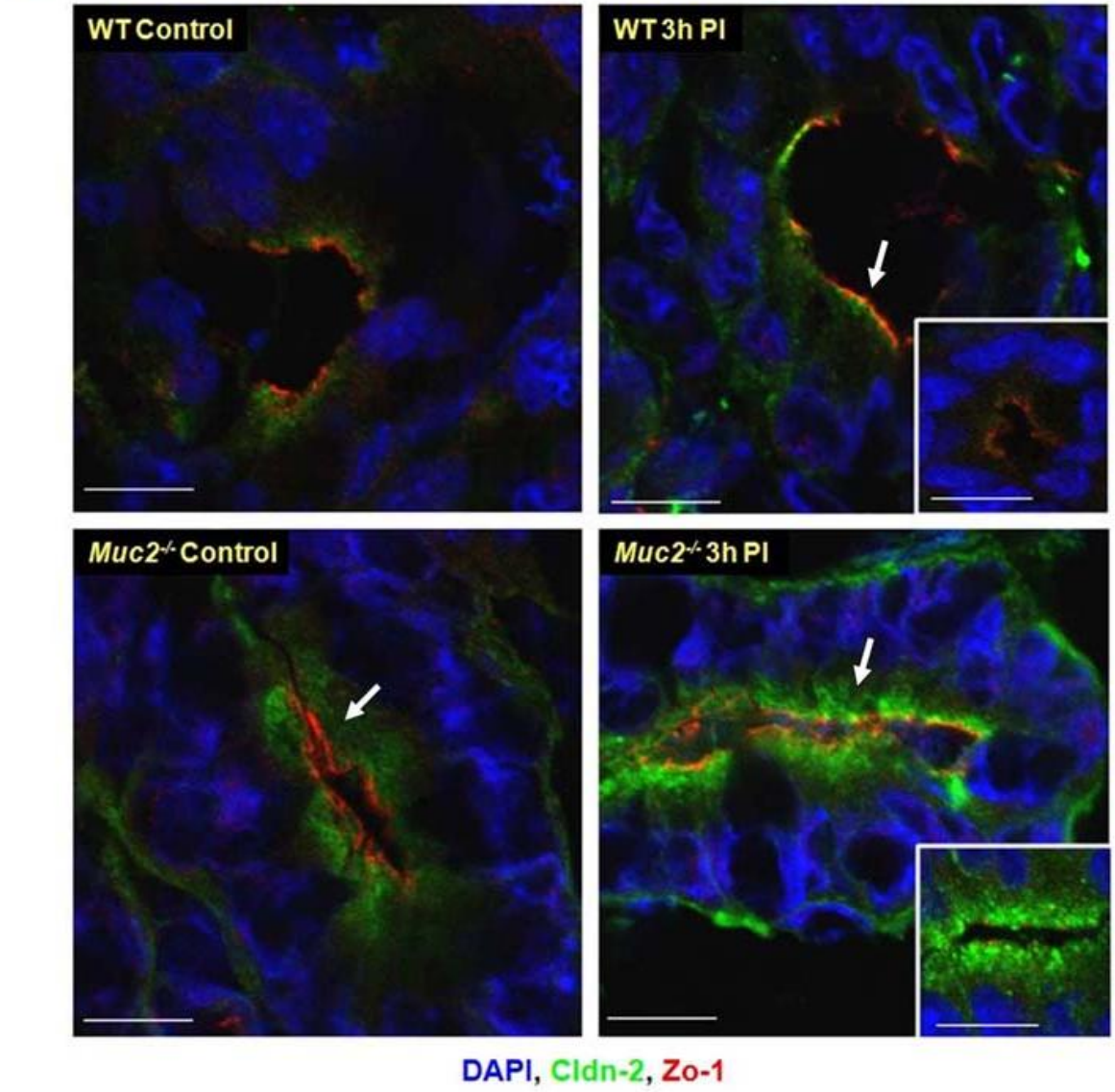


Figure 6-9: *Eh* induced modest changes in *Cldn* expression. The gene expression of *Cldn-1, -3, -4, -5, -8* and *-10* was examined in tissue samples obtained from WT and *Muc2*$^{-/-}$ mice at 1h, 3h and 6h p.i. Data is expressed as fold change compared to sham animals. Although modest changes were observed in *Cldn* expression in infected animals, changes were neither time-dependent nor statistically significant (NS) in most cases. *p<0.05, **p<0.01, ***p<0.001. n = 6 animals, repeated twice.

These findings were confirmed by confocal immunofluorescence in *Eh* infected *Muc2*$^{-/-}$ colons which revealed that Cldn-2 protein expression was markedly increased (Fig. 6-10A) throughout the cytoplasm at 3 and 6h p.i. (inset), while Zo-1 expression was undetected. In WT, the modest increase in Cldn-2 expression was found co-localized with Zo-1 (Fig. 6-10A) at the apical cell surface and at 6h p.i. (inset), both Cldn-2 and Zo-1 were reduced. In infected WT there was a modest increase in Ocln expression co-localized with Zo-1 at 3h and 6h p.i. However, in *Muc2*$^{-/-}$ mice, Ocln levels were significantly reduced at 3h p.i. and Zo-1 appeared disorganized and by 6h p.i. (inset), the expression of both of these TJ proteins were reduced (Fig. 6-10B). These findings suggest that WT mice in the presence of an intact mucin barrier have well organized Cldn-2 and Ocln proteins, and the latter was modestly increased following infection. In *Muc2*$^{-/-}$ mice however, the TJ proteins were basally over-expressed and either increased (Cldn-2) or decreased (Ocln and Zo-1) following *Eh* infection, which may explain the increased secretory responses observed in these animals.


A
WT Control
WT 3h PI
Muc2-/- Control
Muc2-/- 3h PI
DAPI, Cldn-2, Zo-1

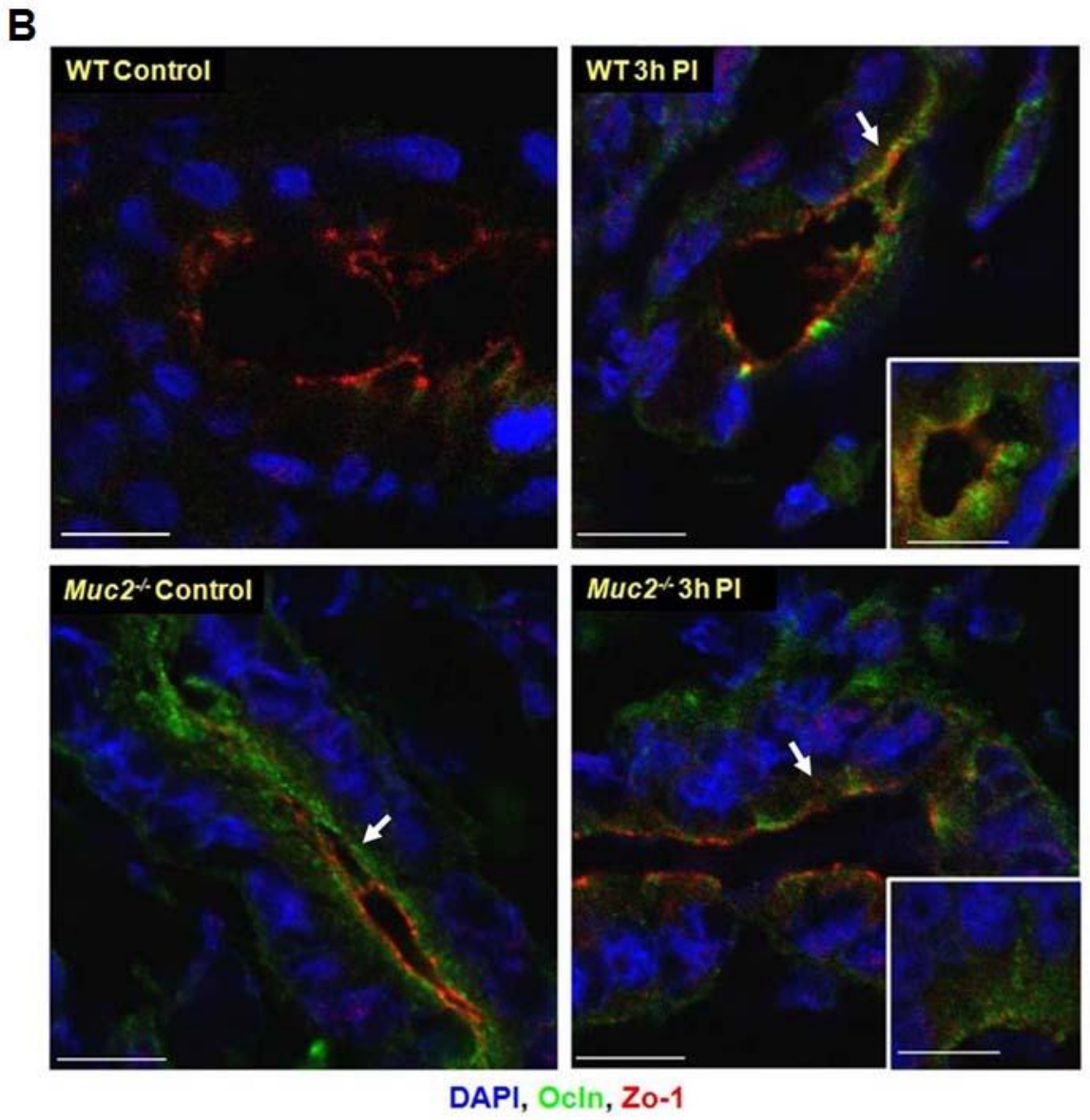


Figure 6-10: *E. histolytica* alters tight junction proteins in *Muc2*$^{-/-}$ mice. Intestinal permeability in *Muc2*$^{-/-}$ animals was assessed for claudin-2 (Panel **A**) and occludin (Panel **B**). Panel **A**. Confocal immunofluorescence shows that *Muc2*$^{-/-}$ mice constitutively express elevated Cldn-2 protein levels as compared to WT, and this is located at the apical surface of the cell away from Zo-1. Following *Eh* in WT animals, Cldn-2 was co-localized with Zo-1 (arrow) at 3h p.i but showed a modest reduction in expression at 6h p.i. (inset). Importantly, the Cldn-2 present at the apical surface was colocalized with Zo-1. In *Muc2*$^{-/-}$ mice at 3 and 6h p.i., (inset), claudin-2 protein was over-expressed at the apical cell surface, with a concomitant loss of Zo-1 evident at both 3h and 6h p.i. (inset). Panel **B**. *Muc2*$^{-/-}$ mice constitutively expressed more Ocln at the apical cell surface, where it was colocalized with Zo-1. A modest increase in Ocln was observed in WT mice at 3 and 6h p.i. (inset), where it was co-localization with Zo-1. In contrast, in *Muc2*$^{-/-}$ mice *Eh* caused a progressive decrease in Ocln expression concomitant with a loss of Zo-1 at 3h and more pronounced at 6h p.i. (inset). DAPI, Cldn, Ocln and Zo-1 are shown in their respective color. Scale bar represents 50μm.

E. histolytica infections cause paracellular serum protein leakage in Muc2$^{-/-}$ mice

As *Muc2*$^{-/-}$ are more susceptible to *Eh*-induced pathological responses as evidenced by gross pathology scores, robust secretory responses and altered TJ permeability, we sought to determine the composition of the luminal exudates obtained from these animals. As shown in Figure 6-11, in addition to more fluid secretions there was a time-dependent increase in serum albumin leakage in *Muc2*$^{-/-}$ as compared to their WT counterparts, which was most striking at 6h p.i. (Fig. 6-11, $p<0.01$). Interestingly, the same was not true for serum IgA or IgG levels indicating that the increased movement of albumin was the result of paracellular leakage of serum proteins into the intestinal lumen. To determine if there was altered intestinal permeability we quantified the movement of the inert probe FITC-dextran in the serum of animals infected with *Eh* for 3h p.i. Surprisingly, whereas FITC-dextran was somewhat higher (not significant) in *Eh* infected WT mice, *Muc2*$^{-/-}$ animals showed a decrease in FITC levels (Fig. 6-12).

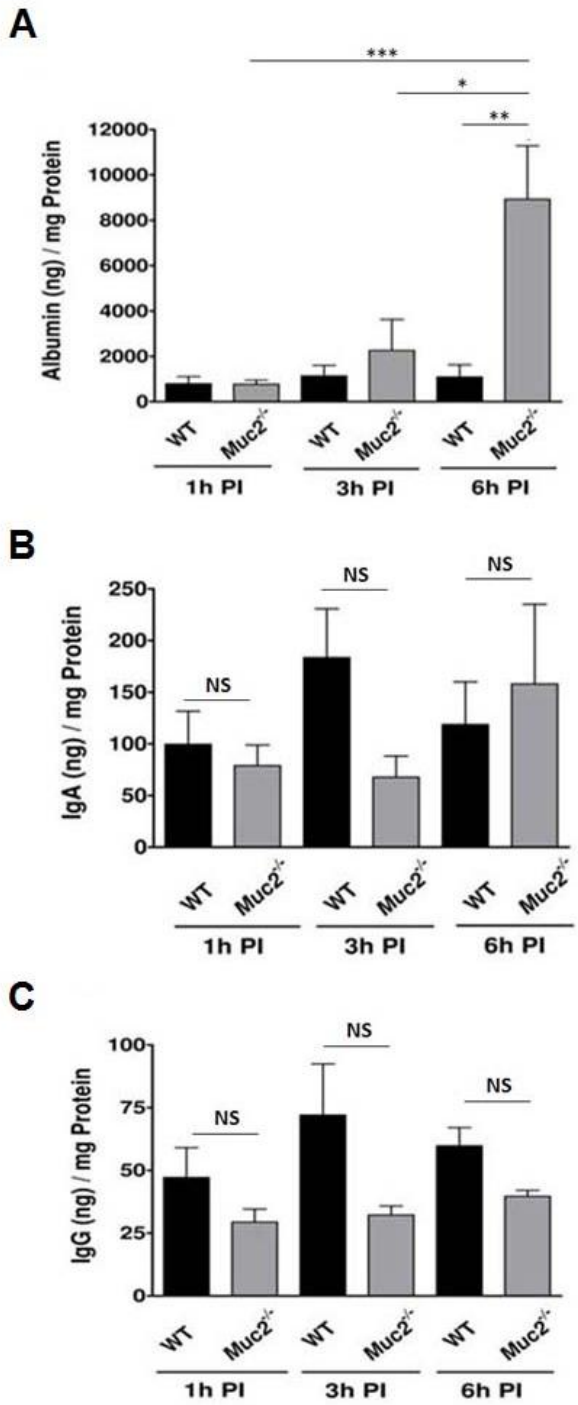


Figure 6-11: *E. histolytica* induces serum albumin leakage in *Muc2*$^{-/-}$ mice. Serum albumin (A), IgA (B) and IgG (C) levels in colonic loop exudates following *Eh* infection were quantified by ELISA. A marked, time dependent increase in serum albumin leakage into the intestinal lumen was observed in *Muc2*$^{-/-}$ mice (*p<0.05, **p<0.01, ***p<0.001), with no similar alterations in serum IgA or IgG levels. Curiously the same was not observed in WT mice for any of the proteins examined at the time points investigated. For all histograms, n=4-6 animals per group, repeated twice. NS = not significant.

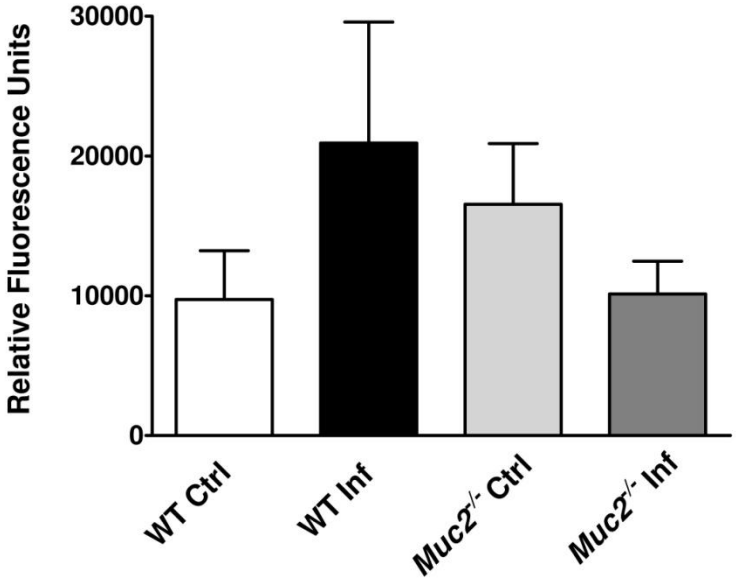


Figure 6-12: *Eh* infection did not alter intestinal permeability to FITC-dextran. 1 x 10^6 *Eh* trophozoites were injected in a colonic loop together with 10mg FITC-dextran in 100uL PBS, and FITC levels were measured in the serum after 3h p.i. *Eh* induced a modest though not significant (NS) increase in FITC levels in WT mice, but the same was not true of *Muc2*$^{-/-}$ animals. WT mice: n = 5, *Muc2*$^{-/-}$: n = 4.

Identification of the E. histolytica virulence factor involved in altering intestinal permeability

As *Eh* infection elicited a hyper-secretory response in *Muc2*$^{-/-}$ it was of interest to identify the parasite virulence component involved in this response. Based on the results obtained in Figures 6-7 and 6-8, we used *Il-1β* gene expression to evaluate pro-inflammatory responses and *Cldn-2* and *Ocln* expression as markers for alteration in TJ permeability. For this study we used *Muc2*$^{-/-}$ mice as the colonic response was robust in these animals. As expected, live *Eh* was most effective at up regulating *Il-1β* mRNA expression however, equivalent amounts of lysed (Eh Lys) or fixed *Eh* (Eh Glut) inhibited expression by 89 and 95%, respectively (Fig. 6-13). Similarly lysed/fixed *Eh* decreased

Cldn-2 and *Ocln* expression but not to the same extent as with *Il-1β* (Fig. 6-13). As live *Eh* evoked a pro-inflammatory response and altered TJ proteins in the absence of direct parasite-cell contact, and *Eh* cysteine proteinases can stimulate pro-inflammatory responses in *Muc2*$^{-/-}$ colons[28], we speculated that these might be the candidate virulence factor involved in inducing these effects. Consistent with our previous findings, Figure 6-13 A-C shows that live *Eh* pre-treated with the specific cysteine proteinase inhibitor E64, markedly reduced *Il-1β*, *Cldn-2* and *Ocln* mRNA expression. Moreover, as EhCP5 is the major proteinase released by live *Eh*[8], it was not surprising that *EhCP-A5*$^{-/-}$ deficient ameba was refractory for stimulating *Il-1β, Cldn-2* and *Ocln* mRNA expression in *Muc2*$^{-/-}$ mice (Fig. 6-14).

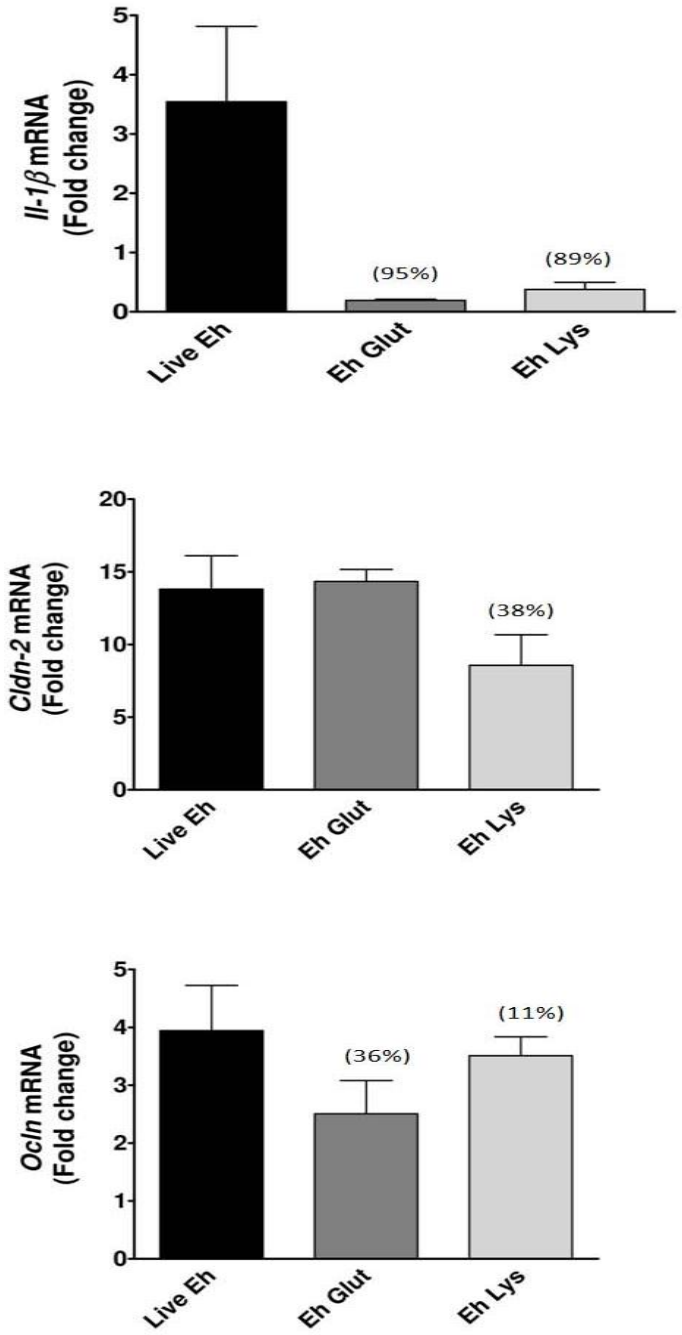


Figure 6-13: Live but not fixed or lysed *Eh* induce pro-inflammatory and TJ protein changes in *Muc2*$^{-/-}$ mice. Colonic loops in *Muc2*$^{-/-}$ mice were inoculated with live *Eh* (Live *Eh*), glutaraldehyde fixed amebae (*Eh* Glut) or an equivalent amount of fresh freeze/thaw amebic lysates (*Eh* Lys). Live amebae, but not glutaraldehyde fixed or fresh amebic lysates induced a robust *Il-1β* pro-inflammatory response and to a lesser extent *Cldn-2* and *Ocln* gene expression. Values in parentheses are % reduction compared to live *Eh*. n=3 for glutaraldehyde fixed and amebic lysates, n=10 for WT amebae.

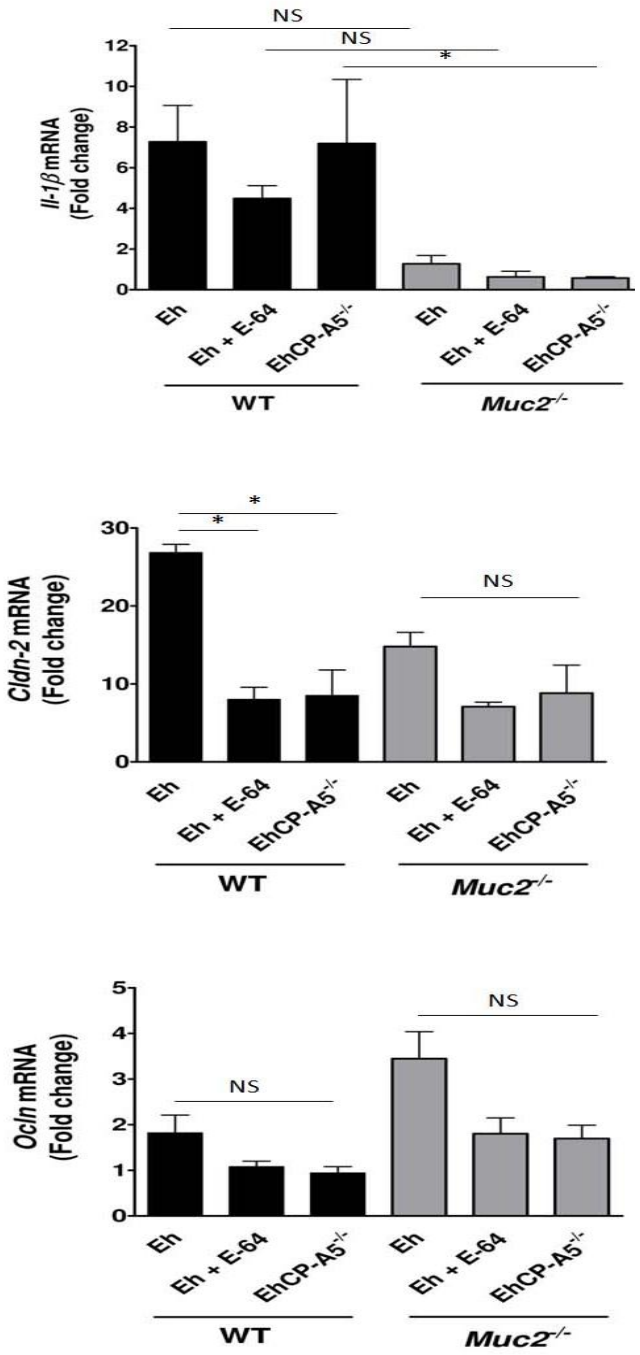


Figure 6-14: Virulent *Eh* but not *EhCP-A5*$^{-/-}$ induces pro-inflammatory and tight junction protein gene expression in *Muc2*$^{-/-}$ mice. WT and *Muc2*$^{-/-}$ mice were infected with *Eh* or *Eh* pre-treated for 12h with the cysteine protease inhibitor E-64 (*Eh* + E-64) and *EhCP-A5*$^{-/-}$ for 3h p.i., and *Il-1β*, *Cldn-2* and *Ocln* gene expression was examined. All gene expression data is expressed as fold change compared to sham controls. *Eh*: n=4, *Eh*+E-64: n=4, *EhCP5-A5*$^{-/-}$: n=4. *p<0.05, NS = not significant. The gross pathology scores were similar to those depicted in Figure 1C. Even though E64 treated *Eh* and *EhCP-A5*$^{-/-}$ elicited an overall lower gross pathology score in WT and *Muc2*$^{-/-}$ mice the values were not significantly different due to the low sample size.

To confirm if *Eh*CP5 altered TJ proteins, we used confocal immunofluorescence microscopy to visualize Cldn-2, Ocln and Zo-1. In WT mice infected with *Eh*, Cldn-2 expression remained the same while Ocln was increased and Zo-1 was lost whereas, in response *EhCP-A5*$^{-/-}$ there were no changes. Furthermore, *Eh* induced Cldn-2 expression at the apical cell surface concomitant with a loss of Zo-1 in *Muc2*$^{-/-}$ was not seen with *EhCP-A5*$^{-/-}$ (Fig. 6-15 A). Curiously, a loss of Ocln and Zo-1 was also observed in these mice infected with *Eh* at 3h p.i., and although this was absent with *EhCP-A5*$^{-/-}$, the degradation of Zo-1 was less pronounced (Fig. 6-15 B). Thus, EhCP5 appear to be a major virulence factor responsible for eliciting both pro-inflammatory responses and alterations in TJ proteins.

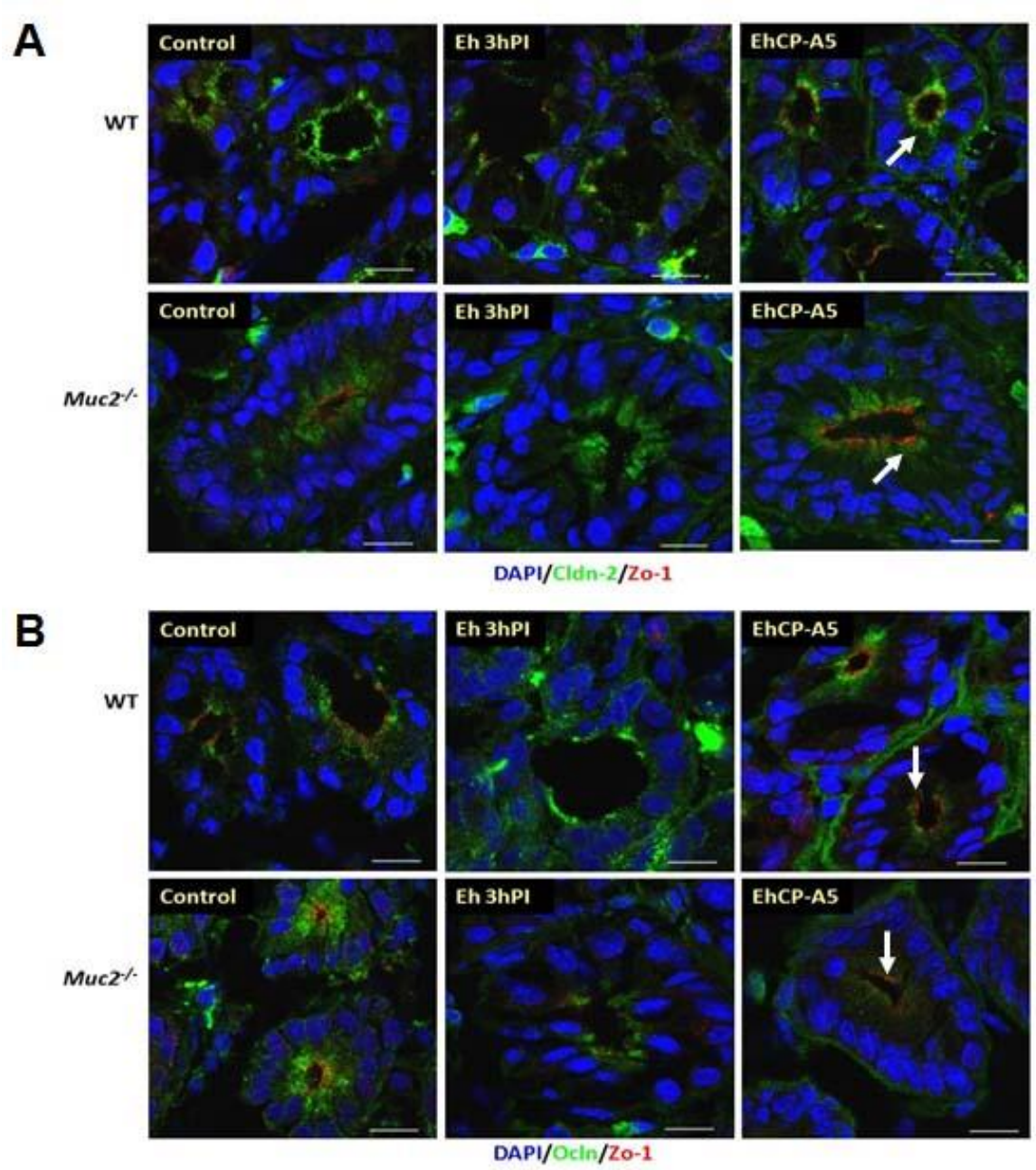


Figure 6-15: *EhCP-A5*$^{-/-}$ does not alter tight junction proteins. Mice were infected with virulent *Eh* or *EhCP-A5*$^{-/-}$ in a colonic loop for 3h and localization of the TJ proteins Cldn-2 (Panel A), Ocln (Panel B) and Zo-1 were examined. *Eh* induced no change in Cldn-2 in WT mice though a modest increase was observed in *Muc2*$^{-/-}$ animals (arrow). In both cases, these changes were concomitant with a loss of Zo-1 and this was attenuated in mice infected with *EhCP-A5*$^{-/-}$. Although a different pattern was observed in Ocln that increased in WT but decreased in *Muc2*$^{-/-}$ mice, the changes in Zo-1 remained consistent. Scale bar represents 50µm.

Discussion

The colonic mucus layer is the first line of innate host-defence against intestinal amebiasis and *Eh* interactions with MUC2 may hold the key to understanding disease pathogenesis. *Eh* binds epithelial cells as a prerequisite for host-cell cytolysis and disease pathogenesis, and this is inhibited when parasites are incubated with purified human colonic mucins[23]. We have shown that EhCP5 cleaves MUC2 at the C-terminus to disrupt the mucin polymer and to compromise barrier integrity[29]. Curiously, Muc2 lacks the critical amino-acid residues susceptible to EhCP5 degradation, which may explain why rodents are resistant to intestinal amebiasis. In WT mice with an intact mucin barrier, *Eh* colonizes the mucus layer but cannot degrade it. However it is unclear how *Eh* interacts with the colon in the absence of this barrier. Our studies show that with an intact mucus layer, mucin secretion is rapidly induced following *Eh* infections, and this engulfs invading trophozoites preventing parasites from binding the epithelia. Curiously, *Eh* also elicited a heightened pro-inflammatory response characterized by elevated levels of Il-1β. However, in the absence Muc2, *Eh* induced a robust hyper-secretory response associated with movement of water into the colon, a differential pro-inflammatory response characterized by elevated Tnf-α and Ifn-γ, altered expression of TJ proteins and increased intestinal permeability to serum proteins. Importantly, in the absence of this protective mucus layer there were no signs of epithelial damage, suggesting that other innate responses are present in *Muc2*$^{-/-}$ mice that prevent colonization and/or epithelial destruction. Previous studies have shown that aberrant mucin expression is observed in colitis in *Muc2*$^{-/-}$ mice, where it is thought to serve a protective role[25]. Our studies support these findings that in the distal colon of *Muc2*$^{-/-}$ mice, *Muc6* gene expression was

present at low levels where it was localized to the base of the crypts in the AB-positive staining region[25]. In response to *Eh*, a time-dependent increase in *Muc6* gene expression was observed in both WT and *Muc2*$^{-/-}$ mice suggesting that this may represent an unappreciated innate immune response towards *Eh*.

A curious finding was that *Eh* induced a robust differential pro-inflammatory response in WT and *Muc2*$^{-/-}$ mice that correlated with changes in Il-1β, Tnf-α, Ifn-γ and Il-13 expression. In WT, *Eh* induced elevated levels of only the pro-inflammatory cytokine Il-1β, while in *Muc2*$^{-/-}$ animals, in addition to Il-1β, Tnf-α, Ifn-γ and Il-13 proteins were all increased in colonic exudates despite modest changes in mRNA expression. It is well known that IECs produce Il-1β *in vivo* following injury, and *Il-1β* gene expression plays a critical role in the early events leading to inflammation[30]. It is unclear however, in our system, whether the increased Il-1β expression is itself protective or if it induces protection mediated by other cytokines. Interestingly, in *Muc2*$^{-/-}$ mice the increased gross pathology and robust secretory responses were concomitant with elevated levels of Il-1β, Tnf-α, Ifn-γ and IL-13. Thus, it is likely that this increase represents an aberrant and uncontrolled pro-inflammatory response. Previous studies have shown that *Muc2*$^{-/-}$ mice constitutively express increased levels of pro-inflammatory cytokines, chemokines and immunoglobulins, suggesting that these animals may have ongoing low-grade inflammation. Furthermore, a deficiency in Muc2 has been shown to result in colonic bacteria in direct contact with epithelial cells, which may lead to elevated levels of pro-inflammatory cytokines[31], and may contribute to the onset and perpetuation of colitis[2, 25]. In addition, *Muc2*$^{-/-}$ mice have increased bacterial penetration prior to DSS induced

colitis[32], suggesting that epithelial integrity is already compromised in these animals. In light of these reports, it appears that the robust increase in pro-inflammatory cytokines observed here may be aberrant responses characteristic of a hyper-active immune system, therefore suggesting that not only does Muc2 act as a physical barrier to prevent colonization, but it also protects against robust acute inflammatory responses toward mild injury.

In *Eh* infection it is unclear what causes alteration in gut permeability to water, ions or protein leakage. It is known that elevated levels of pro-inflammatory cytokines are associated with alterations in TJ permeability; however, the precise mechanism underlying this effect is uncertain. Serosal addition of Tnf-α to HT-29/B6 colonic cells resulted in an 81% decrease in TER, reduced complexity and depth of TJ strands, and increased sodium and mannitol flux[33]. Similarly, Ifn-γ reduced TER in T84 monolayer after 48h[34] with redistribution of Ocln, Cldn-1, Cldn-4 and Jam-A away from the TJs via protein endocytosis[35]. Furthermore, a synergistic effect was observed when cells were treated with a combination of Ifn-γ and Tnf-α[35]. While the mechanisms are not clear, it appears that Tnf-α and Ifn-γ promote barrier dysfunction by altering TER and TJ permeability through decreased expression of the barrier tightening claudins (Cldn-1,-5 and -7) and Ocln[36]. Thus, it is tempting to speculate that the pro-inflammatory responses observed in *Muc2*$^{-/-}$ mice may be responsible for the alterations in gut permeability. Indeed, previous studies have shown that IECs secrete TNF-α which may explain the elevated levels of this cytokine in the intestinal lumen, and TNF-α is a chemotactic and chemokinetic stimulus for *Eh*[37]. Furthermore, TNF-α is associated with gut

inflammation[38] and the diarrheal response typically observed in *Eh* infections in children[39]. Thus it is likely that Tnf-α is an important player in driving the secretory responses observed in these studies.

An emerging concept in the movement of water, ions and high molecular weight molecules between serosal and luminal cell surfaces is the pore and leak pathway[40]. The pore pathway, a high capacity paracellular route of transport, is size and charge selective allowing movement of molecules 4Å or less. It is thought to be under the control of the claudins expressed[40], and Il-13 has been shown to activate this pathway[40]. The leak pathway however, is a low capacity channel that allows the flux of larger ions and molecules regardless of their charge, and Zo-1 and Ocln have been implicated in the control of this pathway[40]. Curiously, the TNF-α induced changes in barrier integrity have been associated with alterations in this pathway[40]. Furthermore, pathogens can increase intestinal permeability by altering TJ localization and function and activation of these pathways, and we have previously shown that the intestinal barrier is compromised in *Muc2*$^{-/-}$ mice infected with *Citrobacter rodentium*[41]. Here we show for the first time that *Eh* alters TJ permeability *in vivo*, by increasing Cldn-2 expression and localization, which may be the result of increased Il-13 expression while at the same time altering Ocln and Zo-1 expression which may be mediated by changes in TNF-α. Claudin-2 is a small protein (20-27kDa) localized to the crypts but it is rarely expressed in the normal epithelium. It is increased in inflamed tissue such as Celiac disease[42] and UC[43], and is associated with increased ion and water transport across the membrane and decreased TER[44]. Occludin is a 65kDa protein, and siRNA silencing increased the flux of large

probes including mannitol and dextran, and the expression of Cldn-2 in enterocytes[45]. Ocln expression is down regulated by Il-1 by an NF-κB-dependent mechanism[46], and TNF-α has been shown to induce Ocln internalization. The function and stability of claudin/occludin are post-transcriptionally modified and phosphorylation of these proteins leads to increased protein internalization, indicative of either increased protein turnover or disruption of the interaction between scaffolding proteins[47], including Zo-1. Both claudin and occludin associate with the plasma membrane protein Zo-1, a 220kDa protein linked to the actin cytoskeleton. Interestingly, studies *in vitro* have demonstrated that *Eh* trophozoites can increase paracellular permeability through the degradation of Zo-1[48].

Here we show that *Eh* infection differentially regulates the expression of TJ proteins depending on the presence or absence of an intact mucin barrier. In WT mice, we hypothesize that the elevated levels of Cldn-2 opened the pore pathway facilitating the movement of water and ions into the intestinal lumen hydrating the mucus layer. Once hydrated, this layer effectively traps *Eh* trophozoites, thereby preventing direct contact with the underlying IECs. Furthermore, Eh degrades the barrier forming protein Zo-1, which would normally lead to increased intestinal permeability through the leak pathway. In the presence of an intact mucin barrier however, *Eh* increases Ocln expression which was localized to the TJ, where it reinforces the barrier and prevents increased epithelial permeability and penetration by other potential pathogens. In *Muc2*$^{-/-}$ mice however, *Eh* induces a markedly different response. Our data suggests that the high basal and increased Cldn-2 expression at the apical surface of enterocytes could lead to increased

flux of water and ions in a similar manner to that induced in WT mice. However, in the absence of an intact mucin barrier, the markedly reduced expression of both Ocln and Zo-1 following *Eh* infection in *Muc2*$^{-/-}$ animals leads to a loss of barrier integrity facilitating the movement of serum proteins into the intestinal lumen. In addition, our data demonstrate that changes in intestinal permeability in the absence of direct contact, is primarily mediated by the virulence factor EhCP5 secreted by live parasites.

In summary, using *Eh* as a model of a non-invasive pathogen in WT and *Muc2*$^{-/-}$ animals, we have unravelled novel findings on how colonic Muc2 plays a critical role in both luminal and epithelial barrier functions. In WT mice with an intact mucin barrier, *Eh* stimulated mucin secretion and pro-inflammatory cytokines aimed at sequestering the parasites away from the epithelium. However, in *Muc2*$^{-/-}$ mice, *Eh* induced an aberrant pro-inflammatory response concomitant with altered expression of Cldn-2, Ocln and Zo-1 leading to activation of the pore and leak pathways, resulting in a robust secretory response presumably aimed at flushing out the parasites. While the net effect may function to prevent parasite colonization and/or invasion in *Muc2*$^{-/-}$ animals, the mechanisms by which this was achieved may have contributed to the initial pathologic responses. Thus, our study has defined for the first time an unrealized mechanistic role for Muc2 in conferring epithelial barrier functions in innate host-defences against *Eh*.

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SECTION V: DISCUSSION & FUTURE DIRECTIONS

CHAPTER 7: DISCUSSION

The gastrointestinal tract is exposed to a plethora of antigenic substances daily, including food and nutrients, and is faced with the monumental task of separating the good from the bad. This segregation is largely accomplished by the intestinal epithelium.

Intestinal epithelial barrier function is multilayered and involves host and bacterial proteins present in the lumen, the mucus layer and peptides embedded in it, and the epithelial cells and tight junctions between them. At the center of this triad is the mucus layer, composed of the secreted mucin, MUC2. *In vitro*, MUC2 protects epithelial cells by acting as a luminal barrier to prevent binding of pathogenic agents directly to the cell surface. *In vivo*, Muc2 protects against attaching and effacing bacteria and it facilitates clearance of parasites following nematode infections. In addition, Muc2 plays a role in protection against colon cancer since *Muc2*$^{-/-}$ mice are prone to spontaneously develop adenomas and adenocarcinomas within the first year of life. Therefore it is clear that Muc2 plays a role in protection at the luminal surface.

However the role of Muc2 as it pertains to other components of epithelial barrier function remains unclear. Prior to these studies, it was not known whether Muc2 exerted other effects on intestinal barrier function. Specifically, it was not clear how Muc2 was involved in normal GI function and motility or if it played a role in health as well as

disease. Although Muc2 was important at the luminal surface, it was unclear if it directly acted on bacteria present in the lumen, the epithelial cells or the tight junctions or if it simply served as a protective but essentially inert wall on the surface IEC. The purpose of the studies outlined in this thesis is intended to address these concerns.

Using WT mice with an intact mucin barrier, and those with a partial (*Muc2*$^{+/-}$) or complete loss (*Muc2*$^{-/-}$) of this defence mechanism, the role of Muc2 in heath and disease was investigated. The results of the studies conducted for this thesis show that Muc2 is critical in maintaining intestinal epithelial barrier function *in vivo*, and when this layer is disrupted or the integrity of the barrier compromised, animals are more susceptible to chemical and/or pathological injury.

The Role of Muc2 in Intestinal Barrier Function

Healthy controls were examined to determine if Muc2 played a role in intestinal morphology or barrier function in the absence of disease. In the absence of Muc2, the microbial composition was altered resulting in the depletion of the *Bacteroides* and *Firmicutes* phyla which are normally resident in the murine colon. Histological examination of tissue samples from WT and *Muc2*$^{-/-}$ animals further revealed that intestinal morphology was altered. In particular a thickened muscle layer with longer crypts was evident in *Muc2*$^{-/-}$ animals. In addition, *Muc2*$^{-/-}$ mice appeared to be devoid of goblet cells or other PAS positive staining mucins. To assess whether or not these changes had an impact on epithelial barrier function, GI motility and intestinal permeability was examined. In the absence of Muc2, upper GI transit time remained the

same but total gut transit was significantly reduced indicative of increased movement through the colon. Intestinal permeability as assessed by uptake of FITC dextran or fractional sucralose excretion was increased in *Muc2*$^{-/-}$ mice as early as one month after birth, and these remained elevated at 5 months of age. Taken together these findings demonstrate that Muc2 is an essential component of epithelial barrier function in healthy individuals.

The Role of Muc2 on Disease Progression and Recovery

Since increased permeability is associated with intestinal injury, either as a cause or a consequence of disease, it was of interest to determine if in the absence of an intact mucin barrier animals were more susceptible to models of acute injury. The DSS model of colitis was chosen since it provides an ideal model to investigate the disease process from onset, through progression into recovery.

Muc2$^{-/-}$ mice were extremely sensitive to DSS as evidenced by rapid weight loss, increased disease activity index scores and mortality in these animals, even at dosages as low as 0.75% (5d). WT animals however tolerated dosages of 4% DSS (5d) and *Muc2*$^{+/-}$ animals did not die on 3% DSS (5d) though significant weight loss was observed, clearly demonstrating that during intestinal injury, even an incomplete mucus layer still provides some protective benefit. Additionally, the disease onset, at least in severe colitis was much more rapid in the absence of a mucus layer. When treated with DSS for 5d, *Muc2*$^{-/-}$ animals lost weight as of Day 3 leading to significant weight loss and mortality necessitating the premature termination of experiments. In contrast, WT mice also

treated for 5d lost some weight at Day 4 but a significant decline was not observed until DSS was removed, demonstrating a role for Muc2 in disease onset. In addition in the absence of Muc2, injury was more diffuse and affected the full length of the colon. Loss crypt architecture, thickening of the muscle layer, ulceration and complete denuding of the epithelium were evident in these animals, even after DSS was removed for 5d. Damage in the WT colon however was primarily restricted to the distal colon with normal epithelium and increased mucin secretion evident in more proximal regions less affected by DSS. This suggests that Muc2 may also be involved in reducing the extent of damage induced by noxious agents. In addition, at milder levels of colitis, recovery was impaired in the absence of Muc2. Indeed, although these animals lost the same amount of weight as their WT counterparts they had regained less by Day 15, and the colon length did not improve during recovery. These data clearly demonstrate that Muc2 mucin plays an important role in all phases of disease pathogenesis. Here we have shown that Muc2 delays disease onset, protects against severe injury, and facilitates recovery *in vivo*.

Since the epithelial barrier is composed of bacteria present in the intestinal lumen and IEC, in addition to the mucus layer, it was of interest to determine if in the absence of Muc2 these other components could exert beneficial effects. Since the probiotic mixture VSL#3 acts on all levels to promote barrier function *in vivo*, it was of interest to determine if augmenting this natural host defence in *Muc2*$^{-/-}$ animals could reduce the severity of disease. WT and *Muc2*$^{-/-}$ mice were treated once daily with VSL#3 by oral gavage before, during and after disease induction. At none of the doses used did VSL#3 alter the disease onset, progression or recovery in either WT or *Muc2*$^{-/-}$ animals in terms

of weight loss or DAI scores. VSL#3 did not change the expression of the pro-inflammatory cytokines associated with disease onset or recovery, or any of the growth factors examined indicating that at least in this model VSL#3 did not play a beneficial role. This data suggests that strengthening of the existing defence mechanisms in the absence of Muc2 may not be sufficient to alter disease onset, progression or recovery.

While previous studies have shown that in the absence of Muc2 animals are more susceptible to infection, they have focused on the protective function of Muc2 in the intestinal lumen but failed to examine the effect of Muc2 on other components of barrier function.

The Role of the Mucus layer in Disease Prevention

The intestinal parasite *E. histolytica* causes significant morbidity and mortality in the developing world and this is largely due to diarrhea induced by the parasite. Furthermore, *Eh* produces lipid mediators which act on various components of the TJ to increase intestinal permeability. Since epithelial barrier function was compromised in *Muc2*$^{-/-}$ animals, it was of interest to examine the early responses of these animals to acute parasitic injury, and to determine if other protective mechanisms existed in these animals to prevent excessive injury. The present findings indicate that *Muc2*$^{-/-}$ mice were significantly more susceptible to *Eh* and this occurred as early as 1h p.i. In the absence of an intact mucus layer, the parasite did not bind to or destroy IECs, but rather induced an exaggerated inflammatory response, altered expression of TJ and ultimately led to aberrant protein secretion. Furthermore, all of the responses induced in *Muc2*$^{-/-}$ animals

served to suspend the parasite in the fluid filled intestinal lumen where it was unable to bind to and destroy epithelial cells, suggesting that in these animals some as yet unidentified mechanisms of innate host defence may be at play to protect against invasion.

The research presented in this thesis has made significant contributions to the field of mucin biology and our understanding of the role of the mucus layer *in vivo*. Firstly we have shown that Muc2 is important in maintaining barrier function and reducing intestinal permeability in healthy individuals. Secondly, in the absence of this protective mechanism, not only is susceptibility to disease increased, but mechanisms to control disease onset and progression and those that facilitate recovery may also be compromised. Thirdly, in the absence of this barrier, probiotics that enhance other aspects of barrier function are of little benefit. Finally we have shown that in the absence of a mucin barrier, animals are more susceptible to pathogenic infections leading to robust inflammatory responses and alterations in tight junction permeability.

While it is clear that further investigation needs to be done to explore in more detail the various components of intestinal epithelial barrier function *in vivo*, it is clear that the mucus layer is an integral part of this barrier. This research has major implications on human health and disease. Pathogens possess a variety of virulence factors that destroy the mucus layer or components of it, thereby compromising the integrity of this barrier, and this is further compounded by co-infections, only some of which may result in noticeable symptoms. The data presented here indicate that when the integrity of the

mucus layer is weakened, this could have an impact on epithelial barrier function by altering the composition of the intestinal microflora, reducing the thickness and therefore the level of protection offered by this layer or reducing barrier integrity by acting on the IEC and TJ. In the context of IBD the thickness of the mucus layer is reduced in patients with ulcerative colitis, and its integrity is altered in Crohn's disease. This could result in increased susceptibility to secondary GI infections in these individuals, and this problem may be compounded by biologic therapies currently in use to manage the symptoms of the primary disease. Therefore, it is clear that strategies to strengthen this barrier are necessary as are therapeutics that fortify, rather than destroy this essential host defence.