Persistent enteric mycobacterial infection sensitizes mice to intestinal epithelial injury

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Persistent enteric mycobacterial infection sensitizes mice to intestinal epithelial injury

by

Charles Scott Johnson

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Veterinary Pathology

Program of Study Committee:
Jesse Hostetter, Major Professor
Douglas Jones
Judith Stabel
Michael Wannemuehler
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Iowa State University
Ames, Iowa
2010
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First and foremost, I would like to thank my advisor Dr. Jesse Hostetter. Although it may sound corny, he has been an advisor in the truest sense of the word. He has not only tolerated my near perpetual juvenile attitude but has always provided advice that invariably helped me when I was struggling to find answers both in my personal life and during my graduate and residency training. Additionally, my graduate training greatly benefited from the assistance of Drs. Wannemuehler, Jones, Stabel and Ackermann. Interacting with Dr. Wannemuehler’s lab group helped facilitate both my understanding of our mouse model and resolve technical problems I encountered throughout my research project. I always appreciated Dr. Jones’ willingness to take the time to engage in a conversation with me about my random questions about science that helped remind me that biology is not a static and linear discipline. I am grateful for Dr. Stabel’s prompt and timely answers to my technical questions concerning M. paratuberculosis during the initial stages of my research project. I would also like to thank Dr. Ackermann for allowing me to monopolize Jack’s time when I was having difficulty with quantitative real-time PCR.

My residency and graduate training was made all the more enjoyable because of the talented and personable group of residents that I have had the good fortune to interact with over the years. Drs. Meyerholz, Simutis, and Greenlee’s reassuring advice was greatly appreciated while studying for boards. Furthermore, I have always valued Dr. Meyerholz’s unwavering support and willingness to help whenever I needed it and teaching me how to feed chocolate to squirrels. My cubicle neighbor and fellow lab mate Dr. Plattner is a man who I admire for his religious conviction; and, even though he always felt guilty about asking for my help with his project, I always enjoyed the opportunity to “wrestle” with the calves. Dr. Shannon Hostetter’s playful attitude and wit were always a joy to be around and I am thankful for Dr. Madison’s
friendship during some of the more difficult times of my personal life. Lastly, I’d like to acknowledge Elise Huffman for her help and unparalleled skill in the lab and for always reminding me that the details really do matter.

For myself, I have to admit trying to develop an animal model for a research project was, for the most part, an enjoyable experience. Moreover, my residency and graduate training has been a constant reminder that the art of writing really does lay in thrift and that remaining objective and not being married to preconceived biases are essential when evaluating clinical or research data. Perhaps more importantly, my graduate training has reminded me of the importance of patience, listening, and persistence and led me on a path of becoming more self-aware. I would like to dedicate this thesis to my mother and sister who have endured hardships in their lives that I hope I will never have to face and whose friendship and support have sustained me throughout my life.
General Introduction: Statement of the Problem

Alterations in intestinal homeostasis: Inflammatory bowel disease

Maintenance of intestinal homeostasis in the context of a diverse and abundant intestinal bacterial flora is a complex process that requires coordination between multiple components of the adaptive and innate immune system and elements of the mucosal barrier.[1] Failure of the innate immune response, such as a primary defect in intestinal macrophages, or, for example, a breakdown of mucosal barriers following injury to enterocytes can potentially serve as a catalyst for initiating chronic, adaptive immune response mediated inflammation.[2, 3] Furthermore, infection with, or exposure to, microbial pathogens can cause a shift in this regulatory network and tip the balance in favor of inflammation, with the potential to exacerbate pre-existing inflammation in disorders of chronic inflammation, such as inflammatory bowel disease (IBD).[4]

Chronic inflammation with disruption of intestinal homeostasis is the hallmark lesion in both of the distinct clinical phenotypes of the inflammatory bowel diseases, Crohn’s disease (CD) and ulcerative colitis (UC).[5] The pathogenesis of these disorders is thought to be multifactorial, with environmental factors, deregulation of the host immune response, the normal intestinal flora, and the genetic background of the host all considered being important factors that interact to result in the characteristic clinical and pathologic features of these diseases.[6] Although defects in these broad categories are thought to be essential for the manifestation of the inflammation observed in IBD, the precise casual mechanisms leading to
the initiation of inflammation of these disorders is not understood, and, more specifically, it is not completely clear how or if functional alterations in components of the innate immune system, such as intestinal macrophages, contribute to pathogenesis of IBD.

*Intestinal macrophages as key effectors in maintaining intestinal homeostasis:*

Intestinal macrophages play a critical role in maintaining mucosal homeostasis.[7] Therefore, it is essential to understand how changes in macrophage phenotype, secondary to infections or genetic mutation, alters the microenvironment of the intestine and whether or not these changes influence the susceptibility of the host to subsequent intestinal injury. With respect to their homeostatic role, resident intestinal macrophages have a unique functional phenotype. They can eliminate potential pathogens while at the same time preventing harmful inflammation.[8] This anti-inflammatory phenotype of human intestinal macrophages is defined by the absence of innate response receptors such as receptors for LPS (CD14) and CR4 (CD11c/CD18) and the inability to produce pro-inflammatory cytokines in response to inflammatory stimuli.[8] Despite this dual protective role of intestinal macrophages, it is clear that they can contribute to the pathogenesis of mucosal injury, such as in IBD where it has been documented that large amounts of IL-1β are produced almost exclusively by intestinal macrophages.[9, 10] Although these studies demonstrate that intestinal macrophages have important homeostatic or potentially pathogenic roles in the intestinal mucosa, there is a gap in our knowledge about the ability of a chronic intestinal infection of macrophages to cause a shift in macrophage effector function which subsequently impairs the host response to mucosal injury.
The influence of microbial pathogens on intestinal homeostasis:

Experimental studies in which colitis does not develop in germ-free mice indicates that the normal intestinal flora plays an important, if not essential, role in the development or maintenance of intestinal inflammation.[11] Some studies suggest that this may represent a type of autoimmune response since neonatal rats colonized by bacteria develop tolerance to bacterial antigens, presumably due to intra-thymic processing that causes the host to recognize the bacteria “self” antigens.[12] Although these reports highlight the importance of the intestinal flora in experimental models of IBD, the significance of any single infectious agent is questionable. Several epidemiologic studies and animal models of IBD suggest that microbial pathogens may have a role in initiating or exacerbating pre-existing inflammation in IBD. The most convincing support for the role of a specific intestinal pathogen in IBD has been demonstrated in patients with ileal CD, where an increased number of patients are colonized by an adherent/invasive strain of *E. coli* (AIEC).[13] It has been reported that AIEC was recovered from 36% of mucosal biopsy specimens from patients with early postresection recurrent CD relative to 6% of normal control ileal biopsy specimens.[14, 15] This strain of *E. coli* is able to both replicate and persist within macrophages and enhance macrophage secretion of TNF-α.[16] Experimentally, superimposed exposure to low-level microbial pathogens can exacerbate intestinal inflammation. For example, exacerbation of chemically mediated intestinal inflammation has been shown following acute luminal exposure to *Bacteroides fragilis*, AIEC, and *Hymenolepis diminuta*.[17-19] These studies emphasize the potential for microbial infections to alter intestinal mucosal homeostasis and the need to further define the mechanisms associated with microbe induced disturbances in
intestinal inflammation. In a broader sense, since large numbers of macrophages in the intestine are strategically placed in the lamina propria adjacent to abundant numbers of immunostimulatory bacteria, it is imperative to understand how infections of intestinal macrophages affects their function, in particular, their response following exposure to the normal flora or its products.

This literature review will focus on components of the intestinal mucosal barrier, with an emphasis on macrophages in the context of IBD and how infections with low level pathogens, and to a lesser extent non-microbial factors, may cause a positive or negative shift in the response of the mucosa to injury.

Specific Aims

Our primary goal was to understand how a subclinical intestinal infection with *Mycobacterium avium ss paratuberculosis* (*Map*) would influence subsequent acute mucosal injury. To address this goal, we utilized a mouse model of intestinal *Map* infection.[20, 21] BALB/c mice were infected with *Map* strain K10 and subsequently administered a low dose of dextran sulfate sodium (DSS) in the drinking water to induce mild intestinal injury. Our central hypothesis was that persistent intestinal mycobacterial infection sensitizes the host to enhanced intestinal injury. The following specific aims were pursued to test this hypothesis: 1) determine if subclinical intestinal *Map* infection influences subsequent DSS mediated intestinal inflammation (Chapter 2); 2) define how *Map* infection influences the characteristic DSS mediated intestinal immune profile and determine if *Map* infected mice had a *Map*-specific immune response (Chapter 3); 3) determine how infection with *Map*
alters the phenotype and function of intestinal macrophages following injury to the intestinal mucosa or in vitro stimulation with LPS (Chapter 4).

Dissertation Organization

This dissertation is organized in the alternative format with the first chapter being a general introduction and literature review (Chapter 1). This is followed by three manuscripts (Chapters 2-4) which have been prepared for submission to the journal of Experimental Biology and Medicine, Clinical and Experimental Immunology, and the Journal of Leukocyte Biology, followed by general conclusions. References are cited at the end of each chapter.

References


Chapter One: Literature Review

*Intestinal homeostasis and the role of the mucosal barrier:*

The intestinal mucosal barrier is a complex cellular and biochemical structure that has multiple physiologic roles, including nutrient, electrolyte and fluid absorption and, perhaps most importantly, it serves to limit and regulate exposure of the host innate and adaptive immune system to the abundant bacterial and dietary antigens in the adjacent intestinal lumen.[1] This barrier is composed of multiple cell types and their associated secreted or cell surface products, such as mucus and tight junctions and whose permeability is influenced by interactions with both the contents of the intestinal lumen and the innate and adaptive immune cells in the adjacent lamina propria.[2] Given the importance of disturbances in intestinal permeability in diseases such as IBD, it is essential to try and understand the potential triggers associated with alterations in the mucosal barrier that can initiate or promote the characteristic chronic inflammation that is the hallmark of IBD.[3]

*Intestinal permeability and intercellular junctional complexes*

The microbial diversity and bacterial load in the intestinal lumen is large, with increasing concentrations of bacteria from the relatively sterile gastric lumen to the large intestine which contains up to $10^{11}$ to $10^{12}$ bacteria per gram in the cecum and colon.[4] Enterocytes, mucus and tight junctions all have an essential role in regulating intestinal permeability and preventing translocation of these bacterial antigens across the mucosal barrier. There are two principal routes by which material can traverse the mucosal barrier. One route is transcellular, in which molecules pass directly through epithelial cells, with the
other being the paracellular route which is regulated by intercellular junctional complexes and allows material to pass between enterocytes.[1] Disruption of the mechanisms regulating passage of antigens via these routes can result in increased permeability and passage of antigens potentially promoting the development of or maintenance of mucosal inflammation.[3, 5, 6] The importance of enhanced intestinal permeability in the pathogenesis of IBD is highlighted in studies that have demonstrated familial associated increased intestinal permeability in first degree relatives of patients with CD and in a case report describing increased intestinal permeability in a patient prior to the development of CD.[7, 8] Therefore, it is critical to understand the normal physiology of the mucosal barrier and mechanisms associated with maintaining its integrity that prevent unwanted exposure of immune cells to luminal bacterial antigens.

Enterocyte associated intercellular junctional complexes are essential regulators of intestinal permeability, with defects in their structure or function predisposing the host to intestinal inflammation.[3] Intercellular junctional complexes are cell surface associated structures located at the lateral apical and lateral margins of the enterocyte and include tight junctions, adherens junctions, and desmosomes.[9] These structures are composed of transmembrane proteins which are linked to the intracellular actin cytoskeleton and relative to the adherens junctions and desmosomes, which are thought to be primarily responsible for the mechanical linkage between adjacent cells; the tight junctions have an additional role in selectively regulating paracellular ion transport.[10-14]

The major structural components of the adherens junctions include the transmembrane glycoprotein E-cadherin that is bound intracellularly to catenin proteins. The
catenins, in turn, link the adherens junction complex to the intracellular cytoskeleton.[11] The adherens junctions not only function in physically linking adjacent cells, cadherin-catenin complexes also have an important role in forming desmosomes, proliferation and migration.[15, 16] The importance of the cadherins in intestinal homeostasis is highlighted in a study where mouse enterocytes containing a dominant negative N-cadherin mutation. This resulted in alterations in the rates of cell migration and programmed cell death and the development of intestinal inflammation.[17]

In contrast to the adherens junctions, the tight junctions are composed of four different transmembrane proteins, including claudins, occludin, tricellulin, and junctional adhesion molecules (JAMs), with the functional integrity of the tight junction complex being modulated by phosphorylation of these proteins.[18-20] Similar to the transmembrane proteins of the adherens junction, proteins of the tight junction, such as occludins and claudins are linked to the cytoskeleton via a distinct group of proteins, the zonula occludens (ZO).[21, 22] Alterations or loss of proteins of the tight junction such as ZO-1 and JAM-A can result in increased permeability leading to translocation of luminal bacterial antigens. An example of which occurs following treatment of mice with DSS.[23] This change in ZO-1 presumably occurs before the development of inflammation and is not a consequence of the intestinal inflammation induced by DSS treatment. Although individual proteins of the tight junction have an important role in adhesion and paracellular permeability, the absence of a single component of the complex does not always result in a dysfunctional phenotype as demonstrated in mice lacking occludin which still have normal tight junction formation, numbers, and barrier function.[24]
Further support for the importance of enterocytes and their associated intercellular junctional complexes in maintaining the mucosal barrier is provided by numerous animal models that have elucidated diverse mechanisms that can promote intestinal permeability. For example, in IL-10 knockout mice, increased enterocyte apoptosis can be induced by treatment with a non-steroidal anti-inflammatory drug such as piroxicam, resulting in increased permeability and inflammation secondary to exposure to the normal flora in the intestinal lumen.[25] Furthermore, different cytokines such as TNF-α and IFNγ can directly enhance the permeability of the intestinal mucosa by interfering with the integrity of the tight junction complexes. For instance, IFNγ can increase paracellular permeability by promoting the disassembly of the tight junction proteins via a macropinocytosis or an endocytosis mediated mechanism that involves a Rho-associated kinase.[26, 27] With respect to TNF-α mediated alterations of the tight junction, in vitro incubation of intestinal epithelial cells with increasing concentrations of TNF-alpha can cause a decrease in claudin-1 which was speculated to be associated with relocation of ZO-1.[28] It has also been demonstrated that these two cytokines can synergistically act to alter the intestinal epithelial barrier by increasing the expression of myosin light chain kinase (MLCK) with phosphorylation of myosin II regulatory light chain (MLC).[29] The phosphorylation of MLC promotes opening of the tight junction via contraction of peri-junctional actin-myosin filaments.[30-32] Moreover, another innate cytokine, namely IL-1β, is capable of altering tight junctions by a similar mechanism. It does so via activation of NF-κβ, resulting in an increase in the expression of myosin L chain kinase.[33, 34]
**The role of enterocytes in the mucosal barrier:**

Enterocytes are the initial cellular component of the host that provides not only a physical barrier to the luminal bacterial antigens but they also participate in a dynamic interaction with the intestinal flora to either promote or inhibit the inflammatory response. A key molecule in determining whether or not an inflammatory response will occur following interaction with the intestinal flora is NF-κβ. For example, by way of TLR2 and TLR4, colonic epithelial cells in germ-free mice colonized with either *Bacteroides vulgatus* or *Enterococcus faecalis* respond to this interaction with temporary NF-κβ activation, with perpetual activation of NF-κβ being associated with deficiency of IL-10 as shown in mice monoassociated with *Enterococcus faecalis*. In addition to IL-10, NF-κβ signaling is thought to be modulated by lamina propria derived TGF-β, as suggested by increased SMAD signaling and corresponding decreased NF-κβ activation following bacterial colonization of intestinal epithelial cells.

The NF-κβ pathway can also be differentially influenced by the properties of the stimulating microbe. For instance, pathogenic and non-pathogenic intestinal bacteria are capable of either activating or suppressing this key nuclear transcription factor. In a polarized model of intestinal epithelial cells, it has been shown that the pathogen *Salmonella typhimurium* activates Iκβα kinase in a Ca^{2+} dependent manner resulting in the translocation of NF-κβ. This ultimately results in the production of the neutrophil chemoattractant IL-8. In contrast, it has been shown, again with a polarized model of human epithelia, that a non-virulent strain of Salmonella is capable of inhibiting Iκβα degradation which prevents activation of the Iκβα/NF-κβ. This is accomplished by preventing polyubiquination of Iκβα,
thus it avoids degradation by the proteasome.[38] In addition to the different pathogenic properties of a particular bacterium influencing the activation of NF-κβ, the spatial location of TLRs can influence whether or not NF-κβ will be activated; even with the same TLR. In the case of TLR9, which has both apical and basolateral expression in enterocytes, stimulation of the basally located TLR9 results in activation of NF-κβ due to Iκβα degradation. However, NF-κβ activation is prevented if apical TLR9 is activated which causes accumulation of ubiquinated Iκβα.[39]

NF-κβ appears to have opposing functions in the intestine. In addition to its well-documented pro-inflammatory properties, NF-κβ has a seemingly contradictory protective role in maintaining the integrity of the intestinal mucosa. One example of this protective effect was demonstrated in mice which develop spontaneous colitis following conditional deletion of Iκβ-kinase γ (Iκγ-NEMO), which is necessary for NF-κβ signaling, in their intestinal epithelial cells.[40] Protective mechanisms associated with NF-κβ signaling include both functions in epithelial repair and secretion of products that alter the immune response. For example epithelial NF-κβ can promote repair of epithelial damage, as demonstrated in a mouse model of defective TLR signaling where there is decreased mucosal hyperplasia in conjunction with bacteremia and the production of type 1 interferons.[41]

Intestinal epithelial secreted products such as thymic stromal lymphopoitetin (TSL) are capable of dampening chronic intestinal inflammation by inhibiting dendritic cell production of IL-12/23.[42] Moreover, TSL may be associated with suppression of pro-inflammatory cytokines such as IL-1β or altering the development of intestinal macrophages. This is based on studies indicating TSL functions to limit inflammation in a mouse model of
DSS mediated intestinal inflammation and the suggestion by some author’s that it may be involved in the phenotypic maturation of intestinal macrophages.[43-45] These studies emphasize that enterocytes are not restricted in only acting as a physical barrier but also are participants in a dynamic relationship with the overlying microflora in the intestinal lumen.

**Intestinal mucus:**

Intestinal goblet cell produced mucus is a component of the mucosal barrier that perhaps gets overlooked compared to disturbances in the mucosal immune system in the pathogenesis of IBD.[46] However, several studies in patients with IBD demonstrate alterations in this protective barrier. Reductions in goblet cell numbers and a corresponding thinning of the mucus layer have been described in patients with IBD.[46] Moreover, another study demonstrated that approximately 40% of patients with IBD had anti-goblet cell autoantibodies.[47]

Intestinal mucus is an O-glycosylated, carbohydrate rich material that contains proteins such as Muc2 and Muc3 whose expression within the intestinal tract varies.[48, 49] For example, Muc3 is predominantly expressed in the small intestine relative to Muc2 which is expressed in both the small and large intestine.[49] Likewise, there are spatial and regional differences in how the intestinal microflora interacts with the mucus. For instance, bacteria are generally not present in mucus of the distal to mid colon, in contrast to the proximal colon and cecum where there may be mucosal and luminal associations of bacteria.[50] Therefore, disruption of this delicate barrier may promote the development of mucosa associated biofilms, which are present in up to two thirds of patients with IBD, or possibly allow opportunistic pathogens to interact with the intestinal epithelium. Experimental
disruption of this barrier, for example in mice deficient in the Muc2 protein, is associated with the development of colitis and increased expression of IL-1β and TNF-α.[46] Therefore, these studies confirm that intestinal mucus is protective and its absence or thinning may allow for the association of bacteria with the mucosa which may serve as a trigger for development of intestinal inflammation.

Normal flora:

As highlighted in the previous sections, the mucosal barrier plays an important role in preventing the normal bacterial flora from gaining access to the mucosal immune system. The immensely complex and abundant normal flora can have a profound effect on the physiology of the host in terms of their production of various metabolites following metabolism of undigested carbohydrates, mucus and exfoliated epithelial cells which can influence the immune system, energy balance and epithelial function of the host.[51-53] For example, bacteria contain a variety of enzymes that are capable of metabolizing xenobiotic compounds and are capable of synthesizing vitamins, short-chain fatty acids and methane.[54] Bacterial numbers increase in number from the relatively sterile stomach to the densely populated colon, with the proximal intestine containing a greater percentage of aerobes compared to the relatively large number anaerobes within the colon.[4] In total there are up to $10^{13}$ to $10^{14}$ total microorganisms and 15,000 to 36,000 individual species within the intestinal tract, with approximately 99% of the bacteria belonging to one of 4 bacterial divisions, which are the Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes.[54-56] The Firmicutes, which contain the clostridial XIV and IV groups are the most abundant, with
Bacteriodetes accounting for the next largest percentage of bacteria, and the Proteobacteria, which includes *Escherichia coli*, accounting for the smallest number of bacteria.[54]

The significance of the normal bacterial flora in experimental models of IBD is emphasized by studies where colitis does not develop in germ-free mice.[57] Further support for the importance of the intestinal microbial flora in intestinal inflammation is provided by the C3H/HeJ Bir mouse model of spontaneous colitis in which CD4+ T-cells reactive to antigens of the normal bacterial flora can cause intestinal inflammation following adoptive transfer to SCID mice and based on the observation that antibiotics and probiotics can treat or prevent IBD.[58, 59]

In humans, shifts in the composition of the bacterial flora have been defined in patients with UC and CD, but it is not clear if there are disease specific changes relative to UC and CD. Frank et al, described alterations in different phyla of bacteria, with decreased numbers of Bacteroidetes and Firmicutes with increases in Actinobacteria and Proteobacteria.[56] However, it is not apparent if these changes are a secondary phenomenon or involved in the initiation of intestinal inflammation. A bacterial induced colonic epithelial nutrient deficiency represents a plausible disease associated mechanism following shifts in the composition of the intestinal microflora. For example, the production of short chain fatty acids by bacteria of the intestinal microflora serves as nutrient source for colonic epithelial cells, with decreased concentrations of short chain fatty acids being detected in the patients with IBD.[60] Deficiencies in nutrients could be further compounded by alterations in the production of hydrogen sulfide which could interfere with the utilization of short chain fatty acids by epithelial cells.[61]
The role of opportunistic pathogens in IBD and models of enhanced intestinal inflammation

Although the normal intestinal flora is an important contributing factor in IBD, the significance of any single infectious agent is questionable. The most convincing support for the role of a specific intestinal pathogen in IBD has been demonstrated in patients with ileal CD, where an increased number of patients are colonized by an adherent/invasive strain of *E. coli* (AIEC) that can persist within epithelial cells and macrophages and has been shown to enhance macrophage secretion of TNF-α.[62, 63] AIEC was recovered from 65% and 36% of mucosal biopsy specimens from patients with chronically inflamed ileal resections or early postresection recurrent CD, respectively, compared to 6% of normal control ileal biopsy specimens.[64, 65] The ability of AIEC to colonize the ileum in CD is dependent on the expression of a type I pilus which allows adherence to an AIEC induced epithelial receptor CEACAM6, the induction of which is mediated by cytokines such as TNF-α and IFNγ.[66] Flagellin of AIEC represents another important virulence factor of the LF82 strain of *E. coli* and has been speculated to be essential for AIEC enhanced intestinal inflammation following DSS treatment, which is associated with increased expression of IL-1β and IL-6.[67]

In addition to AIEC, there has also been an association between exposure to *Mycobacterium avium ss paratuberculosis* (*Map*) and CD.[68] *Map* is an enteric pathogen of adult ruminants that causes chronic weight loss and diarrhea following a prolonged incubation period.[69] It is an obligate intracellular pathogen of macrophages, with a characteristic lesion associated with *Map* infection being transmural thickening of the terminal ileum by a large numbers of macrophages.[69] The belief that *Map* infection may have a role in the pathogenesis of CD has been tenuously extrapolated from the presence of
grossly similar lesions seen in patients with Crohn’s disease when compared to the lesions of bovine Johne’s disease.[70] Exposure to Map through the food supply may not be a rare event; for example, Map has been detected in commercially pasteurized milk, during the manufacture and ripening of cheddar cheese, and following sub-pasteurization heat treatment of milk used for cheese manufacture.[71-73] Furthermore, the detection of Map DNA within the lesions of Crohn’s patients, the isolation of Map organisms from the blood of patients with CD, and the detection of Map antigen specific antibody within the serum of Crohn’s patients provides circumstantial evidence that Map plays a role in the pathogenesis of Crohn’s disease. [74-79] What is more, an open-label drug trial has been conducted in patients with Crohn’s disease in which complete clinical remission was observed following treatment with anti-mycobacterial drugs.[80] However, contradictory reports exist on the significance of Map in the pathogenesis of CD. For example, some studies have not detected Map 16s rRNA in patients with CD or UC; and, even though a subset of CD patients may have genetic defects that affect innate immune function; namely, the NOD2 mutation, which may predispose patients to an intracellular pathogen such as Map, there is a lack of an association between NOD2 polymorphisms and Map infection.[56, 81, 82]

Although previous studies provide evidence for an association between Map exposure and CD, there is limited experimental data from animal models to support the claim that Map infection is essential for the development or maintenance of inflammation in CD. Exposure to Map can exacerbate disease in an IL-10 deficient mouse model of spontaneous intestinal inflammation.[83] The Map exposed mice in this model had increased weight loss and higher colonic lesion scores following oral challenge with Map strain Ben (ATCC
43544), with enhanced production of Ag85B/MPT59 peptide 25 specific TNF-α and IFNγ from both serum and cells isolated from the Peyer’s patches and mesenteric lymph nodes.[83] The author’s proposed that since Ag85B/MPT59 peptide 25 is conserved across several mycobacterial species, that Map may function as an immunomodulator that stimulates T-cells in a manner similar to a superantigen, suggesting that the enhanced disease observed in these mice is not unique to Map. Several other investigators have also presented data suggesting Map may act as a modifying variable in CD patients by functioning as a modulator of the immune response, but not in a cause effect relationship with the disease.[84, 85] In particular, Map exposure in CD patients has been associated with enhanced secretion of TNF-α, which is therapeutically important since anti-TNF-α therapy is often effective in patients with CD.[86] It is also possible that exposure to or infection with Map promotes increased production of IL-1β in a subset of genetically susceptible individuals, for example in individuals with a defect in autophagy related killing.[87, 88] Following in vitro infection or exposure of bovine and murine macrophages with Map or Map cell wall components there is increased production of IL-1β.[89, 90] Furthermore, in vivo Map infection is associated with expression of IL-1β.[91] It has been suggested that IL-1β plays an important role in the pathogenesis of IBD as evidenced by enhanced secretion of IL-1β by mononuclear cells isolated from the inflamed mucosa from patients with Crohn’s disease and increased IL-1β secretion by murine macrophages which contain the most common susceptibility allele for Crohn’s disease which is in the Nod2 locus.[92, 93] Moreover, there is a positive correlation between IL-1β levels and the degree of intestinal inflammation in Crohn’s disease. [94]
Microbial pathogens have also been linked to reactivation or exacerbation of inflammation in IBD patients.\[95, 96\] However, only a limited numbers of studies have examined the role of specific pathogens as causes of enhanced intestinal inflammation and, more specifically, even fewer studies have examined the role of occult or subclinical infections on the development of intestinal inflammation. In one study it was noted that there is an increased prevalence of active cytomegalovirus (CMV) infection in patients with IBD.\[97\] It is speculated that this represents reactivation of latent infection. In support of a potential role for CMV, a virus known to infect intestinal macrophages, in reactivation or exacerbation of inflammation in IBD, it has been shown that latent cytomegalovirus infection sensitizes mice to a worsening of disease following DSS mediated intestinal injury.\[98, 99\] These authors established subclinical infection based on the detection of viral DNA in various tissues, including the intestines, in the absence of clinical signs of disease such as weight loss and gross or occult colonic or rectal blood and the lack of viral induced histologic lesions. After the administration of DSS, enhanced disease was demonstrated as evidenced by increased weight loss and enhanced gross and histologic lesion scores. These authors also reported increased levels of antibodies to commensal bacterial antigens which they suggested was due to enhanced gut permeability. They proposed that the exacerbation of disease was due to CMV mediated increases in gut permeability that promoted antigen-specific responses and enhanced the innate immune response following disruption of the intestinal mucosa by DSS.

Additional studies have shown that prior exposure to minimally pathogenic enteric microbes, such as *Hymenolepis diminuta*, *Bacteroides fragilis*, *Candida albicans* or AIEC
exacerbates subsequent oxazolone or DSS mediated intestinal inflammation.[67, 100, 101] With respect to *Hymenolepis diminuta*, mice were infected and then following a brief incubation period were given oxazolone to induce intestinal injury. The combination of *H. diminuta* and oxazolone resulted in both enhanced clinical disease and increased production of eosinophil peroxidase (EPO), IL-13, IL-4 and IL-5. The proposed mechanism associated with the enhanced disease was an additive Th2 response, with the reasoning being that both *Hymenolepis diminuta* and oxazolone both induce a Th2 polarized immune response, suggesting that infection with *Hymenolepis diminuta* primed the immune response for enhanced disease.[100] Interleukin-13 mediated increases in enterocyte apoptosis were suggested as one of the specific mechanisms for the observed exacerbation of intestinal injury. In contrast, the enhanced disease observed in BALB/c mice infected with an AIEC strain of *E. coli*, a pathogen known to infect macrophages, was associated with increased expression of IL-1β and IL-6, with enhanced disease thought to be mediated by AIEC flagellin as indicated by increased expression of its receptors, TLR5 and IPAF, and the absence of enhanced disease in mutants that lacked flagella.[67]

Bacterial products such as unmethylated cytosine-guanosine dinucleotides (CpG) motifs of bacterial DNA or filamentous hemagglutinin antigen (FHA) can also positively or negatively influence the progression of concurrent intestinal inflammation. In the study examining the influence of CpG motifs of bacterial DNA, CpG-ODN was inoculated intraperitoneally during treatment with DSS which resulted in enhanced intestinal injury as demonstrated by increased weight loss, decreased colon lengths and increased histologic lesion scores.[102] The mechanism for enhanced disease is not known but these investigators
did demonstrate that intraperitoneally inoculated digoxigenin labeled CpG-ODN that CpG-ODN was delivered to the lamina propria of the intestine and to the mesenteric lymph nodes, thereby indicating that CpG-ODN could have a local effect in the intestine following intraperitoneal injection.[102] In contrast to the deleterious effect of CpG, subcutaneous administration of FHA has been shown to promote the production of IL-10 and TGF-β in mesenteric lymph nodes and the Peyer’s patches, the increase of which was associated with amelioration of intestinal inflammation experimentally induced by the transfer of CD45RB<sup>hi</sup> cells.[103] In total, these studies suggest that specific microbial pathogens or their products can alter intestinal mucosal homeostasis and predispose the host to enhanced intestinal injury.

*Non-microbial causes of enhanced intestinal permeability and inflammation:*

Although the normal bacterial flora or, potentially, individual pathogenic species of bacteria contribute to the initiation or progression of intestinal inflammation in IBD, it is important to note that there are non-microbial or environmental factors that can exacerbate pre-existing or subsequent intestinal inflammation. Factors such as oral supplementation of iron and psychosocial stress can contribute to the enhancement of chemically induced intestinal inflammation. Iron supplementation is often required in chronic inflammatory colitis due to anemia and has been associated with exacerbation of disease activity.[104] It is speculated that poorly absorbed iron reaches the colon and via the Fenton reaction reacts with neutrophil derived free radicals, which can, via lipid peroxidation of cell membranes, exacerbate pre-existing intestinal lesions.[105] Addition of iron to the diet of mice being administered DSS have worse intestinal inflammation relative to mice given DSS only.[106]
The addition of iron was associated with increased IL-1β, myeloperoxidase and inflammatory lesion scores, which was ameliorated with anti-oxidant supplementation.[106] Psychosocial stressors also can exacerbate DSS mediated intestinal inflammation. This was demonstrated by both overcrowding and imposing social defeat on mice prior to the administration of DSS.[107] Social defeat was defined as the repeated placement of mice into a resident male mouse’s cage for a prolonged period of time. The mice exposed to the stressors had more severe weight loss, increased lesion scores and elevated production of TNF-α, IL-6 and IFNγ from cells isolated from the mesenteric lymph nodes.

Non-steroidal anti-inflammatory drugs (NSAIDs) can also interfere with the normal functioning of the intestinal mucosal barrier. Side effects associated with NSAID treatment can include reactivation of quiescent IBD or the development of gastrointestinal ulcers and perforations.[108-110] These side effects are not only associated with the inhibition of cyclooxygenase, but also with damage caused by generation of nitric oxide and oxygen radicals.[111-113] Experimentally, aspirin induced increases in gastric epithelial permeability are associated with decreased expression of claudin-7, with NSAIDs also being shown to alter permeability by interfering with oxidative phosphorylation, that in conjunction with suppression of cyclooxygenase, compromises the integrity of the tight junction.[114, 115] Lastly, alcohol consumption is associated with increased intestinal permeability. Acetaldehyde, a byproduct of alcohol consumption, in rats is associated with increased intestinal permeability and enhanced endotoxin translocation.[116] This association is supported by in vitro studies where exposure of Caco2-cells to acetaldehyde is associated
with phosphorylation of E-cadherin and ZO-1 and resulting increased permeability of the monolayer.\[19\]

*The role of macrophages in host homeostasis and inflammation*

Intestinal mucosal macrophages are continual targets for both constituents of the normal flora and enteric pathogens, thus it is imperative to understand their homeostatic functions and mechanisms that can cause a shift in their phenotype to promote injurious inflammation. Therefore, the next section of this review will focus first on the general biology of macrophages and then transition to a more focused analysis on the homeostatic and potentially pathogenic profiles of intestinal macrophages.

*Macrophage development*

Although resident tissue macrophages have specialized functions that are dependent on the organ in which they ultimately reside, they all have a common origin in a bone marrow derived progenitor cell which gives rise to the blood monocyte. Blood monocytes originate from a progenitor cell that, at least in mice, is capable of differentiating into a macrophage or dendritic cell and is CD115+, CD117+, CX3CR1+ and Lin−.[117] In both humans and mice, after these macrophage progenitor cells are released into the systemic circulation as blood monocytes they have been phenotypically subdivided into “non-inflamatory” and “inflamatory” monocytes, primarily based on their expression of chemokine receptors.[118] It is this differential chemokine profile which presumably influences the selective migration of these two subsets of monocytes. The “non-inflamatory” monocytes are presumed to develop into resident tissue macrophages and are
CCR2\(^{-}\), Gr1\(^{-}\), and CX3CR1\(^{+}\), while the “inflammatory” monocytes of mice are relatively short lived and characterized by intermediate expression of CX3R1 and being CCR2\(^{+}\), Gr1\(^{+}\).[118] Therefore, the “inflammatory” monocytes migrate to areas where there is increased expression of pro-inflammatory chemokines, such as CXCR2 (IL-8) and MCP-1 (the CCR2 ligand) while “non-inflammatory” monocytes are chemotactically attracted to peripheral tissues that express CX3CR1.[119] After these monocytes home to the peripheral tissues and develop into fully differentiated macrophages, their potential to proliferate locally is different. For example, Kupffer cells in the liver and alveolar macrophages in the lungs are capable of local proliferation in contrast to the non-proliferative potential of intestinal macrophages.[120, 121] These studies highlight the dynamic phenotypic properties of monocytes which presumably correlate with the final tissue dependent functions of macrophages.

**Macrophages as key mediators of inflammation and the innate immune response**

Resident macrophages are strategically located throughout the body in various host tissues and organs to optimally perform their roles in host homeostasis and as key effectors in the innate and adaptive immune response. It is important to remember that regardless of their location, macrophages are phagocytic cells whose physiologic functions are not exclusively restricted to host immune defense. Macrophages play a vital role in various homeostatic functions such as in the clearance of senescent red blood cells in the red pulp of the spleen and in the removal of apoptotic cellular debris in the thymus.[122, 123] These functions are mediated by multiple receptors, for example, phosphatidylserine and scavenger receptors, and often occur in the absence of obvious inflammation.[124] Although these functions are
often neglected in discussions of macrophages, their role as potent effectors of the host immune response is perhaps more important. For example, macrophages kill bacteria. This effector function is enhanced in the context of an adaptive immune response, with recognition of bacteria being achieved via cell surface associated receptors such as the complement receptor 3 (CR3) which results in phagocytosis and formation of the phagolysosome.[125] It is within the phagolysosome that lysosomal NADPH oxidases and other enzymes promote the generation of antimicrobial substances, such as superoxide anion, hydrogen peroxide, and nitric oxide that are directly toxic to bacteria.[125]

In addition to their antimicrobial effects, macrophages have an equally important role as key cellular mediators of inflammation. In contrast to the non-inflammatory profile of macrophages while performing homeostatic functions, macrophages can exert potent pro-inflammatory effects following infection with bacterial pathogens or exposure to necrotic cellular debris derived from various injurious stimuli, for example following physical trauma.[126] These pro-inflammatory stimuli are detected by macrophages as a “danger signal” and initiates a response that often culminates in inflammation.[126] Some of these extracellular “danger signals” include histones, DNA, and heat-shock proteins, which are detected by various receptors, for example, intracellular pattern recognition receptors (PRRs) and Toll-like receptors (TLRs).[127, 128] Following interaction with receptors, such as TLRs, this extracellular signal is often transmitted to the cell nucleus via an adapter protein called myeloid differentiation primary response gene 88 (MyD88) which ultimately allows for the translocation of an activated transcription factor to the nucleus of the cells, for example nuclear factor-κβ (NF-κβ) which promotes the transcription of pro-inflammatory
cytokines such as IL-1β. The production of the pro-inflammatory cytokines IL-1, IL-6 and TNF-α causes both local and systemic responses in the host. Locally, these factors can cause vasodilatation and increased vascular permeability and through the production of IL-8 can recruit inflammatory cells, such as neutrophils to the site of inflammation.

Moreover, these molecules act systemically as endogenous pyrogens and induce the generation of acute-phase proteins from the liver, including C-reactive protein and mannose binding lectin.

*Functional classification of macrophages*

As with the extensive efforts to categorize and define the functional profiles of T-cells in the adaptive immune response, there have been attempts to develop a classification system in which macrophages are subdivided based on their predominant function. Previously, macrophages associated with a Th1 or Th2 polarized immune response were classified into relatively static categories as classically, M1, or alternatively, M2, activated. Classically activated macrophages are the product of a Th1 skewed cell-mediated immune response characterized by the production of IFNγ, with their primary function being to kill intracellular pathogens. On the other hand, macrophages in a Th2 polarized environment, defined by the presence of IL-4 and IL-13, develop into alternatively activated macrophages which often express arginase and mannose receptor (CD206). However, this system lacks sufficient flexibility to account for the dynamic properties of macrophages. A recent report has proposed a new classification system which places activated macrophages into a relatively plastic scheme where there is overlap and expansion of the number of functions of macrophages. Macrophages within this system are
defined as being regulatory, wound-healing, or, as previously used, classically activated macrophages. The definition of a classically activated macrophage in this system is similar to the original classification scheme and highlights some additional features of this population of cells.

As described previously, classically activated macrophages are effector cells generated in the presence of a cell mediated Th1 polarized immune response that require IFNγ and TNF-α to be activated.[132, 135] The initial source of the IFNγ can come from natural killer cells (NK), with sustained production of IFNγ being derived from antigen-specific Th1 CD4+ T-cells.[136] Ultimately, the signal from these cytokines are transmitted to the nucleus via transcription factors that include signal transducer and activator of transcription molecules.[137, 138] An exception to this pathway of activation involves IFNβ, which can take the place of IFNγ.[139] After activation, classically activated macrophages acquire enhanced antimicrobial properties, including expression of inducible nitric oxide synthase (iNOS), which results in the production of anti-microbial nitrogen radicals, and respiratory burst induced increases in the production of antimicrobial oxygen radicals and superoxide anion.[140, 141] Although these molecules are effective antimicrobials, they are non-specific and can cause extensive collateral tissue damage and contribute to the pathogenesis of immune-mediated diseases, such as rheumatoid arthritis.[142] These unintended detrimental effects can be further compounded by the generation of Th17 T-cells whose formation can be promoted by classically activated macrophages following the production of IL-6 and IL-23.[143, 144] The deleterious effects of Th17 cells are mediated by the production of IL-17 that induces the recruitment of neutrophils.[145]
In addition to responding to an adaptive immune response by developing into potent anti-microbial effector cells, macrophages are also capable of modifying the immune response in their role as regulatory macrophages. They accomplish this primarily through the production of the immunoregulatory cytokines IL-10 and TGF-β and require two signals for their generation. These two signals can include IgG immune complexes and a TLR agonist.[146] These signals generate a population of regulatory macrophages that produce IL-10 and decrease the production of IL-12. Phagocytosis of apoptotic cellular debris is another stimulus that can generate TGF-β producing macrophages which can inhibit the production of pro-inflammatory cytokines.[124] In contrast to the subsequently described wound-healing macrophages, regulatory macrophages do not promote the production of extracellular matrix but do express high levels of the co-stimulatory molecules CD80 and CD86, which enables them to present antigens to T-cells.[147] These distinct properties of regulatory macrophages emphasize the potentially divergent roles that macrophages can have in the immune response, ranging from modulating the overall immune response via IL-10 production to functioning as a highly effective anti-microbial effector cell.

Further highlighting the dynamic functional properties of macrophages is the observation that they can function in the response to tissue injury as wound healing macrophages. These cells previously were described as alternatively activated macrophages; however, since this is not the only other pathway for macrophage activation, it has been suggested that this name is misleading. The key cytokines for generating and sustaining this subset of macrophages is IL-4 and IL-13, prototypical cytokines of a Th2 type adaptive immune response.[147, 148] However, at least experimentally, it has been demonstrated that
IL-4 can be produced by mast cells and basophils in an innate immune response following injury to tissue.[149]

Although anti-helminthic properties have been ascribed to this population of macrophages, a primary role of these cells is in the production of extracellular matrix, which if overproduced can lead to excessive fibrosis, as demonstrated in experimental schistosomiasis infection.[150] Exposure of resident macrophages to IL-4 promotes the formation of wound healing macrophages that express the enzyme arginase, which, in turn, generates ornithine from arginine. The final product of this response is the formation of extracellular matrix via production of polyamines and collagen.[148] In contrast to the anti-microbial properties of classically activated macrophages, wound healing macrophages have seemingly contradictory abilities in either being able to restrict the growth or be susceptible to pathogens such as *Mycobacterium tuberculosis* and Cryptococcus neoformans. *In vitro* exposure of macrophages to IL-4 or IL-13 results in less production of pro-inflammatory cytokines, decreased generation of nitrogen and oxygen free radicals, and impaired killing of intracellular pathogens, further implying that the term activated is inappropriate.[147] In support of this population of macrophages being more susceptible to certain intracellular infections, it has been demonstrated that macrophages treated with IL-4 are more susceptible to infection with *Mycobacterium tuberculosis* because of inhibition of autophagy related killing.[151] Additionally, Cryptococcus neoformans grows at a higher rate in mice that overproduce IL-13, in contrast to resistance to infection observed in mice that lack IL-13.[152] With that said, there is evidence that these cells have a role in the clearance of helminths, but it is not known if this is due to a direct or indirect inhibitory effect of these
macrophages. The anti-helminthic properties are thought to be due to the generation of chitinase and chitinase-like molecules, such as stabilin-interacting chitinase-like protein and acidic mammalian chitinase.[153, 154]

Lastly, the functional ability of a macrophage is not fixed, thus making it difficult to define its phenotype. This can occur because macrophages can co-express markers characteristic for different subsets of macrophages. For example, the molecule resistin-like molecule-α (RELMα) is expressed by both regulatory and wound-healing type macrophages. If macrophages are treated in vitro with IL-4 they have characteristics of wound-healing macrophages and express RELMα.[155] After these cells are treated with immune complexes, they assume the phenotype of regulatory macrophages whereby they produce IL-10 and decrease the production of IL-12 yet still express RELMα.[147] Furthermore, in vivo it has been suggested that macrophages are capable of shifting their phenotype in different diseases. For example, it has been suggested that macrophages through their pro-inflammatory properties have the potential to contribute to the pathogenesis of neoplasia; but, following progression of the neoplastic mass, the characteristics of macrophages can change to a regulatory phenotype presumably due to exposure to a tumor associated environmental trigger such as hypoxia.[156, 157] [158, 159] In summary, these functional properties of macrophages affirm that these cells are not one dimensional anti-microbial effector cells of the host immune response but are also capable of modulating the immune response and can play a role in the host response to injury.
The role of macrophages in intestinal homeostasis:

Macrophages are dynamic effector cells, whose function is modulated and defined by its local environment. This is equally true for intestinal macrophages which play a critical role in maintaining mucosal homeostasis. Therefore it is essential to study the biology of intestinal macrophages and understand how local environmental factors such as exposure to microbial pathogens or how macrophage specific genetic mutations alters intestinal macrophage phenotype and whether these changes translate to increased susceptibility to intestinal injury.

Intestinal macrophages are strategically situated in the lamina propria of the intestinal tract where they can interact with and eliminate bacteria that penetrate the overlying mucosal barrier. The morphologic features of the more numerous macrophages in the superficial lamina propria are distinct from the macrophages in the deep lamina propria. For example, consistent with intestinal macrophage ability to efficiently phagocytose material, macrophages in the subepithelial lamina propria have prominent vesicles and abundant cytoplasm. Although these differences in morphologic features exist, it is not clear if there are differences in phenotype or function of macrophages within different regions of the intestine or within different levels of the intestinal wall. Of the total number of mononuclear cells in the intestinal tract, approximately 10% are macrophages, with greater numbers in the colon.

Because of the relatively large number of intestinal macrophages and their potential to function as potent pro-inflammatory cells it is critical to tightly regulate their behavior to prevent unwanted injurious activation. To accomplish their primary dual function in
eliminating bacteria without generating pro-inflammatory cytokines, intestinal macrophages have evolved to have both antimicrobial and anti-inflammatory properties. For example, although intestinal macrophages have the ability to actively phagocytose and kill bacteria they do not function as antigen-presenting cells because of the lack or low expression of CD40, CD80 and CD86.[164] [45, 165] Moreover, they are deficient in a wide range of receptors that are commonly associated with the production of the pro-inflammatory cytokines IL-1β, IL-6, TNF-α, and IL-8; they lack the Fc receptors CD16, CD32, and CD64 for IgG; CD14, a receptor for LPS, and do not express the Fc receptor for IgA CD89.[45, 166] Additionally, they lack multiple complement receptors, such as CR3 (CD11b/CD18) and CR4 (CD11c/CD18).[45] The absence of these receptors correlates with an inability of intestinal macrophages to produce pro-inflammatory cytokines following stimulation with muramyl dipeptides, LPS and heat-killed *Staphylococcus aureus* or phagocytosis of latex bleads.[45, 121] Also, they generally do not express the triggering receptor on myeloid cells-1 (TREM-1), the activation of which enhances expression of receptors such as CD40 and CD86 and the production of pro-inflammatory cytokines.[167-169] The protective properties of intestinal macrophages are highlighted in a study where macrophages were either systemically or selectively depleted in the intestines. Depletion of intestinal macrophages correlated with increased neutrophil infiltration and enhanced DSS mediated intestinal inflammation.[170] In addition to displaying an anti-inflammatory profile, intestinal macrophages are capable of modulating the immune response by both inhibiting the formation of potentially pathogenic Th17 cells and promoting the formation of regulatory T-cells.[171]
The unique anti-inflammatory profile of intestinal macrophages is supported by both the overlying intestinal epithelium and stroma. For instance, in an experimental system utilizing intestinal epithelial spheroids, these spheroids promoted the development of macrophages which had decreased IL-1β production following stimulation with LPS and had decreased expression of the following surface receptors: CD11b, CD11c, CD14 and CD16.[172] Although a basement membrane exists between intestinal epithelial cells and macrophages in the underlying lamina propria, it is porous enough to allow interaction between these two cell types.[173] The intestinal stroma also promotes the development of the intestinal macrophage phenotype, with the immunomodulatory cytokines TGF-β and IL-10 having a primary role in their development.[45, 167, 174] With respect to TGF-β, this is based on the observation that incubation of stromal cell culture conditioned medium with anti-TGF-β prevented blood monocytes from developing a refractory inflammatory profile.[45] The importance of IL-10 in regulating intestinal macrophage function is highlighted in mouse models in which IL-10 is absent or IL-10 signaling is disrupted.[175, 176] For example, in IL-10−/− mice IL-12 and IL-23 secreting macrophages are associated with the development of spontaneous colitis.[175] The depletion of these macrophages prevents inflammation. Additionally, disruption of macrophage associated IL-10 signaling by interfering with Stat3 results in colitis.[176] As exemplified by these IL-10 knockout mouse models, despite the protective roles of intestinal macrophages, such as in the clearance of bacteria, it is clear that macrophages can contribute to the pathogenesis of intestinal inflammation. Moreover, if monocytes are recruited to these sites of inflammation they likely do not differentiate into the “normal” intestinal phenotype and may further exacerbate
inflammation due to the presence of luminal bacterial flora or antigens if there is a breach in the mucosal barrier.

Clinical studies in IBD patients have also highlighted the ability of macrophages to contribute to the pathogenesis of intestinal inflammation. It has been documented that, unlike normal intestinal macrophages, macrophages in the inflamed intestines of patients with IBD express co-stimulatory molecules such as CD80 and CD86 and are almost the exclusive source of large amounts of IL-1β.[93, 165, 177] In addition, macrophages in these foci of intestinal inflammation have been shown to express TREM-1 and CD14, with increased numbers of TREM-1 expressing macrophages in patients with active IBD having a corresponding increase in the production of IL-1β and TNFα.[168, 178] The importance of TNF-α and macrophages in the pathogenesis of IBD is exemplified by the success of anti-TNF-α therapy in patients with CD where it is thought that part of the success of this therapy is attributed to the elimination of TNF-α producing macrophages.[179] Although macrophages are significant sources of cytokines in IBD, potentially pathogenic effector molecules of macrophages are not limited to cytokines. For example, intestinal macrophages from patients with IBD have increased expression of tissue degrading cathepsins, with amelioration of colitis being described following their inhibition in a chemically induced mouse model of colitis.[180]

*Abberant macrophage associated production of IL-1β:*

Intestinal macrophages are a primary source of the pro-inflammatory cytokine IL-1β in IBD.[93, 177] The transcription of IL-1β is mediated by NF-κβ and is translated as an inactive pre-cursor that requires processing for generation of the mature cytokine.[181, 182]
It is the inflammasome, a multi-protein complex, that promotes the processing of IL-1β by activating interleukin converting enzyme (ICE) or caspase-1, which, in turn, acts on pro-IL-1β to form IL-1β.[183] Experimentally, in a study emphasizing the importance of ICE in the formation of mature IL-1β, there is a marked reduction in IL-1β with a corresponding reduction in DSS mediated intestinal inflammation in ICE knockout mice.[184] The pro-inflammatory properties of IL-1β include stimulation of liver produced acute phase proteins, increased expression of adhesion molecules on endothelial cells; and, perhaps most importantly with respect to IBD, it also can promote enhanced gut permeability through increased expression of myosin light chain kinase, resulting in disruption of tight junctions.[29, 33, 130]

Intrinsic defects of intestinal macrophages can compromise intestinal homeostasis and aberrantly increase the production of IL-1β. A macrophage associated mutation in the nucleotide-binding oligomerization domain 2 (NOD2) protein, which is an intracellular pattern recognition receptor stimulated by muramyl dipeptide, have been described in patients with CD.[185, 186] An additional macrophage associated defect in CD has revealed alterations in autophagy related genes, namely, autophagy-related 16-like 1 (Atg16L1), with several authors proposing interactions between the seemingly disparate NOD2, autophagy and the inflammasome pathways resulting in a macrophage immunodeficiency and potential increases in IL-1β and IL-18 production.[87, 88] Defects and the inter-relatedness of the autophagy and NOD2 pathways have recently been described.[187] Normally, NOD2 binds muramyl dipeptide and subsequently interacts with the autophagocytic process, which is involved in the removal of damaged organelles and defense against intracellular pathogens,
by bringing ATG16L1 to the plasma membrane and promotes the formation of the autophagolysosome. This ultimately leads to major histocompatibility class II (MHC II) being loaded with antigen and clearance of bacteria. Mutations of either NOD2 or ATG16L1 prevent this process from occurring, with defects in autophagy potentially leading to deregulated macrophage inflammasome activity as shown in mice with a mutation in ATG16L1 that have increased IL-1β and IL-18 secretion.[87]

Macrophage associated increases in the production of IL-1β in CD may be associated with constitutive activation of the inflammasome, with inflammasome activity being indirectly influenced, as mentioned before, by defects in the NOD2 pathway.[188, 189] [88] A component of the inflammasome, NACHT-, LRR- and pyrin domain-containing proteins (NALP), and NOD2 are members of a relatively new family of PRRs called Nod-like receptors (NLRs).[190] Structurally, these PRRs are defined by a C-terminal LRR domain; an N-terminal effector domain that can be a caspase recruitment domain (CARD), a pyrin domain (PYR) or a baculovirus inhibitor of apoptosis protein repeat (BIR); and an intermediary nucleotide binding domain (NOD/NACHT).[191] The CARDS are involved in protein-protein interactions, the nucleotide binding domain mediates self oligomerization which is important for the activation of downstream effector molecules, and the LRRs are involved in binding bacterial products, in particular muramyl dipeptide (MDP), a structural component of bacterial peptidoglycan.[188] Although the inflammasome shares a similar subunit with NOD2, it is an independent molecular entity that can be composed of a different combination of proteins making up distinct inflammasomes.[192] Assembly of the inflammasome is associated with activation of the P2X7 receptor which results in a decrease
of intracellular potassium.[193, 194] Originally, the inflammasome was structurally defined by apoptosis-associated speck-like protein containing a CARD (ASC), NALP1, caspase-5 and caspase-1.[195] A more recently described inflammasome brings together two caspase-1 molecules and is composed of NALP-3 (cryoporin) and two adapter proteins cardinal (CARD8) and ASC which associate with the two caspase-1 molecules.[196] It is expressed by macrophages and can potentially respond to multiple stimuli, including *Staphylococcus aureus*, adenosine triphosphate (ATP), and uric acid crystals produced from dying cells.[197-199] Following generation of the mature IL-1β by caspase-1, it needs to be secreted from the cell. The mechanisms of its release, relative to its production, are less well defined. It is thought that calcium influx concurrent with the activation of multiple phospholipases promotes the secretion of mature IL-1β, with another mechanism involving activation of the P2X7 receptor with ATP.[200, 201]

In contrast to the inflammasome, NOD2 is an intracellular sensor of bacteria that can contribute to the generation of IL-β via NF-κβ mediated signaling and activation of caspase-1 or indirectly influence the activity of the inflammasome through interaction with the autophagy pathway.[87, 187, 202] NOD2 was the first susceptibility gene associated with CD and found to be on chromosome 16q12, with the defect being characterized as a frameshift mutation in the leucine rich region of the NOD2 gene that was caused by a cytosine insertion, 3020insC.[203, 204] The resulting protein product was predicted to encode a truncated protein, with its expression being increased in patients with CD.[203] The NOD2 protein detects the GM-Di muropeptide (GlcNAc-MurNAc-L-Ala-D-Glu), which is a muramyl dipeptide (MDP) present in almost all bacteria; the exception being bacteria that do
not contain peptidoglycan in their cell wall, such as *Mycoplasma spp.*\[205, 206\] It is expressed by monocytes and its signaling results in the translocation of the transcription factor NF-κβ to the nucleus with transcription of, but not limited to, proIL-1β.\[202, 207\] Translocation of NF-κβ to the nucleus is accomplished through the interaction of NOD2 with a RIP-like interacting CLARP kinase receptor interacting 2 (RICK) protein which is a serine/threonine kinase that phosphorylates inhibitor of NF-κβ kinase (IKK).\[207, 208\]

It is not entirely apparent if the NOD2 mutation *in vivo* results in increased or decreased function with respect to activation of NF-κβ. Experiments have been conducted in mice that contain the most common susceptibility allele of NOD2, the 3020insC.\[92\] Bone-marrow derived macrophages from these mice exhibit elevated NF-κβ expression and IL-1β secretion following stimulation with MDP and had more severe intestinal inflammation following induction of colitis with dextran sodium sulfate. However, other studies have suggested that NOD2 disease variants have a loss of function phenotype based on the observation of decreased IL-1β following stimulation with muramyl dipeptide or peptidoglycan.\[209\]

In conclusion, although the aforementioned studies demonstrate that intestinal macrophages have important homeostatic or potentially pathogenic properties, additional studies are warranted to better define whether or not chronic intestinal infections of macrophages can cause a shift in macrophage effector function that disrupts the mucosal barrier or impairs the host response to mucosal injury.
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Chapter Two: Persistent enteric mycobacterial infection enhances sensitivity to acute mucosal injury

A paper to be submitted to the journal of *Experimental Biology and Medicine*

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Abstract

Intestinal macrophages are dynamic effector cells which play a critical role in maintaining mucosal homeostasis. Superimposed acute exposure to low-level microbial pathogens, such as adherent/invasive strain of *E. coli* (AIEC), that survive within intestinal macrophages can exacerbate experimental intestinal inflammation. However, it is not known how occult intestinal infections alter the response of the intestinal mucosa to subsequent intestinal injury. The aim of this study was to evaluate how persistent subclinical intestinal infection with *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) would influence subsequent acute dextran sulfate sodium (DSS) mediated intestinal inflammation. BALB/c mice were infected intraperitoneally with *Map*. Following an incubation period of 90 days, mice were administered 2% DSS in the drinking water for 6 days. Prior to and during treatment with DSS mice were evaluated for clinical signs of disease and body weights were recorded. At the termination of the experiment, body weights, frequency of rectal blood, gross and histologic cecal lesions were evaluated and tissues were collected for isolation of *Map*. Subclinical and persistent intestinal *Map* infection was established based on the absence of both weight loss and rectal blood. Following treatment with DSS, *Map* infected mice had increased weight loss, increased frequency of rectal blood, and exacerbation of gross lesions and increased cecal lesion scores. Also, there was a significant reduction in *Map* isolated from the small intestines of *Map* infected and DSS treated mice. In conclusion, subclinical *Map* infection sensitizes the host to enhanced acute DSS mediated intestinal inflammation.

Key words: Dextran sulfate sodium, *Mycobacterium avium* subsp. *paratuberculosis*, subclinical infection, enhanced inflammation
Introduction

Intestinal mucosal macrophages are continual targets for enteric pathogens, thus it is imperative to understand mechanisms that balance macrophage effector function with mucosal homeostasis. For example, in Crohn’s disease (CD), a chronic inflammatory disease of the gastrointestinal tract, a disorder of macrophage function resulting in impaired bacterial clearance and cytokine secretion has been described, with some investigators suggesting that CD represents a primary immunodeficiency of macrophages.[1, 2] What is less clear is what role, if any, intestinal pathogens that target macrophages have in CD and, more specifically, what impact subclinical intestinal infections have on the homeostasis of the intestinal mucosa.

The most convincing support for the role of a specific intestinal pathogen in IBD has been demonstrated in patients with ileal CD, where an increased number of patients are colonized by an adherent/invasive strain of E. coli (AIEC).[3] It has been reported that AIEC was recovered from 36% of mucosal biopsy specimens from patients with early postresection recurrent CD relative to 6% of normal control ileal biopsy specimens.[4, 5] This strain of E. coli is able to both replicate and persist within macrophages and enhance macrophage secretion of TNF. [6]

There has also been an association between exposure to Mycobacterium avium ss paratuberculosis (Map) and CD.[7] Map has been detected by in situ hybridization, PCR and bacterial isolation in patients with CD.[8-10] However, contradictory reports exist on the significance of Map in the pathogenesis of CD. For example, some studies have not detected Map 16s rRNA in patients with CD or UC; and, even though a subset of CD patients may
have genetic defects that affect innate immune function; namely, the NOD2 mutation, which may predispose patients to an intracellular pathogen such as Map, there is a lack of an association between NOD2 polymorphisms and Map infection.[11-13]

Limited studies have examined the role of occult infections on the development of intestinal inflammation. It has been noted that there is an increased prevalence of active cytomegalovirus (CMV) infections in patients with IBD.[14] It is speculated that this represents reactivation of a latent infection. In support of a potential role for CMV in IBD it has been shown that mice with pre-existing subclinical CMV infection have enhanced disease following dextran sulfate sodium (DSS) mediated colitis.[15] Additional studies have demonstrated that prior exposure to opportunistic pathogens, such as Hymenolepis diminuta, Bacteroides fragilis or an adherent/invasive strain of E. coli exacerbates subsequent oxazolone or DSS mediated intestinal inflammation.[16-18] In addition to specific pathogens, several studies have indicated that extra-intestinal exposure to bacterial products, such as unmethylated cytosine-guanosine dinucleotides (CpG) or staphylococcal enterotoxin B, may also influence intestinal inflammation.[19, 20] Collectively, these studies suggest that specific microbial pathogens or their products can alter intestinal mucosal homeostasis and predispose the host to enhanced intestinal injury.

Considering the ongoing potential for intestinal exposure to pathogenic and environmental species of mycobacteria, we set out to understand how exposure to Map would impact mucosal homeostasis. Our hypothesis was that exposure to Map would enhance acute mucosal injury. To address this goal, we utilized a mouse model of intestinal Map infection.[21, 22] BALB/c mice were infected with Map for 90 days followed by
exposure to a low dose of DSS in the drinking water to induce mild intestinal injury. In these mice we evaluated clinical signs of disease and development of mucosal pathology before and after intestinal injury. Our results demonstrate that subclinical intestinal mycobacterial infection amplifies the severity of DSS mediated intestinal inflammation as demonstrated by enhanced clinical disease, exacerbation of gross lesions and increased lesion scores.

Materials and Methods

Animals.

Conventionally reared 6-8 week-old male BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME) weighing approximately 22-24 grams and were housed in isolation at the Iowa State University College of Veterinary Medicine biosafety level II animal care facility. All animal experiments were approved by the Institutional Animal Care and Use Committee of Iowa State University.

Bacterial inoculum and infection.

The Map strain K10 was a clinical isolate obtained from the National Animal Disease Center (Ames, IA) and maintained in Middlebrook 7H9 broth supplemented with mycobactin J. Logarithmic growth-phase bacteria were washed and resuspended in sterile saline for inoculation. Bacterial concentration was determined by measuring absorbance at 540 nm, comparing the absorbance optical density to the standard curve and adjusted to a final concentration of 1x10^8 colony-forming units. Controls were given a sham inoculation of saline. The Map inoculum used in these studies was shown to have greater than 90% viability via fluorescein diacetate staining and flow cytometric analysis prior to inoculation.
addition, challenge inocula were confirmed negative for contaminants by streaking onto sheep blood agar plates 24 h prior to inoculation.

Experimental design.

In three separate experiments, 10-11 mice per treatment group were given a single intraperitoneal injection of *Map* or sham inoculated with saline. Following inoculation, body weights of the mice were measured at weekly intervals and feces were periodically collected for isolation of *Map*. At approximately 3 months post-inoculation, acute intestinal injury was chemically induced by administering a suboptimal (as discussed below) concentration of 2% DSS (MP Biomedicals, Solon, OH) in the drinking water for 6 days. We established this dose by determining the lowest DSS concentration in the drinking water that would induce minimal but perceptible microscopic lesions in the cecal and colonic mucosa of DSS only treated mice. Using a pilot study we tested doses that ranged from 1-5% DSS, increasing by 0.5% DSS in each treatment group. 2% DSS induced consistent mild multifocal villus elongation with minimal erosions of the mucosal epithelium. This dose was selected for all experiments in this study. While the mice were administered DSS, they were weighed daily, evaluated for clinical signs of disease, such as the presence of rectal blood and huddling, on day 6 all of the mice were euthanized with CO2 and necropsies were preformed. At necropsy, body weight and colon and cecal length were measured. Additionally, sections of spleen, liver, small and large intestine were collected for histopathology and isolation of *Map*. Additionally, saline washes from the abdominal cavity were collected and inoculated onto 7H10 agar for isolation of *Map*.
Histopathology.

At necropsy, samples of liver, spleen, small intestine, mesenteric lymph node, cecum, and colon were fixed in 10% neutral buffered formalin, processed by routine methods, embedded within paraffin wax and sectioned. Sections of tissue (5 µm) were stained with hematoxylin and eosin. Cecal histologic lesion scores were generated based on the severity of mucosal epithelial erosion and hyperplasia, the degree of lamina propria inflammatory cell infiltrate, and edema (Table 1).

Acid-fast staining (Ziehl-Neelsen method).

Slides were deparaffinized and rehydrated in distilled water. Carbol fuchsin solution was added for 45 seconds while microwaving at 450 W. Slides were rinsed in 0.5% acid alcohol for decolorization, rinsed for 1 minute in tap water then counterstained in methylene blue for 1 minute.

Culture of M. avium ss paratuberculosis from tissues and feces.

Sections of spleen, liver, small intestine, and colon were collected for isolation of Map. Each piece of tissue was weighed and homogenized in 0.75% hexadecylpyridinium chloride solution and allowed to stand overnight at room temperature to decontaminate the cultures. Sediments from individual tissue homogenates (100 µl) were inoculated onto Middlebrook 7H10 agar. Additionally, the peritoneum was washed with saline and 100 µl was plated onto Middlebrook 7H10 agar. After 12 weeks of incubation at 37°C, the number of colonies was counted. Fecal pellets were collected from each group of mice at approximately 14 day intervals until the end of the experiment. The fecal pellets were
homogenized in 1 ml of 0.05% Tween 80, diluted, decontaminated and plated on supplemented 7H10 agar as described above.

Statistical analysis.

Changes in body weight were analyzed using an ANOVA model with repeated measures. *Map* infection or saline inoculation, DSS or water administration, day and their interactions were considered as fixed effects, with animal being the subject of repeated measures. On each day, the treatment group means were compared first with an overall F-test and then followed by post-hoc Tukey-Kramer’s t-test for multiple comparisons if the F-test was significant. A p-value of < 0.05 was considered significant. For all other parameters, treatment group means were compared first with an overall F-test and then followed by post-hoc Student’s t-test with group mean differences considered significant if the p-value was < 0.05. Data are presented as the mean value ± standard error of the mean except where stated otherwise.

Results

Clinical disease and gross lesions

In order to establish *Map* infection we inoculated BALB/c mice with 10^8 CFU of *Map* strain K10 by a single intraperitoneal injection. Mice infected with *Map* transiently lost up to 1 gram of their body weight within the first 7 days after inoculation relative to mice given a sham inoculation with saline (data not shown). However, mice recovered their body weights and by the end of the 90 day incubation period all treatment groups had similar body weights. Other than transient weight loss, inoculation with *Map* did not cause signs of clinical disease.
To evaluate clinical disease we monitored body weight and rectal bleeding. Significant weight loss was only identified in the Map + DSS mice and occurred on day 6 of DSS administration (Fig. 1, p < 0.03). Rectal bleeding (Table 2) occurred only in treatment groups given DSS and coincided with onset of weight loss. There was a greater frequency of rectal blood in Map + DSS mice; 40% of Map + DSS mice had rectal blood compared to 14% of DSS only mice.

A consistent feature in the murine DSS colitis model is cecal and colonic shortening (atrophy) and this was used as a measure of gross pathology in this model (Fig. 2).[23] Cecal atrophy was most severe in the Map + DSS group (Fig. 3, **, p < 0.0001). DSS only had significant cecal shortening relative to the negative control and the Map K10 only group (Fig. 3*, p <0.03). Map only mice had cecal lengths similar to the negative control. We observed similar trends in the colon for gross and histologic lesions, with significant shortening of the colon in Map + DSS mice compared to all other treatment groups; however, we chose to focus our analysis on the cecum because it had more severe and consistent lesions.

Cecal histologic lesion scores

Microscopic lesions were typical of the DSS mucosal injury model and were defined by a multifocal proliferative to ulcerative typhlitis and colitis (Fig. 4). In the DSS only group there was mild multifocal gland proliferation and rare mucosal ulceration. The histologic score was not significantly different between the DSS only and the negative control and Map only groups. In contrast, the lesions were more severe and widespread in the Map + DSS group. (Fig. 5, *, p < 0.0013). Inoculation with Map alone did not induce histologic lesions. Lesions within the large intestine have been reported for BALB/c mice infected with Map.
19698; however, lesions were not prominent until 32 weeks after infection and did not specify whether they were located in the cecum.[24]

*M. avium subsp. paratuberculosis* tissue colonization

The distribution of *Map* in tissues was similar to that reported previously for *Map* infected BALB/c mice.[22] *Map* was isolated from spleen, liver, peritoneum, small intestine and large intestine, with the greatest concentration of bacteria being isolated from the spleen (Fig. 6). *Map* was not isolated from uninfected control mice. DSS administration had a significant effect on *Map* load only in the small intestine where DSS administration corresponded to lower CFU in *Map* infected mice (Fig. 6,* p = 0.043). There was a trend for decreased numbers of *Map* CFU in the colon of mice given DSS, but this did not reach statistical significance. *Map* was not isolated from the feces from any of the treatment groups.

*Acid-fast stains of tissues*

Acid-fast bacilli were present within foci of granulomatous inflammation in the liver, spleen, peritoneum, cecum, and small intestine. Consistent with the isolation results there was a greater density of acid-fast bacilli in the spleen, liver, and at the site of inoculation in the peritoneum compared to the small and large intestine. In the small and large intestine low numbers of acid fast bacilli were identified within the mucosa. In the large intestine acid fast bacilli were most commonly associated with gut associated lymphoid tissue. Acid-fast bacilli were present in cells with macrophage morphology.
Discussion

We set out to explore how occult intestinal infection would influence intestinal homeostasis, specifically enteric mycobacterial infection. This is relevant considering the frequency of enteric mycobacterial infection in HIV patients, and the potential for opportunistic infections in individuals with persistent intestinal inflammation, such as those with IBD.[25] In this study we have demonstrated that in our model subclinical Map infection sensitizes mice to enhanced DSS mediated intestinal injury. Mice infected with Map and treated with DSS had increased weight loss and frequency of rectal blood, increased cecal lesion scores and more severe cecal atrophy. These observations suggest that mycobacterial infection alters the mucosal response to injury and potentially influences repair mechanisms.

Map infection alone resulted in persistent infection but did not induce clinical disease as indicated by the absence of weight loss and lack of rectal blood. Infection was successfully established as demonstrated by the isolation of Map from the spleen, liver, small and large intestines, and peritoneum; the demonstration of acid-fast bacilli from tissues in which bacteria were isolated, which in the small and large intestines were often present in the gut associated lymphoid tissue (GALT); and the presence of foci of granulomatous inflammation in the spleen, liver and peritoneum. Even though bacteria were isolated and acid-fast bacilli were demonstrated in both the small and large intestines, areas of granulomatous inflammation were not present in these tissues. The bacterial numbers recovered from the small and large intestine were low, which is consistent with prior studies in the BALB/c mouse.[26] This condition bears similarities to occult abdominal Mycobacterium avium
infection in humans where disease is absent and bacterial burdens are low.[25] Collectively, we believe these findings support the use of this model to better understand how occult intestinal infections alter the mucosal response to subsequent intestinal injury.

When enteric pathogens are recognized the mucosal immune response must control pathogen proliferation and dissemination, yet minimize inflammation and injury. It has become clear that intestinal macrophages play a critical role in maintaining the balance between mucosal defense and homeostasis.[27] With respect to their homeostatic role, resident intestinal macrophages have a unique functional phenotype. They can eliminate potential pathogens while at the same time preventing excessive inflammation.[28] This anti-inflammatory phenotype of human intestinal macrophages is defined by the absence of innate response receptors such as receptors for LPS (CD14) and CR4 (CD11c/CD18) and the inability to produce pro-inflammatory cytokines in response to inflammatory stimuli.[28] Despite this dual protective role of intestinal macrophages, it is clear that macrophages can contribute to the pathogenesis of mucosal injury, such as in IBD where it has been documented that large amounts of IL-1β are produced almost exclusively by intestinal macrophages.[29, 30] Although these studies demonstrate that intestinal macrophages have important homeostatic or potentially pathogenic roles in the intestinal mucosa, it is unclear whether or not pathogens that target macrophages can cause a shift in macrophage effector function that impairs the host response to mucosal injury. In this study we have demonstrated that an occult infection with a pathogen targeting intestinal macrophages is capable of augmenting subsequent intestinal injury. In future studies it is our aim to try and determine if
Map infected macrophages have an enhanced pro-inflammatory profile following injury to the intestinal mucosa.

Regardless of the trigger utilized to initiate intestinal inflammation, superimposed acute exposure to low-level microbial pathogens, some of which target intestinal macrophages, can exacerbate intestinal inflammation. To the authors’ knowledge, only one other study, similar to the current one, has been described in which latent cytomegalovirus infection, a virus known to infect intestinal macrophages,[31] sensitized mice to a worsening of disease following treatment with DSS.[15] Although infection was established in these mice based on the detection of viral DNA in various tissues, including the intestines, clinical signs of disease such as weight loss and gross or occult colonic or rectal blood were not observed, in addition to the absence of viral induced histologic lesions. After the administration of a similar dose of DSS used in the current study, enhanced disease was demonstrated in those mice subclinically infected with CMV, as evidenced by increased weight loss and enhanced gross and histologic lesion scores. One other study has evaluated the affect of Map in a model of intestinal injury. Increased weight loss and increased colonic lesion scores following oral challenge with live Map, strain Ben (ATCC 43544), has been demonstrated in an IL-10 deficient mouse model of spontaneous colitis.[32] In this same study there was enhanced production of Ag85B/MPT59 peptide 25 specific TNF-α and IFNγ from both serum and cells isolated from the Peyer’s patches and mesenteric lymph nodes. The author’s proposed that since Ag85B/MPT59 peptide 25 is conserved across several mycobacterial species, that Map may function as an immunomodulator that stimulates T-cells in a manner similar to a superantigen, suggesting that the enhanced disease observed in these
mice is not unique to Map. Unlike the current study, colonization of the intestine with Map was not demonstrated. The absence of IL-10, a key immune regulatory cytokine, [33] is a confounding factor which is not present in our model where immunocompetent BALB/c mice were used. Additionally, augmented chemically mediated intestinal inflammation has been shown following acute luminal exposure to Bacteroides fragilis, AIEC, and Hymenolepis diminuta.[16-18] However, unlike the current study where we have demonstrated enhanced disease in mice with chronic intestinal mycobacterial infection; these models are not able to address how subclinical infection affects the mucosal response to injury.

Even though oral exposure to Map is presumably the natural route of infection in cattle,[34] we chose to inoculate mice intraperitoneally in order to establish more consistent colonization of the intestine. Previous studies in mice have achieved intestinal colonization after 6 or 18 weeks following intraperitoneal inoculation in immunocompetent BALB/c mice.[22, 24] In contrast, following inoculation by the intragastric route in flora defined mice, colonization of the cecum by Map could not be demonstrated after the first week post-inoculation.[26] Only if germ-free mice were orally inoculated could consistent intestinal colonization be established in immunocompetent mice.[26] In our study intestinal colonization was demonstrated by isolation of bacteria from both the small and large intestines and by demonstration of acid-fast bacilli that were often present in gut-associated lymphoid tissue (GALT). In addition to the intestines, and, consistent with previous studies in BALB/c mice, the greatest concentration of bacteria was isolated from the liver and spleen and was not significantly affected by treatment with DSS.[24] However, a smaller number of
bacteria were isolated from the small intestines with a trend, but not statistically significant, for smaller numbers to be isolated from the large intestine in those mice treated with DSS. This result was not anticipated and may partially be explained by an enhanced non-specific immune response induced by DSS. Dextran sulfate has been demonstrated in colonic macrophages in a DSS mediated mouse model of colitis.[35] Moreover, it has been shown that DSS-pulsed macrophages promote the proliferation of T-cells from colitic mice.[36] However, we did not observe, by routine histologic stains, a relative increase in the number of lymphocytes in the lamina propria of the small intestine or hyperplasia of the GALT. Additional studies need to be conducted to precisely define the influence of DSS on the replication of *Map.*

Most studies utilizing the DSS model of intestinal injury evaluate its effect on the colon.[15, 35] Although the colon was significantly affected in our model, we chose to focus on the cecum because initial experiments demonstrated more severe gross and histologic lesions in the cecum and were able to demonstrate consistent colonization of the cecum by both bacterial isolation and demonstration of acid-fast bacilli in the cecal lymphoid tissue. Involvement of the cecum is not uncommon following treatment with DSS and, the cecum itself, appears to play a central role in the development of experimental colitis. In particular, the GALT in the distal tip of the cecum, or sometimes referred to as the appendix of the mouse, may be an important factor in the development of colitis. Following experimental removal of the murine appendix, clinical disease and lesions associated with DSS administration are improved.[37] In support of this observation, it has been noted in humans that removal of the appendix reduces the risk of developing ulcerative colitis.[38] The
mechanism associated with this protective effect is not known, but functional differences between lymphoid tissue of the Peyer’s patches and appendix have been demonstrated, and it has been suggested that the appendix is the major priming site for cells involved in the development of experimental colitis in the TCRα deficient mouse model of inflammatory bowel disease.[39]

We believe our model can be utilized to better understand how persistent infection with a low-level microbial pathogen, such as Map, disrupts the regulation of intestinal homeostasis and tips the balance in favor of enhanced inflammation following injury to the intestine. Enhanced disease in our model may be associated with Map mediated exacerbation of inflammation, disruption of repair mechanisms, or a combination of both. Future studies with this model will define the immune profile of the locally injured intestinal mucosa and attempt to characterize how chronic mycobacterial infection modifies the function and phenotype of intestinal macrophages, which are critical components of an elaborate regulatory system that maintains the intestine in a non-inflammatory state despite the presence of large numbers of bacteria in the adjacent intestinal lumen.

Acknowledgements

We thank Elise Huffman for technical assistance. This work was funded through a Iowa State University research grant and the Iowa Healthy Livestock Initiative.
References:


### Table 1

Cecal lesion scoring system

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Scoring Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterocyte hyperplasia</td>
<td>Ratio of crypt depth to width</td>
</tr>
<tr>
<td></td>
<td>Rising score from 0 to 3 (no hyperplasia → severe hyperplasia)</td>
</tr>
<tr>
<td>Inflammatory score</td>
<td>Denotes cellular infiltrates in the lamina propria or cellular exudates overlying</td>
</tr>
<tr>
<td></td>
<td>areas of mucosal ulceration</td>
</tr>
<tr>
<td></td>
<td>Rising score from 0 to 3 (small → large numbers of inflammatory cells)</td>
</tr>
<tr>
<td>Mucosal erosions</td>
<td>Rising score from 0 to 3 (no erosions → ulceration)</td>
</tr>
<tr>
<td>Edema</td>
<td>Rising score from 0 to 3 (no edema → marked submucosal edema)</td>
</tr>
<tr>
<td>Distribution</td>
<td>Rising score from 0 to 3</td>
</tr>
</tbody>
</table>

(0 = lesion is not present; 1 = < 25%; 2 = 25 – 75%; and 3 = > 75% of the section is affected)

Parameters are scored (0 → 3) + distribution (0 → 3) resulting in a maximal total histological score of 24.
Table 2.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Severity of Rectal Blood</th>
<th>% with Rectal Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Map only</td>
<td>-</td>
<td>0%</td>
</tr>
<tr>
<td>DSS only</td>
<td>+</td>
<td>14%</td>
</tr>
<tr>
<td>Map + DSS</td>
<td>++/++++</td>
<td>40%</td>
</tr>
</tbody>
</table>

Severity and % of mice with rectal blood that were either infected with *Map* or inoculated with saline after the administration of DSS or water. A greater percentage of *Map + DSS* mice had rectal blood compared to all other treatment groups. Data are represented as the % of affected mice from three separate experiments including 22 DSS only mice, 25 *Map + DSS* mice, 8 *Map* only mice.
Fig. 1. Change in body weight in mice inoculated with saline (Control); infected with Map only (Map only); inoculated with saline and treated with DSS (DSS only); and mice infected with Map and treated with DSS (Map + DSS). Map + DSS mice lost a statistically significant amount of body weight relative to all other treatment groups (*, p < 0.03). Data are represented as the mean ± standard error of the means from three separate experiments including 22 DSS only mice, 25 Map + DSS mice, 8 Map only and 9 control mice.
Figure 2. Representative pictures demonstrating cecal atrophy. *Map* only (A); DSS only (B); and *Map* + DSS (C). Note that there is also shortening of the colon and a reduction in the number of formed fecal pellets in the mice administered DSS.
Fig. 3. Cecal lengths in mice inoculated with saline (Control); infected with *Map* only (*Map* only); inoculated with saline and treated with DSS (DSS only); and mice infected with *Map* and treated with DSS (*Map + DSS*). The average cecal length in *Map + DSS* mice was shorter relative to all other treatment groups (**, p < 0.001). The average cecal length for DSS only mice was shorter relative to the control and *Map* only mice (*, p < 0.03). Data are represented as the mean ± standard error of the means from three separate experiments including 22 DSS only mice, 25 *Map + DSS* mice, 8 *Map* only and 9 control mice.
Figure 4. Representative photomicrographs of ceca from Map + DSS (A), Map only (B), DSS only (C) and Control (D) mice. Note the ulceration of the mucosa and the cecal lumen containing neutrophils intermixed with blood in the Map + DSS mouse. There is moderate hyperplasia and submucosal edema in the DSS only mouse. Lesions were not identified in the mucosa of the DSS only and Control mice. HE; Bar = 100 µm.
Fig. 5. Cecal histologic lesion scores in mice inoculated with saline (Control); infected with *Map* only (*Map* only); inoculated with saline and treated with DSS (DSS only); and mice infected with *Map* and treated with DSS (*Map* + DSS). Cecal lesion scores were significantly higher in *Map* + DSS mice relative to all other treatment groups (*, p < 0.0013). Data are represented as the mean ± standard error of the means from three separate experiments including 22 DSS only mice, 25 *Map* + DSS mice, 8 Map only and 9 control mice.
Fig. 6. Log colony forming units per gram of tissue in mice infected with K10 Map and given either DSS or water. A greater concentration of K10 Map was isolated from the small intestine from Map K10 only mice relative to Map K10 + DSS mice (*, p = 0.043).
Chapter Three: Immune responses associated with intestinal mucosal injury in mice

subclinically infected with *Mycobacterium avium* subsp. *paratuberculosis*

A paper to the journal of *Clinical and Experimental Immunology*

By

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Abstract

Microbial pathogens have been linked to reactivation or exacerbation of inflammation in IBD patients. However, only a limited number of studies have examined the role of specific pathogens as a cause of enhanced intestinal inflammation; while fewer studies have examined the role of occult infections in the development of intestinal inflammation. We have previously demonstrated that subclinical and persistent enteric mycobacterial infection sensitizes BALB/c mice to enhanced intestinal injury. Our primary aims for this study were to define the intestinal and systemic immune cytokine profiles in our Map enhanced intestinal injury model and to determine if Map infected mice had an antigen specific immune response. To address these aims, we analyzed adaptive and/or innate cytokine expression patterns in the intestines and spleen and evaluated mice for Map specific antibody. We also infected a subset of SCID/C.B-17 (SCID) mice with Map and treated them with DSS to determine if Map enhanced intestinal disease is independent of the adaptive immune response. BALB/c mice infected with Map had antigen specific production of IgG2a and IgG1 antibody and Map specific IFNγ and IL-17 production from cells isolated from the spleen. In mice infected with Map and treated with DSS we did not observe an increase in intestinal IFNγ, IL-17 or IL-4 mRNA, or an increase in Map-specific production of the same set of cytokines in cells isolated from the cecal tonsil. However, Map infected and DSS treated mice had increased expression of IL-1β. Similar to BALB/c mice, SCID mice treated with DSS and infected with Map had enhanced clinical disease and increased intestinal expression of IL-1β; but, unlike BALB/c mice, had enhanced TNF-α expression. In conclusion, our results confirm that Map infected mice had an antigen specific immune
response and that Map enhanced intestinal disease is associated with increased activation of the innate immune response.

Key words: Dextran sulfate sodium, IL-1β, *Mycobacterium avium* subsp. *paratuberculosis*, subclinical infection, enhanced inflammation

**Introduction**

Acute exposure to various environmental factors may be associated with the periods of reactivation or exacerbation of inflammation in both patients with IBD and in experimental animal models of intestinal inflammation. For example, the administration of iron has been associated with relapse of IBD and has been shown to exacerbate dextran sulfate sodium (DSS) mediated intestinal inflammation.[1, 2] Moreover, acute exposure to an adherent-invasive strain of *Escherichia coli* (AIEC) can enhance experimental intestinal inflammation.[3] However, these models do not address the question of how chronic and subclinical microbial infection influences mucosal homeostasis, and inflammation in IBD.

Both enhanced activation of the adaptive and innate immune response are thought to play an important role in the pathogenesis of IBD.[4] In terms of the adaptive immune response, Crohn’s disease (CD), a clinical phenotype of IBD, is defined by a polarized Th1 immune response with increases in IFNγ and IL-12.[4] The adaptive immune response can also be amplified in experimental models of IBD where it has been shown that infection with *Hymenolepis diminuta* increases IL-4 expression in the TNBS model of colitis.[5] The importance of the innate immune response is highlighted by the success of anti-TNF-α therapy in the treatment of CD.[6] Moreover, other cytokines of the innate immune system,
such as IL-1β, are thought to play an important role in the pathogenesis of IBD as evidenced by its increase in the inflamed mucosa from patients with CD.[7, 8] Although adaptive and innate cytokines are elevated in IBD, it is not clear how an underlying infection with an opportunistic pathogen; in particular, one that targets macrophages, influences the characteristic immunopathology in IBD.

We have previously reported that subclinical and persistent mycobacterial infection in BALB/c mice exacerbates subsequent DSS mediated intestinal inflammation, as demonstrated by enhanced clinical disease, exacerbation of gross lesions and increased lesion scores. In this study we set out to define the intestinal and systemic immune profiles in our *Map* enhanced intestinal injury model. We hypothesized that an additive Th1 immune response was associated with *Map* enhanced intestinal inflammation. Our rationale for this hypothesis was that acute DSS mediated colitis in the BALB/c mouse is defined by a Th1 cytokine profile and because *Map* infected macrophages is capable of producing a similar profile of cytokines.[9, 10] To address our goal, we analyzed adaptive and innate intestinal and systemic cytokine expression patterns and evaluated mice for *Map* antigen specific antibody. We next infected a subset of SCID/C.B-17 (SCID) mice with *Map* to determine if enhanced intestinal disease would occur in the absence of an adaptive immune response. Our results demonstrate that *Map* enhanced intestinal inflammation is associated with increased activation of the innate immune response; in particular, expression of IL-1β.
Materials and methods

*Animals*

Conventionally reared 6-8 week-old male BALB/c and C.B-17 *scid* (SCID) mice were purchased from The Jackson Laboratory (Bar Harbor, ME), weighed approximately 22-24 grams, and were housed in micro-isolation units that were individually ventilated in a positive pressure Thoren unit caging system at the Iowa State University College of Veterinary Medicine biosafety level II animal care facility. All animal experiments were approved by the Institutional Animal Care and Use Committee of Iowa State University.

*Bacterial inoculum and infection*

The *Map* strain K10 was a field isolate obtained from the National Animal Disease Center (Ames, IA) and maintained at 37°C in Middlebrook 7H9 broth supplemented with mycobactin J. Logarithmic growth-phase bacteria were washed and resuspended in sterile saline for inoculation. Bacterial concentration was determined by measuring at 540 nm, comparing the absorbance optical density to the standard curve and adjusted to a final concentration of 1x10⁹ colony-forming units/ml. Controls were given a sham inoculation of saline. The *Map* inoculum used in these studies was shown to have greater than 90% viability via fluorescein diacetate staining and flow cytometry analysis prior to inoculation. In addition, challenge inocula were confirmed negative for contaminants by streaking onto sheep blood agar plates 24 h prior to inoculation.
Experimental design

Tissues from BALB/c utilized in this study were part of a previously reported experiment (Chapter 2) in which three separate experiments were conducted with 10-11 mice per treatment group. Two separate experiments were performed with SCID mice, with 4-5 mice per treatment group. In all experiments, mice were assigned to one of two groups and either given a single intraperitoneal sham injection of saline or Map. Following inoculation, body weights of the mice were measured at weekly intervals and feces were periodically collected for isolation of Map. At approximately 3 months post-inoculation, acute intestinal injury was chemically induced by administering a suboptimal concentration of 2% dextran sulfate sodium (DSS; MP Biomedical, Solon, OH) in the drinking water for 6 days. We established this dose by determining the lowest DSS concentration in the drinking water that would induce minimal but perceptible microscopic lesions in the cecal and colonic mucosa. Using a pilot study we tested doses that ranged from 1-5% DSS, increasing by 0.5% DSS in each treatment group. 2% DSS induced consistent mild multifocal villus elongation with minimal erosions of the mucosal epithelium. This dose was selected for all experiments in this study. While the mice were administered DSS, they were weighed daily and on day 6 all of the mice were euthanized with CO₂ and necropsies were performed. At necropsy, body weights and colon and cecal length were measured. Additionally, sections of spleen, liver, small and large intestine, and mesenteric lymph node were collected for histopathology and/or isolation of Map.
Histopathology

At necropsy, samples of liver, spleen, small and large intestine, and mesenteric lymph node were fixed in 10% neutral buffered formalin, processed by routine methods, embedded within paraffin wax and sectioned. Sections of tissue (5 µm) were stained with hematoxylin and eosin. Cecal histologic lesion scores were generated on the severity of mucosal epithelial erosion and hyperplasia, the degree of lamina propria inflammatory cell infiltrate, and edema (Table 1).

Acid-fast staining (Ziehl-Neelsen method)

Slides were deparaffinized and rehydrated in distilled water. Carbol fuchsin solution was added for 45 seconds while microwaving at 450 W. Slides were rinsed in 0.5% acid alcohol for decolorization, rinsed for 10 minutes in tap water then counterstained in methylene blue for 10 minutes.

Culture of M. avium subsp paratuberculosis from tissues and feces

Sections of spleen, liver, and small and large intestine were collected for isolation of Map. Each piece of tissue was weighed and homogenized in 0.75% hexadecylpyridinium chloride solution and allowed to stand overnight at room temperature to decontaminate the cultures. Sediments from individual tissue homogenates (100 µl) were inoculated onto Middlebrook 7H10 agar. Additionally, the peritoneum was washed with saline and 100 µl was plated onto Middlebrook 7H10 agar. After 12 weeks of incubation at 37°C, individual colonies were counted. Fecal pellets were collected from each group mice at approximately 10-14 day intervals until the end of the experiment. The fecal pellets were homogenized in 1
ml of 0.05% Tween 80, diluted, decontaminated and plated on supplemented 7H10 agar as described above.

*Preparation of Map antigen*

*Map* was cultured in Middlebrook 7H9 broth at 37°C supplemented with mycobactin J. The bacterial concentration was determined by measuring at 540 nm, comparing the absorbance optical density to the standard curve. Bacteria were pelleted by centrifugation at 3500g for 20 minutes and subsequently washed twice with PBS. The pellet was then resuspended in PBS and sonicated on ice with a probe sonicator. Sonication consisted of three cycles of 10 minute bursts at 18W (amplitude 60%) on ice with 10 minute chilling periods between sonication steps. Debris was removed by centrifugation at 12000g for 5 minutes. The supernatants were saved and stored at -70°C until used. The protein concentration of the supernatant was determined by using the BCA protein assay (Thermo Scientific, Rockford, IL). Additionally, the supernatant was confirmed negative for contaminants by streaking onto a sheep blood agar plate.

Map-specific antibody detection by ELISA.

*Map*-specific IgG1 and IgG2a antibody responses in the sera of BALB/c mice infected with *Map* or sham inoculated with saline were measured by ELISA. Briefly, 96-well Immulon 2HB microtiter plates (Fisher Scientific, Hanover Park, IL) were coated with 2.5 µg/ml of *Map* K10 sonicate in coating buffer (sodium carbonate-bicarbonate buffer) overnight at 4°C. Plates were washed two times and blocked with 1% BSA in TBS (Tris buffered saline) for 2 h at room temperature. Plates were then washed four times and
individual serum samples diluted at 1:100 in sample diluent (TBS/Tween 20/BSA) were added. After 1 h of incubation at room temperature, the plates were washed four times, and the concentrations of IgG subclass antibodies were determined following the addition of avidin-horseradish peroxidase detection antibody, goat anti-mouse IgG1 (Bethyl Laboratories Inc., Montgomery, TX) at 1:150,000 or goat anti-mouse IgG2a (Bethyl Laboratories Inc., Montgomery, TX) at 1:50,000 for 1h at room temperature. The plates were then washed five times and TMB substrate was added for 12 minutes. Stop reagent was then added and plates read at 450nm to obtain OD values.

*Spleen and cecal tonsil cell isolation*

Cells isolated from the spleen and cecal tonsil from a total of 6 mice per group was individually analyzed for the production of *Map*-specific IFNγ, IL-4, and IL-17. At day 6 of DSS administration mice were euthanized with CO2 inhalation and their spleens and cecal tonsils were aseptically removed and transported in RPMI 1640 for cell isolation. Single cell suspensions were obtained by homogenization. Cells were then washed, resuspended in culture medium and counted, with more than 90% cell viability via trypan blue exclusion. Complete medium was prepared by adding 10% fetal bovine serum, 0.5 mM 2-mercaptoethanol, penicillin G (100U/ml), streptomycin (100 µg/ml), amphotericin B (250 ng/ml), 2 mM L-glutamine, and gentamicin (25 µg/ml) to RPMI 1640 medium. Spleen and cecal tonsil cells were incubated at 1 x 10^6 cells/ml and 1 x 10^5 cells/ml, respectively. Cells were stimulated with either *Map* antigen (10 µg/ml), concanavalin A (5 µg/ml; ConA; Sigma, St. Louis, MO), or medium alone and maintained at 37°C, 5% CO2 for 3 days, with
cell culture supernatants from duplicate wells being collected and stored at -70°C until analyzed.

Cytokine ELISA

Commercially available ELISA kits (eBioscience, San Diego, CA) were used for the detection of IL-4, IFN-γ, and IL-17 protein in splenic and cecal tonsil cell culture supernatants. The appropriate recombinant cytokine protein was used as a positive control to generate the standard curve. The detection limit was 4 pg/ml, 15 pg/ml, and 4 pg/ml for IL-4, IFN-γ, and IL-17, respectively. Results are expressed as the mean (± SEM) pg/ml, calculated from 2-3 mice per group from two separate experiments and tested individually.

Quantitative real-time reverse transcriptase PCR (qRT-PCR)

Samples of cecum from each treatment group of mice were analyzed for the expression of a panel of cytokines (Table 2). The primer sequences were designed by using Primer Express Software (Applied Biosystems, Foster city, CA). Possible genomic DNA contamination in the RNA samples was eliminated by using Turbo DNA-free (Ambion, Austin, TX). A SuperScript III first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA) was used for cDNA synthesis. A Sybr green PCR system (Applied Biosystems, Foster city, CA) was used in the two-step real-time PCR, and the signal was detected by using a GeneAmp 5700 sequence detection system. Prior to final qRT-PCR sample analyses, a test plate was run using a mixture of cDNA samples serially diluted for each target of interest to identify the specific cDNA sample dilution range at which qRT-PCR inhibition no longer existed; and the extended dilution range of the standard curves
within which all targets exhibited amplification efficiencies between 80 and 100%. cDNA samples were diluted to their ideal concentration when added to the final reaction mixtures for qRT-PCR. The Ct values were gathered at an appropriate threshold for each different target using the GeneAmp 5700 SDS software, and relative quantitative analysis was performed using our own custom Excel files. Relative mRNA expression quantity for each qRT-PCR target was calculated using the equation: relative quantity = 10 \((\text{Ct}-b)/m\). Relative quantity values for each target were calculated using the slope (m) and y-intercept (b) of the line generated for each target’s standard curve. Final relative values were normalized to beta actin and dissociation curve analysis was conducted as a control for each target gene.

**Statistical analysis**

Changes in body weight were analyzed using an ANOVA model with repeated measures. *Map* infection or saline inoculation, DSS or water administration, day and their interactions were considered as fixed effects, with animal being the subject of repeated measures. On each day, the treatment group means were compared first with an overall F-test and then followed by post-hoc Tukey-Kramer’s t-test for multiple comparisons if the F-test was significant. A p-value of < 0.05 was considered significant. For all other parameters, treatment group means were compared first with an overall F-test and then followed by post-hoc Student’s t-test with group mean differences considered significant if the p-value was <0.05. Data are presented as the mean value ± standard error of the mean except where stated otherwise.
Results

*Cecal cytokine gene expression in BALB/c mice*

To determine if there were unique cytokine profiles in the ceca of the different treatment groups of BALB/c mice, we used qRT-PCR to evaluate the gene expression of a panel of cytokines associated with the adaptive and innate immune response. Cecal homogenates from Map + DSS mice had a significant increase in the expression of IL-1β (Fig.1,**, p < 0.016); however, they did not have a significant increase in the expression of any of the markers of the adaptive immune response, namely, IFNγ, IL-4 or IL-17 (Fig. 2). In contrast to the Map + DSS mice, DSS only mice had a significant increase in the expression of TNF-α (Fig. 1,***, p < 0.016), IL-4 and IFNγ (Fig. 2 *, **, p < 0.013 and p < 0.001, respectively). The expression of all innate cytokines in the Map + DSS and DSS only groups was higher relative to their respective controls (Fig. 1, *, p < 0.0004). Control and Map only mice did not have a significant increase in the expression of any of the cytokines evaluated. All data from each individual animal were normalized to the data for the internal control β-actin, which was expressed at similar levels within each treatment group.

*Cytokine production from cells isolated from the spleen and cecal tonsil*

In addition to evaluating cytokine expression by qRT-PCR, we performed ELISA assays to determine if there was antigen specific production of IFNγ, IL-4 and IL-17 by measuring cytokine secretion from cells isolated from the spleen and cecal tonsil. We chose to isolate cells from the cecal tonsil because it is thought to play an essential role in the development of DSS mediated intestinal inflammation.[11] Cells isolated from the spleens of
Map + DSS and Map only mice had a significant increase in the production of antigen-specific IFNγ (Fig. 3, *, ** p < 0.0001). Additionally, Map + DSS mice had a significant increase in the production of IL-17 (Fig. 4, *, p < 0.009). Production of antigen-specific IL-4 was not different for any of the treatment groups (Fig. 5). In contrast to the spleen, there was not antigen-specific production of any of the cytokines in cells isolated from the cecal tonsil in mice infected with Map, regardless of whether they were given DSS or not. Control and DSS only mice did not produce antigen-specific IFNγ, IL-4, or IL-17.

Map specific antibody responses

We wanted to determine if mice infected with Map had Map antigen specific antibody responses and assess whether the response was associated with a Th1 or Th2 polarized immune response by measuring antigen-specific IgG1 and IgG2a antibody. To accomplish this we collected blood at day 6 of DSS treatment and analyzed the serum by ELISA. Both Map + DSS and Map only mice produced antigen specific IgG1 and IgG2a compared to the control and DSS only mice (Fig. 6, *, **, *** p < 0.0001). Map-specific IgG1 and IgG2a antibody responses were decreased in those mice treated with DSS, with the decrease being statistically significant for Map specific IgG2a (**, p = 0.0005). Antigen-specific antibody was not detected in the DSS only and control mice.

Clinical disease, gross pathology, cecal histologic lesion scores in SCID mice

Results of clinical disease, gross pathology and histologic lesion scores for BALB/c mice were reported previously (Chapter 2). In order to establish Map infection in SCID mice, one of two groups was inoculated with 1 x 10⁸ CFU of Map strain K10 or a separate group
was given a sham inoculation of saline by a single intraperitoneal injection. Mice infected with Map transiently lost up to 0.5 grams of their body weight within the first 7 days after inoculation relative to sham inoculated mice (data not shown). However, mice regained the weight they had lost and by the end of the incubation period all treatment groups had similar body weights. Other than transient weight loss, inoculation with Map did not cause signs of clinical disease.

In DSS treated mice, to evaluate clinical disease we monitored body weight and rectal bleeding. Significant weight loss was identified in the Map + DSS SCID mice and occurred on days 5 and 6 of DSS administration. At day 6 Map + DSS lost more weight relative to all other treatment groups (Fig. 7, *, p < 0.03), while at day 5 they had lost more weight compared to the Control and Map only groups (Fig. 7, *, p < 0.05). DSS only mice lost more weight relative to the control at day 5 (Fig. 7, **, p < 0.04). Rectal bleeding (Table 3) occurred only in treatment groups given DSS and coincided with onset of weight loss. There was a greater frequency of rectal blood in Map + DSS mice; 100% of Map + DSS mice had rectal blood compared to 50% of DSS only mice.

A consistent feature in the murine DSS colitis model is cecal and colonic shortening (atrophy) and this was used as a measure of gross pathology in this model.[12] Cecal atrophy was most severe in the Map + DSS group (Fig. 8, *, p < 0.001) compared to all other treatment groups. Map only mice had cecal lengths similar to the negative control. We observed similar trends in the colon for gross and histologic lesions, with significant shortening of the colon in Map + DSS mice compared to all other treatment groups; however,
we chose to focus our analysis on the cecum because it had more severe and consistent lesions.

Microscopic lesions were typical of the DSS mucosal injury model and were defined by a multifocal proliferative to ulcerative typhlitis and colitis. In the DSS only group there was mild multifocal gland proliferation and rare mucosal ulceration. The histologic score was significantly higher between the DSS only and the control group (Fig. 9, *, p = 0.02). In contrast, the lesions were more severe and widespread in the Map + DSS group (**, p < 0.0001). The control and Map only mice had similar cecal lesion scores.

**M. avium subsp. paratuberculosis tissue colonization**

The distribution of Map in tissues was similar to that reported previously for SCID mice.[13] Map was isolated from spleen, liver, peritoneum, and small and large intestines from mice infected with Map, with the greatest concentration of bacteria being isolated from the spleen and being too numerous to count (Fig.10). Map was not isolated from any tissues from the uninfected control animals. DSS administration did not have a significant effect on bacterial load in any of the tissues; however, there was a trend for decreased bacteria in the small intestines of Map + DSS mice, but this did not reach statistical significance. Map was not isolated from the feces from any of the treatment groups.

**Acid-fast stains of tissues**

Acid-fast bacilli were present within foci of granulomatous inflammation in the liver, spleen, peritoneum, cecum, and small intestine. Consistent with the isolation results there was a greater density of acid-fast bacilli in the spleen, liver, and at the site of inoculation in
the peritoneum compared to the small and large intestine. In all tissues examined acid-fast bacilli were identified within aggregates of cells with macrophage-like morphology.

**Cecal cytokine gene expression in SCID mice**

To determine if the innate cecal cytokine pattern was similar to BALB/c mice, we used qRT-PCR to evaluate cecal gene expression. Cecal homogenates from \( \text{Map} + \text{DSS} \) mice had a significant increase in the expression of IL-1\( \beta \) (Fig. 11, *, \( p < 0.0009 \)) and TNF-\( \alpha \) (**, \( p < 0.0062 \)). As expected there was no expression of markers of the adaptive immune response, namely, IFN\( \gamma \), IL-4 and IL-17. Control and \( \text{Map} \) only mice did not have a significant increase in the expression of any of the cytokines evaluated. All data from each individual animal were normalized to the data for the internal control \( \beta \)-actin, which was expressed at similar levels within each treatment group.

**Caspase-1 Expression**

Considering the high expression of IL-1\( \beta \) in both BALB/c and SCID mice infected with \( \text{Map} \) and given DSS, we wanted to determine if caspase-1 expression, which is necessary for the processing of IL-1\( \beta \) from pro-IL-1\( \beta \) to mature IL-1\( \beta \), was increased. There was a significant increase in the expression of caspase-1 in \( \text{Map} + \text{DSS} \) BALB/c mice (Fig. 12, **, \( p < 0.0001 \)). There was a similar trend in the \( \text{Map} + \text{DSS} \) SCID mice, but this increase was not statistically significant. In SCID mice, DSS only mice had a significant increase in the expression of caspase-1 (Fig. 12, *, \( p < 0.0004 \)). Control and \( \text{Map} \) only SCID and BALB/c mice had a similar level of caspase-1 expression.
Discussion

We have previously shown that persistent enteric mycobacterial infection sensitizes BALB/c mice to enhanced acute mucosal injury. The results from the current study demonstrate that \textit{Map} infected mice developed a systemic antigen specific immune response. However, enhanced sensitivity to DSS was independent of adaptive immune cytokines, which is supported by the sensitivity of \textit{Map} infected SCID mice to DSS. A common feature when BALB/c and SCID mice were used in this model was increased expression of IL-1\(\beta\). Collectively, these results indicate that \textit{Map} enhanced intestinal injury is not dependent on the adaptive immune response but is associated with increased activation of the innate immune response.

\textit{Mycobacterium avium} ss. \textit{paratuberculosis}, an intracellular pathogen of macrophages, is an appropriate model organism that can be utilized to determine if a subclinical and chronic infection can sensitize or prime the mucosa for an aberrant immune response following injury to the intestinal mucosa.[14] Other investigators and our lab have previously demonstrated that \textit{Map} is capable of establishing subclinical and persistent intestinal infection in the BALB/c mouse.[15] This is relevant because \textit{Map} has been proposed to act as an immunomodulating factor in CD.[16]

\textit{Map} infection in the BALB/c mouse results in an antigen-specific immune response.[17] Likewise, in \textit{Map} infected mice we observed splenic antigen specific IFN\(\gamma\) and IL-17. Splenic production of \textit{Map}-specific IFN\(\gamma\) has been reported previously in the BALB/c mouse.[18] However, \textit{Map} antigen specific IL-17 has not been described, although \textit{Map} specific T-cell clones have been isolated from patients with CD that produce IL-17.[19] Even
though there was a trend for increased IL-4, it did not reach statistical significance.

However, despite an immune response to Map, infection did not result in detectable clinical disease in our model. This model may certain aspects of “occult” infections by weak pathogens, including M. avium, in predisposed individuals. To further define the antigen specific immune response, we also measured Map specific antibody production. We observed both antigen specific IgG2a and IgG1 antibodies in Map infected mice. This is consistent with previous vaccine studies in C57/BL6 or BALB/c mice.[17, 20] The predominance of IFNγ produced in cells isolated from the spleen relative to IL-4 is consistent with IgG2a production.[21] However, we also observed significant, but not greater, production of IgG1. A trend towards IgG1 production in the Th2 prone BALB/c mouse may be expected but we did not observe increased expression of IL-4 in either the intestine or spleen.[22] This is consistent with previous studies where low splenic expression of IL-4 was demonstrated in Map infected mice.[20] We cannot exclude the possibility that there was an increase in other cytokines associated with IgG1 production, such as IL-5 and IL-6.[23, 24] An unanticipated observation was a decrease in both IgG1 and IgG2a in mice infected with Map and given DSS. This may be associated with protein loss through the areas of intestinal ulceration; however, to confirm this possibility serum protein levels will need to be measured. In total, these observations establish that Map infected mice had an antigen specific immune response. However, a polarized immune bias was not clearly demonstrated. Mice had both antigen specific IgG2a and IgG1 production despite the marked increase in antigen specific IFNγ.
We observed divergent adaptive and innate immune cytokine profiles in the cecal homogenates of mice infected with *Map* and/or exposed to DSS. We had hypothesized that the combination of *Map* infection and exposure to DSS would result in amplification of adaptive immune cytokines; yet increased IL-4 and IFNγ expression was restricted to mice treated with DSS only. Although increased expression of IFNγ is associated with short term DSS treatment in the BALB/c mouse, enhanced IL-4 expression is not characteristic; however, its increase has been reported previously in BALB/c mice treated with DSS alone.[9, 25] Furthermore, it has been proposed that a Th2 immune response is protective in the DSS mediated model of colitis.[26]

To assess if mice had an intestinal antigen-specific immune response we evaluated cells isolated from the cecal tonsil because it plays an important role in DSS mediated intestinal inflammation, as demonstrated by amelioration of disease following its removal.[11] Additionally, it is the cecum where we saw the most consistent and severe lesions. In agreement with the gene expression data, we did not demonstrate cecal antigen specific production of IFNγ, IL-17 or IL-4 in *Map* infected mice. We cannot exclude the possibility that there was increased expression of the immunoregulatory cytokines IL-10 and TGF-β or cytokine independent mechanisms involved, for example CTLA-4 dampening the adaptive immune response.[27] Only one other study, to the authors’ knowledge, has evaluated cytokine expression in the intestines of mice exposed to *Map*. That study utilized IL-10 deficient mice on a B6 background that develop spontaneous colitis under conventional housing conditions.[28] The author’s demonstrated an increase in IFNγ positive CD4+ T-cells and NK/NKT cells in the intestinal mucosa of mice exposed to live *Map*. There
are some distinct differences between these two models. For example, intestinal colonization was not demonstrated in the IL-10 deficient mice and these mice lack IL-10 an essential immunoregulatory cytokine, a cytokine which may have played a role in our model. Lastly, differences in these two models may be associated with the inherent Th1/Th2 immune biases of these two inbred strains of mice.[22]

Enhanced innate immune activation with increases in IL-1, TNF-α and IL-6 is also a feature of acute DSS exposure in the BALB/c mouse.[9, 29] We identified increased expression of a similar panel of cytokines in mice given DSS alone. However, superimposed intestinal injury in Map infected mice generated a unique cecal cytokine profile. These mice had augmentation of the innate immune response, as indicated by increased cecal expression of IL-1β which was dependent on injury to the intestinal mucosa. Generation of mature IL-1β requires processing by caspase-1, whose activation is mediated by the inflammasome.[30] We observed increased expression of caspase-1 in Map infected mice given DSS which, similar to IL-1β, was dependent on injury to the mucosal barrier. Map infected macrophages may have directly generated IL-1 or contributed through increased recruitment of neutrophils, which are able to produce caspase-1 and IL-1β following exposure to LPS.[31] By analogy, Mycobacterium tuberculosis infected mouse macrophages are capable of recruiting neutrophils via the production of KC, a neutrophil chemoattractant.[32].

To confirm that Map enhanced intestinal inflammation was independent of the adaptive immune response; we infected a subset of SCID mice. Our results show that Map infected and DSS treated SCID mice had a similar disease phenotype as the BALB/c mice. They had more severe clinical disease, exacerbation of gross lesions, and increased cecal
lesion scores relative to all of the other treatment groups. Furthermore, similar to our observation in the BALB/c mice, Map infected and DSS treated SCID mice had increased expression of both IL-1β and caspase-1. However, ceca from these mice also had increased expression of TNF-α, the increase of which was dependent on exposure to DSS. This is consistent with in vitro data that demonstrates LPS primed Map infected macrophages produce increased amounts of TNF-α.[33] Differences in bacterial burden, which was higher in SCID mouse intestines, may influence TNF-α expression [34, 35] Similar to the BALB/c mice, there was also increased expression of caspase-1 in SCID Map infected and DSS treated mice; however, caspase-1 expression was greater in DSS only treated SCID mice. This may reflect differences in the character of the lesions, for example the rate of apoptosis or temporal variations in caspase-1 expression in the two groups of mice.[36] Collectively, the data from both the SCID and BALB/c mouse studies suggests that Map enhanced intestinal inflammation is independent the adaptive immune response and is associated with innate immune activation.

Although the disease phenotype was similar for both the SCID and BALB/c Map infected and DSS treated mice, suggesting that there was a common pathway, perhaps mediated by IL-1β, for the development of the lesions in these two models, we recognize that there may be mechanistic differences resulting in the same outcome. For example, we cannot rule-out that enhanced disease in BALB/c mice was mediated by an increased antibody response to the commensal flora, which has been demonstrated in Helicobacter bilis and cytomegalovirus models of IBD.[37, 38] Furthermore, we cannot exclude the possibility that
disruption of regulatory T-cell function was a feature of Map enhanced disease in the BALB/c mouse.

In conclusion, we have shown that Map infected mice have an antigen specific immune response and that Map infection in combination with DSS treatment results in a distinct cytokine profile relative to mice infected with Map only or treated with DSS alone. We believe our model can be utilized to better understand how a persistent infection with a low-level microbial pathogen, such as Map, disrupts the regulation of intestinal homeostasis and predisposes the host to enhanced intestinal injury and inflammation. Future studies will try and define a mechanism for how infection with Map promotes increased innate immune activation, with an emphasis on understanding how Map infection alters IL-1β production.

Acknowledgements

We thank Elise Huffman for technical assistance. This work was funded through a Iowa State University research grant and the Iowa Healthy Livestock Initiative.

References:


Table 1
Cecal lesion scoring system

<table>
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<tr>
<th>Parameters</th>
<th>Scoring Criteria</th>
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<tr>
<td>Enterocyte hyperplasia</td>
<td>Ratio of crypt depth to width&lt;br&gt;Rising score from 0 to 3 (no hyperplasia $\rightarrow$ severe hyperplasia)</td>
</tr>
<tr>
<td>Inflammatory score</td>
<td>Denotes cellular infiltrates in the lamina propria or cellular exudates overlying areas of mucosal ulceration&lt;br&gt;Rising score from 0 to 3 (small $\rightarrow$ large numbers of inflammatory cells)</td>
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<tr>
<td>Mucosal erosions</td>
<td>Rising score from 0 to 3 (no erosions $\rightarrow$ ulceration)</td>
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<tr>
<td>Edema</td>
<td>Rising score from 0 to 3 (no edema $\rightarrow$ marked submucosal edema)</td>
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<tr>
<td>Distribution</td>
<td>Rising score from 0 to 3 (0 = lesion is not present; 1 = $&lt; 25%$; 2 = $25 – 75%$; and 3 = $&gt; 75%$ of the section is affected)</td>
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Parameters are scored (0 $\rightarrow$ 3) + distribution (0 $\rightarrow$ 3) resulting in a maximal total histological score of 24.
Table 2. Primer sequences for qRT-PCR

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<td>β-actin</td>
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### Table 3.

<table>
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<th>Treatment</th>
<th>Severity of Rectal Blood</th>
<th>% with Rectal Blood</th>
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<tr>
<td>Map only</td>
<td>-</td>
<td>0%</td>
</tr>
<tr>
<td>DSS only</td>
<td>+</td>
<td>50%</td>
</tr>
<tr>
<td>Map + DSS</td>
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Severity and % of rectal blood in SCID mice infected with *Map* or inoculated with saline after the administration of DSS or water. A greater percentage of *Map* K10 + DSS mice had rectal blood compared to all other treatment groups. Data are from two separate experiments including 6 *Map* + DSS mice, 4 *Map* only and 4 DSS only mice.
Fig. 1  Cecal cytokine mRNA expression in BALB/c mice inoculated with saline (Control); infected with *Map* only (*Map only*); inoculated with saline and treated with DSS (DSS only); and mice infected with *Map* and treated with DSS (*Map + DSS*). IL-1β mRNA expression was significantly increased in *Map + DSS* treated mice relative to all other treatment groups (**, p < 0.016). The expression of TNF-α was significantly elevated in DSS only mice compared to all other groups (***, p < 0.016). Furthermore, the expression of all cytokines in the *Map + DSS* and DSS only groups was higher relative to their respective controls (*, p < 0.0004). Data are represented as the mean ± standard error of the means from one of two separate experiments, including 7 *Map + DSS* mice, 2 *Map only*, 5 DSS only mice and 2 control mice. β-actin was used as an internal control, with target gene mRNA expression being normalized to β-actin mRNA within the same tissue.
Fig. 2  Cecal cytokine mRNA expression in BALB/c mice inoculated with saline (Control); infected with *Map* only (*Map only*); inoculated with saline and treated with DSS (DSS only); and mice infected with *Map* and treated with DSS (*Map + DSS*). IL-4 and IFNγ mRNA expression was significantly increased in DSS only treated mice relative to all other treatment groups (*, **, p < 0.013 and p < 0.001, respectively). Data are represented as the mean ± standard error of the means from one of two separate experiments, including 7 *Map + DSS* mice, 2 *Map only*, 5 DSS only mice and 2 control mice. β-actin was used as an internal control, with target gene mRNA expression being normalized to β-actin mRNA within the same tissue.
Fig. 3 Induction of IFNγ following stimulation of spleen cells with Map sonicate from BALB/c mice inoculated with saline (Control); infected with Map only (Map only); inoculated with saline and treated with DSS (DSS only); and mice infected with Map and treated with DSS (Map + DSS). IFNγ secretion was significantly higher in Map + DSS and Map only mice stimulated spleen cells mice relative to all other treatment groups (*, **, p < 0.0001). Data are represented as the mean ± standard error of the means from two separate experiments including 4 Map + DSS mice, 2 Map only, 4 DSS only mice and 2 control mice.
Fig. 4  Induction of IL-17 following stimulation of spleen cells with *Map* sonicate from BALB/c mice inoculated with saline (Control); infected with *Map* only (*Map* only); inoculated with saline and treated with DSS (DSS only); and mice infected with *Map* and treated with DSS (*Map* + DSS). IL-17 secretion was significantly higher in *Map* + DSS stimulated mice relative to all other treatment groups, with the exception of the *Map* only stimulated mice (*, p < 0.009). Data are represented as the mean ± standard error of the means from two separate experiments including 4 *Map* + DSS mice, 2 *Map* only, 4 DSS only mice and 2 control mice.
Fig. 5 Induction of IL-4 following stimulation of spleen cells with *Map* sonicate from BALB/c mice inoculated with saline (Control); infected with *Map* only (*Map* only); inoculated with saline and treated with DSS (DSS only); and mice infected with *Map* and treated with DSS (*Map + DSS*). There are no statistically significant differences in IL-4 secretion between any of the treatment groups. Data are represented as the mean ± standard error of the means from two separate experiments including 4 *Map + DSS* mice, 2 *Map* only, 4 DSS only mice and 2 control mice.
Fig. 6 Measurement of serum IgG1 and IgG2a antibody responses to whole cell sonicates of *Map* in BALB/c mice. The magnitude of the antibody response was determined by ELISA as described in Section 2. The average OD value is significantly higher for both IgG1 and IgG2a in mice infected with *Map* only and *Map* + DSS compared to the control and DSS only groups (***, **, * p < 0.0001). Additionally, the average OD value of *Map* + DSS IgG2a is significantly lower compared to *Map* only IgG2a (*, p = 0.0005). Data are presented as pooled group means ± standard errors of the mean derived from one of two experiments.
Fig. 7. Change in body weight in SCID mice inoculated with saline (Control); infected with Map only (Map only); inoculated with saline and treated with DSS (DSS only); and mice infected with Map and treated with DSS (Map + DSS). Map + DSS mice lost a statistically significant amount of body weight relative to all other treatment groups (*, p < 0.03) at day 6 and at day 5 (*, p < 0.05) compared to the control and DSS only groups. DSS only mice lost more weight relative to the control at day 5 (**, p < 0.04). Data are represented as the mean ± standard error of the means from two separate experiments including 6 Map + DSS mice, 4 Map only, 4 DSS only mice and 4 control mice.
Fig. 8 Cecal lengths in SCID mice inoculated with saline (Control); infected with *Map* only (*Map* only); inoculated with saline and treated with DSS (DSS only); and mice infected with *Map* and treated with DSS (*Map + DSS*). The average cecal length in *Map + DSS* mice was shorter relative to all other treatment groups (*, p < 0.001). Data are represented as the mean ± standard error of the means from two separate experiments including 6 *Map + DSS* mice, 4 *Map* only, 4 DSS only mice and 4 control mice.
Fig. 9 Cecal histologic lesion scores in SCID mice inoculated with saline (Control); infected with *Map* only (*Map only*); inoculated with saline and treated with DSS (DSS only); and mice infected with *Map* and treated with DSS (*Map + DSS*). Cecal lesion scores were significantly higher in *Map + DSS* mice relative to all other treatment groups (**, *p* <0.0001). Additionally, DSS only mice had higher lesion scores relative to the control (*, *p* = 0.02). Data are represented as the mean ± standard error of the means from two separate experiments including 6 *Map + DSS* mice, 4 *Map only*, 4 DSS only mice and 4 control mice.
Fig. 10. Log colony forming units per gram of tissue in SCID mice inoculated with saline (Control); infected with Map only (Map only); inoculated with saline and treated with DSS (DSS only); and mice infected with Map and treated with DSS (Map + DSS). There were no significant differences between any of the tissues in any of the treatment groups. Data are represented as the mean ± standard error of the means from two separate experiments including 6 Map + DSS mice and 4 Map only mice. TNTC = too numerous to count.
Fig. 11 Cecal cytokine mRNA expression in SCID mice inoculated with saline (Control); infected with Map only (Map only); inoculated with saline and treated with DSS (DSS only); and mice infected with Map and treated with DSS (Map + DSS). IL-1β and TNF-α mRNA expression was significantly increased in Map + DSS treated mice relative to all other treatment groups (*, p < 0.0009 and ** p < 0.0062). Data are represented as the mean ± standard error of the means from one of two separate experiments, including 3 Map + DSS mice, 2 Map only, 2 DSS only mice and 2 control mice. β-actin was used as an internal control, with target gene mRNA expression being normalized to β-actin mRNA within the same tissue.
Fig. 12  Cecal caspase-1 mRNA expression in BALB/c and SCID mice inoculated with saline (Control); infected with Map only (Map only); inoculated with saline and treated with DSS (DSS only); and mice infected with Map and treated with DSS (Map + DSS). For BALB/c mice, caspase-1 was significantly increased in Map + DSS treated mice relative to all other treatment groups (**, p < 0.0001). For SCID mice, Caspase-1 was significantly increased in DSS only treated mice relative to all other treatment groups (*, p < 0.0004). For BALB/c mice, data are represented as the mean ± standard error of the means from one of two separate experiments, including 7 Map + DSS mice, 2 Map only, 5 DSS only mice and 2 control mice. β-actin was used as an internal control, with target gene mRNA expression being normalized to β-actin mRNA within the same tissue.
Chapter Four: Persistent mycobacterial infection sensitizes mucosal macrophages to a pro-inflammatory phenotype following intestinal epithelial injury

A paper to be submitted to the *Journal of Leukocyte Biology*

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Abstract

The function of intestinal macrophages is modulated and defined by its local environment. Therefore, it is essential to understand if exposure to an intracellular pathogen can cause a shift in macrophage phenotype. We have previously demonstrated that persistent enteric *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) infection in SCID mice exacerbates dextran sulfate sodium (DSS) mediated intestinal inflammation. Enhanced disease is associated with increased expression of IL-1β, suggesting macrophages are an important effector cell in our model. Our primary aim was to define the IL-1β expression profiles in *Map* infected macrophages. To accomplish this, we analyzed both *in vivo* and *in vitro* IL-1β protein expression in *Map* infected macrophages. Additional aims included evaluating the functional phenotype, the total number of F4/80+ cells and inflammasome mediated IL-1β production in *Map* infected macrophages. By immunohistochemistry, *Map*-infected macrophages had similar expression of pro-IL-1β that was not affected by DSS treatment. However, macrophages infected with *Map* and exposed *in vitro* to LPS had augmented IL-1β production which was not dependent on the NALP3 inflammasome. *Map* antigen positive cells from *Map* infected and DSS treated mice also had increased iNOS expression and greater numbers of F4/80+ cells. In conclusion, these observations indicate that *Map* infection sensitizes macrophages to a pro-inflammatory phenotype defined by increased IL-1β and iNOS expression following injury to the intestinal mucosa.

Key words: Macrophage, IL-1β, inflammasome, *Mycobacterium avium* subsp. *paratuberculosis*, iNOS
Introduction

Excessive activation of the innate immune system is thought to play an important role in the pathogenesis of the inflammatory bowel diseases (IBD). This is highlighted by the success of anti-TNF-α therapy in the treatment of Crohn’s disease (CD).[1] Moreover, other cytokines of the innate immune system, such as IL-1β, also appear to have an important role, as evidenced by enhanced secretion of IL-1β by mononuclear cells isolated from the inflamed mucosa from patients with Crohn’s disease.[2] Even though it has been shown that intestinal macrophages play a protective role in an experimental model of colitis, these cells represent potential sources of these pro-inflammatory cytokines; therefore, it is critical to tightly regulate their function to prevent unwanted injurious inflammation and emphasizes the need to understand the triggers that may cause a shift in their phenotype to promote inflammation.[3]

Although the evidence indicates that pro-inflammatory cytokines are involved in the evolution of the intestinal inflammation in IBD, it is not clear how an infection with an opportunistic pathogen that targets macrophages impact mucosal macrophage function. This is important considering the continuous exposure of the intestine to opportunistic pathogens and predisposition of certain populations to enteric infections. Mycobacterium avium ss. paratuberculosis (Map) has a tropism for intestinal macrophages and historically has been incriminated as having a role in CD. Mounting evidence indicates that Map is not the primary etiology of CD.[4] However, recent studies have suggested that Map may act as an immune modulator in susceptible individuals thereby promoting mucosal inflammation initiated by other causes. [5] In support of this hypothesis, oral exposure to Map, without intestinal
colonization, is capable of augmenting intestinal inflammation in IL-10 deficient mice.[6] These authors demonstrated enhanced T-cell immunoreactivity to a conserved mycobacterial antigen (Ag85), suggesting that exposure to various non-tuberculous mycobacterial (NTM) species have the potential to influence intestinal inflammation. This is relevant because environmental exposure is reported and underscores the need to understand how exposure to such intracellular pathogens may modulate the phenotype of intestinal macrophages.[7]

We have previously reported that subclinical and persistent enteric Map infection sensitizes mice to exacerbation of DSS mediated intestinal inflammation. Exacerbation of disease in our model is associated with enhanced activation of the innate immune response; which includes high IL-1β expression. In the current study we set out to understand how Map infection influences intestinal mucosal macrophage phenotype and function in an environment of epithelial injury. We analyzed both in vivo and in vitro IL-1β protein expression in Map infected macrophages. In cultured macrophages we explored cellular pathways culminating in inflammasome activation and IL-1β secretion. The inflammasome is essential for caspase-1 activation which, in turn, is necessary for processing of pro-IL-1β into mature IL-1β.[22] To accomplish this aim, we infected mouse macrophages that were deficient in different components of the inflammasome. Based on the in vitro ability of Map to prime macrophages for enhanced TNF-α expression following exposure to LPS, we hypothesized that Map infection would prime macrophages for increased expression of IL-1β, with its secretion requiring a second signal, such as exposure to commensal bacteria.[8] To evaluate the functional phenotype of Map infected macrophages, we examined the expression of inducible nitric oxide synthase (iNOS), a marker associated with classic
macrophage activation and enhanced killing of intracellular pathogens; and arginase, an enzyme characteristic of alternatively activated as well as wound-healing macrophages.[9, 10] We hypothesized that there would be minimal iNOS expression relative to arginase which would be expected to be increased since Map infected macrophages do not characteristically express iNOS and because SCID macrophages are prone to express arginase.[11-13] Our results demonstrate that macrophages in our in vitro and in vivo Map infection models have a pro-inflammatory profile defined by the enhanced production and secretion of IL-1β and increased expression of iNOS.

Materials and methods

Animals

Conventionally reared 6-8 week-old male C.B-17 scid (SCID) mice were purchased from The Jackson Laboratory (Bar Harbor, ME), weighed approximately 22-24 grams, and were housed in micro-isolation units that were individually ventilated in a positive pressure Thoren unit caging system at the Iowa State University College of Veterinary Medicine biosafety level II animal care facility. All animal experiments were approved by the Institutional Animal Care and Use Committee of Iowa State University.

Experimental design

Tissues from SCID mice utilized in this study were part of a previously reported study. In brief, two separate experiments were performed with 4 and 5 mice per Map infected and saline treatment group, respectively. In each experiment, mice were assigned to one of two study groups and either given a single intraperitoneal sham injection of saline or
*Map*. Following inoculation, body weights of the mice were measured at weekly intervals and feces were periodically collected for isolation of *Map*. At approximately 3 months post-inoculation, acute intestinal injury was chemically induced by administering a suboptimal concentration of 2% dextran sulfate sodium (DSS; MP Biomedicals, Solon, OH) in the drinking water for 6 days. We established this dose by determining the lowest DSS concentration in the drinking water that would induce minimal but perceptible microscopic lesions in the cecal and colonic mucosa. While the mice were administered DSS, they were weighed daily and on day 6 all of the mice were euthanized with CO₂ inhalation and necropsies were performed.

**Bacterial Inoculum and Infection**

The *Map* strain K10 was a clinical isolated obtained from the National Animal Disease Center (Ames, IA) and maintained at 37°C in Middlebrook 7H9 broth supplemented with mycobactin J. Logarithmic growth-phase bacteria were washed and resuspended in sterile saline for inoculation. Bacterial concentration was determined by measuring at 540 nm, comparing the absorbance optical density to the standard curve and adjusted to a final concentration of 1x10⁸ colony-forming units. Controls were given a sham inoculation of saline. The *Map* inoculum used in these studies was shown to have greater than 90% viability via fluorescein diacetate staining and flow cytometry analysis prior to inoculation. In addition, challenge inocula were confirmed negative for contaminants by streaking onto sheep blood agar plates 24 h prior to inoculation.
**Immunohistochemistry**

**IL-1β, iNOS, and arginase/Map combined and F4/80 only staining**

Before staining, slides were deparaffinized, rehydrated, and processed for immunohistochemistry (IHC). Antigen retrieval was accomplished by microwaving slides in a pH 10 buffer for 5 minutes for iNOS; 0.1% triton X-100 in PBS for 20 minutes at RT for IL-1β; microwaving in pH 6 citrate buffer for 5 minutes for arginase; and 2% protease in Tris buffer for 20 minutes at RT for F4/80. Slides were then blocked with 10% normal goat serum at room temperature. Primary antibodies were used at the following dilutions and incubated overnight at 4°C: F4/80 at 1:1,000 (eBioscience, San Diego, CA); arginase at 1:6,000 (Fitzgerald Industries; Concord, MA); IL-1β at 1:500 (Santa Cruz Biotechnology, Inc; Santa Cruz, CA); and iNOS at 1:1,500 (Upstate, Billerica, MA). Biotinylated goat anti-rabbit IgG at 1:400 or goat anti-rat IgG F(ab’)2 fragment secondary antibodies at1:250 were added and incubated at room temperature for 30 minutes. HRP-streptavidin diluted 1:200 was added to the slides for 15 minutes. DAB was added to the slides until optimal staining developed (10-15 minutes). Following staining for iNOS, arginase, and IL-1β, Map antigen staining was performed. To accomplish this, we used a polyclonal rabbit anti–*M. bovis* antibody that has extensive cross-reactivity with Map and is a useful tool for the identification of Map.[14] Rabbit anti–*M. bovis* (Dako Cytomation, Carpinteria, CA) was added at 1: 4,000 for 30 minutes at RT. Alkaline phosphatase–streptavidin diluted 1:200 was added to the slides for 15 minutes. Vector Red Substrate was then added until optimal staining developed (8-10 minutes).
Evaluation of stained slides

We restricted our analysis to ceca from SCID mice so that a sufficient number of *Map* antigen positive macrophages could be evaluated. The number of ceca evaluated included samples from 3 controls, 3 DSS only, 3 *Map* only infected, and 3 to 4 *Map* infected and DSS treated mice. A previously described method was used for evaluation of IL-1β, arginase, and iNOS immunoreactivity and was adapted for use in this study.[11, 15] All *Map* antigen positive 40x fields of the cecum were scored for the frequency and intensity of immunoreactivity for IL-1β, arginase, and iNOS in *Map* infected mice. For DSS only and control mice at least 10 40x random fields were evaluated for iNOS and IL-1β immunoreactivity. Because arginase expression was randomly scattered throughout the entire lamina propria of the cecum in all treatment groups, arginase immunoreactivity in DSS only and control mice was restricted to focal collections of mononuclear cells. The distribution of cell staining was scored on a scale of 0–3 (0 = 0%, 1 = < 25%, 2 = 25–75%, and 3 > 75% immunoreactive cells). The intensity of the cell staining was also scored on a scale from 0–3 (0 = no staining, 1 = mild intensity, 2 = moderate intensity, and 3 = intense). There was a maximum score of 6 per 40x field evaluated. For evaluation of F4/80 immunoreactivity, all positive F4/80 cells within the cecum were counted for each animal.

Bone-marrow derived macrophages (BMDM) and infection with Map

Femurs and tibias from NALP3-, ASC-, and caspase-1- deficient mice were kindly provided by Dr. Sutterwala (University of Iowa, Carver College of Medicine). The generation of NALP3-, ASC-, and caspase-1- deficient mice has been described.[16-18] Caspase-1 deficient mice were backcrossed onto the C57BL/6 genetic background for 10
generations. ASC- and NALP3-deficient mice were backcrossed onto the C57BL/6 genetic background for nine generations. Age and sex-matched C57BL/6 mice purchased were used as WT controls. Bone marrow cells were obtained by flushing the femurs and tibias from mice with complete tissue culture medium (CTCM) on ice. Complete tissue culture medium was prepared by adding 10% fetal bovine serum, 0.05 mM 2-mercaptoethanol, penicillin G (100U/ml), streptomycin (100 µg/ml), 2 mM L-glutamine, and 25mM Hepes to DMEM. Viability of cells was assessed by trypan blue exclusion and total cells were adjusted to approximately 15-20 x 10^6 cells and plated in 150x15 mm petri dishes with L929 conditioned medium containing DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, and 0.1 mM non-essential amino acid mix and maintained at 37°C, 5% CO₂ in a humidified incubator. At day 2 the supernatants of bone marrow cells were supplemented with additional L929 conditioned medium. At day 6, medium was removed from the petri dish and replaced by ice cold PBS, incubated on ice for 20 minutes, and macrophages were detached using a cell scraper. BMDM were pelleted, resuspended in CTCM and 5 x 10^5 cells/well were plated in 24 well plates. The next day, medium was replaced with CTCM without penicillin/streptomycin and cells were infected with Map with a final multiplicity of infection of 10:1. Cells were then incubated for 4 hours, washed with PBS and then stimulated with LPS at 1µg/ml in CTCM without penicillin and streptomycin. Supernatants were subsequently harvested at 24 and 48 hours and frozen at -70°C until analyzed for IL-1β.
**IL-1β ELISA**

A commercially available ELISA kit (eBioscience, San Diego, CA) was used for the detection of IL-1β protein in cell culture supernatants. The appropriate recombinant cytokine protein was used as a positive control to generate the standard curve. The detection limit was 8 pg/ml. Results are expressed as the mean (± SEM) pg/ml.

**Statistical analysis**

For all parameters, treatment group means were compared first with an overall F-test and then followed by post-hoc Student’s t-test with group mean differences considered significant if the p-value was <0.05. Data are presented as the mean value ± standard error of the mean except where stated otherwise.

**Results**

**Intestinal F4/80 immunoreactivity in mice with persistent mycobacterial infection**

Macrophages are a prominent component of the inflammatory infiltrate in SCID mice treated with DSS and are a target cell of the intracellular pathogen Map. [19, 20] Consequently, we wanted to assess the relative number of macrophages within the intestinal mucosa of mice exposed to DSS and persistently infected with Map. To achieve this, we performed immunohistochemistry with the macrophage specific F4/80 antibody. [21] Map + DSS mice had a greater number of F4/80 positive cells per 40x field examined relative to all other treatment groups (Fig.1, **, p < 0.0001). Also, DSS only mice had greater numbers of F4/80 positive cells compared to Control and Map only mice (*, p < 0.0009). Individual
F4/80 positive cells were primarily present in the edematous submucosa subjacent to lesions in the overlying mucosa or surrounding cecal crypts (Fig. 6, A).

**IL-1β protein expression and secretion from Map infected macrophages**

We have previously reported that IL-1β mRNA is increased in the cecal mucosa of SCID mice infected with Map and given DSS; therefore, one of our goals was to determine if there were differences in IL-1β immunoreactivity between the different treatment groups of mice, focusing where applicable on Map infected macrophages. To accomplish this we used a dual immunohistochemical staining method to label IL-1β and Map antigens. In cecal sections, cytoplasmic Map antigen was abundant and confined to cells with macrophage type morphology. In Map infected mice we evaluated dual labeling cells (Map + IL-1β) and in non-infected mice we evaluated all macrophages. The IL-1β antibody used in this study was specific for pro-IL-1β. In Map infected mice, pro-IL-1β immunoreactivity was restricted to the cytoplasm of Map antigen positive cells. Pro-IL-1β and Map antigen immunoreactivity was not observed in macrophages from any of the DSS only or control mice. We observed similar levels of pro-IL-1β in both Map only and Map + DSS mice (Fig. 2). However, pro-IL-1β immunoreactivity for both Map + DSS (Fig. 2, **, p < 0.0001) and Map only (Fig. 2, *, p = 0.0002) mice was higher relative to the control and DSS only groups. Representative pro-IL-1β immunoreactivity is shown in Figure 6 (B).

The observation of similar pro-IL-1β immunoreactivity between Map only and Map + DSS mice suggested that Map infection may prime macrophages for enhanced IL-1β production. We hypothesized that disruption of the mucosal barrier by DSS and exposure to normal floral antigens, such as LPS, would serve as the trigger for secretion of mature IL-1β.
To address this hypothesis, we utilized an *in vitro* model system where we infected C57BL6 mouse macrophages (WT) with *Map* and subsequently exposed them to *E. coli* LPS. *Map* infected and LPS exposed macrophages had augmented IL-1β production relative to all other treatment groups (Fig. 3, *) at 24 and 48 hours post-infection. Although smaller in magnitude compared to *Map* infected and LPS exposed macrophages, *Map* infection and LPS exposure alone also resulted in IL-1β production.

We next set out to define pathways of IL-1β secretion using macrophages from C57BL6 mice deficient in components of the inflammasome pathways. The inflammasome is essential for caspase-1 activation which, in turn, is necessary for processing of pro-IL-1β into mature IL-1β.[22] The inflammasome is a dynamic molecular entity and is composed of different components to achieve activation of caspase-1 depending on the inciting stimuli. The NALP3 inflammasome is associated with caspase-1 activation following infection with *Mycobacterium tuberculosis*; therefore, we used mouse macrophages deficient in NALP3 and an adaptor protein associated with this complex, ASC, to determine if this pathway was sufficient for IL-1β production following infection with *Map*.[23] Mouse macrophages deficient in NALP3 and infected with *Map* and/or exposed to LPS were still capable of producing IL-1β. However, deficiency of the adaptor protein ASC resulted in a marked reduction in IL-1β, with minimal production at 48 hours in *Map* infected and LPS exposed macrophages. Similarly, there was a marked reduction in IL-1β in caspase-1 deficient macrophages, yet at 48 hours there was capase-1 independent production of IL-1β in *Map* infected and LPS exposed macrophages.
Functional phenotype of Map infected intestinal macrophages

We performed immunohistochemistry for arginase, a marker of wound-healing and alternative activated macrophages, and iNOS, a marker associated with classic macrophage activation and enhanced killing of intracellular pathogens, to define the functional phenotype of Map infected intestinal macrophages.[9, 24] We used a dual label for Map/arginase and focused on Map infected macrophages as described for IL-1β detection. We focused on co-localized Map with iNOS or arginase antigen positive cells in Map infected mice. Positive iNOS immunoreactivity in DSS only mice was confined to the cytoplasm of apical enterocytes associated with areas of mucosal ulceration and erosion. For Map infected mice cytoplasmic iNOS positive staining was confined to Map infected cells. iNOS immunoreactivity was not observed in control mice. Map + DSS infected mice had a higher iNOS score relative to all other treatment groups (Fig.4, **, p < 0.002). Map only mice had higher iNOS immunoreactivity scores compared to DSS only and control mice (*, p < 0.002).

In Map infected mice, iNOS immunoreactivity was not observed in non-infected macrophages. There was diffuse and randomly scattered cytoplasmic arginase immunoreactivity throughout the lamina propria of the intestinal mucosa in mice of all treatment groups. Labeling occurred within Map infected and non-infected macrophages. In Map infected mice, evaluation of arginase immunoreactivity was confined to Map antigen positive foci. Assessment of arginase immunoreactivity in DSS only and control mice was limited to well defined aggregates of mononuclear cells, with morphology similar to gut associated lymphoid tissue of conventional mice. In Map infected mice, there was no difference in
arginase immunoreactivity in Map infected or non-infected macrophages and the graph depicts scoring for Map infected cells only (Fig. 5). Map infection did not affect arginase immunoreactivity relative to control mice. There was a trend for decreased arginase immunoreactivity in DSS only mice but this did not reach statistical significance. Representative arginase and iNOS staining is shown in Figure 6 (C, D, respectively).

Discussion

We set out to understand how subclinical Map infection in the context of acute mucosal injury potentiates innate immune responses. We accomplished this by evaluating the functional phenotype and expression of IL-1β in Map infected macrophages and by examining the relative number of macrophages in Map enhanced intestinal lesions. This is relevant because Map has been suggested to act as an immune modulator, but not as a primary pathogen, in CD.[5] With support from our in vitro studies, our results suggest that Map infected macrophages in our in vivo model have a pro-inflammatory profile defined by the enhanced expression of IL-1β and iNOS, which corresponds with an increase in the number of lamina propria macrophages. Combined with the observation that DSS treatment did not affect the expression of pro-IL-1β in Map infected mice, our results indicate that Map infection promotes a shift towards classic macrophage activation, which is sustained and potentially amplified following interaction with a second stimulus, namely, LPS. Moreover, it appears that Map infection primes the uninjured mucosa for augmented macrophage recruitment following an intestinal insult.

Intestinal macrophages are dynamic effector cells which play a critical role in maintaining mucosal homeostasis.[25] Map represents an appropriate model organism for
opportunistic infections that can be used to better understand how subclinical infections alter intestinal macrophage phenotype and whether these changes translate to increased susceptibility to intestinal injury. Limited numbers of studies have specifically addressed how subclinical infections of intestinal macrophages affect inflammation in a model of intestinal injury. Cytomegalovirus (CMV), a virus known to infect intestinal macrophages, is one of the few pathogens that has been studied in a model of experimental colitis.[26] Onyeagocha et al. have shown that latent CMV infection sensitizes mice to exacerbation of disease following DSS mediated intestinal injury.[27] It was proposed that increased clinical disease was due to CMV mediated increases in gut permeability that promoted an enhanced innate immune response following disruption of the intestinal mucosa by DSS. Although CMV is capable of infecting intestinal macrophages, it has a relatively broad cellular tropism, for example, it is able to infect epithelial and endothelial cells.[28] In contrast, Map replication is primarily restricted to macrophages, thereby providing a model to specifically explore how infection of intestinal macrophages alters intestinal homeostasis.[19]

The findings of the current study are unique in that we have demonstrated that subclinical Map infection of intestinal macrophages is capable of sensitizing the intestinal mucosa for an enhanced innate immune response following acute mucosal injury. This is based on the observation that Map infection in the uninjured intestine can promote the expression of iNOS, in conjunction with our in vitro observation that Map infection augments IL-1β following exposure to LPS. In the current study we observed similar levels of pro-IL-1β in Map infected mice with and without DSS administration. This suggests that Map infection promotes increased synthesis of pro-IL-1β and storage in the cytoplasm. We
hypothesized that LPS prompts full inflammasome activation and secretion of IL-1β. To examine this possibility, we infected macrophages *in vitro* with *Map* then exposed them to LPS to mimic translocation of commensals across a damaged mucosal barrier. We observed significant increases in IL-1β secretion into the culture supernatants from macrophages that were infected with *Map* and exposed to LPS. Even though previous studies and the current study demonstrate that *Map* infection alone is sufficient for IL-1β production, our results support the hypothesis that both production of mature IL-1β and its secretion is enhanced by the combination of *Map* and LPS.[29, 30] Support for our observation of LPS enhanced expression of IL-1β by *Map* infected macrophages is provided by a study in which LPS was shown to enhance TNF-α expression by *Map* infected macrophages.[8] However, the precise mechanism associated with *Map* enhanced IL-1β expression remains to be defined. *Map* infection may have primed macrophages for enhanced production of IL-1β by promoting a basal level of IL-1β expression, with LPS serving as the trigger for its enhanced activation and secretion. Piccini et al have recently reported that various microbial components, including LPS, are competent to induce both the processing and secretion of IL-1β.[31] This mechanism was proposed to be mediated by extracellular release of endogenous ATP from monocytes. Following ATP release there is autocrine stimulation of the P2X7 receptor, causing K+ efflux and phospholipase A2 activation, both of which are necessary for the processing and secretion of IL-1β.

Since *Map* infection alone is capable of eliciting IL-1β production, we wanted to try and identify how *Map* induces its production. Formation of mature IL-1β requires processing mediated by caspase-1 whose activation, in turn, requires assembly and activation of a
multiprotein complex called the inflammasome.[22, 32] Originally, the inflammasome was structurally defined by apoptosis-associated speck-like protein containing a CARD (ASC), NACHT- LRR- and pyrin domain-containing protein 1 (NALP), caspase-5 and caspase-1.[33] A more recently described inflammasome brings together two caspase-1 molecules and is composed of NALP-3 (cryoporin) and two adapter proteins cardinal (CARD8) and ASC.[34] We chose to evaluate the NALP3 inflammasome because it is activated by *Mtb.[23]* Macrophages deficient in the NALP3 inflammasome were still capable of producing IL-1β indicating that an alternative inflammasome platform is involved for *Map* mediated IL-1β production. However, the ASC component of the NALP3 inflammasome is necessary because there was minimal production of IL-1β in macrophages lacking this protein, thus suggesting that an ASC associated inflammasome is important, such as NALP1 or NALP2.[33, 35] The ability of *Map* infected and LPS stimulated macrophages to produce IL-1β in the absence of caspase-1 was unexpected. However, caspase-1 independent production of IL-1β has been described. One mechanism involves matrix metalloproteinases (MMP), which can be induced by both LPS and *Map* stimulated macrophages.[36, 37] Moreover, caspase-1 independent production of IL-1β has been described in a mouse model of *Mtb* infection.[38]

Free radical injury of the intestinal mucosa is thought to play a role in IBD as indicated by increases in nitric oxide and iNOS in patients with IBD and based on the amelioration of DSS mediated colitis following decreases in nitric oxide.[39, 40] *Map* infected macrophages had an augmented activated phenotype following injury to the intestinal mucosa characterized by an increase in the expression of iNOS. We hypothesized
that there would be minimal expression of iNOS. *Map* infected bovine macrophages do not characteristically express iNOS *in vitro* and *in vivo*; however, its expression has been demonstrated in peripheral blood mononuclear cells of subclinically cattle and in hepatic foci of granulomatous inflammation in *Map* infected athymic nude mice.[11, 13, 41][42] This increase may have been due to superimposed exposure to intestinal antigens, such as LPS, following injury to the mucosal barrier.[43] Augmented iNOS levels may also be partially explained by the increases in IL-1β we observed since it is capable of increasing iNOS expression.[44] *Map* infection did not affect arginase expression, a marker of wound healing macrophages.[9] To the authors’ knowledge, there are no other reports on arginase expression in *Map* infected macrophages. There may be reciprocal regulation of iNOS and arginase in *Map* infected cells. The increase in iNOS expression may have caused a decrease in arginase expression, since both enzymes compete for the same substrate, L-arginine.[45] In contrast to the *Map* infected mice, there was a trend for decreased arginase expression in DSS only mice in which arginase would have been expected to be increased because it has been demonstrated that exposure to LPS increases arginase expression in SCID macrophages.[12]

Higher doses of DSS induce colitis in SCID mice, and macrophages likely play an important role in this model.[46, 47] Our observation of increased expression of both a macrophage associated cytokine, IL-1β, and iNOS following infection with an intracellular pathogen of macrophages suggests that this cell type is an important effector cell in our model. We observed increased numbers of F4/80 positive cells, a marker of mouse macrophages, in SCID mice treated with DSS, with the greatest numbers being observed in
Map + DSS treated mice. The increase in F4/80 positive cells was primarily restricted to the edematous submucosa and cecal crypts and did not appear to be associated with foci of Map localization. A previous study with a different macrophage marker has demonstrated that macrophages predominate in the SCID DSS model relative to neutrophils.[20] Although we did not evaluate chemokine levels, their expression was likely elevated in foci of inflammation. By analogy, TNF-α mediated macrophage production of chemokines is an important mechanism for macrophage recruitment in mouse models of Mtb.[48, 49] Previously, we have documented increased TNF-α expression in Map infected and DSS treated SCID mice. Our results imply that subclinical Map infection potentiates the active recruitment of macrophages to sites of intestinal injury.

Collectively, our observations from this study suggest that Map infection causes a shift in intestinal macrophage effector function which results in enhanced pro-inflammatory properties defined by increased secretion of IL-1β following exposure to LPS and increased expression of iNOS. This builds on and characterizes a possible mechanism associated with our previous studies where we have shown that Map infection is associated with enhanced innate immune activation in a model of enhanced intestinal injury. Furthermore, our studies indicate that Map induced production of IL-1β is not dependent on the NALP3 inflammasome, and may involve a caspase independent mechanism, but not in Map infected and LPS treated macrophages. We believe this model represents an important tool that can be used to advance our understanding of how subclinical infection of macrophages alters their homeostatic promoting anti-inflammatory profile.
Acknowledgements

We thank Elise Huffman for technical assistance. This work was funded through a Iowa State University research grant and the Iowa Healthy Livestock Initiative.

References:


Fig. 1 Average number of F4/80+ cells per 40x field in the ceca from mice inoculated with saline (Control); infected with Map only (Map only); inoculated with saline and treated with DSS (DSS only); and mice infected with Map and treated with DSS (Map + DSS). The number F4/80+ cells was significantly higher in Map + DSS relative to all other treatment groups (**, p < 0.0001). Also, the number of F4/80+ cells was greater in DSS only mice compared to Map only and Control mice (*, p < 0.0009). Data are represented as the mean ± standard error of the means from two separate experiments including 4 Map + DSS mice, 3 Map only, 3 DSS only mice and 3 control mice.
**Fig. 2** Average pro-IL-1β immunoreactivity score per 40 x field in the ceca from mice inoculated with saline (Control); infected with *Map* only (*Map* only); inoculated with saline and treated with DSS (DSS only); and mice infected with *Map* and treated with DSS (*Map* + DSS). The average pro-IL-1β reactivity was not different between *Map* only and *Map* + DSS mice. The average pro-IL-1β immunoreactivity score for both *Map* only (*, p = 0.0002) and *Map* + DSS (*, p < 0.0001) mice was higher compared to DSS only and control mice. Data are represented as the mean ± standard error of the means from two separate experiments including 3 *Map* + DSS mice, 3 *Map* only, 3 DSS only mice and 3 control mice.
Fig. 3 Average IL-1β production in C57BL6 Asc −/−, Nalp3 −/−, Caspase 1 −/− deficient and wild-type (WT) macrophages infected with Map for 4 hours and subsequently exposed to LPS at 1µg/ml. In wild-type macrophages, Map infected and LPS exposed macrophages had the greatest amount of IL-1β production. Deficiency of Asc, Caspase-1, or Nalp3 did not affect Map infected and LPS exposed macrophage secretion of IL-1β; however, Map only and LPS only mediated production of IL-1β was affected by the absence of Asc and Caspase-1. Cell culture supernatants were collected at 24 and 48 hours and measured for IL-1β production by ELISA. Duplicate wells were assayed and results are expressed as the mean ± standard error of the means and are representative of one experiment.
Fig. 4 Average iNOS immunoreactivity score per 40x field in the ceca from mice inoculated with saline (Control); infected with Map only (Map only); inoculated with saline and treated with DSS (DSS only); and mice infected with Map and treated with DSS (Map + DSS).

iNOS immunoreactivity was only observed in macrophages infected with Map. The average iNOS immunoreactivity was significantly higher in Map + DSS mice compared to all other treatment groups (**, p < 0.002). The average iNOS immunoreactivity score for Map only mice was higher compared to DSS only and control mice (*, p-value < 0.002). Data are represented as the mean ± standard error of the means from two separate experiments including 3 Map + DSS mice, 3 Map only, 3 DSS only mice and 3 control mice.
Fig. 5 Average arginase immunoreactivity score per 40x field in the ceca from mice inoculated with saline (Control); infected with *Map* only (*Map* only); inoculated with saline and treated with DSS (DSS only); and mice infected with *Map* and treated with DSS (*Map* + DSS). The average arginase immunoreactivity was not different between any of the treatment groups. Data are represented as the mean ± standard error of the means from two separate experiments including 3 *Map* + DSS mice, 3 *Map* only, 3 DSS only mice and 3 control mice.
Fig. 6 Representative photomicrographs from mice infected with *Map* and treated with DSS demonstrating F4/80 (A), *Map* and pro-IL-1β (B), arginase (C), and iNOS immunoreactivity (D) in the ceca of SCID mice infected with *Map* and treated with DSS. F4/80 immunoreactivity was primarily confined to the submucosa and cecal crypts. Immunoreactivity for *Map*, F4/80, pro-IL-1β, arginase, and iNOS was present within the cytoplasm of cells with macrophage type morphology. F4/80, pro-IL-1β, arginase, and iNOS stains are brown, whereas the *Map* stain is red. Bar = 50 µm.
General Conclusions

Our central hypothesis for this thesis was that persistent intestinal mycobacterial infection sensitizes the host to acute intestinal injury. *Mycobacterium avium* subsp. *paratuberculosis* is an appropriate model organism to address this hypothesis since it can establish subclinical intestinal infection in the mouse and because of its tropism for macrophages. This allows for the evaluation of the relationship between pathogen-induced alterations in macrophage effector function and its consequences on intestinal homeostasis.[1, 2] Other *in vivo* models examining the interaction between chronic infection and mucosal injury are few. The best example is provided by a single study conducted with cytomegalovirus (CMV).[3, 4] In this study subclinical CMV infection exacerbated subsequent DSS mediated intestinal inflammation. However, unlike Map, the cellular tropism of CMV is broad and not restricted to macrophages.[5] It is relevant to use Map in our model because it has been proposed to act as an immunomodulator in Crohn’s disease and because intestinal macrophages are consistently exposed to low level pathogens such as non-tuberculous mycobacteria.[6, 7] Collectively, the results we have generated from this thesis support the central hypothesis and suggest that persistent enteric mycobacterial infection can prime the intestinal mucosa for an excessive innate immune response following acute mucosal injury.

*Persistent enteric mycobacterial infection enhances sensitivity to acute mucosal injury*

In Chapter 2 our primary goal was to determine if chronic intestinal *Map* infection would influence subsequent DSS mediated intestinal inflammation. We hypothesized that
exposure to Map would sensitize the host to acute mucosal injury. To address this goal, we utilized a mouse model of intestinal Map infection.[2, 8] BALB/c mice were infected with Map for 90 days followed by a low dose of DSS in the drinking water to induce mild intestinal injury. In these mice, we evaluated clinical signs of disease, such as changes in body weight and frequency of rectal blood, and assessed gross and histologic cecal lesions. Our results support the hypothesis that subclinical and chronic intestinal mycobacterial infection amplifies acute mucosal injury. This was demonstrated by increased weight loss, increased frequency of rectal blood, and exacerbation of gross and histologic cecal lesions in mice infected with Map and treated with DSS. An additional observation was decreased Map burden in the small intestines of DSS treated mice, and we hypothesize that this was related to macrophage activation following DSS treatment. Based on demonstration of viable Map and acid-fast bacilli in the lymphoid tissue of the cecal tonsil, a tissue that plays a central role in the development of DSS-mediated intestinal inflammation, in conjunction with the fact that Map is an intracellular pathogen of macrophages, our results support a hypothesis that excessive activation or dysregulation of the mucosal immune system was responsible for the enhanced disease in our model.[9]

Immune responses associated with intestinal mucosal injury in mice subclinically infected with Mycobacterium avium subsp. paratuberculosis

In Chapter 3 we set out, to define the intestinal and systemic immune responses that were associated with sensitivity of Map-infected mice to DSS in our model. We hypothesized that an additive Th1 immune response was associated with Map enhanced intestinal inflammation. The rationale for this hypothesis was that acute DSS mediated colitis in the BALB/c mouse is defined by a Th1 cytokine profile and because Map infected macrophages
is capable of producing a similar profile of cytokines.[10, 11] To address our goals, we analyzed adaptive and innate intestinal and systemic cytokine expression patterns and evaluated mice for Map antigen specific antibody. We infected a subset of SCID/C.B-17 (SCID) mice with Map to determine if enhanced intestinal disease would occur in the absence of an adaptive immune response.

Our results confirm that Map infected mice had antigen specific production of adaptive immune cytokines, namely IFNγ and IL-17, in addition to the production of antigen specific IgG2a and IgG1 antibody. However, we observed enhanced activation of the innate rather than adaptive immune response Map infected BALB/c mice treated with DSS. An innate mechanism was supported by infecting SCID mice with Map followed by DSS treatment, which resulted in similar clinical disease and mucosal pathology. A common feature of the disease in both BALB/c and SCID mice was increased expression of IL-1β. We did not observe an increase in cecal IFNγ, IL-17 or IL-4, nor did we observe Map antigen specific production of these cytokines in lymphoid cells isolated from the cecal tonsil of BALB/c mice infected with Map and treated with DSS.

Although Map infected and DSS treated SCID and BALB/c mice had a similar clinical phenotype and had increased expression of IL-1β, there were differences identified between these two mouse strains. Ceca from Map infected SCID mice had increased expression of TNF-α which was dependent on exposure to DSS. Similar to the BALB/c mice, there was also increased expression of caspase-1 in SCID Map-infected and DSS-treated mice; however, caspase-1 expression was greater in DSS only treated SCID mice. This may reflect differences in the character of the lesions, for example the rate of apoptosis or
temporal variations in caspase-1 expression in the two groups of mice.[12] In total, the results from this study indicate that Map infection primes the mucosa for secretion of innate cytokines.

*Persistent mycobacterial infection sensitizes macrophages to a pro-inflammatory phenotype following intestinal epithelial injury*

The results from Chapter 3 indicated that Map mediated enhanced intestinal disease was not dependent on the adaptive immune response, but was associated with increased activation of the innate immune response; more specifically, increased IL-1β. In Chapter 4, we set out to understand how Map infection in the context of acute mucosal injury, promotes an augmented innate immune profile. Because Map infection was limited to mucosal macrophages, we focused our studies on macrophages as a key source of inflammatory cytokines. To accomplish our goal, we chose to define how Map infection alters macrophage activation phenotype and influences expression of IL-1β. We analyzed both in vivo and in vitro IL-1β protein expression in Map infected macrophages and assessed the ability of Map to mediate inflammasome IL-1β production. Based on the in vitro ability of Map to induce the expression of IL-1β in mouse macrophages, we hypothesized that Map infected macrophages would have an increase in both the expression and secretion of IL-1β.[11, 13]

To evaluate the functional phenotype of Map infected macrophages, we examined the expression of iNOS; a marker associated with classic activation and enhanced killing of intracellular pathogens, and arginase, an enzyme characteristic of alternative activation and wound-healing macrophages.[14, 15] We hypothesized that there would be minimal iNOS expression relative to arginase which would be expected to be increased since Map infected
macrophages do not characteristically express iNOS and because SCID macrophages are prone to express arginase.[16-18] An additional aim was to determine if Map enhanced intestinal lesions contained increased numbers of macrophages. By immunohistochemistry, Map infected macrophages had similar expression of pro-IL-1β that was not affected by DSS treatment. However, macrophages infected with Map and exposed \textit{in vitro} to LPS had augmented IL-1β production which was not dependent on the NALP3 inflammasome. Map infected and DSS treated mice also had increased iNOS expression and greater numbers of F4/80+ cells. iNOS expression was not identified in non-infected macrophages. In summary, these observations indicate that Map infection promotes a basal level of IL-1β production thereby priming macrophage for IL-1β enhanced secretion following interaction with a second stimulus, namely, LPS.
Proposed model for Map enhanced intestinal injury

The results from this thesis support the following model (Figure 1) where Map infection of intestinal macrophages promotes a basal level of IL-1β and iNOS expression thereby priming macrophages for enhanced production of these molecules following interaction with a second stimulus, namely, LPS, from the intestinal lumen. Mechanistically, IL-1β may compound DSS-mediated injury through disruption of tight junctions via up-regulation of myosin light chain kinase, while increased iNOS expression may be associated with free radical mediated injury following the formation of reactive nitrogen intermediates such as NO₂⁻, NO₃⁻ and ONO₂⁻ or as an immunomodulator.[19] Moreover, these effects may be further augmented by Map-mediated increases in the total number of macrophages recruited to sites of intestinal inflammation.

Figure 1
Recommendations for Future Research

Our observations suggest that Map enhanced activation of the innate immune system can sensitize the mucosa to DSS mediated inflammation. This is based on the observation of increased IL-1β expression in both BALB/c and SCID mice which had a similar disease phenotype and the absence of increases in markers of the adaptive immune response. Additionally, results from our last set of experiments indicate that Map infection primes macrophages for a pro-inflammatory profile after exposure to components of the intestinal lumen, such as LPS. Even though these results suggest a mechanism associated with Map enhanced intestinal disease, its confirmation requires further research.

Although we demonstrated increased IL-1β mRNA expression by qRT-PCR, it would be critical to confirm that there is a corresponding increase in IL-1β protein levels. This could be accomplished measuring IL-1β secretion in colonic explants. To validate that IL-1β plays a central role in the pathogenesis of our model it would also be important to block IL-1β with a monoclonal antibody or disrupt its binding to target cells, with the hypothesis that disease would be ameliorated. Alternatively, to substantiate that IL-1β producing macrophages are key effector cells in our model, these cells could be isolated from Map infected ceca, expanded in vitro and transferred to SCID mice to determine if enhanced disease occurred following DSS treatment. It would also be important to neutralize TNF-α since this cytokine was increased, in conjunction with IL-1β, in our SCID mouse model of Map enhanced disease.

If IL-1β is shown to be essential for enhanced disease, a more precise mechanism for IL-1β enhanced disease could be explored. IL-1β is capable of altering intestinal permeability
via activation of NF-κβ which results in an increase in the expression of myosin light chain kinase (MLCK). [20, 21] Increased MLCK phosphorylates myosin II regulatory light chain (MLC) which promotes opening of the tight junction via contraction of peri-junctional actin-myosin filaments. [22-24] An in vitro transwell system could be used evaluate this possibility by placing Map infected macrophages in one well and intestinal epithelial cells in an adjacent chamber and then assess if MLCK expression is enhanced. If it is, then an IL-1β antibody could be used to confirm that IL-1β mediates this change.

There may be multiple mechanisms responsible for enhanced IL-1β in Map infected and DSS treated mice. Map infected macrophages may have indirectly mediated this increase in IL-1β through increased recruitment of neutrophils, which are able to produce IL-1β following exposure to LPS. [25] We observed a semi-quantitative increase in cecal lesion associated neutrophils in Map infected and DSS treated mice (data not shown). By analogy, Mycobacterium tuberculosis infected mouse macrophages are capable of recruiting neutrophils via the production of KC, a neutrophil chemoattractant. [26] We could isolate Map infected macrophages from the intestines or infect macrophages in vitro to determine if they had increased production of KC. Map infected macrophages may have also had increased expression of TLR receptors, such as TLR4, which has been shown in the ileum of Map infected sheep. [27] Enhanced TLR expression on Map infected macrophages could amplify the pro-inflammatory response following exposure to LPS which itself can increase both the expression and activation of caspase-1 and IL-1β. [28-30] This possibility could be evaluated by examining TLR expression by immunohistochemistry or flow cytometry on in vitro or in vivo Map infected macrophages.
Although the results from our experiments support a role for excessive activation of the innate immune system; in particular increased IL-1β, as a cause for the *Map* enhanced intestinal inflammation, it is not possible to rule-out the existence of more important or at least contributory mechanisms in our model. There was a difference in intestinal bacterial load between SCID and BALB/c mice, with greatest numbers of *Map* being present in SCID mice. Although there was this difference in bacterial burden, *Map* infected and DSS treated SCID and BALB/c mice had the same disease phenotype, suggesting that there may be alternative adaptive or regulatory immune mechanisms involved in the BALB/c mouse model of enhanced intestinal disease. The relatively small number of *Map* infected cells in BALB/c mice was often present in the cecal tonsil or gut associated lymphoid tissue, implying that *Map* altered the adaptive immune response; however, we did not identify increases in markers of the adaptive immune response in the cecum. With that said, it would be beneficial to assess the immune response in the mesenteric lymph nodes, with the hypothesis being that cells from this site would have a similar cytokine profile to that in the cecum.

We did not extensively evaluate the humoral immune response. *Map* infection in DSS treated BALB/c mice may have been associated with an enhanced humoral immune response to the intestinal flora. Aberrant host immune responses to the intestinal flora have been demonstrated in a *Helicobacter bilis* model of intestinal inflammation and in a subclinical CMV model of enhanced intestinal disease.[3, 31] To answer this question we could evaluate serum for antibodies reactive to the intestinal flora, with the greatest titers presumably being present in *Map* infected and DSS treated mice. If a population of intestinal flora reactive B-
cells were identified, these cells could be transferred into SCID mice to determine if they exacerbated DSS mediated disease. Alternatively, a population of CD4+CD25+ regulatory T-cells may have been generated which then persisted when Map bacterial loads were reduced. Antigen specific regulatory T-cells have been demonstrated in bovine paratuberculosis and persistence of regulatory T-cells in the presence of low antigen has been demonstrated previously. [32, 33] Loss of regulatory T-cell function in a model of enhanced intestinal disease has been described. For example, a previous study in a SAMP1/YitFc mouse model of ileitis demonstrated that B-cells are associated with exacerbation of intestinal inflammation, which is not antibody dependent. These authors demonstrated that mesenteric lymph node B-cell numbers correlated with the severity of ileitis and that B-cells mediated enhanced intestinal inflammation by interfering with regulatory T-cell function. [34] To examine this possibility, we could quantify the number of T-regulatory cells and assess if there was expansion of B-cells in the cecal mucosa or mesenteric lymph nodes. The hypothesis would be that B-cell proliferation in the mesenteric lymph node would correlate with increased disease severity in Map infected and DSS treated mice and correspond with a decrease in number or functioning of regulatory T-cells.

Additional unanticipated results could be further investigated with in vitro Map infected and LPS stimulated macrophages. We observed increased iNOS in Map infected and DSS treated mice. One plausible explanation for the increase in iNOS is that superimposed exposure to intestinal antigens, such as LPS, enhanced expression of iNOS. [35] The augmented iNOS levels may also be partially explained by the increases in IL-1β. [36] Exposure of Map infected macrophages to LPS and/or IL-1β would be expected
to increase nitric oxide. To further confirm that increased iNOS is associated with enhanced disease in our model, nitric oxide levels in colonic explants from Map infected and DSS treated mice could be evaluated.

To further define a mechanism associated with Map and LPS enhanced in vitro production of IL-1β, the effect of ATP on enhanced IL-1β secretion could be assessed. We would hypothesize that ATP production and its release is increased in Map infected and LPS exposed macrophages since endogenously released ATP from LPS exposed macrophages is necessary for processing and secretion of IL-1β in vitro.[37] Presumably, since Map is also a TLR agonist, this response would be further augmented in Map infected and LPS treated macrophages.[38] To evaluate the importance of ATP, we could we could utilize ATPase to determine if enhanced IL-1β secretion is abolished by the elimination of ATP. Lastly, Map and LPS associated increases in IL-1β were not significantly affected in NALP3 knockout macrophages. However, ASC was necessary for increased IL-1β; ASC is associated with the NALP1/2 inflammasome.[39] Therefore, future studies utilizing macrophages deficient in these inflammasome proteins could be performed to determine if they are critical for Map mediated production of IL-1β.

There were decreased numbers of Map in the small intestines of DSS treated BALB/c mice which may be due to an enhanced non-specific immune response induced by DSS. It has been shown that DSS-pulsed macrophages promote the proliferation of T-cells from colitic mice.[40] Additional immunohistochemical studies could be performed to define the activation status of Map infected BALB/c macrophages; with the hypothesis that Map infected macrophages in DSS treated BALB/c mice would have enhanced expression of
iNOS. It is also possible that DSS had a direct inhibitory effect on \textit{Map} replication, which could be evaluated in \textit{Map} infected macrophages \textit{in vitro}.

Since F4/80 positive cells were elevated in \textit{Map} infected and DSS treated mice, it would be interesting to assess the impact of preventing recruitment of macrophages to sites of DSS mediated inflammation. The hypothesis would be that \textit{Map} enhanced DSS intestinal inflammation would be ameliorated by preventing infiltration of this population of cells, but not completely abolished since \textit{Map} infected macrophages alone appear to have an enhanced pro-inflammatory profile which could promote intestinal inflammation. Furthermore, since increased numbers of F4/80 positive cells appeared to be associated with cecal crypt epithelium, the role of macrophages in the proliferative or repair response in DSS mediated intestinal injury could be assessed since it has been shown that crypt epithelial associated activated macrophages play an important role in the reparative response following DSS mediated intestinal injury.[41] Similar studies to those performed Chapters 2 and 3 could be performed, but after 6 days of DSS treatment, administer water for a period of a few weeks to allow for repair to occur.

References:


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