Provocateurs and triggers: induction of antigen-specific responses to the colonic resident microbiota and susceptibility to colitis

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Provocateurs and triggers: induction of antigen-specific responses to the colonic resident microbiota and susceptibility to colitis

by

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These works are dedicated to my mother, my heart and my soul. All that I am or ever hoped to be, I owe to my mother.
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CHAPTER 1. General Introduction

Dissertation Organization

This dissertation follows the new format of thesis organization in which manuscripts are included as the main component of the dissertation. The first chapter encompasses the general introduction and literature review providing detailed experiments by other research groups with the intent to introduce the reader to topics reported on in the dissertation. Since the thesis is one complete document, the manuscripts have been formatted and included as chapters two, three, and four. These manuscripts will be in the process of submission for publication. The entire document is tied together in the last chapter discussing the results of all three manuscripts.

Literature Review

Inflammatory Bowel Disease

The gastrointestinal (GI) tract is commonly thought of as the organ with the largest surface area (100 m^2) in the body to digest and absorb 500 kg of food per year, however a function that perhaps is less thought of is its role as the physical interface between the immune system and the antigens of daily life. The GI tract is colonized with a plethora of beneficial commensal bacteria. This is a beneficial relationship because the microbiota live in a nutrient rich environment consisting of ingested food and assist in the breakdown of compounds, like cellulose for which humans do not have digestive enzymes. However, the GI tract is also exposed to environmental dangers like pathogenic bacteria that have varying capabilities to invade and to colonize the intestinal mucosa. The constant bombardment of food and microbes creates the necessity for a delicate balance between defending against pathogens while maintaining the beneficial relationship of commensal bacteria. An imbalance or defect in multiple areas of innate or adaptive immunity may result in chronic inflammatory bowel disease.

Gastrointestinal diseases have a significant economic impact costing the U.S. between 1.8 to 2.6 billion dollars per year (1). Inflammatory bowel disease (IBD) is a chronic inflammation of the GI tract. The exact etiology of IBD is unknown, but is thought to be multi-factorial. Previous studies indicate that genetics and environment strongly associate with the development of an aberrant immune response resulting in IBD (2-4). The main diseases commonly associated with IBD are Crohn’s disease (CD) and ulcerative colitis (UC). Crohn’s disease is a transmural inflammation that can manifest in any area of the GI tract; however, CD typically affects the distal ileum (5). Lesions are usually multifocal and are associated with an increased infiltration of macrophages that contribute to granuloma development. Some CD patients also experience fistulas or obstructing strictures (6). Ulcerative colitis lesions are superficial and tend to only affect the colon. These lesions are associated with neutrophil infiltration into the lamina propria and a depletion of goblet cell mucin is also observed histologically. Aphthous or linear ulcers form over Peyer’s patches and lymphoid
nodules and are characterized by purulent secretions which cause the colon to frequently empty, resulting in diarrhea. Other symptoms that patients with CD or UC may experience include vomiting, crampy abdominal pain, rectal bleeding, and weight loss. One in 300 people experience these symptoms, but typically not until the second to third decade of their life and 30% of IBD cases are diagnosed during childhood (7). Incidence rates and prevalence of UC or CD are highest in Europe and the US, while the lowest rates are observed in South America, southeast Asia, Africa, and Australia (8). Therefore, it has been hypothesized that environmental influences such as diet, improved sanitation, and pollution affect the incidence of IBD in western societies causing many to speculate that the hygiene hypothesis may contribute to disease development (9, 10). Although IBD can affect males and females equally, the Jewish population has a greater susceptibility to CD than other ethnic groups and they are three to five times more likely to get UC (8).

**Pathogenesis**

IBD is a multi-genetic disease in mice and humans that is hypothesized to result from a defect in multiple components of the immune response in immune competent individuals (11). For example, a defect in innate immunity, toll-like receptors (TLRs) results in priming of CD4 T cells resulting in IBD (12). A deficiency in T regulatory cells or an excessive amount of effector T cells also results in IBD (13-20). Although an idiopathic disease, the most agreed upon hypothesis contributing to IBD is that an inappropriate immune response develops in reaction to microbial antigens due to persistent infection, dysbiosis, and/or genetic defects that affect microbial clearance and epithelial barrier function (21, 22). The adaptive immune response that develops to the microbial invasion is inadequate, resulting in excessive recruitment of effector cells and dysregulation of local immunity, rather than alleviation or elimination of the underlying cause of disease.

**Importance of Resident microbiota/Environment**

The importance of the commensal microbiota in the pathogenesis of IBD is apparent from many studies. Biopsy samples from CD patients have a greater concentration and thicker layer of bacteria associated with the mucosal epithelium compared to healthy individuals (23, 24). Another example of the significance of the microbiota in relation to IBD is the amelioration of symptoms in patients treated with broad spectrum antibiotics like metronidazole or ciprofloxacin (25-27). Probiotics are living bacteria that have been shown to promote the health of the host by enhancing the epithelial barrier and by altering proinflammatory cytokine expression of epithelial cells (28, 29). Cocktails containing species of *Bifidobacterium*, *Streptococcus* and/or *Lactobacillus* prevent the relapse of UC patients (30). However, probiotics have been shown to have the most benefit in cases of pouchitis (22). Furthermore, 50% of CD patients have positive serological responses to bacterial components including flagellin, OmpC (outer membrane porin C of *E. coli*), I2 (microbial sequence homolog in *Pseudomonas fluorescens* of the tetR bacterial transcription factor family), or *Saccharomyces cerevisiae* (31). Research on identical twins show that only 50% that share susceptible genes for IBD develop Crohn’s disease, indicating that genetics are not the sole reason for IBD development and implicating a significant role for environmental factors in the development of IBD (32). Laboratory research has also demonstrated the significance of the enteric flora to the development or onset of colitis (21, 33-38). There are numerous animal
models of IBD including genetic knockouts, transgenic, spontaneously occurring, or chemically induced models of colitis. Although dissimilar, they share a common theme: depletion of the enteric flora results in elimination/amelioration of clinical signs of disease. For example, IL10⁻/⁻ mice spontaneously develop clinical signs of colitis when housed in conventional conditions; however, in a germ free environment, IBD does not occur, unless the mice are reconstituted with a bacterial flora (34). Others have reasoned for the importance of the enteric flora by developing IBD hypotheses based on various premises including persistent infection, dysbiosis, defective clearance of microbes, and defective epithelial barrier as discussed below (22).

Persisting infection.

Chronic infection with pathogens has been hypothesized to predispose patients to IBD. One of the most notorious bacterial agents implicated in causing IBD is *Mycobacterium avium* subspecies *paratuberculosis* because it causes colitis in cattle (Johne’s disease). Johne’s disease is a chronic wasting intestinal disease that occurs in wild and domesticated animals with granulomatous lesions primarily in the small intestine. *M. avium* was first cultured from granulomas of CD patients in the 1980’s (39). Other reports have identified *M. avium* in blood and other body fluids from CD patients (40, 41). Serum antibody levels to *M. avium* are elevated in CD patients indicating presence of this organism (42). A recent study reported that *M. avium* infections exacerbates colitis in IL10⁻/⁻ mice (43). Mucosal CD4⁺ T cells from IL10⁻/⁻ mice administered *M. avium subsp. paratuberculosis* proliferated greater and secreted elevated antigen specific IFN-γ that CD4⁺ from IL10⁻/⁻ mice alone. Arguments against *M. avium* being the causative agent of IBD are that comparisons between the clinical signs and pathological responses of Johne’s and Crohn’s disease are different (44). CD is segmental in nature and symptoms include ulcerations, bleeding, stricturing, adhesions, perforations and abscesses, whereas these are not clinical signs associated with Johne’s disease (45). Also, there have been no documented cases of *M. avium* being transmitted from infected cattle to humans (46). However, human *M. avium* has been transferred into goats and one animal was shown to have intestinal inflammation 5 months postinfection. Acid-fast bacilli were not isolated back from the intestine, but a single organism was detected in the mesenteric lymph node (39). Although this may fulfill Koch’s third postulate, more research needs to be performed to confirm if *M. avium* causes CD. In a five year clinical trial, there was no benefit or amelioration of disease when 130 patients with active CD were given anti-tuberculous chemotherapeutic agents (47). Other agents that have been implicated in IBD pathogenesis but do not have substantial data include *Pseudomonas* species, *Listeria monocytogenes*, *Helicobacter* species and measles (4, 5, 22, 48)

Dysbiosis.

Compared to human somatic and germ-line cells, the human microbiota outnumber human cells by ten fold. The enormity of this microbial community begs consideration when investigating human health and disease. This combined microbiome is a mutualistic relationship between our enteric flora and gastrointestinal tract. Humans provide the nutrient rich environment for bacteria to multiply and in exchange the bacteria assist in the degradation of food, metabolic activities, and also contribute to the development of the innate and adaptive immune systems. Because the commensal microbiota contribute to the development of the gut and
immune response, it is not outrageous to hypothesize that they may also contribute to the breakdown of intestinal health and aberrant immune response associated with IBD. One hypothesis suggests that there is an imbalance in the ratio of beneficial to harmful bacteria in the gastrointestinal tract of individuals with IBD. Examples of beneficial bacteria include *Lactobacillus* species, *Bifidobacterium* species, selected *E. coli* strains and *Streptococcus salivarius*. Aggressive bacteria include *Bacteroides* species, *Enterococcus faecalis*, adherent/invasive or toxigenic *E. coli*, *Eubacterium* and *Peptostreptococcus* species, *Fusobacterium varium*, and *Helicobacter* species. Factors that may contribute to dysbiosis include the use of antibiotics, diet, and potentially the impact of refrigeration in the 20th century or the “cold chain hypothesis” (49). Antibiotic therapy seems a natural choice to have contributed to dysbiosis since opportunistic infections may occur following treatment, however, treatment only transiently changes the microbial flora and when treatment is discontinued the original state or composition of the microbioa returns (50). There is also evidence that diet may contribute to dysbiosis. Infants that were breastfed had a disparate microflora compared to formula fed infants. Breastfeeding increased numbers of *Bifidobacteria* whereas bottle fed infants had a slightly higher percentage of *E. coli* and *Clostridium difficile* (51). Contributions of the cold chain hypothesis to dysbiosis has received mixed reviews. Hugot et al hypothesize that bacteria that can survive at lower temperatures, called psychrotropic bacteria and may have increased due to modern refrigeration (52). Some of these bacteria include *Listeria monocytogenes, Yersinia enterocolitica, Clostridium botulinum,* and *Bacillus cereus*. However, another study did not find any differences between CD patients and controls in regards to the age at which they first experienced refrigerated foods (53). Other factors can also influence the composition of the intestinal flora including geography, illness, emotional stress, and age (54-58). Compared to healthy humans, patients with CD have a decrease in microbial diversity including increased numbers of *Enterobacteriaceae* and decreased Firmicutes (including *Clostridium XIV*) (59). Swidsinski’s and colleagues reported that the diversity of adherent bacteria in IBD patient’s was very low and the main adherent bacteria in the biofilm of IBD patients was *Bacteroides fragilis* (60). Effects of an imbalance in harmful vs beneficial bacteria include the contribution of metabolic constituents to epithelial health. Butyrate, an energy substrate for epithelial cells, is produced by various species of *Clostridium* and *Bacteroides*. Therefore, decreased members of these genera may negative impact epithelial health and integrity. Another example of the importance of flora to intestinal health is shown by the fact that germfree mice renew their epithelium at a slower rate than flora-bearing mice (61). This thought has lead to probiotic and prebiotic studies that increase the beneficial bacteria like *Lactobacillus* species or beneficial nonabsorbed carbohydrates like fructose that promote the growth of beneficial bacteria like *Bifidobacterium*. Commensal bacteria contribute to the innate immune response by altering the expression of angiogenin-4, a bacteriocidal protein that kills Gram-negative bacteria. Contributions to adaptive immunity include the expansion of germinal centers and diversity of antibody repertoire and increasing production of IgA (62).
Genetic defects

IBD is a multigenic disease with a diversity of genes implicated in its pathogenesis. However, when comparing the penetrance of IBD for monozygous twins, they only have a 20-50% chance of developing CD, and only a 6-14% chance of developing UC (32). While important, these observations indicate that genetics are not the sole indicator or predictor for IBD development and that environmental triggers are likely to have an impact. Although abundant, the genetic aberrations contributing to IBD progression are categorized into 3 main classes: defective clearance of microbial organisms, aberrations in mucosal barrier and aberrant activation/regulation of immunity.

Defective Clearance of microbial organisms

NOD2/CARD15 (nucleotide-binding oligomerization domain 2/caspase-recruitment domain 15) located on chromosome 6, is a cytosolic receptor that recognizes bacterial components and can initiate innate immune responses. NOD2 mutations or polymorphisms are strongly correlated with 25 to 35% of Crohn’s disease cases but not with ulcerative colitis (21). Monocytes, DCs, and epithelial cells express this intracellular receptor. Activation of NOD2 through binding of muramyl dipeptide (MDP), a product of peptidoglycan degradation, activates NF-kB or the mitogen-activated protein kinase (MAPK) pathway (63). Mice that are deficient for NOD2 do not spontaneously develop IBD; however, they are susceptible to bacterial infections (64). When intragastrically gavaged with *L. monocytogenes*, NOD2−/− mice had greater numbers of bacteria in the liver and spleen compared with wildtype mice. This lack of innate protection was attributed to a defect in NF-kB activation in Paneth cells, thus decreasing production of antimicrobial peptides (64). Another group developed mice with a NOD2 knockin (KI) mutation at position 2939 (similar to the human NOD2 mutation 3020) (65). NOD2 KI mice were more susceptible to DSS-induced colitis compared to wildtype mice and were characterized by greater NF-kB activity. When macrophages were stimulated with MDP, they had greater NF-kB activation and IL-1β secretion than controls. This model may be more consistent with human CD since patients have elevated NF-kB activity. Since MDP can also be recognized by TLR2 and activate NF-kB through MyD88, it was hypothesized by Watanabe *et al* that TLR2 and NOD2 signaling may be linked (66). Recent studies showed that pretreating mice with MDP, thus upregulating NOD2 expression, protected them from DSS or TNBS-induced colitis (66). This protection was mediated through suppression of multiple TLR pathways including TLR2, 3, 4, 5, and 9 and required IRF4. Furthermore, the ability of MDP pretreatment to protect mice from DSS-induced colitis was lost when NOD2 knockout mice were used in these studies (66). NOD2 has recently been linked with adaptive immunity in the induction of IL-17 cells from T cells. Van Beelen *et al* (2007) showed that NOD2 activation by peptidoglycan stimulated human DCs to secrete IL-18 and IL-23. These activated DCs induced the production of IL-17 by memory T cells (67). Likewise, DCs harvested from human CD patients having mutations in NOD2 that were primed with MDP and Pam3CSK4 and incubated with T cells were unable to induce production of IL-17 compared to NOD2 sufficient DCs. Cumulative results indicate that aberrant NOD2 expression results in susceptibility to IBD (68).
Another manifestation of mutations in NOD2 is Blau syndrome, a rare disorder characterized by skin rashes, arthritis, and/or uveitis (69). Indicating that NOD2 mutations are not the solitary reason behind CD development, but may factor into the understanding of the etiology. NEMO (Ikb kinase-γ) is a regulator of NF-kB activity and when this gene is knocked out in intestinal epithelial cells mice develop severe pancolitis (70). Other characteristics include decreased secretion of antimicrobial peptides and increased bacterial translocation indicating that NEMO is essential to gut barrier health and integrity. Other genes associated with IBD that function to clear microbes are related with autophagy. ATG16L1 (Autophagy-related 16-like gene) is expressed in intestinal epithelium, APCs, CD4/8 T cells, B1 cells, and memory B cells. ATG16L1 and IRGM (immunity-related guanosine triphosphatase-human homologue of mouse Irgm/Lrg47) are associated with increased risk for Crohn’s disease (71-73).

**Defective barrier**

Genes that contribute to defective epithelial barrier function have also been implicated in the susceptibility to IBD. DLG5 encodes an epithelial scaffolding protein and a single-nucleotide polymorphism in DLG5 has been associated with individuals who also have the risk-associated NOD2/CARD15 alleles and Crohn’s disease (74). The multidrug resistance gene (MDR1) encodes P-glycoprotein 170, an efflux drug transporter, and deletion of this gene results in colitis in mice (21, 75). Although MDR1a−/− mice develop a spontaneous colitis, research from a recent Dutch study indicated that there is no association between the human equivalent polymorphism, (exon 26, C3435T) and IBD (76). Prostaglandin receptor EP4 (PTGER4) is expressed by intestinal epithelial cells and regulates barrier function. Polymorphisms in EP4 have also been associated with CD in a recent genome-wide association study (77). Other genes associated with altered epithelial barrier functions include Gai2, Muc2, and N-cadherin. The α subunit of the Gi2 protein (on lymphocytes, APCs, gastrointestinal epithelial cells and other cell types) regulates adenylate cyclase activity and facilitates Th1 mediated colitis in Gi2a deficient mice. Mucus is an important barrier protecting the epithelium from luminal contents and Muc2 is a goblet cell-derived secretory mucin. Muc2 is downregulated in CD patients (78) and Muc2−/− mice are deficient in goblet cells and develop spontaneous colitis (79). Mice that are transgenic/chimeric expressing a dominant negative mutant of the cell adhesion molecule N-cadherin in intestinal epithelial cells develop chronic colitis characterized by a breach in epithelial permeability (80).

**Aberrant activation/regulation of immunity.**

Dysregulated immune responses are thought to contribute to IBD development and it is hypothesized that aberrant immune responses arise as a result of a defect in one or more of the following mechanisms; epithelial barrier & health, APC sampling (antigen presentation), regulation of innate/adaptive immunity, and/or excessive generation of effector T cells (22). Aside from the mucus layer, epithelial cells provide the first line of defense as a physical shield against the plethora of antigens in the gut lumen, a chemical barrier by the secretion of antimicrobial peptides by Paneth cells, and an immunological shield, engaging innate receptors to microbial ligands. In addition to the role of the epithelium in clearance of bacteria and permeability mentioned above, another important role is the recognition of microbial components through innate receptors. Innate mechanisms
that have been implicated in driving IBD include TLRs, NOD2, and pyrin-domain containing proteins that recognize components of bacterial cells to initiate secretion of downstream inflammatory cytokines (81). A simplistic form of innate immunity is the recognition of structures with repeated patterns. One class of pattern recognition receptors (PRRs) are TLRs, transmembrane proteins that recognize bacterial products and viral nucleic acids. Although the elucidation of TLRs is not complete, they aid in defense on epithelial cells and APCs by inducing antimicrobial peptides, chemokines, and cytokines through NF-κB activation (82). There are prophylactic effects of TLR agonists in murine colitis models, but mutations in these receptors can have detrimental effects as well (83). For example, TLR2−/− TLR4−/−, TLR9−/− and MyD88−/− mice are susceptible to DSS-induced colitis. Mice that have TLR deficiencies and leaky epithelia develop chronic inflammation characterized by excessive infiltration of CD4+ T cells. NF-κB activation mediated through TLR signaling is beneficial to epithelial homeostasis, but exacerbation can be detrimental. For example, Deletion of IκB kinase γ (NEMO) resulted in spontaneous colitis (70). The location of the TLRs and downstream signals can also result in differential immunological effects. Stimulation of TLR9 by synthetic DNA on the basolateral side of the epithelium activated NF-κB, whereas apical stimulation blocked NF-κB signaling due to inhibiting the breakdown of ubiquinated IκBα (84). Peroxisome proliferative-activated receptor γ (PPARγ) is an intracellular receptor that inhibits NF-κB activation and is highly expressed in adipocytes, macrophages, T and B cells and colonic epithelial cells (85). Polymorphisms in the PPARγ gene have been linked to increased susceptibility to ileitis development in the SAMP1/YitFC mouse model of spontaneous colitis (86). Defective PPARγ activity has also been linked to UC in that downregulation of PPARγ expression in colonic epithelium is observed (87, 88).

**APC sampling.**

When most strains of mice that are often used as animal models of IBD are housed under germfree conditions, they do not develop clinical signs of colitis (37, 38, 89, 90). However, if they are reared under SPF or conventional housing conditions, they develop severe colitis. Therefore, the presence of an enteric flora plays a critical role in the development and severity of IBD. In this regard, it is reasonable to hypothesize that a defect in sampling (i.e., antigen processing and presentation) of antigens derived from the microbiota may predispose to IBD. There are three main routes for the bacterial antigens to be sampled and presented to the immune system. The most studied pathway is entry of the microorganism through M cells overlying Peyer’s patches. M cells are polarized epithelial cells specialized for antigen transport that phenotypically differ from other epithelial cells located in the follicular associated epithelium (FAE). The apical membrane has no brush border, but microdomains instead to facilitate transcytosis. The basolateral membrane is deeply invaginated to form an unusual pocket (91). This pocket is full of T and B cells and macrophages and shortens the distance that the transcytosed antigen in the vesicle has to travel to be phagocytosed and presented to lymphocytes (91, 92).

The second method of APC sampling has been shown by Rescigno et al to be mediated by immature DCs extending their dendrites between and beyond the apical surface of epithelial cells to sample luminal bacteria while secreting tight junction proteins to preserve epithelial integrity (93). The third method to sample bacteria
is by the use of neonatal Fc receptors (FcRn). FcRn functions to transport IgG in the milk across the intestinal epithelium and into the bloodstream which serves to maintain serum IgG levels (94, 95). Recently, basolaterally expressed FcRn was shown to transport IgG from the lamina propria through epithelial cells and to the lumen to bind bacterial antigens. Following capture of antigens, the FcRn transports the package back to the lamina propria to be phagocytosed by DCs, and processed for antigen presentation to T cells (96, 97). This leads to activation and differentiation of CD4⁺ T cells into Th1 effectors to kill the invading bacteria or Th2 effectors to aid in differentiation of B cells into plasma cells that secrete antigen-specific IgG. Because bacterial antigen sampling is the initial phase in development of aberrant bacterial-specific T cells, these pathways may need to be studied in more depth. Recently some studies have shed light on how the second sampling pathway may be manipulated to cause or ameliorate IBD. Dendritic cells that were cultured in the presence of vasoactive intestinal peptide (VIP) secreted IL-10 and induced regulatory T cell development (secreted IL-10 and TGF-8). Injection of VIP-stimulated DCs ameliorated TNBS induced colitis as shown by reduced gross and histological scores, reduced weight loss and decreased production of inflammatory cytokines and chemokines (98). DCs have also been associated with the induction of IBD. A CD11c⁺ F4/80⁺ population that secretes IL-23 has been implicated in granuloma formations in an experimental IBD model that was dependent upon the presence of an intestinal microbiota (99). Furthermore, antigen sampling aberrations have also been implicated in predisposing individuals to IBD. Specifically, an increased risk for the development of IBD has been linked to mutations in the IBD3 locus on chromosome 6p, encompassing the major histocompatibility complex (MHC). There are strong associations between HLA and CD or UC (2, 90). There is an inverse correlation between UC and CD with respect to HLA-DR2 mutations in that there is a positive association between HLA-DR2 and UC genes and a negative association with CD genes (48).

**Regulation of innate/adaptive immunity**

Considering the total microbial load, 10^{13}-10^{14} organisms, the diversity of 1800 genera species that colonize the human gut, and the constant bombardment of food and environmental antigens, it is amazing that the immune system can enforce tolerance in the homeostatic state. IBD patients have broken tolerance shown by increased T cell reactivity and serum responses to bacterial antigens (31, 100, 101). Peripheral tolerance is primarily mediated by regulatory T cells (Tregs). Since the discovery of “suppressor T cells” in the 1970’s, research has advanced our knowledge tremendously (102). Regulatory CD4⁺ T cells are a population of lymphocytes that are able to suppress both innate and adaptive immunity (20, 103, 104). Suppression of harmful inflammatory responses is important to maintain homeostasis, however elevated Tregs in peripheral blood and tissues have been associated with cancers (105, 106). Likewise, a lack of or defect in Tregs is associated with autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, EAE and IBD (107). Therefore, a balance of regulation and effector functions is required to maintain homeostatic conditions. T regulatory cells are categorized into naturally occurring and induced/adaptive Tregs. Naturally occurring Tregs develop in the thymus and make up 5-10% of mouse peripheral T cells, however, only 1-2% of human peripheral T cells. Cell surface molecules to identify this population are unfortunately shared by other cell types. For example, Tregs are typically
characterized as CD4+CD25+GITR+CTLA4+ among others, however, CD25, GITR and CTLA4 are activation markers that can be upregulated on effector non-suppressive cells. The hunt for new cell surface markers to identify Tregs is underway. The discovery of the mutation in Foxp3 that is responsible for the autoimmune disease, IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked), identified Foxp3 as a transcription factor solely for T regulatory cells. Tregs have been shown to have multiple mechanisms of suppression including cell-cell contact (108, 109), death by granzyme B/perforin (110), depleting other effectors of IL-2 to induce apoptosis (111), and disrupting metabolic activity by inducing intracellular or extracellular release of adenosine nucleosides (112). Tregs have also been shown to induce indoleamine 2,3-dioxygenase (IDO) in DCs that influence Tregs by upregulating heme oxygenase-1 (HO-1) through 3-hydroxyanthranilic acid (HA), a byproduct of tryptophan metabolism (113). Induced Tregs include the Tr1 and Th3 populations and the primary mechanism of suppression is mediated by IL-10 and TGF-β. Recently, a new member of the IL-12 family, IL-35 has been shown to be required for Treg suppression (114). Although it has not been established if Tregs protect against human IBD, there is clear evidence of protection in mouse models. Powrie et al showed that transfer of naïve T cells (CD45RBhi) into SCID or RAG−/− induced colitis. Transfer of memory CD45RBlo cells would protect against colitis that was associated with IL-10 and TGF-β (14-16). Tregs have also been shown to cure established colitis (115). CD103+ DCs have been shown to induce Tregs through TGF-β and retinoic acid (116). Transfer of Foxp3+ Tregs can prevent or reverse established colitis. Mechanisms of suppression are thought to depend upon TGF-β, IL-10, IL-2, and CTLA-4. CTLA-4 inhibits T cell activity by outcompeting CD28 for binding to B7 on the APC. The impact of Tregs in immune homeostasis is manifested when genes critical for their suppressive functions are depleted in mouse models. For example, CTLA-4−/− and TGF-β−/− develop a fatal multi-organ autoimmune disease and IL-10R−/−, SMAD 3−/− (mediates TGF-β signaling), or IL-2−/− induce colitis. IL-2 is required for maintenance and generation of T regs. IL-10−/− mice are disease free if reared in germfree conditions, however, develop colitis if housed in specific pathogen free (SPF) conditions indicating that the enteric flora drives colitis. The ability of B cells to secrete IL-10 in an inflammatory environment and to downregulate IL-18 supports their role in the induction of peripheral tolerance (117). B cells were also shown to be protective in the non-obese diabetic mouse model of diabetes (NOD mice). LPS treated B cells express FAS ligand and secrete TGF-β. When LPS treated B cells are transferred into prediabetic NOD mice, diabetes is prevented through inducing apoptosis of Th1 cells and downregulating APCS by TGF-β (118). Furthermore, colitis induced by transfer of pathogenic CD3 T cells from Gai2−/− into Rag−/− is prevented if wildtype MLN B cells are cotransferred. This protection is associated with an increase in regulatory CD4+CD8α− cells (119). Further investigation will reveal whether B cells have a direct or indirect effect on T regulatory cells in the future and this interaction may aid in the design of therapeutics.

**Excessive effector T cell function**

The daunting task of the gut to maintain a state of hypo-responsiveness to commensals, yet mount an efficient T effector and/or antibody response to pathogens is mystifying. These immunological responses must be tightly regulated to prevent unwanted inflammation, therefore, alterations in this regulation may predispose
individuals to IBD. Naïve T cells migrate throughout secondary lymphoid tissues until encountering their cognate antigen. Upon activation and maturation into effector T cells, they are now able to migrate into peripheral tissues to secrete antigen specific antibody or cytokines. Migration is enabled due to the upregulation of chemokine receptors on the cell surface. T cells that home to the gut upregulate CCR9 and α4β7. CCR9 is a receptor for CCL25, expressed on IELs and endothelial cells in the lamina propria. The intestinal venules express mucosal addressin cell adhesion molecule-1 (MAdCAM-1) and following binding to the integrin α4β7, on T cells, induces extravasation through the tissue. Activated T cells have a tropism for the tissue that the draining lymph node is in. For example, T cells that are activated in the MLN or Peyer’s patches home to the gut whereas T cells activated in cutaneous lymph node’s migrate to the skin (120). Depending on the environment, CD4 T cells may differentiate into Th1, Th2, Th17 or regulatory effector cells (121). Th1 cells predominantly secrete IFN-γ and IL-12 to control intracellular infections, whereas Th2 cells aid in humoral immunity by secreting IL-4, 5, 9, or 13. Interleukin 17 produced by Th17 cells recruit neutrophils, stimulate endothelial cells, macrophages, epithelial cells, and fibroblasts to secrete IL-1, IL-6, TNF-α, NOS-2, metalloproteases and chemokines in response to external bacterial and fungal infections (121). Induced Foxp3+ regulatory T cells can upregulate CD25 and cytotoxic T-lymphocyte antigen-4 (CTLA-4) and secrete IL-10 or TGF-β in response to inflammatory cytokines (122). A fine-tuned balance of all T cell lineages are required to maintain homeostasis, and an immune imbalance may trigger IBD in susceptible individuals. CD4+ T cells have been implicated in IBD due to a report of patients having anti-CD4 antibodies (123). Also treatment of CD patients with anti-TNF antibodies increased CD4 T cell apoptosis as lesions decreased indicating that TNF secreting effector cells may be contributing to the pathogenesis of CD (124). Previous reports of IBD pathogenesis state that CD is predominantly Th1 driven having increased IFN-γ and IL-12 (p70) secretion. Alternatively, UC is mainly Th2 driven due to increases in IL-4 and IL-13. Recently, CD etiology has expanded to include IL-17 and IL-23. Naïve T cells develop into Th17 cells in the mouse, in the presence of TGF-β and IL-6, compared to IL-1 and IL-6 in humans. Mice deficient in TGF-β do not have Th17 cells and there is increasing interest in the role that regulatory cells play in the development of Th17 cells. Expansion of IL-17 cells requires IL-23 (p19) and recent evidence suggests that IL-21 regulates Th17 cells (125). IL-23 (p19 & p40) contributes to Th1 mediated colitis in the following models: IL-10−/− mice (126, 127), transfer of CD45Rbhi cells (127), induction of pathogenic antigen-specific T cells (128), colonization of immune deficient mice with Helicobacter hepaticus (129), and CD40 mediated activation of innate immunity (130). Th17 popularity has increased due to the finding of increased IL-17 in patients with autoimmune diseases like RA, MS, asthma, and IBD (131, 132). While IL-12 and IFN-γ are required for killing intracellular bacteria, IL-17 and IL-23 are required for killing extracellular bacteria and fungi (121). Yen et al showed mice possessing depletions in both IL-10 /IL-12 (p35), but not IL-10/IL-23 (p19) developed spontaneous IBD indicating that disease manifestation required the presence of IL-23 (126). There are many knockout or transgenic animals models resulting in spontaneous IBD (see Table 1). Many have hypothesized that the target of these pathogenic CD4+ T cells is the intestinal flora since there are many knockout or transgenic mouse models that have ameliorated or no clinical
signs of IBD when housed in SPF or germfree environments. Cong et al showed that CD4+ T cells from C3H/HeJ Bir mice that spontaneously develop colitis, were reactive to enteric bacterial antigens (133). Transfer of the reactive CD4 T cells into C3H/HeSnJ scid/scid mice induced colitis indicating that the enteric flora drives aberrant effector T cells in the pathogenesis of IBD.

<table>
<thead>
<tr>
<th>Innate immunity</th>
<th>Adaptive immunity</th>
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<tbody>
<tr>
<td>Mdr1α, A20, NEMO, Tir8, NFATc2, STAT3, GPX1/2, XBP1, Muc2 and Keratin8, NCAD and GFAP-HA</td>
<td>IL-2, IL-2Rα, IL-2Rβ, Gai2, Smad3, TNF(ARE), CBI-b, IL-10, TGFB, CdCS, TCRα, WASP, and Runx2, HLA-B27, CD3ε, IL-7, gp39, LIGHT, STAT4, IL-15</td>
</tr>
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Table 1. Spontaneous and transgenic models of IBD mediated by genes associated with innate or adaptive immunity (134).

**Epithelial Defense in Response to Microbial Interactions**

There are many defenses against microbes within the many physiological layers of the gut. Epithelial cells lining the GI tract are an innate barrier of defense, protecting the lamina propria cells from the contents of the lumen. The role of these innate sensors is critical in maintaining the homeostasis of the gut since most of the initial interactions between commensal and pathogenic microbes occur through the epithelial cells. Since the enteric flora are critical to IBD development, knowledge of epithelial and microbial interactions is important in the discovery of therapeutics and prevention of disease.

A single layer of polarized epithelial cells, spanning 200 square meters acts as a mechanical, chemical and biological barrier to the environmental antigens of the lumen. A thick glycocalyx lines the epithelium and prevents adhesion by mucus. Tight junction extracellular adhesive proteins including occludins, claudins and jam associate with zonula occludens, zonula adherens and macula adherens to make up the paracellular barrier of the epithelium. A breach in permeability and altered tight junctions have been associated with IBD (135).

**Innate receptor recognition of enteric flora: PAMPs-NF-κB.**

Pathogen associated molecular patterns (PAMPs) are conserved structures on bacteria and trigger innate signaling cascades after binding pathogen recognition receptors (PRRs). PAMPs include LPS, peptidoglycan, lipoteichoic acid, flagellin, non-methylated DNA and double stranded RNA. Epithelial cells express PRRs including TLR2, 3, 4, 5 and and TLR9. Healthy individuals have a low expression of TLR indicating a hyporesponsiveness to luminal flora; however, elevated TLR expression is associated with IBD. A recent study found a greater expression of TLR2 and TLR4 in colonic biopsy samples from children with either freshly diagnosed or relapsed IBD (136). Mice having a depletion in TLR2, 4, 9, or MyD88 genes have a greater sensitivity to acute DSS induced colitis that wildtype controls indicating the importance of NF-κB signaling in
epithelial health. Some T cell mediated colitis models including IL-2\(^{-/-}\) and CD4\(^{+}\)CD62L\(^{+}\) transfer models are also predisposed to IBD in TLR9/-/- and MyD88/-/- mice (83). Interestingly, when TLR agonists are prophylactically given by oral or systemic routes in various acute and chronic models of colitis, mice were protected from colonic inflammation (83). One downstream pathway of TLR signaling is NF-\(\kappa\)B activation and proinflammatory cytokines including TNF-\(\alpha\), IL-1\(\beta\), IL-12 (p40) and IL-23 (p19) that are dependent upon NF-\(\kappa\)B activation are upregulated in IBD patients (137). NF-\(\kappa\)B expression ensures homeostasis by controlling antimicrobial peptide secretion, cell survival, and permeability. Innate regulation of NF-\(\kappa\)B activity is apparent following subsequent TLR ligation resulting in reduced NF-\(\kappa\)B activity. The reduction of NF-\(\kappa\)B activity is through multiple inhibitors of NF-\(\kappa\)B including toll interacting protein (Tollip), IRAK-M, single immunoglobulin IL-1 receptor (SIGIRR), A20, NOD2, and PPAR\(\gamma\). Although PAMPs are shared by both commensals and pathogenic bacteria, the structure is altered. Commensal Gram-negative bacteria have a lipid-A component that is pentacylated and pathogenic bacteria have a hexacylated lipid A, thus enhancing the agonistic affinity to the LPS receptor in pathogenic bacteria (138). However, some bacteria have evolved strategies to hide by reducing acetylation of lipid A. Salmonella contains a bacterial deacetylase, PagL, that deacetylates its lipid A (139).

**Antimicrobial peptides.**

Other innate mechanisms of the host in recognition of the microbiota include secretion of antimicrobial peptides. Paneth cells in the crypts of the small intestine are the primary producer of \(\beta\)-defensins (HD5 and HD6) in response to NOD2 activation, however, \(\beta\)-defensins can be made by epithelial cells in other types of tissues including skin, respiratory tract and GI tract (140). Defensins are a class of small and cationic antimicrobial peptides that kill bacteria by disrupting their cell membrane. Crohn’s disease patients with NOD2 mutations have decreased production of \(\beta\)-defensins indicating that NOD2 regulates this function. Human \(\beta\)-defensin-1 (HBD1) is constitutively secreted in the colon and small intestine whereas \(\beta\)-defensin-2 (HBD2) is an NF-\(\kappa\)B target inducible by bacterial stimulation under inflammatory conditions in the colon. HBD3 is an interferon-\(\gamma\) regulated gene secreted by the colon and small intestine, that has been reported to be upregulated in UC (141). Cathelicidin LL37/hCAP18 in humans in constitutively produced by epithelial cells in the colon. One study reported that stimulation of TLR2 and TLR4 by LPS and peptidoglycan upregulated \(\beta\)-defensin production demonstrating the protective features of the epithelium (142).

**Apoptosis of IECs.**

The rapidly proliferating intestinal epithelium adapts to environmental conditions by turning over every 24-96 hours when new cells proliferate and migrate up the crypts to replace apoptosed cells. Although commensal bacteria are not invasive, pathogenic bacteria can infect cells and disrupt this proliferation-apoptotic cycle. Some bacteria, like enteroinvasive E. coli, can induce apoptosis of epithelial cells that can potentially benefit the host by depleting infected cells or the bacteria by delaying signals to innate immune cells to destroy the bacteria (143). Other bacteria have virulence factors that allow invasion and or damage of the epithelial cell.
Following colonization with a pathogenic organism, the epithelial cell responds by inducing apoptosis or inflammation, depending on which is more beneficial for survival of the host.

**Interaction of bacterial virulence factors on epithelium.**

Not all bacteria have to be invasive to induce damage to epithelial cells. *Vibrio cholerae* and enterotoxigenic *E. coli* (ETEC) secrete toxins following endocytosis, that bind to G proteins and activate adenylyl cyclases. Upregulation of cAMP activates protein kinase A and ion transporters leading to diarrhea (144). Another example of a powerful virulence factor is invasin. *Yersinia* express this surface attachment factor that binds to integrins on the apical side of M cells in the gut. Binding of invasin to integrins induces bacterial uptake into the cell by actin rearrangement. Many diseases manifest following this invasion tactic including gastroenteritis by *Y. pseudotuberculosis* and *Y. enterocolitica* and the plague by *Y. pestis*. *Helicobacter pylori* has virulence factors including type IV secretion systems that aid in attachment. *H. pylori* contains an omp-related protein, BabA that binds to the blood group antigen Lewis b (Le^b^) which is produced by the pit and surface mucous cells of the stomach. Once attached, *H. pylori* produces CagA and the vacuolating toxin, VacA. VacA functions to block the endosome pathway, thus inhibiting intracellular trafficking. Mutations in CagA result in less IL-8 secreted from *H. pylori* infected gastric epithelial cells and CagA+ specimens from gastric biopsies in patients had increased IL-8 compared to CagA- strains (145, 146). Type III secretion systems (TTSS) are common in gram negative bacteria and mediate the transport of bacterial effector proteins into the cell cytosol of eukaryotic cells by interacting with the actin cytoskeleton and disrupting signal transduction pathways. Many bacteria have been reported to have TTSS including *Yersinia, Salmonella, Shigella, Escherichia, Pseudomonas, Bordetella, Burkholderia*, and *Chlamydia* (147). Pathogenicity islands encode the genes for TTSS and bacteria that have mutations in these genes lack pathogenicity. Bacterial flagella are produced in bacteria to promote motility and thus aid in colonization, adhesion, invasion, and secretion of virulence factors (148). Recently, IgG specific for flagella from the *Clostridium* phylogenetic cluster XIVa has been demonstrated in the serum of patients with CD and from a murine colitis model (149, 150).

**Epithelial secretion of chemokines as a microbial defense.**

Epithelial cells secrete many chemokines and cytokines in response to microbes. Following infection, the epithelium undergoes activation that results in increased NF-κB or AP-1 expression leading to proinflammatory or chemokine secretion (151). CCL5/RANTES (regulated upon activation, normal T-cell expressed and secreted), and CCL2/MCP-1 (monocyte chemotactic protein-1) are CC chemokines that can recruit monocytes and promote transepithelial migration of TCRαβ^+^ and TCRγδ^+^ T cells (152). CC chemokines including RANTES and MCP-1 have been reported to be elevated in active colitis and correlate with increased leukocyte infiltration in colonic tissue (153-156). CXCL8 (IL-8) is a neutrophil attractant secreted from the basolateral side of the intestinal epithelium in response to invading bacteria or bacterial colonization. Following epithelial secretion of CXCL8, neutrophils will translocate across the epithelium and enter the gut lumen, where they can secrete antimicrobial peptides. IL-8 has been implicated in the pathogenesis of IBD due to elevated levels of protein observed in active inflammatory tissues of IBD patients (157). The murine homologue of IL-8 is MIP-2
CCL20 (MIP3α) is a dendritic and T cell chemoattractant, constitutively secreted by the epithelium overlaying Peyer’s patches that is upregulated following inflammation. Epithelial cells secrete many other chemokines to recruit monocytes and T cells including MCP-1, CCL22, CCL9, CCL10, CCL25 and CCL28 (159-162).

**Effect of microbiota on gut homeostasis.**

The commensal flora is required to maintain homeostasis in the gut. Colonization of the gut flora begins directly after birth as bacteria multiply becoming more specialized and diverse. As stated previously, the enteric flora benefit the human host in many ways. For example, *E. coli, Bacteroides spp.* and *Clostridium spp.* deconjugate and dehydroxylate bile acids (163). *Eubacterium* converts cholesterol to coprostanol and *Clostridium ramosum* converts bilirubin to urobilinogen (164, 165). Vitamin K is also produced by many bacteria including *Lactobacillus* and *Bifidobacterium* (166). Germfree mice display epithelial deformities including: hypoplasia of intestinal villi, shorter crypts, fewer cells, cuboidal morphology of enterocytes, lack of development of lymphoid follicles, luminal mucus inspissation (don’t have flora to degrade secreted mucus glycoproteins). However, re-colonization with enteric flora reverse these conditions (167). Oral gavage of germfree mice with *Salmonella typhimurium* induced a two-three fold increase in M cells, crypt depth and enterocyte migration after seven days (168). Enteric flora also provide competition of niches and nutrients to protect against pathogenic bacteria. Germfree bacteria have an enlarged cecum compared to conventionally colonized animals, which is partially resulting from expansion of undegraded mucus due to a lack of bacteria that produce glycoside hydrolases like *Peptostreptococcus micros, Ruminococcus* and *Bifidobacterium* (169, 170).

Homeostasis of the adaptive immune system is also influenced by the commensal flora. Germfree mice produce less IgA and have less circulating B and T cells compared to conventional flora mice (62). The commensal flora also affects the GALT in that colonization of germfree mice with flora induces a larger number of αβ TCR intraepithelial lymphocytes (171). A preimmune antibody repertoire also depends upon the presence of the flora by promoting somatic diversification (172).

**Induction of tolerance**

**Central tolerance**

Central tolerance is defined as the prevention of immunological responses to self antigen induced in developing lymphocytes. This process occurs in the thymus for T cells and the bone marrow for B cells. Following TCR rearrangement and positive recognition of MHC/peptide in lymphocyte development, cells are faced with negative selection. Negative selection ensures deletion of cells with a high affinity for self peptide and mechanisms include clonal deletion, anergy, receptor editing, or development of regulatory cell lineages (173). These processes are highly regulated and autoimmune diseases can arise if mutations occur in key genes. For example, mutations in the autoimmune regulator (AIRE) gene inhibit clonal deletion and result in autoimmune polyendocrinopathy-candidiasis-ectodermal-dystrophy syndrome (APECED) (174). Another autoimmune disease, immunodysregulation, polyendocrinopathy and enteropathy, X-linked syndrome (IPEX) occurs due to a mutation in forkhead box P3 (FOXP3), a transcription factor required for T regulatory
development (175). The body has also conceived a secondary measure to induce tolerance in the scenario that cells escape primary lymphoid tissues and migrate to the periphery to encounter alternate self proteins: peripheral tolerance.

**Peripheral/Oral tolerance**

This phenomenon was described almost 100 years ago by Besredka referring to anaphylactic sensitization to milk proteins (176). Immunological non-responsiveness to antigens that were previously administered by the oral route is referred to as oral tolerance. This form of peripheral tolerance was thought to evolve to prevent unwanted responses to dietary antigens and commensal flora. Oral tolerance has been observed in animal models by diminished immunological responses to antigen that was repeatedly ingested in large doses followed by systemic immunization with the same antigen (177). There are two reported mechanisms of oral tolerance. First, oral antigens repeatedly given at low quantities induce regulatory cell driven tolerance. Mice orally challenged with myelin basic protein (MBP) were protected from experimental autoimmune encephalomyelitis (EAE). Mesenteric lymph node CD4+ cells were cloned from these orally tolerized mice and were found to secrete TGF-β and IL-10 (178). Another study found that cells from Peyer’s patches of MBP orally tolerized mice secreted TGF-β and suppressed proliferation of other cells in vitro and protected mice from EAE following cell transfer (179). Other studies have shown that oral antigens induce increased percentages of CD4+CD25+ T cells that express CTLA4 or Foxp3 and secrete IL-10 and TGF-β. When transferred into naïve mice, these cells can transfer tolerance (180, 181). The regulatory cytokines secreted from T regulatory cells act by bystander suppression, therefore, while the antigen to induce regulation must be specific, the downstream effects of suppression are not. This mechanism of suppression has enabled researchers to harness the power of T regulatory cells for therapies for autoimmune diseases like arthritis, EAE and diabetes (182). A second mechanism of oral tolerance is that a single, high dose of antigen induces deletion or anergy (183-185). However, recent work indicates that the “anergic” T cells may still be capable of secreting suppressive cytokines signifying an overlap of oral tolerance mechanisms (186). Other cells that secrete suppressive cytokines may also contribute to maintaining tolerance including CD8+ T cells, γδ T cells, NKT cells, B cells, macrophages, immature DCs and plasmacytoid DCs (187-191). CD8+CD28− T cells with regulatory characteristics expand after interaction with epithelial cells in the gut and are lacking in patients with active IBD indicating that these cells may contribute to IBD (189, 192). IL-10 secreting B cells that have a lower expression of CD80/86 have also been shown to be protective in colitis (193). Plasmacytoid DCs have been shown to contribute to T regulatory differentiation (188). Other less conventional suppressors like neuropeptides include vasoactive intestinal peptide (VIP), glucocorticoids, oestrogens, cortistatin, urocortin and adrenomedullin have been reported to contribute to T regulatory cell development either directly, or indirectly through DC maturation with LPS as well (98, 194).

**Mucosal Inflammation-breaking tolerance**

Three factors account for the maintenance of tolerance to the commensal microbiota: characteristics of the flora, the epithelium, and the lamina propria immune cells. For example, an impaired ability of bacteria to
attain and invade the mucosa; having defective sensors of molecular PAMPs; and immune cells that produce anti-inflammatory cytokines including TGF-β and IL-10 all promote tolerance (195, 196). Deregulation of these attributes may contribute to inflammation and development of IBD. Evidence for a breach in tolerance contributing to IBD is supported by experiments from IBD patients. Compared to healthy individuals, T cells from IBD patients proliferate and secrete cytokines in response to antigens from their fecal extracts (101). Furthermore, mononuclear cells from healthy, non-colitic mice do not proliferate in response to their own bacterial antigens, but greatly proliferate to bacterial antigens from other mice (197). However, during inflammation induced by TNBS colitis, mice have broken tolerance as displayed by proliferation in response to enteric flora that is ameliorated with either anti-IL-10 or anti-IL-12 treatment (197). Additional evidence that IBD patients have broken tolerance to the flora is evident by increased antibodies to enteric flora constituents (198).

**Objectives of Dissertation**

In conclusion, review of the literature indicates that while progress has been made towards the pathogenesis of IBD, the exact mechanisms addressing why occurrences typically develop in the second-third decade of life and how the resident flora contributes to the development of IBD still need to be determined. Furthermore, differentiation by the mucosal immune response between commensal and pathogenic organisms may play a key role in why probiotic treatments have controversial effects in IBD therapy. Therefore, the mechanisms of initial host responses to novel provocateurs may elucidate the etiology of IBD.

The specific aims of this dissertation were to 1) develop a multiple-hit model of colitis to characterize the adaptive antigen-specific immune responses following the disruption of homeostasis of the resident microbiota following an environmental inflammatory trigger in the development of IBD, 2) determine whether the increased sensitivity to DSS was a unique feature of *H. bilis* colonization, and 3) evaluate the induction of chemokines and proinflammatory cytokines from murine epithelial cells by *H. bilis* or *E. coli* exposure.

**References**


CHAPTER 2. *Helicobacter bilis* Colonization Induces Susceptibility to DSS-induced Colitis

A paper to be submitted to *Infection and Immunity*

Abigail Henderson, Zhiping Liu, Andrea Dorn, Jesse Hostetter, Albert Jergens, and Michael Wannemuehler

**Abstract**

Inflammatory bowel disease is a chronic inflammation of the gastrointestinal tract (GI) that develops in part as a result of an aberrant immune response to enteric bacteria in the GI tract. Prior studies indicate that *Helicobacter bilis* colonization of immune competent, defined-flora (DF) mice induced antigen-specific responses to their resident flora, in the absence of colitis. We hypothesize that *H. bilis* colonization of DF mice perturbs mucosal homeostasis and, thus, alters tolerance to the commensal flora and increases the host’s sensitivity to an inflammatory insult. Defined flora mice were colonized with *Helicobacter bilis* for up to 20 weeks prior to treatment with low-dose (1.5%) dextran sulfate sodium (DSS). Mice were evaluated for gross and histological lesions as well as cellular and humoral responses in comparison to controls. In comparison to DSS treated or *H. bilis* colonization alone, mice dually treated with *H. bilis* and DSS developed significantly (p < .05) increased gross and histopathological lesions including cecal atrophy, decreased colon length, and histological inflammation. Serum IgG2a responses to antigens derived from the enteric flora were increased compared to controls. Evaluation of the proximal colon indicated an increase in mucosal IL-17, IFN-γ, and TNF-α after colonization and/or DSS treatment. The magnitude of antigen-specific T cell proliferation increased with time, as Foxp3 expression declined; however, lesions did not increase in severity. Susceptibility to DSS-induced colitis was associated with increased antigen-specific reactivity to the enteric flora and mucosal expression of inflammatory cytokines. In conclusion, these studies demonstrate that perturbation of the microbial ecology following colonization with a mildly pathogenic bacteria, such as *H. bilis*, may induce antigen-specific Th1 and Th17 immune responses, thus, predisposing the host to colitis following exposure to an otherwise innocuous inflammatory insult.
Introduction

Inflammatory bowel disease (IBD) is a chronic inflammation of the gastrointestinal tract primarily composed of ulcerative colitis (UC) and Crohn’s disease (CD). The exact etiology of IBD is unknown, but is thought to be multi-factorial. Previous studies indicate that genetics and environment strongly associate with the development of an aberrant immune response resulting in IBD (1, 2). There is much evidence for the impact of the enteric flora in the development of IBD. Biopsies taken from patients with chronic IBD indicate a denser bacterial population and thicker mucosal layer in lesions compared to healthy individuals (3, 4). Furthermore, 50% of CD patients have positive serological responses to bacteria like Saccharomyces cerevisiae or bacterial components including Cbir flagellin, OmpC, and I2 (5). Murine models of IBD reiterate the significance of the gut flora (6-10). Models including genetic knockouts, cell transfer, and chemically induced models of disease are very diverse, yet they share a common theme: depletion of flora ameliorates or eliminates clinical signs of disease. For example, IL10−/− mice spontaneously develop clinical signs of colitis when housed under conventional conditions; however, in a germ free environment, IBD does not occur unless the mice are reconstituted with a bacterial microbiota (11, 12). Other evidence for the importance of the enteric flora can be shown by twin studies. The occurrence of IBD that manifests in identical twins that share an IBD susceptible locus is only 50% (13). This suggests that a genetic predisposition only accounts for half of the IBD outbreaks and environmental factors may account for the other half of disease. Lastly, clinical therapies for IBD patients include the administration of broad spectrum antibiotics like metronidazole and ciprofloxacin (14-17). While antibiotics do not cure the disease, they deplete species of the enteric flora and lead to a temporary improvement of IBD. Collectively, this evidence indicates that the enteric flora plays an vital role in the development of IBD (18-20).

Although IBD is associated with detrimental immune responses to the enteric flora, it is likely that there are multiple factors associated with the communication between the host and microbial environment and deficiencies in these interactions may manifest in colitis. It has been hypothesized that ‘multiple hits’ or defects in the innate, adaptive, or regulatory immune responses are required to induce susceptibility to IBD in immune competent individuals (21). To develop a multiple hit model of colitis in immune competent, defined flora mice, we introduced Helicobacter bilis as a novel provocateur to the microbial ecology. H. bilis is a Gram-negative, microaerophilic, spiral to curved rod. This fusiform bacterium has 3-14 flagella and periplasmic fibers wrapped around the cell and has been isolated from the liver, intestine, and bile of aged, inbred mice associated with hepatic lesions (22). Antibody responses to Helicobacters species other than H. pylori have been detected in serum from IBD patients (23). H. bilis has also been associated with IBD-like lesions in immunocompromised mice (24-26). For example, SCID mice colonized with H. bilis had clinical signs of typhlitis by 12 weeks post-infection (24). Previous experiments from this laboratory indicated that H. bilis colonization of immune competent, defined flora (DF) C3H mice, induced immune reactivity to the resident commensal flora (27). Furthermore, it has been reported that T cells reactive to antigens from the commensal flora can mediate chronic IBD (9). Recent microarray data indicated that H. bilis significantly increased gene expression associated with
proteolysis, glycoprotein synthesis, protein metabolism and immune response and significantly downregulated fatty acid metabolism, lipid metabolism and carboxylic acid metabolism genes (Zhiping Liu-submitted for publication). Although \textit{H. bilis} colonization results in mild lesions, it is conceivable that the gene expression changes and newly induced immune reactivity may increase host susceptibility to an environmental stressor/inflammatory insult. We hypothesized that the introduction of a novel bacterial provocateur into the established intestinal flora will perturb host mucosal homeostasis, inducing aberrant immune responses to the resident flora (hit #1-\textit{H. bilis}), resulting in increased vulnerability to an environmental/inflammatory insult/stresser (hit #2-dextran sulphate sodium (DSS)). IBD patients typically have relapses in inflammatory episodes between periods of quiescence. These active inflammatory episodes tend to correspond to stressful life events (28, 29). Evidence for the effects of stress on the gastrointestinal tract include alterations in intestinal permeability, increased PMN infiltration and epithelial dysfunction including increased macromolecular protein uptake (29-31). Stress has been shown to enhance colitis in rats and may predispose a person to IBD (32, 33).

DSS has been commonly used to induce acute or chronic colitis in mice as the resultant clinical signs are similar to human colitis (diarrhea, weight loss, bloody stool) (34-39). The acute phase of inflammation induced by DSS results in macrophage infiltration. Lesion restitution is characterized by the recruitment of lymphoid cells (34). Administration of DSS results in exposure of luminal contents to lamina propria cells and the severity of clinical signs positively correlates to the dose of DSS. Furthermore, since clinical signs of DSS are similar to the effects that stress can have on the gut including increased permeability and PMN infiltration, we wanted to use this chemical as an inflammatory stressor/trigger to expose innate immune cells to luminal antigens. While previous studies have indicated that colonization with specific bacteria can trigger IBD in immunocompromised mice, this is the first study to show that colonization with a novel, enteric bacterial provocateur can predispose the host to colitis in an immuno-competent, ‘multi-hit’ mouse model (40).

To test the hypothesis that a novel microbial provocateur may predispose an immuno-competent host to IBD, defined flora mice were colonized with \textit{H. bilis} for 3-20 weeks at which time they were treated with a low dose [i.e. environmental/inflammatory trigger] of DSS. The results of these studies demonstrate that \textit{H. bilis} colonization induced antigen specific immune reactivity to the enteric flora including antibody responses, increased lesion scores, and increased inflammatory cytokines that may contribute to susceptibility to DSS exposure. \textit{H. bilis} colonization increased sensitivity to low dose DSS manifested by significantly (p < .05) elevated macroscopic and histological scores that were associated with enhanced host immune responses as demonstrated by development of antigen-specific T cell proliferation.
Materials & Methods

Animals

Six- to eight-week old male and female gnotobiotic C3H/HeN:Tac mice harboring a defined flora consisting of the altered Schaedler’s flora (ASF 356: Clostridium spp. Cluster XIV; ASF 360: Lactobacillus acidophilus; ASF 361: Lactobacillus murinus; ASF 457: Mucispirillum schaedleri; ASF 492: Eubacterium plexicaudatum; ASF 500: Low-G+C-content gram-positive; ASF 502: Clostridium spp. Cluster XIV; ASF 519: Bacteroides distasonis) were originally obtained from Taconic Farms (Albany, NY) and are now bred and maintained under gnotobiotic conditions at ISU. All mice were certified free of Helicobacter prior to use and mice were routinely screened for all eight ASF members. Antigen presenting cells (APCs) were also prepared from defined flora, C3H/HeN:Tac mice certified free of Helicobacter. All experiments were approved by Iowa State University Animal Care Committee.

Experimental Design

Defined flora mice were separated into four groups of five to six mice per group: 1) Non-colonized only, 2) Non-colonized + DSS, 3) *H. bilis* colonized only and 4) *H. bilis* colonized + DSS. Groups three and four were inoculated by oral gavage with > 2 x 10^7 *H. bilis* CFU in 0.3 ml of Brucella broth three times over a 3-day period. *Helicobacter* colonization was confirmed by species-specific fecal PCR one to two weeks after inoculation and at necropsy by urease broth. Following 3-20 weeks postinfection (PI), groups two and four received filter sterilized 1.5% DSS (molecular weight=36-50 kilodaltons, MP Biomedicals, Irvine, CA) in deionized drinking water for five days, followed by four days of regular sterile water. Groups one and three received sterile water throughout the entire experiment. Immediately following the four day restitution period, mice were euthanized. Cecae were blindly scored as 0=healthy, normal control, 1=mild atrophy, 2=apparent mucus and atrophy, 3= severe atrophy, no cecal contents, overt mucus and 4= 3 + blood and colon length was measured. Colonic tissue samples were collected and fixed in 10% buffered formalin and submitted for routine histopathological evaluation. Mesenteric lymph nodes (MLN) were collected and enriched for CD4+ T cells using nylon wool columns (41). Spleen cells were harvested from DF donor mice one day prior to necropsy for antigen presenting cells (APCs) and incubated with ASF antigens (200 µg/ml) overnight following mitomycin C treatment (Fisher). APCs were plated at a 1:1 ratio with CD4+ T cells and stimulated with the following treatments: APC (no stimulant), pooled APC (all 8 ASF antigens), or anti-CD3/28 (2/.5µg/ml) for 72 hours. Supernatants were collected and analyzed by multiplex fluorescent bead assay for the following cytokines: IL-2, IL-6, IL-10, IL-12 (p40), IL-17, IFN-γ, and TNF-α. In addition, samples were assessed for antigen-specific CD4+ T cell proliferation and serum antibody responses and proximal colon was assessed by quantitative RT-PCR analysis for gene expression.
**Preparation of Bacterial Antigens**

Each member of the ASF (ASF360, ASF361, ASF356, ASF457, ASF492, ASF500, ASF502, and ASF519) were grown in anaerobically prepared medium as previously described (27). Bacterial antigens (e.g. all eight ASF strains) used for ELISA or for stimulation of lymphocyte cultures were prepared from the lyophilized cells. The cell suspension was placed on ice and sonicated for 5 minutes to prepare whole cell sonicates (WCS). WCS were sterilized by UV light irradiation and stored at –20 °C until use. Sterility of each individual WCS was confirmed bacteriologically.

**Gene Expression via Real Time RT-PCR**

Total RNA was extracted from 20 mg of proximal colon using the RNAeasy Mini Kit (Qiagen) as described by the manufacturer. The RNA samples were quantified by ND-1000 spectrophotometer (Nanodrop technologies) and treated with TURBO DNA-free DNase at 2U/µl (Ambion) to eliminate genomic DNA contamination. 5 µg of RNA was reverse transcribed into cDNA by SuperScript™ III First-Strand Synthesis System (Invitrogen, San Diego, CA). cDNA was amplified using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen). The following PCR conditions were used: 95°C for 10 minutes followed by 40 cycles of amplification (95°C for 10 seconds, 56°C for 5 seconds, and 72°C for 10 seconds). Primers for glyceraldehydes-3-phosphate dehydrogenase (GAPDH−forward primer: 5’- TCACCACCATGGGAAGGC-3’, reverse primer: 5’-GCTAAGACAGGTTGGTGCA-3’), IFN-γ ( forward primer: 5’- TCAAGTGGCATAGATGTGGAAAGAA-3’, reverse primer: 5’-TGGCTCTCTGCAGGATTTTCATG-3’), TNF-α ( forward primer: 5’- CATCTTTCTCAAATTGAGTGACA-3’, reverse primer: 5’-TGGGAGTAGACAAAGGTACAACC-3’ ) and IL-17A (forward primer: 5’-CTGAGAGCTGCCCTTTAC-3’, reverse primer: 5’-CCACACCCACCAGGATCTTC-3’) were used. The qRT-PCR was performed using Applied Biosystems GeneAmp™ 5700 Sequence Detection System. Standard curves for each gene were made using a series of cDNA dilutions. The relative RNA quantity of each gene was normalized to GAPDH (42).

**Histopathology**

Sections of cecum and colon were fixed in 10% buffered formalin. The tissues were routinely processed, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin. Tissues sections from each mouse were scored for severity of mucosal epithelial damage, glandular alterations, and magnitude/character of the lamina proprial cellular infiltrate. Histological sections were evaluated blindly by J.M. Hostetter and scores ranged from 0 (healthy) to 5 (diseased) for each parameter of erosion and inflammation reported by a previously defined scoring system (27).

**Analysis of Ag-Specific Serum Antibodies by ELISA**

Serum antibody levels in mice were determined by enzyme linked immunoabsorbant assay (ELISA). Briefly, 96-well plates (Costar-3590) were coated with 100 µl/well of whole cell sonicate (WCS) at 10 µg/ml derived
from the individual members of the altered Schaedler’s flora (ASF) and *H. bilis* diluted in PBS (pH 7.4) overnight at 4°C. Plates were washed three times in PBS/Tween 20 (.05%) and blocked with 280 µl/well 2% gelatin diluted in PBS/.05%Tween 20/2% FBS for two hours at room temperature. Plates were washed three more times with PBS/.05%Tween 20 prior to addition of 100 µl/well serum samples diluted 1:200 in PBS/Tween/2% FBS overnight at 4°C. Plates were washed five times with PBS/.05%Tween 20. Then, 100 µl of 1:2000 diluted alkaline phosphatase -conjugated goat anti-mouse IgG (H&L), IgG1 or IgG2a (Southern Biotechnology) in PBS/Tween/2% FBS was added per well and incubated for 2 hours at room temperature. Prior to washing five times with PBS/.05%Tween 20, wells were developed for a colormetric reaction using 100 µl of p- nitrophenyl phosphate (Sigma 104) (1 mg/ml) at room temperature. Absorbance was measured using an ELISA reader (405 nm).

**Analysis of Ag-Specific Serum Antibodies by SDS-PAGE & Western blots**

Duramide pre-poured 12% tris-glycine gels (Cambrex) were loaded with Precision Plus protein standards dual ladder (Biorad) or 7 µg of ASF WCS that had been boiled in SDS-PAGE buffer for 10 min at 90°C and run in a electrophoresis unit at 125 volts for approximately 1.5 hours. Following electrophoresis, gels were either prepared for western blots (below) or fixed in deionized H2O/50% methanol/14% acetic acid for 30 minutes and rinsed in deionized H2O. Gels were incubated on rocker in Gelcode Blue (company) overnight at 4°C and washed several times in deionized H2O.

After running the gel as described above, it was washed briefly in deionized H2O and equilibrated in transfer buffer (25mM Tris, 192 mM glycine, 20% methanol and deionized H2O) for approximately five minutes. PVDF membrane was pre-wet in methanol to prevent gel swelling and keep proteins absorbed to the membrane and sandwiched between blot paper and gel prior to loading into Cambrex cassettes. Cassettes were placed into a transfer unit with an ice block and run at 150 mAmps (constant currant) for 90-120 minutes. Membranes were washed three times in PBS/Tris/Tween20 and blocked in PBS/Tris/Tween20/1% gelatin overnight at 4°C. Primary antibody (1:250 dilution of pooled serum from *H. bilis* colonized mice) was incubated with membranes for 2 hours at room temperature. Membranes were washed three times in PBS/Tris/Tween20 and then incubated with secondary antibody (anti-IgG2a at 1:1000 dilution) for 2 hours at room temperature. Membranes were washed three times in PBS/Tris/Tween20 and incubated in substrate (Naphthol AS-MX/Fast red/Tris solution) at room temperature until desired color was reached (approximately 20 minutes) and placed in deionized H2O to stop the reaction.
**Preparation of Antigen Presenting Cells (APCs)/Mitomycin C Treatment**

Spleens were recovered from three adult C3H/HeOuJ mice and homogenized to prepare single cell suspensions in complete RPMI 1640, (Cellgro) containing 5% FBS, 1M HEPES, essential amino acids, non-essential amino acids, 1 mM sodium pyruvate, (2 mM) L-glutamine, penicillin-streptomycin, 25 µg/ml gentamycin and 175 µl 2-β-mercaptoethanol. Antigen pulsed APCs were prepared as described by Cong *et al* (43). Briefly, two separate aliquots of 20 x 10^6 cells/5 ml were placed into two 15 ml tubes along with 200 µg/ml (total protein content) of all eight ASF WCS (25 µg/ml each) added to one aliquot or medium alone was added to the other aliquot of APCs and both were incubated overnight at 37ºC. The APC preparations were then incubated in the presence of mitomycin C (50 µg/ml final concentration) at 37ºC for 30 to 45 minutes. The cell suspensions were washed five times using RPMI supplemented with 5% fetal calf serum (FCS) and antibiotics (41).

**T cell Enrichment**

T cells were enriched using nylon wool columns as previously described (41). Briefly, 0.6 g nylon wool was packed to the 6 ml line of a 10 ml syringe and autoclaved. Columns were then incubated with pre-warmed FCS supplemented hank’s balanced salt solution (HBSS) for 1 hour at 37 ºC. Pre-warmed medium was added to wash the column prior to the addition of 1 x 10^8 MLN cells. After addition of the cells, the columns were incubated at 37 ºC for 1 hour and cells were eluted through a 22-gage needle at 1 drop per second. Collected cells were centrifuged at 1000 rpm for 10 min and resuspended at 2 x 10^6/ml in complete medium. The recovered cells were analyzed by flow cytometry to determine the percentage of CD3^{+} T cells in the preparation (> 90 %). For antigen-specific responses, T cells were added to control and antigen-pulsed APCs at a ratio of 1:1 in flat bottom plates (Costar 3595).

**Proliferation/Cytokine Secretion by LUMINEX 100 technology**

Following 72 hour T cell/APC culture, 100 µl of cell supernatants were collected for cytokine analysis by the Luminex 100 technology and 100 µl of fresh complete medium was added back to cells. Cells were then pulsed with 10 µl of 50 µCi/ml ^3^H-thymidine for seven hours prior to harvesting. Incorporation of ^3^H-thymidine was quantified by a scintillation counter.

**Colonic Explants**

Colons were fileted open and 3 mm biopsy punches were extracted and placed into 96 well flat bottom plates (Costar 3595) in 200 µl complete RPMI 1640, 1X (Cellgro) plus 50 µg/ml gentamycin and 2X penicillin-streptomycin per well per punch. Three punches were biopsied from each mouse. Cultures were incubated at 37 ºC for 48 hours prior to recovery of supernatants and cytokine analysis.
**Statistical Analysis**

Data is displayed as mean +/- SEM. Non-parametric group comparisons were analyzed by Kruskal-Wallis tests. Parametric group comparisons were analyzed by analysis of variance (ANOVA). Pairwise group comparisons having equal variances were analyzed using t-tests.

**Results & Discussion**

**Dual treatment of *H.bilis* colonization and DSS administration increased disease severity**

In order to investigate the pathogenesis of IBD, a multiple hit model of colitis was developed. IBD is characterized by periods of quiescence interspersed by periods of active, chronic inflammation. To determine if an environmental insult could induce active inflammation following colonization with a novel organism, DSS was utilized. As a measure of an environmental/inflammatory trigger to induce active inflammation, a low dose of DSS was desired to expose lamina propria cells to luminal contents. Defined flora mice (C3H:HeN:Tac) were used for these experiments to assess antigen specific responses to the enteric flora in the development of IBD. To define a low dose of DSS to engage mild inflammatory responses, titration experiments were performed by administration of DSS that would not induce lesions and graded doses of DSS were added to drinking water for five days, followed by four days of restitution (Fig 1). A dose of 1.5% DSS was chosen for the multiple-hit model in DF mice that did not cause overt cecal or colonic damage, yet resulted in mild histological inflammatory scores in the colon, including macrophage and polymorphonuclear cell infiltrate (data not shown).

Defined-flora C3H:HeN:Tac mice bearing ASF were colonized with *H.bilis* for 12 weeks. At which time they were exposed to a low dose (1.5%) of DSS in drinking water for five days immediately preceding four days of restitution. Mice were euthanized and tissues collected. Mice that received the dual treatment of *H. bilis* colonization and DSS administration had significantly greater (*p* < .05) cecal scores and shorter colon lengths compared to any other treatment group, indicating that the combined treatments exacerbated the observed colitis (Fig 2 & 3).

To further investigate lesions of treated mice, proximal colon sections were prepared for histological analysis. In sections of colon from mice that were colonized with *H. bilis* and treated with DSS there were severe changes characterized by macrophage infiltration, ulceration, and marked mucosal hyperplasia (Fig 4B). Lesions in colonic epithelium tended to be multifocal in nature and in some sections, inflammation extended deeply into the tunica muscularis. In comparison to DSS treatment alone, *H. bilis* colonization with DSS treatment resulted in greater colonic scores (*p* < 0.05) than that for any other group, implicating the detrimental attributes of *H. bilis* colonization, prior to DSS treatment.
**H. bilis Colonization Results in Antigen-specific Responses to Resident Flora**

Broken immune tolerance to normal flora bacterial components in patients with IBD has been manifested by increased serum IgG to (44-46). Previous experiments demonstrated the induction of serum responses to antigens derived from members of the altered Schaedler flora (ASF) at 3, 6, and 10 weeks after colonization with H. bilis (47). To determine if serum specific antibody responses to enteric flora antigens plateau after 12 weeks similar to lesion severity, serum was assessed for IgG2a antibodies via ELISA. Following 3, 12, and 20 weeks of H. bilis colonization serum was collected and assessed for IgG2a reactivity to each ASF antigen. H. bilis colonized mice had elevated IgG2a antibodies to ASF 356, 360, 361, 457, 492, 500, 502, and H. bilis WCS antigens compared to non-colonized controls, suggesting a loss of tolerance to the microbiota and an increase in Th1 immune reactivity (Fig 5). Responses to *Clostridium* spp (ASF 356 and 502) were elevated and have been associated with Cluster XIV flagellin antigens. These results support findings from Elson and colleagues that colitic mice and CD patients have elevated serological responses to flagellin proteins from the commensal flora (48, 49). This suggests that H. bilis colonization results in a Th1 mediated breach in tolerance to the enteric flora. To further assess the increased IgG2a responses to the flora, western blots were performed using WCS of ASF antigens loaded on SDS-PAGE gels and blotted with serum from H. bilis colonized mice at 3, 12, and 20 weeks (Fig 6). Immunoblot analysis demonstrated that the number of antigens recognized by serum antibodies increased in number and intensity following H. bilis-colonization over time. Furthermore, molecular weight bands at 55-60 kb may correspond to flagellin recombinant protein, but further experiments needs to be performed (49). IgG1 responses were weak to non-detectable (data not shown). These results may be indicative of epitope spreading.

CD4 T cells have been implicated in the development of IBD (50). The development of antibody reactivity to H. bilis antigen and the increase over time to antigens of the commensal flora prompted us to investigate if H. bilis increased the T cell reactivity to the flora. MLN T cells were cultured with APCs that had been primed overnight with antigens from the enteric flora (ASF) or no antigen. T cells from H. bilis colonized animals, but not from control animals proliferated to the enteric flora indicating that colonization with a bacterial provocateur can induce antigen-specific immune reactivity to the enteric flora that may contribute to susceptibility to an environmental trigger (Fig 7).

**Mucosal Inflammatory Cytokine Expression is Elevated in the H. bilis Dual Group**

IL-17 has been associated with multiple human autoimmune disorders and IBD (51-54). Likewise, IL-17A has been shown to mediate intestinal inflammation in *H. hepaticus* infected mice administered anti-IL-10R monoclonal antibodies (55). To determine if there is an association between the induced antigen-specific reactivity to the commensal flora and IL-17A in the mucosa, we assessed IL-17A gene expression in proximal colon tissue and IL-17A protein in colonic explants following H. bilis colonization and DSS treatment. H. bilis colonized mice that were DSS treated induced IL-17A mRNA expression in proximal colonic tissue that was
associated with elevated protein for IL-17A from colonic explants (Fig 8). This finding indicates that IL-17A may modulate inflammation in the dually treated group. The induction of IgG2a serum responses indicated an elevated Th1 response following *H. bilis* colonization, therefore, we assessed expression of inflammatory cytokines locally in the proximal colon. IFN-γ (Α) and TNF-α (Fig 9B) RNA expression in the dual treatment group was elevated compared to control groups (Fig 9). This suggests that *H. bilis* colonization is changing the mucosal environment such that a low dose DSS insult results in inflammation. Other groups have reported the detrimental effects of IFN-γ and TNF-α on epithelial cells *in vitro* (56, 57). Although we cannot directly say that these inflammatory cells affected the colonic epithelium, histological analysis revealed that enterocytes along the luminal surface of the colonic glands within the *H. bilis* dual treatment groups presented with attenuated low cuboidal morphology compared to the tall columnar adjacent enterocytes (Fig 9C). The changes in cellular morphology were indicative of acute cellular injury that might precede enterocyte death and loss. The glands associated with these cellular changes were hypercellular and lacked goblet cells indicating a hyperplastic response.

**Mucosal Foxp3 Elevated in *H. bilis* Dual Treatment Group**

Regulatory T cells actively suppress the proliferation and cytokine production of effector T cells. To further define the mechanism behind the increase in immunological responses to the enteric flora, we hypothesized that *H. bilis* may be altering T regulatory cell function, resulting in less regulation and increased host responsiveness. Foxp3 is a transcription factor for regulatory T cell development, maintenance, and function and mice that do not express Foxp3 develop a fatal autoimmune disease characterized by hyperresponsive CD4+ T cells (58). *H. bilis* colonization mice treated with DSS displayed elevated levels of Foxp3 expression in the proximal colon at three and 12 weeks post-infection (Fig 10). Increased T regulatory cells at the site of infection may be beneficial for the host to prevent overt inflammation, however, this may also be beneficial for the pathogen, to sustain colonization.

**DSS Treatment and *H. bilis* Colonization Increased Bacterial Invasion Within Colonic Tissue**

To confirm *H. bilis* colonization prior to DSS administration, fecal samples were collected and DNA was extracted for PCR analysis by using primers for the 16s ribosomal RNA. To determine if the *H. bilis* and DSS dual group was more severe due to *H. bilis* attachment to the epithelium, thus increasing its pathogenicity, colonic samples were harvested and fixed in gluteraldehyde for analysis by electron microscopy. *H. bilis* organisms were found in the lumen in close proximity to the mucosa (Fig 11-A), within the interepithelium (Fig 11B-C), and occasionally within the colonic tissues (Fig 11D). The results of the PCR analysis and the electron photomicrographs indicated that *H. bilis* did not transiently colonize, but persisted. The close proximity of the organism within the gland or in the epithelial cells suggests an increased probability of APC sampling and bacterial translocation, contributing to disruption of mucosal homeostasis.
Conclusion

The human resident flora in the gastrointestinal tract contains 100 times more microbial genes than the human genome (59). Although this mutualistic relationship with our microbiome aids in metabolizing of nutrients and benefits epithelial health and immunity, consequences of a break-down in tolerance can be detrimental. A chronic inflammatory response to the enteric flora has been associated with IBD, although it has not been clearly identified if this response develops prior to clinical IBD or is a consequence of IBD (9, 43, 49, 60-62). Therefore, development of a model that is capable of assessing the kinetics of antigen-specific responses and the importance of these immune responses against individual members of the microbiota will aid in the understanding of the mechanisms regulating the development of IBD. The pathogenesis of IBD has not been shown to be caused by a single organism, but instead there are multiple possibilities that likely contribute to IBD susceptibility and severity. Previous results from this laboratory indicate that H. bilis colonization of DF immunocompetent mice results in a multitude of upregulated genes involving the immune response, proteolysis, glycoprotein synthesis and protein metabolism and significantly downregulated genes including several involved in fatty acid metabolism, lipid metabolism and detoxification (Zhiping Liu-submitted for publication). Although H. bilis did not induce overt mucosal inflammation during the course of these studies, we hypothesize that the disruption in the microbial ecology by the introduction of a novel organism predisposes the host to react more vigorously to exposure to an environmental trigger/stressor at a later time. In contrast to bacteria that transiently colonize the GI tract, persistent colonization with a bacterial provocateur would be especially problematic for the susceptible host. Using defined-flora mice to develop a provocateur-trigger model of IBD (two hit model) allows for the chronological investigation of changes in antigen-specific immunity, and the mechanisms affecting the onset of clinical signs that are not simply the consequence of inflammation following the onset of overt clinical signs associated with disease. Another advantage of this provocateur-trigger model is the ability to assess these responses in immune competent mice. Understanding the initial immune responses following the introduction of an environmental trigger (ex: DSS) may reveal evidence as to the mechanisms associated with the onset of disease and allow the rational design of strategies that will prevent or control IBD.

Following colonization of DF mice with H. bilis for 12 weeks and subsequent exposure to a low dose of DSS, the magnitude of gross and histological lesions are significantly more severe than those of mice treated solely with DSS or colonized with H. bilis. Likewise, the combined treatments of H. bilis colonization and DSS administration resulted in an increase in cecal score and decreased colon length compared to controls, DSS alone, and H. bilis alone. The magnitude of these macroscopic changes was associated with the severity of the histological changes (infiltration of mononuclear cells, increased hyperplasia, ulceration and epithelial erosion) within the colonic mucosa of mice in the dual treated group.

A break in tolerance to antigens derived from the enteric flora is thought to occur during IBD episodes (60). Loss of tolerance has been manifested by increased serum IgG directed against bacterial components in patients with IBD (44-46). Assessment of serum antibody responses in the present study indicated that H. bilis
colonization increased IgG2a production to the majority of the ASF organisms by 12 weeks post-colonization (Fig 5). The *Clostridium* subphylum is the most represented bacteria in human intestinal mucosa and IgG serum antibodies to clostridial flagellin (Cbir) are increased in patients with IBD (46, 62). In the absence of DSS treatment, mice colonized with *H. bilis* presented with greater IgG2a response to ASF 356 and 502 *Clostridium* species, suggesting that these responses are similar to those observed in human IBD patients and C3HHeJBir mice (62). In addition, this observation suggests that the enhanced susceptibility to low dose DSS-induced colitis is dependent upon or associated with the induction of a Th1 response to members of the enteric flora.

Proinflammatory cytokines can be detrimental to the health of epithelial cells. Specifically, IFN-γ and TNF-α have been shown to change the cellular morphology of, effect barrier function of, and reduce chloride secretion by epithelial cells (56). These cytokines have also been shown to increase the production of reactive oxygen species (ROS) in colonic epithelial cells (57). Dual treatments of *H. bilis* colonization and DSS administration result in a 3.2 fold increase of mucosal IFN-γ expression and a 1.5 fold increase in TNF-α expression in the proximal colon compared to control groups. To determine if the proinflammatory cytokines induced within the colonic mucosa of mice dually treated with *H. bilis* and DSS correlated with changes in epithelial integrity and morphology, proximal colon sections were stained with hematoxylin and eosin (H & E). The colonic epithelium from mice dually treated with *H. bilis* and DSS presented a flattened epithelial morphology and increased numbers of infiltrating macrophages/neutrophils within the lamina propria that may have contributed to mucosal hyperplasia.

The discovery of Th17 cells expanded previous concepts proposing that Th1, Th2, and T regulatory lineages were the sole effector cells for the immune response. The development of Th17 cells in murine tissues requires TGF-β and IL-6, although maintenance requires IL-23 (63-67). Many articles have addressed the inflammatory characteristics of IL-17 following the onset of autoimmune diseases like EAE and IBD (51-54, 66, 68). For example, IBD patients have elevated IL-17A expression in colonic mucosa (51) and in mice, clinical signs of TNBS-induced colitis including weight loss, IL-6 production, myeloperoxidase activity, and local macrophage inflammatory protein-2 induction are ameliorated in IL-17R knockouts (69). The induction of IL-17 responses in immune competent animals following the onset of colitis has not been previously demonstrated. *H. bilis* colonized mice treated with DSS had elevated mucosal expression of IL-17A-specific mRNA and increased amounts of IL-17 protein was secreted from colonic explants. This finding supports previous observations that local synthesis of IL-17 may contribute to chronic IBD episodes. In addition, exposure of DF mice to DSS treatment resulted in the increased secretion of IL-6 from colonic explants. We hypothesize that *H. bilis* colonization results in the recruitment of Tregs that secrete TGF-β. The combination of TGF-β and the proinflammatory cytokine IL-6 ultimately results in effector Th 17 cells (64).

We hypothesized that the mechanism of increasing antigen-specific immune reactivity to the commensal microbiota following 3, 12, and 20 weeks *H. bilis* colonization was due to decreased T regulatory cells in the local environment. However, Foxp3 expression in the proximal colon indicated an increased expression in the dual group compared to either treatment group alone (data not shown). Based on the increase
in Foxp3 mRNA expression in the colonic mucosa, we speculate that *H. bilis* colonization resulted in the recruitment of Treg cells to the lamina propria of the colon to downregulate adaptive immune responses to the ASF and/or *H. bilis*; however, additional experiments will be required to confirm this. A consequence of recruitment and activation of Treg cells would be to sustain *H. bilis* colonization by dampening adaptive immunity. Other pathogens including *H. pylori* and *Bordetella pertussis* have been shown to recruit Tregs to sustain colonization (70, 71). Patients with CD have also been shown to have increased Foxp3+CD4+ cells in the intestinal mucosa that retain suppressive functions ex vivo (72). Interestingly, as immune reactivity increases over time including elevated serum antibody responses, Foxp3 expression within the mucosa of the *H. bilis* colonized mice decreases. Although we cannot say that this is directly due to a decrease in Foxp3, we speculate that Treg cells hinder the resolution of the infection. In addition, other regulatory mechanisms may potentially be in place to prevent overt inflammation. Alternatively, the increased reactivity to the flora over time may suggest that the T regulatory cells are converting into Th17 cells that may be modulating damage. Following *H. bilis* colonization, preliminary data (not shown) indicated that the presence of IL-17-specific mRNA in the colonic mucosa increased over time. Other groups have shown that T regulatory cells convert into IL-17 producing cells when cultured with IL-6 (73).

Following the colonization with a bacterial provocateur, immune competent mice colonized with a defined flora of eight bacteria were shown to be predisposed to colitis and were characterized as having upregulated immune reactivity to antigens derived from the resident flora. The newly induced immune reactivity to the commensal flora alone was not deleterious to the health of *H. bilis* colonized mice as these mice did not develop overt inflammation; however, in the context of an inflammatory insult, the onset of IBD is triggered. Utilizing an experimental model that employed a provocation-trigger treatment regimen resulted in the upregulation of Th1 and Th17 immune responses that may contribute to the pathogenicity of disease. To confirm that the IL-17 secreting and antigen-specific proliferating CD4+ T cells are pathogenic, future experiments involve the transfer of either IFN-γ and IL-17 secreting mesenteric lymph node CD4+ T cells or IFN-γ and IL-17 secreting lamina propria lymphocytes (LPLs) from *H. bilis* colonized animals into naïve, defined flora mice (*Helicobacter* free). If the mild colitic insult imparted by low dose DSS is sufficient to induce disease, it could be inferred that the ability of the transferred LPLs or CD4+ from *H. bilis* colonized mice will predispose the host to DSS sensitivity mediated by immune activation by antigens from the resident bacterial flora. In conclusion, *H. bilis* colonization of DF mice resulted in immune reactivity to the commensal flora that increased susceptibility to DSS-induced colitis. These results signify that humans colonized with a novel provocateur are predisposed to an environmental insult later in life.
Figures

Figure 1. 1.5% DSS induces minimal clinical signs of IBD in defined flora mice. Defined flora mice were administered graded doses of DSS in drinking water for five days preceding four days of restitution. Measurement of parameters including percent weight change from day 0 (A), cecal score (B), and colon length (C) indicated that 1.5% DSS induced minimal clinical signs of colitis. Ceca were blindly scored using a scale from 0 (healthy) to 4 (severe macroscopic lesions). Data is presented as the mean ± SEM. Statistical significance indicated is p < .05*, p < .01 **. n =5 per treatment group. Representative of two experiments.

Figure 2. Dual treatment group displayed increased macroscopic lesions of colitis. Defined flora mice were colonized with H. bilis for 12 weeks and/or treated with 1.5% DSS in drinking water for five days followed by four days restitution. Gross scores including cecal score and colon length were measured. The photographs depict representative ceca and colons from each of the four treatment groups. It should be noted that the colons from the dual treatment group displayed decreased cecal/colonic contents, thickened and shortened colon length (numbers on upper right corner), cecal atrophy, blunting and presence of blood.
Figure 3. *H. bilis* colonization results in increased susceptibility to DSS-induced colitis. Defined flora mice were colonized with *H. bilis* for 12 weeks and/or administered 1.5 % DSS. Ceca were blindly scored as previously described using a scale from 0 (healthy) to 4 (severe macroscopic lesions) (A) and colon length was measured (B). Data is presented as the mean ± SEM. Statistical significance indicated is p < .05*, p < .01**. n = 5 to 10 mice per treatment. Results are representative of eight independent experiments following *H. bilis* colonization.
Figure 4. *H. bilis* and DSS dual treatments increased colonic disease scores. Following 12 weeks post-infection with *H. bilis* or DSS treatments mice were euthanized, proximal colon was histologically scored for inflammation and erosion parameters as described in Materials and Methods (A). Hematoxylin and eosin staining of proximal colon depicting infiltration of mononuclear cells, crypt hyperplasia, with loss of goblet cell differentiation and gland dropout in *H. bilis* and DSS treated group (E) compared to control (B), DSS alone (D), or *H. bilis* colonized only (C). n = 5 per treatment group. Data is presented as the mean ± SEM. Statistical significance indicated is p < .05*. Representative of eight independent experiments.
Figure 5. *H. bilis* colonization increased IgG2a antibody response over time to *H. bilis* & ASF antigens. Serum samples from *H. bilis* colonized mice and non-colonized mice were assessed by ELISA for reactivity to whole cell lysates derived from enteric flora whole cell lysates of the resident microbiota (ASF 356: *Clostridium* spp. Cluster XIV; ASF 360: *L. acidophilus*; ASF 361: *L. murinus*; ASF 457: *M. schaedleri*; ASF 492: *E. plexicaudatum*; ASF 500: Low-G+C-content gram-positive; ASF 502: *Clostridium* spp. Cluster XIV; ASF 519: *B. distasonis*, *H. bilis*, and *S. epidermidis* (S.epi)). Absorbance (OD) was measured spectrophotometrically (405 nm). Data is presented as the mean ± SEM of the optical density (OD) for each treatment group of mice. n = 5 mice per treatment group, representative of 3 independent experiments. Differences in the serum antibody response to each of the individual ASF antigens is indicated by the presence of different letter. *p* ≤ 0.05.
Figure 6. *H. bilis* induced broader IgG2a serum antibody responses to commensal microbiota over time. Evaluation of serum antibody reactivity to whole cell lysates of the ASF was performed by immunoblot analysis. Panel A: serum samples from control defined flora (DF) mice; Panel B: serum from DF 3 weeks postcolonization with *H. bilis*; Panel C: serum from DF mice 12 weeks postcolonization with *H. bilis*; Panel D: serum from DF mice 20 weeks postcolonization with *H. bilis*. Lane 1: Precision Plus protein standards; Lanes 2 – 9: altered Schaedler flora (ASF) antigens (356: *Clostridium* spp. Cluster XIV; 360: *L. acidophilus*; 361: *L. murinus*; 457: *M. schaedleri*; 492: *E. plexicaudatum*; 500: Low-G+C-content gram-positive; 502: *Clostridium* spp. Cluster XIV; 519: *B. distasonis*); lane 10: *S. epidermidis*; Lane 11: *H. bilis*. Each lane was loaded with 7 µg of total protein from the specified bacterial lysates, separated electrophoretically using 12% tris-glycine gels, and immunoblots blots were developed using pooled serum samples from 5 mice per treatment group as described in Materials and Methods.
Figure 7. *H. bilis* colonization induces antigen specific T cell proliferation. MLN T cells were enriched from mice colonized with *H. bilis* for 12 weeks and stimulated with splenic antigen presenting cells that had been stimulated with pooled ASF antigens at 1:1 ratio of APC: T cells for 72 hours as described in Materials and Methods. Proliferation was assessed via \(^3\)H-thymidine incorporation. Data is presented as the mean ± SEM. Stimulation index (SI) was calculated by dividing counts per minute of stimulated cultures by control values (APC no antigen). \(n = 3\) per treatment group. * \(p ≤ .06\), ** \(p ≤ .02\). Results are representative of three independent experiments.

Figure 8. Mucosal IL-17A is Elevated in *H. bilis* Dual Treatment Group. (A) Real-time RT-PCR analysis for IL-17A-specific mRNA and (B) analysis of supernatants from ex vivo colon cultures for IL-17A protein secretion. Samples were collected from defined flora mice colonized with *H. bilis* and/or treated with 1.5% DSS. RT-PCR data is displayed as fold changed normalized to GAPDH. Data is presented as the mean ± SEM. Statistical significance between groups is indicated by different letters. Data are representative of at least two independent experiments. \(n = 5\) mice per treatment group.
Figure 9. Mucosal Proinflammatory Expression Elevated in Dual Treatment Group. Real-time RT-PCR analysis of IFN-γ (A) or TNF-α (B) in the proximal colon following colonization of H. bilis colonized defined flora mice for 12 weeks and/or 1.5% DSS treatment. (C) Hematoxylin and eosin staining of proximal colon following dual treatments of H. bilis colonization and DSS administration illustrates attenuation of the outer enterocytes of the glands (arrow). Data is presented as the mean ± SEM. n = 5 mice per treatment group. Data are representative of two independent experiments.
Figure 10. Mucosal Foxp3 expression decreased over time. Real-time RT-PCR Foxp3 analysis of proximal colon tissue following *H. bilis* colonization for 3, 12 or 20 weeks. Data is displayed as fold change after normalization to GAPDH. Data is presented as the mean ± SEM. Data are representative of at least two independent experiments. n = 5 mice per treatment group.
Figure 11. Colonic localization of *H. bilis* following colonization of defined flora mice. Electron microscopic analysis of proximal colon from defined flora mice colonized with *H. bilis* for 12 weeks and treated with 1.5% DSS. *H. bilis* was observed in the lumen (A), within the glands (B-C) (the inset in panel B is shown in C) and within the epithelial cells (D).
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References


CHAPTER 3. Nature of Provocateur and Degree of Inflammatory Insult Modulates DSS-induced Colitis

A paper to be submitted to *Microbes and Infection*

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**Abstract**

While the etiology of inflammatory bowel disease (IBD) is unknown, host genetics, the intestinal microbiota, and aberrant immune responses have been implicated as contributing factors. However, the initial provocation and subsequent inflammatory trigger(s) have yet to be discovered. Although there is evidence that IBD patients respond immunologically to antigens derived from their enteric microbiota, no specific microbial agent has been identified as the sole initiator/provocateur of IBD. Our laboratory has previously shown that colonization of defined-flora (DF) (i.e., gnotobiotic) mice with *Helicobacter bilis* induced immune reactivity to the commensal enteric flora. It was hypothesized that the nature of the provocateur (i.e., virulence potential) influences the manner in which the host reacts to its microflora, thus, modulating the susceptibility to subsequent colitic insults (i.e., triggers). To test this hypothesis, groups of 6-8 week old, DF C3H/HeN:Tac mice bearing the altered Schaedler flora (ASF) were colonized with a non-pathogenic strain of *Escherichia coli*. As a measure of susceptibility to mucosal inflammation, these mice were treated for five days with a low dose (1.5%) or high dose (2.5%) of dextran sulfate sodium (DSS) in their drinking water at 3 or 12 weeks post-colonization with *E. coli*. Mice were euthanized 6 days after cessation of the DSS treatment and evaluated for gross and histological lesions, cytokine-specific mRNA expression, humoral and cell-mediated immune responses. In contrast to DF mice colonized with *H. bilis*, colonization of DF mice with *E. coli* did not significantly increase the susceptibility to low-dose DSS; however, the susceptibility to typhlocolitis following exposure to high-dose DSS was markedly increased as evidenced by the induction of macroscopic and histological cecal lesion scores. Similar to control mice, *E. coli* colonization of the DF mice alone induced no macroscopic lesions. Interestingly, DF mice colonized with *E. coli*, developed ASF antigen-specific CD4+ T cell proliferative responses following *in vitro* stimulation that was not dependent upon DSS exposure. The treatment of *E. coli* colonized mice with 2.5% DSS resulted in the induction of ASF-antigen-specific IL-17A secretion from mesenteric lymph node CD4+ T cells and cecal mucosal expression of IL-17A-specific mRNA. Depending on the severity of a subsequent inflammatory insult, these studies demonstrated that introduction of a non-pathogenic, commensal strain of *E. coli* could also serve as a mucosal provocateur and that its impact on
the colonic mucosal homeostasis (e.g., gene expression, aberrant immunity) is a critical factor that predisposes to the onset of IBD.

**Introduction**

The human gastrointestinal (GI) tract is the organ with the largest surface area \((100 \text{ m}^2)\) in the body and must accommodate the digestion and absorption of 500 kg of food per year. The GI tract also harbors a diverse microbiota \((10^{13} \text{ organisms/gram contents})\) that aid in immune system development/maturation and nutrient metabolism (1). However, a GI tract function that perhaps is less well considered is the role that the intestinal epithelium serves as the physical interface between the host and the environmental components located in the GI tract lumen. In this regard, the GI tract serves as a barrier for environmental dangers like pathogenic bacteria that attempt to colonize and invade the epithelial layer. In order to prevent the onset of intestinal inflammation, the GI tract has developed regulatory mechanisms that function to maintain mucosal health and immunological tolerance in the face of the constant borage of food and microbial components while maintaining the beneficial relationship of commensal bacteria. Inflammatory bowel disease (IBD) is a chronic inflammation of the GI tract that is hypothesized to result from the immune system attacking the enteric flora. Previous experiments from this laboratory indicated that development of DSS-induced colitis in immune competent, defined flora (DF) mice required the prior colonization with a bacterial provocateur, *Helicobacter bilis*. *H. bilis* is a mildly pathogenic, Gram-negative, motile, microaerophilic bacteria that has been associated with IBD-like lesions in immunocompromised mice (2-4). Colonization of DF mice with *H. bilis* resulted in disruption of mucosal homeostasis as evidenced by changes in profile of mucosal gene expression and the induction of antigen-specific cellular and humoral immune responses to the commensal flora. Additionally, *H. bilis*-colonized DF mice developed typhlocolitis following exposure to an otherwise innocuous low dose of DSS that was characterized by increased mucosal expression of IFN-γ- and IL-17-specific mRNA. Because the development of IBD is closely associated with the presence of a complex microbiota and IBD induction has not been ascribed to a single organism, it was hypothesized that the nature of the provocateur (virulence attributes) in conjunction with a downstream environmental insult/trigger (e.g., DSS) determines the severity of the resultant inflammatory response. When considering a multi-hit model of IBD, it will be critical to evaluate the range of host responses to different provocateurs in the context of subsequent colitic insults or triggers. To determine if susceptibility to DSS-induced colitis resulted simply as a result of colonization of the GI tract with a novel organism, DF mice were colonized with a commensal strain of *Escherichia coli*. These studies support previous observations that colonization of DF mice with a novel organism (*H. bilis or E. coli*) disrupts mucosal homeostasis and increases their susceptibility to a mild to moderate colitic insult/trigger.
Materials & Methods

Animals

Six- to eight-week old C3H/HeN:Tac mice naturally colonized with a defined flora (altered Schaedler’s flora: ASF 356: Clostridium spp. Cluster XIV; ASF 360: Lactobacillus acidophilus; ASF 361: Lactobacillus murinus; ASF 457: Mucispirillum schaedleri; ASF 492: Eubacterium plexicaudatum; ASF 500: Low-G+C-content gram-positive; ASF 502: Clostridium spp. Cluster XIV; ASF 519: Bacteroides distasonis) were originally obtained from Taconic Farms (Albany, NY) and subsequently bred and maintained in gnotobiotic isolators at Iowa State University. All mice were certified free of E. coli spp prior to use in these studies. Following colonization with E. coli all animals were housed (2-4 mice/cage) in gnotobiotic isolators. Mice were fed an irradiated rodent diet and autoclaved water. All experiments were approved by Iowa State University Animal Care Committee.

Experimental Design

Defined flora mice of mixed gender were separated into 4 groups: 1) Non-colonized, 2) Non-colonized + DSS, 3) E. coli colonized alone, and 4) E. coli colonized + DSS. Groups three and four were inoculated by oral gavage with > 2 x 10^7 E. coli CFU in 0.3 ml of Brucella broth three times over a 3-day period. E. coli infection status was confirmed by fecal cultures on EMB agar plates. E. coli was evaluated for virulence factors (cvaC, iron, ompTp, hlyF, etsA, iss, aerJ, ireA, papC, cdtA, cdtC, cdtB) by PCR. Three-12 weeks postinfection (PI), groups two and four and six received either low (1.5%) or high (2.5%) DSS (MW=36-50kD, ICN) in deionized drinking water for five-six days, followed by three-four days with regular sterile water. Groups one and three received sterile water only throughout the entire experiment. Immediately following the restitution period from DSS, mice were euthanized with CO2 and necropsied. Tissue samples were fixed in 10% buffered formalin and submitted for routine histopathological evaluation. Mesenteric lymph nodes (MLN) were collected and negatively sorted for CD4 T cells by MACS magnetic beads and MS separation columns (Miltenyi Biotech). In order to generate antigen presenting cells (APCs), spleen cells were harvested from SPF C3H mice one day prior to necropsy and incubated with ASF antigens (200 µg/ml) overnight. Following mitomycin C treatment (Fisher) (see Chapter #2) APCs were inoculated into microtiter plates along with CD4 T cells at a 1:1 ratio and assigned to one of seven treatments: APC-NS (no stimulant), APC-Pool (pooled ASF antigens), APC-457/519, APC-492/360/361, APC-356/500/519, APC-Ec or APC-Se (E. coli or Staphylococcus epidermidis, respectively), or anti-CD3/28 and incubated for 72 hours. Prior to the addition of 3H-thymidine, an aliquot of the culture supernatant was collected and analyzed by a multiplex fluorescent bead assay for the following cytokines: IL-2, IL-6, IL-10, IL-12(p40), IL-17, IFN-γ, and TNF-α. In addition, samples were assessed for antigen-specific serum antibody responses and tissue from the proximal colon was analyzed by quantitative RT-PCR for gene expression.
**Preparation of Bacterial Antigens**

Each member of the altered Schaedler’s flora (ASF360, ASF361, ASF356, ASF457, ASF492, ASF500, ASF502, and ASF519) was grown in bacteriological medium and cultured at 37°C for five to seven days under anaerobic conditions. Cells will be grown in either reinforced clostridium medium (RCM) supplemented with 5% calf serum (ASF360, ASF361, ASF 356, ASF 502, and ASF519) or Schaedler’s broth supplemented with 5% sheep serum (ASF457, ASF492, and ASF500). Because many of these organisms are extremely oxygen sensitive (EOS), all inoculations were performed in an anaerobic chamber (Bactron). Commensal *E. coli* was isolated from a conventional flora-bearing C3H mouse and grown aerobically in Brucella broth at 37°C for approximately 4-7 hours (McFarland scale #6, ca 5 x 10^8) and tested for purity prior to inoculation into DF mice. Cells were harvested from the broth by centrifugation, washed in buffer, and lyophilized. Bacterial antigens (e.g. all eight ASF strains) used for in ELISA or for in vitro stimulation of lymphocyte cultures were prepared from the lyophilized cells. The cell suspensions were placed on ice and sonicated for 5 minutes to prepare whole cell sonicates (WCS). WCS was sterilized by UV light irradiation and stored at -20°C until use. Sterility of each individual WCS was confirmed bacteriologically.

**Gene Expression via Real Time RT-PCR**

Total RNA was extracted from 20 mg of proximal colon by RNAeasy Mini Kit (Qiagen). The RNA samples were quantified by ND-1000 spectrophotometer (Nanodrop technologies) and treated with TURBO DNA-free DNase (Ambion) to eliminate genomic DNA contamination. RNA was reverse transcribed into cDNA by SuperScript™ III First-Strand Synthesis System (Invitrogen, San Diego, CA). cDNA was amplified using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen). The following PCR conditions were used: 95°C for 10 min followed by 40 cycles of amplification (95°C for 10 s, 56°C for 5 s, and 72°C for 10 s). Primers for GAPDH (forward primer: 5’- TCACCACCATGGAGAAGGCG-3’, reverse primer: 5’-GCTAAGCAGTTGGTGTGCA-3’), IFN-γ (forward primer: 5’- TCAAGTGGCATAAGATGTGAGAA-3’, reverse primer: 5’-GCTAAGCAGTTGGTGTGCA-3’), TNF-α (forward primer: 5’-CATCTTCTCAAAATTCCGATGGACAA-3’, reverse primer: 5’-TGGGAGTAGACACAGGTACAACC-3’) and IL-17A (forward primer: 5’-CTGGCTCTGCTGGCCCTTTC-3’, reverse primer: 5’-CCACCCACCACCATCTTC-3’) were used. The real-time quantitative PCR was performed using Applied Biosystems GeneAmp™ 5700 Sequence Detection System. Standard curves for each gene were made using a series of cDNA dilutions. The relative mRNA quantity of each gene was normalized to GAPDH (5).

**Histopathology**

Sections of cecum and colon were fixed in 10% buffered formalin. The tissues were routinely processed, embedded in paraffin, sectioned at 5 µm and stained with hematoxylin and eosin. Tissues sections from each mouse were scored for severity of mucosal epithelial damage, glandular alterations, and magnitude/character of the lamina propria cellular infiltrate. Histological sections were evaluated blindly by J.M. Hostetter, Department of Veterinary Pathology, Iowa State University and the scores for individual parameters ranged from 0 (healthy)
to 5 (diseased) for both erosion and inflammation (6).

**Analysis of Antigen-Specific Serum Antibodies.**

Antigen-specific serum antibody responses were measured by enzyme linked immunosorbant assay (ELISA). Serum antibody levels in mice were determined by enzyme linked immunosorbant assay (ELISA). Briefly, 96-well plates (Costar-3590) were coated with 100 µl/well of whole cell sonicate (WCS) at 10 µg/ml derived from the individual members of the altered Schaedler’s flora (ASF) and *H. bilis* diluted in PBS (pH 7.4) overnight at 4ºC. Plates were washed three times in PBS/.05%Tween 20 and blocked with 280 µl/well 2% gelatin diluted in PBS/.05%Tween 20/2% FBS for two hours at room temperature. Plates were washed three more times with PBS/.05%Tween 20 prior to addition of 100 µl/well serum samples diluted 1:200 in PBS/Tween/2% FBS overnight at 4ºC. Plates were washed five times with PBS/.05%Tween 20. Then, 100 µl of 1:2000 diluted alkaline phosphatase -conjugated goat anti-mouse IgG (H&L), IgG1 or IgG2a (Southern Biotechnology) in PBS/Tween/2% FBS was added per well and incubated for 2 hours at room temperature. Prior to washing five times with PBS/Tween 20 (.05%), wells were developed for a colorimetric reaction using 100 µl of p-nitrophenyl phosphate (Sigma 104) (1 mg/ml) at room temperature. Absorbance was measured using an ELISA reader (405 nm).

**Preparation of Antigen Presenting Cells (APCs)/Mitomycin C Treatment**

Spleens were recovered from three adult C3H/HeOuJ mice and homogenized to prepare single cell suspensions in complete RPMI 1640, (Cellgro) containing 5% FBS, 1M HEPES, essential amino acids, non-essential amino acids, 1 mM sodium pyruvate, (2mM) L-glutamine, penicillin-streptomycin, 25 µg/ml gentamycin and 175 µl 2-β-mercaptoethanol. APCs were pulsed with antigen using a method adapted from Cong et al (7). Two separate aliquots of 20 x 10⁶ cells/5 ml were placed into two 15 ml tubes with 200 µg/ml (protein content) of combined ASF antigens added to one aliquot or media alone to the other aliquot and both were incubated overnight at 37ºC. The APC preparations were then incubated in the presence of mitomycin C (50 µg/ml final concentration) at 37ºC for 30 to 45 minutes. The cell suspensions were washed five times using RPMI supplemented with 5% fetal calf serum (FCS) and antibiotics (8).

**Proliferation/Cytokine Secretion via LUMINEX**

T cell and APCs were cultured for 72 hrs, 100 µl of cell supernatants were collected for cytokine analysis by the Luminex 100 technology and 100 µl of fresh complete medium was added back to cells. The culture was then pulsed with 10 µl of 50 µCi/ml ³H-thymidine for seven hours prior to harvesting. Incorporation of ³H-thymidine was quantified by a scintillation counter.
Colonic tissues were fileted open and 3 mm biopsy punches were extracted and placed into 96 well flat bottom plates (Costar 3595) in 200 µl complete RPMI 1640, (Cellgro) plus 50 µg/ml gentamycin and 2X penicillin-streptomycin per well per punch. Three punches were biopsied from each mouse. Cultures were incubated at 37 °C for 48 hours prior to recovery of supernatants and cytokine analysis.

Statistical Analysis

Data is displayed as mean ± SEM. Non-parametric group comparisons were analyzed by Kruskal-Wallis tests. Parametric group comparisons were analyzed by analysis of variance (ANOVA). Pairwise group comparisons having equal variances were analyzed using t-tests.

Results & Discussion

Three week E. coli colonization of defined flora mice does not increase susceptibility to low-dose DSS-induced colitis

Prior experiments from this laboratory indicated that H. bilis colonization of DF mice resulted in antigen-specific cellular and humoral responses to the commensal microbiota that was exacerbated by low-dose (1.5%) DSS and characterized by increased mucosal expression and protein secretion of proinflammatory cytokines. To determine if these responses were uniquely associated with H. bilis colonization, DF mice were colonized with a non-pathogenic, commensal strain of E. coli that was originally isolated from a conventional colony of C3H mice. Three weeks after E. coli colonization, mice were administered 1.5% DSS in their drinking water. After five days of exposure to 1.5 % DSS and four days of restitution on normal drinking water, mice were euthanized and evaluated for the presence of colitis. Ceca and colons were excised and scored for clinical signs of colitis. Mice that received the dual treatment of E. coli colonization and 1.5% DSS presented with similar cecal scores and colon lengths as control DF mice (Fig 1 and 2). These macroscopic observations were consistent with the histological scores (Fig 3) indicating that colonization with commensal E. coli did not predispose the DF mice for the development of colitis in response to 1.5% DSS.

E. coli colonization enhanced CD4+ T cell proliferation to commensal flora

In contrast to healthy individuals, IBD patients, have increased reactivity to bacterial components from their commensal flora. To address if antigen-specific responses are developed following E. coli colonization towards the commensal flora in absence of gross and histological lesions, humoral and cellular responses were analyzed. Serum collected from three week E. coli colonized animals was assessed for total IgG antibodies to altered Schaedler’s flora (ASF) whole cell sonicates. ELISA results indicated that no IgG antibodies were generated to the commensal flora (Fig 4). Although E. coli colonization did not induce antibodies reactive to the flora, CD4+ T cells that were harvested from MLN proliferated to antigen presenting cells that were primed with ASF.
antigens (Fig 5). Predominantly, CD4+ proliferation from the dual treated group was in response to ASF 356/500/502 antigens. These results suggest that T cell proliferation to enteric flora antigens may not be a consequence of lesion development, but a prelude to clinical signs of IBD.

**Increased length of time following *E. coli* colonization does not increase susceptibility to 1.5% DSS**

To determine if the *E. coli* induced T cell reactivity to the commensal flora required more time to induce susceptibility to low-dose (1.5%) DSS, DF mice were colonized with the commensal strain of *E. coli* for 12 weeks. After 12 weeks, separate groups of control and *E. coli*-colonized DF mice were administered 1.5% DSS in their drinking water for five days followed by four days restitution. Gross clinical signs of colitis did not increase with time of *E. coli* colonization and were similar to clinical signs at three weeks. *E. coli* colonization did not increase cecal scores or decrease colon length following administration of 1.5% DSS and histological evaluation revealed similar findings (Fig 6-8).

**CD4+ T cell antigen-specific proliferation to commensal flora in absence of lesions**

To determine if T cell proliferation to commensal flora was only transiently induced as a consequence of *E. coli*, T cell proliferation was assessed 12 weeks post-colonization. Similar to three week post-colonization, the proliferative response of MLN CD4+ T cells to the ASF antigens was also increased in absence of disease (Fig 10). At 12 weeks compared to 3 weeks, CD4+ T cells from the dual treated group proliferated in response to ASF bacterial antigens including ASF 457/519 and ASF 492/360/361; however, serum antibodies were not generated to ASF bacterial antigens indicating that insufficient ASF antigen reached or persisted within the lymph node to induce antibody responses (Fig 9). Similar to responses to ASF antigens, CD4+ T cell responses to *E. coli* antigen were greater at 12 weeks post-colonization compared to those at three weeks.

**E. coli protects against low-dose DSS induced colitis**

To assess the local cytokines secreted in response to colonization, colonic explants were collected and incubated for 48 hrs at 37 °C in complete medium. Supernatants were harvested and assessed for cytokine production from colonic explants. Results showed that explants obtained from the dual *E. coli* colonized and 1.5% DSS treated group secreted a reduced amount of the inflammatory cytokines including IFN-γ, IL-6, and IL-17A compared to explants recovered from mice treated with DSS alone (Fig 11). Comparisons of MLN CD4+ cells indicated that *E. coli* colonized mice treated with DSS had greater IL-10 secreting CD4+ cells compared to DSS only treated DF mice in response to pooled ASF, 457/519, and *E. coli* antigens (Fig 12). This indicates that *E. coli* may protect against low-dose DSS-induced colitis.

**Commensal *E. coli* does not protect against high-dose DSS**

There are many probiotic studies reporting the beneficial attributes of *E. coli* in preventing/ameliorating disease.
For example, colonization of conventional mice with the Nissle 1917 strain has been shown to increase antibacterial peptides, prevent and/or improve acute colitis, and prevent chronic colitis (9, 10). Because *E. coli* decreased proinflammatory cytokines and increased IL-10 secreting CD4+ T cells, we hypothesized that these responses may be protective against a greater inflammatory insult. Defined flora mice were colonized with *E. coli* for three weeks prior to administration of a dose of 2.5% DSS in drinking water for six days preceding three days of restitution. In contrast to the results obtained with 1.5% DSS, DF mice colonized with *E. coli* and treated with 2.5% DSS developed clinical signs of colitis including lack of formed fecal pellets, shortened colon, and watery mucoid contents, but also presented with severe cecal atrophy, cecal emptying and gained less weight than other treatment groups (Fig 13-16). The gross lesions were consistent with the increase in severity of histological lesions predominantly in the ceca and characterized by the infiltration of mononuclear and numerous polymorphonuclear cells (Fig 13-16). Increased cecal severity may be due to the increase in antigenic load in the ceca.

**High dose DSS does not induce humoral reactivity to commensal flora**

The administration of a greater inflammatory insult and the consequence of increased severity of lesions prompted an examination of the humoral response to the ASF in the dual *E. coli* plus 2.5% DSS group. Similar to the mice in the three week *E. coli* colonization and low-dose DSS group, the dual group did not increase their serum antibody responses to commensal flora members (Fig 17); however, CD4 T cell proliferation after stimulation with ASF antigens was comparable to that at 3 weeks post-colonization (data not shown).

**Local inflammatory cytokines are elevated in response to *E. coli* and high-dose DSS**

To determine how the local environment responded to the dual treatment group of *E. coli* colonization and high-dose DSS, 3 mm diameter colonic explants were placed into complete medium and supernatants were collected after 48 hours. Results indicated that the dual group had elevated IL-6 and IL-17A compared to the other treatment groups and the dual group had elevated IFN-γ compared to the dual group with a low-dose of DSS (Fig 18). This indicates that commensal *E. coli* colonization may predispose to IBD under a high inflammatory trigger. IL-17A has been implicated in many autoimmune inflammatory diseases including rheumatoid arthritis, multiple sclerosis, asthma, and IBD (11). DF mice colonized with *E. coli* and treated with 2.5% DSS increased MLN CD4+ antigen specific IL-17A secretion to commensal flora species.

**Conclusions**

The microbiota of the human gut play a pivotal role in the development of IBD. The fact that there are 10 to 100 times more bacterial cells occupying the human body compared to somatic host cells clearly demonstrates the necessity for a controlled relationship between the host and its microbiota in order to maintain GI tract harmony (1). An uncontrolled and persistently excessive T cell effector response to bacterial antigens has been implicated in the development of IBD (7, 12, 13). Although a single bacterial species has not been
implicated as the specific inducer of IBD, patients with active CD have a decrease in diversity of bacterial species within the colon. Patients have also been reported to have an accumulation of various \textit{E. coli} strains at inflamed tissue sites (14-16). Previous experiments indicated that colonization of defined flora mice with a bacterial provocateur, \textit{H. bilis}, induced susceptibility to a low-grade (1.5\% DSS) inflammatory trigger. Although \textit{H. bilis} is mildly pathogenic, we questioned whether colonization of DF mice with any novel organism could disrupt mucosal homeostasis and induce susceptibility to a low-grade colitic insult. A commensal \textit{E. coli} strain previously isolated from conventional flora C3H mice was confirmed to be negative for the following virulence factors: cvaC, iroN, ompTp, hlyF, etsA, iss, aerJ, ireA, papC, cdtA, cdtC, and cdtB by PCR. Defined flora mice were colonized with \textit{E. coli} for three weeks and then administered a low dose (1.5\%) of DSS. Dually treated mice did not develop clinical or histological signs of IBD; however, the mesenteric lymph node had CD4$^+$ T cells that proliferated following stimulation with antigens derived from the eight members of the ASF. It was hypothesized that the presence of T cells reactive to the ASF antigens contribute to the predisposition to colitis and that a longer colonization time would manifest an increased sensitivity to the colitic insult. To test this hypothesis, mice were colonized for 12 weeks prior to DSS treatment; however, similar results were obtained. To determine how the local environment was aiding in the bacterial-specific T cell proliferation, the profile of cytokine production was examined from MLN CD4$^+$ T cells and colonic explant cultures. Low-dose DSS treatment alone increased IFN-$\gamma$, IL-6, IL-12/23(p40) and IL-17 in colonic explants relative to control DF mice. However, the MLN cells recovered from DF mice receiving the dual \textit{E. coli} and DSS treatment presented with reduced amounts of these cytokines compared to DSS alone. Following \textit{E. coli} colonization, CD4$^+$ T cells recovered from the MLN secreted more IL-10 in response to in vitro stimulation with bacterial antigens that did CD4$^+$ T cells from control mice. Based on these observations, it was hypothesized that this strain of \textit{E. coli} induced probiotic or cytoprotective mechanisms that ameliorated or attenuated DSS-induced colitis. Other research has shown that treatment of dendritic cells with muramyl dipeptide, a component of peptidoglycan degradation, upregulates NOD2 and protects against DSS- and TNBS-induced colitis (17). To further evaluate the potential protective benefits of the commensal \textit{E. coli} strain, DF mice were treated with 2.5 \% DSS in their drinking water three weeks after colonization. In contrast to the results observed using 1.5 \% DSS, mice that received the dual treatment displayed even greater lesion severity including typhlocolitis than the 2.5 \% DSS treatment alone. The cecum was markedly more affected in the dual group as evidenced by the significant increase in the histological and macroscopic lesion scores in comparison to those for the colon, which may correspond to increased antigenic load in the cecum relative to the colon. Analysis of cytokine responses revealed enhanced secretion of IFN-$\gamma$, IL-6, and IL-17 in the high dose dually treated group compared to the low dose (1.5\%) DSS dually treated group. In comparison to \textit{E. coli} colonization alone and low-dose DSS treatment, the MLN CD4$^+$ T cells from DF mice colonized with \textit{E. coli} and exposed to the higher dose of DSS induced a greater concentration of antigen-specific IL-17 in response to in vitro stimulation with antigens from the commensal flora. Likewise, assessment of the local production of cytokines within the cecal tissue showed elevated expression of mucosal IL-17A in the dually treated group compared to
DSS alone. Although colonic explants form *E. coli*-colonized DF mice treated with the low-dose of DSS also demonstrated increased amounts of mucosal IL-17A compared to the other groups, the fold change was much greater when the mice were treated with the higher dose of DSS in conjunction with *E. coli* colonization. IBD patients have been shown to have elevated levels of IL-17A mRNA in colonic mucosa and IL-17 secreting T cells (11, 18). IL-17 has been reported to be important for the clearance of pathogenic bacteria such as *Klebsiella pneumoniae*, *Citrobacter rodentium*, and *Borrelia burgdorferi* (19-21). Although the *E. coli* strain used in these studies does not possess virulence attributes characteristic of invading or attaching pathogens, the combination of colonization along with the exposure to 2.5 % DSS may disrupt tight junctions in epithelial cells to increase permeability, thus allowing *E. coli* to “translocate” across the mucosal epithelium. IL-17 producing T cells may also be induced due to processing *E. coli* by DCs. van Beelen et al (2007) showed that NOD2 activation stimulated human DCs to upregulate IL-1β and IL-23, resulting in production of IL-17 by memory Th17 cells (22). Likewise, DCs harvested from human CD patients having mutations in NOD2 that were primed with MDP and Pam3CSK4 and incubated with T cells were unable to induce production of IL-17 compared to NOD2 sufficient DCs. The development and implementation of a multi-hit model of colitis may allow for the incremental characterization of lesion development in response to varying inflammatory insults. For example, commensal *E. coli* is not detrimental to our health under homeostatic conditions; however, colonization may facilitate enhanced sampling of the commensal flora, thus, priming the immune response to a downstream environmental trigger/stressor. AS IBD patients experience periods of quiescence followed by active inflammatory flares, this multi-hit model may allow us to elucidate the disease related mechanisms that are responsible for the “peaks” and “valleys” associated with active IBD. The use of commensal bacteria as probiotic treatments has been the focus of much attention; however, results have been contradicting and no probiotic “cure” has been established for IBD. Studies using *Lactobacillus* or *Bifidobacterium* species as a maintenance treatment in UC have shown that treatment with *lactobacilli* did not change the relapse rates in patients; in contrast, another study found that patients that were administered bifidobacterial-derived supplements had fewer relapses (23, 24). Comparing relapse rates of 327 patients using no other commonly prescribed medications for IBD, it was observed that there was no difference in the rate of relapses when comparing administration of the commensal *E. coli* Nissle 1917 strain to an anti-inflammatory agent, mesalazine (25). Others have found that *E. coli* Nissle 1917 is just as effective as mesalazine in preventing relapses over 12 weeks (26). One reason for the variation in results related to the efficacy of probiotic treatments may be due to how the commensal organism interacts with the intestinal mucosal and how these interactions may or may not predispose the host for periods of increased sensitivity to inflammatory stressors. Variability in patient’s exposure to a potential plethora of environmental insults may greatly influence the efficacy of various *E. coli* strains as probiotics, similar to results in this study. Therefore, care should be taken to thoroughly evaluate the effects of probiotic bacteria on mucosal health in the context of varying inflammatory insults in order to develop safe and efficacious strategies to enhance gut health.
Figures

**Figure 1.** 3 week *E. coli* colonization does not enhance DSS-induced colitis. Mice were colonized for three weeks prior to low dose, 1.5% DSS treatment in drinking water. Colon and ceca were harvested and measured for colon length (inset) and gross cecal score. A) control, B) 1.5% DSS, C) *E. coli*, D) *E. coli* + 1.5% DSS. Gross photos demonstrate that the dual treatment group did not display more severe clinical signs than other treatment groups. Photographs are representative of each treatment group. n = 5 to 8 mice per treatment group. Representative of three independent experiments.
Figure 2. *E. coli* colonization does not affect gross clinical signs of DSS-induced colitis. Defined flora mice were colonized with commensal *E. coli* for three weeks prior to administration of 1.5% DSS. Ceca and colon were harvested at necropsy and measured for gross clinical signs of IBD. A) colon length (cm), B) cecal score (0-7). The horizontal line represents the mean. n = 6 to 9 per treatment group. Representative of three independent experiments. There were no significant differences between treatment groups.

Figure 3. No histological difference between *E. coli* dual group and control groups. Proximal colon and ceca were preserved in formalin at time of necropsy, paraffin embedded and cut into 5 µm sections. Tissues were scored upon mucosal height, necrosis, edema, inflammation, and erosion. A) colonic score, B) cecal score. The horizontal line represents the mean. n = 6 to 9 mice per treatment group. Representative of three independent experiments. There were no significant differences between treatments.
Figure 4. *E. coli* colonization does not increase antigen-specific IgG (H&L) to commensal flora. Serum from three week *E. coli* colonized mice and/or treated with 1.5% DSS was assessed by ELISA for reactivity to enteric flora whole cell lysates of the resident microbiota (ASF 356: *Clostridium* spp. Cluster XIV; ASF 360: *L. acidophilus*; ASF 361: *L. murinus*; ASF 457: *M. schaedleri*; ASF 492: *E. plexicaudatum*; ASF 500: Low-G+C-content gram-positive; ASF 502: *Clostridium* spp. Cluster XIV; ASF 519: *B. distasonis*), and *E. coli* antigen. Absorbance (OD) was measured spectrophotometrically (405 nm). Data is presented as a mean ± SEM of the optical density (OD) for each treatment group of mice. * p ≤ 0.05 compared to control and DSS only. Representative of three independent experiments.
Figure 5. MLN CD4$^+$ T cells from *E. coli* colonized DF mice proliferate to antigens derived from the commensal flora. Three weeks after *E. coli* colonization and/or 1.5% DSS treatment, CD4$^+$ T cells were recovered from mesenteric lymph node and stimulated with splenic antigen presenting cells that had been primed with antigens from the resident microflora (ASF 356: *Clostridium* spp. Cluster XIV; ASF 360: *L. acidophilus*; ASF 361: *L. murinus*; ASF 457: *M. schaedleri*; ASF 492: *E. plexicaudatum*; ASF 500: Low-G+C-content gram-positive; ASF 502: *Clostridium* spp. Cluster XIV; ASF 519: *B. distasonis*, or pooled ASF WCS). T cells and APCs were mixed at a ratio of 1:1 and incubated for 72 hours. Proliferation was assessed via $^3$H-thymidine incorporation. Data is represented by mean ± SEM of counts per minute. n = two per treatment group. Representative of two independent experiments.
Figure 6. 12 week *E. coli* colonization does not enhance susceptibility to 1.5% DSS. Following 12 weeks of *E. coli* colonization and/or low-dose, 1.5% DSS, proximal colon and ceca were harvested at euthanasia and scored based upon gross and histological evaluations. Colon length and gross cecal appearance (left) and hematoxylin and eosin stained sections of colon (right). A, D)-controls, B, E) 1.5% DSS, C, G) *E. coli* colonized, D, H) *E. coli* and 1.5% DSS treated. Photos were representative of mean treatment scores. n = 7 to 9 per treatment group. Representative of two experiments.
Figure 7. 12 week *E. coli* colonized and 1.5% DSS treated dual group does not induce macroscopic signs of IBD. Defined flora mice were colonized with commensal *E. coli* for 12 weeks and/or administrated 1.5% DSS in drinking water. Ceca were blindly scored as 0=healthy to 7=disease (A) and colon length was measured (B) for gross clinical signs of IBD. Data is represented by mean ± SEM. n = 7 to 9 mice per treatment group. There were no significant differences between treatments. Results are representative of two experiments.

Figure 8. No histological evidence of lesions in dual *E. coli* colonized and low-dose DSS treated mice. Following 12 weeks of *E. coli* colonization, and/or 1.5% DSS treatment, proximal colon and cecal sections were scored based upon crypt and villus height, necrosis, inflammation, erosion, ulceration, mucosal hyperplasia and edema. 0=healthy and 35=diseased A) colon score, B) cecal score. Data is represented by mean ± SEM. n = 7 to 9 mice per treatment group. There were no significant differences between treatments. Results are representative of two experiments.
Figure 9. *E. coli* colonization and DSS treatment does not induce antibody response to resident flora. Serum from 12 week *E. coli* colonized mice and/or treated with 1.5% DSS was assessed by ELISA for total IgG reactivity to enteric flora whole cell lysates of the resident microbiota (ASF 356: *Clostridium* spp. Cluster XIV; ASF 360: *Lactobacillus acidophilus*; ASF 361: *L. murinus*; ASF 457: *M. schaedleri*; ASF 492: *E. plexicaudatum*; ASF 500: Low-G+C-content gram-positive; ASF 502: *Clostridium* spp. Cluster XIV; ASF 519: *B. distasonis*), *E. coli* antigen or non-specific *Staphylococcus epidermidis* antigen. Absorbance (OD) was measured spectrophotometrically (405 nm). Data is represented by mean ± SEM of the optical density (OD). n = 10 to 15 mice per treatment group. Results are representative of two experiments for each treatment group of mice. Significance is indicated p < .05*.
Figure 10. *E. coli* and DSS treatment increased antigen specific CD4⁺ T cell proliferation. Following 12 weeks of *E. coli* colonization and/or 1.5% DSS treatment, mesenteric lymph node T cells were enriched from mice colonized with *H. bilis* for 12 weeks and stimulated with splenic antigen presenting cells (primed with resident microflora: ASF 356: *Clostridium* spp. Cluster XIV; ASF 360: *L. acidophilus*; ASF 361: *L. murinus*; ASF 457: *M. schaedleri*; ASF 492: *E. plexicaudatum*; ASF 500: Low-G+C-content gram-positive; ASF 502: *Clostridium* spp. Cluster XIV; ASF 519: *B. distasonis*) at 1:1 ratio for 72 hours. Proliferation was assessed via ³H-thymidine incorporation. Data is represented by mean ± SEM. n = one to three pooled CD4⁺ T cells per treatment group. Significance within stimulation groups is indicated by different letters. Results are representative of two experiments for each treatment group of mice.
Figure 11. Mucosal inflammatory cytokines are decreased in *E. coli* and 1.5% DSS dual treatment group. Ex vivo colon cultures (assessed by LUMINEX technology) of A) IFN-γ, B) IL-12/23, C) IL-6 (p40), and D) IL-17A protein analysis from defined flora mice colonized with *E. coli* for 12 weeks and/or treated with 1.5% DSS. Data is represented by mean ± SEM. n = 7 to 9 mice per treatment group. Results are representative of two experiments for each treatment group of mice. For each treatment group, statistical significance is indicated by different letters.
Figure 12. *E. coli* colonization increased IL-10 production in mesenteric lymph node. Mesenteric lymph node CD4⁺ T cell secretion of IL-10 following stimulation with splenic antigen presenting cells (primed with resident microflora: ASF 356: *Clostridium* spp. Cluster XIV; ASF 360: *L. acidophilus*; ASF 361: *L. murinus*; ASF 457: *M. schaedleri*; ASF 492: *E. plexicaudatum*; ASF 500: Low-G+C-content gram-positive; ASF 502: *Clostridium* spp. Cluster XIV; ASF 519: *B. distasonis* or pool of all 8 ASF) at 1:1 ratio (T cell: APC) for 72 hours from 12 week *E. coli* colonized defined flora mice compared to non-colonized. Data is represented by mean ± SEM. n = one to three pooled CD4⁺ T cells from each treatment group. Representative of two experiments.
Figure 13. *E. coli* and high dose DSS treatments induce greater severity of disease. Defined flora mice were colonized with *E. coli* for three weeks and/or treated with 2.5% DSS in drinking water. Gross scores including cecal score and colon length were measured. Gross photos demonstrate that the dual treatment group had decreased cecal/colon contents, thicked and shorted colon length (numbers on upper right corner), cecal atrophy, and blunting. Photos representative of each treatment group. A) Control, B) 2.5% DSS treated, C) *E. coli* colonized, D) *E. coli* colonized and 2.5% DSS treated. n = 5 to 10 mice per treatment group. Representative of two independent experiments.
**Figure 14. E. coli colonization and high dose DSS induce typhlocolitis.** Defined flora mice were colonized with *E. coli* for three weeks and/or administered 2.5 % DSS. Weight gain/loss was evaluated (A), Ceca were blindly scored as 0=healthy to 7=disease (B) and colon length was measured (C). Data is represented by mean ± SEM. n = 5 to 12 mice per treatment group. Representative of two independent experiments. Statistical significance is indicated by different letters.

**A**

![Graph A](image_url)

**B**

![Graph B](image_url)

**C**

![Graph C](image_url)
Figure 15. *E. coli* colonization and high dose (2.5%) DSS induced greater lesion severity in cecum. Photomicrographs of proximal colon (right) and ceca sections (left) following three weeks of *E. coli* colonization and/or 2.5% DSS stained with hematoxylin and eosin. A, E) control, B, F) DSS, C, G) *E. coli*, D, H) *E. coli* + 2.5% DSS. Photomicrographs representative of mean treatment scores from each group, depicting enhanced severity of cecal tissue characterized by gland dropout, inflammatory mono/polynuclear infiltrate and mucosal hyperplasia in (D). n = 5 to 12 per treatment group. Representative of two experiments.
Figure 16. *E. coli* & High Dose DSS Enhance Histological Cecal Score. Histological scores following 3 weeks of *E. coli* colonization, and/or 2.5% DSS treatment, proximal colon (B) and cecal sections (A) were scored were based upon crypt and villus height, necrosis, inflammation, erosion, ulceration, mucosal hyperplasia and edema. 0=healthy and 35=diseased Data is represented by mean ± SEM. Statistical significance is indicated by different letters. n = 5 to 12 per treatment group. Scores are representative of two independent experiments.
Fig 17. E. coli colonization and high dose DSS do not elevate serum antibodies to commensal flora members. Serum from three week E. coli colonized mice and/or treated with 2.5% DSS was assessed by ELISA for total IgG reactivity to enteric flora whole cell lysates of the resident microbiota (ASF 356: Clostridium spp. Cluster XIV; ASF 360: L. acidophilus; ASF 361: L. murinus; ASF 457: M. schaedleri; ASF 492: E. plexicaudatum; ASF 500: Low-G+C-content Gram-positive; ASF 502: Clostridium spp. Cluster XIV; ASF 519: B. distasonis), E. coli antigen or non-specific Helicobacter hepaticus antigen. Absorbance (OD) was measured spectrophotometrically (405 nm). Data is represented by mean ± SEM of the optical density (OD) for each treatment group of mice. Statistical significance indicated is p < .05*. n = 5 to 10 mice per treatment group. Results representative of two independent experiments.
Figure 18. *E. coli* and high DSS dual group induces elevated inflammatory cytokines locally. Ex vivo colon cultures (assessed by LUMINEX technology of A) IFN-γ, B) IL-17A, C) IL-6 (p40), D) IL-12/23(p40) protein analysis from defined flora mice colonized with *E. coli* and/or treated with 2.5% DSS. Data is represented by mean ± SEM. n = 5 to 10 mice per group. Statistical significance indicated by different letters.
Figure 19. Greater Inflammatory Dose positively correlates with antigen specific IL-17 expression to resident flora. (A, B) Mesenteric lymph node CD4+ T cell secretion of IL-17A following stimulation with splenic antigen presenting cells (primed with resident microflora: ASF 356: Clostridium spp. Cluster XIV; ASF 360: L. acidophilus; ASF 361: L. murinus; ASF 457: M. schaedleri; ASF 492: E. plexicaudatum; ASF 500: Low-G+C-content gram-positive; ASF 502: Clostridium spp. Cluster XIV; ASF 519: B. distasonis) at 1:1 ratio for 72 hours from 3 week E. coli colonized and 2.5% DSS (A) or 1.5% DSS (B) compared to non-colonized. Data is represented by mean ± SEM (C, D) Mucosal IL-17A expression is elevated in cecum following 3 week E. coli colonization and 2.5% DSS (C) or 1.5% DSS (D). Data is represented as fold change compared to GAPDH. n = 2 to 12 per treatment group. Statistical significance is indicated by different letters.
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References


CHAPTER 4. Predisposition to Colitis: Differential Responses of Intestinal Epithelial cells Following Exposure to *Helicobacter bilis* in Comparison to *Escherichia coli*

A paper to be submitted to *Microbes and Infection*

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**Abstract**

While the etiology of inflammatory bowel disease (IBD) is unknown, genetics, microflora, mucosal permeability, and aberrant immune responses have been implicated. The interactions between luminal bacteria and host epithelial cells that contribute to the development of IBD are not well characterized. In the absence of mucosal inflammation, colonization of the intestinal epithelium with bacterial provocateurs may increase host sensitivity to colitis. Our laboratory has previously shown that defined flora mice colonized with *Helicobacter bilis*, unlike a commensal *Escherichia coli*, developed antibody responses to the resident flora and became susceptible to colitis induced by a low dose of dextran sulphate sodium (DSS). Interestingly, both the pathogenic *H. bilis*, and the commensal *E. coli*, induced antigen-specific CD4+ T cell responses to the commensal microbiota. By using a greater inflammatory stressor (2.5% DSS), *E. coli* colonized mice were predisposed to a severe typhlocolitis or cecitis. The interaction between luminal bacteria and host epithelial cells are likely central to the induction of colitis. We hypothesized that changes in host-susceptibility to colitis induced following colonization with a bacterial provocateur is compartmentalized to the epithelium. The present studies were designed to define responses of an intestinal epithelial cell line (MODE-K) colonized with either *H. bilis* or *E. coli* that would elucidate the differential ability of these organisms to effect changes in mucosal homeostasis and predispose defined flora mice to the onset of colitis. A murine epithelial cell line, MODE-K, was exposed to viable *H. bilis* or *E. coli* or whole cell sonicates (WCS) for 12 to 48 hours. Cells and supernatants were collected and evaluated for cytokine secretion and chemokine RNA expression. Cell viability following addition of the bacteria to cell culture was measured by an MTT assay and indicated that both *H. bilis* and *E. coli* were cytotoxic for MODE-K cells following 12 hours of cell culture (p < .05). After as little as 12 hours, MODE K cells exposed to *E. coli* expressed elevated levels of the proinflammatory cytokine, IL-6 compared to that induced by *H. bilis*. However, *H. bilis* increased expression of CXCL9 and MIP-2. *E. coli* incubation of MODE-K cells for 24 hours resulted in similar secretion of chemokines as *H. bilis* colonization including MCP-1 and RANTES. However, live bacteria were not required to induce these chemokines as WCS also increased production of these cytokines. Collectively, these data indicate that although *H. bilis* and *E. coli* differ in virulence attributes, the direct effects of their interactions with epithelial cells are minimal indicating that predisposition to an inflammatory insult may depend upon other innate/adaptive cell populations resident to or infiltrating the mucosa. Results suggest that bacterial colonization of the mucosal epithelium provokes innate
responses that contribute to the induction of mucosal inflammation. These studies are correlate with in vivo observations (chapter #3) demonstrating that the nature of the bacterial provocateur is a critical factor affecting the predisposition of the host to a colitic insult

**Introduction**

Inflammatory Bowel Disease (IBD) is a chronic inflammation of the gastrointestinal tract and that results in patients suffering with abdominal pain, nausea, diarrhea and vomiting. Although genetics, a breach in epithelial permeability, and aberrant immunity to members of the resident microbiota have been implicated in the etiology of IBD, it is widely accepted that an inappropriate response to the commensal flora drives the pathogenesis. For example, rodent models of IBD including IL-2−/−, IL-10−/−, T cell receptor alpha deficient mice and HLA-B27 rats maintained in germ-free conditions do not develop IBD; however, colonization with commensal bacteria results in the development of colitis in these mice and rats (1-4). Furthermore, human IBD patients develop serological responses to bacterial components including antibodies to the outer membrane porin C (OmpC) of *Escherichia coli* (5, 6). Previous studies from this laboratory have demonstrated that colonization of defined flora mice with a novel organism predisposed defined flora mice to DSS-induced colitis. The nature of the provocateur (e.g., virulence attributes) may determine the degree of susceptibility; however, both a pathogenic and commensal bacteria were shown to be able to induce antigen-specific CD4+ T cell reactivity to the commensal flora indicating and supporting evidence of the complex nature of the responses associated with the induction and/or maintenance of IBD.

To determine if *Helicobacter bilis* or *E. coli* preferentially exacerbate the susceptibility to DSS-induced colitis is due to the initial bacteria-epithelial cell interactions, an intestinal cell line was utilized to analyze chemokine and cytokine expression following exposure of MODE K cells to these two bacterial species. Results indicated that *H. bilis* and *E. coli* have different effects on MODE-K epithelial cells. The differential modulation of epithelial genes and proteins following treatment with a pathogen or commensal indicate initial responses may be critical to the development of host responses to chronic provocateurs.

**Materials & Methods**

**Cell Culture**

MODE-K cells are a polarized duodenal epithelial cell line from C3H/HeJ mice, immortalized with SV40 (7). Cells were and maintained in DMEM (high glucose-Cellgro) supplemented with 10% FBS, penicillin/streptomycin, 25 µg/ml gentomycin, L-glutamine, non-essential amino acids, 1 mM Na-pyruvate, and 2 mg/ml insulin. Cells were plated in a 24-well flat bottom plates (Costar 3512) at 8 x 10⁴ cells per well and incubated at 37°C in 5% CO₂. After 12-20 hours, when cells developed a flat and adherent morphology and were approximately 70-80% confluent, bacteria were added at a 1:200 ratio of cells: bacteria (8 x 10⁴cells/well). Bacteria were enumerated using a Petroff-Hausser chamber. Whole cell sonicates (WCS) were added at 59 µg/ml of protein, which was equivalent to a 1:200 dilution by BCA assay. Cultures were incubated at 37°C in
5% CO$_2$ for 12-24 hours and 100 µl of supernatant was collected and replaced with fresh media at each time point. After 12 hours of cell culture, supernatants were completely extracted off of MODE-K cells and cells were dissociated from plates by adding .5 ml of non-enzymatic cell dissociation solution (1X) (Sigma) to each well. Plates were incubated at 37ºC in 5% CO$_2$ for 10 minutes and then firmly tapped against bench top to dissociate cells. Cells were washed in media and resuspended in RINAlater for RNA extraction.

**Bacterial Preparation**

*H. bilis* was cultivated on modified Columbia broth agar plates containing, Isovitalex (BD), yeast extract (BD), casamino acids (Difco) and 10% bovine blood. 1-2 mls of sterile bruccella broth containing 20% newborn calf serum was overlayed on each plate. Plates were under microaerophilic conditions using a gas mixture containing 90 % N$_2$, 5% CO$_2$ and 5% H$_2$ and incubated at 37ºC for 12-24 hours. Microscopic evaluation of *H. bilis* was performed by wet mount to assess viability based upon degree of darting motility. Blood agar plates were inoculated with *H. bilis* and incubated aerobically prior to experimental assays to check for contamination. *E. coli* was cultivated on blood agar plates and inoculated into Brucella broth for 5-8 hours. *H. bilis* and *E. coli* were counted using a Petroff-Hauser chamber and prepared at 8 x 10$^6$ bacteria/ml. One ml of bacteria (8 x 10$^6$) was added to each well containing MODE-K cells. Whole cell sonicates were prepared as previously described (8). Briefly, WCS was sterilized by UV light irradiation and stored at –20 ºC until use. Sterility of each individual WCS was confirmed by culturing on blood agar plates overnight. WCS were added at 59 µg/ml (protein concentration equivalent to 1:200 cells: live bacteria by BCA assay).

**Analysis of Cell Cytotoxicity**

Cell viability was measured by the ability of mitochondrial dehydrogenases to cleave the tetrazolium ring of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) (Sigma). Cleavage of the tetrazolium ring results in the formation of insoluble formazan crystals that were dissolved by acidified isopropanol. The resultant color change (purple solution) was measured by a spectrophotometer at 570 nm following subtraction of the background absorbance measured at 690 nm.

**Chemokine and Cytokine Analysis Utilizing Real Time Quantitative PCR**

Total RNA was extracted from MODE-K cells using the RNAeasy Mini Kit (Qiagen) as described by the manufacturer. The RNA samples were quantified by ND-1000 spectrophotometer (Nanodrop technologies) and treated with TURBO DNA-free DNase at 2U/µl (Ambion) to eliminate genomic DNA contamination. 5 µg of RNA was reverse transcribed into cDNA by SuperScript™ III First-Strand Synthesis System (Invitrogen, San Diego, CA). cDNA was amplified using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen). The following PCR conditions were used: 95°C for 10 minutes followed by 40 cycles of amplification (95°C for 10 seconds, 56°C for 5 seconds, and 72°C for 10 seconds). Primers for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (forward primer: 5’- TCACCACCACATGGAGAGGC-3’, reverse primer: 5’-GCTAAGCAGTTGGTGTTGCA-3’) MIP-2 (forward primer: 5’-GAGTACCCCTGTGGGACATT-3’, reverse
primer: 5’-ACGAAAGGCATGACAAAC-3’), CXCL9 (forward: 5’-TTTCTTTTGGGCATC-3’, reverse primer: 5’-AGCATCGTGCATTCCAT-3’) and IL-6 (forward primer: 5’-CTGAGCTGCCCCTTAC-3’, reverse primer: 5’-CCACACCCCACGGCATCTC-3’) were used. The qRT-PCR was performed using Applied Biosystems GeneAmp™ 5700 Sequence Detection System. Standard curves for each gene were made using a series of cDNA dilutions. The relative RNA quantity of each gene was normalized to GAPDH (9).

**Chemokine Analysis Using LUMINEX Technology**

Following 12-24 hours of MODE-K incubation with bacteria or WCS, 100 µl of cell supernatants were collected for cytokine analysis by the Luminex 100 technology. A mouse chemokine kit was purchased (Millipore) to evaluate GM-CSF, MCP-1 and RANTES proteins.

**Statistical Analysis**

Data is displayed as mean ± SEM. Non-parametric group comparisons were analyzed by Kruskal-Wallis tests. Parametric group comparisons were analyzed by analysis of variance (ANOVA). Pairwise group comparisons having equal variances were analyzed using t-tests.

**Results & Discussion**

**Commensal E. coli and H. bilis induce cell cytotoxicity**

*In vivo* experiments from defined flora mice colonized with *E. coli* or *H. bilis* indicated that both organisms increased the susceptibility of DF mice to DSS-induced colitis, a lesser dose of DSS (1.5 %) was required to induce inflammation in *H. bilis* colonized DF mice compared to the 2.5 % dose of DSS used to induce colitis in *E. coli* colonized DF mice. To determine if *H. bilis* exposure was differentially affecting the health of the colonic epithelium, a viability assay was performed following bacterial treatment of MODE-K cells following 12-48 hours of incubation with live *H. bilis* or *E. coli*. Results indicated that *H. bilis* colonization significantly (p < .05) increased cell cytotoxicity following 12, 24, or 48 hours compared to cells incubated with medium alone or *E. coli* treated cells (Fig 1). Exposure of MODE-K cells to *E. coli* also decreased cell viability indicating that both bacteria negatively affect mitochondrial health.

**Viable bacteria not required for secretion of MCP-1 and RANTES**

The acute phase of IBD is characterized by the infiltration of neutrophils into the lamina propria of the colon or small intestine, whereas the chronic stages include the infiltration of neutrophils, T cells, macrophages, mast cells and eosinophils (10). The recruitment of cells to effector sites involves activation of the endothelium, rolling, tethering, and migration to sites of inflammation. Dysregulation in any of these processes may contribute to IBD development. IBD patients have elevated expression of CC chemokines in inflamed tissues and human colonic epithelial cell lines have been reported to secrete and express IL-8, MCP-1 and GM-CSF in
response to bacterial invasion (11-13). To determine if H. bilis recruits monocytes early in colonization, contributing to its virulence, CC chemokines including monocyte chemotactic protein-1 (MCP-1), and regulated upon activation, normal T-cell expressed and secreted (RANTES) were assessed from supernatants collected from MODE K cells following bacterial colonization for 12-24 hours. Granulocyte-macrophage colony-stimulating factor (GM-CSF) was also assessed. The addition of live H. bilis to MODE-K cells decreased production of MCP-1 and RANTES compared to cells only, however, H. bilis whole cell sonicates (WCS) increased their production indicating that MCP-1 and RANTES production does not require the presence of viable bacteria (Fig 2). H. bilis increased GM-CSF levels above WCS stimulation or E. coli stimulation. Interestingly, levels of MCP-1 and RANTES were similar between H. bilis and E. coli treated cells indicating that an additional cell type may be required to induce susceptibility. E. coli WCS also increased the levels of MCP-1 and RANTES above concentrations with live E. coli suggesting that toll-like receptor activation may drive the initial recruitment of monocytes and granulocytes.

Commensal E. coli increased epithelial IL-6 expression
IL-6 is a potent, multifunctional cytokine that plays an important role in acute inflammation and IBD patients have elevated levels of IL-6 in their serum (14). To determine if MODE-K cells expressed IL-6 following treatment with H. bilis or E. coli, cells were assessed for IL-6 expression by quantitative RT-PCR. Results indicated that E. coli increased IL-6 expression in MODE-K cells compared to H. bilis treated cells or WCS.

H. bilis evoked MIP-2 and CXCL9 Synthesis
The human CXC chemokine, IL-8 is a potent neutrophil attractant secreted in response to bacterial stimulation and products (15, 16). IL-8 has been implicated in the pathogenesis of IBD due to elevated levels of protein observed in active inflammatory tissues of IBD patients (17). The murine homologue of IL-8 is MIP-2 (18) and quantitative RT-PCR results indicated the H. bilis exposed MODE-K cells expressed significantly (p < .05) elevated MIP-2 and CXCL9 RNA compared to E. coli treated or WCS (Fig 4). CXCL9 (monokine induced by IFN-γ (MIG)) is expressed on the surface of epithelial cells, amongst others and is a chemoattractant for neutrophils, T and B cells (19-21).
Conclusions

The gastrointestinal tract contains a single layer of epithelial cells that serves to provide a physical, mechanical, and chemical barrier between the host and the luminal contents. These cells make the distinction between food antigens, pathogens, and commensal organisms on a daily basis. It is also quite clear that there is cross-talk between the mucosal epithelium and the intestinal microbiota. Previous experiments indicated that a pathogen, *H. bilis* and a commensal, *E. coli* differentially induced susceptibility to DSS-induced colitis following colonization of defined flora mice. We hypothesized that this increase in host susceptibility was likely initiated by interactions between the colonizing microorganism and the epithelium, which constituted the initial bacterial provocation. To test this hypothesis, an intestinal cell line, MODE-K was exposed to *H. bilis*, *E. coli*, or WCS. Initial experiments indicated that in vitro exposure of MODE-K cells to *H. bilis* significantly (P ≤ 0.05) decreased cell viability compared to controls. Interestingly, *E. coli* treated cells also reduced cell viability compared to control cells. Other research groups have reported that colonization of a human commensal *E. coli* on a colonic cell line increased ultrastructural changes in mitochondria morphology of the epithelium, increased permeability of epithelial barrier, and mediated cytoskeletal and tight junction protein rearrangements. The metabolic stress/cytotoxicity induced by *E. coli* or *H. bilis* on the epithelium may enhance internalization of commensal bacteria explaining why antigen-specific T cells were generated to the commensal flora in vivo following *E. coli* colonization even in the absence of detectable inflammation.

Chemokines mediate recruitment of inflammatory cells to mucosal tissues in active IBD, however, they are a double edged sword in host defense in as much that they are critical for the recruitment of innate cells to sites of inflammation to prevent spread of invading pathogens. In contrast, the excessive infiltration of granulocytes, monocytes and macrophages has a negative impact on epithelial and tissue health, thus, contributing to the pathogenesis of IBD. CCL5/RANTES, and CCL2/MCP-1 are CC chemokines that can recruit monocytes and promote transepithelial migration of TCRαβ+ and TCRγδ+ T cells. CC chemokines including RANTES and MCP-1 have been reported to be elevated and correlate with increased leukocyte infiltration in colonic tissue during active colitis. Supernatants from MODE-K cells treated with WCS of *H. bilis* or *E. coli* contained elevated MCP-1 and RANTES proteins compared to treatment with live *H. bilis* or *E. coli*. MCP-1 has also been reported to promote Th2 immunity as evidenced by the fact that MCP-1 deficient mice are incapable of generating Th2 responses. Previous in vivo experiments with *H. bilis* and *E. coli* colonization of defined flora mice demonstrated an elevated Th1/Th17 phenotype indicated by IgG2a antigen-specific antibodies and cytokine secretion by CD4+ mesenteric lymph node cells. Therefore, speculative evidence suggests that the lack of a balanced Th2 immune response following colonization with *H. bilis* and *E. coli* in vivo may correlate with the initial decrease of MCP-1 levels in the epithelium in vitro.

IL-6 is a critical cytokine in acute inflammation and has multiple functions in innate and adaptive immunity. IL-6 has also been shown to activate NF-kB activity in epithelial cells, which leads to increased...
expression of adhesion molecules (34) and promotes T cell growth and B and T cell differentiation. MODE-K cells were assessed for expression of IL-6 following treatment with H. bilis or E. coli live organisms or WCS. Results indicated that both H. bilis and E. coli live bacteria elevated epithelial IL-6 expression significantly (p < .05) compared to control cells or WCS. Therefore, the initial IL-6 response of the epithelium to a commensal E. coli or pathogenic H. bilis treatment is indifferent. It has been reported that the combination of IL-6 and TGF-β, can induce the Th17 cell lineage (35). Previous experiments (Chapters 2 & 3) indicated that E. coli and H. bilis induced mucosal IL-17 responses in vivo including antigen-specific induction of IL-17A by CD4+ T cells recovered from the draining mesenteric lymph node and recovery of mRNA from the proximal colon and cecal tissues. Therefore, acute expression of IL-6 in the epithelium may predispose to chronic in vivo IL-17 production. IL-17A has been shown to increase chemokine expression and neutrophil recruitment in the lung and blocking IL-17A prevented Th17 cell-induced neutrophilia (36). To determine if H. bilis or E. coli affected the ability of epithelial cells to recruit neutrophils, we measured the induction of the murine homologue of human IL-8, MIP-2. IL-8/MIP-2 is secreted in vitro in response to bacterial stimulation and in vivo has also been detected in crypt abscesses and at the base of intestinal ulcers (17). H. bilis treated MODE-K cells expressed significantly elevated (p < .05) MIP-2 compared to E. coli or WCS treated cells. The differential upregulation of MIP-2 by H. bilis suggests that it is recognized as a threat by the epithelium compared to E. coli, which may trigger downstream adaptive immunity. A recent report indicated that a nonpathogenic E. coli was described to decrease TNF-α induced IL-8 expression from an epithelial cell line (37). Furthermore, polymorphisms in CXCL9 are associated with Crohn’s disease in children (38) and adults (39). H. bilis significantly (p < .05) increased CXCL9 expression compared to E. coli or WCS treated MODE-K cells indicating that in vivo, the host may be responding by activating neutrophils as well as lymphocytes to the site of colonization.

It has been demonstrated that epithelia exposed to live H. bilis or E. coli bacteria display reduced cell viability. As previously shown (26), the metabolic stress associated with the presence of these bacteria may induce increased uptake of commensal organisms, which may indicate why E. coli induces antigen-specific CD4+ T cell reactivity to the commensal flora (i.e., increased antigen uptake and processing). The epithelium may perceive H. bilis as threatening and induce increased chemokines synthesis including MIP-2, CLCL9, and GM-CSF. The cross-talk between E. coli and the epithelial cells induced IL-6 expression suggesting that laminal proprial lymphocytes may be activated or predisposed to a IL-17 maturation pathway. The differential modulation of epithelial genes and proteins following treatment of MODE K cells with a pathogen or commensal indicated that the acute responses may be critical to the development of host responses to the persistent colonization of the GI tract with bacterial provocateurs.
Figure 1. Epithelial exposure to *H. bilis* and *E. coli* induces cytotoxicity. MODE-K cells were incubated with medium alone, live *H. bilis* or live *E. coli* for 12 to 48 hours prior to assessment of cell viability by the MTT assay. Data is represented by mean ± SEM. Statistical significance within treatment groups is indicated by different letters. n = three to nine per treatment group. Representative of two independent experiments.
Figure 2. Viable bacteria not required to increase secretion of MCP-1 and RANTES. MODE-K cells were incubated with *H. bilis*, *E. coli* or whole cell sonicates for 24 hours. Supernatants were collected and assessed for secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF) (A-B), monocyte chemotactic protein-1 (MCP-1) (C-D), and regulated upon activation, normal T-cell expressed and secreted (RANTES) (E-F). Data is represented by mean ± SEM and n= three to nine per treatment. Groups identified by different letters are statistically different from one another. Data representative of two independent experiments.
Figure 3. Commensal *E. coli* increased IL-6 expression. Quantitative RT-PCR analysis of IL-6 expression following exposure of MODE-K cells to live bacteria (1:200) or whole cell sonicates (WCS) for 12 hours. Data are expressed as fold change compared to GAPDH. Treatment groups identified with different letters are statistically different. \( n = \) three to nine per treatment group. Data representative of two independent experiments.

Figure 4. *H. bilis* induces expression of MIP-2 and CXCL9 mRNA. Quantitative RT-PCR was performed using RNA samples isolated from MODE-K cells that had been incubated with live *H. bilis*, *E. coli* or whole cell sonicates (WCS) of each organism for 12 hours showing elevated MIP-2 (A) and CXCL9 (B). Data are expressed as fold change compared to GAPDH. Data is represented as the mean ± SEM and \( n = 8 \) per treatment group. Treatment groups identified with different letters are statistically different. \( n = \) three to nine per treatment group. Data representative of two independent experiments.
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Inflammatory bowel disease (IBD) is a chronic inflammation of the gastrointestinal tract primarily comprised of Crohn’s disease (CD) and ulcerative colitis (UC). Although the etiology of IBD has not been completely established, genetics, aberrant immunity, and the environment have been implicated (1). However, it is believed that the influence of these factors on the development of IBD is contingent upon the presence of a resident microbiota and that an inappropriate immune response develops in reaction to microbial antigens due to persistent infection, dysbiosis, and/or genetic defects that affect microbial clearance and epithelial barrier function (2, 3). Although the importance of the resident flora has been studied in multiple animal models of IBD, the complications of genetic knockouts obscure the conclusions (4-9). For example, the 50% of IL-2−/− mice that do not die by 4-9 weeks after birth develop highly activated B and T cells and elevated antibodies in addition to pancolitis. However, if reared in germfree housing conditions, intestinal inflammation is delayed, but not absent (10). Because IL-2−/− mice lack regulatory T cells, the development of IBD is an expected outcome. The multi-factoral nature of the pathogenesis of IBD is driven by the hypothesis that defects in one or more aspects of the immune response contributes to disease (11). For example, a defect in innate immunity, toll-like receptors (TLRs), results in priming of CD4+ T cells resulting in disease (12). A deficiency in T regulatory cells or an excessive amount of effector T cells also results in IBD (13-20). Therefore, it has been hypothesized that ‘multiple hits’ or defects in the innate, adaptive, or regulatory immune responses are required to induce susceptibility to IBD in immune competent individuals (11). To be able to address how the resident flora contributes to an aberrant immune response in the development of IBD, one of the specific aims for this work was to develop a multiple-hit model of IBD in immune competent mice in order to characterize the resultant antigen-specific immune responses following the onset of colitis. To develop a multiple-hit model of colitis in immune competent, defined flora mice, we introduced Helicobacter bilis as a novel, bacterial provocateur into the stable microbiota of defined flora mice. Human IBD patients have been shown to develop immune responses to Helicobacter species other than H. pylori (21). In addition, H. bilis has also been associated with IBD-like lesions in immunocompromised mice (22-24). For example, SCID mice colonized with H. bilis developed clinical signs of typhlitis by 12 weeks post-infection (22). Previous experiments from this laboratory indicated that H. bilis colonization of immune competent, defined flora (DF) C3H mice, induced immune reactivity to the resident commensal flora (25) and microarray data indicated that H. bilis significantly upregulated genes associated with proteolysis, glycoprotein synthesis, protein metabolism and immune response and significantly downregulated fatty acid metabolism, lipid metabolism and carboxylic acid metabolism genes (Zhiping Liu-submitted for publication).

IBD patients typically have relapses in inflammatory episodes or flares between periods of quiescence. These active inflammatory episodes tend to correspond to stressful life events (26, 27). Stress has been shown to enhance colitis in rats and may predispose a person to IBD as well (28, 29). To induce environmental stress on the gut, we employed a low dose of dextran sulphate sodium (DSS) as the colitic insult or trigger to induce colitis. DSS has been commonly used to induce acute or chronic colitis in mice as...
the resultant clinical signs are similar to human colitis (diarrhea, weight loss, bloody stool) (30-35). We hypothesized that the introduction of a novel bacterial provocateur into the established intestinal flora would perturb host mucosal homeostasis, inducing aberrant immune responses to the resident flora (hit #1-\textit{H. bilis}), resulting in increased vulnerability to an environmental/inflammatory insult/ stressor (hit #2- dextran sulphate sodium (DSS) (Fig 1). Results indicated that, in comparison to DSS or \textit{H. bilis} colonization alone, mice dually treated with \textit{H. bilis} and DSS developed significantly (p < .05) increased clinical signs of disease including gross lesion scores, histological scores, and decreased colon length. Serum IgG2a responses to antigens derived from the enteric flora were increased compared to controls. Evaluation of tissue recovered from the proximal colon indicated an increase in mucosal IL-17, IFN-γ, and TNF-α after colonization and/or DSS treatment compared to other treatment groups. The magnitude of antigen-specific T cell proliferation increased with time, as Foxp3 expression declined; however, lesions did not increase in severity. Susceptibility to DSS-induced colitis correlated with the increased antigen-specific reactivity to the enteric flora and mucosal expression of inflammatory cytokines. In conclusion, these studies demonstrate that perturbation of the microbial ecology following colonization with a pathogenic bacteria may lead to abnormal Th1 and Th17 immunity, thus, predisposing the host to an inflammatory insult.

The innate and adaptive immune components of the gastrointestinal tract must maintain a multitude of complex functions including the differentiation between food, pathogenic, and commensal antigens on a daily basis. Complex mechanisms of tolerance are established to maintain homeostatic unresponsiveness to members of the microbiome including bacterial specific regulatory T cells, antimicrobial peptides, immunoglobulin A, and mechanisms of negative and positive selection in the thymus (20, 36). Disruption of these mechanisms may result in the generation of antigen-specific effector responses generated to members of the resident flora (37-41).

Therapeutic treatments of IBD include the administration of broad spectrum antibiotics including metronidazole and ciprofloxacin (42). The increase in microbial resistance to antibiotics has generated interest in developing alternate treatments, including probiotics (43, 44). Probiotics are beneficial bacteria that aid in the metabolism of nutrients, breakdown long chain fatty acids to provide butyrate to maintain the health of the epithelium among other functions and are hypothesized to rescue disbiosis conditions potentially contributing to IBD development. Although much hype has been generated towards the use of probiotic therapies, results are inconclusive. More specifically, commensal \textit{E. coli} strains have been utilized for probiotic therapies and have resulted in mixed reviews (45-49). In this regard, our second specific aim was to determine whether the increased sensitivity to DSS was a unique feature of \textit{H. bilis} colonization. Defined flora (DF) mice were colonized with a pathogenic \textit{H. bilis} or commensal \textit{E. coli} for three or 12 weeks prior to administration of DSS. In contrast to previous \textit{H. bilis} experiments, colonization of DF mice with \textit{E. coli} did not significantly increase the susceptibility to low-dose DSS (1.5 %); however, the susceptibility to typhlocolitis following exposure to a higher dose of DSS (2.5 %) was markedly increased. Gross and histological cecal scores of colonic tissues recovered from DF mice following colonization with
E. coli and high dose DSS were elevated as compared to similarly colonized mice treated with low dose DSS. Similar to control mice, E. coli colonized mice displayed no macroscopic lesions. As with H. bilis, DF mice colonized with E. coli developed ASF antigen-specific CD4+ T cell proliferative responses following in vitro stimulation that did not require DSS exposure. The treatment of E. coli colonized mice with a high-dose (2.5 %) DSS resulted in the induction of ASF-antigen-specific IL-17A secretion and cecal mucosal expression of IL-17A-specific mRNA. These results demonstrate that introduction of a novel bacterial species into the resident flora can affect mucosal homeostasis even though the bacterial strain is low in pathogenicity. Based on the context of complex interactions, the nature of the introduced provocateur and its impact on the colonic mucosa (e.g., gene expression, aberrant immunity) are critical factors that predispose the host to the onset of IBD (Fig 2) and caution should be taken when utilizing a commensal organism as probiotic therapy.

Epithelial cells are the first line of defense against microbial invaders. There are multiple mechanisms involved in the maintenance of tolerance to commensal bacteria including properties of the specific bacteria, characteristics of the epithelium, and characteristics of the immune cells in the lamina propria (50, 51). For example, some reports have shown that some intestinal epithelial cell lines express minimal amounts of TLR4 on their plasma membranes although they make detectable amounts of TLR4-specific mRNA (52). Depending on the degree of the inflammatory trigger, both pathogenic and commensal organisms were shown to differentially induced susceptibility to DSS-induced colitis. In this regard, the third specific aim was designed to evaluate the induction of chemokines and proinflammatory cytokines from murine epithelial cells exposed to H. bilis or E. coli. An intestinal epithelial cell line, MODE-K was exposed to live or whole cell sonicates of H. bilis or E. coli. Cell viability following addition of the bacteria to cell culture was assessed via MTT assay and indicated that both H. bilis and E. coli were cytotoxic for MODE-K cells following 12 hours of cell culture (p < .05). As previously shown (48), The metabolic stress associated with the presence of these bacteria may induce increased uptake of commensal organisms, which may indicate why E. coli induces antigen-specific CD4+ T cell reactivity to the commensal flora (i.e., increased antigen uptake and processing). Alternately, an increase in epithelial apoptosis may contribute to a breach in tolerance, explaining enhanced CD4+ responses to the resident microbiota (50). Consistent with mild inflammation observed in DF mice colonized with H. bilis, the exposure of MODE-K cells to H. bilis induced increased chemokine synthesis including MIP-2, CXCL9, and GM-CSF relative to that induced by E. coli. In contrast, the result of cross-talk between E. coli and the epithelial cells induced IL-6 expression suggesting that laminal proprial lymphocytes may be activated or predisposed to a IL-17 maturation pathway. Previous data (not shown) indicated that E. coli colonization of DF mice resulted in elevated mucosal IL-17A as early as three weeks indicating that epithelial responses may contribute towards the generation of IL-17 responses characterized in vivo. Results suggest that bacterial colonization of the mucosal epithelium provokes innate responses that contribute to the induction of mucosal inflammation. The differential modulation of gene expression and cytokine secretion following
treatment of MODE K cells with *H. bilis* or *E. coli* indicated that the acute responses may be critical to the development of host responses to the persistent colonization of the GI tract with bacterial provocateurs.

Collectively, these results have demonstrated the importance of the enteric flora in the development of IBD in an immune competent animal. Although *H. bilis* colonization increased reactivity towards a greater number of bacterial epitopes of the resident microbiota over time, the lesions do not become more severe following 12 or 20 weeks post-colonization. Consistent with human IBD, flares do not become worse with time indicating that this multi-hit mouse model may be realistic of clinical cases. The discovery that a commensal *E. coli* was able to predispose to DSS-induced colitis indicates that scientists should not be trying to discover a single complex bacterial species as the sole cause of IBD. Continued research should be spent determining the mechanism of how the initial host-bacterial interactions contribute to a breach in tolerance to the resident flora, resulting in aberrant immune responses that predispose to IBD.
Figure 1. Development of a multiple-hit model of IBD. Immune competent, defined flora C3H/HeN:Tac mice harboring the altered Schaedler’s flora were colonized with a novel bacterial provocateur, *H. bilis*. Three to 20 weeks after *H. bilis* colonization, low-dose (1.5%) dextran sulphate sodium (DSS) was administered in drinking water. The combinatory treatments resulted in clinical signs of colitis.
Figure 2. A pathogenic and commensal organism differentially predispose to IBD. An immunological threshold must be crossed in order to develop inflammatory bowel disease (IBD). Colonization of defined flora mice with *Helicobacter bilis* followed by administration of 1.5% of dextran sulphate sodium (DSS) induced clinical signs of IBD. However, the same low dose of DSS does not induce disease following the introduction of a novel *Escherichia coli* commensal into the resident flora. Interestingly, *E. coli* is able to predispose the host to typhlocolitis following administration with a high dose (2.5%) DSS. These data indicate that depending upon the virulence attributes of a novel provocateur and the degree of the inflammatory trigger/stressor, the host may be rendered susceptible to IBD.

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