ABSTRACT

LENNON, ELIZABETH MORE. Investigating the Pathogenesis of Inflammatory Bowel Disease: The Influence of Early Life Stress and the Role of the Mast Cell in the IL10⁻/⁻ Model. (Under the direction of Adam Moeser.)

The inflammatory bowel diseases (IBDs), debilitating intestinal diseases that affect between 1-1.5 million people in the United States, appear to be the result of a complex interaction between genetic predisposition, environmental factors, and a dysregulated immune response to the intestinal microbiota, but the pathogenesis is not well understood. No cure is available, so this disease requires lifelong treatment that carries significant side effects. Psychological stress contributes to disease flares, and the brain-gut-mast cell axis has been implicated. We have developed a model of early life stress that exacerbates colitis in the IL10⁻/⁻ model.

Mast cells (MCs) are thought to be involved in the pathogenesis of IBD, as their numbers and products are altered in IBD patients. MCs play a key role in intestinal health: they regulate the intestinal barrier and clear bacteria that may breach the compromised intestinal barrier in IBD. Additionally, they conduct surveillance of the environment for bacteria and antigens through surface Fce receptors as well as pattern recognition receptors such as toll-like receptors (TLRs), and regulate and orchestrate immune responses through interaction with other cell types such as T lymphocytes.

However, the exact role of the MC in IBD is not fully defined. MCs have a deleterious role in acute chemical detergent colitis models. In contrast, we have demonstrated that MCs play a protective role in spontaneously occurring colitis in IL10⁻/⁻ mice on a C57Bl/6 background. IL10⁻/⁻ mice that also lack MCs (double knockout, DKO: (IL-10⁻/⁻ x
Kit	extsuperscript{Wsh/Wsh} have more severe colitis than MC-sufficient IL10	extsuperscript{−/−} mice. DKO mice have increased colitis scores, mucosal hypertrophy and enhanced colonic TNF production, which can be ameliorated by repletion with bone marrow-derived MCs (BMMCs). These findings indicate a novel protective role of MCs in spontaneous colitis.
Investigating the Pathogenesis of Inflammatory Bowel Disease: The Influence of Early Life Stress and the Role of the Mast Cell in the IL10⁻/⁻ Model

by
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A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Comparative Biomedical Sciences

Raleigh, North Carolina
2015

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BIOGRAPHY

Elizabeth Lennon was born in Bethlehem, Pennsylvania in 1980. She graduated from Saucon Valley High School in 1998, and then went on to complete her Bachelor of Science degree in Animal Sciences from the University of New Hampshire in Durham, NH in 2002. During her studies at the University of New Hampshire, Liz assisted with a research project monitoring growth and estrus cycles of dairy heifers. Following her graduation, she took a position as the serology laboratory supervisor at the New Hampshire Veterinary Diagnostic Laboratory. In 2003, she moved to Raleigh, North Carolina to attend veterinary school at North Carolina State University. During her veterinary school training, Liz sought out numerous research opportunities in order to continue her training as a clinician scientist. Following veterinary school, Liz completed a small animal rotating internship in small animal medicine and surgery at the University of Pennsylvania, and then accepted a position in the clinician scientist training program at North Carolina State University, which combines a small animal internal medicine residency with a PhD program. In 2011, Liz completed her residency and became board certified in small animal internal medicine by the American College of Veterinary Internal Medicine. She then began working full time on her graduate research in the laboratory of Dr. Adam Moeser in the Comparative Biomedical Sciences department with a focus in the area of cell biology. The focus of her graduate research was in the role of psychological stress and mast cells in the pathogenesis of inflammatory bowel disease, which is the work presented here.
ACKNOWLEDGMENTS

I would like to thank my mentor, Dr. Adam Moeser, for the opportunity to work in his laboratory and for his mentorship and guidance throughout the duration of my PhD program. I would also like to thank Dr. Jody Gookin for her tremendous support through my residency and PhD program. She has given me advice and helped me throughout the last 6.5 years more times than I can count, and she served as a role model for me since I was a veterinary student. I appreciate her hard work and excellent example of a clinician scientist. I would also like to thank Dr. Sam Jones for his support and for directing the T32 program, which made it possible for me to attend graduate school, and he and Anthony Blikslager for serving on my PhD committee and for their feedback.

I would also like to thank the members and former members of the Moeser laboratory, in particular Dr. Amelia Gibson and Laura Edwards, for their friendship, advice, and encouragement as well as help with my experiments.

I would also like to thank my parents, Linda and Jerry Lennon, who have always given me their love and support. I would also like to thank my friends and family for being there for me.
TABLE OF CONTENTS

LIST OF TABLES ........................................................................................................................................... vii
LIST OF FIGURES ......................................................................................................................................... viii

CHAPTER 1: The Influence of Stress on Inflammatory Bowel Disease ................................................. 1

Literature Review ......................................................................................................................................... 1

Inflammatory bowel disease: Significance ................................................................................................. 1

Psychological Stress in the Pathogenesis of Inflammatory Bowel Disease ................................. 1

Psychological Stress in IBD Models ........................................................................................................... 4

Early Life Stress Triggers Persistent Colonic Barrier Dysfunction and Exacerbates Colitis in Adult IL-10−/− Mice ................................................................................................................................. 5

Abstract .................................................................................................................................................... 5

Introduction .................................................................................................................................................. 6

Materials and Methods ............................................................................................................................. 8

Mice ......................................................................................................................................................... 8

Neonatal Maternal Separation ..................................................................................................................... 9

Sample collection ........................................................................................................................................ 9

Histology ..................................................................................................................................................... 9

Cytokine analysis ......................................................................................................................................... 10

Intestinal Barrier Function ......................................................................................................................... 11

Mast cell counts and assessment of mast cell product tryptase ................................................................. 12

Gel electrophoresis and Western blotting ..................................................................................................... 12

Statistics ................................................................................................................................................... 13

Results ....................................................................................................................................................... 13

Neonatal maternal separation exacerbates histologic colitis in IL-10 deficient mice ......................... 13

Neonatal maternal separation causes increased colonic proinflammatory cytokine production in IL-10 deficient mice .................................................................................................................... 14

IL-10 deficiency and neonatal maternal separation stress compromise intestinal barrier function ................................................................................................................................. 14

Transepithelial short circuit current (Isc) ................................................................................................. 14

Mast cell activity ........................................................................................................................................ 15

Discussion .................................................................................................................................................. 15

Acknowledgments ..................................................................................................................................... 19
Results .......................................................................................................................... 45

Mast cells are protective against spontaneous colitis .............................................. 45

Fecal scoring and fecal % water ............................................................................. 46

Assessment of intestinal barrier function ............................................................... 46

Cytokine analysis ...................................................................................................... 47

Germ free mice .......................................................................................................... 47

Real time PCR Array for Mouse Cytokines/Chemokines ........................................ 48

BMP protein and gene expression .......................................................................... 48

Mast cell- CD4+ T cell coculture ............................................................................ 48

Discussion .................................................................................................................. 49

Figures ....................................................................................................................... 54

REFERENCES ............................................................................................................ 70
LIST OF TABLES

TABLE 1: PCR Array .................................................................................................................. 69
LIST OF FIGURES

CHAPTER 1

Figure 1-1: Experimental Timeline .................................................................................................................. 20
Figure 1-2: NMS exacerbates colitis in IL10<sup>−/−</sup> mice................................................................. 21
Figure 1-3: NMS stress exacerbates colitis scores and colon length. ......................... 22
Figure 1-4: Colonic cytokine levels are increased in IL-10<sup>−/−</sup> mice subjected to
NMS .......................................................................................................................................................... 23
Figure 1-5: NMS and IL-10 deficiency alter colonic barrier function......................... 24

CHAPTER 2

Figure 2-1: Bone marrow-derived mast cells .............................................................. 54
Figure 2-2: Confirmation of mast cell repletion.............................................................. 55
Figure 2-3: Mast cell deficiency exacerbates colitis in IL10<sup>−/−</sup> mice...................... 56
Figure 2-4: Mast cell deficiency exacerbates colitis....................................................... 57
Figure 2-5: Mast cell deficiency causes mucosal hypertrophy................................. 58
Figure 2-6: Fecal scoring and fecal percent water ......................................................... 59
Figure 2-7: DKO mice demonstrate increased intestinal permeability as
measured by 4KDa FITC-dextran flux............................................................................ 60
Figure 2-8: Spontaneous colonic cytokine production ............................................. 61
Figure 2-9: Spontaneous colonic TNF production........................................................ 62
Figure 2-10: DKO mice in germ-free conditions do not develop colitis................ 63
Figure 2-11: TNF production in germ free mice.......................................................... 64
Figure 2-12: Representative PCR array analysis of genes for 82 cytokines and chemokines................................................................. 65

Figure 2-13: BMP is present in BMMCs. ......................................................... 66

Figure 2-14: BMP7 protein expression is detectable in mucosal scrapes from IL10−/− and DKO mice. ................................................................. 67

Figure 2-15: Long term exposure of mast cells to LPS dampens proinflammatory cytokine production from coculture..................................................... 68
CHAPTER 1: The Influence of Stress on Inflammatory Bowel Disease

Literature Review

Inflammatory bowel disease: Significance

The inflammatory bowel diseases (IBDs), Crohn’s disease (CD) and ulcerative colitis (UC) affect 1-1.5 million people in the United States, resulting in an estimated 6.3 billion dollars in annual healthcare costs. (Kappelman et al., 2008) IBD is a debilitating and incurable disease which has a significant impact on quality of life; many patients experience severe abdominal pain, the need for multiple surgeries to resect severely affected areas of intestine, and psychological impacts. (Graff, Walker, & Bernstein, 2009; Lix et al., 2008; Walker, Graff, Dutz, & Bernstein, 2011) Complications of IBD can include fistulas, abscesses, and increased risk of colorectal cancer. (Arora et al., 2010; Cerqueira & Lago, 2013; Koganei, Sugita, Tatsumi, Futatsuki, & Kimura, 2012; Lakatos et al., 2012; Vatn, 2009) These diseases have no cure and require lifelong treatment. Medical management largely consists of immunosuppression, which can result in life threatening complications and unwanted side effects. (Cerqueira & Lago, 2013; Hudesman, Lichtiger, & Sands, 2013; Lan, Patil, & Shen, 2013; Long, Martin, Sandler, & Kappelman, 2013; Louis, 2013; Norgard et al., 2013)

Psychological Stress in the Pathogenesis of Inflammatory Bowel Disease

Stress is defined as an acute threat to the homeostasis of an organism, either real (physical) or perceived (psychological). (Bhatia & Tandon, 2005). Psychological stress has an impact on disease activity in human IBD, with the majority of patients reporting a link between flares of their illness and stress. (Mawdsley & Rampton, 2005) However, measuring
stress in humans is challenging because stress is individually interpreted. Despite these challenges, studies from people with IBD provide numerous links between stress and initiation and/or flares of IBD. Adverse life events, chronic stress, and depression are associated with increased likelihood of relapse in IBD. (Bitton et al., 2008; Mardini, Kip, & Wilson, 2004) In one analysis, the relationship between major life events and the risk of developing UC was evaluated in newly diagnosed patients and matched hospital controls. Almost half of the UC patients reported a stressful life event within 12 months before diagnosis compared to only 11% of the controls. (Tocchi et al., 1997) Another study of 200 newly diagnosed CD patients compared stressful life event occurrences in CD patients to patients with acute gastrointestinal illnesses and a community sample of blood donors. CD patients experienced significantly more life events compared with controls and had higher stress severity ratings. (Lerebours et al., 2007) The majority of studies considering the relationship between stressful events and disease flares have reported positive associations, although other well designed studies have failed to show an association. In a prospective study, 62 patients with inactive UC were tracked for 4 to over 5 years. Higher perceived stress levels at baseline significantly increased the risk of disease exacerbation. Also, higher chronic perceived stress tripled the risk of exacerbation over an 8-month period. (Levenstein et al., 1994) Another study evaluated whether there was a relationship between stress levels and mucosal inflammation in asymptomatic UC patients. Indeed, the results of this study showed that symptomatic patients were more likely to report major life events in the previous 6 months. However, the finding that asymptomatic patients with endoscopically confirmed inflammation reported significantly higher levels of chronic stress than asymptomatic
patients without mucosal inflammation provides strong supporting evidence for a role of stress in inflammatory disease activity. (Levenstein et al., 2000) More recently, 101 patients with inactive CD were seen every 3 months until relapse or for up to a year. One third of these patients relapsed over the observation period. Perceived stress in conjunction with a particular type of less adaptive coping (i.e. avoidance) significantly predicted earlier time to relapse. (Bitton et al., 2008) Chronic stress was found to be strongly predictive of subsequent IBD relapse and immune system activation, suggesting that there may be cumulative damaging effects of stress over time as well. (Miller, Cohen, & Ritchey, 2002)

The function of the stress response is to maintain homeostasis. Stress-induced alterations in gastrointestinal inflammation may be mediated through changes in the hypothalamic-pituitary-adrenal axis function, changes in epithelial barrier permeability, alterations in bacterial epithelial interactions, and via induction of stress responses in mucosal inflammatory cells. (Mawdsley & Rampton, 2005) Stress-induced changes at the level of sympathetic nervous system mediators can modulate the release of pro-inflammatory agents. Neuropeptides released during stress such as corticotrophin releasing factor (CRF) and substance P may alter the activity of immune and inflammatory cells. Both physical and psychological stressors have been found to increase intestinal permeability. (Gareau, Jury, & Perdue, 2007; Santos et al., 1999) Where some stress induced pathways may be deleterious, others may be protective such as the anti-inflammatory effects of stress-induced glucocorticoid release.
**Psychological Stress in IBD Models**

Psychological stress can also impact IBD in experimental models. In one study, self-limiting colitis was induced in mice by treatment with the inducer of chemical colitis DNBS. Colitis was reactivated by a combination of acute and restraint stress with a sub-threshold dose of DNBS, but not by DNBS alone. (Gareau et al., 2007; Qiu, Vallance, Blennerhassett, & Collins, 1999) In another model, stress reduced colonic mucin production and increased colonic permeability. (Gareau et al., 2007) Mast cell activation in a model of crowding stress was demonstrated to cause intestinal barrier dysfunction, one of the key initiating events in IBD (Vicario et al., 2010).

Loss of intestinal epithelial barrier function (increased intestinal permeability) is a central factor in IBD pathogenesis, allowing excessive translocation of luminal bacteria and antigens into sub-epithelial tissues, triggering a vicious cycle of chronic intestinal inflammation and further intestinal barrier injury. (Benjamin, Makharia, Ahuja, Kalaivani, & Joshi, 2008; Bruewer, Samarin, & Nusrat, 2006; Secondulfo et al., 2001) Several studies have demonstrated that psychological stress can negatively influence intestinal barrier function (Gareau, Silva, & Perdue, 2008; Kiliaan et al., 1998; Meddings & Swain, 2000) and thus may be an important link between stress and IBD flare-up. The exact role of stress-induced barrier disruption in IBD flare-ups has not been directly investigated due in large part to the lack of relevant animal models of stress-induced colitis.
Early Life Stress Triggers Persistent Colonic Barrier Dysfunction and Exacerbates Colitis in Adult IL-10−/− Mice

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Abstract

Background—It has become increasingly evident that disease flares in the human inflammatory bowel diseases (IBD) are influenced by life stress. It is known that life stress can trigger disturbances in intestinal barrier function and activate proinflammatory signaling pathways, which are important contributors to intestinal inflammation and clinical disease; however, the exact mechanisms of stress-induced IBD exacerbations remain to be elucidated. Here we present a model of early life stress-induced exacerbation of colitis in IL-10−/− mice.

Methods—C57Bl/6 wild type and IL-10−/− mice were exposed to neonatal maternal separation (NMS) stress on postnatal days 1-18 and reared under normal conditions until 10-
12 weeks of age. At this time, histopathology, colitis scores, intestinal barrier function, proinflammatory cytokine expression and mast cell activity were evaluated.

Results—NMS increased the severity of colitis in IL-10⁻/⁻ mice, indicated by greater colitis scores and colonic proinflammatory cytokine concentrations. NMS and IL-10⁻/⁻ increased colonic permeability; however, NMS alone did not induce colitis. Increased mast cell activation and colonic tryptase release were observed in IL-10⁻/⁻ mice exposed to NMS, indicating mast cell activation.

Conclusions—This study demonstrates that colitis in IL-10⁻/⁻ mice can be exacerbated by NMS stress. The precise mechanisms of enhanced colitis severity in NMS IL10⁻/⁻ mice are unclear but persistent defects in intestinal barrier function likely play a contributing role. NMS serves as a novel model to investigate the mechanisms by which early life stress influences the development and course of IBD in adulthood.

Introduction

The pathogenesis of IBD is multifactorial in nature, involving host genetic susceptibility, dysregulated immune responses to the enteric microbiota, and environmental influences. Life stress has been studied as a possible environmental trigger of IBD exacerbations. In one analysis, 45% of UC patients reported a stressful life event within 12 months of diagnosis compared to only 11% of matched hospital controls. (Tocchi et al., 1997) Other studies have corroborated these results, linking coping behavior, chronic stress, and relapse in IBD. (Bitton et al., 2008; Lerebours et al., 2007; Levenstein et al., 1994; Levenstein et al., 2000; Miller et al., 2002) Although epidemiological analyses demonstrate a link
between stress and IBD, the pathophysiological mechanisms through which stress impacts the onset and clinical course of IBD are not understood. Early life stressful events, including inadequate parental bonding and low maternal care have become increasingly scrutinized as a possible risk factor for the development of IBD later in life, but the biologic link between early life stress and development of IBD remains poorly understood. (Agostini, Rizzello, Ravegnani, Gionchetti, Tambasco, Ercolani et al., 2010; Agostini, Rizzello, Ravegnani, Gionchetti, Tambasco, Straforini et al., 2010; Ercolani et al., 2010) Infancy, childhood, and adolescence are critical periods in development that are characterized by increased vulnerability to stressors; therefore, exposure of the developing brain and intestine to adverse environmental factors may have long-lasting effects on the individual that may influence disease development and activity in adulthood. (Agostini et al., 2010) Neonatal maternal separation (NMS) stress is a documented model of early life stress which results in long-term alterations in behavior as a consequence. (Fabricius, Wortwein, & Pakkenberg, 2008; George, Bordner, Elwafi, & Simen, 2010) The pathogenesis of IBD includes loss of intestinal epithelial barrier function and a consequent increase in intestinal permeability. These factors allow excessive translocation of luminal bacteria and antigens into subepithelial tissues, triggering a vicious cycle of chronic intestinal inflammation and further intestinal barrier injury. (Benjamin et al., 2008; Bruewer et al., 2006) Several animal studies have demonstrated that psychological stress negatively influences intestinal barrier function. (Gareau et al., 2008; Kiliaan et al., 1998; Meddings & Swain, 2000) In rodent and porcine animal models, early life stress resulted in permanent alterations in central adaptive stress responses (Plotsky et al., 2005) and chronic disturbances in intestinal barrier
Thus, intestinal barrier disturbances may serve as an important link between stress and IBD flare-ups. The exact role of stress-induced barrier disruption causing the exacerbation of IBD has not been investigated in animal models of naturally occurring colitis.

The IL-10−/− mouse develops spontaneous colitis and therefore serves as a model commonly utilized to study IBD. Conventionally reared IL-10−/− mice on the C57Bl/6 background develop a mild, patchy colitis with incomplete disease penetrance;(Berg et al., 1996) therefore, this represents an ideal model to study the role of stress on the initiation and exacerbation of colitis. The objective of this study was to investigate whether early life stress (NMS) influences the development of colitis in IL-10−/− mice. We hypothesized that NMS would induce chronic disturbances in barrier function and increase colonic inflammation in IL-10−/− mice.

**Materials and Methods**

**Mice**

Wild type and IL-10−/− mice on a C57Bl/6 background were obtained from the Jackson Laboratories, Bar Harbor, ME, and were maintained in a specific pathogen free environment at the University of North Carolina, Chapel Hill. Litters were randomly assigned to one of two groups: neonatal maternal separation stress (NMS) or control (normally reared). All animals were housed in accordance with guidelines from the American Association for Laboratory Animal Care and Research Protocols and experiments were
approved by the Institutional Animal Care and Use Committee of the University of North Carolina.

**Neonatal Maternal Separation**

NMS was performed as previously described by Chung. (Chung et al., 2007) Briefly, pups in the NMS group were removed from dams and placed in isolated warmed cages for 180 minutes daily for postnatal days 1-18 (Figure 1-1). Following isolation, pups were returned to the cage containing their dam and littermates and were left undisturbed other than routine bedding changes.

**Sample collection**

Mice were sacrificed at 10 - 12 weeks of age and colonic samples were immediately collected and divided for Ussing chamber experiments, histopathology, and colonic explant cultures.

**Histology**

Colonic tissue sections were fixed in 10% buffered formalin, embedded in paraffin, and 4-μm-thick sections were stained with hematoxylin and eosin using standard techniques. Spontaneous colitis scoring was adapted from the criteria reported by Berg et al, as previously described. (Berg et al., 1996) All histological scores were determined by a single pathologist (LB) who was blinded to the experimental protocols. Mucosal hypertrophy score was assigned as follows: a clinical light microscope (Olympus BX45) equipped with a high resolution digital camera (Olympus DP2-BSW version 2.2) was used to measure the colonic epithelial thickness in microns. Well oriented crypts from 5 separate colon sections per mouse (n=5-6 mice/experimental group) and 6 randomly selected mucosal areas within each
section were measured from the basement membrane of the crypt base to the mucosal section using the arbitrary line tool and the 20× objective. The average mucosal thickness of each of the 5 colon sections was expressed as a percentage of wild type colon epithelial thickness. The percentages were then re-coded into a mucosal hypertrophy score (0 to 4) as follows: 0 = 90-125%; 1 = 125-150%; 2 = 151-175%; 3 = 176-200% and 4 = >200 and summed for final mucosal hypertrophy score. Inflammation was characterized for cell types as follows:

Additional sections were stained following standard immunohistochemical procedures for CD3 as previously described (de Jong et al., 2001) and areas of inflammatory infiltrate, when available, were targeted for examination in each of the 5 colon sections. At high magnification (40× objective), 100 cell count differentials were performed with CD3 positive lymphocytes counted as T. Macrophages, neutrophils and eosinophils were identified based on morphology. Cell types were represented as percentage of inflammatory cells present.

**Cytokine analysis**

Intestinal tissue explant cultures—Sections of the colon were collected and processed for IL-12 p40 as previously described, (Hegazi et al., 2005) using a cytokine specific ELISA immunoassay kit according to manufacturer's instructions (BD Biosystems, Franklin Lakes, NJ). Quantitative real-time PCR, using SYBR Green Master Mix (Applied Biosystems, Bedford, MA), was performed on an HT-7900 (Applied Biosystems, Bedford, MA), as previously published. (Sheikh et al., 2011)

The following primer sequences were used: β-actin: forward, 5′-AGCCATGTACGTTCCATCCAG-3′, reverse, 5′-TGCGGTAGGGAGAGCATAG-3′; Ifng: forward, 5′-CTTCCTCATGGCTTTCTG-3′, reverse, 5′-
ACGCTTATGGCTGATGG-3’; Il12b: forward, 5’-
CGCAAGAAAAGAGATGAAGGAG-3’, reverse, 5’-
TTGCATTTGGACTTCGGTAGATG-3’;
Il17a: forward, 5’-AACCGTTCACGTCACCCTGGA-3’, reverse, 5’-
TGGTCCAGCTTTCCCTCAGCA-3’, tnf: forward, 5’-TGAGATCCATGCCGTTGG-3’,
reverse, 5’- ACCCTCACACTCATCTTCTC-3’; Il6: forward, 5’-
GAAATGATGGATGCTACCAACTG-3’, reverse, 5’-
CTCTCTGAGGACTCTGGCTTTG-3’; Il1b: forward, 5’-
CTCAATGGACAGATATCAACCAAC-3’, reverse, 5’-GGCTGTGCGTCTTTTATTAC-3’

**Intestinal Barrier Function**

Ussing chamber experiments—Segments of proximal colon were harvested and immediately placed in oxygenated (95% O2-5% CO2) Ringer solution. Tissues were then mounted in 0.3 cm2 aperture Ussing chambers (Physiologic Instruments, Inc, San Diego, CA) as described previously. After a 15-minute equilibration period on chambers, short circuit current ($I_{sc}$), an index of electrogenic ion transport, was monitored by a multichannel voltage–current clamp (model VCC MC6, Physiologic Instruments, San Diego, CA) at 1 minute intervals over a 60-minute period and expressed as microamperes (μA) per square centimeter. Mucosal-to-serosal flux of (FITC)-dextran (4 kDa; Sigma-Aldrich, St. Louis, MO) (FD4) were performed as an index of paracellular permeability. After a 15 min equilibration, FD4 (0.25 mM) was added to the mucosal side of Ussing chamber-mounted tissues. The FD4 was allowed to equilibrate for 15 min, after which 100 μL samples (in
triplicate) were collected from the serosal side of tissues at 15 minute intervals and transferred into a 96 well assay plate. The presence of FD4 was assayed by measurement of fluorescence intensity using an fMax Fluorescence Microplate Reader (Molecular Devices, Sunnyvale, CA) and concentrations were determined from standard curves generated by serial dilution of FD4. FD4 flux across colonic tissues was determined by dividing the final concentration of FD4 in the serosal chamber in the by the initial FD4 concentration of the mucosal chamber and was expressed as % FD4 permeability.

**Mast cell counts and assessment of mast cell product tryptase**

Formalin-fixed, paraffin-embedded tissue (n=4 mice/experimental treatment) was mounted on slides and stained with the mast cell granule stain toluidine blue. Lamina propria mast cell counts were performed by an investigator blinded to experimental treatments. Mast cell counts were derived by averaging the number of mast cells observed in 10 high power fields per tissue slide and were expressed as number of mast cells per high power field. The animal served as the experimental unit for statistical analysis.

**Gel electrophoresis and Western blotting**

Supernatants from colonic explants were stored at −70°C before SDS-PAGE analysis. Aliquots were thawed at 4°C, and protein analysis was performed (Pierce BCA protein assay kit, Thermo Scientific, Rockford, IL). Aliquots (amounts equalized by protein concentration) were mixed with an equal volume of 2× SDS-PAGE sample buffer and boiled for 10 minutes. Western immunoblots for tryptase were performed using a mouse mast cell tryptase primary antibody (Chemicon International, Temecula, CA) as previously described. (Moeser, Ryan, Nighot, & Blikslager, 2007) Quantitative results were obtained by scanning the
resulting images and densitometric analysis using QuantityOne software (Bio-Rad Laboratories, Hercules, CA).

**Statistics**

Data were reported as mean ± SE based on experimental number (n). Data were analyzed using a standard 2-way ANOVA (SigmaStat, Jandel Scientific, San Rafael, CA) with stress and genotype as the main effects. A post-hoc Tukey's test was used to determine differences between experimental groups. Histology scores were compared using a Mann-Whitney U test. p<0.05 was considered significant and p values ≥ 0.05 and ≤0.1 were reported as trends. Cytokine data were analyzed with a Mann-Whitney U test using GraphPad Prism (Graphpad software, San Diego, CA).

**Results**

**Neonatal maternal separation exacerbates histologic colitis in IL-10 deficient mice**

In IL-10−/− mice, NMS resulted in more severe colitis, characterized by marked colonic epithelial hyperplasia as well as increased frequency and severity of inflammatory aggregates within the lamina propria and submucosa compared to controls (Figure 1-2). The inflammatory infiltrate consisted primarily of lymphocytes, with macrophages and neutrophils representing less than 3% and 1% of the inflammatory cell infiltrate, respectively. Mice subjected to NMS had higher histological scores and decreased colon length compared to controls (Figure 1-3). Crypt abscesses were not a prominent feature of the colitis in these mice. Mice exposed to NMS displayed a trend towards higher percentage of T-lymphocyte infiltration (p=0.05) than control mice. Mucosal hypertrophy was evident in control and
NMS mice, but was similar between the two groups. There was no evidence of histological inflammation in the NMS group in wild type mice.

_**Neonatal maternal separation causes increased colonic proinflammatory cytokine production in IL-10 deficient mice**_

NMS resulted in markedly elevated colonic IL-12p40 levels in colonic explant cultures compared to controls, as assessed by ELISA (Figure 1-4A). This elevation was also present at the level of mRNA (Figure 1-4B). Colonic Ifng mRNA was also significantly elevated in IL10-/- mice subjected to NMS compared to control mice (Figure 1-4C). Colonic levels of IL-1β, IL-6, IL-17, and TNFα were not significantly different between the two groups, although these cytokines trended higher in the NMS group (Figure 1-4, D-G).

_**IL-10 deficiency and neonatal maternal separation stress compromise intestinal barrier function**_

IL-10 deficiency and NMS had deleterious influences on colonic epithelial barrier function in the present study. In colonic mucosa from control (no NMS), IL-10-/- mice exhibited elevated colonic permeability (p<0.05; Figure 1-5) compared with wild type mice. NMS induced elevations in colonic permeability to FD4 in both wild type and IL-10-/- mice.

IL-10-/- NMS mice exhibited the highest colonic permeability of all the experimental groups.

_**Transepithelial short circuit current (I_{sc})**_

IL-10-/- mice displayed a trend for lower baseline colonic I_{sc} compared with wild type controls (p<0.01). Wild type mice subjected to NMS exhibited elevated I_{sc} (p<0.05) values compared with respective, normally-reared controls (Figure 1-6). Colonic mucosa from
IL-10⁻/⁻ mice previously subjected to NMS exhibited a trend towards elevated $I_{sc}$ values compared to normally reared IL-10⁻/⁻ controls (p=0.058).

**Mast cell activity**

Mast cells have been previously demonstrated to mediate intestinal barrier dysfunction and contribute to chemical-induced colitis;(Moeser et al., 2007; Santos, Yang, Soderholm, Benjamin, & Perdue, 2001; Snoek et al., 2012) therefore, we evaluated the effects of NMS on colonic mast cell activity in IL-10⁻/⁻ mice. Colonic lamina propria mast cell counts, as assessed by toluidine blue staining, displayed a trend towards a decrease in mice exposed to NMS (p=0.10), indicative of increased mast cell activation and granule release. Consistent with these results, tryptase concentrations in colonic explant supernatants were elevated (p<0.05) in IL-10⁻/⁻ mice exposed to NMS compared with normally reared mice.

**Discussion**

The results of this study demonstrate that NMS stress in IL-10⁻/⁻ mice triggered the development of severe colitis which was sustained later in life. NMS exacerbated colitis in IL-10⁻/⁻ mice, which was associated with marked defects in colonic epithelial function, elevated proinflammatory cytokines, and evidence for enhanced mast cell activation.

Overall, these findings indicate that NMS in the IL-10⁻/⁻ mouse represents a novel model for studying the effect of stress on the pathogenesis of IBD. In this study, normal reared IL-10⁻/⁻ mice exhibited elevations in colonic permeability compared with wild type controls demonstrating that IL-10 deficiency plays a role in baseline regulation of intestinal
barrier function. These findings are supported by Madsen et al (1999) who showed that Sv/Ev 129, IL-10–/– mice exhibited a higher baseline intestinal permeability compared with controls and this preceded colitis development. (Madsen et al., 1999) A subsequent study by Arrieta et al (2009) showed that administration of the zonulin antagonist (AT-1001) significantly reduced small intestinal permeability and colitis indicating that intestinal barrier dysfunction is a primary defect associated with colitis in IL-10–/– mice. (Arrieta, Madsen, Doyle, & Meddings, 2009) In this study, we showed that NMS induced long-term increases in colonic permeability in wild type and IL-10–/– mice; however, colitis was observed only in IL-10–/– mice subjected to NMS. These findings support the “two-hit hypothesis” implicated in inflammatory bowel disease, in which a single factor, such as genetic predisposition, barrier dysfunction, or dysbiosis, is not sufficient to induce significant IBD. (Kaser, Zeissig, & Blumberg, 2010)

The precise mechanisms by which NMS resulted in long-lasting exacerbations in colitis in IL-10–/– mice are unclear. In addition to causing increased intestinal permeability and more severe colitis in IL-10–/– mice, NMS also caused an increase in intestinal permeability without inducing colitis in wild type mice. Therefore, it is likely that the enhanced colonic permeability observed in NMS-exposed mice results in increased luminal antigen and adjuvant translocation across the intestinal epithelium, thus aggravating colitis in IL-10–/– mice. Intestinal barrier dysfunction is a critical event that precedes inflammation in IBD patients and animal models. (Arrieta et al., 2009) In patients with Crohn's disease, increased intestinal permeability is present and precedes inflammation and can predict relapse. (Bjarnason, MacPherson, & Hollander, 1995; Meddings, 1997) The presence of
intestinal barrier dysfunction in relatives of Crohn's disease patients suggests that increased permeability may be a single early defect that may precede the development of overt disease or may be a contributing factor to disease. (D’Inca et al., 2006; Hilsden, Meddings, & Sutherland, 1996; Hollander et al., 1986; Peeters et al., 1997) In the current model, increased intestinal permeability could be a cause or effect of the increase in colitis scores in IL-10/− mice. Further studies are necessary to determine if the increased colitis is preceded by an increase in intestinal permeability.

Alternatively, enhanced colitis in stressed IL-10/− mice could be due to the direct influence of stress mediators on intestinal immune system. For example, peripheral stress mediators, such as corticotrophin releasing factor (CRF) have been implicated as contributing to intestinal inflammation independent of intestinal barrier dysfunction. (la Fleur, Wick, Idumalla, Grady, & Bhargava, 2005) For example, CRF receptors are expressed on a variety of cell types in the intestine, including mast cells and macrophages. (Chatzaki et al., 2004; Santos et al., 1998; Wallon et al., 2008) CRF signaling on these cells may contribute to IBD either by triggering intestinal barrier dysfunction or by initiating direct release of pro-inflammatory mediators that in turn result in barrier dysfunction. (Kokkotou et al., 2006; Santos et al., 1998; Saruta et al., 2004; Theoharides et al., 1998) Although there were only modest effects of NMS on measures of mast cell activation as evidenced by (1) a trend for decreased toluidine blue-stained mast cells, an indicator or intracellular loss (exocytosis) of mast cell granule (Augusto, Lunardi, & Vugman, 1990) and (2) elevated colonic tryptase release, these data are in line with a well-established of the mast cell in mediating intestinal barrier dysfunction (Santos et al., 2001) and colitis. (Barreau, Ferrier, Fioramonti, & Bueno,
Mast cell tryptase has been shown to induce multiple effects that can contribute to colitis including breakdown of intestinal epithelial barrier function via tight junction protein disruption and neutrophil recruitment. (Hamilton et al., 2011; Jacob et al., 2005) Given that we only assessed mast cell activation, the functional role of the mast cell in this model remains to be fully understood.

In the present study, IL-10 deficiency and NMS induced alterations in $I_{sc}$, an index of net electrogenic ion transport. Baseline $I_{sc}$ was elevated by NMS in wild type mice and displayed a trend towards increase in IL-10$^{-/-}$ mice, which is consistent with previous studies utilizing NMS in rats. (Santos et al., 2001; Zareie et al., 2006) Previous studies demonstrated that elevated $I_{sc}$ induced by NMS was not observed in mast cell deficient rats suggesting that NMS induced mast cell activation was responsible for the chronically elevated $I_{sc}$. (Santos et al., 2001) Although others have demonstrated altered $I_{sc}$ in several models of colitis, the direct role of altered $I_{sc}$ remains unclear. (Perez-Navarro, Ballester, Zarzuelo, & Sanchez de Medina, 2005)

In summary, stress is a major factor triggering the onset of IBD but the exact mechanisms remain unclear. Here we present a novel animal model to investigate the mechanisms by which early life stress influences development of IBD in adulthood. Future research into the mechanisms through which stress influences IBD will provide insight into the mechanism of stress-induced IBD onset or IBD flares and potentially reveal therapeutic targets that control IBD development and relapse; therefore, this novel NMS/IL-10$^{-/-}$ model
may serve as a useful model to study the influence of early life stress on the development of IBD in human patients.

**Acknowledgments**

This work was supported by the NIH K08 DK084313 (AJM), R01 DK54452 (SEP), and P30 DK34987 (AJM and SEP, Immunotechnologies Core and Histology Core). Nitsan Maharshak is supported by the Crohn's and Colitis Foundation of America Research Fellowship Award and the American Physicians Fellowship for Medicine in Israel. Elizabeth Lennon is supported by the Ruth L. Kirschstein National Research Service Award T32 RR024394 as part of North Carolina State University's Comparative Medicine and Translational Research Training Program.
Figures

Figure 1-1: Experimental Timeline.
**Figure 1-2: NMS exacerbates colitis in IL10⁻/⁻ mice.** Colon, hematoxylin and eosin staining: top row: x2 magnification; bottom row: x60 magnification. NMS IL-10⁻/⁻ mice display marked epithelial hyperplasia compared with control. Increased frequency and severity of inflammatory aggregates within the lamina propria and submucosa of the NMS mouse (B and D) compared with the control mouse (A and C).
Figure 1-3: NMS stress exacerbates colitis scores and colon length. IL-10−/− mice (n = 3–10 per group) were either exposed (NMS group) or not exposed (control group) to NMS. (A) Colitis was more severe in NMS group as assessed by histologic colitis scores (p < 0.01). (B) Colon length was significantly decreased in IL-10−/− mice exposed to NMS (p < 0.04).
Figure 1-4: Colonic cytokine levels are increased in IL-10−/− mice subjected to NMS. (n = 5–10 per group). IL-12p40 (A; P < 0.003), IL-12b mRNA (B; P < 0.001), and IFNg (C; P < 0.05). IL-1b, IL-6, IL-17, and TNF-α were not significantly different from control mice (D–G).
Figure 1-5: NMS and IL-10 deficiency alter colonic barrier function. NMS caused significant increases in mucosal-to-serosal fluorescein isothiocyanate (FITC)–dextran flux in wild-type and IL-102/2 mice (b, c: P < 0.05). Effects of NMS and IL-10 deficiency were additive in causing increased permeability (c) (n = 8 per group).
CHAPTER 2: Mast cells play a protective role in a model of spontaneous colitis

Literature Review

Mast cell biology

Mast cells are a unique granule-containing cell type. They are derived from myeloid cells, exit the bone marrow as CD34+/CD117+ stem cells with minimal granulation, and differentiate into mature mast cells in their tissue of residence. (Gurish et al., 1995; Kirshenbaum et al., 1999; Kitamura, 1989) They are long-lived cells (Padawer, 1974) that reside in almost all tissues, but they are present at markedly higher concentrations at tissue-environment interfaces, where they are likely positioned for pathogen defense and environmental surveillance. (Kawakami & Galli, 2002; Kitamura, 1989; Marshall, 2004; St John & Abraham, 2013) Mast cells contain a wide variety of mediators, some of which are stored in cytoplasmic granules for immediate release upon stimulation and others which are synthesized de novo (Voehringer, 2013).

The contents of mast cell granules are made up of five main subtypes: lysosomal enzymes, biogenic amines, cytokines and growth factors, proteases, and proteoglycans. These granules can be exocytosed upon stimulation, often by binding of a ligand to a mast cell’s cell surface receptor, for example, IgE binding to an Fcε receptor.

Lysosomal enzymes in mast cell granules include β-hexosaminidase, a commonly used marker of mast cell degranulation that normally functions for carbohydrate turnover, other enzymes involved in carbohydrate degradation including β-glucuronidase, N-acetyl-β-glucosaminidase, and β-D-galactosidase, as well as the cysteine proteases Cathepsins B,
C, D, E, and L. (Dragonetti et al., 2000; Henningsson et al., 2005; Schwartz, Austen, & Wasserman, 1979; Schwartz & Austen, 1980; Schwartz, Lewis, Seldin, & Austen, 1981; Wolters, Laig-Webster, & Caughey, 2000) The cathepsins perform normal lysosomal functions but also are important for processing some of the important mast cell serine proteases including pro-tryptase, pro-chymase, and pro-carboxypeptidase A3. (Dragonetti et al., 2000; Henningsson et al., 2005; Wolters et al., 2000)

The important biogenic amines present in mast cells include histamine and serotonin. (Benditt, Wong, Arase, & Roeper, 1955; Kushnir-Sukhov, Brown, Wu, Kirshenbaum, & Metcalfe, 2007; Riley, 1953; Sjoerdsma, Waalkes, & Weissbach, 1957) Histamine is a powerful inducer of bronchoconstriction, vasodilation, and increased vascular permeability, the mediator for which mast cells are probably best known. (Riley, 1953; Sjoerdsma et al., 1957) Histamine is present in all subtypes of mast cells in all species, while serotonin is present in high levels in rodent mast cells but only low levels in human mast cells. (Benditt et al., 1955; Kushnir-Sukhov et al., 2007; Riley, 1953)

Although mast cells can synthesize a wide variety of cytokines and growth factors, only a few are stored preformed in the mast cell granule to be secreted at a moment’s notice. These include TNF, interleukin-4 (IL4), basic fibroblast growth factor, vascular endothelial growth factor, nerve growth factor, TGFβ, IL5, IL6, IL15, and stem cell factor. (Boesiger et al., 1998; Bradding et al., 1992; Bradding et al., 1993; Gordon & Galli, 1990; Grutzkau et al., 1998; Leon et al., 1994; Lindstedt et al., 2001; Reed, Albino, & McNutt, 1995; S. Zhang et al., 1998)
Mast cells also contain a large number of proteases. Of these, the most important are mast cell-specific proteases including the serine proteases tryptase and chymase, as well as the metalloproteinase carboxypeptidase A3.(Benditt & Arase, 1959; Glenner & Cohen, 1960; Haas, Heinrich, & Sasse, 1979) Humans have a single type of tryptase and chymase, while the situation in rodents is more complex. The murine counterparts of chymase are mouse mast cell protease-1 (mMCP-1), mMCP-2, mMCP-4, and mMCP-5, while the counterparts of tryptase are mMCP-6 and mMCP-7.(McNeil et al., 1992; D. S. Reynolds et al., 1990; D. S. Reynolds, Gurley, Austen, & Serafin, 1991) Only a single gene for carboxypeptidase A3 is expressed in both rodents and humans.(Haas et al., 1979; Wernersson & Pejler, 2014) Tryptase and chymase are important for some of the most critical and unique mast cell functions, and both can have either proinflammatory or anti-inflammatory functions. For example, mast cell chymase can activate an IL1β precursor to its active form, and mast cell proteases can contribute to airway hyperreactivity.(Mizutani, Schechter, Lazarus, Black, & Kupper, 1991; Williams & Galli, 2000; Yu et al., 2006) On the other hand, mast cell chymase has antiinflammatory actions in dust mite-induced airway inflammation, presumably by degrading IL-33 and decreasing sensitization.(Waern, Lundequist, Pejler, & Wernersson, 2013) Furthermore, mast cell proteases can degrade proinflammatory cytokines, limiting inflammation.(Nelissen et al., 2014; Zhao, Oskeritzian, Pozez, & Schwartz, 2005) Therefore, the contribution of mast cell proteases to a pro- or anti-inflammatory state are likely situational and highly complex.

Lastly, mast cells contain proteoglycans, specifically serglycins. (Abrink, Grujic, & Pejler, 2004) Serglycins, such as heparin and chondroitin sulfates, have a major role in
storage of proteases and amines, and are responsible for the metachromatic staining of mast cells.(Abrink et al., 2004; Forsberg et al., 1999; Humphries, Wong, Friend, Gurish, & Stevens, 1999; Humphries et al., 1999) Proteoglycans consist of a protein core with polysaccharide chains of glycosaminoglycans bound to it.

**Mast cell classification**

Mast cells are classically characterized by their serine protease contents, but these differ between species and between types of mast cells. In humans, mast cells that contain only tryptase, typically referred to as MC\(_T\), are present on mucosal surfaces such as the intestinal mucosa or airway epithelium.(Irani, Schechter, Craig, DeBlois, & Schwartz, 1986; Metcalfe, Baram, & Mekori, 1997) Mast cells that contain both tryptase and chymase, as well as carboxypeptidase A3 as MC\(_{TC}\) and are present in the intestinal submucosa, peritoneum and skin.(Irani et al., 1986; Metcalfe et al., 1997) In mice, mucosal mast cells contain the chymases mMCP-1 and mMCP-2, while connective tissue mast cells contain the chymases mMCP-4 and mMCP-5, tryptase like proteases mMCP-6 and mMCP-7, and carboxypeptidase A3.(Pejler, Abrink, Ringvall, & Wernersson, 2007; Pejler, Ronnberg, Waern, & Wernersson, 2010; Wernersson & Pejler, 2014)

Mucosal mast cells have low histamine content but express high levels of cysteinyl leukotrienes, while connective tissue mast cells have high histamine content and high amounts of prostaglandin D2.(Heavey et al., 1988; Metcalfe et al., 1997) However, this classification scheme is dated and probably overly simplistic.(Wernersson & Pejler, 2014)
**Mast cells: function**

Mast cells are innate immune cells that are best known for their role in allergy and anaphylaxis. However, mast cells also regulate the intestinal barrier, participate in defense against pathogens, and orchestrate inflammation.(Echtenacher, Mannel, & Hultner, 1996; Groeschwitz et al., 2009; Jacob et al., 2005; McNeil, Adachi, & Stevens, 2007; Sutherland, Olsen, McKinstry, Villalta, & Wolters, 2008) Mast cells are critical players in directing immune responses; they contain toll-like receptors on their cell surface and have the capability to phagocytize bacteria.(Matsuguchi, 2012; Saluja, Delin, Nilsson, & Adner, 2012; Sandig & Bulfone-Paus, 2012; Wesolowski, Caldwell, & Paumet, 2012) Mast cells’ granules contain a wide variety of mediators, and in response to a variety of signals, they can respond in a wide variety of ways, from selectively secreting a single mediator, to explosively degranulating.(Gilfillan & Metcalfe, 2011) Mast cells are understudied in IBD, in part because these cells are difficult to study mechanistically due to their low numbers in tissue and relative inaccessibility compared to other cell types. In addition, mature, differentiated mast cells produce very low amounts of mRNA compared to other cell types, further complicating the study of these cells.(Douaiher et al., 2014) Additionally, mast cells can be difficult to detect using routine formalin fixation and hematoxylin and eosin staining methods, so they are less likely to be visualized on routine biopsy samples.(Hamilton, Frei, & Stevens, 2014)

**Mast cells: development and experimental models**

Mast cells are derived from cells of hematopoietic origin. In humans, they are derived from CD34+/CD117+ pluripotent stem cells in the bone marrow.(Kirshenbaum et al., 1999)
Maturation of these pluripotent stem cells into mature mast cells requires that stem cell factor binds to KIT (CD117) on the cell surface, leading to KIT dimerization and transautophosphorylation. (Opatowsky et al., 2014) The two most well-described mouse models which have been used to study in vivo mast cell biology and function rely on defective expression of KIT on the cell surface of the mast cell (Kit\(^{W/W-v}\)) or an upstream mutation of c-kit that prevents its catalytic activity (Kit\(^{Wsh/Wsh}\)). (Voehringer, 2013)

Although these models have been critical in developing an understanding of the functions of mast cells, there are limitations to the interpretation of these studies since c-kit is also expressed on hematopoietic progenitor cells and innate lymphoid cells. The Kit\(^{W/W-v}\) mice demonstrate anemia and neutropenia, decreased basophil numbers, impaired development of γδ-T cells in the gut, loss of melanocytes, male sterility, lack of interstitial cells of Cajal, and reduced pacemaker activity in the intestine. (Feyerabend et al., 2011; Lantz et al., 1998; Mancardi et al., 2011) However, the reduced pacemaker activity in the intestine is actually due to inflammation caused by mast cell deficiency rather than by lack of the interstitial cells of Cajal, since the motility can be restored by blockade of COX-2 and iNOS without affecting c-kit expression. (Winston, Chen, Shi, & Sarna, 2014)

In order to overcome these confounding factors, the Kit\(^{Wsh/Wsh}\) mouse was next introduced. This model was a significant improvement over the Kit\(^{W/W-v}\) mouse, since these mice do not demonstrate anemia, are fertile, and have normal basophil numbers. However, they do have cardiac hypertrophy due to loss of the corin protein, and they have loss of melanocytes and decreased numbers of interstitial cells of Cajal, although some interstitial cells of Cajal are still present in the deep muscular plexus layers of the small intestine. (Iino,
Horiguchi, & Nojyo, 2009; Voehringer, 2013) These mice demonstrate neutrophilia and mild splenomegaly.(Nigrovic et al., 2008)

Newer models of mast cell deficiency have been introduced that do not rely on c-kit mutations. The Mcpt5-Cre mouse is an inducible knockout model that causes deficiency of connective tissue mast cells but not mucosal mast cells or basophils.(Dudeck et al., 2011) The “Hello KITty” Cpa3-Cre inducible knockout mouse results in knockout of 90% of connective tissue and mucosal mast cells, but also deletes 60-80% of basophils and causes anemia and neutrophilia.(Lilla et al., 2011) The Chm-Cre mouse can be used to mark mast cells, except for those in the skin, peritoneum, bone marrow, and heart, but does not cause deletion.(Musch, Wege, Mannel, & Hehlgans, 2008) The “Cre-Master” Cpa3Cre mouse has a knock-in of Cre before the first exon of Cpa3, which causes a constitutive deletion of all connective tissue and mucosal mast cells, along with 60% deletion of basophils. This knock-in also marks 90% of T cells and 5% of B cells, but does not delete them.(Feyerabend et al., 2011) Therefore, many models of mast cell deficiency are now available to characterize the in vivo activities of mast cells, but many confounding factors can make the differentiation of cause-and-effect difficult. In particular, differentiation of function between mast cells and basophils can be difficult using many of the models. Mast cell repletion is a powerful tool that can be used to ensure that an effect is mast cell-specific.

**Inflammatory bowel disease: Pathogenesis**

IBD has a complex pathogenesis which is thought to occur from an interaction between genetic, environmental, and immune factors, and particularly a dysregulated immune response to an altered enteric microbiota.(De Schepper, De Man, Moreels,
Research into IBD pathogenesis has historically focused on the adaptive immune system, but more recently, the innate immune system has gained increasing recognition for its important role in the initial stages of disease; however, the precise cell types and mechanisms remain poorly understood. Mast cells are an area of research that has historically been understudied in IBD, but are increasingly being recognized for their role in IBD.

**Inflammatory bowel disease: the role of the mast cell**

Mast cell numbers are increased in CD and UC patients, they have long been recognized to play an important role in the pathogenesis of IBD. (Dvorak, Monahan, Osage, & Dickersin, 1980; King, Biddle, Bhatia, Moore, & Miner, 1992; Nishida et al., 2002; Sasaki, Tanaka, & Kudo, 2002) Specifically, mast cell numbers are increased in the colorectal mucosa, lamina propria, and submucosa of patients with IBD, and are more frequently observed to be degranulated or activated in IBD patients compared to controls. (De Schepper et al., 2008; He, 2004; Rijnierse, Nijkamp, & Kraneveld, 2007) Additionally, IBD patients have increased expression of mast cell products such as tryptase and histamine, substance P, and TNF. (Barbara, Stanghellini, De Giorgio, & Corinaldesi, 2006; Raithel et al., 2001; Sasaki et al., 2002) Jejunal histamine secretion is increased in active Crohn’s disease, and antigen-stimulated mast cells isolated from resected colons of patients with UC have increased capacity for histamine secretion upon stimulation. (Fox, Lichtenstein, & Roche, 1993; Knutson, Ahrenstedt, Odlind, & Hallgren, 1990)

One intriguing study investigated the line of demarcation between abnormal and healthy tissue in patients with UC, and concluded that about half of UC patients had a
marked increase in mast cell numbers at the line of demarcation between normal and abnormal tissue, suggesting that the mast cell may play a critical role in either initiating or suppressing spread of inflammation. (King et al., 1992) However, the specific role of the mast cell in IBD has not been fully characterized.

**Mast cells in IBD models**

In order to further elucidate the role of the mast cell in IBD, researchers have turned to experimental models. To date, mast cells have primarily been investigated in chemical colitis models, and they appear to play a deleterious role. (Araki, Andoh, Fujiyama, & Bamba, 2000; Cho et al., 2011; Eliakim, Karmeli, Okon, & Rachmilewitz, 1992; Hamilton et al., 2011; Iba, Sugimoto, & Kamei, 2002; Ishida et al., 2008; Isozaki et al., 2006; Rijnierse, Koster, Nijkamp, & Kraneveld, 2006) Mast cell proteases increase in serum and tissue in response to DSS colitis in mice. (Iba et al., 2002) In both TNBS and DSS-induced colitis, mast cell deficiency ameliorates colitis in rats and mice, and mast cell protease-6 is required for DSS and TNBS colitis in mice. (Araki et al., 2000; Hamilton et al., 2011; Rijnierse et al., 2006) Additionally, pharmacological mast cell stabilization (ketotifen), and tryptase and chymase inhibition (nafamostat and NK3201, respectively) decrease mucosal damage in TNBS colitis. (Cho et al., 2011; Eliakim et al., 1992; Ishida et al., 2008; Isozaki et al., 2006) In one study, mast cells appear to be important in recovery after DSS colitis induction in rats; mast cell numbers and degranulation were markedly elevated following initiation of DSS colitis, but while mast cell protease I and degranulation quickly decreased as colitis resolved, mast cell protease II remained elevated for a prolonged period of time, suggesting that this protease may play a role in recovery from colitis. (Iba, Sugimoto, Kamei, & Masukawa,
Unfortunately, the preliminary findings from this study have not been followed up with a more comprehensive assessment of mast cells’ function in the recovery from colitis, although mast cells are known to be important for wound healing in other sites. (Douaiher et al., 2014) A description of worsening colitis in the IL10−/− mouse in the absence of mast cells has been described, providing evidence for a beneficial role of the mast cell, however, this role was not confirmed to be mast cell-specific by repletion, and the report focused largely on exacerbation of colitis by infectious or pharmaceutical agents. (Chichlowski, Westwood, Abraham, & Hale, 2010)

Despite these findings in rodent chemical colitis models, the translation of the results of studies using chemical colitis models in mice to human medicine has been disappointing; mast cell blockade using cromolyn or ketotifen has not been a successful treatment for either CD or UC. (Binder et al., 1981; Franchi, Meneghelli, Seminara, Spadini, & Bonomo, 1982; Hovdenak, Halvorsen, Nordgard, Schjonsby, & Sigstad, 1986; Rampton, Brown, Causon, & Sahib, 1982; Babb, 1980; Buckell, Gould, Day, Lennard-Jones, & Edwards, 1978; Dronfield & Langman, 1978).

Serious limitations of these animal models include the fact that chemical injury does not replicate the slow onset of intestinal barrier dysfunction and slow progressive sensitization to bacterial and dietary antigens that is known to occur in spontaneous IBD.

**Divergent role of mast cells in inflammation**

An additional explanation for the failure of mast cell stabilizers in clinical trials of human IBD is that while mast cells are critical mediators of the inflammatory response, they play divergent roles in inflammation. Although they are classically thought of as a
proinflammatory cell type which produces histamine, results in vasodilation and releases powerful vasoactive substances which can result in wheals (Church & Clough, 1999), mast cells have a wide range of functions including promoting bacterial clearance and anti-inflammatory functions (Malaviya, Ikeda, Ross, & Abraham, 1996; Sayed, Christy, Quirion, & Brown, 2008). Mast cell tryptases and mast cell-derived TNF-α are critical in bacterial defense and clearance and survival from sepsis, and mast cells recruit neutrophils that phagocytose and kill bacteria. (Malaviya et al., 1996; Sutherland et al., 2008; Thakurdas et al., 2007) In the absence of mast cells, the ability of neutrophils to phagocytize and kill bacteria is significantly compromised. (Echtenacher et al., 1996; Malaviya et al., 1996)

**Anti-inflammatory role of the mast cell**

In addition to their well-recognized proinflammatory roles, mast cells also have critical anti-inflammatory functions. Mast cells are required for allograft tolerance through induction of regulatory T cells and influence on dendritic cells. (de Vries, Elgueta, Lee, & Noelle, 2010; de Vries et al., 2011; Lu et al., 2006a) Mast cell tryptase is a serine protease that functions to degrade cytokines and matrix metalloproteinases and aids in bacterial defense. (McNeil et al., 2007) Following long term incubation with LPS, mast cells induce regulatory T cell differentiation in vitro. (Nie et al., 2012; W. Zhang et al., 2010) Mast cells are also activated in response to intestinal helminth infection, and placebo controlled clinical trials of helminth infection in humans with IBD have shown that exposure to intestinal helminths improves symptoms of IBD. (Summers, Elliott, Urban, Thompson, & Weinstock, 2005) possibly by shifting the immune response to one dominated by IL4+/IL10+ CD4 T cells. (Elliott, Summers, & Weinstock, 2007)
**IBD Models: Beyond Chemical Colitis**

Mast cells could potentially have a different effect in human IBD and the IL10 deficient model of colitis than in the chemical colitis models (protective vs. deleterious) because the chemical colitis models work in a different mechanism than spontaneously occurring colitis. A severe form of childhood-onset IBD results from genetic defects in IL10, the IL10 receptor, or its signaling pathway (Begue et al., 2011; Glocker et al., 2009; Glocker et al., 2010; Moran et al., 2013) which is a similar pathogenesis to the IL10−/− mouse. Chemical colitis models, which cause sudden catastrophic barrier dysfunction that triggers inflammation, do not accurately recapitulate the slow, progressive onset and occurrence of IBD. The IL10−/− spontaneous model of IBD more closely replicates the onset and pathogenesis of IBD than chemically induced detergent colitis. Mast cells have divergent actions, and while they may be deleterious in chemical colitis, they may have a different action in the slowly occurring immune dysregulation, and bacterial exposure that occurs in the IL10 deficient mouse.

**Microbiota**

The intestinal microbiota is well known to be critical in the pathogenesis of IBD. (Sartor, 2008) IL10−/− mice require the presence of bacteria for the pathogenesis of colitis; germ-free IL10−/− mice do not develop colitis. (Sellon et al., 1998) Early treatment of IL10−/− mice with antibiotics, particularly those that target gram-negative bacteria, can ameliorate colitis. (Madsen et al., 2000) Similarly, in humans, significant improvement in severe CD and UC is noted when fecal flow is surgically diverted from the intestinal lumen
Mast cells are important players in bacterial infections and mucosal surveillance. They contain Toll-like receptors (TLRs) on their cell surface and can phagocytose bacteria and act as antigen-presenting cells. Similar to the method by which they play a key role in orchestrating immune responses to helminths, we propose that they play a role in crosstalk between the lamina propria immune system and the microbiota. Long term exposure of mast cells to LPS results in induction of regulatory T lymphocytes, which are critical in intestinal immune tolerance to the microbiota. Additionally, alterations in signaling of complement component 5, a product of mast cells as well as other cell types, has been demonstrated to alter the cutaneous microbiota.

**Mast cell-T cell interaction**

Mast cells are documented to induce T regulatory cells (Tregs). Previous studies have demonstrated that presence of mast cells are critical for allograft tolerance, through the induction of Tregs. This occurs via the production of TGFβ1 as well as through cell-cell contact.
Mast Cells Play a Protective Role in a Spontaneously-Occurring Model of Inflammatory Bowel Disease

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Introduction

The inflammatory bowel diseases (IBDs), Crohn’s disease (CD) and ulcerative colitis (UC) affect 1-1.5 million people in the United States, resulting in an estimated 6.3 billion dollars in annual healthcare costs. (Arora et al., 2010; Cerqueira & Lago, 2013; Kappelman et al., 2008; Koganei et al., 2012; Lakatos et al., 2012; Vatn, 2009) These diseases have no cure and require lifelong management with significant side effects from treatment. Medical management largely consists of immunosuppression, which can result in life threatening complications and unwanted side effects. (Cerqueira & Lago, 2013; Hudesman et al., 2013; Lan et al., 2013; Long et al., 2013; Louis, 2013; Norgard et al., 2013) Given that intestinal mast cell numbers are increased in CD and UC patients, they have long been recognized to play an important role in the pathogenesis of IBD. (Dvorak et al., 1980; King et al., 1992; Nishida et al., 2002; Sasaki et al., 2002) Specifically, mast cell numbers are increased in the colorectal mucosa, lamina propria, and submucosa of patients with IBD, and are more frequently observed to be degranulated or activated in IBD patients compared to controls. (De Schepper et al., 2008; He, 2004; Rijnierse et al., 2007) Additionally, IBD patients have
increased expression of mast cell products such as tryptase and histamine, substance P, and TNF. (Barbara et al., 2006; Fox et al., 1993; Knutson et al., 1990; Raithel et al., 2001; Sasaki et al., 2002)

To date, mast cells have primarily been investigated in chemical colitis models, and they appear to play a deleterious role. (Araki et al., 2000; Cho et al., 2011; Eliakim et al., 1992; Hamilton et al., 2011; Iba et al., 2002; Ishida et al., 2008; Isozaki et al., 2006; Rijnierse et al., 2006) However, mast cell stabilization has not been an effective treatment for IBD, so the translational value of these studies has been called into question. (Binder et al., 1981; Franchi et al., 1982; Hovdenak et al., 1986; Rampton et al., 1982)

A description of worsening colitis in the IL10−/− mouse in the absence of mast cells has been described, providing evidence for a beneficial role of the mast cell, however, this role was not confirmed to be mast cell-specific by repletion, and the report focused largely on exacerbation of colitis by infectious or pharmaceutical agents. (Chichlowski et al., 2010) The objective of this study was to determine the role of the mast cell in a model of spontaneously-occurring inflammatory bowel disease.

Materials and Methods

Experimental groups

IL10−/− mice on a C57Bl/6 background (IL10−/−) and mice that lack mast cells in addition to IL10 (IL-10−/− x KitWsh/Wsh) (DKO, C57Bl/6 background) were used in this study. In separate experiments, DKO mice were repleted with bone marrow-derived mast cells at 4 weeks of age as previously described and compared with DKO and IL10−/− mice at 20-24
weeks of age. (Jin et al., 2011) Mice were euthanized by CO₂ inhalation at 20-24 weeks of age. n=5-15/group. All animals were housed in accordance with guidelines from the American Association for Laboratory Animal Care and Research Protocols and experiments were approved by the Institutional Animal Care and Use Committee of North Carolina State University.

**Differentiation and repletion of bone marrow-derived mast cells**
Bone marrow derived mast cells (BMMCs) were generated as previously described. (Jin et al., 2011) Briefly, bone marrow stem cells were cultured in the presence of IL3 (5ng/ml) and stem cell factor (5 ng/ml), (R&D Systems) for 8 weeks with weekly culture media changes, after which purity of mast cells was assessed by staining with toluidine blue and visualization using light microscopy (Figure 2-1). Mast cell purity as assessed by toluidine blue staining, and confirmed by performing staining for c-kit and FcεR1 with analysis by flow cytometry (Figure 2-1) is typically >98% in our laboratory using these procedures. DKO mice were repleted with BMMCs at 4 weeks of age by intraperitoneal injection of 1x10⁷ cells as previously described. (Jin et al., 2011) Mast cell repletion was performed with BMMCs derived from both wild-type and IL10⁻/⁻ mice to demonstrate whether the anti-inflammatory activity of the mast cell is dependent on IL10. Successful repletion was confirmed histologically at the time of euthanasia (16-20 weeks following repletion) by microscopic examination of toluidine blue-stained sections of intestine. Mice that did not have identifiable mast cells within any section of the colon were excluded from analysis.
**Colitis scoring**

Colonic tissue sections were fixed in 10% buffered formalin and embedded in paraffin, and 4-μm-thick sections were stained with hematoxylin and eosin using standard techniques. Spontaneous colitis scoring was adapted from the criteria reported by Berg et al, as previously described. (Berg et al., 1996) Briefly, colonic tissue sections were fixed in 10% buffered formalin and embedded in paraffin, and 4μm-thick sections were stained with hematoxylin and eosin using standard techniques. Five sections of hematoxylin/eosin stained intestine were examined using a clinical light microscope (Olympus BX45), equipped with a high-resolution digital camera (Olympus DP2-BSW, version 2.2) and scored on a scale from 0-4 by a single blinded veterinary pathologist (LBB). Mucosal (epithelial) hypertrophy was assessed by measuring the colonic epithelial thickness in micrometers. Well-oriented crypts from 5 separate colon sections per mouse, and 6 randomly selected mucosal areas within each section were measured from the basement membrane of the crypt base to the mucosal section using the line tool and the 20x objective.

**Fecal scoring and fecal % water**

Fecal pellets were collected from the distal colon and rectum of mice following euthanasia. Feces were weighed and placed in a drying oven for 12 hours, after which time the dry weight was determined. Fecal % water was determined by dividing the dry weight by the wet weight and multiplying by 100. Fecal scores were performed by examining the feces present in the rectum of each mouse and using a modified Bristol Stool Scale to score the feces.
**Assessment of intestinal barrier function**

Segments of proximal colon were harvested and immediately placed in oxygenated (95% O\textsubscript{2} to 5% CO\textsubscript{2}) Ringer solution. Tissues were then mounted in 0.3-cm\textsuperscript{2} aperture Ussing chambers (Physiologic Instruments, Inc, San Diego, CA) as described previously. (Argenzio, Henrikson, & Liacos, 1988) Mucosal-to-serosal flux of (FITC)-dextran (4 kDa; Sigma-Aldrich, St. Louis, MO) (FD4) was assessed as an index of paracellular permeability. After a 15-minute equilibration, FD4 (0.25 mM) was added to the mucosal side of Ussing chamber-mounted tissues. The FD4 was allowed to equilibrate for 15 minutes, after which 100 mL samples (in triplicate) were collected from the serosal side of tissues at 15-minute intervals and transferred into a 96-well assay plate. The presence of FD4 was assayed by measurement of fluorescence intensity using an fMax Fluorescence Microplate Reader (Molecular Devices, Sunnyvale, CA), and concentrations were determined from standard curves generated by serial dilution of FD4.

**Colonic explant culture**

Colonic sections were collected and processed as previously described. (Hegazi et al., 2005) Colonic tissue samples were cut into small fragments and incubated for 24 hours in cell culture media at 37°C, 5% CO\textsubscript{2}. Supernatants were collected and stored at -80°C until analysis.

**Cytokine ELISA**

IL12p40, IL6, and TNF concentrations were determined in colonic supernatant samples using commercially available sandwich ELISA kits (BD Biosciences, Franklin Lake, NJ).
**Germ free mice**

DKO mice were derived into germ free conditions by embryo transfer at the Gnotobiotic Core, University of North Carolina, Chapel Hill. Mice were euthanized at 20-24 weeks of age for evaluation of colitis and were compared to IL10−/− C57Bl/6 mice also housed in germ free conditions. Mice housed in germ free conditions were euthanized at 20-24 weeks of age.

**Real-Time PCR Array for Mouse Cytokines/Chemokines**

RNA was extracted from rinsed colon samples that had been snap-frozen in liquid nitrogen and stored at -80°C. Tissues were homogenized and RNA was extracted using a commercially available kit (RNeasy, Qiagen, Valencia, CA) and was analyzed with a spectrophotometer. RNA was subjected to DNase treatment (RNase-free DNase kit, Qiagen, Valencia, CA) and then was reverse transcribed using a commercially available kit (RT2 First Strand, Qiagen, Valencia CA) followed by PCR amplification. Samples were analyzed using the RT2 Profiler Array for Mouse Cytokines/Chemokines (Qiagen, Cat #PAMM-150Z, Valencia, CA) according to the manufacturer’s instructions in a LightCycler 480 (Roche Life Sciences, Indianapolis, IN) to quantify expression of genes encoding 82 mouse inflammatory cytokines and chemokines. Gene expression was normalized to five housekeeping genes included with each experiment. PCR controls and RT controls were included with each experiment. Data were analyzed and fold changes were calculated using commercially available software (SA Biosciences, http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php website).
**BMP Western blotting**

Colonic mucosal scrapes were collected from IL10\(^{-/-}\) and DKO mice at 24-28 weeks of age and were frozen at -80\(^\circ\)C until analysis. Samples were homogenized and protein was extracted in RIPA buffer containing phosphatase and protease inhibitors according to standard protocols. Protein quantification was performed using a commercially available kit (Pierce BCA protein assay kit, Thermo Scientific, Rockford, IL). Aliquots of protein homogenates (amounts equalized by protein concentration) were mixed with an equal volume of 2× SDS-PAGE sample buffer and boiled for 10 minutes, after which gel electrophoresis was performed. Western immunoblots for BMP-7 were performed using a mouse anti-rabbit monoclonal BMP-7 primary antibody (Abcam, Cambridge MA) at a dilution of 1:1000. Secondary detection was accomplished using a donkey anti-rabbit IgG secondary antibody (Cell Signaling Technologies, Danvers, MA). Quantitative results were obtained by scanning the resulting images and densitometric analysis using Image Lab software (Bio-Rad Laboratories, Hercules, CA) and were normalized to β-actin (Cell Signaling Technologies, Danvers MA).

**RT-PCR for BMP 2, 4, 6**

RNA was extracted from rinsed colon samples that had been snap-frozen in liquid nitrogen and stored at -80\(^\circ\)C. Tissues were homogenized and RNA was extracted using a commercially available kit (RNeasy, Qiagen) and was analyzed with a spectrophotometer. Quantitative RT-PCR was performed using SYBR-green master mix (Roche) and samples were analyzed using a LightCycler 480 (Roche Life Sciences, Indianapolis, IN). The following primer sequences were used: BMP2;   Forward: 5’-
GGGACCCGCTGTCTTCTAGT-3'; Reverse, 5’-ACGGCTTCTTCGTGATGGAA-3’;
BMP4: Forward, 5’-CCTGTTCGTGTCCCCACTGAA-3’, Reverse, 5’-
GGTCAAAACATTTGCACGTAAG-3’; BMP6: Forward, 5’-
GGTCGTGAGAGCTTGTGGTT-3’; Reverse, 5’-ACAAGCCTTCCAGCTTCCCTG-3’

**Coculture of Mast Cells and T cells**

BMMCs were derived as described above. CD4+ T cells were isolated from the spleen of wild-type mice using a commercially available T cell isolation kit (iMag, BD Biosciences). Mast cell activation and stimulation will be achieved by incubation in the presence or absence of cecal bacterial lysates, LPS, or peptidoglycan and then mast cells were washed several times and then were placed into co-culture with naïve CD4+ T lymphocytes at a 1:2 ratio of MC:T cells. After 72 hours, supernatants and cell pellets were collected from the coculture system. Supernatants were analyzed using commercially available sandwich ELISA kits (BD Biosciences).

**Statistics**

Data were analyzed using a t-test or one way ANOVA as appropriate. A post-hoc Tukey's test was used to determine differences between experimental groups. \( p<0.05 \) was considered significant. Statistical analysis was performed using GraphPad Prism (Graphpad software, San Diego, CA).
**Results**

*Mast cells are protective against spontaneous colitis*

Colonic histopathology was examined by routine hematoxylin and eosin staining in wild-type, IL10<sup>−/−</sup>, and DKO mice, as well as in DKO mice that were repleted with BMMCs to demonstrate gain-of-function. Successful mast cell repletion was confirmed by evaluating toluidine blue-stained sections of colon in repleted mice (Figure 2-2). Consistent with previous reports, C57Bl/6 IL10<sup>−/−</sup> mice displayed a mild, patchy colitis with incomplete penetrance. IL10<sup>−/−</sup> mice that also lack MCs (DKO: (IL-10<sup>−/−</sup> x Kit<sup>Wsh/Wsh</sup>) had more severe colitis than MC- sufficient IL10<sup>−/−</sup> mice (p < 0.005, Figure 2-3 and 2-4). The colitis was characterized by diffuse inflammatory cell infiltrate in DKO mice, compared to smaller aggregates of lymphoid cells (“de novo lymphoid follicles”) in IL10<sup>−/−</sup> mice. The inflammation consisted primarily of infiltration of lymphoid cells, with few neutrophils. In some mice, the inflammatory cell infiltrate crossed through the muscularis layer of the intestine (Figure 2-3B). Additionally, mucosal hypertrophy was present in DKO mice, which displayed increased mucosal height compared to IL10<sup>−/−</sup> mice, a characteristic change seen in colitis (p < 0.05, Figure 2-5). The more severe colitis in DKO mice compared to IL10<sup>−/−</sup> mice suggests that mast cells play a protective role in spontaneous colitis. In order to demonstrate that the exacerbation in colitis was mast cell-specific, DKO mice were repleted with BMMCs, which markedly reduced colitis (p < 0.005, Figure 2-3C) and resulted in similar colitis scores to MC-sufficient IL10<sup>−/−</sup> mice (Figure 2-4). Repletion with BMMCs derived from either wild-type (Figure 2-4, p<0.05) or IL10<sup>−/−</sup> (Figure 2-4B, p < 0.01) both ameliorated colitis, indicating that the anti-inflammatory effect of the mast cell is not dependent on IL10.
**Fecal scoring and fecal % water**

DKO mice had higher fecal scores (p < 0.001, Figure 2-6) and higher fecal % water content (p < 0.05, Figure 2-6) than IL10\(^{-/}\) mice, indicating more severe diarrhea in the absence of mast cells, indicative of more severe colonic disease in DKO mice.

**Assessment of intestinal barrier function**

DKO mice had increased colonic mucosal-to-serosal flux of the 4 kDa FITC-dextran (FD4) molecule based on Ussing chamber experiments, compared to IL10\(^{-/}\) mice, which is indicative of intestinal permeability. The increased FD4 flux was prevented by repletion with BMMCs. (p < 0.02, Figure 2-7).

**Cytokine analysis**

Spontaneous colonic cytokine production was assessed by ELISA in supernatants from colonic fragment cultures. DKO mice had significantly elevated production of IL12p40 (p < 0.05, Figure 2-8A) and TNF (p < 0.005, Figure 2-9) compared to IL10\(^{-/}\) mice. Repletion of DKO mice with BMMCs from either wild-type (p < 0.005, Figure 2-9A) or IL10\(^{-/}\) (p < 0.005, Figure 2-9B) ameliorated the increased TNF, but not IL12p40 (Figure 2-9A). IL6 concentrations were not significantly different between IL10\(^{-/}\), DKO, and repleted DKO mice (Figure 2-8).

**Germ free mice**

In order to investigate the role of the intestinal microbiota in the setting of mast cell deficiency and colitis, IL10\(^{-/}\) and DKO mice were raised in a germ free environment. Unfortunately, only 2 DKO mice survived past weaning age to be evaluated for colitis.
Analysis of histopathology revealed that there was no colitis or mucosal hypertrophy present in IL10\(^{-/-}\) or DKO mice in germ free conditions (Figure 2-10). Neither IL10\(^{-/-}\) nor the 2 surviving DKO mice had evidence of colitis when maintained in germ free conditions, indicating that the intestinal microbiota is required for colitis in mast cell-deficient IL10\(^{-/-}\) mice, as has been previously reported for mice deficient in IL10 alone (Sellon et al., 1998).

Both IL10\(^{-/-}\) and DKO mice raised in a germ free environment had extremely low spontaneous colonic production of TNF compared to mice raised in SPF environments (Figure 2-11). In IL10\(^{-/-}\) mice, spontaneous colonic TNF production was significantly decreased compared to SPF IL10\(^{-/-}\) and DKO mice (p < 0.002); however, statistical testing of the TNF results of germ free DKO mice could not be performed due to the fact that only two mice were able to be included in the analysis.

**Real-Time PCR Array for Mouse Cytokines/Chemokines**

In order to gain a broad overview of cytokine and chemokine regulation in DKO versus IL10\(^{-/-}\) mice, a real-time PCR array was performed on colon tissue samples which evaluated 82 different cytokines and chemokines. A large number of cytokines and chemokines were dysregulated in DKO mice compared to mice that lacked IL10 alone (Figure 2-14A), and these dysregulated cytokines and chemokines were largely returned to normal or near-normal by mast cell repletion (Figure 2-14B). A list of some of the most importantly and significantly altered cytokines and chemokines can be found in Table 1, but the most dramatically altered were the bone morphogenetic proteins (BMPs) 2, 4, 6, and 7. IL4, 5, and 9 were not significantly altered between IL10\(^{-/-}\) and DKO mice.
**BMP protein and gene expression**

BMP7 protein expression was detectable by Western blot in cultures of BMMCs (Figure 2-13A), and BMP-2, -4, and -6 gene expression was detectable using RT-PCR (Figure 2-13B). BMP7 was detectable in mucosal scrapes from both IL10\(^{-/-}\) and DKO mice (Figure 2-14).

**Mast cell- CD4+ T cell coculture**

Mast cell- CD4+ T cell cocultures were performed to evaluate mast cells’ influence on proinflammatory cytokine production in CD4+ T cells by pre-exposing mast cells to LPS for varying amounts of time (short term=24 hours; long term= 6 weeks). Short term pre-exposure of mast cells to LPS resulted in increased proinflammatory cytokine production compared to mast cells’ cytokine production alone (Figure 2-15). However, long term incubation of mast cells with LPS dampened proinflammatory cytokine production during coculture, indicating that tolerance is induced when mast cells are exposed to LPS long term.

**Discussion**

The results of this study indicate that MCs play a protective role in spontaneously occurring colitis (IL10\(^{-/-}\) mouse model). Mast cell-deficient, colitis-susceptible DKO mice had exacerbated colitis compared to mast cell-sufficient IL10\(^{-/-}\) mice, and this effect was ameliorated by repletion of mast cells into DKO mice. Additionally, repletion of DKO mice with either wild type or IL10\(^{-/-}\) BMMCs both resulted in decreased colitis, indicating that the protective effect of the mast cell is independent of mast cell IL10.
In addition to the increased colitis scores, DKO mice also had increased mucosal height compared to IL10−/− mice, and this effect was also ameliorated when DKO mice were repleted with BMMCs. The etiology of mucosal hypertrophy in conjunction with colitis is unknown, but is a common finding that increases with colitis severity. (Berg et al., 2002; Kuhn, Lohler, Rennick, Rajewsky, & Muller, 1993; Matta et al., 2012)

Mast cell deficient IL10−/− mice also had increased colonic production of TNF and IL12p40 compared to mast cell sufficient IL10−/− mice, consistent with the increased colitis scores. Interestingly, mast cell repletion only ameliorated the increase in TNF and did not affect IL12p40 levels. Several explanations are possible for this finding. First, mast cells were repleted in DKO mice at 4 weeks of age, so it is possible that an irreversible change occurred prior to that period, such as an impact on immune development or the intestinal microbiota resulting from the absence of mast cells, which, in turn, predisposed the mice to having increased IL12p40 throughout the remainder of their life. Secondly, it is possible that the etiology of the increased IL12p40 was the result of another abnormality resulting from abnormal KIT signaling in the DKO mice, independent of the mast cell. (Nigrovic et al., 2008) IL6 production was not different between IL10, DKO, or repleted DKO mice.

Together, these results indicate that mast cells differentially regulate cytokine production in the setting of colitis.

DKO mice had increased intestinal permeability compared to IL10−/− mice, which is not surprising given the increased severity of their colitis. Mast cell repletion ameliorated the increased intestinal permeability, likely by decreasing inflammation. Mast cells can have a variety of effects on regulation of the intestinal barrier, but mast cell degranulation is most
commonly reported to increase intestinal permeability (Chichlowski et al., 2010; Groschwitz et al., 2009; Jacob et al., 2005).

The increased colitis scores and proinflammatory cytokine production in DKO mice compared to IL10\(^{-/-}\) indicate that mast cells are playing a protective role in spontaneous colitis, but the mechanism is unclear. Mast cells can have both pro- and anti-inflammatory actions. There are four main mechanisms by which mast cells could be protective in IBD, based on previous literature: through 1) interaction with the microbiota including aiding in bacterial defense (Chehoud et al., 2013; Malaviya et al., 1996; Sutherland et al., 2008); 2) induction of T-regulatory cells (Gan et al., 2012; Lu et al., 2006b), 3) orchestration of immune responses (Leveson-Gower et al., 2013; Sayed et al., 2008), or 4) serine protease-mediated degradation of proinflammatory cytokines or other proinflammatory molecules such as alarmins (Nelissen et al., 2014; Roy et al., 2014).

In order to gain insight into the role of the intestinal microbiota in initiating inflammation in DKO mice, we attempted to derive this strain into germ free conditions. Two mice did survive past weaning age, which allowed us to document lack of colitis and minimal production of TNF in these mice by 6 months of age, at which time their counterparts in specific pathogen free environments have developed moderate to severe colitis. These results indicate that similar to IL10\(^{-/-}\) mice, the intestinal microbiota are required for development of colitis in DKO mice. We were unfortunately unsuccessful in maintaining this strain in a germ-free environment, so were unable to proceed with gnotobiotic studies to isolate the effect of particular components of the microbiota. Future studies are necessary to further assess the role of the microbiota in regulation of colitis in the setting of mast cell deficiency.
We next performed a PCR array for 82 different genes encoding cytokines and chemokines in order to broadly evaluate the cytokine environment in DKO compared to IL10\(^{-/+}\) mice. While numerous cytokines and chemokines were dysregulated in DKO mice and restored by mast cell repletion, some of the most significantly altered were the BMP proteins. BMP 2, 4, 6, and 7 were all significantly decreased in DKO mice compared to IL10\(^{-/-}\). BMP proteins are well-known for their role in inhibition of stem cell division in the colon (A. Reynolds et al., 2014), so loss of this protein may serve as an explanation for the mucosal hypertrophy and epithelial hyperplasia seen in DKO mice, and will require further study.

Additionally, BMP proteins are important in inflammation. BMP-2, -4, and -7 have been demonstrated to have anti-inflammatory activity in the stomach of mice (Takabayashi et al., 2014), and BMP-6 and -7 are protective in renal fibrosis (Dendooven et al., 2011; Lv et al., 2014; Yang et al., 2013). BMP-7 is anti-inflammatory in chemical colitis models (Hao et al., 2012; Marie et al., 2003). The anti-inflammatory mechanism of the BMPs is not completely understood, but has been demonstrated to decrease IL2 production in the Jurkat T-cell line (Yoshioka, Ono, Osaki, Konishi, & Sakaguchi, 2012).

BMP protein and gene expression was detectable in BMMCs, which is a novel finding. BMP7 protein was also detectable in colonic mucosal scrapes from IL10\(^{-/-}\) and DKO, and further studies will quantify BMP7 protein levels between these two groups of mice.

We had hypothesized that the cytokine/chemokine array would reveal skewing toward a so-called Th1 or Th17 cytokine profile, with decreases in the Th2-type cytokines that are classically thought of as being present in mast cell activation, such as IL4 and IL5. Surprisingly, these cytokines were not significantly altered in mast cell deficiency and a
strongly Th1 or Th17 profile was not evident. Instead, we were able to identify particular mediators that were most impacted by mast cell deficiency in the setting of colitis.

Lastly, we have demonstrated that pre-exposure of mast cells to bacterial products such as LPS can induce later proinflammatory cytokine production in a coculture model. Short term pre-incubation of mast cells with LPS, followed by washing and then coculture with CD4+ T cells, results in increased TNF production. However, long-term pre-incubation of mast cells with LPS suppresses TNF production in coculture, indicating that mast cells are inducing a tolerance mechanism following long term exposure to LPS. Mast cells are critical in the maintenance of allograft tolerance (de Vries et al., 2010; de Vries et al., 2011; Lu et al., 2006b) and can induce T regulatory cells in vitro, but studies have not examined the effect on cytokine production (Nie et al., 2012; W. Zhang et al., 2010). We hypothesize that the decrease in TNF production with long term exposure of mast cells to LPS is due to induction of T regulatory cells by mast cells that have been exposed to LPS long term. Because intestinal mucosal mast cells are likely chronically exposed to LPS in IBD patients with a compromised intestinal barrier, we propose that dampening of proinflammatory cytokine production through induction of T regulatory cells may be a key factor in the protective role of the mast cell, but future studies are needed to confirm this hypothesis.

In summary, mast cells have a key protective role in a model of spontaneous colitis. Mast cells have many potentially protective functions, and future work will attempt to elucidate the mechanism of the protective effect of the mast cell in inflammatory bowel disease. Implications for this novel finding could include improved therapeutic strategies for treatment of inflammatory bowel disease.
Figures

**Figure 2-1:** Bone marrow-derived mast cells A. Transmission electron microscopy; B. Toluidine blue staining; C. Flow cytometry for c-kit and FcεR1.
Figure 2-2: Confirmation of mast cell repletion.
**Figure 2-3:** Mast cell deficiency exacerbates colitis in IL10−/− mice. Colon, hematoxylin and eosin staining, 200x. A: Representative image from an IL10−/− mouse, B: double knockout mouse, C: double knockout mouse repleted at 4 weeks of age with bone marrow-derived mast cells.
Figure 2-4: Mast cell deficiency exacerbates colitis. IL10\(^{-/-}\) mice that additionally lacked mast cells (DKO) had worse colitis than mice that lacked IL10 alone. DKO mice that were repleted intraperitoneally with bone marrow-derived mast cells (BMMCs) at 4 weeks of age had colitis scores that were similar to the level of IL10\(^{-/-}\) mice. A. Mast cell repletion with wild-type BMMCs (\*, p < 0.005, n=7-12/group). B: Mast cell repletion with IL10\(^{-/-}\) BMMCs (\*, p < 0.01, n=10-22/group).
Figure 2-5: Mast cell deficiency causes mucosal hypertrophy. Hypertrophy is prevented by mast cell repletion (*, p < 0.05, n=10-11/group).
**Figure 2-6**: Clinical disease as assessed by fecal scoring and fecal percent water content as a marker of diarrhea. A. Fecal scores were higher in DKO mice than IL10\textsuperscript{+/−} (p < 0.001); B. Fecal % water was higher in DKO mice compared to IL10\textsuperscript{+/−} (p < 0.05). n=10-11/group.
Figure 2-7: DKO mice demonstrate increased intestinal permeability as measured by 4KDa FITC-dextran flux. Mast cell repletion with wild-type BMMCs ameliorates the intestinal barrier dysfunction induced by mast cell deficiency. n=10-23 mice/group, p <0.02).
Figure 2-8: Colonic cytokine production. A. DKO mice had increased spontaneous colonic production of IL12p40 compared to IL10\(^{-/-}\) mice (p < 0.05), but repletion of DKO mice did not impact IL12p40 concentration. IL6 was similar between all 3 groups of mice. n=7/group.
Figure 2-9: Spontaneous colonic TNF production is increased in DKO mice compared to IL10−/−, and is ameliorated by mast cell repletion of DKO mice. A. Colonic TNF production, mice repleted with wild type BMMCs. (p < 0.005); B. Colonic TNF production, mice repleted with IL10−/− BMMCs. (p < 0.005). n=7-18/group.
Figure 2-10: IL10−/− and DKO mice in germ-free conditions do not develop colitis. A. Representative image from an IL10−/− at 6 months of age housed in germ-free conditions. B. Representative image from a DKO mouse at 6 months of age housed in germ free conditions.
Figure 2-11: TNF production in germ-free mice. IL10<sup>−/−</sup> and DKO mice raised in germ free (GF) conditions have very low spontaneous production of TNF compared to conventionally raised (Conv) specific pathogen free IL10<sup>−/−</sup> and DKO mice. (p<0.002), n=3-7/group (GF DKO excluded from analysis due to n=2).
Figure 2-12: Representative PCR array analysis of genes for 82 cytokines and chemokines. A. IL10^{−/−} vs. DKO mouse demonstrating a large number of cytokines and chemokines that are altered between the two genotypes. Each cytokine or chemokine is represented by a single dot. Black dots between the two lines have similar expression between IL10^{−/−} and DKO mice. Red dots above the lines represent cytokines or chemokines that are increased in DKO mice compared to IL10^{−/−}, while green dots below the lines represent cytokines or chemokines that are decreased in DKO mice compared to IL10^{−/−}. B. IL10^{−/−} vs. DKO mouse that has been repleted with BMMCs at 4 weeks of age.
Figure 2-13: BMP is present in BMMCs. A. BMP7 Western blot demonstrating presence of BMP7 in BMMCs. B. RT-PCR demonstrating presence of bmp-2, -4, and -6 in BMMCs.
Figure 2-14: BMP7 protein expression is detectable in mucosal scrapes from IL10⁻/⁻ and DKO mice. A. Densitometric analysis of protein expression, normalized to β-actin. B. BMP7 Western blot in DKO and IL10⁻/⁻ mice.
Figure 2-15: Long term exposure of mast cells to LPS dampens proinflammatory cytokine production from coculture. Short-term mast cell preincubation with LPS results in increased proinflammatory cytokine expression from coculture with CD4+ T cells. However, this effect is prevented with long-term preincubation of mast cells with LPS, indicating that mast cells are promoting tolerance.
**Table 1: PCR Array**

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