THE ROLE OF ENVIRONMENTAL FACTORS IN GUT HOMEOSTASIS AND EXPERIMENTAL INFLAMMATORY BOWEL DISEASE

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by
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ABSTRACT

The etiology of inflammatory bowel disease (IBD) is complex. In addition to genetic factors, IBD onset is proposed to be triggered by environmental exposures. Studies have shown that environmental factors influence the bacterial composition in the gut. Intestinal bacterial flora have also been linked to IBD susceptibility. Using mouse models of colitis, I explored the role of two environmental factors in IBD pathogenesis: diet and vitamin D. The first objective was to evaluate the effect of different dietary intake on intestinal bacterial flora and IBD susceptibility. My study shows that variation in diet resulted in differential susceptibility to dextran-sodium sulfate (DSS)-induced colitis and *Citrobacter rodentium* infection in mice that were not genetically susceptible. In addition, 20% of lactose intake exacerbated the symptoms of DSS-induced colitis. Dramatic changes in gut bacterial composition were induced by dietary feeding in a short period of time. Furthermore, the depletion of bacteria using antibiotics protected mice from colitis regardless of diet. These findings suggest that diet-induced changes in susceptibility to colitis were associated with different varieties and amounts of bacteria present in the gut and that high lactose intake may contribute to the increased susceptibility to colitis. The second objective was to determine whether vitamin D regulates experimental IBD by modulating intestinal bacterial flora. My data show that the increased susceptibility of vitamin D-deficient mice to DSS-induced colitis was mediated by the alterations in gut bacterial flora. Vitamin D deficiency resulted in increased potentially pathogenic bacteria and decreased beneficial bacteria in the gut before colitis induction. In addition to bacterial flora, impaired epithelial barrier and decreased frequency of tolerogenic dendritic cells in vitamin D-deficient mice also predisposed them to DSS-induced colitis.
These results suggest that vitamin D plays a role in regulating gut bacterial flora, intestinal epithelial integrity, and IBD pathogenesis. Lastly, I examined the source and role of extrarenal Cyp27B1 in the immune system. Cyp27B1 is an enzyme critical for vitamin D synthesis. My study shows that CD8+ T cells but not CD4+ T cells produced Cyp27B1 activity. T cells from Cyp27B1 knockout mice showed elevated IFN-γ production post activation compared to wild-type T cells. Cyp27B1 knockout mice were protected from DSS-induced colitis by reconstitution with wild-type bone marrow cells, suggesting an anti-inflammatory role of immune-derived Cyp27B1. This study provides direct evidence of extrarenal Cyp27B1 and vitamin D production in regulating IFN-γ-induced pro-inflammation and experimental IBD. Together, the work presented in this thesis advocates the critical role of environmental factors in gut homeostasis and IBD susceptibility through the regulation of intestinal bacterial flora and host immunity.
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Chapter 1

Introduction
**Inflammatory bowel disease**

Inflammatory bowel disease (IBD) is a chronic inflammation occurring in gastrointestinal tract. There are estimated 1.4 million people in the United States and 4 out of 1000 people worldwide suffering from IBD (1, 2). Patients with IBD show symptoms including abdominal pain, diarrhea, ulcerations, rectal bleeding, and rupture of the bowel. Surgery is often required to remove the affected tissues when the medications can no longer control the inflammation. Moreover, patients with IBD have been reported to have increased risk of developing colonic cancer (3). The chronic inflammation in IBD does not have a medical cure and often require a lifetime health care. The treatments available for IBD patients include drugs for anti-inflammation and immune-suppression as well as antibiotics (4). However, these treatments have side effects and risks. Long-term use of drugs for anti-inflammation and immune-suppression may render the patients vulnerable to infections. Furthermore, antibiotic treatment has the risk for developing antibiotic resistant bacteria. Therefore, an alternative treatment to effectively prevent or control IBD onset is highly desired.
The etiology of IBD

The etiology of IBD is very complex and caused by a combination of genetic and environmental factors. Relatives of IBD patients have 10 times higher risk in developing IBD (5). The incidence rate increases to 30 times when siblings have IBD (5). Several IBD associated genetic factors have been identified. Most of the genes are involved in innate or adaptive immunity including NOD2 and ATG16 (6).

Independent of genetic factors, IBD pathogenesis is triggered by environmental exposures even in genetically predisposed subjects. The concordance rate between identical twins is only 50% for Crohn’s disease and 14% for ulcerative colitis (6), implicating the role of environmental factors in IBD pathogenesis. Epidemiology studies suggest a correlation between IBD incidence and dietary changes or sunlight exposure (7, 8). For instance, IBD is more prevalent in the westernized countries (9), and IBD incidence increases in people migrating into western countries (8). Increasing incidence of IBD has been reported in countries such as Japan and China that previously had low prevalence rates (8). Moreover, higher incidence of IBD was reported in people living in northern latitudes with less sunlight exposure than those who live in southern latitudes (7). However, there is yet no thorough study of the mechanisms by which environmental factors regulate IBD susceptibility.

IBD and intestinal microbiota

There is accumulating evidence suggesting the role of intestinal bacteria in IBD. The inflammatory condition in IBD may be the consequence of the overreaction to normal harmless bacteria due to the dysfunction of immune tolerance, or of the overgrowth of
specific pathogenic bacteria as a result of immunodeficiency (10-12). Studies suggest that alterations in intestinal flora (dysbiosis) contribute to the predisposition to IBD. For instance, antibiotic treatment and administration of probiotics have been reported to ameliorate the symptoms of IBD, presumably due to the elimination of pathogenic bacteria and the anti-inflammatory response induced by beneficial bacteria (13-15). It has also been shown that germ-free mice develop worse DSS-induced colitis compared to conventionally raised mice due to the lack of both beneficial and pathogenic bacteria (16). Furthermore, NOD2, a pattern recognition receptor, is one of the genetic factors that have been associated with IBD (17). Mutation of NOD2 has been shown to reduce the secretion of antimicrobial peptides by paneth cells and bacterial-killing activity (18). In addition, NOD2-deficient mice were found to have increased intestinal bacteria and impaired ability in preventing the colonization of pathogens, suggesting the role of bacterial regulation and IBD. On the other hand, environmental factors such as diet and antibiotics may alter the bacterial composition after the initial colonization (19), indicating the modulation of intestinal microflora by environmental factors may influence the susceptibility of the hosts to IBD.

**Intestinal microbiota**

Our gastrointestinal tract contains ten times more microorganisms than the human cells (20). Among intestinal microorganisms, bacteria represent the major population in the intestine. Due to the acid, bile, and pancreatic secretions and the propulsive motility in upper intestinal tract, the colonization of bacteria increases from the distal small intestine and becomes abundant in the colon, which reaches $10^{11}$ to $10^{12}$ of bacteria in a gram of
colonic content (20). Except for the bacteria in cecum which has higher ratio of aerobes, most of the intestinal microbiota is anaerobic (21), and Bacteroidetes and Firmicutes are the largest divisions among the species of intestinal bacteria. *Bifidobacterium*, *Lactobacillus*, and other lactic acid producing bacteria are beneficial bacterial species, while some species of *Clostridia*, *Staphylococci*, and *Bacteroides* are potentially pathogenic (22). It is essential to maintain the equilibrium between beneficial and pathogenic bacteria, and the disruption of the microflora may lead to pathogenesis.

During initial colonization of host gastrointestinal tract, bacteria create a favorable environment for themselves to prevent the colonization of the other bacteria later (23). Thus, early exposure of intestinal bacteria is considered to be a key determinant of the bacterial composition later in life, and the effect could last for years (24).

**Fermentation of intestinal microbiota**

Most of the intestinal microbiota is generally harmless in healthy hosts and performs beneficial functions to the hosts. Some carbohydrates such as fiber, starch, and lactose that cannot be digested by the hosts are fermented by the intestinal microbiota (25). This process is called saccharolytic fermentation, which ferments carbohydrates into short chain fatty acids including acetic, butyric, and propionic acids. These short chain fatty acids function as energy and nutrients that can be utilized by the hosts in colonic epithelium, muscle or liver (25). Furthermore, intestinal bacteria also has metabolic effects to promote the absorption of iron, calcium, and magnesium as well as synthesis of vitamin K, biotin, and folate for the host (20). It has been shown that the microbiota promotes lipid storage through the inhibition of fasting-induced adipose factor (26).
Studies have found that germ-free mice are resistant to obesity following the consumption of high-fat diet and require more intake of calories in order to maintain regular body weight of conventionalized mice (26, 27). The composition shift between Bacteroidetes and Firmicutes with reduced Bacteroidetes and increased Firmicutes has also been linked to obesity (28).

**Intestinal microbiota and the development of the immune system**

The initial colonization of intestinal bacteria has been suggested to play an important role in the early development of immune system during postnatal stage (29). There is mounting evidence that infants delivered by cesarean section had higher incidence of atopy (30, 31); moreover, healthy and allergic infants appeared to have different microbial composition (32). Early bacterial colonization is likely a crucial signal to stimulate the maturation of the immune system. Insufficient stimulation or altered microbial composition may influence the postnatal development of balanced immunity. In particular, imbalance of T helper (Th)-1 and Th-2 responses are related to the onset of Th-1 mediated autoimmune diseases (IBD and experimental autoimmune encephalomyelitis) and Th-2 mediated diseases (allergy) (32-34). Primary exposure of the microbial ligands also plays a role in the development of host’s immune tolerance to bacterial as well as food antigens. It has been demonstrated that germ-free mice were able to develop OVA-induced oral tolerance only after reconstitution of intestinal microflora to neonates but not to adult mice (32). Induction of peripheral tolerance results in unresponsiveness to specific antigens, and it is required to prevent the overreaction to self or environmental antigens. Failure to induce tolerance may trigger the uncontrolled
expansion or imbalance development of a particular subset of immune cells and further lead to the pathogenesis of autoimmune disease and allergy.

Studies using germ-free or gnotobiotic mice revealed significant contributions of the commensal microbiota on modulating the host’s immune system and intestinal morphology. Studies have shown that short chain fatty acids function to stimulate the mucosal epithelial cells, and germ-free mice show reduced proliferation and differentiation of epithelial cells (35). Compared to conventional mice, mice under germ-free conditions also show defects in the development of immune system, characterized by smaller Peyer’s patches, shorter crypts with less crypt cells, fewer TCRαβ+ intraepithelial lymphocytes (IELs), lower levels of serum immunoglobulins, and no inducible lymphoid follicles such as small isolated lymphoid follicles (ILFs) (21, 36, 37). Conventionalization of the germ-free mice with normal intestinal microflora successfully elicited the development of gut-associated lymphoid tissues (GALT), such as the expansion of TCRαβ+ IELs particularly those that express CD8αα, the proliferation of immunoglobulin-producing B cells in the lamina propria (LP), the formation of ILFs and the induction of secretary immunoglobulin A (21, 37, 38). Furthermore, it has been demonstrated that colonization of segmented filamentous bacteria (SFB) of the small intestine was able to restore the mucosal morphology and function in germ-free mice and partially increased the number of TCRαβ+ IELs to the level of conventional mice (39).

It has been a challenge to identify the strain of intestinal microbes because many of them are not cultivable, and results from conventional quantitative culture method may be biased by different culture conditions. However, in more recent years, the utilization of metagenomic analysis enables a thorough investigation on the correlation between the
components or stains of intestinal bacteria and immune cells. Bacterial DNA derived from gut microflora was reported to mediate the equilibrium between pro-inflammatory Th-17 cells and regulatory T cells (40). *Bacteroides fragilis* has been demonstrated to show immunoregulatory function in *Helicobacter*-induced colitis. Secretion of polysaccharides including polysaccharide A by *Bacteroides fragilis* was reported to inhibit the pro-inflammatory cytokine such as IL-17 but promote the anti-inflammatory cytokine IL-10 (41). On the other hand, studies suggest that ATP produced by the bacteria promote the accumulation of Th-17 (42), and SFB induces Th-17 cell development in the LP (43).

**Intestinal microbiota and host mucosal immunity**

Intestinal mucosa is the first site to have the direct contact with the intestinal microbiota, and there is only a layer of epithelium between the host’s immune system and the commensal flora (Fig. 1-1). Besides epithelial cells, M cells, paneth cells, and goblet cells, dendritic cells can extend their dendrites to directly sense the luminal contents and interact with the bacteria (44). The innate immune response distinguishes the intestinal bacteria by pattern-recognition receptors (PRRs): Toll like receptors (TLRs) and nucleotide-binding oligomerization domain (Nod). Once the epithelial cells detect the pathogens, they secrete humoral mediators such as immunoglobulin A, antibacterial peptides, chemokines or cytokines to initiate innate and adaptive response. M cells can also transport the antigens to the antigen-presenting cells such as dendritic cells or macrophages. The antigen-presenting cells carry the bacterial antigens and induce adaptive response in Peyer’s patches or mesenteric lymph node (45).
The intestinal immune system develops pro-inflammatory responses against pathogenic bacteria but not against commensal bacteria, raising a question how the beneficial bacteria evade the host’s immune system. Although the mechanism is not yet fully understood, studies have demonstrated some approaches used by the commensal bacteria to evade the host’s immune system. For instance, commensal bacteria were shown to mimic host cell membrane proteins, inhibit NFκB pro-inflammatory response, and downregulate pro-inflammatory cytokines (46-49).

The intestinal inflammation of IBD may be due to overreaction to normally harmless bacteria or overgrowth of pathogenic bacteria (10-12). Studies suggest that alterations in intestinal flora (dysbiosis) contribute to the predisposition to IBD. For instance, antibiotic treatment and administration of probiotics have been reported to ameliorate the symptoms of IBD (13-15). Additionally, germ-free mice either do not develop IBD (50) or show exacerbated IBD symptoms compared to conventionally raised mice (16).

**Vitamin D**

Vitamin D is a type of steroid hormone that is found in the diet or synthesized from 7-dehydrocholesterol when the skin is exposed to UV light (51). The inactive vitamin D undergoes hydroxylation in the liver to become the major circulating form 25(OH)D₃, which is further converted in the kidney to the active form of vitamin D, 1,25(OH)₂D₃. The enzyme that catalyzes 25(OH)D₃ into 1,25(OH)₂D₃ is called 1α-hydroxylase, which is encoded by the Cyp27B1 gene (52). 1,25(OH)₂D₃ binds to the nuclear vitamin D receptor (VDR) and forms a heterodimer with retinoid X receptor (RXR). The heterodimer complex then interacts with vitamin D response elements (VDRE) to
regulate gene transcription critical for calcium and phosphate homeostasis. Since the expression of the VDR was identified in immune cells, accumulating evidence suggest an immunoregulatory role of vitamin D during inflammatory disease.

**Vitamin D and extrarenal Cyp27B1**

Early experiments established that the kidney is the major organ to express Cyp27B1 enzyme. It was shown that nephrectomized animals and humans failed to produce measurable 1,25(OH)_{2}D_{3}. Under pathological conditions, several studies have reported extrarenal Cyp27B1 expression particularly during chronic inflammation. In 1981, an anephric sarcoidosis patient showed definitively that 1,25(OH)_{2}D_{3} was produced extrarenally. Macrophage from the anephric sarcoidosis patient but not other patients with lung disease was identified as the source of the extrarenal Cyp27B1 in vitro. Human monocytes/macrophages and dendritic cells have also been reported to express Cyp27B1 mRNA and protein in vitro upon stimulation with pathogen-associated molecular patterns including TLR4 ligand or TLR1/2 ligands. Renal Cyp27B1 production is mainly regulated by parathyroid hormone (PTH), calcium, and phosphate. When circulating calcium or phosphate level decreases, PTH is secreted to induce Cyp27B1 production from kidney proximal tubule cells for 1,25(OH)_{2}D_{3} synthesis. 1,25(OH)_{2}D_{3} then stimulates calcium or phosphate absorption from the intestine or promotes the release of calcium or phosphate from the bone. Elevated 1,25(OH)_{2}D_{3} also negatively regulates Cyp27B1 production. In contrast to renal Cyp27B1, PTH stimulation or the levels of calcium and phosphate did not affect extrarenal Cyp27B1 in macrophages. Alternatively, extrarenal
Cyp27B1 has been shown to be induced by inflammatory stimuli particularly IFN-γ and LPS through signaling pathways including JAK/STAT, p38 MAPK, and NK-κB in monocytes (62).

**Vitamin D and immune system**

Vitamin D has been shown to regulate the development and function of both innate and adaptive immune cells. It has been demonstrated that 1,25(OH)₂D₃ inhibited the differentiation and maturation of dendritic cells (DCs) as well as the pro-inflammatory cytokine secretion of macrophages and DCs, specifically IL-12, TNF-α, IL-1β, and IL-6 (74-77). Conversely, 1,25(OH)₂D₃ treatment resulted in increased IL-10 secretion in DCs, and 1,25(OH)₂D₃ treated DCs were able to suppress alloreactive T cells and stimulate the induction of regulatory T cells (76). In addition to innate immunity, 1,25(OH)₂D₃ has been reported to suppress the induction and cytokine secretion of Th-1 and Th-17 in a direct and indirect manner (75, 78-80). In contrast, vitamin D promotes the development and function of regulatory iNKT cells, and this subset is reduced in VDR KO mice (81). Together, vitamin D is an immunoregulator that suppresses pro-inflammatory responses and bolsters regulatory T cells and IL-10 secretion.

**Vitamin D and antimicrobial functions**

1,25(OH)₂D₃ directly induces the expression of antimicrobial peptides (β-defensin and cathelicidin) in human innate immune cells upon activation of TLRs (82). In addition, 1,25(OH)₂D₃ indirectly triggers the expression of antimicrobial peptides, β-defensin 2 by
upregulating the transcription of the NOD2 gene in human monocytes and epithelial cells (83, 84). Given that antimicrobial peptides regulate the microbial composition and IL-17-producing cells in LP (85), vitamin D may function through a similar pathway.

**Vitamin D and IBD**

Vitamin D deficiency has been reported in patients with IBD (86). Studies using animal models have shown that deficiency of vitamin D or vitamin D receptor exacerbates symptoms of autoimmune diseases including IBD, multiple sclerosis, and type-1 diabetes, whereas supplementation of vitamin D attenuated the symptoms of diseases and protected the mice (87-89). IBD is driven by the pro-inflammatory subsets, Th-1 and Th-17 cells, characterized by their cytokine production of IFNγ and IL-17 (75, 87). IL-10 is an important anti-inflammatory cytokine, and IL-10 KO mice have been shown to spontaneously develop IBD when they are exposed to conventional microflora (90). Double knockout of IL-10 and VDR has been shown to accelerate the onset of IBD in mice. No IL-10/VDR double knockout mice survive by 8 weeks of age, while IL-10 KO mice had 100% survival rate (91). Likewise, more severe colitis was induced in recombination activating gene (Rag) KO mice by the transfer of VDR KO derived CD4+ CD45RB^{high} T cells compared to that of WT CD4+ CD45RB^{high} T cells (91). Together, these studies suggest the critical role of vitamin D in regulating IBD.
In this thesis, the role of two environmental factors in IBD pathogenesis was investigated using mouse models of colitis. The first objective was to evaluate the effect of different dietary intake on intestinal bacterial flora and IBD susceptibility (Chapter 2). The second objective was to determine whether vitamin D regulates experimental IBD by modulating the composition of intestinal bacterial flora (Chapter 3). Finally in Chapter 4, I examined the source and role of extrarenal Cyp27B1 in immune system during inflammation.
Figure 1-1. Intestinal bacteria and host mucosal immunity.

Epithelial cells and dendritic cells constantly sense the luminal contents by pattern recognition receptors (PRRs). Once pathogens are detected, humoral mediators such as immunoglobulin A, antibacterial peptides, chemokines, or cytokines are secreted to initiate innate and adaptive responses.

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Chapter 2

Dietary intake predisposes hosts to inflammatory bowel disease via modulation of intestinal microbiota

DGGE and histological pictures were contributed by Rhonda Smith. Metagenomic analysis of 454 pyrosequencing was performed by Dr. Istvan Albert.
Abstract

Different dietary habits may underlie distinct predisposition to inflammatory diseases between individuals. The effect of diet on inflammatory bowel disease (IBD) was tested using mice that are not genetically susceptible. Three very diverse diets were used: Chow (CD), Teklad diet (TD), and purified diets (PD) made in our laboratory. The TD-fed mice were the most susceptible to dextran sodium sulfate (DSS)-induced colitis followed by the CD-fed mice, and the PD-fed mice were the least susceptible. There are multiple differences in the composition of these three diets. In particular, the TD contained higher lactose and fiber than the PD. Addition of extra lactose (20%), fiber (5%), or both lactose and fiber to the PD increased the susceptibility of the mice to DSS colitis, indicating the association between high intake of lactose and fiber and IBD pathogenesis. The changes in susceptibility were associated with differences in the variety and amounts of bacteria present in the gastrointestinal tract. Additionally, Citrobacter rodentium infection caused increased bacterial diversity and delayed secondary clearance in the TD-fed mice, suggesting that the dietary-induced bacterial changes resulted in the impaired ability of the commensal flora to outcompete with pathogens. Taken together, our study provides direct evidence of the dietary effect on IBD pathogenesis independent of genetic factors. These findings further highlight the potential of using dietary manipulation in disease prevention and treatment.
Introduction

It is estimated that 1.4 million people in the United States and 4 out of 1000 people worldwide suffer from inflammatory bowel disease (IBD) (1,2). Symptoms of IBD include fatigue, abdominal pain, diarrhea, weight loss, and rectal bleeding. IBD has no medical cure and requires a lifetime of medication. In severe cases, surgery is necessary to remove the inflamed tissues. In recent years, increased risk of developing colorectal cancer has also been reported in patients with IBD due to the chronic gastrointestinal inflammation (3). The etiology of IBD is yet unclear; however, it is proposed to be caused by a combination of genetic and environmental factors.

Several lines of evidence suggest that IBD pathogenesis is triggered by environmental exposures even in genetically predisposed persons. IBD is more prevalent in the western countries (4). However, increasing incidence of IBD has been reported in countries such as Japan and China that previously had low prevalence rates (5). Moreover, increased IBD cases have been found in people migrating into western countries (5). These epidemiology studies suggest a correlation between increased incidence and changes in dietary habits towards a western diet (6). Diet that contains high fat and protein has been linked to the pathogenesis of metabolic syndrome including atherosclerosis and inflammatory diseases (7,8). Epidemiological or case-control studies have been done to investigate the causal relationship between diet and IBD. Although dietary treatments have shown beneficial effects on IBD (9), the evidence is lacking to support diet as a risk factor of IBD pathogenesis. Studies on human subjects have encountered difficulties in patient recall of their dietary intake before IBD diagnosis and in drawing conclusions from patients with genetic variation (5,6). Considering that diet consumption varies from
person to person, different dietary habits may underlie distinct predisposition to inflammatory diseases between individuals.

The intestinal inflammation of IBD may be due to over reaction to normally harmless bacteria or overgrowth of pathogenic bacteria (10-12). Studies suggest that alterations in the intestinal flora (dysbiosis) contribute to the predisposition to IBD. For instance, antibiotic treatment and administration of probiotics have been reported to ameliorate the symptoms of IBD (13-15). Additionally, germ-free mice either do not develop IBD (16) or show exacerbated IBD symptoms compared to conventionally raised mice (17). Initial colonization of the bacterial flora is a key determinant of the major bacterial composition, but it has been suggested that environmental factors such as diet and antibiotics may alter the bacterial composition after the initial colonization (18). Modulation of intestinal microflora by environmental factors may influence the susceptibility of the hosts to IBD.

Several mouse models have been used to study IBD including dextran sodium sulfate (DSS)-induced colitis and *Citrobacter rodentium* infection. DSS is a chemical that causes damage of the epithelial barrier, leading to intestinal inflammation (19). The symptoms of DSS-induced colitis include weight loss, colonic bleeding and shrinkage. As recombinase-activating gene (Rag) knockout (KO) mice lacking T and B cells develop DSS symptoms (20), innate immune cells particularly macrophages and dendritic cells (DCs) are critical for the pathogenesis of DSS-induced colitis. Short-term DSS administration induces acute colonic inflammation, and mice normally recover after removal of DSS treatment (19). *C. rodentium* are bacteria from the Proteobacteria phylum. Infection with *C. rodentium* in mice is similar to enteropathogenic *Escherichia coli* (EPEC) infection in humans (19). The symptoms of *C. rodentium-*
induced colitis include diarrhea and intestinal epithelial thickening. Immunoglobulin production has been shown to play a critical role in clearance of *C. rodentium* (21). After primary infection, the host develops memory response against *C. rodentium*. A recent study has reported that the colonization of *C. rodentium* is controlled through their competition with gut commensal flora sharing similar food sources (22). The IBD models allow investigations on the association of IBD with intestinal microbiota and host immunity.

The effect of diet on the susceptibility to intestinal inflammation was probed using mouse models of IBD. Three widely used laboratory mouse diets were investigated. There are multiple differences in the composition of the diets particularly in the diversity of carbohydrates. The data show that short-term dietary feeding resulted in dramatic differences in the susceptibility to DSS- or *C. rodentium*-induced colitis. Moreover, high dietary intakes of lactose and fiber significantly increased the susceptibility to DSS colitis in mice, indicating lactose and fiber as key factors contributing to IBD susceptibility. Depletion of bacteria by antibiotics protected mice from colitis regardless of the diet they were on, suggesting that the changes in susceptibility were associated with dietary-induced bacterial alterations in the intestine. Our study provides direct evidence of the dietary effect on IBD pathogenesis independent of genetic factors.

**Methods and Materials**

**Mice and diet**

Age and sex-matched C57BL/6 WT mice were bred and housed at the Pennsylvania State University (University Park, PA). Three diets were used in the experiments. The standard
chow diet (CD, Laboratory Rodent Diet 5001, LabDiet, Quakertown, PA), the purified diet (PD) made in the laboratory (23), and the Teklad diet (TD, Teklad Diet 96348, Harlan Laboratories, Madison, WI) which is a rescue diet for vitamin D receptor knockout mice that has increased amounts of lactose, calcium, and phosphorus. Mice were fed the CD to begin with and switched to the PD or TD for two weeks. For short-term antibiotic experiments, mice were put on PD or TD for two weeks and then administrated broad-spectrum antibiotics (24) (1 g/L neomycin, 1 g/L metronidazole, 0.5 g/L vancomycin, and 1 g/L ampicillin) in drinking water for another two weeks. Mice for long-term antibiotic experiments were generated by breeders treated with broad-spectrum antibiotics and kept on the same antibiotics throughout the experiments. All of the experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the Pennsylvania State University.

**DSS-induced colitis**

Mice were induced with DSS colitis as previously described (25). Briefly, mice were treated with 2.5% DSS (MW= 40 kDa; ICN Biomedicals, Aurora, OH) in drinking water ad libitum for 5 days. Body weight (BW) change was monitored every day, and mice that had lost more than 20% of their BW were sacrificed for humane reasons. Mice were euthanized at day 5 and day 14 post-DSS to assess the colonic length and bleeding. Colonic blood score was evaluated as follows: 0- no visible blood in the entire colon, 1- blood detected in less than 2/3 of the colon, 3- blood visible throughout the entire colon (25). Distal colon was fixed in 4% paraformaldehyde for hematoxylin/eosin staining at Animal Diagnostic Laboratory at the Pennsylvania State University. Histological sections were scored on a scale from 0-11: severity of inflammation (0- no inflammation, 1-
increased number of leukocytes in the mucosa, 2- multiple loci of inflammation, leukocyte infiltration of mucosa and submucosa, 3- extensive leukocytic infiltrate in mucosa, submucosa, ulceration, depletion of mucin-secreting goblet cells, 4- extensive transmural leukocytic infiltrate, crypt abscesses), extent of injury (0- none, 1-mucosal, 2-mucosal and submucosal, 3- transmural), and crypt damage (0- none, 1- basal 1/3 damaged, 2- basal 2/3 damaged, 3- only surface epithelium intact, 4- entire crypt and epithelium lost) (25).

**Citrobacter rodentium infection**

The *C. rodentium* strain ICC169 was a gift received from Dr. Gad Frankel (London School of Medicine and Dentistry, London UK). *C. rodentium* were cultured overnight in LB broth containing 50 μg/ml nalidixic acid (EMD chemicals, Gibstown, NJ), then 5x10⁹ CFU *C. rodentium* in phosphate-buffered saline (PBS) were orally gavaged to each mouse. Fecal samples were collected and homogenized in PBS (0.1 g feces in 1 ml PBS) to monitor fecal shedding. Serial dilutions were plated in triplicate on LB agar plates containing nalidixic acid and cultured overnight at 37 ºC for colony counting. Secondary infection was administered one week post clearance of the primary infection. Mice were sacrificed at peak (day 10) and clearance of primary infection and clearance of secondary infection. Serum and fecal supernatant were collected for ELISAs. Distal colon was fixed for histological analysis as DSS colitis. Distal colon was scored on a scale from 0-8 for inflammation (0-4) and epithelial thickening (0-4) using previously described criteria (26).

**Denaturing gradient gel electrophoresis (DGGE)**
Fecal samples were collected two weeks after dietary feeding or antibiotic treatment. DNA was isolated from fecal samples using QIAamp DNA stool mini kit (Qiagen, Valencia, CA), and 200 ng of fecal DNA was amplified with universal bacterial primers targeting the variable V3 region and conserved regions of the 16S rDNA in 30 μl PCR mixture (27). Primer sequences are as follows: forward 5’-CGCCCGCCGCGCGCGGCGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3’ (GC-clamp); reverse 5’-ATTACCGCGGCTGCTGG-3’.

25 μl/lane of PCR products were then loaded to the perpendicular gel with a linear 30-60% denaturing gradient and run at 70V for 17 h in DCode Universal Mutation Detection System (BioRad, Hercules, CA). PCR products of bacterial DNA isolated from purified cultures of *Clostridium propionicum* (ATCC strain 25522), *Lactobacillus murinus* (ATCC strain 35020), and *Parabacteroides distasonis* (ATCC strain 8503) were also included in the gel as standards. The same standards were used every time to compare the migration of the bands between gels run on different days.

To identify bacterial DNA in the diet, DNA was isolated from PD and TD using QIAamp DNA stool mini kit (Qiagen) and amplified with 16S rDNA primers. PCR products were processed through DGGE as described above. Bacterial bands on the DGGE gel were then excised and incubated with 1xTAE buffer at 37 °C for 5 h. DNA eluted in the buffer was further amplified with PCR using 16S rDNA primers, cloned into pCR4-TOPO vector, and transformed in competent *E. coli* following manufacturer’s instructions (Invitrogen, Carlsbad, CA). Colonies were then picked, and plasmid DNA was isolated. The plasmid DNA was further amplified with 16S rDNA primers and run on DGGE gel with DNA samples from diet to confirm the alignment of bands. Following confirmation,
the plasmid DNA was sent for sequencing to the Genomic Core facility at the Pennsylvania State University. DNA sequences were then compared with GreenGenes (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi) and Ribosomal Database Project (http://rdp.cme.msu.edu/) database using BLASTN program (National Center for Biotechnology Information, Bethesda, MD).

**Metagenomic analysis**

Fecal DNA isolated from the PD- or TD-fed mice with or without antibiotic treatment was sequenced on a 454 Titanium sequencer at the Pennsylvania State University. The data were then sent to Bioinformatics Consulting Center (http://www.bcc.bx.psu.edu) at the Pennsylvania State University for metagenomic analysis. DNA sequences were compared with GreenGenes (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi) and Ribosomal Database Project (http://rdp.cme.msu.edu/) database using BLASTN program (National Center for Biotechnology Information).

**Quantification of total bacteria**

10 ng/sample of fecal DNA was amplified with 16S rDNA primers using SYBR green mix (BioRad) by ABI 7500 Fast RT PCR machine (Applied Biosystems, Carlsbad, CA). Relative 16S rDNA copies were calculated using \( \Delta \Delta C_t \) method \( (2^{\Delta C_t}) \) and then converted to the total relative copies in the amount of DNA isolated per g of feces. The final results were shown as relative fold changes.

**Cell isolation**

Mesenteric lymph node (MLN) cells were isolated by homogenizing MLN using 25 gauge needles. Isolation of intestinal epithelial cells (IEC) and lamina propria lymphocytes (LPL) was performed as previously described (28,29). Briefly, colon was
collected and flushed with HBSS containing 5% FBS to remove fecal matter. Tissue was then opened longitudinally and cut into 0.5 cm pieces followed by three incubations in HBSS containing 5mM EDTA, 1mM DTT, and 5% FBS (Sigma-Aldrich, St. Louis, MO) for 20 min at 37 ºC under 200 rpm rotation. Supernatant containing IEC was collected from the three incubations, then tissue was further incubated in RPMI-1640 containing 1 mg/ml collagenase type 1 (Worthington, Lakewood, NJ), 3 mg/ml dispase II (Roche Diagnostics, Indianapolis, IN), and 10% FBS for 1 h at 37 ºC under 200 rpm rotation to obtain LP cells. IEC and LP samples were further processed through 40%/80% Percoll gradients (Sigma-Aldrich) to enrich lymphocytes. Cells were then stained with FITC CD11b, PE Gr1, PECy7 F480, PE CD103, PECy7 CD11c (BD Biosciences, San Jose, CA), analyzed on a FC500 bench top cytometer (Beckman Coulter, Brea, CA), and further evaluated with Flowjo 7.6.1 software (Tree Star, Ashland, OR).

**ELISA**

Total IgA production in serum and fecal supernatant was measured by mouse IgA ELISA quantitation set (Bethyl Laboratories, Montgomery, TX). For *C. rodentium*-specific IgG and IgA production, 30 µg/ml of sonicated *C. rodentium* protein was coated on 96-well plates (50 µl/well) at 4 ºC overnight. The wells were washed three times with PBS containing 0.05% Tween-20 and blocked with PBS containing 2.5% BSA and 0.05% sodium azide for 1 h at room temperature. Wells were then washed three times and incubated with 50 µl/well of sample for 2 h at room temperature. Wells were again washed five times and incubated for 1 h at room temperature with HRP-conjugated anti-mouse IgA or IgG1 (Bethyl Laboratories). After seven washes, wells were incubated with
50 µl of TMB substrate solution (BD Biosciences, SanDiego, CA) for 15 min, stopped with 50 µl of 2 N H₂SO₄, and read at 405 nm. Two-fold serially diluted serum samples were included to generate a standard curve, and aliquots of the same standard samples were used in every experiment. C. rodentium-specific IgG or IgA were shown as relative concentrations obtained from the standard curve.

**Statistical analysis**

Unpaired Student’s t test, one-way ANOVA with Tukey’s post-tests, and two-way ANOVA with Bonferroni post-tests were used to calculate statistical significance via GraphPad Prism software (GraphPad, La Jolla, CA). *p* < 0.05 is indicated by *, *p* < 0.01 is indicated by **, and *p* < 0.001 is indicated by ***. Error bars represent standard error of the mean.

**Results**

**Dietary effects on DSS-induced colitis**

Three very diverse diets were used to study the dietary effects on intestinal inflammation: CD, TD, and PD. No weight loss was observed as a result of the dietary changes (Fig. 2-1). WT mice on CD lost weight by day 8 post DSS and recovered the weight by day 14 post DSS (Fig. 2-2A). More than 90% of the CD-fed mice were able to survive throughout the experiments (n=13, one death at day 8) (Fig. 2-2B). The PD-fed mice lost significantly less weight than the CD-fed mice following DSS treatment, and all survived throughout the experiments (Fig. 2-2A and 2-2B). The TD-fed mice had the most severe weight loss and only a 10% survival rate by day 8 post DSS (Fig. 2-2A and 2-2B). In addition, colonic shrinkage and bleeding were most severe in the TD, intermediate in the
CD, and least severe in the PD-fed mice (Fig. 2-2C and 2-2D). Histopathology sections showed that distal colons of the TD- or CD-fed mice by day 14 post DSS were severely inflamed, showing increased lymphocyte infiltration, loss of goblet cells, and crypt damage, whereas the PD-fed mice had only slight colonic inflammation in the mucosa (Fig. 2-2E). Collectively, the TD-fed mice were the most susceptible to DSS-induced colitis followed by the CD-fed mice, and the PD-fed mice were the least susceptible (Fig. 2-2).

Similar dietary-induced changes in colitis severity were also observed in Rag KO mice lacking T and B cells (Fig. 2-3A). Compared to WT mice, Rag KO mice exhibited more weight loss post DSS induction (Fig. 2-3A). The CD-fed Rag KO mice lost more than 15% of their original BW by day 8 post DSS and showed slow recovery from weight loss by day 12 post DSS (Fig. 2-3A). In addition, Rag KO mice on the PD showed a mild weight loss and recovered by day 12 post DSS, while the TD-fed Rag KO mice had the most severe weight loss and did not survive more than 6 days post DSS (Fig. 2-3A). The data suggest that the dietary effect on colitis severity was not associated with T and B cells.

**Dietary composition**

To investigate why the three diets caused differential colitis severity, the dietary compositions were assessed. There are multiple differences in the composition of the diets (Table 2-1). The CD had the highest proportion of protein among the three diets, while the PD and TD contained similar amounts of protein (Table 2-1). However, there is no difference in fat among the diets (5 %) (Table 2-1). The biggest difference found was in carbohydrates, with the PD and TD containing a higher proportion of carbohydrates.
than the CD (Table 2-1). The CD had the greatest diversity of carbohydrates, the main source being starch (Table 2-1). The TD had four types of carbohydrates including sucrose, lactose, and starch, and the PD contained mainly sucrose and glucose (Table 2-1). The PD and TD had the same source of fiber (cellulose), but the CD was composed of cellulose, hemi-cellulose, and lignin. The PD had lower fiber than the other two diets, 3% instead of 5% (Table 2-1). Additionally, the TD had double the amount of calcium and phosphorus compared to the PD and CD (Table 2-1). Calories from the PD and TD were provided by similar proportions of protein, fat, and carbohydrate, while calories in the CD were derived from higher protein and lower carbohydrates (Table 2-1). Despite the diversity and sources of dietary components, the metabolizable energy of the three diets was comparable (3.35 - 3.84 kcal/g) (Table 2-1).

To address the effects of selected nutrient changes on colitis severity, the PD was modified to contain the same amount of high lactose (20%) and/or fiber (5%) as the TD. Mice fed the PD containing high lactose (PD HL), high fiber (PD HF), or high lactose and fiber (PD HLF) did not show weight loss following the change in diet prior to DSS. Upon DSS administration, mice fed PD HL or PD HF had significantly more severe weight loss compared to the PD-fed mice (Fig. 2-3B). Furthermore, PD HLF resulted in significantly more weight loss than the PF HL or PD HF (Fig. 2-3B). The colitis symptoms of mice fed PD HL, PD HF, or PD HLF were however not as severe as the CD- or TD-fed mice (Fig. 2-2A), and no mortality was observed. The data suggest that high lactose and fiber in the diet contribute to the increased severity of colitis in the TD-fed mice.

**Dietary changes alters the intestinal bacterial composition**
Next, to understand whether the variability in colitis susceptibility induced by diet was associated with intestinal microbiota, the PD and TD which showed the most and least severe colitis symptoms were further investigated. Bacterial DNA was isolated from the feces of the PD- or TD-fed mice prior to DSS and screened by DGGE fingerprints (Fig. 2-4A). DGGE utilizes a denaturing gradient to separate DNA with different sequences and sizes. Differences within sexes and littersmates were seen in mice fed the same diet (Fig. 2-4A). Dietary feeding induced dramatic changes in bacterial banding patterns regardless of their breeders and sexes (Fig. 2-4A). The data indicate changes in the types and amounts of bacteria as a result of short-term feeding of PD or TD.

454 pyrosequencing further revealed multiple alterations in bacterial phyla and genera (Fig. 2-4B and 2-4C). In particular, the TD-fed mice showed a decrease in Bacteroidetes and an increase in Proteobacteria and Actinobacteria in the feces compared to the PD-fed mice (Fig. 2-4B). Additionally, the TD-fed mice expressed higher *Clostridium sp. ID4* (Firmicutes) and lower *Odoribacter splanchnicus* and *Lactobacillus* (Firmicutes) than the PD-fed mice (Fig. 2-4C). Cluster analysis of the bacterial composition showed that the mice fed the PD were more similar to each other than mice fed the TD (Jaccard similarity measurement). Despite the changes in bacterial composition, total bacterial numbers in the feces were comparable between the PD- and TD-fed mice (Fig. 2-4D).

To rule out the possibility that the differences found in the PD- or TD-fed mice were due to the bacterial DNA contamination in the diet itself, bacterial DNA isolated directly from the PD and TD were examined using DGGE (Fig. 2-4E) and Sanger sequencing. The TD contained bacterial DNA from two types of lactic acid bacteria, *Leuconostoc sp.*
and *Lactococcus lactis subsp. lactis*, and the PD contained only *Lactococcus lactis subsp. lactis* (Fig. 2-4E). These two bacterial strains were very low (< 1%) or undetectable in the fecal DNA from the PD- or TD-fed mice.

**TD feeding induces inflammation of the colon**

The total cell numbers isolated from colonic epithelium (IEC), lamina propria (LP), and mesenteric lymph nodes were comparable between the PD- and TD-fed mice prior to DSS (Fig. 2-5A). No significant difference was found in the frequency of macrophages, monocytes, and neutrophils in the colon LP of the PD- or TD-fed mice (Fig. 2-5B). Significantly higher frequency of CD11b- dendritic cells (DCs) was present in the colon LP of the TD-fed mice compared to their PD counterparts (Fig. 2-5C). Additionally, the frequency of regulatory CD11b-CD103+ DCs significantly decreased in the TD-fed mice (Fig. 2-5C). Histological sections of distal colon from the TD-fed mice showed significantly increased lymphocyte infiltration in the mucosa compared to their PD counterparts (Fig. 2-5D). Moreover, the TD-fed mice had significantly higher total histological scores in colon than the PD-fed mice, due to the increased colonic inflammation and epithelial thickening (Fig. 2-5D). The data show that the TD feeding induces colonic inflammation.

**Antibiotic treatment attenuates colitis symptoms**

Mice continuously treated with antibiotics were fed with the PD or TD. Elimination of the bacterial diversity in both the PD- and TD-fed mice with antibiotics was confirmed by DGGE fingerprints (Fig. 2-6A) and 454 pyrosequencing (Fig. 2-6B and 2-6C). Despite administration of broad-spectrum antibiotics, no reduction in total bacterial numbers were observed in feces of the PD- or TD- fed mice post antibiotics, presumably due to
overgrowth of antibiotic-resistant bacteria (Fig. 2-6D). There were dietary effects on the bacterial flora in the PD or TD-fed mice treated with antibiotics (Fig. 2-6A, 2-6B, and 2-6C). Antibiotic-treated TD-fed mice had only bacteria from the Proteobacteria phylum, while their PD counterparts expressed bacteria from Proteobacteria, Tenericutes, and Firmicutes in the feces (Fig. 2-6B and 2-6C).

Long-term antibiotic treatment decreased colonic inflammation in the TD-fed mice at day 0 without DSS (Fig. 2-5A). Moreover, antibiotic-treated PD- or TD-fed mice were protected from DSS-induced colitis. The PD-fed mice that normally developed mild colitis symptoms showed decreased weight loss, colonic bleeding, and colonic shrinkage after antibiotics (Fig. 2-7A, 2-7B, and 2-7C). DSS treatment was fatal to the TD-fed mice (Fig. 2-7A), whereas antibiotics significantly increased survival rate to 86% (n=7, one death at day 8). Antibiotic-treated TD-fed mice developed milder weight loss, colonic bleeding, and colonic shrinkage (Fig. 2-7A, 2-7B, and 2-7C).

Additional experiments were done using the same antibiotic cocktail but for 2 weeks. Short-term antibiotic treatment resulted in a 2 to 6 fold reduction of bacterial numbers in the intestine compared to the control without antibiotics (Fig. 2-8A). Similar to long-term antibiotics, the TD-fed mice were protected from severe DSS-induced colitis post short-term antibiotics (Fig. 2-8B, 2-8C, and 2-8D). Both long-term and short-term antibiotics significantly reduced colonic inflammation in the TD-fed mice, with decreases in lymphocyte infiltration, colonic injury and crypt damage post DSS (Fig. 2-7D and 2-8E).

**TD results in delayed clearance of secondary Citrobacter rodentium**

The PD- or TD-fed mice were infected with *C. rodentium* to induce colitis. *C. rodentium* peaked at day 9, and all of the mice cleared the infection with the same kinetics (Fig. 2-
No cultivable *C. rodentium* was detected by day 40 (Fig. 2-9A). The mice were then re-infected with a secondary challenge of *C. rodentium* at day 47 after primary infection. The PD-fed mice showed undetectable *C. rodentium* by 6 days post secondary infection, while the TD-fed mice still had *C. rodentium* in the feces, showing the TD-fed mice took significantly longer to clear a secondary infection of *C. rodentium* (Fig. 2-9B). The bacterial loads in the TD-fed mice were also more than 10-fold higher than their PD counterparts on days 1-6 (Fig. 2-9B).

There was no difference in the histological sections of the distal colon in the PD- or TD-fed mice after primary and secondary infection (Fig. 2-9C). Total IgA and *C. rodentium*-specific IgA and IgG in serum were comparable between the PD- and TD-fed mice (Fig. 2-10A and 2-10B). Different bacterial banding patterns were detected between the PD- or TD-fed mice following primary *C. rodentium* infection, indicating dietary-induced bacterial changes post *C. rodentium* colonization (Fig. 2-11).

**Discussion**

Our studies show that different dietary intakes affect colitis susceptibility, providing direct evidence that variation in diet between individuals contribute to predisposition to inflammatory diseases. The dietary effect on inflammation was observed in mice that are not genetically susceptible to colitis, suggesting that a dietary factor may lead to the pathogenesis of inflammatory diseases independent of genetic factors.

Alterations in the bacterial composition were found in the PD- or TD-fed mice prior to colitis induction, suggesting the dietary effect on the composition of bacterial flora in the gut. Additionally, elimination of bacterial diversity or total numbers by antibiotic
treatment rescued the phenotype of the TD-fed mice from fatal DSS symptoms. The susceptibility of mice to colitis was associated with the dietary-induced gut bacterial changes. Despite the protective effect of antibiotics, there are yet differences in the severity of colitis and the bacterial composition between antibiotic treated PD- and TD-fed mice. Additionally, although bacterial flora were also affected by sex and breeder, dietary feeding resulted in dramatic alterations in bacterial composition in the gut. More importantly, the symptoms of DSS-induced colitis were consistently most severe in the TD-fed mice and least severe in the PD-fed mice, regardless of either sex or breeder. Collectively, our data show the dietary effect on intestinal inflammation through modulation of the bacterial flora in the intestine.

In addition to DSS-induced colitis, the TD-fed mice showed delayed clearance of secondary *C. rodentium*-induced colitis. The delayed clearance was not due to impaired host memory response as comparable IgA and IgG levels were found in the PD- or the TD-fed mice at primary and secondary infection. The finding is supported by the study reporting that *C. rodentium* colonization is regulated through their competition with commensal bacteria that share similar food sources but not by the host immune defense (22). The bacterial composition following *C. rodentium* primary colonization showed different bacterial banding patterns between the PD or TD-fed mice. It is yet unclear whether the bacterial changes in the TD-fed mice contributed to the delayed clearance of *C. rodentium* during secondary infection. However, *C. rodentium* primary infection may have decreased the colonization of bacteria that were capable of outcompeting *C. rodentium*, leading to a more susceptible phenotype in the TD-fed mice prior to
secondary infection. These data indicate that dietary-induced bacterial alterations may also contribute to different susceptibilities to pathogen colonization.

Multiple differences were found in PD and TD. In particular, TD contained higher amounts of lactose and fiber than PD. Our data show that PD containing high lactose and/or fiber consistent with TD levels caused more severe DSS-induced weight loss than PD. Mice fed PD containing both high lactose and fiber had even significantly more severe DSS symptoms compared to mice fed PD with either high lactose or high fiber. This finding is supported by the study showing that IBD patients had higher fiber intake before diagnosis compared to controls (30). Additionally, increased consumption of dairy food, which are high in lactose, has also been reported in IBD patients before diagnosis (31). Several other dietary components, such as protein, have been linked to IBD pathogenesis (31). However in our data, the CD did not cause the most severe colitis symptoms despite the fact that it contained the highest proportion of protein compared to PD and TD. Although it is possible that other dietary components might have also contributed to the colitis severity in the TD-fed mice, our findings suggest that dietary intake high in lactose and fiber significantly increase the susceptibility to IBD.

Fermentation by gut bacteria results in the production of short chain fatty acids (SCFA). SCFA production decreases the luminal microenvironment pH, leading to overgrowth of bacteria favoring low pH condition. Lactose and fiber (including cellulose) are fermentable by intestinal microbiota (22). As we expected, the TD-fed mice showed lower pH in the colon compared to the PD-fed mice (TD pH 7 versus PD pH 9, data not shown). This symptom may be caused by the high levels of lactose and fiber in the TD or by the impaired host production of lactate due to the intestinal inflammation. The
fermentation of lactose and fiber by intestinal flora may have resulted in overgrowth of 
bacteria that lead to severe colitis in mice.

In addition to bacterial changes, the TD-fed mice at baseline showed low level of colonic 
inflammation and changes in frequency of macrophages and DCs in the colon compared 
to their PD counterparts. Disruption of bacterial composition by antibiotics decreased the 
inflammatory level in the TD-fed mice, indicating that the inflammation caused in the 
TD-fed mice at baseline was associated with the intestinal bacterial flora. However, it is 
also possible that the dietary effect on host immunity at baseline was too low to be 
detected using our system. Further investigation using germ-free mice will be 
necessary to clarify the direct role of diet in host immunity.

In conclusion, our data show that dietary intake predisposed the hosts to inflammatory 
diseases independent of genetic factors. The differential severity of inflammation was 
associated with dietary induced alterations in bacterial flora in the gastrointestinal tract. 
Together, our findings provide direct evidence suggesting different dietary habits as an 
environmental factor contributing to variation in inflammatory disease susceptibility 
between individuals. Understanding the causal relationship between diet and 
inflammatory diseases highlights the importance of pre-disease diet and provides useful 
insights for dietary manipulation in disease prevention and treatment.
References


luminal bacteria is essential for the development and perpetuation of colitis in Tg(epsilon26) mice. *Gastroenterology* 120:900-13.


Figure 2-1

Figure 2-1. No weight loss prior to DSS during dietary feeding.

Weight change of the PD-, CD-, or TD-fed mice after dietary changes (n=5/group). Data shown are representative of three independent experiments.
Figure 2-2. Diets cause differential severity of DSS-induced colitis.

(A) Weight change of the PD, CD, or TD-fed mice post 2.5% of DSS administration (n=4-6/group). (B) Survival rates of the PD, CD, or TD-fed mice post DSS-induced colitis (n=11-14/group). * Significant difference between PD and TD, † significant difference between CD and TD, ‡ significant difference between PD and CD. (C) Colonic length of the PD, CD, or TD-fed mice at day 0 (n=4/group), day 5 (n=3/group), and day 14 (PD n=11, CD n=8, TD n=1) post DSS. (D) Colonic blood score at day 5 post DSS (n=3/group). (E) Histological scores of distal colon from the PD-, CD-, or TD-fed mice at day 14 post DSS (PD n=4, CD n=2, TD n=1). Data shown are representative or combined data of three independent experiments.
Table 2-1

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Table 2-1. Dietary composition of the PD, CD, and TD.

1 Total carbohydrates of the CD were shown as % by difference. 2 Total carbohydrates of the TD were the numbers subtracted with the moisture in lactose (5 %) and corn starch (9.5 %). 3 Selected minerals. Metabolizable energy was calculated using the Atwater factors: decimal fractions of protein, fat, and carbohydrate x 4,9,4 kcal/g, respectively.
Figure 2-3. High lactose and fiber in diet increase colitis severity.

(A) Weight change of Rag KO mice consuming the PD, PD HL, CD, or TD post 2.5% DSS (n=3-5/group). (B) Weight change of Rag KO mice fed PD, PD HL, PD HF, PD HLF post 2.5% DSS (n=6-16/group). *** Weight loss of PD HL, PD HF, or PD HLF was significantly different from PD values, and weight loss of PD HLF was significantly different from PD HL and PD HF values. Data shown are representative or combined data of three independent experiments.
Figure 2-4
Figure 2-4. Dietary feeding changes intestinal bacterial composition

(A) DGGE fingerprints of fecal DNA from the PD- or TD-fed mice prior to DSS treatment (n=4/group). Mice were males (M) and females (F) from two pairs of breeders (1 and 2). Standards (STD) shown were bacterial DNA from *Parabacteroides distasonis*, *Lactobacillus murinus*, and *Clostridium propionicum*. (B and C) Phylogenetic composition of bacterial phyla (B) and genera (C) present in feces from the PD- or TD-fed mice without DSS induction (n=2/group). (D) Relative fold change of total bacterial number per g of feces from the PD- or TD-fed mice using amplification of 16S rDNA by quantitative real-time PCR (n=8-9/group). (E) Bacterial DNA isolated from PD or TD. Leuco: *Leuconostoc sp.*, Lacto: *Lactococcus lactis subsp. lactis*. Data shown are representative of three independent experiments.
Figure 2-5. TD feeding induces inflammation of the colon.

(A) Total cell numbers in lamina propria (LP), colonic epithelium, and mesenteric lymph node (n=3/group). (B) Frequency of macrophages (CD11b^+F480^+Side scatter^low^), monocytes (CD11b^+F480^+Gr1^+^), and neutrophils (CD11b^+F480^−Gr1^+^) in colon LP from the PD- or TD-fed mice (n=3/group). (C) Frequency of dendritic cells (DCs) in colon LP from the PD- or TD-fed mice (n=3/group). (D) Histological scores and representative sections of distal colon from the PD- or TD-fed mice with or without long-term antibiotics prior to DSS (n=4/group).
Figure 2-6. Depletion of bacterial diversity post broad-spectrum antibiotics.

(A) DGGE fingerprints of the PD- or TD-fed mice with long-term antibiotics (LT ABX) (n=3-4/group). Mice were males (M) and females (F) from the same breeders. (B and C) Phylogenetic composition of bacterial phyla (B) and genera (C) in feces of the PD- or TD-fed mice with or without LT ABX (n=2/group). (D) Relative fold change of total bacterial number per gram of feces from mice with or without LT ABX (n=3-9/group). Data shown are representative of three independent experiments.
Figure 2-7. Elimination of bacterial diversity protects mice from colitis.

(A) Weight change of the PD- or TD-fed mice with or without LT ABX post DSS administration (n=3-4/group). * Significant difference between TD and TD ABX, † significant difference between PD and PD ABX, ‡ significant difference between PD ABX and TD ABX, ‡‡ significant difference between PD and TD. (B) Colonic length at day 0 (n=4/group) and day 5 (n=3-4/group) post DSS. (C) Colonic blood score at day 5 post DSS (n=3-4/group). (D) Histological scores and representative sections of distal colon with or without LT ABX at day 5 post DSS. Data shown are representative of three independent experiments.
Figure 2-8. Bacterial reduction attenuates DSS-induced colitis.

(A) Relative fold change of total bacterial numbers post 2 weeks of short-term (ST) ABX (n=4-9/group). (B) Weight change of the PD- or TD-fed mice with or without ST ABX post DSS (n=3-5/group). *Significant difference between TD and TD ST ABX, # significant difference between PD and TD. (C and D) Colonic blood score (C) and colonic length (D) at day 5 (n=3-4/group). (E) Histological scores and representative sections of distal colon with or without ST ABX at day 5 post DSS. Data shown are representative of two independent experiments.
Figure 2-9. TD causes delayed clearance of secondary *C. rodentium* infection.

(A) *C. rodentium* loads in feces during primary *C. rodentium* infection (n=14/group). (B) *C. rodentium* loads in feces during secondary infection (n=14/group). (C) Histological Scores of distal colon from the PD- or TD-fed mice at primary peak (n=4/group), primary clearance (n=4/group), and secondary clearance (n=14/group). Data are representative or combined data from three independent experiments.
Figure 2-10. Immunoglobulin production following *C. rodentium* infection.

(A) Serum total IgA and *C. rodentium*-specific IgA and IgG at peak and clearance of primary infection (n=4/group). (B) Serum total IgA and *C. rodentium*-specific IgA and IgG at secondary clearance (n=14/group). Data shown are representative or combined data from three independent experiments.
**Figure 2-11. Bacterial banding patterns following primary *C. rodentium* clearance.**

DGGE fingerprints of colonic content from the PD- or TD-fed mice post primary *C. rodentium* clearance (n=4/group). All mice were female (F) and from the same breeders. Standards (STD) shown were bacterial DNA from *Parabacteroides distasonis*, *Lactobacillus murinus*, and *Clostridium propionicum*. 
Chapter 3

Vitamin D-mediated gut homeostasis between bacterial flora and host mucosal integrity protects mice from colitis

Metagenomic analysis of 454 pyrosequencing was performed by Dr. Istvan Albert and Yunfei Li.
Abstract

Vitamin D regulates experimental inflammatory bowel disease. Vitamin D receptor knockout (VDR KO) mice or mice that are deficient in the ability to convert vitamin D to the active form (Cyp27B1 KO) were highly susceptible to dextran sodium sulfate (DSS)-induced colitis. In this study, depletion of gut bacterial flora using broad-spectrum antibiotics protected Cyp27B1 KO and VDR KO mice from DSS colitis, suggesting that the increased susceptibility of Cyp27B1 KO and VDR KO mice to DSS was due to the alterations in gut bacterial flora. 454 pyrosequencing showed that Cyp27B1 KO and VDR KO mice had increased numbers of Bacteroidetes and Proteobacteria phyla and decreased numbers of Firmicutes and Deferribacteres phyla in the gut. In particular, a decrease in normal or beneficial bacteria from the Firmicutes phylum including Lactobacillaceae and Lachnospiraceae was present in Cyp27B1 KO and VDR KO mice. Additionally, the Helicobacteraceae family members from the Proteobacteria were significantly elevated in Cyp27B1 KO mice. Treating Cyp27B1 KO mice with 1,25(OH)\(_2\)D\(_3\) rescued the phenotype of Cyp27B1 KO mice by reducing Helicobacteraceae expression and protecting mice from DSS-induced colitis. Furthermore, Cyp27B1 KO mice expressed significantly lower frequencies of epithelial adherence junction protein E-cadherin and tolerogenic DCs in the colon, and VDR KO mice showed increased gut permeability without DSS induction. Together, our data suggest that vitamin D plays a role in regulating the gut bacterial composition, and that 1,25(OH)\(_2\)D\(_3\) or VDR deficiency causes impaired epithelial integrity and dysbiosis, leading to increased susceptibility to IBD. Our findings broaden our understanding of the
mechanism by which vitamin D regulates IBD and may have implications for other inflammatory diseases that have also been linked to gut bacterial flora.

**Introduction**

Vitamin D is a type of steroid hormone that plays a role in maintaining calcium and phosphate homeostasis. More recently, vitamin D has been recognized as an important immunoregulator. Vitamin D can be found in the diet or synthesized from 7-dehydrocholesterol in the skin after exposure to UVB light (1). The active form of 1,25(OH)$_2$D$_3$ is synthesized from the hydroxylation of 25(OH)D$_3$ by 1α-hydroxylase enzyme, which is encoded by the Cyp27B1 gene (2). 1,25(OH)$_2$D$_3$ then binds to the nuclear vitamin D receptor (VDR) to regulate gene transcription (3). Mice that lack the Cyp27B1 gene are not able to synthesize the active vitamin D and therefore become 1,25(OH)$_2$D$_3$ deficient (4).

Vitamin D deficiency has been reported in patients with inflammatory bowel disease (IBD) (5). Studies using animal models of IBD have shown that deficiency of VDR or vitamin D exacerbated IBD symptoms (6-9). Cyp27B1 knockout (KO) and VDR KO mice are highly susceptible to dextran sodium sulfate (DSS)-induced colitis (7), an animal model of IBD that causes mucosal epithelial cell damage, with symptoms including weight loss and colonic bleeding and shrinkage (10). Interleukin-10 KO mice spontaneously develop IBD (9), while double knockout of IL-10 and VDR accelerates the onset of IBD and led to high mortality rate at early age (8). Likewise, more severe IBD developed in recombination-activating gene KO mice by the transfer of VDR KO derived
CD4+ naïve T cells compared to wild-type (WT) T cells (8). These studies suggest the critical role of vitamin D in regulating IBD.

The balance between beneficial and pathogenic intestinal bacteria is proposed to be essential to maintain the homeostasis of the host immune system. Disruption of the gut microflora or the overgrowth of potential pathogenic bacteria (dysbiosis) has been reported in patients with IBD (11). In many cases, increased Proteobacteria and Actinobacteria and decreased Lachnospiraceae from Firmicutes phylum were present in the intestine of IBD patients compared to healthy controls (11). Treatments with antibiotics or probiotics ameliorate symptoms of IBD (12-15), suggesting the association between intestinal microbiota and IBD pathogenesis.

Intestinal microbiota have been shown to modulate the host immune system. For instance, bacterial DNA derived from gut microflora has been shown to mediate the equilibrium between pro-inflammatory Th17 cells and regulatory T cells (16). Secretion of polysaccharide A by Bacteroides fragilis inhibits the pro-inflammatory cytokine interleukin (IL)-17 but promotes the anti-inflammatory cytokine IL-10 (17). Additionally, Helicobacter pylori had been shown to induce intestinal inflammation in immunocompromised hosts (18). Recently, it has been demonstrated that segmented filamentous bacteria (SFB) induces the development of Th17 cells in lamina propria (LP) (19). The gut bacterial flora is a regulator of inflammation in the intestine.

Host mucosal immunity is critical for the gut homeostasis. Luminal bacteria are separated from host immune system by a layer of intestinal epithelium. Mucus layers covering intestinal epithelium act as the frontline of defense to prevent bacteria from breaching the intestinal wall (20). Trefoil family factor 3 (TFF3) secretion by goblet cells is important
to stabilize the mucus layer (21). Epithelial cell-to-cell junctions such as E-cadherin adherence form another barrier to prevent bacterial penetration to LP (22). In addition to epithelial cells and goblet cells, dendritic cells (DCs) are able to extend their dendrites to constantly monitor bacteria in the lumen (23). The intestinal bacteria are recognized by pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (20). Once the epithelial cells or DCs detect the pathogens, innate and adaptive immune response are initiated to secrete humoral mediators such as antimicrobial peptides (AMPs) and immunoglobulin A (IgA) (20). Dysregulation of intestinal mucosal immunity may lead to imbalance between beneficial and potentially pathogenic bacteria in the gut.

1,25(OH)_{2}D_{3} has been demonstrated to induce the expression of antimicrobial peptides (β-defensin 2 and cathelicidin) in human innate immune cells upon activation of TLRs or NOD2 receptor (24-26). α-defensins have been reported to regulate the gut bacterial composition (27). Despite evidence of the role of vitamin D in IBD and the association of IBD with gut bacterial flora, it is unknown whether vitamin D directly controls the composition of the gut microflora.

In this study, the role of 1,25(OH)_{2}D_{3} and the VDR in regulating intestinal microbiota and IBD was investigated. Our data show that 1,25(OH)_{2}D_{3} supplemetation ameliorated DSS-induced colitis in Cyp27B1 KO mice, and that depletion of gut bacterial flora protected Cyp27B1 KO and VDR KO mice from severe DSS symptoms. Furthermore, deficiency in Cyp27B1 or VDR altered the composition of the normal bacterial flora before DSS, with increased Bacteroidetes and Proteobacteria and decreased Firmicutes and Deferrribacteres phyla compared to their WT littermates. In particular, an increase in
pathogenic bacteria such as Helicobacteraceae from Proteobacteria and a decrease in normal or beneficial bacteria such as Lactobacillaceae and Lachnospiraceae from Firmicutes were present in Cyp27B1 KO and VDR KO mice. Additionally, Cyp27B1 KO mice had significantly lower frequencies of E-cadherin positive epithelial cells and tolerogenic DCs in colon, and VDR KO mice showed increased gut permeability without DSS induction. Taken together, our data suggest that vitamin D plays a role in regulating the gut bacterial composition, and that 1,25(OH)_{2}D_{3} or VDR deficiency causes impaired epithelial integrity and dysbiosis, leading to increased susceptibility to IBD.

Methods and Materials

Mice and diet
Age and sex-matched C57BL/6 WT, Cyp27B1 KO, and VDR KO mice were produced and housed at the Pennsylvania State University (University Park, PA). Cyp27B1 or VDR heterozygote breeders were used to generate WT and KO mice. The breeders were fed a rescue diet with high lactose (Teklad Diet 96348, Harlan Laboratories, Madison, WI). After weaning, all mice were switched to the same purified diet made in the laboratory that contains vitamin D (28). For some experiments, Cyp27B1 KO mice were supplemented daily with 50ng of 1,25(OH)_{2}D_{3} in diet as previously described (29), two weeks prior to and during DSS induction. For antibiotic experiments, mice were administered broad-spectrum antibiotics (30) (1 g/L neomycin, 1 g/L metronidazole, 0.5 g/L vancomycin, and 1 g/L ampicillin) in drinking water two weeks prior to and throughout DSS induction. All of the experimental procedures were reviewed and
approved by the Institutional Animal Care and Use Committee at the Pennsylvania State University.

**DSS-induced colitis**

Colitis was induced with DSS as previously described (7). Briefly, mice were administrated 3.5% DSS (MW= 40 kDa; ICN Biomedicals, Aurora, OH) in drinking water ad libitum for 5 days and resumed on water for 5 additional days. Body weight (BW) was tracked every day, and mice that had lost more than 20% of their BW were euthanized for humane reasons. Colonic length and blood score were measured following sacrifice at day 5 and day 10 post DSS. Blood score was assessed as follows: 0- no visible blood in the entire colon, 1- blood detected in less than 2/3 of the colon, 3- blood visible throughout the entire colon (7). Distal colon was fixed in 4% paraformaldehyde and sent for hematoxylin/eosin staining at Animal Diagnostic Laboratory at Pennsylvania State University. Colon sections were scored on a scale from 0 to 11: severity of inflammation (0- no inflammation, 1- increased number of leukocytes in the mucosa, 2- multiple loci of inflammation, leukocyte infiltration of mucosa and submucosa, 3- extensive leukocytic infiltrate in mucosa, submucosa, ulceration, depletion of mucin-secreting goblet cells, 4- extensive transmural leukocytic infiltrate, crypt abscesses), extent of injury (0- none, 1-mucosal, 2- mucosal and submucosal, 3- transmural), and crypt damage (0- none, 1- basal 1/3 damaged, 2- basal 2/3 damaged, 3- only surface epithelium intact, 4- entire crypt and epithelium lost) (7).

**Denaturing-gradient gel electrophoresis (DGGE)**

Fecal samples were collected before and after antibiotic treatment. DNA was isolated from fecal samples using QIAamp DNA stool minikit (Qiagen, Valencia, CA), and 200
ng of fecal DNA was amplified with universal bacterial primers targeting the variable V3 region and conserved regions of the 16S rDNA in 30 µl PCR mixture (31). Universal bacterial primer sequences are as follows: forward 5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGCTACGGAGGCAGCAG-3' (GC-clamp); reverse 5'-ATTACCGCGGCTGCTGG-3'. 25 µl/lane of PCR products were then loaded to the perpendicular gel with a linear 30-60% denaturing gradient and run at 70V for 17 h in DCode Universal Mutation Detection System (BioRad, Hercules, CA). Denaturing gradient in DGGE separates DNA with the different sequence or size, and each DGGE band typically represents one type of bacteria.

**Metagenomic analysis**

Fecal DNA isolated from Cyp27B1 WT and KO littermates or VDR WT and KO littermates without DSS treatment was separated by barcodes and sequenced on a 454 Titanium sequencer at the Pennsylvania State University. The data were then sent to Bioinformatics Consulting Center (http://www.bcc.bx.psu.edu) at the Pennsylvania State University for metagenomic analysis. Briefly, sequencing reads from sequencer were first processed by MOTHUR (32). Cyp27B1 samples started with 556,232 reads, and VDR samples started with 254,704 reads. Raw reads were filtered to have an average quality over 20 and with a length longer than 100bp, and about 20% reads from the total were removed from the initial filtering. Reads from the same genotype were then merged together into a total of four data groups and again filtered to remove duplicated reads from each group (an additional 20% of the reads). At the end of the quality filtering steps, the final reads were Cyp27B1 for 315,803 and 191,684 for VDR, and the read counts for each group spanned from 91,927 for the lowest covered sample to 158,558 reads for the
highest coverage. Taxonomical classification was performed with the rdp_multiclassificier (33) using the RDP taxonomy. A statistical model based on Pearson Chi-Square Goodness of Fit test was then applied for statistical analysis.

**Bacterial quantification**

For total bacterial numbers, 10 ng/sample of fecal DNA was amplified with 16S rDNA primers using SYBR green mix (BioRad) by ABI 7500 Fast RT PCR machine (Applied Biosystems, Carlsbad, CA). Relative 16S rDNA copies were calculated using \( \Delta \Delta C_t \) method (\( 2^{(C_t_{sample} - C_t_{ctrl})} \)) and then converted to the total relative copies in the amount of DNA isolated per g of feces. The final results were shown as relative fold changes. To measure bacteria in the small intestine, 2 cm of terminal small intestine was excised and flushed with PBS to remove the luminal contents. DNA was then isolated from the small intestine with QIAamp DNA stool mini kit (Qiagen). 10 ng/sample of DNA was amplified with 16S rDNA primers or SFB primers and calculated as above. For some experiments, 10ng/sample of fecal DNA was amplified with group-specific Helicobacteraceae primers. The abundance of Helicobacteraceae was determined using standard curves with serial dilutions of purified PCR products amplified using the same Helicobacteraceae specific primers. Group-specific bacterial primer sequences were as follows:

<table>
<thead>
<tr>
<th></th>
<th>forward</th>
<th>reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helicobacteraceae</td>
<td>5’-GGCTATGACGGGTATCCGGC-3’</td>
<td>5’-GCCGTGCAGCACCTGTTTTC-3’</td>
</tr>
<tr>
<td>SFB</td>
<td>5’-GACGCTGAGGCATGAGAGCA-3’</td>
<td>5’-GACGGCACGGATTGTTATTC-3’</td>
</tr>
</tbody>
</table>
Quantitative real-time PCR (Q-PCR)

Total RNA was isolated from colon tissues of mice without DSS treatment following manufacturer’s instructions (QIAGEN, Valencia, CA). cDNA was synthesized with the TaqMan reverse transcription reagents kit (Applied Biosystems, Carlsbad, CA) and amplified for cathelin-related antimicrobial peptide (CRAMP), β-defensin 3, angiogenin 4, RegIII-γ, mucin 1-4, TFF3, TLR2, TLR4, and NOD2 with SYBR green mix (BioRad) by ABI 7500 Fast RT PCR machine (Applied Biosystems). Expression levels were calculated using ΔΔCt method and normalized with 18S rRNA. Primer sequences were as follows:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence (5' -&gt; 3')</th>
<th>Reverse Sequence (5' -&gt; 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRAMP</td>
<td>forward 5' - CTTCAACCACAGCAGTCCTCCTAGACA-3'</td>
<td>reverse 5' - TCCAGGTCCAGGAGACGTA-3'</td>
</tr>
<tr>
<td>β-defensin 3</td>
<td>forward 5' - GCTAGGGAGCACTTTGTTGC-3'</td>
<td>reverse 5' - TTGTTTAGAAAGGAGGCA-3'</td>
</tr>
<tr>
<td>Angiogenin-4</td>
<td>forward 5' - CTCTGGTCAGAATGTTAAGGTACGA-3'</td>
<td>reverse 5' - GAAATCTTTAAAGGCTCCTGG-3'</td>
</tr>
<tr>
<td>RegIII-γ</td>
<td>forward 5' - TTCCCTGCCTCCCATGATCAAAA-3'</td>
<td>reverse 5' - CATCCACCTCTGTGGTTCA-3'</td>
</tr>
<tr>
<td>mucin 1</td>
<td>forward 5' - TGCCAGTGCCGCGAAAGAG-3'</td>
<td>reverse 5' - GCGAAACCTCCTCATAGGGGC-3'</td>
</tr>
<tr>
<td>mucin 2</td>
<td>forward 5' - GCTGACGAGTGTTGGTGTAATTG-3'</td>
<td>reverse 5' - GATGAGTGGCAGACAGGAGAC-3'</td>
</tr>
<tr>
<td>mucin 3</td>
<td>forward 5' - CGTGGTGCAATCTCGAGGAATGG-3'</td>
<td>reverse 5' - CGGCTCTATCTCTACGCTCCTCC-3'</td>
</tr>
<tr>
<td>mucin 4</td>
<td>forward 5' - CAGCAGCCAGTGGGAGACAG-3'</td>
<td>reverse 5' - CTCAGACACAGCCAGGAAACTC-3'</td>
</tr>
<tr>
<td>TFF3</td>
<td>forward 5' - CCTGGTTTGCTGGTGCTCTCTG-3'</td>
<td>reverse 5' - GCCACGGTTGTTACACTGCTG-3'</td>
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<tr>
<td>TLR2</td>
<td>forward 5' - TCTGGGCTCTGAGGAGATGTAATTT-3'</td>
<td>reverse 5' - AGAGTCCAGGTGAGGTGTCG-3'</td>
</tr>
<tr>
<td>TLR4</td>
<td>forward 5' - GCAATGTCTCTGCGCAGGTGA-3'</td>
<td>reverse 5' - CAAGGGGATAAAGAAGCTGAGA-3'</td>
</tr>
<tr>
<td>NOD2</td>
<td>forward 5' - CGACATCTCCCACAGGTTGAATCC-3'</td>
<td>reverse 5' - GGCACCTGAAATGTTGACATTTG-3'</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>forward 5' - CATTCAACCAGCTGGGCTCCTATCC-3'</td>
<td>reverse 5' - CCTCGCTCTCCTTGGGA-3'</td>
</tr>
</tbody>
</table>
Cell isolation

Isolation of intestinal epithelial cells (IEC) and lamina propria lymphocytes (LPL) was performed as previously described (34,35). Briefly, the colon was collected and flushed with HBSS containing 5% FBS to remove fecal matter. Tissue was then opened longitudinally and cut into 0.5 cm pieces followed by three incubations in HBSS containing 5mM EDTA, 1mM DTT, and 5% FBS (Sigma-Aldrich, St. Louis, MO) for 20 min at 37 ºC under 200 rpm rotation. Supernatants containing IEC were collected from the three incubations, then tissues were further incubated in RPMI-1640 containing 1 mg/ml collagenase type 1 (Worthington, Lakewood, NJ), 3 mg/ml dispase II (Roche Diagnostics, Indianapolis, IN), and 10% FBS for 1 h at 37 ºC under 200 rpm rotation to obtain LP cells. IEC and LP samples were further processed through 40%/80% Percoll gradients (Sigma-Aldrich) to enrich lymphocytes. Cells were then stained with FITC B220, PE IgA, FITC E-cadherin, APC CD45, PE CD103, and PECy7 CD11c (BD Biosciences, San Jose, CA), analyzed on a FC500 bench top cytometer (Beckman Coulter, Brea, CA), and further evaluated with Flowjo 7.6.1 software (Tree Star, Ashland, OR).

FITC dextran permeability assay

In vivo intestinal permeability assay was performed as previously described (36). Mice were removed from food and water for 4 h and then inoculated with 0.4 mg of FITC dextran (4000 Da, Sigma) per g of BW by oral gavage. Sub-mandibular blood was collected 4 h post inoculation and centrifuged at 14,000 g for 5 min after 1 h at room temperature. FITC dextran in the serum was measured by fluorometer at 525 nm. A
standard curve with serial dilutions of FITC dextran in PBS was measured to determine the concentration in the serum.

**Statistical analysis**

Two-tailed Student’s t test, one-way ANOVA with Bonferroni post-tests, and two-way ANOVA were used for most of the experiments. Pearson Chi-Square Goodness of Fit test was applied for metagenomic analysis. \( p < 0.05 \) is indicated by *, \( p < 0.01 \) is indicated by **, and \( p < 0.001 \) is indicated by ***. Error bars represent standard error of the mean.

**Results**

1,25(OH)\(_2\)D\(_3\) supplementation protects mice from DSS-induced colitis

Cyp27B1 WT and KO mice were treated with 3.5% DSS to study the role of 1,25(OH)\(_2\)D\(_3\) in DSS-induced colitis. While WT mice showed only mild weight loss and recovered by day 10 post DSS, Cyp27B1 KO mice exhibited significantly more severe weight loss and no sign of recovery by day 10 (Fig. 3-1A). In contrast to Cyp27B1 KO mice, 1,25(OH)\(_2\)D\(_3\) treatment beginning two weeks before DSS induction rescued Cyp27B1 KO mice from severe DSS-induced weight loss (Fig. 3-1A). Compared to WT mice, severe colonic bleeding was observed in Cyp27B1 KO mice at day 5 post DSS, whereas 1,25(OH)\(_2\)D\(_3\) treatment significantly decreased colonic bleeding in Cyp27B1 KO mice (Fig. 3-1B). Consistent with DSS symptoms, Cyp27B1 KO mice had higher levels of colonic inflammation at day 10 post DSS, with increased lymphocyte infiltration and injury in the mucosa and submucosa and more than 1/3 basal crypt damage compared to their WT counterparts. 1,25(OH)\(_2\)D\(_3\) treatment partially decreased the extent of colonic inflammation in Cyp27B1 KO mice, particularly crypt damage (Fig. 3-1C).
1,25(OH)_{2}D_{3} supplementation ameliorates severe DSS-induced colitis in Cyp27B1 KO mice.

**Increased susceptibility to DSS in Cyp27B1 KO and VDR KO mice is associated with gut microbiota**

The depletion of gut bacteria by antibiotics was confirmed using PCR with universal primers for bacterial 16S rDNA (2-4 fold reduction) (Fig. 3-2A). No weight loss was observed in mice during antibiotic treatment prior to DSS induction (Fig. 3-2B). While WT mice showed mild weight loss, antibiotic-treated WT mice had no sign of weight loss post DSS induction (Fig. 3-2C). Similarly, antibiotic treatment of Cyp27B1 KO mice significantly protected mice from weight loss compared to Cyp27B1 KO mice which exhibited continuous weight loss (Fig. 3-2C). Cyp27B1 KO had more colonic bleeding compared to their WT counterparts at day 5 post DSS, whereas antibiotic treatment significantly decreased their colonic bleeding (Fig. 3-2D). Additionally, antibiotic treatment significantly decreased colonic inflammation in Cyp27B1 KO mice at day 10 post DSS, showing a similarly low extent of lymphocyte infiltration, colonic injury, and crypt damage to WT mice (Fig. 3-2 E).

As with Cyp27B1 KO mice, VDR KO mice were highly susceptible to DSS-induced colitis with continuous weight loss by day 10 post DSS, while their WT counterparts exhibited only mild weight loss (Fig. 3-3A). Antibiotic-treated VDR WT and KO mice were both protected from weight loss post DSS induction (Fig. 3-3A). Additionally, while VDR WT and KO mice showed colonic bleeding and shrinkage at day 10 post DSS, antibiotic treatment reduced the colonic bleeding and colonic shrinkage in VDR WT and KO mice at day 10 post DSS (Fig. 3-3B). Together, these results demonstrate
that depletion of intestinal microbiota by antibiotics protected Cyp27B1 KO and VDR KO mice from severe DSS-induced colitis.

**Deficiency in Cyp27B1 or VDR causes alterations in gut bacterial composition**

DGGE fingerprints were used to examine gut bacterial composition in mice prior to DSS (Fig. 3-4). Despite the different bacterial banding patterns seen in mice from different sexes and breeders, there were multiple bacterial bands consistently present or absent in Cyp27B1 KO and VDR KO mice compared to their own WT littermates, indicating potential alterations in bacterial composition in Cyp27B1 KO and VDR KO mice (Fig. 3-4). Additionally, two weeks of antibiotic treatment effectively shifted the bacterial banding patterns and decreased numbers of bacterial bands in both WT and KO mice (Fig. 3-4), confirming the disruption of bacterial composition in mice post antibiotic treatment.

WT and KO littermates from the same breeders (heterozygote) were co-housed to exclude the maternal and environmental factors that may influence the colonization of bacterial flora. Metagenomic analysis confirmed alterations in normal bacterial composition in the feces of Cyp27B1 KO and VDR KO mice compared to their WT littermates (Fig. 3-5A-C and 3-6A-B). Distinct differences in bacterial composition were also found between Cyp27B1 WT and VDR WT mice that were from different breeders and cages (Fig. 3-5A and 3-5B). Cluster analysis of the bacterial composition by Jaccard similarity coefficients showed that Cyp27B1 WT mice were more similar to their Cyp27B1 KO littermates than VDR WT mice. Firmicutes and Bacteroidetes were the dominant phyla followed by Proteobacteria in mice from Cyp27B1 or VDR breeders, but differences in the relative abundance of bacterial phyla were seen in mice of different
backgrounds (Fig. 3-5C). In contrast to mice from Cyp27B1 background that had a higher proportion of Firmicutes relative to Bacteroidetes, mice from VDR background showed similar proportion of Firmicutes and Bacteroidetes (Fig. 3-5C). The Proteobacteria phylum was more abundant in mice from Cyp27B1 background compared to mice from VDR background (Fig. 3-5C). There was low proportion of TM7 or Fusobacteria present only in mice from Cyp27B1 or VDR background, respectively (Fig. 3-5C).

**Increased potentially pathogenic and decreased beneficial bacteria in Cyp27B1 KO and VDR KO mice**

Deficiency of Cyp27B1 or VDR causes a significant increase in Bacteroidetes and Proteobacteria and a decrease in Firmicutes and Deferribacteres compared to Cyp27B1 WT or VDR WT mice, respectively (Fig. 3-6A). Bacterial family members from Firmicutes phylum were significantly decreased in Cyp27B1 KO and VDR KO mice including the beneficial bacterial flora Lactobacillaceae, Ruminococcaceae, and Lachnospiraceae (Fig. 3-6B). Proteobacteria phylum contained a variety of gram negative pathogens including Helicobacteraceae. Cyp27B1 KO mice showed significantly higher Helicobacteraceae compared to their WT littermates, whereas 1,25(OH)₂D₃ supplementation decreased the level of Helicobacteraceae in Cyp27B1 KO mice (Fig. 3-6C). Additionally, antibiotic-treated Cyp27B1 KO mice had no detectable Helicobacteraceae (Fig. 3-6C). SFB has been shown to induce T helper 17 cells in the small intestine, but their colonization was only found in mice derived from Taconic Farms but not from Jackson Laboratory (19). The WT mice in our colonies had no SFB detectable on the surface of small intestine (Fig. 3-6D). Collectively, Cyp27B1 or VDR
deficiency causes alterations in gut bacterial composition, with increased potentially pathogenic bacteria and decreased beneficial bacteria.

**Impaired epithelial barrier in Cyp27B1 KO mice**

Bacteria do not have the vitamin D receptor; therefore, the regulatory effect of vitamin D on the gut bacterial flora must be due to the vitamin D-mediated effects on the host. To study how \(1,25(\text{OH})_2\text{D}_3\) regulated the intestinal bacterial composition, several antibacterial mediators and pattern recognition receptors (PRRs) for gut mucosal defense were assessed. First, antibacterial peptides with differential specificity in bacterial killing were measured: murine cathelicidin CRAMP, \(\beta\)-defensin 3, angiogenin-4, and RegIII-\(\gamma\). CRAMP and \(\beta\)-defensins are effective in killing both gram positive and gram negative bacteria (37-39), while angiogenin-4 and RegIII-\(\gamma\) preferentially target gram-positive bacteria (40-42). No significant differences in these antibacterial peptides were detected between the colons of Cyp27B1 WT and KO mice (Fig. 3-7A). Next, to compare the mucus production, mucin 1-4 and TFF3 expression levels were measured, but comparable expression of mucins and TFF3 were found between WT and Cyp27B1 KO colons (Fig. 3-7B). Similar frequencies of IgA positive plasma cells was also found in colonic lamina propria (LP) of Cyp27B1 WT and KO littermates by flow cytometric analysis (Fig. 3-7C). Additionally, colonic expression of PRRs including TLR2, TLR4, and NOD2 was not altered in Cyp27B1 KO mice compared to their WT littermates (Fig. 3-7D).

E-cadherin which is critical for cell-to-cell junctions in colon epithelial layer was measured. Compared to WT littermates, Cyp27B1 KO mice showed significantly decreased frequencies of E-cadherin positive epithelial cells and E-cadherin positive
leukocytes in the colon epithelial layer (Fig. 3-8A and 3-8B). The mean fluorescence intensity of E-cadherin positive cells were also significantly lower in Cyp27B1 KO mice compared to their WT littermates, showing lower E-cadherin levels in the colonic epithelial layer in Cyp27B1 KO mice (Fig. 3-8C). Intestinal permeability in WT and Cyp27B1 KO mice was then examined by FITC dextran \textit{in vivo} inoculation. No significant increase in FITC dextran level was found in the serum of Cyp27B1 KO mice compared to their WT littermates prior to DSS (Fig. 3-8D). After 5 days of DSS induction, Cyp27B1 KO mice showed slightly higher permeability of FITC dextran compared to their WT counterparts (Fig. 3-8D). In contrast to Cyp27B1 KO mice, VDR KO mice without DSS induction had significantly increased FITC dextran level in serum compared to their WT littermates (Fig. 3-8E). The data show that deficiency in Cyp27B1 or VDR resulted in impaired epithelial integrity, with loose junctions of E-cadherin in colonic epithelium or increased intestinal permeability in the gut.

**Decreased recruitment of tolerogenic DCs in Cyp27B1 KO mice**

E-cadherin is a ligand for CD103 for the recruitment of DCs and intraepithelial cells (43). To determine if the number of CD103+ tolerogenic DCs were affected by low expression of E-cadherin in Cyp27B1 KO mice, frequencies of DCs and CD103+ DCs in mice without DSS induction were measured using flow cytometric analysis. The frequency of total DCs (CD11c+ cells) in colonic epithelium of Cyp27B1 KO mice was significantly lower as compared with their WT counterparts (Fig. 3-9A and 3-9B). The percentage of CD103+ tolerogenic DCs in epithelium was similarly low between Cyp27B1 WT and KO mice (Fig. 3-9A and 3-9B). Different from colonic epithelium, similar frequency of total DCs was found in colonic LP between Cyp27B1 WT and KO mice (Fig. 3-9C).
Cyp27B1 KO mice had a lower percentage of CD103+ tolerogenic DCs in colon LP compared to their WT littermates (Fig. 3-9C). Deficiency in Cyp27B1 resulted in decreased DCs of epithelium and tolerogenic DCs of LP in the colon.

**Discussion**

Using DSS-induced colitis as an animal model of IBD, our studies provide direct evidence showing that vitamin D regulates intestinal microbiota and the susceptibility to IBD. First, deficiency in Cyp27B1 or VDR in mice without DSS induction resulted in alterations in normal bacterial composition. Consistent with our findings showing increased potentially pathogenic bacteria and decreased beneficial bacteria in Cyp27B1 KO and VDR KO mice, similar changes in bacterial flora have also been reported in human IBD patients with increased Proteobacteria and decreased Lachnospiraceae from Firmicutes (11). The data suggest the association of vitamin D deficiency with human IBD and the role of these particular bacteria on IBD pathogenesis. Second, depletion of bacteria protected Cyp27B1 KO and VDR KO mice from severe DSS colitis, suggesting that the susceptibility of Cyp27B1 KO and VDR KO mice to DSS is related to certain types of bacteria present in the intestine. Furthermore, the results that 1,25(OH)_{2}D_{3} supplementation decreased the Helicobacteraceae numbers and protected Cyp27B1 KO mice from severe colitis indicate the high susceptibility to DSS colitis in Cyp27B1 KO mice may be driven by the presence of increased Helicobacteraceae.

Cyp27B1 WT and VDR WT mice were both less susceptible to DSS-induced colitis. Consistent changes in bacterial flora were detected in Cyp27B1 KO and VDR KO mice compared to their WT littermates, suggesting the effect of vitamin D on gut bacterial
flora regardless of the other background factors. It is not elucidated yet whether the dysbiosis in IBD patients is the cause or effect of intestinal inflammation. Our data showing that mice deficient in Cyp27B1 or VDR exhibited changes in bacterial flora before DSS induction and that pretreatment of antibiotics protected mice from DSS colitis suggest that the bacterial alterations is a direct cause of susceptibility to IBD rather than consequences of IBD pathology.

Antibiotic treatment has been shown to exacerbate DSS-induced colitis symptoms, due to the significant weight loss from dehydration of the mice (44). In our study, mice were all fed purified diet made in agarose gel that contained more moisture content compared to standard chow pellet. No weight loss was observed during antibiotic treatment before DSS induction, and mice were protected from DSS colitis. In addition to diet differences, the contradictory data may be also due to differences in the bacterial flora in the two different institutes. Antibiotics have been also shown to have direct effect on the host cells (45). Our studies do not exclude the possibility that the protection in Cyp27B1 KO and VDR KO mice by antibiotics is due to the effect on the host by antibiotics. Further investigation using germ-free mice will be necessary to confirm the causality between gut bacterial flora and colitis susceptibility of vitamin D-deficient mice.

Intestinal epithelial integrity is critical for the host defense against bacterial intrusion (22). DSS treatment on VDR KO mice increased colon permeability in vitro and damaged epithelial tight junctions (46). Our data show that VDR KO mice had significantly increased gut permeability without DSS induction, and Cyp27B1 KO mice expressed lower frequency of adherence junction protein E-cadherin and tolerogenic DCs in colon before DSS treatment, suggesting that impaired epithelial integrity in Cyp27B1
KO and VDR KO mice may cause increased susceptibility to DSS colitis. Increased gut permeability has been also reported in patients with IBD and their relatives who do not have clinical symptoms (47), indicating that dysfunction of epithelial barrier contributes to predisposition to IBD but additional environmental exposures might be required to trigger the IBD pathogenesis. Consistently, Cyp27B1 KO and VDR KO mice do not develop IBD without stimulation. It is not clear whether the impaired epithelial barrier in Cyp27B1 KO and VDR KO mice was the result of bacterial alterations in the gut, as it has been reported that pathogenic bacteria can breach epithelial barrier and promote their colonization (48). A study using colonic cell cultures reported the effect of vitamin D promotes E-cadherin junctions in vitro (46), suggesting vitamin D regulates epithelial integrity in a direct manner.

1,25(OH)_{2}D_{3} induces the production of AMPs, cathelicidin and β-defensins, in human innate immune cells (24). However, others (49) and our study show that murine cathelicidin and β-defensins were not affected by vitamin D, indicating the different regulatory system of AMPs between mice and humans. A decrease in angiogenin-4 had been reported in full vitamin D-deficient mice (50). However, our data show comparable angiogenin-4 expression between Cyp27B1 WT and KO colons, indicating that vitamin D may regulate AMPs in a manner independent of 1,25(OH)_{2}D_{3}. Several other antibacterial mediators including IgA and mucus secretion were tested here, yet no significant differences were found between Cyp27B1 WT and KO mice. It is possible that 1,25(OH)_{2}D_{3} regulates AMPs in mice that are yet unknown. In addition, the bacterial alterations in Cyp27B1 KO mice might be due to the dysregulation in a combination of bacterial mediators instead of a single factor.
In conclusion, our findings suggest that vitamin D plays a role in regulating gut bacterial composition and epithelial integrity to prevent IBD. Deficiency in 1,25(OH)_{2}D_{3} or VDR causes disturbances in the intestinal microbiota and impaired epithelial barrier, contributing to increased susceptibility to IBD. Our findings broaden our understanding of the mechanism by which vitamin D mediates IBD and suggest vitamin D supplementation as a potential therapeutic agent for IBD.
References


Figure 3-1. 1,25(OH)₂D₃ treatment ameliorates severity of DSS-induced colitis.

(A) Percentages of original body weight change in WT, Cyp27B1 KO (Cyp KO), and 1,25(OH)₂D₃-treated Cyp KO (Cyp KO +1,25D₃) post 3.5% DSS treatment (n=7-9/group). * Significant difference between WT and Cyp KO mice or Cyp KO +1,25D₃ and Cyp KO mice. (B) Colonic blood score at day 5 (n=5-7/group) and day 10 (n=7-9/group) post DSS. (C) Histological scores of distal colon from mice at 10 post DSS (7-9/group). Data shown are representative of three independent experiments.
Figure 3-2. Antibiotics protect Cyp27B1 KO mice from DSS-induced colitis.

(A) Relative fold change of total bacterial 16S rDNA gene copies in feces two weeks before and after antibiotics (ABX) (n=4/group). n.s., not significant. (B and C) Percentages of original body weight change prior to DSS (B) and post DSS (C) (n=3-5/group). * Significant difference between WT and Cyp KO mice or ABX-treated Cyp KO mice (Cyp KO + ABX) and Cyp KO mice. # significant difference between WT and ABX-treated WT mice (WT + ABX). (D) Colonic blood score at day 5 post DSS (n=4-6/group). (E) Histological scores of distal colon at day 10 post DSS (n=2-8/group). Data shown are representative of three independent experiments.
Figure 3-3. Antibiotic treatment protects VDR KO mice from severe DSS.

(A) Percentages of original BW change post DSS (n=4-9/group). (B and C) Colonic blood score (B) and colonic length (C) at day 10 post DSS with or without ABX. (n=3-5/group)
Figure 3-4

(A and B) DGGE fingerprints shown are bacterial banding patterns of Cyp27B1 WT and KO littermates (A) and VDR WT and KO littermates (B) before and after two weeks of antibiotic treatment (ABX). Littermates were females (F) and males (M) from different pairs of breeders (1-5).
Figure 3-5. Alterations in gut bacterial composition in Cyp27B1 KO and VDR KO.

(A and B) Pie charts show the proportions of bacterial phyla present in feces from Cyp WT and KO littermates (A) and VDR WT and KO littermates (B) prior to DSS. (C) Relative abundance of bacterial phyla present in feces from Cyp27B1 WT and KO littermates or VDR WT and KO littermates prior to DSS. DNA samples were amplified with 16S rDNA universal primers and sequenced by 454 pyrosequencing (n=2-4/group).
Figure 3-6. Deficiency of Cyp27B1 or VDR causes dysbiosis.

(A) Fold change of bacterial phyla in Cyp KO or VDR KO mice relative to their Cyp WT or VDR WT littermates, respectively. Values of Cyp KO or VDR KO were significantly different from values of Cyp WT or VDR WT mice. VDR KO Bacteroidetes $p < 0.05$, all the others $p < 0.001$. (B) Fold change of bacterial family members from Firmicutes phylum in Cyp KO or VDR KO mice relative to their WT littermates. Values of Cyp KO or VDR KO were significantly different from values of Cyp WT or VDR WT mice, $p < 0.001$. (C) Helicobacteraceae copy numbers in fecal DNA of Cyp WT and KO mice with or without 1,25(OH)$_2$D$_3$ or ABX by Q-PCR (n=3-9/group). (D) 16S rDNA and SFB expression in distal small intestine of Cyp WT and KO littermates by Q-PCR (n=4/group). N.D., not detected.
Figure 3-7

(A) Expression of murine cathelicidin (CRAMP), β-defensin 3 (mBD-3), angiogin-4 (ANG-4), and Reg III-γ in colon by Q-PCR (n=5-7/group). (B) Frequency of IgA positive cells in colon lamina propria (LP). (C) Expression of mucin 1-4 and trefoil family factor 3 (TFF3) in colon using quantitative PCR (n=4-5/group). (D) Expression of TLR 2, TLR 4, and Nod 2 receptors in colon by Q-PCR (n=5-7/group).
Figure 3-8. Intestinal epithelial integrity in Cyp27B1 KO and VDR KO mice.

(A) Representative dot plots of E-cadherin (Ecad) positive epithelial cells (Ecad+CD45-) and Ecad positive leukocytes (Ecad+CD45+) in intestinal epithelial cells (IEC) from colon. (B) Frequency of Ecad+CD45- and Ecad+CD45+ colon IEC (n=3/group). (C) Mean fluorescence intensity (MFI) of Ecad positive cells in colon IEC (n=3/group). (D) Intestinal permeability of FITC Dextran in Cyp WT and KO before and after 5 days of DSS (n=3/group). (E) Intestinal permeability of FITC Dextran in VDR WT and KO mice (n=5/group).
Figure 3-9

Figure 3-9. Decreased colonic dendritic cells in Cyp27B1 KO mice.
(A) Representative dot plots of CD11c and CD103 staining on colon IEC by flow cytometry. (B and C) Frequency of CD11c+ dendritic cells (DCs) and CD11c+CD103+ DCs in colon IEC (B) and LP lymphocytes (LPL) (C) (n=3/group).
Chapter 4

Expression and regulation of Cyp27B1 during inflammation

Chapter adapted from the manuscript entitled:

“Expression and regulation of Cyp27B1 during inflammation.”

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The data represented in figures 4-2C, 4-5, and part of table 4-1 are the contributions of Kaitlin McDaniel. All the ELISA data are the work of Veronika Weaver.
Abstract

The active form of vitamin D, 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] is synthesized by 1α-hydroxylase, which is encoded by the Cyp27B1 gene. The kidney is established to be the organ that expresses 1α-hydroxylase. Recent studies reported the expression of 1α-hydroxylase in extrarenal tissues including the immune system in vitro. In this study, transgenic mice that have replaced the Cyp27B1 gene with the bacterial lacZ reporter gene under the control of the Cyp27B1 promoter were used to investigate the signals by which Cyp27B1 activity is regulated in the immune system. A variety of stimuli including LPS, PMA/ionomycin, and α-CD3 were used to stimulate splenocytes and bone marrow derived macrophage (BMDM) in vitro. Only α-CD3 stimulation of splenocytes resulted in the induction of Cyp27B1 promoter activity with elevated IFN-γ production. CD8+ T cells but not CD4+ T cells were further confirmed to be the source of Cyp27B1 post activation. In vivo, the Cyp27B1 transgenic mice were challenged with LPS, but Cyp27B1 promoter activity was not found in extrarenal tissues. During chronic airway inflammation, 38% of IL10/Cyp27B1 double knockout (DKO) mice showed Cyp27B1 promoter activity in the lungs. Whole mount staining for Cyp27B1 promoter activity in mice with inflammatory bowel disease (IBD) showed spotty staining of the intestines in about 30% of Cyp27B1 KO mice and DKO mice. Cyp27B1 KO mice reconstituted with wild-type (WT) bone marrow (BM) cells were protected from IBD, indicating a role for hematopoietic derived 1,25(OH)₂D₃ production. Our data suggest that the immune system does produce 1,25(OH)₂D₃, and murine IFN-γ-secreting T cells
are induced to express Cyp27B1 activity in vitro. These findings provide new insights into the immunoregulatory function of vitamin D in infectious and autoimmune diseases.

**Introduction**

Vitamin D is a member of the steroid hormone superfamily and is either found in the diet or synthesized in the skin after exposure to UVB light (1). Vitamin D that is either ingested or produced in the skin is inactive and hydroxylated in the liver to become 25(OH)D₃, which is the major circulating form of vitamin D. 25(OH)D₃ is further converted into the active form of vitamin D [1,25(OH)₂D₃] by the 1α-hydroxylase enzyme (Cyp27B1 gene) (2). 1,25(OH)₂D₃ binds to the nuclear vitamin D receptor (VDR) to regulate gene transcription crucial for calcium and phosphate homeostasis (3). Since the expression of the VDR was identified in immune cells (4,5), accumulating evidence suggest an immunoregulatory role of vitamin D particularly during inflammatory disease.

Early experiments established that the kidney is the major organ to express Cyp27B1 (6). Experimentally, it was shown that nephrectomized animals and humans prior to transplantation failed to produce measurable 1,25(OH)₂D₃ (7,8). More recently, several different groups have reported Cyp27B1 mRNA by RT-PCR or protein by polyclonal antibody staining in extrarenal tissues such as the skin, gastrointestinal tract, and bone during normal physiological conditions (9-11). Conversely, others have shown that Cyp27B1 was expressed only in the kidney of healthy animals and humans (8,12-14) and the placenta of pregnant females (15). More recently, transgenic Cyp27B1 KO mice that express the lacZ gene under the control of the Cyp27B1 promoter confirmed the activity
of Cyp27B1 only in the kidney and placenta of healthy animals (16). Thus, there is yet no strong evidence suggesting Cyp27B1 activity in tissues besides kidney and placenta under normal physiological conditions.

Under pathological conditions, several studies have reported extrarenal Cyp27B1 expression particularly during chronic inflammation. Granulomatous diseases such as sarcoidosis and Crohn’s disease have provided suggestive evidence for Cyp27B1 expression in the immune system (12,17-19). Hypercalcemia commonly occurs in granulomatous disease even though vitamin D status is low, and furthermore resolution of hypercalcemia occurs with the use of corticosteroids and the resolution of the granulomatous diseases (20). In 1981, an anephric sarcoidosis patient showed definitively that 1,25(OH)2D3 was produced extrarenally (21). Macrophage from the anephric sarcoidosis patient but not other patients with lung disease was identified as the source of the extrarenal Cyp27B1 in vitro (22). Human monocytes/macrophages and dendritic cells have also been reported to express Cyp27B1 mRNA and protein in vitro upon stimulation with pathogen-associated molecular patterns (PAMPs) including toll-like receptor (TLR) 4 ligand or TLR1/2 ligands (23-27). However, many of these reports were based on in vitro studies that did not measure enzymatic activity of extrarenal Cyp27B1 (28).

Renal Cyp27B1 production is mainly regulated by parathyroid hormone (PTH), calcium, and phosphate (29,30). When circulating calcium or phosphate level decreases, PTH is secreted to induce Cyp27B1 production from kidney proximal tubule cells for 1,25(OH)2D3 synthesis (2).1,25(OH)2D3 then stimulates calcium or phosphate absorption from the intestine or promotes release of calcium or phosphate from the bone (31). Elevated 1,25(OH)2D3 also negatively regulates Cyp27B1 production (32). In contrast to
renal Cyp27B1, PTH stimulation or the levels of calcium and phosphate did not affect extrarenal Cyp27B1 in macrophages (33-36). Alternatively, extrarenal Cyp27B1 has been shown to be induced by inflammatory stimuli particularly IFN-γ and LPS through signaling pathways including JAK/STAT, p38 MAPK, and NK-κB in monocytes (24). Together, the studies suggest that extrarenal Cyp27B1 production can be induced by inflammatory stimuli in a manner independent of renal Cyp27B1 regulation. Here, the inflammatory signals inducing Cyp27B1 activity in the immune system was investigated using the transgenic Cyp27B1 KO mice with the bacterial LacZ reporter under the control of the Cyp27B1 promoter (16). In vitro, α-CD3 stimulation significantly elevated IFN-γ production and Cyp27B1 promoter activity in splenocytes, suggesting the induction of Cyp27B1 in T cells. Purified T cell culture further confirmed CD8+ but not CD4+ T cells are the source of Cyp27B1 post activation. Whole mount staining for Cyp27B1 promoter activity was detected in 27-38% of Cyp27B1 KO or IL-10/Cyp27B1 double knockout (DKO) mice with chronic airway inflammation or IBD. Cyp27B1 KO mice reconstituted with WT BM were protected from DSS colitis, providing the evidence for a critical role of Cyp27B1 in hematopoietic cells. Together, our data suggest that the immune system does produce 1,25(OH)₂D₃, and IFN-γ-secreting T cells are induced to express Cyp27B1 activity in vitro.
Methods and Materials

Mice and diet
Age and sex-matched C57BL/6 WT, IL-10 KO, Cyp27B1 KO, and DKO mice were produced and housed at the Pennsylvania State University (University Park, PA). WT and IL-10 KO mice were fed standard chow (Laboratory Rodent Diet 5001, LabDiet, Quakertown, PA), and Cyp27B1 KO and DKO mice were put on a rescue diet with high lactose (Teklad Diet 96348, Harlan Laboratories, Madison, WI). Prior to IBD experiments, all mice were switched to the same purified diet made in the laboratory that contains vitamin D (37). All of the experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the Pennsylvania State University.

Cell culture
Spleens were homogenized and lysed with red blood cell lysis buffer to obtain single-cell suspensions. Splenocytes were then stimulated with or without 0.5 μg/ml of LPS, or 10 ng/ml PMA and 2.5 μg/ml ionomycin, or 10 mg/ml ConA (Sigma-Aldrich), or 0.5 μg/ml α-CD3 without coating (BD Pharmingen, San Diego, CA) in RPMI 1640-C containing 10% FBS (Equitech-Bio, Inc, Kerrville, TX), 2 mM L-glutamine, 5 mM β-mercaptoethanol (Invitrogen, Carlsbad, CA), and 10 μg/ml gentamycin (Teknova, Hollister, CA). For some experiments, CD4+ or CD8+ T cells from splenocytes were purified using mouse CD4 or CD8 cell recovery column kit (Cedarlane Laboratories Ltd, Burlington, NC) and stimulated with coated 5 μg/ml α-CD3 and 5 μg/ml α-CD28 (BD Pharmingen). The purity of CD4+ or CD8+ T cells was >80% or >60%, respectively.
For growing BMDM, BM cells were isolated from the femurs and cultured in DMEM supplemented with L929 conditioned media (M-CSF) for 7 days (38). BMDM were then stimulated with or without 0.1 µg/ml of LPS in DMEM containing 10% FBS, sodium pyruvate, nonessential amino acids (Mediatech, Inc, Manassas, VA), 2 mM L-glutamine, and 10 µg/ml gentamycin (Teknova) (DMEM). After stimulation, cell culture media was collected for cytokine detection, and cells were used for β-galactosidase luciferase assay. Production of interleukin (IL)-1β, IL-6, IFN-γ, IL-4, and IL-17 (BD Biosciences, San Jose, CA) were measured by ELISAs following the manufacturer’s instructions.

**Acute stimulation in vivo**

For some mice, LPS (16 mg/kg) was injected intraperitoneally. Serum was collected for cytokine detection, and livers and spleens were collected for β-galactosidase luciferase assay 24 h post stimulation. Colitis was induced with DSS as previously described (39). Briefly, mice were administrated 3.5% DSS in drinking water ad libitum for 5 days and resumed on water. Body weight (BW) of mice was tracked every day, and mice that had lost more than 20% of their BW were euthanized for humane reasons. Colonic length and blood score were measured following sacrifice. Blood score was assessed as follows: 0- no visible blood in the entire colon, 1- blood detected in less than 2/3 of the colon, 3- blood visible throughout the entire colon. Kidneys, small intestines (SI), and colons collected at day 5 post DSS were used for whole mount staining of β-galactosidase. Distal colons were fixed in 4% paraformaldehyde and sent for hematoxylin/eosin staining at Animal Diagnostic Laboratory at Pennsylvania State University. Colon sections were scored
blindly by two investigators on a scale from 0 to 10 for severity of inflammation (0-3), extent of injury (0-3), and crypt damage (0-4) (39).

**Chronic airway inflammation**

*Saccharopolyspora rectivirgula* (SR, ATCC 29024) was cultured and processed as described (31). Samples were quantified for protein by BCA protein assay (Thermo Scientific, Rockford, IL) and then heat killed for 2 h at 90 ºC. Heat killing was confirmed by the lack of growth of SR and negative β-galactosidase activity by X-gal staining. Mice were intranasally inoculated under light anesthesia with 150 µg of heat killed SR or sterile PBS for 3 consecutive days for 5 weeks. Mice were sacrificed 24 h after the last inoculation (31). Lungs were fixed in 4% paraformaldehyde for histopathology as described above and scored blindly by two investigators on a scale from 0-5 as previously described (40).

**Chronic IBD**

IL-10 KO mice develop spontaneous chronic inflammatory bowel disease (IBD) (41,42). WT, Cyp27B1 KO, IL-10 KO, and DKO mice were sacrificed to collect weight of SI and colons to calculate the ratio of the SI or colon to the BW. SI and distal colons were then fixed in 4% paraformaldehyde for histopathology as described above. Intestinal sections were scored blindly by two investigators on a scale from 0 to 8 for inflammation (0-4) and epithelial thickening (0-4) using previously described criteria (43). For β-galactosidase activity, IL-10 KO and DKO mice were treated with piroxicam (0.24 g/kg diet) (Sigma-Aldrich) for 3 days to trigger rapid onset of IBD as previously described.
Kidneys, SI, and colons were then collected and used for whole mount staining of β-galactosidase.

**BM transplantation**

CD45.1 WT derived BM donor cells were transferred into sublethally irradiated CD45.2 WT and Cyp27B1 KO recipient mice intravenously. DSS colitis was induced in mice 2 months after transfer. Donor cell reconstitution was determined by flow cytometry using antibodies to CD45.1 to identify the donor derived leukocytes. Blood was collected from the heart following sacrifice, and red blood cells were lysed. Intestinal intraepithelial lymphocytes (IEL) isolation was performed as previously described (45). Cells were stained with PE CD45.1, FITC CD45.2, and PECy5 TCRβ (BD Biosciences), analyzed on a FC500 bench top cytometer (Beckman Coulter, Brea, CA), and further evaluated with Flowjo 7.6.1 software (Tree Star, Inc., Ashland, OR).

**β-galactosidase activity**

Whole kidney, lung, and liver tissues were homogenized and lysed for protein extraction. β-galactosidase activity from the protein samples was measured by β-galactosidase luciferase reporter system (Applied Biosystems, Foster City, CA). Protein assays were done to quantitate the amount of protein in the sample using BCA protein assay (Thermo Scientific). Luciferase activity was normalized to the amount of protein. Cyp27B1 WT and IL-10 KO were used as negative controls for β-galactosidase luciferase assays.

Whole organ staining of kidney, lung, and intestinal tissues for β-galactosidase were done as follows. Heart perfusion with PBS and 4% paraformaldehyde was performed on the mice following sacrifice. Fecal matter in SI and colons were thoroughly flushed out with
PBS. Tissues were cut longitudinally and stained with X-gal (Invitrogen) as previously described (16). Briefly, tissues were fixed in 4% paraformaldehyde at 4 °C for 2 h and then rinsed with permealization buffer for 3 times. Tissues were then stained in 1mg/ml of X-gal at 37 °C overnight. After staining, tissues were rinsed with PBS and fixed in 4% paraformaldehyde for observation. Cyp27B1 WT and IL-10 KO mice were used as negative controls for X-gal staining.

Statistical analysis

For statistical analysis, unpaired Student’s t test and two-way ANOVA were used. $P < 0.05$ is indicated by *, $p < 0.01$ is indicated by **, and $p < 0.001$ is indicated by ***. Error bars represent standard error of the mean.

Results

In vitro stimulation with PMA/ionomycin or LPS fails to induce Cyp27B1

Elevated IFN-γ and IL-4 were produced by from WT and Cyp27B1 KO splenocytes 24 h and 48 h post PMA/ionomycin stimulation (Fig. 4-1A and 4-1B), whereas unstimulated controls at the same time points showed undetectable IFN-γ and IL-4. Despite the activation stimulated by PMA/ionomycin, Cyp27B1 promoter activity was not induced over background in Cyp27B1 splenocytes at either 24 h or 48 h post stimulation (Fig. 4-1B). LPS was used to stimulate splenocytes and BMDM from WT and Cyp27B1KO in vitro (Fig. 4-1C-F). Upregulation of interleukin (IL)-1β and IL-6 was detected 24 h and 48 h post LPS stimulation (Fig. 4-1C and 4-1E). Cyp27B1 KO splenocytes produced significantly higher amounts of IL-6 (Fig.4-1C) and BMDM overproduced IL-1β.
compared WT cells at 48 h post LPS (Fig. 4-1E). Cyp27B1 was not induced in splenocytes and BMDM following LPS stimulation of the Cyp27B1 KO samples (Fig. 4-1D and 4-1F). In addition, Cyp27B1 promoter activity was not detectable in unstimulated and PMA/ionomycin- or LPS-stimulated splenocytes and BMDM (data not shown). The data suggest that PMA/ionomycin and LPS are not able to induce Cyp27B1 activity in murine immune cells in vitro.

**T cell activation induces Cyp27B1**

Cyp27B1 KO splenocytes stimulated with α-CD3 produced significantly higher amounts of IFN-γ than WT splenocytes at 48 h post stimulation, while there was no difference in the amount of IL-17 (Fig. 4-2A). Cyp27B1 promoter activity was low at 24 h, while α-CD3 significantly induced Cyp27B1 promoter activity at 48 h post stimulation (Fig. 4-2B). IL-4 production was undetectable in all of the cultures (data not shown). There was no cytokine production or Cyp27B1 promoter activity detectable in unstimulated splenocytes at the same time points (data not shown). The data demonstrate that T cells from Cyp27B1 KO mice overproduce IFN-γ, and T cells can be induced to produce Cyp27B1 activity at 48 h post stimulation.

To investigate which type of T cells produces Cyp27B1, CD4+ or CD8+ T cells purified from WT and Cyp27B1 KO splenocytes were stimulated with α-CD3 and α-CD28 for 48 h (Fig. 4-2C and 4-2D). Cyp27B1 promoter activity was not induced over background in CD4+ T cells, whereas Cyp27B1 promoter activity was significantly elevated in CD8+ T cells from Cyp27B1 KO mice at 48 h post stimulation (Fig. 4-2C). No Cyp27B1
promoter activity was detected in unstimulated CD4+ or CD8+ T cells at 48 h (data not shown). CD8+ T cells but not CD4+ T cells produce Cyp27B1 activity post activation.

**Cyp27B1 activity during acute intestinal inflammation**

LPS injection resulted in increased serum IL-1β and IL-6 production of WT and Cyp27B1 KO mice compared to controls (Fig. 4-3A). Cyp27B1 promoter activity was detected in the kidneys but not in the lungs and livers from Cyp27B1 KO mice challenged with LPS (Fig. 4-3B). WT and Cyp27B1 KO mice were injected with *L. monocytogenes* intraperitoneally. Again, Cyp27B1 promoter activity was detected only in the kidneys but not in the spleens or livers post stimulation (data not shown). Acute systemic inflammation did not induce Cyp27B1 activity.

Next, Cyp27B1 promoter activity during acute DSS-induced intestinal inflammation was assessed. In contrast to WT mice which showed only mild weight loss, Cyp27B1 KO mice exhibited severe weight loss and no sign of recovery after 10 days post DSS (Fig. 4-4A). Severe colonic bleeding was also observed in the colons from Cyp27B1 KO mice at day 5 and 10 compared to WT mice (Fig. 4-4B). In accordance with the DSS symptoms, the histopathology of the distal colons showed increased lymphocyte infiltration, colonic injury, and crypt damage in Cyp27B1 KO mice compared to WT mice (Fig. 4-4C). The data are consistent with the report suggesting that Cyp27B1 KO mice were more susceptible to DSS-induced colitis (10).

Whole mount staining for Cyp27B1 promoter activity showed no positive staining in the colons and the small intestines (SI) of Cyp27B1 KO mice without inflammation (Fig. 4-4D). DSS-induced intestinal inflammation resulted in spotty induction of Cyp27B1 promoter activity in both the colons and the distal SI of Cyp27B1 KO mice but not in
their WT counterparts (Fig. 4-4E). However, the frequency of positive staining was 27% in the colons and distal SI post DSS colitis (Table 4-1). The induction was sporadic and not associated with the severity of DSS symptoms in Cyp27B1 KO mice.

**Induction of Cyp27B1 during chronic airway inflammation**

Hypersensitivity pneumonitis induced by heat-killed *Saccharopolyspora rectivirgula* (SR) caused chronic and granulomatous inflammation in the lungs of WT, Cyp27B1 KO, IL-10 KO, and DKO mice (Fig. 4-5A). IL-10 KO and DKO mice showed significantly more severe airway inflammation compared to Cyp27B1 KO mice (Fig. 4-5A). β-galactosidase luciferase assay for homogenized lungs from PBS- or SR-inoculated WT, Cyp27B1 KO, IL-10 KO, and DKO mice did not show significant increase in Cyp27B1 promoter activity (Fig. 4-5B). Whole mount staining for Cyp27B1 promoter activity showed no detectable Cyp27B1 promoter activity in the lungs of Cyp27B1 KO mice, whereas positive staining was detected in the lungs of 38% of DKO mice following chronic SR induced airway inflammation (Fig. 4-5C and Table 4-1). No positive staining was found in the lungs of PBS-inoculated Cyp27B1 KO and DKO mice (Fig. 4-5C). Cyp27B1 is produced during a severe airway inflammation.

**Chronic gastrointestinal inflammation induces Cyp27B1**

WT and Cyp27B1 KO mice did not develop intestinal inflammation without stimulation. In contrast to WT and Cyp27B1 KO mice, IL-10 KO and DKO mice developed IBD spontaneously with enlarged colons and SI were observed macroscopically in IL-10 KO and DKO mice by 8 to 10 weeks of age (colon/BW%: IL-10 KO 3.3 ± 0.4, DKO 4.3 ± 0.5; SI/BW%: IL-10 KO 9.0 ± 1.0, DKO 14 ± 2.1, Fig. 4-6A). DKO mice had more
severe lymphocyte infiltration and tissue thickening in the SI and colons than IL-10 KO mice (Fig. 4-6A and Fig. 4-6B).

IL-10KO and DKO mice were treated with piroxicam to trigger rapid onset of IBD in a synchronized fashion. Similar to the outcomes of DSS colitis, spotty staining of Cyp27B1 promoter activity was induced in the SI and the colons of DKO mice but not of their IL-10 KO counterparts (Fig. 4-6C). However, the induction was also sporadic, and the frequency of positive staining was 33% in the colons and SI (Table 4-1). The data indicate that intestinal inflammation may be one of the stimuli that induce extrarenal Cyp27B1.

**WT BM cells protect Cyp27B1 KO from DSS-induced colitis**

Following BM transplantation, WT-WT and WT-Cyp KO had comparable reconstitution rates in the blood (WT-WT 92 ± 3, WT-Cyp KO 95 ± 1, Fig. 4-7A). Reconstitution in SI intestinal intraepithelial lymphocytes (IELs) was similar between WT-WT and WT-Cyp KO but less effective as the blood (WT-WT 43 ± 4, WT-Cyp KO 43 ± 5, Fig. 4-7A). No weight loss in BM chimeras was observed prior to DSS induction (Fig. 4-7B). In contrast to Cyp27B1 KO mice that had severe weight loss and no recovery post DSS administration, Cyp27B1 KO mice reconstituted with WT BM (WT-Cyp KO) only exhibited mild weight loss and recovered to their original BW after 14 days post DSS (Fig. 4-7C). Cyp27B1 KO mice had the most severe colonic bleeding at day 5 and day 14 among the groups, while WT-Cyp KO mice were protected and showed only mild symptoms (Fig. 4-7D). Additionally, WT-Cyp KO mice had less severe inflammation, colonic injury, and crypt damage than Cyp27B1 KO (Fig. 4-7E). WT BM cells rescued the phenotype of Cyp27B1 KO mice and protected the mice from severe DSS colitis.
Discussion

T cell stimulation induced Cyp27B1 activity in vitro, suggesting that T cells are the source of Cyp27B1 in mice. This finding is consistent with a study indicating that activated T cells expressed Cyp27B1 mRNA and converted 25(OH)D$_3$ into 1,25(OH)$_2$D$_3$ (46). Our study further identified that CD8$^+$ T cells but not CD4$^+$ T cells produce Cyp27B1 activity. IFN-γ production has been shown to induce Cyp27B1 in macrophages (47); 1,25(OH)$_2$D$_3$ inhibits cytokine production of IFN-γ (48). Furthermore, IFN-γ promotes the differentiation of CD8$^+$ cytotoxic T cells, T helper-1 cells, and classic inflammatory macrophages (49,50). Our finding that T cells from Cyp27B1 KO mice overproduced IFN-γ suggests that the intracrine or paracrine production of 1,25(OH)$_2$D$_3$ by T cells might be a mechanism by which IFN-γ is turned off to selectively regulate inflammatory response by CD8$^+$ cytotoxic T cells, T helper-1 cells, and inflammatory macrophages.

The strongest evidence for a role of immune-derived Cyp27B1 is the protection of Cyp27B1 KO mice by WT BM. In the chimeric mice, the only source of 1,25(OH)$_2$D$_3$ would be the BM derived cells. Vitamin D is known to regulate the balance between pro-inflammatory and regulatory T cells to control IBD (51). Given that CD8$^+$ T cells are able to synthesize Cyp27B1 in vitro, WT BM derived T cells are a likely source of the Cyp27B1 and 1,25(OH)$_2$D$_3$ in mice during disease. This study provides the evidence of the in vivo role of extrarenal Cyp27B1 in regulating intestinal inflammation.

IL-10 KO mice develop spontaneous chronic IBD and are susceptible to hypersensitivity pneumonitis (41,42). Our data show that Cyp27B1 deficiency resulted in significantly more severe experimental IBD and hypersensitivity pneumonitis. Additionally, Cyp27B1
activity was detected in inflamed intestinal and lung tissues. Extrarenal Cyp27B1 expression has been reported in colons without inflammation (10), conflicting with our study which failed to show induction in extrarenal tissue of Cyp27B1 KO mice without inflammation. The same transgenic Cyp27B1 KO mice have been shown to express Cyp27B1 promoter activity in kidney and placenta but not in other extrarenal tissues during normal physiological condition (16). The frequency of Cyp27B1 promoter activity induction was low during both intestinal and airway inflammation. Extrarenal Cyp27B1 was detected in patients with granulomatous disease, particularly sarcoidosis (21). The type of inflammation induced in the experimental IBD and hypersensitivity pneumonitis models may not have replicated the inflammatory conditions observed in human granulomatous disease. Experimental IBD does not develop granulomas, and human IBD does. Additionally, there is no a good model of sarcoidosis in mice, and disease in the hypersensitivity pneumonitis model shows scattered and poorly formed granulomas (52,53).

PMA, calcium ionophore or TLR stimulation has been shown to induce Cyp27B1 expression in immune cells, particularly monocytes/macrophages and dendritic cells in vitro (23-26,36,54). Our data indicate no induction of Cyp27B1 promoter activity post PMA/ionomycin, LPS, or L. monocytogenes stimulation despite the elevated pro-inflammatory response. There are several possibilities why our finding was not consistent with other’s reports. Firstly, Cyp27B1 promoter activity might have been too low to be detectable using our β-galactosidase luciferase assay. Secondly, most of the studies of extrarenal Cyp27B1 induced by PMA, calcium ionophore, or PAMPs were done using human cells (24-26,36,54). Our findings in mice may indicate a different regulatory
system of Cyp27B1 in mice compared to human. Recent reports have demonstrated that
genes can be regulated by genetic regions outside the promoter (55). It is possible that the
signaling of PAMPs or PMA/ionomycin in macrophages does induce Cyp27B1, but the
region important for control might have been deleted in the construction of the Cyp27B1
KO mice. However in murine T cells and renal cells, the regulatory elements were
present in the Cyp27B1 KO mice.

In summary, our study discovered the induction of Cyp27B1 through activation on CD8+
T cells possibly for suppression of IFN-γ-induced pro-inflammatory response. This finding
enables us to further investigate the role of local production of Cyp27B1 and
1,25(OH)₂D₃ in T cell-mediated diseases. Our data also shows a critical role of Cyp27B1
in controlling the inflammation, possibly through local synthesis of 1,25(OH)₂D₃. The
evidence that Cyp27B1 KO mice reconstituted with WT BM cells were protected from
colitis further support the in vivo role of extrarenal Cyp27B1 in controlling disease in the
gut. These findings extend our understanding of the immunoregulatory function of
vitamin D and may have implications for therapeutic intervention of infectious and
autoimmune diseases.
References


Figure 4-1. LPS and PMA/ionomycin fail to induce Cyp27B1 *in vitro*.

(A and B) Production of IFN-γ and IL-4 (A) and β-galactosidase (β-gal) activity (B) in splenocytes from WT and Cyp27B1 (Cyp) KO 24 h and 48 h post PMA/ionomycin stimulation (n=4-11). Data shown are combined data from three independent experiments. (C and D) Production of IL-1β and IL-6 (C) and β-gal activity (D) in splenocytes 24 h and 48 h post LPS stimulation (n=3-4/group). (E and F) Production of IL-1β and IL-6 (E) and β-gal activity (F) in BMDM 24 h and 48 h post LPS stimulation (n=3-4/group). Data shown are representative of two to three independent experiments.
Figure 4-2. T cells produce Cyp27B1 \textit{in vitro}.

(A and B) Production of IFN-\(\gamma\) and IL-17 (A) and \(\beta\)-gal activity (B) of splenocytes from WT and Cyp KO mice 24 h and 48 h post \(\alpha\)-CD3 stimulation (n=3/group). Data shown are representative of three independent experiments. (C) \(\beta\)-gal activity of CD4+ or CD8+ T cells purified from splenocytes 48 h post \(\alpha\)-CD3 stimulation (n=6-9/group). Data shown are combined data of two independent experiments. Figures 4-2C is the contribution by Kaitlin McDaniel.
Figure 4-3. Cyp27B1 is not induced during LPS-induced acute inflammation.

(A and B) Serum IL-1β and IL-6 (A) and β-gal activity (B) of kidney, lung, and liver in WT and Cyp KO 24 h post intraperitoneal inoculation with PBS or 16 mg/kg of LPS (n=4/group). Values from WT counterparts were considered as background. Data shown are representative of three independent experiments.
Figure 4-4. Induction of Cyp27B1 during acute DSS-induced colitis.

(A) Percentage of BW change of WT and Cyp KO mice post DSS treatment (n=3-4/group). (B) Colonic blood scores at day 5 and day 12 post DSS in WT and Cyp KO mice (n=3-4/group). (C) Histological scores at day 12 and representative sections of the distal colon from WT and Cyp KO mice at day 5 and day 12 post DSS (n=3-4/group). (D and E) Whole mount staining for β-gal activity in the kidney, colon, and SI of WT and Cyp KO mice before (D) and after DSS treatment for 5 days (E). Blue staining indicates positive staining of β-gal. Data shown are representative of three independent experiments (n=22/group).
Figure 4-5. Cyp27B1 induction during SR-induced airway inflammation.

(A) Histopathological scores of lungs from WT, Cyp KO, IL-10 KO, and DKO mice post SR inoculations (n=3/group). (B) β-gal luciferase assay for β-gal activity of kidney and lung of PBS- or SR-inoculated mice (n=4-10/group). (C) Whole mount staining for β-gal activity in the kidney and lung of PBS- or SR-inoculated mice (n=4-10/group). Blue staining indicates positive staining of β-gal. Data shown are combined data from three to six independent experiments. This figure is contributed by Kaitlin McDaniel.
**Figure 4-6.** Cyp27B1 induction during chronic intestinal inflammation.

(A) The ratio of the colon or SI weight to the BW and histopathological scores of colon and SI of WT, Cyp KO, IL-10 KO and DKO mice (n=4/group). The results are presented as means ± SEMs. * Significant difference compared with DKO, # significant difference compared with IL-10 KO. (B) Representative histological sections of the SI and colon of IL-10 KO and DKO mice with IBD. (C) Whole mount staining for β-gal activity in the kidney, colon, and SI of IL-10 KO and DKO mice (n=9/group). Blue staining indicates positive staining of β-gal. Data shown are representative of three independent experiments.
Table 4-1

<table>
<thead>
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<th>Stimuli</th>
<th>Route of Administration</th>
<th>Genotype</th>
<th>Tissues</th>
<th>Cyp27B1 Induction</th>
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<td>Cyp27B1 KO</td>
<td>Lung</td>
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<td>DKO</td>
<td>Intestines</td>
<td>3/9, 33%</td>
</tr>
</tbody>
</table>

Table 4-1. *In vivo* Cyp27B1 induction during inflammation.

Cyp27B1 induction is presented as numbers of mice showing β-gal activity per numbers of mice tested and frequency of positive β-gal activity (shown in percentages). SR data were the work of Kaitlin McDaniel.
Figure 4-7. WT BM cells protect Cyp KO mice from DSS colitis.

(A) Reconstitution rate of CD45.1 WT BM cells in the blood and IELs of CD45.2 WT or Cyp KO recipient mice at day 5 post DSS (n=7/group). (B) Percentage of BW change on WT, Cyp KO mice, WT-WT, and WT-Cyp KO mice prior to DSS (n=7-12/group). (C) Percentage of BW change post 3.5% DSS treatment (n=4-10/group). (D) Colonic blood scores at day 5 and day 14 of DSS (n=3-10/group). (E) Representative histopathological sections of the distal colon from WT-Cyp KO and Cyp KO mice at day 5 and day 14 post DSS. Data shown are representative of two independent experiments.
Chapter 5

Summary and conclusions
The role of two environmental factors (diet and vitamin D) in experimental IBD pathogenesis was investigated in this thesis. In Chapter 2, the dietary effect on IBD susceptibility and intestinal bacterial flora was evaluated. Although dietary treatments have shown a beneficial effect on IBD symptoms (1), evidence is lacking to support diet as a risk factor of IBD development. I show here that short-term dietary changes resulted in differential susceptibility to dextran-sodium sulfate (DSS) or *Citrobacter rodentium*-induced colitis in mice. Notably, the dietary effect on inflammation was observed in mice that were not genetically susceptible to colitis, providing evidence that diet contributes to pathogenesis of experimental IBD independent of genetic factors. Addition of extra lactose (20%), fiber (5%), or both in diet significantly increased the susceptibility to DSS-induced colitis, suggesting high intake of dietary lactose and fiber as key factors increasing IBD susceptibility. The finding on lactose is supported by a study reporting that IBD patients had increased consumption of dairy food high in lactose before diagnosis (2). While consumption of vegetables or fruits that contains high fiber was proposed to be beneficial to IBD patients (3), studies also reported higher fiber intake in IBD patients before diagnosis (4). Lactose and fiber are fermentable by intestinal microbiota, decreasing the luminal pH and leading to overgrowth of bacteria favoring low pH condition (5). These alterations in the bacterial composition may be related to the increased susceptibility to DSS, which is supported by my finding that gut bacterial composition dramatically changed after short-term dietary feeding. In addition, depletion of bacteria by antibiotics protected mice from colitis regardless of which diet they were on, suggesting that the dietary-induced changes in susceptibility to colitis were associated
with alterations in the compositions and amounts of gut bacteria. Short-term dietary changes affect the gut bacterial composition and the susceptibility to experimental IBD.

I investigated the role of vitamin D in the intestinal microbiota and IBD in Chapter 3. Vitamin D is an essential dietary vitamin and has been shown to regulate experimental IBD (6). My data show that the increased susceptibility of vitamin D receptor knockout (VDR KO) or 1,25(OH)₂D₃-deficient mice (Cyp27B1 KO mice) to DSS-induced colitis was associated with alterations in gut bacterial flora. Cyp27B1 KO and VDR KO mice without DSS treatment had increased numbers of potentially pathogenic bacteria such as Proteobacteria and decreased numbers of commensal or beneficial bacteria including Lactobacillaceae and Lachnospiraceae present in the gut. In addition, the Helicobacteraceae from the Proteobacteria phylum were significantly elevated in Cyp27B1 KO mice. Supplementing Cyp27B1 KO mice with 1,25(OH)₂D₃ reduced Helicobacteraceae numbers and protected mice from DSS-induced colitis. Consistent with my findings, similar changes in bacterial flora have also been reported in human IBD patients, with increased Proteobacteria and decreased Lachnospiraceae from Firmicutes (7). These results suggest the association of vitamin D deficiency with human IBD and the role of these particular bacteria on IBD pathogenesis. In addition to intestinal bacterial flora, the increased susceptibility of Cyp27B1 KO and VDR KO mice to colitis may also be a consequence of impaired epithelial integrity, which is critical for the host defense against bacterial intrusion (8). Decreased frequency of epithelial adherence junctions was detected in Cyp27B1 KO mice, and increased gut permeability was found in VDR KO mice without DSS induction. The impaired epithelial integrity
causes invasion of host tissues by pathogenic bacteria in the gut, triggering pro-inflammation. The breach in the intestinal epithelial barrier may also result in overgrowth of pathogenic bacteria, leading to dysbiosis in Cyp27B1 KO and VDR KO mice. In addition to the decreased epithelial E-cadherin expression, Cyp27B1 KO colon had decreased recruitment of CD103+ tolerogenic dendritic cells in Cyp27B1 KO colon. Together, these findings suggest that vitamin D regulates intestinal inflammation by maintaining the homeostasis of gut bacterial composition and intestinal epithelial integrity.

Finally in Chapter 4, the role of extrarenal Cyp27B1 expression during inflammation was examined. While Cyp27B1 enzyme is known to be expressed in the kidney for 1,25(OH)2D3 synthesis, studies suggest the expression of Cyp27B1 in extrarenal tissues including the immune system. However, many of these reports were based on in vitro studies that did not measure enzymatic activity of extrarenal Cyp27B1 (9). Using the transgenic Cyp27B1 KO mice with the Cyp27B1 gene being replaced by bacterial LacZ reporter under the control of the Cyp27B1 promoter (10), my data show that T cell activation significantly elevated IFN-γ production and Cyp27B1 activity in Cyp27B1 KO splenocytes, suggesting the induction of Cyp27B1 in T cells. Furthermore, CD8+ T cells but not CD4+ T cells were identified as the source of Cyp27B1 activity. IFN-γ production has been shown to induce Cyp27B1 in macrophages (11), and 1,25(OH)2D3 inhibits cytokine production of IFN-γ (12). Additionally, IFN-γ promotes the differentiation of CD8+ cytotoxic T cells, T helper-1 cells, and classic inflammatory macrophages (13,14).
The finding that T cells from Cyp27B1 KO mice overproduced IFN-γ suggests the intracrine or paracrine production of 1,25(OH)_{2}D_{3} by T cells as a mechanism by which IFN-γ is turned off to selectively regulate inflammatory response by CD8+ cytotoxic T cells, T helper-1 cells, and inflammatory macrophages. *In vivo*, whole mount staining for Cyp27B1 promoter activity in lungs or intestines was detected in a low frequency (27-38%) of Cyp27B1 KO or IL-10/Cyp27B1 double knockout mice with chronic lung inflammation or colitis. As extrarenal Cyp27B1 was detected in patients with granulomatous disease, particularly sarcoidosis (15), the type of inflammation induced in the experimental IBD and hypersensitivity pneumonitis may not have replicated the inflammatory conditions observed in human granulomatous disease. The strongest evidence for a role of immune-derived Cyp27B1 is the protection of Cyp27B1 KO mice by WT bone marrow transfer. Cyp27B1 KO mice are not able to synthesize 1,25(OH)_{2}D_{3}; therefore, the only source of 1,25(OH)_{2}D_{3} would be the BM derived cells. Given that CD8+ T cells are able to synthesize Cyp27B1 *in vitro*, WT BM derived T cells are a likely candidate of the Cyp27B1 and 1,25(OH)_{2}D_{3} in WT-Cyp KO chimeras during intestinal inflammation. Collectively, this study provides evidence of the role of extrarenal Cyp27B1 in regulating IFN-γ-induced pro-inflammation and experimental IBD.

The work presented in this thesis emphasizes the critical role of environmental factors in gut homeostasis and IBD susceptibility through their regulation of intestinal bacterial flora and host immunity (Fig. 5-1). These findings highlight the potential of using dietary manipulation and vitamin D supplementation as an alternative treatment of IBD and may
also have implications for other autoimmune diseases that have also been linked to environmental factors and gut microbiota. IBD Treatment or prevention with diet or vitamin D is relatively safe with fewer side effects compared to the anti-inflammatory drugs and antibiotics; however, more questions need to be addressed for clinical interventions. Vitamin D deficiency has been linked to IBD patients, and supplementation of vitamin D shows beneficial effect on animal models of IBD. It remains to be investigated whether vitamin D supplementation has the same beneficial role in regulating IBD symptoms in humans. In addition, future experiments are required to determine the causal relationship between vitamin D deficiency in IBD patients and intestinal epithelial integrity and gut microbiota. I showed that different dietary intakes affect the colitis susceptibility and gut bacterial composition. Besides lactose and fiber that contribute to the increased colitis severity, it is necessary to understand whether other dietary components may also predispose individuals to inflammation. The dietary effect on colitis is likely to be associated with a combination of the proportions and types of dietary components. Furthermore, it is critical to determine the dose and duration of treatment using vitamin D or diet in humans. It is possible in the future to achieve personalized vitamin D or dietary treatment for individuals with different disease severity and gut bacterial composition.
Figure 5-1

Figure 5-1. 1,25(OH)₂D₃, VDR, or diet regulates intestinal inflammation by mediating the balance between beneficial and pathogenic bacteria, intestinal epithelial integrity, and host immune responses.

Effects of 1,25(OH)₂D₃, VDR, or diet on specified factors are shown in arrows. ↑: upregulation, ↓: downregulation, ↑↓: alteration.

+ indicates positive expression of Cyp27B1 or 1,25(OH)₂D₃.

References

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