VITAMIN D REGULATES THE DEVELOPMENT AND FUNCTION OF INTESTINAL T CELLS

A Dissertation in

Pathobiology

by

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ABSTRACT

Vitamin D deficiency has been linked to several autoimmune diseases including inflammatory bowel diseases (IBD). Supplementing mice with vitamin D has been shown to suppress while vitamin D deficiency or lack of the vitamin D receptor (VDR) increases intestinal inflammation in several experimental models of IBD. The intestinal microenvironment needs to be able to respond to invading pathogens with an appropriate immune response and then through the actions of gut specific tolerance-inducing immune cells turn off the response. We hypothesize that vitamin D regulates the development and function of intestinal T cells and that in the absence of vitamin D or the VDR intestinal T cell populations are skewed toward the inflammatory T cell types. The first objective of this research was to evaluate the development of intestinal T cell populations in vitamin D or VDR deficient mice. The second was to determine if potential differences in intestinal T cells are due to a T cell intrinsic need for vitamin D during development or if vitamin D is an environmental factor that affects T cell development or function.

The work presented here reveals a role of vitamin D in maintaining intestinal health by regulating the development and function of intestinal specific T cell populations. Expression of the VDR in T cells is required for proper development of tolerogenic regulatory CD8αα⁺ TCRαβ⁺ intraepithelial lymphocytes. VDR deficiency in the host enhances the development of inflammatory Th17 cells increasing inflammation in the intestine in mouse models of IBD. Vitamin D and signaling through the VDR is required for proper development of intestinal T cells and deficiency leads to increased intestinal inflammation because of reduced tolerogenic and increased inflammatory T cell development.
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Chapter 1

INTRODUCTION
Vitamin D and Inflammatory Bowel Disease

It is estimated that 1 million Americans have inflammatory bowel disease (IBD). IBD occurs more often in northern regions and in North America and Europe approximately 1 in 1000 people are affected by IBD (1, 2). A study in 2006 designed to investigate the prevalence of IBD in Canada showed that 0.5% of Canadians have IBD, the highest percentage reported worldwide (3). In addition the prevalence of IBD is increasing in regions of the world that have traditionally been considered “low incidence areas” (4). Many autoimmune diseases have been linked to vitamin D deficiency. Diseases linked to vitamin D deficiency include multiple sclerosis (MS), type-1 diabetes and IBD (5). In this chapter the immuno-regulatory role of vitamin D and its effect on the pathology of IBD will be reviewed. The epidemiological evidence connecting vitamin D deficiency to IBD severity and the data from animal models of experimental IBD will be discussed. Finally, current treatment options for IBD patients will be reviewed and how vitamin D might be used as an alternative or a supplemental treatment for patients with IBD will be discussed.

The Pathology of IBD

IBD occurs due to complicated interactions between multiple genetic and environmental factors. Crohn’s disease (CD) and ulcerative colitis (UC) are the major forms of IBD. CD and UC are chronic inflammatory disorders of the gastrointestinal tract that are characterized by remitting and relapsing inflammation of the intestinal mucosa (6). These diseases often result in abdominal pain, diarrhea, and fever. As the disease progresses rectal bleeding, weight loss and severe fatigue can occur limiting the quality of life for patients with IBD. Men and women are equally affected by IBD. CD affects teens and young adults with the majority of patients being
diagnosed between the ages of 15 to 35 years and UC affects slightly older individuals since
patients are diagnosed in their mid to late 30’s (7). Because the symptoms of CD and UC are
similar approximately 10% of patients cannot be definitively diagnosed. These cases are termed
indeterminate colitis. Even though CD and UC share similarities there are distinct differences in
their pathology. The inflammation found in CD most commonly involves the terminal ileum of
the small intestine (SI) but can be diffuse affecting areas from the esophagus, and all the way to
the rectum (8). CD is characterized by aggregates or clusters of immune cells, specifically
macrophage and T cells that form granulomas; CD is therefore sometimes described as a
granulomatous disease (8). The inflammation in CD can be patchy or segmental and referred to
as “skip” lesions (8). The lesions can become transmural, affecting the entire thickness of the
intestinal wall. Unlike CD, inflammation in UC typically involves the rectum and extends
proximally in a continuous lesion. Histopathology of UC indicates an increase in white blood
cells in the lamina propria of the colon and the crypts, which often leads to the development of
micro-abscesses (8).

Normally, the body’s immune system protects from invading pathogens such as bacteria, viruses
and fungi but tolerates food antigens and microbes living in the lumen of the intestine. However,
in diseases such as IBD the immune system is inappropriately activated by the microbes found in
the gut. In patients with IBD the inflammation does not resolve but instead persists. A
complicated interplay between genetics and the environment predisposes individuals to the
development of IBD. Current research focuses on understanding how the balance between
bacteria, the host, and the immune system is maintained, so that new strategies to prevent or treat
IBD can be discovered.

**Genetic factors**
There is clear evidence of a strong genetic component to IBD. IBD is significantly more prevalent within families. In fact, 20 to 25% of patients have a close relative with CD or UC (9). People with a biological relative with IBD are 10 times more likely to develop the disease than the general population and that number increases to 30 times more likely if the relative is a brother or sister (10). Advances in genetics research have led to the discovery of several IBD specific genes.

Single nucleotide polymorphisms (SNPs) found in genes of the immune system are prevalent in IBD. SNPs in MHC affect the nature of the immune response, and are associated with IBD as well as other autoimmune diseases because of potential alterations in the ability of the immune response to distinguish between self antigens and foreign antigens (4). Some genetic polymorphisms found in patients with IBD affect the function of the immune system by altering the immunomodulatory cytokines produced by it. Interleukin (IL)-10 is an important suppressive cytokine and SNPs in IL-10 have been associated with UC (11). SNPs in inflammatory cytokines or the receptors of inflammatory cytokines including tumor necrosis factor (TNF)-α, IL-8, IL-12, IL-18R and IL-26 have been described in patients with CD and UC (4, 11-13). Several SNPs associated with IBD play a role in the development of inflammatory T cells. Subsets of these genes encode cytokines or cytokine receptors that affect Th17 differentiation (IL-23 receptor) (4). SNPs have been described that can affect or disrupt the IL-23R signaling cascade including STAT3 and STAT4 (associated with CD and UC respectively) and JAK2 (associated with both) (14). Another IBD susceptibility gene is CTLA4 which can suppress T cell activation (15). SNPs in genes that regulate immunity are common in IBD patients.
One important class of IBD genes help the immune system recognize bacteria. SNPs of nod-like (NOD)2 (also known as CARD15) have been shown to limit the immune system’s ability to recognize bacteria (4). NOD2 encodes a cytosolic microbial molecular pattern-recognition receptor (PRR) and belongs to a large group of innate immune receptors (4). Like NOD2, NOD1 is also a PRR but NOD1 SNPs are more strongly associated with UC than CD (16). Toll-like receptor (TLR) 4 recognizes bacterial lipopolysaccharides (LPS) and helps activate the immune response to invading Gram-negative bacteria. Polymorphisms in the gene encoding TLR4 have recently been shown to be associated with both CD and UC (17). In each of these cases, failure to recognize bacteria properly may result in an abnormal immune response to commensal microflora.

Over 500 SNPs have been discovered in genes of the vitamin D pathway with 470 of the SNPs being found within the VDR gene (18). Through the use of genome wide association studies (GWAS), polymorphism in the VDR gene were shown to increase susceptibility to CD and UC (19, 20). Vitamin D is a transcriptional regulator that targets genes with vitamin D response elements (VDRE) in their promoters. Several IBD associated genes have VDREs. Cytokine genes such as IL-2, IFN-γ, IL-12 and others are transcriptional targets of vitamin D (21).

Sequence analysis of human MHC genes revealed VDREs in the promoter (22, 23). The IBD associated gene NOD2 is regulated by vitamin D and has a VDRE (24). Several genes important in the control of IBD susceptibility have VDREs and therefore are transcriptionally regulated by 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) and the VDR.

In recent years the list of IBD associated genes has increased significantly due to the use of GWAS and additional genes will be added in the near future. Several IBD associated genes have been shown to be targets of vitamin D and VDR polymorphisms have been identified in IBD.
patients. More work is needed to determine whether the genetic changes result in functional alterations that can help explain inflammation in the gastrointestinal tract and regulation by vitamin D.

**Environmental factors**

Genetic polymorphisms only account for 10-20% of the overall risk in CD and even less for UC (25). The concordance rate for IBD development in genetically identical twins is only 20% in UC and 50% in CD (5). These findings indicate that important environmental factors affect the development of IBD.

IBD is largely a disease of the developed world, with the majority of the cases being reported in North America and Europe. These diseases occur more often in urban than in rural areas and within the northern hemisphere IBD is more prevalent in northern verses southern climates (4, 26). The prevalence of IBD worldwide and specifically in countries that were previously considered “low-incidence areas”, such as Japan and India is also increasing (4). The increased prevalence of IBD cannot be explained by genetics. First and second generation immigrants from “low-incidence areas” that have moved to countries with higher incidences adopt risk levels similar or higher than the residents of that country (4).

The environmental factors that may play a role in IBD are poorly defined and may be numerous. The incidence of IBD is higher in industrialized nations and even more so for urban areas within those nations. Environmental factors that may be different in IBD low versus high areas include: diet, life-style, pollution, exposure to potential harmful chemicals and exposure to smoke. Smoking tobacco is one of the most highly associated risk factors for CD but interestingly has been shown to be helpful in patients with UC (27).
One critical environmental factor associated with IBD is bacterial exposure. Bacterial exposure has been shown to either induce or prevent experimental IBD. In one animal model of IBD germfree mice were protected and in another experimental model germfree mice developed more severe disease (11, 28). Antibiotic treatments are of some benefit for treating a subset of patients with IBD (26). Infection with Mycobacterium avium paratuberculosis and several species of Helicobacter are associated with IBD (29, 30). Others have proposed that decreased exposure to microbes in industrialized countries has led to the increased incidence of IBD in the developed world. The mechanism by which some bacteria may help and others may exacerbate IBD is an area of current research.

**Current treatments for IBD**

There is no cure for IBD. Immunosuppressive drugs are used to induce remission but are not used as maintenance drugs because of their toxicity and side effects. For maintenance there are a number of different options ranging from immunomodulators to biological therapies. The use of these newer treatments has resulted in the improved quality of life for patients living with IBD.

5-aminosalicylic acid (5-ASA) and corticosteroids are the standard first line therapies for inducing remission in patients with mild to moderate IBD. 5-ASA has been shown to inhibit the synthesis of prostaglandin, leukotrienes and IL-1β and to suppress the activation of NFκB by TNF-α and IL-1 (31). 5-ASA is a broad anti-inflammatory agent that has been shown to be effective for treating mild to moderate UC but is less effective for CD (32). Glucocorticoids, such as prednisone, non-specifically suppress the immune system and are used to treat moderate to severe CD and UC. Treatment with glucocorticoids is an effective way of inducing remission, however, prolonged use results in steroid dependence or resistance (33). Although glucocorticoids and 5-ASA are effective treatments to induce remission, not all patients respond.
Failure to respond to drug therapy is associated with a worse prognosis, including increased risk of surgery, risk of disability and an increased risk of infection (34).

The development of other immunosuppressive drugs has improved treatment for maintaining remission of IBD and has been shown to be useful for limiting long term use of glucocorticoids (7, 35). Azothioprine, methotrexate and cyclosporin suppress inflammation by limiting T cell activity. It has been demonstrated that azothioprine treatment is effective for inducing remission in patients with mild IBD and maintaining remission (36). Methotrexate inhibits the enzymes involved in the nucleoside synthesis and consequently suppresses T cell activation, proliferation and inhibits the expression of adhesion molecules (37). Treatment with methotrexate is effective in CD but less so for UC (38, 39). Cyclosporin is a widely used immunosuppressant that inhibits T cell activation by suppressing calcium activation pathways via the inhibition of calcineurin (40). Cyclosporin has been shown to be effective for treating patients with severe, steroid-refractory UC but has not proven to be effective in patients with severe CD (41).

Within the last 10 years a number of new biological therapies have been developed and found to be useful to treat IBD. Biologicals target specific aspects of the immune response that contribute to intestinal inflammation. Infliximab (Remicade) and adalimumab (Humira) are some of the first biologics that have been shown to be effective therapy options for patients with IBD (42). Infliximab and adalimumab both are humanized antibodies that block the activity of TNF-α. These TNF-α blocking drugs were first shown to be effective in patients with arthritis. In 1998, the FDA approved the use of infliximab to treat patients with moderately and severely active IBD that do not respond to conventional treatment. One of the main complications that exist with the use of this family of drugs is that patients can develop specific immunity against the therapy and stop responding(43). Since the introduction of the TNF blockers there have been
many other biologicals developed that target other inflammatory cytokines or the cytokine receptors including those for IL-12, IFN-γ, and the IL-6 (44). There are now biologicals that antagonize T cells and some that target leukocyte migration (44, 45). These treatments are very expensive and it is estimated that the use of biologicals to treat IBD results in health care costs of more than 200 billion dollars worldwide (46). There are many drawbacks to the biologics including expense, required medical personnel to administer and increased susceptibility to opportunistic infection and cancer. Presently patients with IBD will require life-long treatment. Because of the expense and risks of all of the current therapies, IBD researchers and patients are interested in exploring alternative strategies to limit intestinal inflammation. One strategy involves modifying the bacteria found in the intestine. Studies that looked at the use of antibiotics produced controversial results. Oral antibiotic treatment can be beneficial in models of IBD by eliminating bacteria and inappropriate immune responses (47). Conversely, the use of antibiotics in one study was associated with an increased risk of inflammation, presumably because good microbial flora was eliminated in the gastrointestinal tract (26). Antibiotic resistance would be an additional barrier to using antibiotics for long-term treatment of IBD. Prebiotics are non-digestible fibers that have been reported to have beneficial effects in IBD. Using prebiotics changes the composition of the intestinal microflora toward more protective bacteria and alters systemic and mucosal immune responses of the host (48). Another way to shift the balance of good verses bad bacteria is the use of probiotics. Probiotics contain viable, defined microorganisms that, when administered in adequate amounts, alter the microflora of the host (48). If found to be effective prebiotics and probiotics hold the promise of treatments that have few side-effects and could be used to augment or even replace some of the maintenance therapies for IBD patients.
The “Hygiene Hypothesis”

The higher incidence of IBD in industrialized countries has lead to the proposal of the “hygiene hypothesis”. The hygiene hypothesis states that due to the use of vaccines, improved sanitation, and reduced rates of infection the immune system does not receive critical signals and as a result autoimmunity increases (49). The immune system overreacts and subsequently fails to shut down inflammation. Improvements in hygiene in developed and developing countries includes vaccination, access to clean water, improved sewer systems, the use of food preservatives, and personal hygiene products such as: antibacterial soap, toothpaste and more recently hand sanitizers (26).

Rural areas have fewer cases of IBD. Several studies have shown that children that live on farms have a lower prevalence of immune mediated diseases including: asthma, allergies and IBD (49, 50). Possible factors of the farm lifestyle that may affect the develop of autoimmunity in children in rural areas include exposure to endotoxins, contact with animals or soil, microbial exposure and diets rich in dairy products (49). Improved hygiene in developed countries limits exposure to previously ubiquitous infectious agents such as several different types of worm (helminth) infections. Helminthes are parasites that infect humans throughout the world and are thought to play an important immunoregulatory role in the intestine. The response of the immune system to helminthes such as *Shistosoma mansoni* and *Trichinella spiralis* has been shown to be protective against experimental models of IBD and in patients with IBD (4, 51). In addition, infection with these organisms results in reduced inflammation and increased production of mucins and water secretion into the lumen of the gut that also reduces inflammation (26). Epidemiological data suggest that helminth infection is inversely correlated
with the economic status of the region as well as incidence of IBD and other immune mediated
diseases (4).

**The “Vitamin D Hypothesis”**

The vitamin D hypothesis suggests that vitamin D status may be an environmental factor
involved in the development of IBD (51). A major source of vitamin D comes from a photolysis
reaction in the skin after exposure to sunlight. Skin pigment, aging, time of day, season and
latitude dramatically affect vitamin D synthesis (52). The incidence of IBD is higher in more
northern regions of the US and Canada. Vitamin D status is especially low during winter months
in areas with the greatest seasonal fluctuation (53). Many of the environmental factors that are
present in high risk areas for IBD may also result in decreased availability of vitamin D. Factors
such as air pollutants and decreased outdoor activity are known to reduce vitamin D synthesis
(51). Intentional avoidance of sunlight exposure of the skin also reduces vitamin D status. In
developed countries people limit skin exposure to sunlight in order to avoid skin cancer. The use
of high SPF sunscreens decrease the risk of skin cancer and reduce the amount of vitamin D
produced in the skin (52).

The other source of vitamin D is the diet. Most diets are limited in natural sources of vitamin D.
Dairy products, egg yolk, and UVB treated mushrooms contain some vitamin D. Oily fish like
salmon or cod are high natural sources of vitamin D (54). Fortification of foods and
supplementation have also been useful for limiting vitamin D deficiency (55). However, more
recently vitamin D deficiency has reemerged in urban areas (55). Obesity also results in lower
vitamin D (56). Obesity in IBD patients is associated with increased severity of the disease (57).
Vitamin D insufficiency and deficiency are common in northern regions of the northern
hemisphere and several studies report an even higher prevalence of insufficiency and deficiency
in adult and pediatric patients with IBD (58). Vitamin D deficiency is common even when the patient is in remission (58, 59). This is probably due to malabsorption, low dietary intake and reduced outdoor activity of the patient. These observations indicate that vitamin D deficiency exists in patients with IBD and may both predispose and exacerbate IBD development.

**The immune response and IBD**

The human body is composed of roughly 100 trillion cells and the lumen of the intestine harbors approximately ten times that many bacteria (60). Many of these bacteria are commensal, living in the intestine but not causing harm. Other bacteria in the intestine are pathogenic and require an appropriate immune response to combat their invasion. The immune system must not react to commensal flora but still clear pathogenic organisms. In addition, once the pathogen is cleared the immune response must be turned off.

In healthy intestine the immune response is balanced and tightly regulated to clear pathogens and then shut off once the infection is cleared (Fig. 1-1). The innate immune system is the first line of defense against an invading pathogen. Innate immune cells such as macrophage and dendritic cells (DC), recognize pathogens via PPRs (toll-like or NOD-like receptors) and become activated. These activated cells then become antigen presenting cells (APC) and will process peptide components of the pathogen and present these antigen peptides to T cells. The type of CD4+ T cell (Th cells) response that is generated will dictate the outcome of the infection (Fig. 1-1).

Effector T cell responses including Th1 and Th17 cells are required to fight many different gastrointestinal infections (Fig. 1-1). In IBD uncontrolled Th1 and Th17 immune responses occur and strategies to suppress the Th1 and Th17 cells effectively suppress experimental IBD. Patients with IBD often have high levels of IFN-\(\gamma\) and increased Th1 T cells in the intestine (61).
The Th17 cells produce two inflammatory cytokines IL-17 and IL-22 that have both been shown to play a pathogenic role in several autoimmune diseases including MS, type-1 diabetes and IBD (62, 63). All IBD is not Th1 and Th17 mediated. Some patients with UC have an increased Th2 response exhibiting increased levels of IL-5 and produce autoantibodies that can in some cases disrupt the integrity of the epithelium or impair the nature of a healthy immune response (64-66). There are a number of T cells that play a role in turning off immune responses in the gut (Fig. 1-1A). The invariant natural killer T (iNKT) cells are early cells that act on the APC and shape the T cell responses. iNKT cells in the intestine become activated by the epithelial cells to produce inhibitory cytokines like IL-10 (67). Upon activation, iNKT cells rapidly produce many cytokines including IFN-γ, IL-4, IL-13, and IL-10. The type of cytokines produced by the iNKT cells dictates the nature of the resulting T cell response (Fig. 1-1, (67)). Activation of iNKT cells protects mice from experimental IBD and reduced numbers of iNKT cells have been shown in experimental models of autoimmunity including MS and type-1 diabetes (68). IBD patients have been shown to have decreased numbers of iNKT cells (Fig. 1-1, (69)). FoxP3+ CD4 T cells (T reg) have been shown to inhibit the proliferation of T cell responses both in vitro and in vivo (Fig. 1-1) (70). Animals that lack T reg cells spontaneously develop experimental IBD (11). The spontaneous development of IBD in mice that lack T regs is due to the inability to turn off T cell responses to the commensal flora since germfree mice do not develop IBD symptoms (11). T regs have been shown to induce programmed cell death or apoptosis in effector T cells, suppress proliferation and produce the suppressive cytokines IL-10 and TGF-β1(70). Transfer of T regs have been shown to suppress experimental IBD in vivo(70). iNKT cells and T regs function to suppress Th1 and Th17 responses in the gastrointestinal tract.

**Intraepithelial lymphocytes**
The gut associated lymphoid tissue harbors gut specific regulatory T cells (Fig. 1-1A). These unique T cells express a homodimeric form of CD8 – CD8αα. CD8αα can be expressed by αβ and γδ T cells and some small populations of non T cells found in the intraepithelial lymphocyte (IEL) compartment (71). Expression of CD8αα is believed to be a marker indicating adaptation of the T cells to the immuno-tolerant intestinal microenvironment. CD8αα is not considered a T cell co-receptor for 2 main reasons: 1) CD8αα has a low affinity for MHC class I molecules and 2) the β subunit of CD8αβ is the receptor required for incorporation of CD8 into the lipid rafts of the TCR complex (72). Instead of acting as a co-receptor, CD8αα has been shown to sequester LAT and p56 (Lck) away for the TCR dampening the resulting TCR signaling cascade (72). CD8αα+ TCRαβ+ T are self-reactive regulatory T cells that are believed to play a role in maintaining tolerance in the intestine (71). CD8αα+ TCRαβ+ T cells can suppress inflammation by producing suppressive cytokines like IL-10 and TGF-β1 (73, 74). Transfer of CD8αα T cells have been shown to suppress experimental IBD (Fig. 1-1) (73).

CD8αα can be expressed alone or co-expressed with the conventional T cell co-receptors CD4 and CD8αβ. Expression of CD8αα on conventional T cells helps adapt the T cell to the tolerogenic intestinal micro-environment by altering the TCR signals received by the cell (72). CD4+CD8αα+ IEL are antigen experienced T helper cells that have migrated to the gut from the mesenteric lymph nodes and then up regulated CD8αα (75). In the T cell transfer model of IBD, co-transfer of these regulatory T cells can suppress inflammation (75).

The developmental origin of CD8αα T cells is a controversial topic. Very low numbers of CD8αα+ TCRαβ+ T can be found in the intestine of athymic nude mice supporting the theory of extrathymic development (76). More recent data indicates that under normal physiological conditions CD8αα+ TCRαβ+ T cells development is thymus dependant. Gangadharan et.al.
showed that CD8αα⁺ TCRαβ⁺ T cells developed from double positive (DP) thymocytes that co-express CD8αα making these progenitors triple positive (TP) (CD4⁺/CD8αβ⁺/CD8αα⁺) (77). At the TP stage, CD8αα⁺ TCRαβ⁺ T progenitors rearrange their TCR and undergo agonist selection (72, 77). The process of agonist selection allows thymocytes that express a self-reactive TCR to survive but reprograms the cell to be regulatory in nature (71). After surviving selection, TP thymocytes down regulate all CD4 and CD8 to become DN TCRαβ⁺ T cells that leave the thymus and migrate directly to the gut (77). DN TCRαβ⁺ T cells upregulate expression of CD8αα after arriving in the IL-15 rich intestinal microenvironment (77).

**Vitamin D regulates T cell responsiveness**

Vitamin D receptors are expressed in all types of immune cells that have been examined. Vitamin D deficiency or VDR KO mice have been shown to have increased susceptibility to several different experimental models of IBD (78). In addition, 1,25(OH)₂D₃ suppresses experimental models of autoimmunity including IBD (79).

Treatment with 1,25(OH)₂D₃ in vitro has been shown to inhibit differentiation and activation of DC (80). 1,25(OH)₂D₃ treated DC stay in a more immature state resulting in reduced antigen presentation and decreased IFN-γ production, and increased production of IL-10 (81, 82). Macrophage development is also inhibited by 1,25(OH)₂D₃ in vitro and treatment of macrophage after activation inhibits the production of IL-12 and TNF-α (83, 84). These effects on macrophage and DC result in reduced Th1 T cell activation. T cells are also direct targets of 1,25(OH)₂D₃. In the absence of other cells, 1,25(OH)₂D₃ inhibits proliferation and IL-2 and IFN-γ production (85, 86). Conversely, CD4 T cells from VDR KO mice overproduce IFN-γ and proliferate twice as much as WT in mixed lymphocyte reactions (78). Treatment of peripheral blood mononuclear cells (PBMC) from IBD patients with physiological levels of
1,25(OH)₂D₃ decreased the production of IFN-γ (87). Vitamin D directly and indirectly limits the development of the Th1 immune response. Th17 cells are also targets of vitamin D.

1,25(OH)₂D₃ treatment of DC results in less of the Th17 inducing cytokines IL-6 and IL-23 (88). Treatment of CD4 T cells under Th17 polarizing conditions (IL-6 and TGF-β1) with 1,25(OH)₂D₃ reduces the number of Th17 cells that develop (89). Human PBMC from IBD patients overproduce IL-17 and treating the PBMC with a vitamin D analog reduces the production of Th17 cells (90). The effects of vitamin D on Th2 responses are less clear. Several groups have shown that 1,25(OH)₂D₃ treatment enhances the production of IL-4 and IL-5 and augmented the expression of the Th2 specific transcription factor GATA-3 (91, 92). However, others have reported that 1,25(OH)₂D₃ treatment has no affect on the expression of Th2 genes and inhibits the production of IL-4 (93).

Vitamin D has been shown to control regulatory T cells including iNKT cells, T reg cells, and CD8αα T cells. Expression of the VDR is required for normal development and function of iNKT cells (94). 1,25(OH)₂D₃ increases iNKT cell cytokine production but had no effect on the numbers of iNKT cells (94). VDR KO mice essentially have no functional iNKT cells (94). The absence of iNKT cells is partially responsible for the susceptibility of VDR KO mice to dextran sodium sulfate induced colitis (unpublished data, (95)). The ability of 1,25(OH)₂D₃ to suppress several experimental autoimmune diseases occurs via the induction of T reg cells (96). 1,25(OH)₂D₃ has been shown in vitro to increase T reg numbers (97). Vitamin D enhances the development and function of iNKT cells and T reg cells. In the absence of vitamin D or the VDR iNKT cell development is defective, resulting in the increased susceptibility of these mice to inflammation in the gut but the role of vitamin D in the development and function of T reg cells and CD8αα T cells has not been investigated.
Experimental models of IBD

Much of our knowledge about the mechanisms involved in IBD comes from experimental animal models of intestinal inflammation. While these models provide insight into the pathogenesis of IBD there is not a mouse model that completely mimics either CD or UC. The aim of these models is to provide tools to researchers that allow for investigation into specific aspects of the diseases. There are several different animal models of IBD. Some result spontaneously following KO of regulatory cytokines or cells, while others are induced following chemical injury or immunization. In each of the models there is interplay between the inability to control inflammation in the gut, the bacteria in the gut and loss of barrier function. Examination of several different experimental models of IBD can give a more comprehensive view of the effects of vitamin D on several important characteristics of the human disease.

Deletion of the suppressive cytokine IL-10 results in spontaneous intestinal inflammation. The intestinal inflammation that develops in IL-10 KO mice, is due to an uncontrolled immune response to the commensal microflora in the intestine (11). IL-10 KO mice that are either vitamin D deficient or VDRKO (VDR/IL-10 double KO) develop severe fulminating colitis that is characterized by epithelial hyperplasia, significant weight loss and premature mortality (78). The severity of the inflammation is associated with an increase in the Th1 response including increased IFN-γ, IL-12, TNF-α and IL-1β (98). In contrast, supplemental 1,25(OH)2D3 prevents inflammation in IL-10 KO mice and when given after the onset of intestinal inflammation can block the progression of the disease by reducing TNF-α through inhibition of several genes of the TNF-α pathway (79, 99).

A T cell driven model of intestinal inflammation develops when naïve CD4 T cells are transferred to mice that lack T and B cells (recombination activating gene (Rag) KO). Rag KO
mice that receive the transferred T cells develop a wasting disease as a result of increased inflammation in the small intestine and colon that is driven by uncontrolled Th1 and Th17 responses (100, 101). Transferring naïve VDR KO CD4 T cells to Rag KO mice increases the severity and induces a more rapid onset of the disease than WT CD4 T cells (78). Co-transfer of several different regulatory cells suppresses IBD in this model (73, 75).

Chemical treatment with either trinitrobenzene sulfonic acid (TNBS) or dextran sodium sulfate (DSS) are ways to induce inflammation in the gastrointestinal tract. 1,25(OH)2D3 was effective at reducing TNBS induced colitis (88). VDR KO mice were extremely susceptible to colitis induced with DSS (95). VDR KO mice had increased mortality following low levels of DSS as a result of severe intestinal inflammation, loss of barrier function and endotoxemia (95, 102).

1,25(OH)2D3 or vitamin D analogs reduced the severity and improved recovery from DSS and TNBS induced colitis (88, 95, 102). In addition to the immunoregulatory functions of vitamin D, vitamin D treatments protected mice from chemical injury to the gut and in part this was a result of improved barrier function in the presence of vitamin D.

Experiments in several different experimental models of IBD show a protective effect for vitamin D. There is one model of IBD that failed to show beneficial effects of vitamin D. IL-2 KO mice develop IBD because of an absence of T reg cells (103). 1,25(OH)2D3 treatment had no effect on IBD symptoms in the IL-2 KO mice suggesting that the ability of 1,25(OH)2D3 to induce T reg cells may be critical for suppression of IBD (103). The success of vitamin D treatment in two chemical injury models, a T cell transfer model and IL-10 KO mice and lack of success in the IL-2 KO model gives important insights into the mechanisms underlying the effects of vitamin D as a regulator of intestinal inflammation.
Several studies in humans and mouse models of IBD have shown that vitamin D has an important role in regulating intestinal inflammation. The underlying mechanism by which vitamin D regulates the immune response has not been investigated. Considering the similarities between iNKT cell and CD8αα T cell development and function, we hypothesize that VDR KO CD8αα T cell development may be impaired. To elucidate the potential differences between WT and VDR KO CD8αα IEL we isolated and characterized the different subsets of intestinal T cells and compared WT and VDR KO IEL. We also evaluated the effect of VDR signaling in the host during T cell development and differentiation. The data represented here show that vitamin D regulates the development and function of gut specific T cell populations as well as Th17 cells. In the absence of vitamin D or signaling through the VDR, these T cell populations do not develop normally and consequently result in increased inflammation.
REFERENCES


gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. *J Immunol* 157:1261-1270.


Fig 1-1. Vitamin D sufficiency results in a healthy intestine. Healthy gastrointestinal tract: DC in the IEL sample the lumen of the intestine looking for harmful bacteria. These cells become activated through recognition of the pathogen-associated membrane patterns (PAMPs) expressed on the bacteria and phagocytose the bacteria for killing and presentation of bacteria-specific antigens to naïve T cells (Th0). In the healthy intestine T cells differentiate into effector T cells under the influence of Th1 (IFN-γ and IL-12), Th2 (IL-4 and IL-10) or Th17 (TGF-β, IL-6 and IL-23) cytokines. In addition, regulatory T cells are induced to help moderate the resulting immune response (iNKT cells). Other regulatory T cells (iNKT, FoxP3+ Tregs and CD8αα+ T cells) serve to shut off effector T cells and halt inflammation in the gut.
Chapter 2

Failure of T Cell Homing, Reduced CD4/CD8αα Intraepithelial Lymphocytes and Inflammation in the Gut of Vitamin D Receptor KO Mice

Chapter adapted from the manuscript entitled:

“Failure of T cell homing, reduced CD4/CD8αα intraepithelial lymphocytes and inflammation in the gut of vitamin D receptor KO mice”

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* This manuscript was a collective effort. The data represented in figures 2-3, 2-4, and 2-5 reflects the contributions of Danny Bruce.
ABSTRACT

Specific pathogen free IL-10 KO mice failed to develop inflammatory bowel disease (IBD), whereas IL-10/vitamin D receptor (VDR) double KO mice developed fulminating IBD. WT CD4 T cells inhibited experimental IBD, while VDR KO CD4 T cells failed to suppress IBD. VDR KO mice had normal numbers and functions of regulatory T cells. The percentages of IL-17 and IFN-γ secreting T cells in the gut of mice reconstituted with WT and VDR KO CD4 T cells were also not different. Instead, there were twice as many CD8αα intraepithelial lymphocytes (IEL) in mice that were reconstituted with WT CD4 T cells than in mice reconstituted with VDR KO CD4 T cells. Furthermore VDR KO mice had reduced numbers of CD8αα IEL, absent CD4/CD8αα populations and as a result, low IL-10 production in the IEL. The lack of CD8αα IEL was due in part to decreased CCR9 expression on T cells that resulted in the failure of the VDR KO T cells to home to the small intestine. We conclude that the VDR mediates T cell homing to the gut and as a result the VDR KO mouse has reduced numbers of CD8αα IEL with low levels of IL-10 leading to increased inflammatory response to the normally harmless commensal flora.
INTRODUCTION

Inflammatory bowel diseases (IBD) that encompass Crohn’s disease and ulcerative colitis are chronic inflammatory conditions of the gastrointestinal tract. Factors that predispose individuals to the development of IBD include genetic, microbial, immunological and poorly defined environmental factors (1). The high prevalence of IBD in North America and Europe where the availability of vitamin D is low, especially during the winter when sunlight exposure is reduced, implicates vitamin D status as a possible environmental factor that contributes to IBD development (2).

The intestinal epithelial layer not only forms a physical barrier to protect the body from invading bacterial pathogens but also contains a highly specialized innate and adaptive immune system (3). Several types of T cells are important for regulation of homeostasis in the gastrointestinal tract and either induce or suppress IBD. Forkhead box (Fox)P3/CD4/CD25 regulatory T cells (T reg) induce apoptosis of effector cells, produce the inhibitory cytokines IL-10 and TGF-β1 and inhibit inflammation in experimental mouse models of IBD (4). IL-17 producing CD4 T cells or Th-17 cells are proinflammatory T cells that are associated with IBD and mice deficient in Th-17 cells are resistant to experimental IBD (5, 6). Intraepithelial lymphocytes (IEL) contain a population of regulatory T cells that express a homodimeric form of CD8, CD8αα (7). Although these CD8αα T cells are self-reactive, they are not self-destructive and have been shown to be inhibitors of inflammation in the gastrointestinal tract (7). One specific subset of IEL, CD4/CD8αα T cells are regulatory and prevent inflammation in T cell transfer models of IBD (8). These CD8αα T cells in the gut spontaneously produce a number of cytokines including IL-
that is known to be important for inhibition of experimental IBD (8). Together these T cell subsets maintain gastrointestinal homeostasis.

The discovery of the vitamin D receptor (VDR) in cells of the immune system and the presence of the $1\alpha$ 25(OH)vitamin D3 hydroxylase in dendritic cells and macrophages suggests that locally-produced $1,25(\text{OH})_2\text{D}_3$ has regulatory autocrine and paracrine properties at the site of inflammation (9). Synthesis of active vitamin D requires the $1\alpha$ hydroxylase, which catalyzes the conversion of 25(OH)D$_3$ to $1,25(\text{OH})_2\text{D}_3$. The actions of $1,25(\text{OH})_2\text{D}_3$ are mediated by its binding to the VDR, which acts as a transcription factor to modulate the expression of specific genes in a tissue-specific manner. The VDR is a member of the steroid/hormone superfamily of nuclear transcription factors and is constitutively expressed in a variety of immune cells (10). Resting T cells express low levels of VDR, which are upregulated following activation (11).

The active form of vitamin D ($1,25(\text{OH})_2\text{D}_3$) has been recognized as an immunosuppressive agent that ameliorates the pathogenesis of Th1-autoimmune diseases including IBD (2). Furthermore, vitamin D deficiency and VDR deficiency have been shown to exacerbate experimental IBD in the IL-10 KO mouse, the T cell (CD4/CD45RB$^{\text{high}}$) transfer model and dextran sodium sulfate induced colitis (12-14). The increase in T reg cells caused by $1,25(\text{OH})_2\text{D}_3$ both \textit{in vitro} and \textit{in vivo} has been suggested as a mechanism underlying the ability of $1,25(\text{OH})_2\text{D}_3$ to suppress autoimmunity (15, 16). In addition, genome wide screening techniques suggest that VDR polymorphisms are associated with increased susceptibility to both Crohn’s disease (17) and ulcerative colitis (18) in humans.

Mice lacking the VDR do not develop overt symptoms or present histological evidence of IBD even when they are housed in conventional facilities. IBD is not a vitamin D deficiency disease. However, increased expression of IL-1$\beta$ and TNF-$\alpha$ in the colon of young (5 wk) and old (9
month) VDR KO mice when compared to age matched WT mice suggests that VDR deficiency results in chronic and low grade inflammation in the gastrointestinal tract (13). T cells from VDR KO mice have been shown to express an inflammatory phenotype, respond 2-times higher in a mixed lymphocyte reaction, and to induce a more severe form of T cell induced IBD than their WT counterparts (13). VDR KO mice have heightened immune responses and inflammation in the colon, which suggest that the absence of the VDR predisposes to the development of IBD. Here we sought to determine the effect of the VDR on regulatory T cell populations that would explain the increased susceptibility of the VDR KO mice to multiple models of experimental IBD (12-14).

We show here that IBD induction in the IL-10/VDR double KO (DKO) mice is severe even when the mice are specific pathogen free (SPF) and antibiotic treated. CD4 T cells from SPF DKO mice induce IBD in T and B cell deficient (recombination activation gene; Rag KO) recipients. Conversely IL-10 single KO mice in our SPF colony did not develop overt IBD and CD4 T cells from SPF IL-10 KO mice did not transfer IBD to Rag KO recipients. In addition, we show that while CD4 T reg cell development and function is normal in the VDR KO mice; unfractionated VDR KO CD4 T cells fail to suppress experimental IBD. The failure of VDR KO CD4 T cells to suppress IBD is not due to increased induction of more pathogenic Th17 or Th1 cells in the gut but was associated with the decreased expression of CCR9 and reduced homing of VDR KO T cells to the IEL. The IEL of the VDR KO mice are missing the CD4/CD8αα T cell population and have half as many CD8αα cells that fail to produce IL-10. These data suggest that the susceptibility of the VDR KO mice to inflammation in the gastrointestinal tract comes as a result of decreased homing of T cells to the gut that limits the
number of regulatory CD8αα cells producing IL-10 in the gut. Furthermore, the absence of the VDR and CD8αα IEL result in inflammation to normally non-pathogenic bacterial flora.

**MATERIALS and METHODS**

**Animals**

All of the mice were on the C57BL/6 background all originally from The Jackson Laboratory (Bar Harbor, ME). IL-10 KO, Rag KO, VDR KO, WT and DKO mice (previously described) were bred and maintained in either SPF or conventional animal facilities at The Pennsylvania State University. Conventional animals tested positive for *Helicobacter hepaticus*, but negative for murine viruses, other *Helicobacter* species and all other bacterial pathogens. All mice (SPF and conventional) were confirmed negative for *H. bilis, H. rodentium, H. trogontum* and *H. typhlonius* species (data not shown). Vitamin D deficient and sufficient WT mice were generated exactly as we’ve described previously (12). All procedures were reviewed and approved by the Pennsylvania State University Institutional Animal Care and Use Committee.

**Antibiotic therapy**

Ciprofloxacin (200 mg/L, Mediatech, Inc., Herndon, VA) was added to the drinking water of pregnant VDR KO/IL-10 +/- mice. The pups received the antibiotic via breast milk (ciprofloxacin is secreted in breast milk) and after weaning the mice received the medication in their drinking water.

**Cell purifications and adoptive transfer**

CD4 T cells were isolated from the spleen of mice using Cell Select Columns (Cedarlane, Hornnby, Canada). FACS analysis confirmed that the purity of the T cells was at least 95%. A total of 2.5 x 10⁶ CD4 T cells were injected i.p. into syngenic Rag KO mice. For the co-transfer experiments, Rag KO mice were injected with 1:1 ratio of DKO splenocytes and CD4 T cells
Controls included Rag KO mice that did not receive any cells and Rag KO mice that received only 10^6 DKO splenocytes. Additional Rag KO mice were injected with 10^6 CD4/CD25- sorted (Cytopeia Influx, Seattle, WA) T cells from WT mice. Other Rag KO recipients received 5 x 10^5 CD4/CD25+ T cells co-injected with 10^6 CD4/CD25- T cells. The purity of CD4+ CD25+ and CD4+CD25- T cells was more than 98%. The antibodies were PE-conjugated anti-mouse CD4 (L3T4), FITC anti-mouse CD25 (BD Pharmingen, San Diego, CA). Additional Rag KO recipients (CD45.2) were injected with a 1:1 mixture of 10^6 cells from the MLN or IEL of WT CD45.1 and VDR KO CD45.2 mice. 3 days later the IEL and spleens from the Rag KO recipients were stained with PE-Cy5 anti-TCRβ and FITC anti-CD45.1 cells (BD Pharmingen).

**T reg assay**

2.5 x 10^5 CD4+CD25- T cells from either WT or VDR KO mice were stimulated with CD3 and CD28 antibodies alone or in the presence of decreasing concentrations (1:2 -1:8) of CD25+ T reg from WT and VDR KO mice. For proliferation assays, 0.4 µCiH3 thymidine (ICN, Costa Mesa, CA) was added to the cultures for the last 18h of the 72h incubation.

**IBD severity**

Colitis development was monitored by weight curves, observation of stool consistency, SI/BW ratios, LI/BW ratios, histopathology scores, anal bleeding, rectal prolapse, and death. The SI/BW and LI/BW ratios have previously been shown to be objective measures of IBD severity (12). Mice were sacrificed before they lost 20% of their body weight. The SI and colon were removed, fixed in formalin and sent to the Penn State University Animal Diagnostic Laboratories for paraffin embedding and H&E staining. Sections were scored blindly by two observers on a scale of 0 to 4 for inflammation and 0-4 for epithelial thickening exactly as we’ve described (13).
Total histopathology score ranged from 0-8. For cell transfer experiments mice were characterized as Normal - indistinguishable from uninjected control Rag KO mice (histopathology score=1-2); Mild- slight epithelial hyperplasia and increased number of leukocytes in the mucosa (histopathology score= 3-5); or Severe- marked epithelial cell hyperplasia and extensive transmural leukocytic infiltrate, crypt abscesses (histopathology score=6-8).

**Isolation of IEL**

SI was removed and flushed with Hank’s containing 5% FBS and the Peyer’s patches were removed. The intestine was cut into 0.5 cm pieces. The pieces were incubated twice in media containing 0.15 µg/ml dithiotreotol (Sigma) stirred at 37°C for 20 min. Supernatants were collected and the IEL were collected at the interface of 40/80% Percoll gradients (Sigma).

**Intracellular staining and flow cytometry**

Splenocytes and thymocytes were stained with ECD anti-CD4 then fixed and permeabilized using a FOXP3 Fix/Perm Buffer set and directions as provided (Biolegend, San Diego CA). The Fox P3 antibody was conjugated with Alexa 488. IEL were stained with conjugated antibodies; PE anti-CD8β, ECD anti-CD4, and either FITC anti-CD25 and PE-Cy5 anti-CD8α were or FITC anti-CD8α, and PE-Cy5 anti-TCRβ and appropriate isotype controls. For intracellular staining the IEL were stimulated for 12 h with PMA (0.1 µg, Sigma), ionomycin (0.5µg, Sigma) and Brefeldin A (10µg, Sigma) for the final 6 h. Cells were stained with surface markers and then fixed, permeabilized and stained with FITC anti-IFN-γ and PE anti-IL-17 or APC anti-IL10. Antibodies were purchased from BD Pharmingen. Flow cytometry analyses were performed on a FC500 bench top cytometer (Beckman Coulter, Miami, FL). Data was evaluated with WinMDI 2.9 software (Scripps Institute, La Jolla, CA).
Data analysis

Results are expressed as the mean ± SE. Statistical analysis was performed using the unpaired *t* test or ANOVAs (StatView; SAS Institute, Cary, NC). A value of *P* < 0.05 was considered statistically significant.

RESULTS

Antibiotic treatment of SPF DKO mice is ineffective in preventing IBD

SPF DKO mice were compared to SPF IL-10 KO mice for IBD development (Table 2-1). We determined that 100% of the SPF DKO mice developed fulminating IBD and the severity (small intestine (SI)/BW and large intestine (LI)/BW) of IBD was the same compared to data we had collected previously from conventional DKO mice (13). Conversely, SPF IL-10 KO mice were free of IBD symptoms (Table 2-1).

Ciprofloxacin administration to IL-10 KO mice has been shown to markedly reduce bacteria levels and prevent IBD development (19). Ciprofloxacin was administered to SPF DKO mice from birth onwards (19). All of the antibiotic treated DKO mice manifested a fulminating form of IBD. Microscopic evaluation of the gastrointestinal tract of the antibiotic treated SPF DKO mice showed severe inflammation and hyperplasia in the SI (histology score 5.0 ± 0.1) and colon (histology score 6.5 ± 0.3). SI/BW and LI/BW ratios in antibiotic treated SPF DKO mice did not change as a result of the treatment (Table 2-1). Antibiotic treatment of SPF DKO mice did inhibit the kinetics of IBD development and there was a significant delay in the mortality of the antibiotic treated SPF DKO mice (Table 2-1). We concluded that the antibiotic treatment was ineffective for preventing IBD in the SPF DKO mice (Table 2-1).

CD4 T cells from DKO mice transfer IBD
Previous work has shown that transfer of whole splenocytes from conventional DKO mice to Rag KO recipients induced a fulminating and fatal form of IBD (13). IL-10 is a key cytokine produced by T reg cells and critical for the suppression of experimental IBD. Because T reg from IL-10 KO mice are defective, CD4 T cells from conventional IL-10 KO mice induced intestinal inflammation when transferred to conventionally housed Rag KO recipients (Fig. 2-1, (20)). Rag KO mice receiving CD4 T cells from DKO mice developed chronic inflammation, manifested by weight lost, bloody diarrhea, high ratios of SI/BW (10.4 ±0.4) and LI/BW (7.1 ± 0.4), rectal prolapse and death in 8-9 wks. Histological analysis revealed inflammatory infiltrates in all the intestinal layers and severe epithelial hyperplasia of the large intestine (Fig. 2-1). Rag KO recipients of CD4 DKO T cells exhibited significantly more severe disease than the recipients of IL-10 KO CD4 T cells (Fig. 2-1).

Our conventional mouse facilities are positive for *Helicobacter hepaticus* and in order to determine whether the VDR mediated effects depended on the presence of *H. hepaticus*, we repeated the reconstitution experiments using SPF donors and recipients (confirmed negative for *Helicobacter* species). The DKO CD4 T cells from SPF mice transferred colitis to SPF Rag KO mice as was evident by a loss in the BW of Rag KO mice (Fig. 2-2A). Histopathology scores from the Rag KO recipients of the SPF DKO CD4 T cells were 6.8 ± 0.5 in the small intestine and 6.0 ± 0.4 in the colon and confirmed the development of IBD (Fig. 2-2B and 2-2C). However, IBD symptoms took longer to develop (only a 10% drop in BW by 12 weeks) when the CD4 DKO T cell transfer was done in SPF versus conventionally housed mice (Fig. 2-2A and Fig. 2-1). Conversely, Rag KO recipients of CD4 T cells from SPF VDR KO and SPF IL-10 KO mice increased their BW by approximately 10 to 15% (Fig. 2-2A), looked healthy and showed little to no microscopic pathology for 12 wks post-transfer (Fig. 2-2B and 2-2C). CD4+
T cells from DKO mice are highly pathogenic and transfer IBD to leukopenic mice even under SPF conditions.

**Total CD4 T cells from WT mice but not VDR KO mice inhibit IBD**

Next, we investigated the regulatory effects of WT or VDR KO CD4 T cells. Purified WT or VDR KO CD4 T cells containing T reg cells were injected into DKO mice either before or after colitis symptoms had developed. Figure 2-3A shows that WT CD4 T cells suppressed IBD in DKO mice (increase in BW) if transferred before the mice showed obvious symptoms of intestinal inflammation. Interestingly, injection of VDR KO CD4 T cells into asymptomatic DKO mice had an accelerating effect on intestinal pathology and lead to the early death of the mice (Fig. 2-3A). Histopathological analysis of the DKO mice reconstituted with WT CD4 T cells confirmed attenuated inflammation when compared with untreated or VDR KO CD4 T cell injected DKO mice (data not shown). Injection of WT or VDR KO CD4 T cells to DKO mice after they had developed symptoms had no effect on BW or other measures of IBD severity (Fig. 2-3A).

DKO splenocyte transfer into Rag KO mice (13) was used as a second model to test the effect of the VDR on CD4 T cells in IBD. Rag KO mice were either left untreated (no cells injected), given DKO splenocytes only, DKO splenocytes plus WT CD4 T cells, or DKO splenocytes plus VDR KO CD4 T cells. Untreated Rag KO mice grew during the experiment increasing their BW by 25% (Fig. 2-3B). Rag KO mice that received only DKO splenocytes developed IBD and lost 12% of their starting BW during the 10 wks (Fig. 2-3B). CD4 T cells from WT mice suppressed the weight loss in the Rag KO recipients and the mice were as healthy as the untreated controls also gaining 25% of their original BW (Fig. 2-3B). Rag KO mice that received VDR KO CD4 T cells as well as DKO splenocytes gained weight initially and then subsequently lost weight (Fig.
The Rag KO mice that received VDR KO CD4 T cells plus DKO splenocytes ended up weighing the same at the end of the experiment as at the beginning. In addition, one of the Rag KO recipients of VDR KO CD4 T cells plus DKO splenocytes died of severe IBD at 9 wks post-transfer. VDR KO CD4 T cells lack regulatory function in two different IBD models.

**VDR KO mice have normal numbers of functional T reg cells**

Regulatory T cells that express CD4 and CD25 were purified from WT mice and found to express the VDR by RT-PCR (data not shown). T regs (CD4/CD25) that express FoxP3 have been shown to suppress T cell proliferation in vitro and suppress T cell dependent IBD in vivo (4). The percentage of FoxP3+ T reg cells were also determined in the VDR KO and WT mice (Table 2-2). The numbers of T reg cells were not different in either the spleen or thymus of VDR KO and WT mice. In addition, the IEL of VDR KO and WT mice had similar numbers of CD4/CD25 double positive T cells (data not shown). T reg cells were tested *in vitro* for functional suppression of CD4/CD25− T cell proliferation (Fig. 2-4). T reg cells from VDR KO mice were as effective as WT T reg cells in suppressing proliferation of both WT CD25− cells and VDR KO CD25− cells (Fig. 2-4A and 2-4B). T reg cells were sorted from VDR KO and WT mice and tested *in vivo* for suppression of IBD induced by WT CD4+/CD25− T cell transfers to Rag KO mice. CD25− WT T cells induced IBD in Rag KO recipients and the mice lost 15% of their starting BW by wk 6 post-transfer (Fig. 2-4C). Either WT or VDR KO T regs (CD4+/CD25+) suppressed IBD development when they were transferred at the same time as the CD25− T cells (Fig. 2-4C). VDR KO T reg cells are phenotypically and functionally normal.

**Reduced numbers of CD8αα IEL in VDR KO mice**

Because FoxP3+ T regs were normal in the VDR KO host, we next examined the composition of the T cells found in the IEL of the Rag KO recipients shown in Fig. 2-3B. The total numbers of
IEL and the percentage of CD4 T cells recovered from the IEL of the three groups of Rag KO recipients was not different (data not shown and Fig. 2-5A). Rag KO recipients of DKO splenocytes plus WT CD4 T cells had the lowest percentage of CD8αβ cells and the highest percentage of CD8αα single positive, and CD4/CD8αα double positive cells in the IEL (Fig. 2-5A). Rag KO recipients of only DKO splenocytes or DKO splenocytes plus VDR KO CD4 T cells had low levels of CD8αα and CD4/CD8αα and high levels of CD8αβ cells in the IEL (Fig. 2-5A).

IEL from WT mice express the VDR (data not shown). We found that the total number of IEL isolated, the percentage of CD4 and the percentage of CD8αβ T cells were not different in the VDR KO and WT IEL (data not shown and Fig. 2-5B). However, the percentage of VDR KO CD8αα and CD8αα+ TCRαβ+ IEL was about half that in the WT IEL (Fig. 2-5B and data not shown). More importantly, the CD4+ CD8αα+ TCRβ+ population was missing in the VDR KO IEL (Fig. 2-5B). In order to determine whether the CD8αα T cells were ligand responsive, vitamin D deficient and vitamin D sufficient WT mice were generated, and the CD8αα T cells were compared. Vitamin D deficient WT mice had 19.0 ± 1.3 % CD8αα+ TCRαβ+ and 0.4 ± 0.1 CD4+ CD8αα+ TCRβ+ IEL versus 36.7 ± 4.2 CD8αα+ TCRαβ+ and 3.7 ± 0.3 CD4+ CD8αα+ TCRβ+ IEL in the vitamin D sufficient WT mice. There were reduced numbers of CD8αα T cells in the IEL of VDR KO, vitamin D deficient WT and Rag KO mice reconstituted with VDR KO CD4+ T cells.

Normal IL-17 but reduced IL-10 in VDR KO IEL

IL-17 and IFN-γ production is associated with increased IBD symptoms. IEL from Rag KO recipients of DKO splenocytes only produced IFN-γ and no detectable IL-17 (Fig. 2-7A). The
Rag KO recipients that received either VDR KO or WT CD4 T cells plus DKO splenocytes made IFN-γ and IL-17 (Fig. 2-7A). There was no difference in the percentage of cells that made IFN-γ, IL-17 or both IFN-γ and IL-17 in the IEL of Rag KO recipients of the VDR KO or WT CD4 T cells (Fig. 2-7A). IL-10 production is protective in experimental IBD. Intracellular staining for IL-10 in WT IEL showed that 13.6% of the cells produced IL-10 while only 0.8% of the VDR KO IEL produced IL-10 (Fig. 2-7B). The majority of the IL-10 produced was from the CD8αα IEL in both the WT and VDR KO mice (Fig. 2-7B). Therefore, the amount of IL-10 secretion in the IEL corresponded to the numbers of CD8αα cells that are present.

**Reduced CCR9 and T cell homing of VDR KO T cells**

Mesenteric lymph node (MLN) and IEL cells were stained for the two gut homing receptors integrin α4β7 and CCR9. The level of integrin α4β7 staining was similar in cells from the MLN and IEL even when TCR/CD4 T cells were gated on (data not shown). CCR9 expression was significantly higher (P= 0.017) on CD4/TCR positive T cells in the WT MLN (8.3 ± 0.3 MFI) than the VDR KO MLN (6.5 ± 0.3 MFI). CCR9 staining of the CD4+ CD8αα+ TCRβ+ IEL was also significantly higher (P=0.005) in WT (6.8 ± 0.3 MFI) than VDR KO (5.3 ± 0.1 MFI) IEL.

The ability of VDR KO T cells to home to the IEL was tested *in vivo*. Rag KO mice were injected with a 1:1 mixture of CD45.1 WT and CD45.2 VDR KO cells. Staining for T cells (TCRβ) and donor origin (CD45 isotype) was used to identify T cells from VDR KO mice (TCRβ+ and CD45.1+) or WT mice (TCRβ+ and CD45.1+) in the Rag KO recipients. The results were the same whether MLN or IEL cells were transferred and therefore the results were combined. Reconstitution of the spleens of Rag KO mice was 53% VDR KO T cells and 46%
WT T cells and the values were not significantly different (Fig. 2-7C). Reconstitution of the IEL of the Rag KO mice resulted in only 14% VDR KO versus 85% WT T cells (Fig. 2-7C).

**DISCUSSION**

Microbial antigens are well recognized as inducers of inflammation in experimental models of IBD and there is evidence that a similar process occurs in patients with IBD. However, in this study we show that deletion of the VDR in IL-10 KO mice results in a form of experimental IBD that is of a similar severity in conventionally housed (13), SPF and antibiotic treated SPF mice. Conversely, the VDR expressing IL-10 KO mice show minimal symptoms in SPF conditions and are completely disease free when treated with ciprofloxacin. Although IL-10 KO and DKO mice were maintained in the same cages and fed the same antibiotic treated water, only DKO mice developed IBD. DKO mice were also established as a GF colony. GF DKO mice were maintained and found to be outwardly disease free (normal weight, no diarrhea or bleeding) up until 175 d at which time bacteria was found to have contaminated the colony (data not shown). Therefore it seems that like all other models of IBD; bacteria found in the gastrointestinal tract drive the disease in DKO mice. Nonetheless the data show that VDR deficiency renders the IL-10 KO mice hypersensitive to antibiotic resistant otherwise nonpathogenic microbial flora.

To elucidate the potential cell population(s) involved in the induction of colitis in DKO mice, we performed T cell transfer experiments. We show here that the ability of CD4 T cells from DKO mice to transfer intestinal pathology to Rag KO mice is similar in conventional and SPF conditions. Conversely, CD4 T cell transfers from either IL-10 single KOs caused pathology when transferred to Rag KO mice in conventional mice but not in SPF mice. Our findings argue that VDR expression in CD4 T cells is a protective factor that controls bacterial antigen
dependent responses in the gut and suggests that vitamin D status and signaling through the VDR is crucial for controlling inflammation in the gastrointestinal compartment.

T reg cells are important for the maintenance of self-tolerance. Barrat et al. have shown that a combination of 1,25(OH)\textsubscript{2}D\textsubscript{3} and dexamethasone induces IL-10-producing T reg cells in vitro\textsuperscript{(15)}. Furthermore, in vivo-1,25(OH)\textsubscript{2}D\textsubscript{3} treatment of experimental autoimmune diabetes induces a population of CD4/CD25 positive T reg cells that correlates with protection of the mice from diabetes\textsuperscript{(16)}. Here we show that T reg cells develop and are present in normal numbers in VDR KO mice. Furthermore the T regs from VDR KO mice function to suppress proliferation in vitro and IBD in vivo. Therefore, it seems that expression of the VDR is not required for development or function of T regs. However, in the presence of the VDR; 1,25(OH)\textsubscript{2}D\textsubscript{3} can increase the numbers of T reg that are critical for the maintenance of self tolerance\textsuperscript{(16)}.

Paradoxically even though the classical Treg (CD4/CD25 and FoxP3 positive) cells are normal in the VDR KO mice; transfer of unfractionated VDR KO CD4 T cells does not suppress experimental IBD. In addition, production of both IFN-\(\gamma\) and IL-17 in the IEL was the same in Rag KO mice that received VDR KO or WT CD4 T cells. VDR expression is not required for the development or function of FoxP3 T regs or pathogenic IFN-\(\gamma\) or IL-17 secreting IEL.

However, CD8\alpha\alpha IEL cell numbers were reduced and the CD4/CD8\alpha\alpha T cell population was absent in the VDR KO or vitamin D deficient host. In a similar Rag KO transfer model of IBD, Das et. al have shown that CD4/CD8\alpha\alpha T cells are generated from CD4\textsuperscript{+} precursors that have been stimulated with IL-4 (Th2 cells) and not Th1 cells\textsuperscript{(8)}. However, IL-4 does not directly induce CD8\alpha expression in CD4 T cells\textsuperscript{(8)}. It would be interesting to determine whether 1,25(OH)\textsubscript{2}D\textsubscript{3} treatment affected CD8\alpha expression in CD4 T cells. The increased numbers of
CD8αβ T cells are likely of DKO origin and in the absence of competition traffic to the gut and contribute to the pathology.

CD8αα T cells are regulatory cells that suppress IBD symptoms in the gut (8). We show reduced CCR9 expression on T cells from VDR KO mice in the periphery as well as in the IEL population. Reduced CCR9 expression corresponded with a failure of VDR KO T cells to home to the gut in vivo. Since homing of T cells occurs fairly quickly (3-6 h), the presence of fewer VDR KO T cells in the gut of mice after 72h probably reflects differences in homing (based on CCR9), retention and recirculation of VDR KO T cells compared to WT T cells to the IEL.

1,25(OH)2D3 treatment in vitro has been shown to inhibit retinoic acid induced CCR9 expression but to have no effect on CCR9 expression when added alone (21). The IEL and CD8αα expressing IEL from VDR KO mice fail to produce IL-10 that is known to suppress IBD in vivo. Expression of the VDR is important for reacquisition of CD8α expression, local production of IL-10 and homing of the largely CD4/CD8αα T cells to the gut.

Our data shows that normally nonpathogenic bacteria cause IBD in the VDR deficient host. Expression of the VDR is required within the CD4 T cell compartment to prevent gastrointestinal inflammation to the bacteria found there. Classical FoxP3 positive regulatory T cells are normal in the VDR KO mice. Instead our data shows that in the absence of the VDR, CD4 T cells fail to home to the gastrointestinal tract, express CD8αα and produce IL-10. It is therefore the lack of CD4/CD8αα T cells in the gut of VDR KO reconstituted mice that allows inflammation to the normal bacterial flora to go unregulated. We propose that vitamin D is a regulator of the T cell response in the gastrointestinal tract such that the vitamin D available in the environment (either
sunshine or diet) is one of the factors, which regulates the T cell response and controls immunity, inflammation and homeostasis in the gastrointestinal tract.
REFERENCES


Table 2-1

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<th>Genotype</th>
<th>Facilities</th>
<th>IBD incidence</th>
<th>SI/BW (%)</th>
<th>LI/BW (%)</th>
<th>Mortality (d)</th>
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<tr>
<td>IL-10 KO SPF</td>
<td>0/20 (0)</td>
<td>6.3 ± 0.4</td>
<td>4.1 ± 0.4</td>
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<tr>
<td>IL-10 KO SPF/antibiotic</td>
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<td>6.3 ± 0.2</td>
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<td>DKO SPF</td>
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<td>8.3 ± 0.9³</td>
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<td>11/11 (100)</td>
<td>8.8 ± 0.1³</td>
<td>6.5 ± 0.3³</td>
<td>112 ± 3⁴</td>
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Table 2-1: Severe IBD in DKO mice regardless of the composition of the gut microflora.

¹– IBD incidence was defined as mice showing a decrease in body weight of 10%, rectal bleeding or rectal prolapse.
²– IL-10 KO mice kept in SPF conditions did not die prematurely.
³– Values from DKO mice were significantly different from the values from IL-10 KO mice housed under similar conditions, p<0.05.
⁴– SPF/antibiotic-treated DKO mice showed a significant delay in the onset of IBD and resulting mortality compared to SPF DKO mice, p<0.05.
Fig. 2-1: CD4 T cell transfers from VDR KO, IL-10 KO and DKO mice induce IBD symptoms in Rag KO mice. CD4 T cells were sorted from VDR KO, IL-10 KO, DKO and WT mice and injected into Rag KO mice. A) The percent change in BW over time is plotted ± SEM for each group of recipients. * Values were significantly different from WT values, P<0.05. ** DKO values were significantly different than all other groups, P<0.05. B) Representative colonic sections from Rag KO recipients of CD4 T cells were stained and scored blindly for pathology (see methods and (13)). Colon sections were rated WT-normal, VDR KO-normal, IL-10 KO-severe, DKO-severe. C) The percentage of mice that showed normal, mild, or severe symptoms of IBD in the small intestine (SI) and large intestine (LI) are recorded for each group of Rag KO mice receiving CD4 T cells.
Fig. 2-2

A

![Graph showing percentage change in BW over time for each group of recipients. *DKO values were significantly different than VDR KO and IL-10 KO values, P<0.05.](image)

B

Rag KO recipients of CD4 T cells from:

- VDR KO
- IL-10 KO
- DKO

![Representative colonic sections from the Rag KO recipients of CD4 T cells. Colon sections shown were rated: VDR KO-normal, IL-10 KO-normal, and DKO-mild.](image)

C

<table>
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<th>Genotype (n)</th>
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<th>L1%</th>
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<tr>
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<td>VDRKO (5)</td>
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<td>IL-10KO (6)</td>
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<td>40</td>
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<tr>
<td>DKO (5)</td>
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**Fig 2-2: CD4 T cells from SPF DKO mice transfer IBD to SPF Rag KO mice.** CD4 T cells were sorted from SPF VDR KO, IL-10 KO or DKO mice and injected into SPF Rag KO recipient mice. (A) The percentage change in BW over time is plotted ± SEM for each group of recipients. *DKO values were significantly different than VDR KO and IL-10 KO values, P<0.05. (B) Representative colonic sections from the Rag KO recipients of CD4 T cells (scoring system in methods). Colon sections shown were rated: VDR KO-normal, IL-10 KO-normal, and DKO-mild. (C) The percentage of mice that showed normal, mild or severe symptoms of IBD were recorded in the small intestine (SI) and large intestine (LI) for each group of Rag KO mice receiving CD4 T cells.
Fig 2-3: Failure of total CD4 T cells from VDR KO mice to suppress IBD. A) CD4 T cells were purified from WT or VDR KO mice and injected into DKO recipients that showed symptoms of IBD (squares, n=5-6) or that were symptom free (circles, n=3-5). The percentage change in BW over time as a function of age is shown. Symptomatic DKO mice were injected at 4 wks of age and asymptomatic mice were injected at 2 wks of age. B) Changes in BW of recipient Rag KO mice following transfer of no cells, DKO splenocytes, DKO splenocytes plus WT CD4 T cells (WT CD4), or DKO splenocytes plus VDR KO CD4 T cells (VDR KO CD4). Values represent n=3-4 mice per group and one representative of two independent experiments. *At the end of the experiment the VDR KO CD4 group lost significantly more BW than the WT CD4 group and significantly less than the DKO group, P<0.05.
Table 2-2

<table>
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<th>Genotype</th>
<th>Thymus</th>
<th>Spleen</th>
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<tr>
<td>WT</td>
<td>5.8 ± 0.2%</td>
<td>14.4 ± 1.6%</td>
</tr>
<tr>
<td>VDR KO</td>
<td>5.4 ± 0.3%</td>
<td>18.0 ± 0.8%</td>
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Table 2-2. Equal numbers and percentages of CD4 and FoxP3 double positive T cells were isolated from VDR KO and WT mice. Values are the mean ± the SEM of measurements from 5-8 individual mice per group. There was no difference in the numbers of thymocytes or splenocytes isolated from VDR KO and WT mice.
Fig 2-4: T regs from VDR KO mice are functionally normal. CD4+/CD25- and CD4+/CD25+ T cells were isolated from VDR KO and WT mice. A) WT CD25- proliferation was inhibited equally by WT and VDR KO CD4/CD25+ cells at all ratios tested. B) VDR KO CD25- proliferation was inhibited equally by WT and VDR KO CD4/CD25+ cells at all ratios tested. C) VDR KO T regs suppress IBD in the Rag KO mouse. The percentage change in BW of Rag KO mice that did not receive any cells (control, CTRL), only received WT CD4/CD25- T cells (CD25-), received WT CD4/CD25- and WT CD4/CD25+ (WT T reg), or received WT CD4/CD25- and VDR KO CD4/CD25+ (VDR KO T reg). *Rag KO recipients of CD25- cells lost significantly more BW by the end of the experiment than the CTRL, WT T reg or VDR KO T reg Rag KO recipients, P<0.05.
**Fig. 2-5.** CD8αα T cell populations in the IEL. The IEL were characterized by flow cytometry from Rag KO recipients from experiments in Fig. 2-3B and normal WT and VDR KO mice. A) The IEL from Rag KO recipients of DKO splenocytes, DKO splenocytes plus WT CD4 T cells (WT CD4), and DKO splenocytes plus VDR KO CD4 T cells (VDR KO CD4) cells (same mice as in Fig. 2-3B) were isolated and stained for CD4, CD8α and CD8β. * Values in the WT CD4 group were significantly different than the VDR KO CD4 and DKO group, P<0.05. ** Values in the DKO only group were significantly different than the WT or VDR KO group, P<0.05. B) IEL were isolated from 6-8 VDR KO and WT mice and stained for CD4, CD8α, CD8β and TCRβ. * Values in the WT group were significantly different than the VDR KO group, P<0.01. Figure 2-6 shows the isotype controls for staining and representative histograms.
Fig. 2-6: Isotype control and representative histograms for flow cytometry. A) Isotype control staining of IEL (grey) and CD8α, CD8β, CD4 and TCRβ staining of IEL (white). B) Isotype control staining of splenocytes (grey) and FoxP3 staining (white). C) Dot plots of CD8α, and CD8β staining or TCRβ and CD4 staining of IEL
Fig. 2-7. Cytokine production and homing to the IEL. A) Cytokine production was evaluated by intracellular staining for IL-17 and IFN-γ in IEL from Rag KO recipients of DKO splenocytes, DKO splenocytes plus WT CD4 T cells and DKO splenocytes plus VDR KO CD4 T cells (same mice as in Fig. 2-3B). Values represent the values from 5-6 mice per group ± SEM. B) Cytokine production of normal WT and VDR KO mice was evaluated by intracellular staining for IL-10. Histogram shows IL-10 staining: isotype control staining (grey), VDR KO (dotted) and WT (solid). The values in the table represent percent IL-10 secreting in the total IEL and CD8αα IEL of 3 individual mice per group ± SEM. * Values in the WT group were significantly higher than the VDR KO group, P<0.05. C) T cell reconstitution of the spleen and IEL of Rag KO mice following injection of 1:1 mixture of WT (CD45.1) and VDR KO (CD45.2) cells. VDR KO cells were the TCRβ+ and CD45.1- staining cells and WT cells are the TCRβ+ and CD45.1+ cells. * The percent of the T cells from WT and VDR KO mice in the spleen and IEL of Rag KO mice was compared in 7 Rag KO recipients ± SEM and the percent of T cells from the WT mice were significantly higher than the VDR KO mice in the IEL, P<0.001.
Chapter 3

Intrinsic requirement for the vitamin D receptor in the development of CD8αα+ TCRαβ+ T cells
ABSTRACT

Vitamin D receptor (VDR) deficiency results in a cell intrinsic, but selective defect in the development of CD8αα+ TCRαβ+ T cells. The thymic precursors of CD8αα+ TCRαβ+ cells are reduced in VDR KO mice and the precursors are of an immature phenotype. Immature CD8αα+ TCRαβ+ T cell precursors in the gut fail to up regulate the IL-15R and consequently show reduced proliferation and reduced maturation. CD8αα+ TCRαβ+ T cells are gut specific regulatory T cells that have been shown to play a critical role in suppressing intestinal inflammation and maintaining tolerance to the bacteria residing in the lumen of the intestine. VDR deficiency has been shown to enhance susceptibility and increase the severity of inflammation in several models of intestinal disease. The intrinsic defects in VDR KO CD8αα+ TCRαβ+ T cell development may lead to the increased inflammation in the intestine of VDR KO mice.
INTRODUCTION

The human body is comprised of approximately 100 trillion cells and 10 times that many bacteria reside in the lumen of the intestine (1). The intestinal epithelial layer not only forms a physical barrier to protect from invading pathogens but also contains a highly specialized immune system. The gut associated lymphoid tissue (GALT) has evolved to have effector responses to invading pathogens while maintaining tolerance to harmless commensal flora (2). When the balance between effector and tolerogenic response is lost intestinal inflammation can occur like that seen in inflammatory bowel disease (IBD) (2). The intestinal epithelial layer contains intra-epithelial lymphocytes (IEL) that are responsible for maintaining intestinal health. The IEL contains several unique cell types including CD8αα⁺ T cells. Unlike the TCR co-receptor CD8αβ, CD8αα does not act as a co-receptor and T cells that express CD8αα are not MHC I class restricted (3, 4). CD8αα has been shown to bind to the non-classical MHC molecule Thymic Leukemia (TL) antigen with a higher affinity than MHC class I (5). CD8αα⁺ TCRαβ⁺ IEL are self-reactive but not self-destructive and are believed to be regulatory T cells that help to maintain tolerance in the gut (6). In addition, CD8αα⁺ TCRαβ⁺ IEL have been shown to suppress intestinal inflammation in the T cell transfer model of IBD (7). The homodimeric form of CD8 can be expressed on both αβ and γδ T cells in the gut and expression of CD8αα is IL-15 dependent (8, 9). In addition, IL-15 has been shown to induce maturation, enhance survival and proliferation of both CD8αα⁺ TCRαβ⁺ and CD8αα⁺ TCRγδ⁺ IEL (9). The intestine can support lymphopoiesis as is evident by the presence of CD8αα⁺ IEL in athymic nude mice and in irradiated neonatal thymectomized mice reconstituted with bone marrow (BM) (4). More recent data suggests that under normal physiological states the thymus is the major source of CD8αα⁺ TCRαβ⁺ IEL (8). Like conventional T cells CD8αα⁺ TCRαβ⁺ IEL progenitors
develop from double positive (DP) thymocytes (8). DP thymocytes that become CD8αα⁺ TCRαβ⁺ IEL precursors become triple positive (TP) expressing CD4, CD8αβ and CD8αα (8). The development of these self-reactive T cells requires exposure to self-agonist peptides for selection in the thymus like other regulatory T cell populations (4). After surviving agonist selection, CD8αα⁺ TCRαβ⁺ IEL precursors down regulate expression of CD4 and CD8 to become double negative (DN) TCRαβ⁺ thymocytes that express CD5 (8). Unlike conventional T cells, DN TCRαβ⁺ thymocytes egress the thymus and migrate directly to the intestine (10). Upon entering the IL-15 rich environment of the intestine DN TCRαβ⁺ cells down regulate CD5 and become mature CD8αα⁺ TCRαβ⁺ IEL (8).

The vitamin D receptor (VDR) is a member of the steroid hormone family of nuclear receptors (11). The VDR contains a DNA-binding domain that is accountable for the high affinity binding of the active form of vitamin D (1,25 dihydroxyvitamin D3), for dimerization with retinoid X receptor and for binding other transcription factors (11). The heterodimeric complex of VDR and RXR binds to vitamin D response elements and regulates transcription of the target genes (11). Vitamin D is an important modulator of the immune system. Signaling through the VDR has been shown to suppress multiple models of Th1 and Th17 driven autoimmune diseases including IBD (12). Vitamin D can affect T cell function as well as the development of specific T cell populations. In vitro, supplementation with the active form of vitamin D, 1, 25 dihydroxyvitamin D (1,25 D3) limits secretion of IFN-γ by CD4 T cells and promotes IL-5 and IL-10 which favors Th2 responses over Th1 (13, 14). In addition, VDR KO TCRαβ⁺ cells show an impaired ability to migrate to the intestine when adoptively transferred to Rag KO mice (15). VDR deficient mice have normal numbers of conventional CD4 and CD8 T cells in the peripheral lymphoid organs. VDR KO mice have increased proportions of Th1 cells, reduced Th2 responses and fewer iNKT
cells and CD8αα+ TCRαβ+ T cells than WT (15-17). CD4 T cells from VDR KO mice overproduce IFN-γ and proliferate twice as much in mixed lymphocyte reactions (12). VDR KO and vitamin D deficient WT mice have a significant reduction in the number of CD4+ IEL that co-express CD8αα (15).

We show here that intrinsic defects occur during the development of VDR KO CD8αα+ TCRαβ+ T cells that results in the impaired development of these cells in the IEL. VDR KO mice have normal numbers of CD8αα+ TCRγδ+ IEL. There is a significant difference in the percentages and total numbers of T cells expressing TCRαβ in the VDR KO IEL compared to WT. WT bone marrow can reconstitute VDR KO IEL to normal levels but VDR KO CD8αα+ TCRαβ+ IEL fail to develop normally in a WT host. The number of TP thymocytes and the frequency of maturing TCRβ+ TP thymocytes are significantly reduced in neonatal VDR KO mice. The less mature VDR KO DN TCRαβ+ IEL are more prevalent in the IEL, fail to become IL-15R+ and do not mature and proliferate in response to IL-15. Our results suggest that the VDR is an important factor in the development and maturation of CD8αα+ TCRαβ+ IEL.

**MATERIALS and METHODS**

**Mice**

Age- and sex-matched VDRKO and WT C57BL/6 mice were produced at the Pennsylvania State University (University Park, PA). For embryonic thymocytes, timed breedings were performed. Breeding pairs were caged together in the evening and the following morning females were inspected for seminal plugs. Females with plugs were separated to establish embryonic day 0 and monitored for weight gain as an indicator of pregnancy. Experimental procedures received approval from the Office of Research Protection Institutional Animal Care and Use Committee at the Pennsylvania State University.
**T cell isolation and cell culture**

For IEL, the small intestine was removed and flushed with HBSS (Sigma-Aldrich, St. Louis, MO) containing 5% FBS and the Peyer’s patches were removed. The intestine was cut into 0.5-cm pieces. The pieces were incubated twice in media containing 0.15 μg/ml DTT (Sigma-Aldrich) and stirred at 37 °C for 20 min. Supernatants were collected and the IEL were collected at the interface of 40/80% Percoll gradients (Sigma-Aldrich). Thymocytes were prepared with 70 μm nylon strainer and suspended in RPMI 1640 supplemented with 10% FCS (Thermo-Fisher Scientific, Rockford, IL). For in vitro stimulation thymocytes were suspended at 1.0 x 10^6 cells/ml in RPMI 1640 supplemented with 10% FCS (Thermo-Fisher Scientific, Rockford, IL), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, and 5 mM 2-mercaptoethanol (Invitrogen, Carlsbad, CA) and stimulated with 0.5 ng/ml anti-CD3 antibody (BD Bioscience, San Jose, CA) and 100 ng/ml recombinant IL-15 (R&D Systems, Minneapolis, MN).

**Immunofluorescence**

Cells were stained according to standard procedures and analyzed on a FC500 bench top cytometer (Beckman Coulter, Brea, CA). The following antibodies were used: ECD anti-CD4 (Southern Biotech, Birmingham, AL), PE anti-CD8β, FITC anti-CD122, PE anti-CD25, FITC anti-TCRγδ, PE-Cy5 anti-TCRβ, FITC anti-NK1.1, PE anti-CD5, FITC CD45.1 or CD45.2 and PE-Cy7 anti-CD8α and appropriate isotype controls including: FITC IgG1,λ (A110-1), PE-Cy7 IgG2a, κ (R35-95), PE IgG2b, κ (A95-1), FITC IgG2a, κ (G155-178) or PE-Cy5 IgG2, λ (Ha 4/8) (BD Bioscience). Isotype controls were used to set appropriate gating. CD8αα was detected with PE labeled TL-tetramer (T3b) (8). The tetramers were a gift from Dr, Hilde Cheroutre (La Jolla Institute for Allergy and Immunology, La Jolla, CA) and the National Institute of Health.
Tetramer Core Facility (Atlanta, GA). Data were evaluated with WinMDI 2.9 software (Scripps Institute, La Jolla, CA).

**Bone marrow transplantation**

Donor bone marrow cells were isolated and transferred into sub-lethally irradiated CD45 allele mismatched recipients. Mice were allowed 6 weeks to recover and reconstitution of BM was evaluated in the blood by flow cytometry and staining for donor CD45 allele (CD45.1 or CD45.2) expression.

**BrdU incorporation assay**

2 week old mice were injected i.p. with 50μl of 25mg/ml BrdU dissolved in PBS at day 0, 2 and 4 of the experiment. The mice were sacrificed on day 7 and the IEL were analyzed for BrdU incorporation, using a BrdU flow kit (BD Pharmingen) according to the manufacturer’s instructions. PE-IgG1,κ (MOPC-)21 was used as isotype control (BD Pharmingen).

**Statistics**

Bar graphs are represented as mean ± SEM. Data was analyzed by two- tailed unpaired t test using Prism 5.0 statistical software (GraphPad Software, La Jolla, CA). P values of 0.05 or less were considered statistically significant.

**RESULTS**

**Gastrointestinal TCRαβ T cells are reduced in the absence of vitamin D signaling.**

Equal total numbers of cells were isolated from WT and VDR KO IEL (Fig. 3-1A). The frequency of innate immune cells including dendritic cells, macrophage, and NK cells were the same in the IEL of WT and VDR KO mice. The frequency of both CD4+ and CD8αβ+ T cells were not different in VDR KO and WT mice (15). Similarly, staining for the TCRγ chain showed that WT and VDR KO mice had similar frequencies of γδ T cells in the IEL (Fig 3-2A).
Staining for TCRβ showed significantly fewer TCRαβ+ T cells in VDR KO mice (Fig. 3-2A). Over 40% of the IEL in WT mice expressed the TCR αβ compared to only 25% of the VDR KO IEL (Fig. 3-2A). CD8αα can be expressed on both γδ and αβ T cells (6). Of the CD8αα+ IEL 50% were γδ T cells in WT mice (Fig. 3-2B). In the VDR KO IEL the frequency of CD8αα+ TCRγδ+ IEL was higher than WT (Fig. 3-2B). However, the total numbers of CD8αα+ TCRγδ+ IEL in WT and VDR KO mice are the same (Fig. 3-1B). Conversely, the frequency and total cell number of CD8αα+ TCRβ+ T cells in WT IEL were higher than the VDR KO values (Fig. 3-1C and 3-2B). In the absence of the VDR fewer TCRαβ+ and fewer CD8αα expressing TCRαβ+ T cells were present in the gut.

**VDR KO cell intrinsic defect in CD8αα+ TCRαβ+ T cells**

Reciprocal BM chimeras were produced using VDR KO and WT mice. Reconstitution of WT-WT (donor –recipient), VDR KO-WT, and WT-VDR KO mice was over 80% complete in the blood of all mice tested (Fig. 3-2C). Importantly, VDR KO mice were reconstituted with the same efficiency as WT mice (Fig. 3-2C). The IEL of WT mice reconstituted with WT BM (WT-WT) had 43% of the CD8αα+ TCRαβ+ T cells of donor origin (Fig. 3-2D). VDR KO recipients of WT BM (VDR KO-WT) had slightly lower reconstitution with WT donor cells but the values were not significantly different than WT-WT (Fig. 3-2D). Fewer CD8αα+ TCRαβ+ T cells of VDR KO origin were recovered from WT recipients (VDR KO-WT) and the difference was significantly different than both the WT-WT and WT-VDR KO chimeras (Fig. 3-2D). The data suggest that VDR KO BM has a cell intrinsic defect in the generation of CD8αα+ TCRαβ+ T cells that reside in the IEL.

**Early CD8αα+ TCRαβ+ thymic precursors in the VDR KO mice.**
CD8αα+ T cell precursors are first detectable in the thymus at embryonic (e) day 16 of fetal development (8). Expression of CD8αα along with CD8αβ and CD4 result in a TP cell type (8). The TP thymocytes in WT mice make up over 40% of the thymus at e16, peak at e17 (58%) and were only 10% by birth (d1, Fig. 3-3A). Similar frequencies of TP thymocytes with the same kinetics were found in the VDR KO thymus (Fig. 3-3A). Frequencies of double positive (DP) thymocytes were also not different in the fetal thymus of VDR KO and WT mice (data not shown).

From birth to 3 wks of age WT mice maintained the percentage of TP thymocytes (~10%) while the percentage of VDR KO TP thymocytes significantly decreased (Fig. 3-3B). The frequency of TP cells in the thymus then declined to those found in the adult thymus by 6 wks of about 5% and at 6 wks there were equal numbers of TP cells in WT and VDR KO thymuses (Fig. 3-3B). The frequencies of DP thymocytes are not different in WT and VDR KO mice regardless of age (Fig. 2C). Expression of the TCR is a step in the maturation of the CD8αα+ T cells precursors (8). There were more WT TP cells that express the TCRβ receptor than VDR KO TP cells at 2 and 3 wks of age. The kinetics for the appearance of TCRβ+ TP thymocytes was the same in VDR KO and WT mice but the WT mice had a higher frequency of TCRβ+ CD8αα precursors (Fig. 3-3C). By 6 wks when the frequency of TP cells was low and not different between WT and VDR KO mice the expression of TCRβ on TP cells was also not different (Fig. 3-3D).

The first 3 weeks of life are a critical time for the appearance of CD8αα+ TCRαβ+ T cells in the IEL (4). WT IEL contain 20% TCRαβ+ T cells by 1 wk of age and that percentage gradually increased as the mouse matures to the adult levels of 30% (Fig. 3-3E). CD8αα+ TCRαβ+ IEL can be found in small numbers at 1 and 2 wks in WT mice and a significant increase occurs between 2 and 3 wks of age to when over 40% of the TCRαβ+ T cells express CD8αα (Fig. 3-3F). VDR
KO TCRαβ+ IEL fail to appear in the intestine at the same rate as WT T cells and this defect can be seen as early as the first week of life (Fig. 3-3E). The frequency of VDR KO CD8αα+ TCRαβ+ T cells in the gut were the same as WT at 1 and 2 wk of age (Fig. 3-3F). By 3 wks there are lower frequencies of VDR KO CD8αα+ TCRαβ+ IEL than WT and the numbers of the CD8αα+ TCRαβ+ fail to increase further as the mice age (Fig. 3-3F).

**Reduced proliferation of TCRαβ+ IEL in the absence of the VDR.**

At the time of CD8αα+ TCRαβ+ appearance in the IEL (between 2 and 3 wks of age in WT mice, Fig. 3-3F), mice were treated with BrdU to measure the in vivo proliferation of the cells. Total BrdU incorporation of the IEL of WT mice indicated that over 30% of the cells had proliferated (Fig. 3-4A). Only 22% of the VDR KO IEL incorporated BrdU over the same time period (Fig. 3-4A). Incorporation of BrdU by γδ T cells, NK cells, and other non-TCRβ+ T cells was similar in the IEL of VDR KO and WT mice (Fig. 3-4B). The frequency of proliferating TCRαβ+ T cells in WT IEL was twice as high as VDR KO IEL (Fig. 3-4C). Different TCRβ+ subsets proliferated at different rates. In WT mice, the frequency of proliferating DN TCRβ+ T cells was significantly greater compared to CD8αα+ TCRαβ+ T cells (Fig. 3-4C). The frequency of proliferating DN TCRαβ+ from VDR KO mice was not different than CD8αα+ TCRαβ+ T cells (Fig. 3-4C). There was a significant decrease in the frequency of proliferating DN TCRαβ+ and CD8αα+ TCRαβ+ IEL in VDR KO mice compared to WT (Fig. 3-4C). VDR KO CD8αα+ TCRβ+ IEL and their precursors had impaired homeostatic proliferation.

**Increased frequency but decreased numbers of CD8αα+ TCRαβ+ IEL precursors in VDR KO mice.**

CD8αα+ TCRαβ+ IEL mature from DN TCRαβ+ IEL (8). In the WT IEL there was a minor population of less mature DN TCRαβ+ T cells that had not upregulated CD8αα+ in the IEL (Fig.
VDR KO mice had a higher frequency of DN TCRαβ⁺ IEL than WT IEL (Fig. 4A). Because VDR KO mice had fewer TCRαβ⁺ cells the total number of immature T cells was less in the VDR KO IEL than the WT IEL (Fig. 3-5B and 3-5C). The increased frequency of DN TCRαβ⁺ IEL in the VDR KO IEL was not due to an increase in NKT cells since the percentage of TCRαβ⁺/NK1.1⁺ expressing cells was also less in the VDR KO than WT mice (Fig. 3-5D). Immature DN TCRαβ⁺ IEL first express CD5 that is later down regulated as the cell matures and expresses CD8αα (8). Of the DN cells in the IEL of WT mice 25% express CD5 (Fig. 3-5E). Over 42% of the DN IEL in the VDR KO expressed CD5 which was significantly more than the WT DN cells (Fig. 3-5E). As a result of the reduced numbers of TCRαβ⁺ cells the total number of VDR KO CD5⁺ DN TCRαβ⁺ IEL was significantly less than the WT mice (Fig. 3-5F). VDR KO mice had a higher frequency of immature DN TCRαβ⁺ IEL than WT mice.

IL-15 unresponsiveness of VDR KO CD8αα precursors in the VDR KO mice.

IL-15 is required to induce the maturation of CD8αα⁺ TCRαβ⁺ IEL (8, 18). Expression of the IL-15R was measured on T cells in the thymus and IEL by measuring CD122 on the CD25⁻ T cells. The mean fluorescence intensity (MFI) of the IL-15R on DN T cells in the thymus of WT mice was low (Fig. 3-6A). The level of IL15R expressed on WT and VDR KO DN T cells in the thymus was similar (Fig. 3-6A). Expression of the IL-15R in all IEL of WT and VDR KO mice was also low and similar (Fig. 3-6B). The MFI of the IL-15R was slightly higher on VDR KO CD8αα⁺ TCRαβ⁺ IEL than WT but the difference did not reach significance (Fig. 3-6C, P=0.07). The level of expression of IL-15R on WT DN TCRαβ⁺ IEL was significantly higher than VDR KO DN TCRαβ⁺ IEL(Fig. 3-6D, P=0.01). The frequency of IL-15R positive CD8αα⁺ TCRαβ⁺ T cells was low in WT and VDR KO mice. Only 1.7% of WT and 3% of VDR KO CD8αα⁺ TCRαβ⁺ IEL express high levels of the IL-15R (Fig. 3-6E). Conversely, 37% of DN TCRβ⁺ cells
expressed the IL-15R in the WT IEL (Fig. 3-6F). VDR KO DN TCRβ⁺ IEL failed to express the IL-15R (0.6%) (Fig. 3-6F). Exogenously delivered IL-15 induces the expression of CD8αα in activated thymocytes (8). Twenty percent of the cells expressed CD8αα when WT thymocytes were activated and cultured with IL-15 (Fig. 5G). Half as many of the similarly treated VDR KO thymocytes upregulated CD8αα in response to IL-15 (Fig. 5G). The data point to defects in DN cell expression of the IL-15R and intrinsic defects in the response to exogenous IL-15 in the absence of the VDR.

DISCUSSION

We show here that there is a T cell intrinsic defect in VDR KO CD8αα T cell development that is TCRαβ T cell specific. In VDR KO mice, TP thymocyte development was normal during fetal development and in the adult thymus but there are fewer TP thymocytes during the first 3 wks of life. VDR KO DN precursors failed to mature and proliferate under the influence of IL-15 due to the low expression of the IL-15R. These results identify the VDR as an important regulator of homeostasis, development, maintenance and proliferation of CD8αα⁺ TCRαβ⁺ cells in the IEL. The GALT can support extrathymic development of CD8αα⁺ T cells as evident by the discovery that T cells can be found in the IEL compartment of athymic nude mice (Lin et al., 1995). The majority of the T cells found in athymic mice are CD8αα⁺ TCRγδ⁺ T cells but the numbers were 4 fold less than normal euthymic mice and very few CD8αα⁺ TCRαβ⁺ IEL existed in athymic mice (Lin et al., 1995). These discoveries indicated that the thymus is more important for the development of CD8αα⁺ TCRαβ⁺ than CD8αα⁺ TCRγδ⁺ IEL. Under normal physiological conditions TCRγδ⁺ T cell development occurs both thymically and extrathymically during fetal development and continues through adulthood (Hayday and Gibbons, 2008). The discovery that TP cells are thymic precursors of CD8αα⁺ TCRαβ⁺ IEL supports a thymic requirement for these
cells (Gangadharan et al., 2006). The ability of CD8αα⁺ TCRγδ⁺ T cells to develop normally in VDR KO mice suggests that signals specific for CD8αα⁺ TCRαβ⁺ T cell development such as thymic selection may be VDR dependent.

The development of both CD8αα⁺ TCRαβ⁺ and CD8αα⁺ TCRγδ⁺ IEL is severely impaired in the absence of IL-15, IL-15Rα or IL-15/IL-2Rβ (Ma et al., 2009). IL-15 is produced and trans-presented via the IL-15Rα by the epithelial cells of the intestine (Ma et al., 2009). Because VDR KO mice had normal numbers of CD8αα⁺ TCRγδ⁺ IEL and WT CD8αα⁺ TCRαβ⁺ IEL developed normally in a VDR KO host, expression and presentation of IL-15 by intestinal epithelial cells must be adequate for induction of these IL-15 dependent cell types. Expression of the IL-2/IL-15Rβ chain in CD4 T cells is increased when the cells are treated with 1,25-dihydroxyvitamin D3 in vitro (Mahon et al., 2003). We found that VDR KO DN TCRαβ⁺ T cells failed to become IL-15R positive and proliferated less than WT DN T cells. The severe reduction in the level of proliferation in VDR KO DN TCRαβ⁺ reflects the loss of IL-15 signaling. In addition, the maturation of CD5⁺ DN TCRαβ⁺ T cells requires IL-15 and VDR KO IEL contains a higher frequency of CD5⁺ DN TCRαβ⁺ T cells (Gangadharan et al., 2006). The increased percentage of VDR KO DN TCRαβ⁺ T cells that remain in an immature CD5⁺ state also reflects a lack of IL-15 signaling and likely contributed to the overall reduction of the mature CD8αα⁺ TCRαβ⁺ T cells in the IEL of VDR KO mice.

iNKT cell development is also impaired in VDR KO mice (Yu and Cantorna, 2008). CD8αα⁺ TCRαβ⁺ T cells have been called “gut NKT cells” because of their expression of NK receptors (Cheroutre, 2004). Like NKT cells, CD8αα⁺ TCRαβ⁺ T cells express NK receptors including members of the Ly49 family; CD94 and NKR-PI and use the invariant signaling component FcεRIγ as a part of their CD3 complex (Bendelac et al., 2007; Cheroutre, 2004). Both cells have
a memory phenotype and rapidly produce cytokines upon stimulation (Bendelac et al., 2007; Cheroutre, 2004). In addition, both cells can develop in the absence of MHC class I or class II but do require β2 microglobulin (Bendelac et al., 2007; Cheroutre, 2004; Cheroutre and Lambolez, 2008b). An interesting similarity between CD8αα+ TCRαβ+ T cells and NKT cells is their developmental dependence on expression of the NF-κB family transcription factor RelB (Cheroutre, 2004). RelB deficiency is associated with autoimmunity that is believed to be linked to dysfunctional T cell selection in the thymus which allows the survival of conventional T cells that express high affinity receptors that would normally be deleted during the selection process (DeKoning et al., 1997; Valero et al., 2002). The reduced numbers of CD8αα+ TCRαβ+ and NKT cells and the survival of conventional T cells with self-reactive TCR suggests that RelB plays a role in thymic function during agonist selection (Cheroutre, 2004). Interestingly, RelB is transcriptionally regulated by vitamin D and has been shown to have VDR response elements in its promoter in both mice and humans (Griffin et al., 2007). It seems likely that expression of RelB in the thymus may be enhanced by VDR signaling and that in the absence of the VDR RelB expression is reduced.

The process of agonist selection allows T cells carrying “forbidden repertoires” to survive the selection process but reprograms the cell to be regulatory in nature, carrying a self-reactive TCR while maintaining a non-destructive function (Cheroutre, 2004; Lin et al., 1995). Both CD8αα+ TCRαβ+ and NKT cells undergo agonist selection in the thymus as DP cells (Bendelac et al., 2007; Cheroutre, 2004). Each cell type then diverges along a separate developmental pathway. NKT cells that survive selection downregulate CD8 or CD4 and CD8, rapidly expand and upregulate expression of CD44 and NK1.1 (Bendelac et al., 2007). At the DP stage CD8αα+ TCRαβ+ precursors transition to a TP stage in a preTCR signal dependent manner (Gangadharan
et al., 2006). The TP thymocytes complete TCR rearrangement and undergo agonist selection (Gangadharan et al., 2006). After surviving selection, TP cells downregulate all CD4 and CD8 becoming DN TCRαβ⁺ T cells that egress the thymus and continue their maturation in the gut under the influence of IL-15 (Gangadharan et al., 2006). The similarities in the selective requirement for the VDR in CD8αα⁺ TCRαβ⁺ and NKT cells suggests a common mechanism for regulation. Further investigation is needed to determine the role of vitamin D signaling in the thymus during agonist selection.

Vitamin D and signaling through the VDR are important for regulating intestinal health. Vitamin D or VDR deficiency increases the severity of inflammation in several mouse models of intestinal disease (Froicu et al., 2003). The prevalence of Crohn’s disease and ulcerative colitis is higher in northern versus southern climates and urban versus rural areas (Braus and Elliott, 2009; Koloski et al., 2008). These are also factors that correlate with vitamin D deficiency. Several studies have reported deficiencies in adult and pediatric patients with IBD and vitamin D deficiency is common even when the patient is in remission (Katz, 2006; Pappa et al., 2006).

Genome wide association studies have shown that polymorphisms within the VDR gene increase susceptibility to IBD (Dresner-Pollak et al., 2004; Simmons et al., 2000). It seems likely that reduced signaling through the VDR would limit the development of CD8αα⁺ TCRαβ⁺ T cells in the human gut.

In summary, we have shown that there is an intrinsic and specific defect in the development of VDR KO CD8αα⁺ TCRαβ⁺ T cells. TP precursors in the thymus are reduced in neonatal VDR KO mice. Immature DN TCRαβ⁺ T cells fail to upregulate the IL-15R, proliferate less and maintain a more immature phenotype. CD8αα⁺ TCRαβ⁻ T cells are an important regulator of
intestinal immune responses and their failure to mature in VDR KO mice likely contributes to the increased susceptibility of these mice to intestinal inflammation.
REFERENCES

alpha alpha TCR alpha beta and TCR gamma delta intestinal intraepithelial lymphocytes. 
Fig. 3-1: Total T cell numbers in WT and VDR KO IEL. The T cell subsets in the IEL of WT and VDR KO mice were characterized and the total cell numbers of each subset were calculated. A) Total numbers of IEL isolated from WT and VDR KO, n=3. Total number of B) CD8αα+ TCRγδ+ IEL and C) CD8αα+ TCRαβ+ IEL from WT and VDR KO mice, n=3 per group. Values are mean ± SEM.
Fig. 3-2

**Fig. 3-2: Intrinsic defect in VDR KO mice.** The T cell subsets in the IEL of WT and VDR KO mice were characterized by flow cytometry. A) The percentage of $\gamma\delta$ and $\alpha\beta$ TCR+ IEL in WT and VDR KO mice, n=3 per group. B) The percentage of the CD8$\alpha\alpha$ IEL from WT and VDR KO mice that are TCR$\gamma\delta$ or TCR$\alpha\beta$ positive, n=3 per group. C) The percentage of donor derived cells in the blood of recipient mice (Donor→Recipient), n=5 per group. D) Percent of donor derived CD8$\alpha\alpha^+$ TCR$\alpha\beta^+$ IEL in the transplant recipients, n=5 per group. Values are mean ± SEM of 3-5 mice per group. Data represent 1 of 2 experiments.
Fig. 3-3: The development of CD8αα+ TCRαβ+ IEL and thymic precursors in fetal and neonatal VDR KO mice. The percentages of CD8αα+ TCRαβ+ thymic precursors and CD8αα+ TCRαβ+ IEL were evaluated in fetal and neonatal WT and VDR KO mice. A) The percentage of the thymus that is TP (CD4+/CD8αβ+/CD8αα+) in fetal (e16, e17, and e18) and newborn (d1) WT and VDR KO mice, n=4 per group. B) The percentages of TP thymocytes in 1, 2, 3, and 6 wk old WT and VDR KO mice, n=3 per group. C) The frequency of DP thymocytes of WT and VDR KO neonatal mice, n=3 per group. D) The percentage of maturing (TCRβ+) TP cells in WT and VDR KO mice, n=3 per group. E) The percentage of the total IEL of WT and VDR KO mice that express TCRαβ at 1, 2, 3, and 6 wks of age, n=3 per group. F) The percentage of WT and VDR KO CD8αα+ TCRαβ+ IEL in neonatal mice, n=3 per group. These data represent 1 of 2 independent experiments. Values are mean ± SEM. * VDR KO values are significantly different from WT, p<0.05.
Fig. 3-4

A

Total IEL

WT

VDR KO

34.1 ± 2.2%

22.3 ± 0.4% *

B

TCRβ- IEL

WT

VDR KO

60.7 ± 3.0%

61.5 ± 2.3%

C

WT

VDR KO

TCRβ+

36.4 ± 7.8%

15.7 ± 4.8% *

DN TCRβ+

35.1 ± 10.0%

3.0 ± 2.9% *

CD8αα

9.7 ± 1.1%

2.1 ± 0.7% *
Fig. 3-4: Reduced proliferation of VDR KO CD8αα+ TCRαβ+ IEL precursors.
Proliferation was evaluated by BrdU incorporation of the IEL subsets of WT and VDR KO mice between 2-3 wks of age. A) BrdU incorporation of the total IEL from WT and VDR KO mice at 3 wks of age, n=3 per group (p<0.01). B) Total BrdU incorporation of TCRαβ negative IEL from 3 wk old WT and VDR KO mice, n=3 per group. C) The frequency of proliferating TCRβ+, DN TCRαβ+ and CD8αα+ TCRαβ+ IEL in 3wk old WT and VDR KO mice (each gated on TCRβ+ cells first), n=3 per group. One representative histogram or dot plot is shown from 2 independent experiments. The shaded histograms are isotype staining, whereas open histograms are BrdU staining. Values are mean ± SEM of the MFI or percentages. * VDR KO values are significantly different from WT, p<0.05.
Fig. 3-5: Immature CD8αα+ TCRαβ+ precursors in the VDR KO IEL. CD8αα+ TCRαβ+ IEL precursors in the intestine of WT and VDR KO mice were evaluated by flow cytometry. A) The frequency of DN (CD4−/CD8−) TCRαβ+ IEL in WT and VDR KO mice, n=4 per group. B) The total number of TCRαβ+ IEL isolated from WT and VDR KO mice, n=4 per group. C) The total number of DN TCRαβ+ IEL in WT and VDR KO mice, n=4 per group. D) The percentage of NKT cells or DN TCRαβ+ and NK1.1+ cells in the IEL, n=4 per group. The E) frequency and F) total numbers of CD5+ DN TCRαβ+ cells in the IEL of WT and VDR KO mice, n=4 per group. These represent 1 of 2 experiments. Values are mean ± SEM.
Fig. 3-6

A) DN Thymocytes

B) TCRβ⁺ IEL

C) CD8α⁺ TCRβ⁺ IEL

D) DN TCRβ⁺ IEL

E) IL-15R

F) CD8α⁺ TCRβ⁺

G) TL tet

WT

VDR KO

*
Fig. 3-6: Immature CD8αα precursors express low levels of the IL-15R and respond poorly to IL-15 in the absence of the VDR. Cell surface expression of the IL-15R (CD122<sup>+</sup> CD25<sup>-</sup>) on A) DN TCRαβ<sup>+</sup> thymocytes, B) TCRβ<sup>+</sup> IEL, C) CD8αα<sup>+</sup> TCRαβ<sup>+</sup> IEL and D) DN TCRβ<sup>+</sup> IEL, n=3 per group. Values are mean MFI ± SEM. * Values for VDR KO are significantly different from the WT values, p<0.05. The frequency of E) CD8αα<sup>+</sup> TCRαβ<sup>+</sup> IEL and F) DN TCRαβ<sup>+</sup> IEL that express the IL-15R, n=3 per group. These data are 1 representative dot plot from 2 experiments. Values are mean ± SEM. There is a significant difference between WT and VDR KO DN TCRαβ<sup>+</sup> IEL, p<0.001. IL-15 induces CD8αα expression on WT and VDR KO thymocytes in vitro. G) The percentage of WT and VDR KO T cells that are CD8α and TL tetramer<sup>+</sup> after IL-15 treatment, p<0.05. One representative dot plot is shown and gates were set from negative stain and isotype control. Cultures were performed in triplicate. Data represent 1 of 3 experiments. Values represent mean ± SEM percentage for VDR KO and WT cultures.
Chapter 4

Converging Pathways Lead to Overproduction of IL-17 in the Absence of Vitamin D Signaling

Chapter adapted from the manuscript entitled:

“Converging pathways lead to overproduction of IL-17 in the absence of vitamin D signaling.”

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* This manuscript was a collective effort. The data represented in figures 4-4, 4-5, and 4-6 reflect the contributions of Danny Bruce.
ABSTRACT

Multiple pathways converge to result in the overexpression of Th17 cells in the absence of either vitamin D or the vitamin D receptor (VDR). Th17 and inducible regulatory T (iTreg) cells are two cell types that are considered to be alternative cell fates of the same precursor. Both cells are important in the development of autoimmunity in the gut. Here we show that VDR knockout (KO) CD4⁺ T cells readily developed into Th17 instead of iTregs. The increased frequency of Th17 cell induction in VDR KO T cells was in response to all cytokine combinations tested. Furthermore, the VDR KO iTregs were refractory to IL-6 inhibition. Development of T cells in the VDR KO host resulted in more Th17 cells, which induced more severe IBD. VDR KO mice had fewer tolerogenic CD103⁺ dendritic cells that resulted in more Th17 cells. The data collectively demonstrate that Th17 and iTreg cells are direct and indirect targets of vitamin D. Increased propensity for development of Th17 cells in the VDR KO host results in more severe inflammatory bowel disease.
INTRODUCTION

In the healthy intestine there is a balance between protective effector T cell responses to opportunistic infection and tolerogenic T cell responses to harmless commensal flora. When the balance is disrupted chronic intestinal inflammation can occur and results in inflammatory bowel disease (IBD) (1). Th1 and/or Th17 T cells are proinflammatory and are associated with increased severity of inflammation in experimental IBD (2). Several regulatory T cells have been shown to prevent inflammation including naturally occurring Foxp3⁺CD4 regulatory T cells (Treg) and CD8αα T cells (3, 4). Tolerogenic CD103⁺ dendritic cells (DC) present in the mesenteric lymph nodes (MLN) inhibit Th1/Th17 T cell differentiation and drive the development of Tregs (5). Maintaining a balance in these T cell responses is critical for gastrointestinal homeostasis. Control of inflammation in the gut relies on the balance between the Th17 cells and inducible regulatory T (iTreg) cells. Interestingly, these two cells both develop in response to TGF-β1 and therefore Th17 and iTreg are considered to function in part by direct inhibition of each other (6). TGF-β1 addition to CD4 cultures in vitro induces expression of the transcription factor Foxp3 and production of IL-10 (7). Th17 cells develop in the presence of TGF-β1 and IL-6 by suppressing Foxp3 induction and up regulating two other Th17 transcription factors RORγt and RORα (8-10). In vivo, an increase in Th17 cells in the gut is associated with a reciprocal decrease in Treg cells and vice versa (6, 11). It has therefore been suggested that Treg and Th17 cells may represent alternative cell fates of the same precursors. Vitamin D and the vitamin D receptor (VDR) are critical for the maintenance of gastrointestinal homeostasis. Vitamin D is an important regulator of the immune system and deficiency has been linked to increased susceptibility to IBD (12, 13). Vitamin D deficient or VDR knockout (KO) mice are susceptible to several different models of IBD (12, 14). Treating mice with the active
form of vitamin D, 1,25dihydroxyvitamin D₃ (1,25D3) suppresses IBD (15). The increased susceptibility of VDR KO mice to IBD is a result in part to reduced numbers of invariant NKT cells as well as CD8αα T cells in the gut (12, 14, 16). Naturally occurring VDR KO Tregs are present in normal numbers and suppress as well as wild type (WT) Tregs both in vitro and in vivo (14). 1,25D3 treatments in vitro and in vivo have been shown to increase the numbers of Foxp3⁺ Treg cells (17). In addition, the beneficial effects of 1,25D3 in experimental uveitis have been ascribed to reduced production of IL-17 (18).

VDR KO CD45RB^{high} CD4 T cells are more pathogenic than their WT counterparts (12). Here we determined the cause of the increased pathogenicity of VDR KO CD4 T cells (12). Specifically, the role of the VDR and vitamin D in the development and function of Th17 cells both in vitro and in vivo was determined. There was increased differentiation of VDR KO or vitamin D deficient CD4 T cells (compared to WT) into Th17 cells in vitro. Addition of 1,25D3 to CD4 T cell cultures inhibited Th17 cell development. The increased differentiation of VDR KO T cells into Th17 cells also occurred in the presence of just TGF-β1. Conversely, iTreg were more readily induced in WT CD4 T cell cultures. Addition of IL-6 and TGF-β1 to the CD4⁺ T cells from VDR KO mice failed to affect the expression of Foxp3. In vivo, development of WT CD45RB^{high} CD4 cells in the VDR KO host resulted in an increase in the number of IL-17 secreting cells and increased pathogenicity when the cells are transferred to Rag KO mice. In addition, transferring WT CD45RB^{high} CD4 T cells to double VDR and Rag KO (DKO) mice resulted in increased numbers of IL-17 secreting Th cells and a fulminating form of IBD. Fewer CD103⁺ DCs were present in the VDR KO MLN than in WT mice suggesting that the reduced presence of tolerogenic DCs affected the development of Th17 cells in vivo. Converging
pathways in the T cells as well as in the DCs and host antigen presenting cells result in the increased numbers of Th17 cells in the absence of vitamin D or the VDR.

**MATERIALS AND METHODS**

**Mice**

Age- and sex-matched VDRKO, Cyp27B1 KO, Rag KO, DKO and WT C57BL/6 mice were produced at the Pennsylvania State University (University Park, PA). The Cyp27B1 gene is the enzyme that converts 25(OH)D3 to 1,25D3 and therefore Cyp27B1 KO mice are 1,25D3 deficient. For vitamin D deficient mice, breeders were fed synthetic diets that do not contain vitamin D as described previously (19). Cyp27B1 KO mice were a kind gift from Dr. Hector DeLuca (University of Wisconsin, Madison, WI). Experimental procedures received approval from the Office of Research Protection Institutional Animal Care and Use Committee at the Pennsylvania State University.

**T cell isolation and culture**

CD4+ T cells were purified by mouse CD4 cell recovery column kit (Cedarlane Laboratories Ltd, Burlington, NC). The purity of CD4+ T cells was over 90%. For some experiments CD4+CD45RB\textsuperscript{high} cells were sorted using a Cytopeia Influx cell sorter (BD Bioscience, San Jose, CA). CD4+ T cells were cultured in RPMI 1640. Cells were cultured in RPMI 1640 supplemented with 10% FCS (Thermo-Fisher Scientific, Rockford, IL), 2 mM L-glutamine, 100 U/ml penicillin and 100 ug/ml streptomycin, and 5 mM 2-mercaptoethanol (Invitrogen, Carlsbad, CA). Purified CD4+ T cells (2 \times 10^{6}) were stimulated with 5\mu g/ml of anti-CD3 (2C11) and 5 \mu g/ml anti-CD28 (BD Pharmingen). In addition to CD3 and CD28 stimulation some of the cultures contained different combinations of cytokines and neutralizing antibodies. Th0 cultures contained no additions; iTreg cultures contained 5ng/ml of TGF-\beta1, 10\mu g/ml of anti-IL-4, and 1\mu g/ml of anti-
IFN-γ; Th17 cultures contained 10 ng/ml of IL-6, 5ng/ml of TGF-β1, 10µg/ml of anti-IL-4, and 1µg/ml of anti-IFN-γ; additional Th17 cultures contained 100 ng/ml of IL-21, 5 ng/ml of TGF-β1, 10µg/ml of anti-IL-4, 1µg/ml of anti-IFN-γ, and 10µg/ml anti-IL-12; additional Th17 cultures contained 100ng/ml of IL-1α, 20 ng/ml IL-6, 10µg/ml of anti-IL-4, 1µg/ml of anti-IFN-γ, and 10µg/ml anti-IL-12. Cytokines were purchased from (Peprotech, Rocky Hill, NJ) except that TGF-β1 was from (R&D, Minneapolis, MN). Antibodies were from BD Pharmingen. For some cultures 100nM of 1,25D3 dissolved in ethanol or the equivalent amount of ethanol was added to in vitro cultures.

**Bone Marrow transplantation and CD45RB<sup>high</sup> transfer.**

Bone marrow (BM) cells were isolated and transferred into sub-lethally irradiated CD45 mismatched recipients. Mice were allowed 6 weeks to recover and reconstitution of BM was evaluated in the blood by flow cytometry and staining for donor CD45 expression. Splenocytes from transplant recipients were isolated and stained for FITC anti-CD45.1 or CD45.2, PE anti-CD45RB, and PE-Cy5 anti-CD4 (BD Bioscience). Cells were sorted for donor CD45 and CD4 then selected as CD45RB<sup>high</sup>. 4.0 × 10<sup>5</sup> CD4<sup>+</sup> CD45RB<sup>high</sup> T cells were injected into each Rag KO or Rag/VDR DKO mouse. The recipients were monitored for weight loss weekly. Mice were sacrificed before losing 20% of their starting body weight (BW).

**IBD Severity**

Colitis development was monitored by weight curves, observation of stool consistency, histopathology scores, anal bleeding, rectal prolapse, and death. The small intestine (SI) and colon were removed and the tissue was fixed in formalin and sent to the Pennsylvania State University Animal Diagnostic Laboratories for sectioning and staining with hematoxylin and
Sections were scored blindly by two observers on a scale of 0 to 4 for inflammation and 0–4 for epithelial thickening. 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. Epithelial thickening: 0, normal; 1, slight epithelial cell hyperplasia; 2, pronounced epithelial cell hyperplasia (2- to 3-fold increase in crypts); 3, marked epithelial cell hyperplasia (3- to 10-fold increase in crypts); 4, marked epithelial cell hyperplasia (crypts were more than 10-fold greater). Total histopathology score ranged from 0–8.

**Isolation of intraepithelial lymphocytes (IEL)**

The SI was removed and flushed with HBSS (Sigma-Aldrich, St. Louis, MO) containing 5% FBS and the Peyer’s patches were removed. The intestine was cut into 0.5-cm pieces. The pieces were incubated twice in media containing 0.15 μg/ml DTT (Sigma-Aldrich) and stirred at 37 °C for 20 min. Supernatants were collected and the IEL were collected at the interface of 40/80% Percoll gradients (Sigma-Aldrich).

**Intracellular Staining and Flow Cytometry**

Splenocytes and IEL were stained with conjugated antibodies: ECD anti-CD4 (Southern Biotech, Birmingham, AL), PE anti-CD8β or PE anti-CD62L, FITC anti-CD25, PE-Cy5 anti-TCRβ and PE-Cy7 anti-CD8α and appropriate isotype controls (BD Bioscience). To characterize DC, single cell suspensions were stained with FITC anti-CD11b, PE anti-CD103, PE-Cy5 anti-CD8 and PE-Cy7 anti-CD11c or isotype control (BD Bioscience). To eliminate lymphocytes, the high forward scatter/side scatter dot plots were first gated on to determine DC frequency. Selected isotype control staining histograms are shown in Figure 4-1. For intracellular staining cells were
stimulated for 12 h with PMA (0.1 µg), ionomycin (0.5 µg), and brefeldin A (10 µg) (all reagents from Sigma-Aldrich) for the final 6 h. Surface markers were stained, the cells fixed, permeabilized and stained with FITC-labeled anti-IFNγ clone B27, PE-labeled anti-IL-17A (TC11), and Alex488 labeled-Foxp3 (BD Bioscience). Flow cytometry analyses were performed on a FC500 bench top cytometer (Beckman Coulter, Brea, CA). Data were evaluated with WinMDI 2.9 software (Scripps Institute, La Jolla, CA). Supernatants from 72 h cultures were analyzed for the production of IL-10 and IL-17 by ELISAs (BD Bioscience).

**Reverse-Transcription Quantitative PCR**

Total RNA was prepared from differentiated T cells 72h after stimulation RNeasy Mini kit (QIAGEN, Valencia, CA). cDNA was synthesized with the Taqman Reverse kit (Applied Biosystems, Carlsbad, CA). Quantitative RT-PCR was performed on a 7500 Fast Real-Time PCR instrument (Applied Biosystems) with TaqMan Universal PCR Master Mix (Applied Biosystems) for IL-17A, IL-17F, IL-21, IL-22, RORγt and RORα (Applied Biosystems). The data in differentiated CD4⁺ T cells was expressed relative to naive CD4⁺ T cells. The data were expressed as $2^{-\Delta\Delta CT}$ with the ABI 7500 SDS 1.3.1 software.

**Statistics**

Bar graphs are represented as mean ± SEM. A 2-way mixed methods ANOVA was performed or in some cases a two- tailed unpaired $t$ test using Prism 5.0 statistical software (GraphPad Software, La Jolla, CA). P values of 0.05 or less were considered statistically significant.

**RESULTS**

**Increased production of IL-17 in the absence of the VDR.**

It has been shown that TGF-β1 induces iTreg cells when added alone to CD4⁺ T cells (20). Addition of IL-6 plus TGF-β1 to CD4⁺ T cells inhibits iTregs and upregulates Th17 cells (21).
The addition of TGF-β1 and IL-6 to activated CD4+ T cells induced IL-17 production (Th17) in both WT and VDRKO T cells (Fig. 4-2A). VDRKO T cells contained twice as many IL-17 producing cells than WT (Fig. 4-2A). The percentage of IFN-γ producing cells was similar in Th17 cultures from WT and VDRKO mice (data not shown). Very few cells produced both IFN-γ and IL-17 in the WT or VDR KO Th17 cultures. The percentage of IL-17 secreting T cells in VDRKO CD4+ cells cultured with TGF-β1 only (Treg) was also twice that of the WT cells (Fig. 4-2A). Total IL-17 secretion in the Th17 cultures was also significantly higher from VDR KO cells than WT cells (Fig. 4-2B). Additional cytokines produced by Th17 cells, including mRNA for IL-17F and IL-21, were higher from VDR KO than WT Th17 cultures (Fig. 4-2C).

There are other combinations of cytokines that will induce Th17 cells (22). To rule out a problem with either the TGF-β1 or the IL-6 pathway in the VDR KO cells, Th17 cells were induced using alternate cytokine pathways. IFN-γ secretion was similar in WT and VDR KO cultures that included TGF-β1/IL-21 or IL-6/IL-1α (Fig. 4-2D). VDR KO cells overproduced IL-17 secreting cells in cultures that included either TGF-β1/IL-21 or IL-6/IL-1α (Fig. 4-2D). The increase in Th17 cells in VDRKO T cells was not due to upregulation of RORγt or RORα (Supplemental 4-2).

**VDR KO CD4+ iTregs are refractory to IL-6**

The occurrence of naturally occurring Tregs that express Foxp3 in T cells was similar in VDR KO and WT mice (14). Here we determined the ability of T cells to develop into iTreg when TGF-β1 is added to activated T cells. As expected, WT CD4+ T cells were induced to express Foxp3 following culture with TGF-β1 (Fig. 4-4A). Addition of IL-6 greatly inhibited Foxp3 expression in WT CD4+ T cells (from 42% to 6.7%, Fig. 4-4A). Half as many Foxp3+ Treg cells
were induced in VDRKO T cells cultured with TGF-β1 as WT T cells (22% vs 42%, Fig. 4-4A).

Unlike WT T cells, addition of IL-6 with TGF-β1 to VDRKO T cells failed to inhibit Foxp3+ expression (Fig. 4-4A). The Treg production of IL-10 followed Foxp3 expression (Fig. 4-4B). Treg cultures had VDR KO cells producing less IL-10 than WT (Fig. 4-4B) and Th17 cultures had VDR KO cells producing more IL-10 than WT (Fig. 4-4B). There were no cells that expressed both FoxP3 and IL-17 even in the VDR KO cultures (data not shown).

**1,25D3 inhibits IL-17**

WT CD4+ T cells were treated with 1,25D3 in the presence of different polarizing cytokines. Addition of 1,25D3 to WT T cell cultures significantly inhibited Th17 differentiation under Treg and Th17 conditions (Fig. 4-5A). The percentage of Th17-producing cells decreased in the presence of 1,25D3 from 10.8% to 6.8% under Treg condition and 13.8% to 6.8% under Th17 cell culture conditions (Fig. 4-5A). 1,25D3 deficient T cells stimulated under Treg conditions had the same frequency of IL-17 secreting cells as WT cultures (Fig. 4-5B). Under Th17 conditions the 1,25D3 deficient T cells overproduced IL-17 compared to WT cells (Fig. 4-5B). Vitamin D deficient T cells overproduced IL-17 secreting cells under Treg and Th17 conditions (Fig. 4-5C). The magnitude of the increase of IL-17 between 1,25D3 deficient and WT (1.5 fold increase) and vitamin D deficient and WT (2.6 fold increase) was greater in the complete absence of all vitamin D metabolites (vitamin D deficient, Fig. 4-5C).

**The development of IL-17 producing T cells is affected by VDR expression in the host.**

Reciprocal BM chimeras were done to determine the effect of VDR expression on the ability of the T cells to induce IBD when transferred. Donor BM was identified by a difference at the CD45 locus and the following donor-recipient combinations: WT -WT, WT-VDR KO and VDRKO-WT were done. Naïve T cells (CD4/CD45RBhigh) of donor origin were sorted and
injected into Rag KO recipients. Beginning at 2 wks, Rag KO recipients of WT-VDR KO cells weighed less than recipients of the WT-WT, or VDR KO-WT cells (Fig 4-6A). Throughout the experiment the recipients of WT-VDR KO cells weighed less than either the VDRKO-WT or WT-WT recipients (Fig. 4-6A). The recipients of VDRKO-WT or WT-WT naïve cells weighed significantly less than uninjected control (Ctrl) Rag KO mice (Fig. 4-6A). The second experiment was terminated at 3 wks post transfer. IFN-γ producing cells were more numerous at 3 wks post-transfer than at 6 wks in the IEL of recipient mice (Fig. 4-6B). The percentage of IFN-γ secreting cells was similar in Rag KO recipients of all three cell types (Fig. 4-6B). The percentage of cells producing IL-17 was higher after 6 wks than 3 wks post-transfer (Fig. 4-6C). Significantly more IL-17 secreting cells were recovered from Rag KO recipients of WT-VDRKO than either the WT-WT or VDRKO-WT recipients (Fig. 4-6C) and the increased presence of IL-17 secreting cells corresponded with the increased symptoms of IBD in these mice (Fig. 4-6A). IL-17 secreting cells were higher in WT-WT than VDRKO-WT recipients at 3 wks but not 6 wks post-transfer (Fig. 4-6C). There were also increased IL-17 producing cells in the spleen of WT-VDRKO recipients at 6 wks (Fig. 4-6C). The data suggest that expression of the VDR in the host alters the development of WT T cells so that Th17 cells develop more readily.

**More severe IBD in VDR deficient hosts.**

To investigate the role of the VDR in the host during intestinal inflammation naïve WT CD4 T cells were transferred to Rag KO and VDR/Rag DKO mice. DKO recipients began losing weight early and a significant difference was observed by 1 wk post-transfer (Fig. 4-7A). The DKO mice had lost >15% of their body weight (BW) by 3 wk post-transfer (Fig. 4-7A). At 3 wk the Rag KO recipients had not lost weight and did not show outward signs of IBD (Fig. 4-7A). There was some inflammation visible in histopathology sections from the Rag KO recipients of naïve T
cells but the colonic sections of the DKO recipients showed significantly more hyperplasia and inflammation (Fig. 4-7B). Quantification of the severity of the inflammation showed that histopathology scores in the DKO recipients were significantly higher than in Rag KO recipients of naïve T cells (Fig. 4-7C).

**Increased Th17 and fewer tolerogenic DC in the absence of the VDR.**

The IEL and spleen from the DKO and Rag KO mice were analyzed for the numbers of CD4+ T cells that were present and the DKO mice had significantly more T cells than Rag KO recipients (Fig. 4-7D). In addition to having more CD4+ T cells in the VDR KO host fewer of those CD4 T cells maintained a naïve phenotype as determined by the expression of CD62L (Fig. 4-8A).

There were more CD4/CD25+ cells in the Rag KO than the DKO recipient’s IEL (Fig. 4-8B). There was no difference in the percentage of IFN-γ positive CD4+ T cells between Rag KO and DKO recipients (data not shown). IL-17 producing cells were higher in the gut of DKO mice than Rag KO (Fig. 4-8C). In addition, the spleen of DKO mice contained more IL-17 secreting cells than the Rag KO recipients of naïve cells (Fig. 4-8D).

An analysis of the DC populations showed that the MLN of VDR KO mice had normal numbers of total CD11c+ DCs (data not shown) but reduced frequency of tolerogenic CD103+ CD11c+ DCs compared to WT MLN (Fig. 4-8E). In addition, the total number of CD103+ CD11c+ DCs were lower in the VDR KO MLN than the WT MLN (Fig. 4-9A). Rag KO and DKO mice have a larger percentage of DCs than WT and VDR KO mice since they don’t have lymphocytes (Fig. 4-8E and 4-8F). DKO mice had decreased frequencies of CD103+CD11c+ DCs in both the MLN and IEL than Rag KO mice (Fig. 4-8F). The total number of CD103+CD11c+ DCs present in the IEL of DKO mice was lower as well as the frequency of cells compared to Rag KO mice (Fig. 4-8F and Fig. 4-9B). Decreased numbers of tolerogenic DCs are present in VDR deficient mice.
DISCUSSION

In the absence of the VDR or vitamin D, CD4+ T cells made more IL-17. The overproduction of IL-17 was a universal response to multiple IL-17-inducing cytokines. Universal overproduction of IL-17 in response to different cytokine milieus suggests that the VDR affects signals downstream of the different cytokine pathways. Each of these cytokine pathways leads to the upregulation of RORγt and RORα in VDR KO T cells (9). However, the increased potential to develop into Th17 was not associated with a difference in the expression of RORγt or RORα. Conversely, 1,25(OH)2D3 treatment inhibited IL-17 production. Vitamin D and the VDR were negative regulators of Th17 cells and direct inhibitors of IL-17 production.

TGF-β1 induced fewer iTreg in VDR KO CD4+ T cells than WT. IL-6 failed to change the percentage of FoxP3+ T cells in the VDR KO cultures. The data suggest a defect in the induction of iTreg and the inhibition of FoxP3+ in the absence of the VDR. TCR engagement and CD28 signaling recruit several transcription factors including NFAT, cRel, Creb, and STAT5 to FoxP3 promoter (23-25). The expression of FoxP3 is not stable and is affected by the environment. The vitamin A metabolite, retinoic acid (RA) promotes iTreg induction by inducing FoxP3 and promoting TGF-β1 signaling (26). RA induces histone acetylation of the FoxP3 promoter (26).

VDR and the vitamin A receptor both accomplish their biological function through a shared heterodimeric partner, the retinoid X receptor. Interactions between vitamin A and vitamin D are well established and there are reports of both cooperation, and antagonism (27, 28). In this case the effect of the VDR and vitamin D mirror the effect of vitamin A suggesting that perhaps there is cooperation by vitamin A and D in the induction of iTreg. 1,25D3 has been shown to induce FoxP3 expression in vitro (17). VDR KO iTregs were not affected by addition of IL-6 indicating
a problem with iTreg induction but also iTreg suppression. The VDR and vitamin D are important direct regulators of the iTreg/Th17 axis.

In addition to the direct effects of vitamin D on Th17 cells, there are also indirect effects of vitamin D and the VDR on Th17 cells in vivo. The bacterial microflora in the lumen of the intestine affects the development of Th17 cells. Intestinal Th17 cells are absent in germ-free mice (29). Segmented filamentous bacterium are critical for inducing Th17 cells (30).

Development of WT CD4+ T cells in the VDR KO host resulted in more IL-17-producing cells suggesting that expression of the VDR in the host is required for normal Th17 cell development. In vivo, one possibility is that vitamin D and the VDR affect the composition of the microbiota between WT and VDRKO mice that could result in more Th17 cells.

DC subsets have been shown to regulate the balance between pathogenic and protective T cells in the gut. Several subsets of DCs have been described to either increase or decrease the numbers of Th17 cells in the gut (31). Tolerogenic DCs have been suggested to provide RA and to tip the iTReg/Th17 axis in favor of tolerance. Data from humans and mouse models of IBD suggest pathogenic DCs that are CD103− play a role in the pathogenesis of the disease by inducing Th17 cell (31). Conversely, tolerogenic CD103+ DCs have been shown to play an important role in driving the development of Foxp3+ Treg and suppressing the development of inflammatory Th17 cells (32). Fewer tolerogenic DCs are present in the gut and MLN of VDR KO mice. The reduction in CD103+ DCs results in more Th17 cells in mice that don’t express the VDR. Host expression of the VDR induces a balanced Th cell response reducing the Th17 cells in favor of the iTreg cells.

Vitamin D and the VDR are critical regulators of the Th17/iTreg axis. Multiple steps in the development of these T cell responses require vitamin D. Expression of the VDR is critical for
induction of iTreg and suppression of Th17 cells. In the absence of the VDR Th17 cells are overproduced at the expense of iTreg. In vivo, expression of the VDR is critical for the development of tolerogenic DCs that indirectly affect the numbers of Th17 cells. Other indirect regulators of the Th17/iTreg axis might include the microbial flora, levels of RA or regulation of the vitamin A pathway by vitamin D. A convergence of pathways leads to higher amounts of Th17 cells, fewer iTreg, and more severe symptoms of IBD in the absence of the VDR.
REFERENCES


**Fig. 4-1**

A) Isotype for IL-17  
B) Isotype for Foxp3

**Fig. 4-1: Negative control staining for flow cytometry.** Sample dot plots from the analysis of IL-17 and Foxp3 in CD4⁺ T cells shown in figures 4-2, 4, 5, 6, and 8. The samples are gated as CD4 positive. A) IL-17 and B) Foxp3 isotype controls.
Fig. 4-2: **Increased production of IL-17 in the absence of the VDR.** The ability of WT and VDR KO CD4 T cells to differentiate into IL-17 producing T cells in vitro was examined. Treg cultures included TGF-β1 and Th17 cultures contained IL-6 and TGF-β1. A) Dot plot showing IL-17 positive CD4+ T cells. Data shown is one representative of 3 experiments. Numbers indicate percentage of IL-17-producing cells. B) IL-17A secretion in Th17 cultures of WT and VDRKO T cells. Values are the mean of 3 individual experiments ± SEM, *P<0.05. C) CD4+ T cells were differentiated under Th17-inducing conditions and mRNA was analyzed by qRT-PCR after 3 days of differentiation. Values are the mean of 3 experiments ± SEM, * P<0.05. D) Th17 induction by TGF-β1 plus IL-21 or IL-6 plus IL-1α. Numbers indicate percentage of IFN-γ or IL-17 cells in the cultures. Data shown is one representative of two experiments.
Fig. 4-3: Normal expression of RORγt and RORα. CD4+ T cells from WT and VDR KO mice were differentiated under Th17 condition for 72 hrs and examined for expression of RORγt and RORα mRNA by qRT-PCR. Values are the mean of 3 experiments ± SEM.
Fig. 4-4: Fewer iTreg cells that are refractory to IL-6 inhibition in the absence of the VDR. iTreg differentiation by WT and VDR KO CD4 T cells was examined after 72 hr in vitro culture under Treg (TGF-β) or Th17 (TGF-β and IL-6) conditions. A) Foxp3 expression analyzed by intracellular staining. B) IL-10 production analyzed by ELISA. Data shown are one representative of 3 individual experiments ± SEM. * P<0.05
Fig. 4-5: Vitamin D deficiency induces and 1,25D3 treatment inhibits Th17 cells. A.) CD4+ T from WT mice were stimulated in the presence of 25nM 1,25D3 and IL17A analyzed by intracellular staining. Data shown are 1 representative of 3 experiments (mean ± SEM). B) WT and 1,25D3 deficient CD4+ T were stimulated and IL-17A-producing cells were analyzed by intracellular staining. Data shown are 1 representative of 3 experiments (mean ± SEM). C) CD4+ T cells from WT and vitamin D deficient mice were stimulated and analyzed by intracellular staining for IL-17A secretion. Data shown are 1 representative of 3 experiments (mean ± SEM). *P<0.05, **P<0.001
Fig. 4-6: **WT T cells that develop in a VDR KO host following BM transplantation produce more IL-17 and induce more severe IBD.** CD4/CD45RB\textsuperscript{high} T cells of donor origin were isolated from VDR KO and WT BM chimeras. Groups were uninjected control Rag KO (Ctrl), and the following donor → host pairs from the BM transplants: WT-WT, VDR KO-WT (KO-WT), and WT-VDR KO (WT-KO). Donor derived CD4/CD45RB\textsuperscript{high} T cells were injected into Rag KO mice. A) IBD development in Rag KO recipients. B) CD4 T cells were evaluated for IFN-γ production and C) IL-17 production in either the IEL (upper) or spleen (lower) of the Rag KO recipients. Data are mean ± SEM with an n=4 for each group. * P<0.05 and ** P<0.01.
Fig 4-7: Expression of the VDR in the host protects against the pathogenicity of CD4/CD45RB<sup>high</sup> T cells. WT CD4/CD45RB<sup>high</sup> T cells were transferred to Rag KO and DKO recipients. A) The percent change in BW over time is plotted ± SEM for each group, n=4. B) Representative colonic sections of Rag KO (histopathology score of section shown = 4.0) and DKO (histopathology score of section shown = 6.5) recipients. C) Histopathology scores for the Rag KO and DKO recipients of naïve T cells, n=4 per group. D) DKO recipients have increased numbers of CD4<sup>+</sup> T cells in the IEL and spleen, n=4. Values are significantly different, *P<0.05.
Fig. 4-8

A. CD4^+CD62L^+ T cells

B. CD4^+CD25^+ T cells

C. IL-17

D. IL-17

E. CD103^+CD11c^+ cells

F. CD103^+CD11c^+ cells
Fig. 4-8: Increased Th17 and decreased tolerogenic DC in the absence of the VDR. The IEL and splenocytes of Rag KO and DKO recipients of WT CD4/CD45RB<sup>high</sup> T cells (Fig. 4-7) were characterized by flow cytometry. A) CD4/CD62L staining of the IEL of Rag KO and DKO recipients. B) CD4/CD25 staining of the IEL of Rag KO and DKO recipients. C) The percentage of IL-17 secreting T cells in the IEL of Rag KO and DKO recipients. D) The percentage of IL-17 secreting T cells in the spleen of Rag KO and DKO recipients. Values are mean ± SEM. The DC subsets from the IEL and MLN of normal WT and VDR KO or uninjected Rag KO and DKO mice were analyzed by flow cytometry E) Dot plots of the CD103 and CD11c positive cells in the MLN of WT and VDR KO mice. Values are the CD103/CD11c positive of n=4 mice per group ± SEM from 2 experiments. F) The frequency of CD103<sup>+</sup>CD11c<sup>+</sup> DCs in the MLN and IEL of Rag KO and DKO mice. Values are mean ± SEM and represent 2 experiments, n=4 mice per group.
Fig. 4-9: 

**Increased Th17 and decreased tolerogenic DC in the absence of the VDR.** The DC subsets from the IEL and MLN of normal WT and VDR KO or uninjected Rag KO and DKO mice were analyzed by flow cytometry. 

A) Total numbers of the CD103 and CD11c positive cells in the MLN of WT and VDR KO mice. Values are mean ± SEM from 2 experiments, n=4 mice per group. 

B) The total number of CD103+CD11c+ DCs in the IEL of Rag KO and DKO mice. Values are mean ± SEM and represent 2 experiments, n=4 mice per group.
Chapter 5

SUMMARY AND FUTURE DIRECTIONS
SUMMARY

In healthy intestine there is a balance between effector immune responses to pathogenic bacteria and tolerance to commensal flora residing in the lumen. When this delicate balance is lost unwanted inflammation can occur. The immune response must be able to combat invading pathogens, clear the infection and then respond to suppressive signals, returning to a state of tolerance. Crohn’s disease (CD) and ulcerative colitis (UC) are chronic diseases of the gastrointestinal tract in which tolerance is lost (1). The data presented here show that vitamin D and signaling through the vitamin D receptor (VDR) regulates the development and function of both inflammatory and tolerogenic T cells as well as plays a role in the host tissue and the innate immune system’s ability to regulate T cell responses in the intestine.

Genetic and environmental factors play a role in the pathogenesis of inflammatory bowel disease (IBD). Several studies have reported vitamin D deficiencies in adult and pediatric patients with IBD and deficiency is common even when the patient is in remission (2, 3). The deficiency observed in patients is probably due to malabsorption, low dietary intake and reduced sunlight exposure. Vitamin D is a transcriptional regulator that when bound to the VDR, targets genes with vitamin D response elements (VDRE). Through the use of genome wide association studies (GWAS) polymorphism in the VDR gene were shown to increase susceptibility to CD and UC (4, 5). Several IBD associated genes have VDREs (6-8). These findings suggest that vitamin D and VDR polymorphisms are environmental and genetic factors in the pathogenesis of IBD.

The bacteria in the lumen of the intestine are another environmental factor that influences the development and severity of IBD. IL-10 KO mice develop spontaneous IBD and the inflammation that develops in IL-10 KO mice, is due to an uncontrolled immune response to the commensal microflora in the intestine (9). Specific pathogen free (SPF) or antibiotic treated IL-
KO mice do not show outward signs of inflammation. However, when SPF IL-10 KO mice are VDR deficient severe fulminating IBD occurs even when the mice are antibiotic treated, but germ free IL-10 DKO mice show no signs of inflammation. The data indicate that in the absence of the VDR the immune response is hypersensitive to antibiotic resistant but otherwise non-pathogenic bacteria (Fig. 5-1).

The bacterial microflora in the lumen of the intestine also affects the development of Th17 and CD8αα T cells. Intestinal Th17 cells are absent and the number of CD8αα+ TCRαβ+ IEL is significantly reduced in germ-free mice (10, 11). Segmented filamentous bacterium are critical for inducing Th17 cells (12). Transferring WT CD4+ T cells to a VDR KO host resulted in more IL-17-producing cells. Vitamin D and the VDR are critical regulators of the Th17/iTreg axis. Multiple steps in the development of these T cells require vitamin D. Expression of the VDR is critical for induction of iTreg and suppression of Th17 cells. In the absence of the VDR, Th17 cells are overproduced at the expense of iTreg. Therefore it seems likely that in the absence of intestinal microflora IL-10 DKO mice do not develop disease because of the lack of Th17 cells but that in the presence of normally harmless commensal flora and an IEL compartment with reduced tolerogenic CD103+ DC increased inflammatory T cells develop causing the onset of disease.

CD8αα+ TCRαβ+ T cells can suppress inflammation by producing suppressive cytokines like IL-10 and TGF-β1 (13, 14). CD4+ CD8αα+ TCRαβ+ T cells suppress intestinal inflammation by producing IL-10 and TGF-β1 and may prevent the development of Th17 cells by producing IFN-γ, skewing Th0 cell differentiation toward Th1 upon activation (15). Even though IL-10 KO CD8αα T cell function would be limited because of their inability to produce IL-10, production of TGF-β1 could cause a shift in the Th17/Treg axis inducing increased development of Treg
cells in the gut protecting these mice from inflammation induced by the presence of SPF flora. However, due to the reduced number of CD4+ CD8αα+ TCRαβ+ T and CD8αα+ TCRαβ+ T cells when the mouse is VDR deficient this potential protection would be lost allowing severe inflammation to develop (Fig. 5-1).

The role of vitamin D in regulating the immune response to commensal SPF flora needs additional investigation. Evaluating the flora that exists in VDR or vitamin D deficient mice might provide useful information about the target flora that activates the immune response in deficient mice. Investigating the potential alterations of the gut flora may also provide insight into the development of specific immune cell subsets such as CD103+ DC, Th17 and CD8αα in deficient mice.

CD8αα+ TCRαβ+ T cells are self-reactive but not self-destructive (16). Unlike conventional T cells that undergo positive and negative selection in the thymus, CD8αα+ TCRαβ+ T cell precursors undergo agonist selection (17). The process of agonist selection allows T cells that express self-reactive TCR that would normally be deleted to survive (16). The surviving self-reactive T cells are reprogrammed to be regulatory in nature. Several T cell types express self-reactive αβ TCRs that are believed to have undergone agonist selection including: CD8αα+ TCRαβ+ T cells, CD4 FoxP3+ Tregs, iNKT cells and Th17 cells (17-20). VDR KO mice have reduced numbers of iNKT cells (21), reduced CD8αα+ TCRαβ+ T cells, normal CD4 FoxP3 Tregs and increased numbers of IL-17 producing T cells (Fig. 5-1). In addition, WT CD4 T cells that developed in a VDR KO host thymus after BM transplantation induced more severe disease that was mediated by Th17 cells. These data indicate that there is a defect in agonist selection in the thymus of VDR KO mice that potentially skews the self-reactive TCR repertoire away from the regulatory T cell types toward inflammatory Th17.
There are several similarities between the two T cell types, CD8αα^+ TCRαβ^+ T cells and iNKT cells, that are reduced in VDR KO mice. CD8αα^+ TCRαβ^+ T cells express the NK receptors CD94 and NKR-PI and use the invariant signaling component FceRIγ as a part of their CD3 complex (16, 18). NKT and CD8αα^+ TCRαβ^+ T cell development does not require MHC class I or II but does require expression of β2 microglobulin and the NF-κB family transcription factor RelB (16, 18, 22). Interestingly, RelB is transcriptionally regulated by vitamin D and has been shown to have VDREs in its promoter in both mice and humans (23). RelB deficiency is associated with autoimmunity that is believed to be linked to dysfunctional T cell selection in the thymus which allows the survival of conventional T cells that express high affinity receptors that would normally be deleted during the selection process or reprogrammed during agonist (24, 25). It is possible that under VDR deficient conditions in the thymus, RelB is depleted; and in a similar manner to RelB KO mice T cells with high affinity self-reactive TCRs are allowed to survive as inflammatory T cells, like Th17 cells. Additional research is required to investigate VDR regulation of RelB in the thymus and how that interaction may be effecting thymic development of Th17, NKT and CD8αα^+ TCRαβ^+ T cell precursors. An additional potential target of vitamin D that may alter the development of the thymus is the thymic epithelial cells. Maturation of the thymic epithelial cells is driven in part by signaling through the receptor activator of NFκB (RANK) during embryonic and postnatal development of the thymus (26). Expression of RANK ligand in osteoclasts is regulated by vitamin D and the VDR (27). An interesting series of experiments would be to investigate the regulation of RANK ligand by vitamin D in the thymus. Then to evaluate the maturity of thymic epithelial cells in vitamin D or VDR deficient mice and determine how these potential effects alter the development of Th17, NKT and CD8αα^+ TCRαβ^+ T cell precursors.
The data presented here suggest that vitamin D is important for the development of intestine specific cells of the immune system. Given the tendency that IBD patients are vitamin D deficient, it is likely that vitamin D supplementation could be a safe and effective addition to the therapies available to treat or prevent IBD. Unfortunately clinical interventions have not been done to look at the effects of vitamin D on IBD disease in humans. Based on the extensive data in animal models several studies have been proposed and registered at Clinicaltrials.gov using vitamin D or vitamin D analogs as treatments. Issues that need to be addressed include dose of vitamin D, whether it matters what other therapies a patient is on, can patients with either UC or CD benefit. Even in the absence of clinical data it seems reasonable to treat IBD patients with vitamin D as a preventative or maintenance treatment. 1,25(OH)₂D₃ or analogs of 1,25(OH)₂D₃ have been shown to be effective treatments of experimental IBD. It is possible that 1,25(OH)₂D₃ could suppress mild to moderate IBD and perhaps be a corticosteroid sparing drug. The benefits of vitamin D in IBD could be multiple including: reductions in inflammation and the decreased severity of the disease as well as improvements in bone mineral density (28). However, evaluation of vitamin D status has not been established as standard-of-care in patients with IBD. Even when vitamin D deficiency is detected there are no established guidelines for treatment in children or adult IBD patients (2). Additional research is required to establish dosage, duration and efficacy of treatment. More work is needed to determine whether vitamin D and 1,25(OH)₂D₃ could be new alternatives or additions to the arsenal of drugs used to combat IBD.
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Fig. 5-1: Vitamin D deficiency results in intestinal inflammation. Vitamin D deficiency leads to increased DC activation but decreased numbers of tolerogenic CD103+ DC, antigen presentation and cytokine production. Th17 and Th1 cells overproduce IL-17 and IFN-γ with few regulatory cells to shut off the response. Few iNKT cells and CD8αα IEL result in the inability to turn off the Th17 and Th1 cells in the gastrointestinal tract and for IBD to develop.
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Publications


Presentations

• Bruce D and Cantorna MT. Vitamin D is required for the development of CD8αα+ TCRαβ+ intestinal intraepithelial lymphocytes. The 96th annual meeting of the American Association of Immunologists. Seattle,WA., 2009 *Oral presentation and Poster
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