THE ROLE OF VITAMIN D IN MAINTAINING GASTROINTESTINAL HOMEOSTASIS

A Dissertation in
Pathobiology

by

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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

May 2014
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ABSTRACT

The causes of inflammatory bowel disease (IBD) are complex. Besides the effect of genetic factors, environmental factors have been shown to affect the development of IBD. Vitamin D and the vitamin D receptor (VDR) have been shown to play an important role in regulating the immune system in the intestine and the mice deficient in vitamin D or the VDR are more susceptible to different animal models of IBD. I used two mouse models of IBD, one is T cell-transfer model and the other one is Citrobacter rodentium-induced colitis, to explore the role of vitamin D and the VDR in the regulation of mucosal immunity and IBD pathogenesis. The first objective was to determine the effects of vitamin D on CD8αβ+ T cell subsets in experimental IBD. My study showed that co-transfer of VDR knock out (KO) CD8+ T cells with naïve wild-type (WT) CD4+ cells accelerated the development of colitis in recombinant activation gene (Rag) KO recipients. The VDR KO CD8+ T cell pathogenicity resulted from uncontrolled proliferation of CD8+ T cells in general and naïve CD8+ T cells in particular. In addition, VDR KO CD8+ T cells produced more IL-2 than WT CD8+ T cells. My data suggest that the absence of the VDR resulted in the generation of pathogenic CD8+ T cells that contributed to the development of IBD.

The second objective was to determine how the VDR regulates host resistance to C. rodentium and the development of inflammation in the gut. My study showed that VDR KO mice were resistant to C. rodentium infection. Increased expression of RegIIIγ, Ang-4 and Muc2 contributed to the colonization resistance of VDR KO mice. Disrupting the gut microbiota with antibiotics or the absence of acquired immunity in T and B cell double Rag/VDR KO mice eliminated C. rodentium colonization resistance. The results suggest that the VDR plays a role in regulating the gut microbiota and C. rodentium colonization.
Lastly, I used the *C. rodentium* model in vitamin D-sufficient (D+) and deficient (D-) WT or 1,25(OH)\(_2\)D\(_3\) deficient (Cyp27B1, Cyp) KO mice. D+ WT and D+ Cyp KO mice cleared a primary infection with identical kinetics indicating that the ability to produce 1,25(OH)\(_2\)D\(_3\) is not required for clearance of *C. rodentium*. D- WT mice cleared a primary infection more slowly than D+ mice. D- Cyp KO mice developed a severe infection that spread systemically and resulted in the premature lethality of 40% of the mice. The increased susceptibility of the D- Cyp KO mice was associated with increased Ifn-γ and Il-17A in the colon compared to the D- WT and the D+ mice at d14 post-infection. Decreased levels of antibody, CD4+ T cells, IgA+ plasma cells and IL-22-producing innate lymphoid cells (ILC) in D- Cyp KO mice correlated with the increased susceptibility of these mice to *C. rodentium* infection. Although the response of the D+ WT and D+ Cyp KO mice to primary *C. rodentium* infection could not be differentiated, D+ Cyp KO mice cleared a secondary infection with *C. rodentium* more slowly than D+ WT. Overall this study shows that vitamin D and 1,25(OH)\(_2\)D\(_3\) regulate susceptibility and resistance to *C. rodentium* infection. Together, the work presented in this thesis shows the critical role of vitamin D and the VDR in gut homeostasis and host susceptibility to *C. rodentium* infection through the regulation of gut microbiota and host immunity.
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ACKNOWLEDGEMENTS

First I would like to thank my advisor Dr. Margherita Cantorna for her guidance, help and patience during my graduate studies here. It is she who inspires me that science can be fun. She made my life as a graduate student interesting and enjoyable. It has been my great pleasure working in her lab. I am grateful for my committee members: Dr. Na Xiong, Dr. Pamela Hankey, Dr. K. Sandeep Prabhu and Dr. Yu Zhang for giving me valuable suggestions, their encouragement and sharing their knowledge.

I would like to thank my colleagues, Veronika Weaver and Rhonda Smith for their support and assistance of experiments. I am very thankful for the friendship and advice of every past and current lab members. They make the lab a wonderful and comfortable place to work. I would also like to thank all of my fellow graduates in Pathobiology program for the many helpful suggestions.

Last but not least, I would like to give a special thank to my family for their endless love and unconditional support. I would like to also thank my friends for their company and encouragement.
Chapter 1

Introduction
Inflammatory bowel disease (IBD)

IBD is a chronic intestinal inflammation. There are almost 2 million people in the United States and 0.4% people in the world suffering from IBD (1, 2). There are two major forms of IBD, Crohn’s disease and ulcerative colitis (1, 2). The symptoms of IBD include diarrhea, abdominal pain, and rectal bleeding. Patients with IBD are more likely to develop colonic cancer, especially if they are not effectively treated for IBD (3). The major drugs used in the clinic are anti-inflammatory, immune-suppressants and antibiotics (4). However, these treatments have many side effects. Taking immunosuppressive drugs over long periods of time causes patients to be vulnerable to infection and cancer. Therefore, additional therapies for IBD are needed.

The etiology of IBD

IBD are immune-mediated diseases that result because of inappropriate T cell responses to the normal bacterial flora in the gut. The IBD are a complex and heterogeneous family of diseases that involve both genetic and environmental factors. There is a strong genetic component to IBD since, siblings of IBD patients have 30 times higher risks of developing IBD and the relatives of patients with IBD are more likely to develop IBD (5). Genes including NOD2 and ATG16 have been identified as risk factors for IBD development (6). Therefore, genetic factors play an important role in causing IBD.

Besides genetic factors, environmental factors also affect the development of IBD. There are studies showing that diet and sunlight exposure may be associated with IBD (7, 8). For instance, IBD is more prevalent in westernized countries and the occurrence of IBD increased in people who migrate to western countries (8, 9). In addition, IBD is more likely to occur in communities to the far north and south of the equator, which is possibly due to the reduced sunlight exposure
especially in the winter (7). Environmental factors impact the development of IBD. Vitamin D and the gut microbiota are two environmental factors that may play a role in IBD. Vitamin D deficiency is common in patients with IBD even when they are in recovery (10, 11), and in a few studies vitamin D supplementation improved the symptoms of IBD (12-14). In mice that spontaneously develop IBD, vitamin D deficiency promotes intestinal inflammation and disease development (15, 16). These findings indicate the critical role of vitamin D in affecting the development of IBD. In addition, gut microbiota also affect IBD pathogenesis. Changes in the gut microbiota are common in patients with IBD and disrupting the gut microbiota by antibiotics or probiotics is beneficial in some IBD patients (17). However, the mechanisms by which environmental factors affect IBD susceptibility have not been extensively studied.

**IBD and gut microbiota**

The composition of the gut microbiota is a critical environmental factor affecting the development of IBD. The intestinal inflammation in IBD patients may come from the overreaction to normal harmful bacteria or from the overgrowth of specific pathogenic bacteria (18-20). Patients with IBD had more pathogenic bacteria, and less beneficial bacteria in the intestine compared to the healthy controls (18, 19). These changes in gut microbiota resulted in chronic intestinal inflammation (18, 19). Alterations in the composition of the gut microbiota contributed to the predisposition to IBD. For example, treating IBD patients with antibiotics and administration of probiotics reduced the symptoms of disease (21-23). Germ-free mice had more severe DSS-induced colitis compared to the conventional mice due to the lack of commensal bacteria in the gastrointestinal tract (24). In addition, the IBD-associated gene NOD2 is a receptor that recognizes and responds to the microbiota (25). NOD2-knockout mice had alterations in the commensal bacteria and developed more severe experimental colitis (26, 27). The intestinal
microbiota is a critical environmental factor that influences the development of experimental IBD.

**Experimental models of IBD**

Experimental models of IBD have been critical for identifying new mechanisms and therapeutic targets for IBD. Dextran sulfate sodium (DSS) induced colitis results from chemical injury of the gastrointestinal tract (28, 29). DSS is toxic to intestinal epithelial cells and following injury the innate immune system heals the injury (28-30). Several T cell mediated IBD models exist. IL-10 KO mice spontaneously develop a chronic IBD resulting from defective T regulatory cells (29, 31). IL-10 KO mice show an enhanced Th1 and Th17 responses to the gut microbiota because of the lack of IL-10 production and regulatory T cells (29). Naïve CD4+ T cell transferred to immunodeficient T and B cell KO (Rag KO) mice develops IBD because of the rapid proliferation and development of Th17 cells in the gut (29). Infection of mice with *Citrobacter rodentium*, a murine model pathogen for human enteropathogenic *E.coli* infection, can also induce IBD-like disease at the peak of infection (32, 33). Host resistance to *C. rodentium* involves good innate and adaptive immunity (32, 33). For *C. rodentium* infection the host needs to balance the elimination of the bacteria with control of the gut inflammation to prevent development of IBD. Although the animal models do not completely mimic human IBD, they allow us to study the mechanisms of intestinal inflammation and potential therapies for the disease.

**Vitamin D and its metabolism**

Vitamin D is a fat-soluble vitamin that is critical for regulating calcium and phosphate homeostasis (34, 35). Vitamin D is inactive and converted to the active form of vitamin D, 1α,25-
dihydroxyvitamin D3 (1,25(OH)2D3), by the enzyme 1α-hydroxylase Cyp27B1 (34, 36).

1,25(OH)2D3 binds to the VDR to regulate the transcription of target genes (36). There are two forms of vitamin D: vitamin D3, found in humans and animals; and vitamin D2, found in plants (34). Vitamin D3 can be produced from 7-dehydrocholesterol in the skin following UV light exposure (37). The diet also provides vitamin D2 and D3. Vitamin D2 and D3 are converted to 25(OH)D2 and 25(OH)D3 by enzymes produced in the liver (36). The evidence suggests that both 1,25(OH)2D2 and 1,25(OH)2D3 bind with equal affinity to the VDR (34). Fig. 1-1 depicts the pathway of vitamin D metabolism.

Vitamin D produced either in the skin or ingested from diet binds to vitamin D binding proteins and is transported into the liver where it is converted to 25 hydroxyvitamin D3 (25(OH)D3) by 25-hydroxylase, including CYP27A1, CYP2R1, CYP3A4 and CYP2J3 (35, 38). 25(OH)D3 is the major circulating form of vitamin D and is stable and is thus commonly used to determine the vitamin D status in humans (36). 25(OH)D3 then enters the kidney where it is hydroxylated again to 1,25(OH)2D3 by the enzyme 1α-hydroxylase encoded by the CYP27B1 gene (36). In addition to the kidney, CYP27B1 has been shown to be expressed in the skin, bone, colon, parathyroid gland and immune cells (39). 25(OH)D3 and 1,25(OH)2D3 can be hydroxylated further to be inactivated by the 25(OH)-24-hydroxylase CYP24A1 (36).

Vitamin D is quickly converted to 25(OH)D3 and is stable for several weeks (36). Conversely, synthesis of 1,25(OH)2D3 is tightly regulated by several factors including 1,25(OH)2D3, the parathyroid hormone and calcium (40, 41). 1,25(OH)2D3 regulates bone metabolism and mineral homeostasis, and promotes calcium absorption in the intestine (42-44). In turn, calcium affects the activity of several vitamin D hydroxylases including the Cyp27B1 hydroxylase and the level of 1,25(OH)2D3 (40, 41). Cyp27B1 is induced by the hormone parathyroid hormone and down-
regulated by 1,25(OH)$_2$D$_3$ (45). 1,25(OH)$_2$D$_3$ regulates calcium and in turn calcium affects the synthesis of 1,25(OH)$_2$D$_3$.

**The VDR and 1,25(OH)$_2$D$_3$-VDR signaling**

The VDR is a member of a superfamily of nuclear receptors (46). The VDR, retinoic acid, thyroid hormone, and peroxisome proliferator activator receptors show the highest similarity among this superfamily (47). The expression level of the VDR is regulated by the parathyroid hormone, retinoic acid and the glucocorticoids (48). Most importantly, 1,25(OH)$_2$D$_3$ is able to increase the expression of the VDR gene (48). 1,25(OH)$_2$D$_3$ binds to the VDR to form a heterodimer with the retinoid X receptor (RXR) (49). The liganded VDR/RXR heterodimer complex then binds to vitamin D response elements (VDRE) which are present in the promoter region of target genes (50).

The mechanism by which 1,25(OH)$_2$D$_3$ regulates the transcription of target genes is well described. First, the binding of 1,25(OH)$_2$D$_3$ to the VDR releases co-repressors while promoting the binding to the RXR and co-activators (51). The altered VDR then binds to the 3’ segment of the VDRE and RXR binds the 5’ segment together with several other proteins such as CBP/p300, pCAE and SRCs that have histone acetylase activity, resulting in the alteration of the chromatin structure (52). Second, some of the ancillary proteins leave and other proteins such as RNA polymerase II bind the receptor complex to start transcription of target genes (52). A bending in the DNA and phosphorylation is associated with RNA polymerase II binding (53), which finally causes stimulation/suppression of gene transcription by vitamin D. In sum, 1,25(OH)$_2$D$_3$ exerts its regulatory function by binding to the VDR expressed on the target gene.
Vitamin D and immune system

Vitamin D and 1,25(OH)$_2$D$_3$ have been shown to play an important role in autoimmune disease via effects on both innate and adaptive immune responses. Many cell types in the immune system express the VDR, including dendritic cells (DC), macrophages, T cells and B cells, suggesting an immune-regulatory role of vitamin D in inflammatory disease.

1,25(OH)$_2$D$_3$ inhibits DC differentiation and maturation in vitro (54-56). VDR KO mice have reduced numbers of mature DCs in skin-draining lymph nodes (56). 1,25(OH)$_2$D$_3$ reduced the production of IL-12 and increased the synthesis of IL-10 by DCs (57, 58). In addition, these DCs were capable of inducing IL-10-producing CD4+CD25+ regulatory T cells (58). The mechanism by which 1,25(OH)$_2$D$_3$ inhibits IL-12 is by interfering with the nuclear factor NF-κB pathway (58). 1,25(OH)$_2$D$_3$ affects both activation of the NF-κB pathway and binding directly to the IL-12p40 promoter (58, 59). Therefore, 1,25(OH)$_2$D$_3$ exerts an inhibitory role in regulating the development and function of DCs.

1,25(OH)$_2$D$_3$ also exerts effects on monocytes and macrophages. 1,25(OH)$_2$D$_3$ stimulated human monocyte proliferation in vitro (60). Induction of proliferation by 1,25(OH)$_2$D$_3$ was mediated by activation of mitogen-activated protein kinase signaling and by up-regulated expression of C/EBPβ expression (61, 62). In addition, 1,25(OH)$_2$D$_3$ increased the secretion of the bacterial peptide cathelicidin by monocytes and macrophages (63, 64). 1,25(OH)$_2$D$_3$ improved phagocytic and oxidative burst ability as well (63, 64). In addition, 1,25(OH)$_2$D$_3$ inhibited the production of IL-12 and TNF-α in macrophages (65), and VDR KO mice produced more IL-12, TNF-α and IL-1β (66). During differentiation from monocytes to macrophages, cells may acquire the ability to synthesize more 1,25(OH)$_2$D$_3$ (59). Therefore, 1,25(OH)$_2$D$_3$ inhibits inflammatory cytokine production (IL-12, TNF-α and IL-1β) in monocytes and macrophages while promoting the secretion of antimicrobial peptides.
1,25(OH)$_2$D$_3$ inhibits adaptive immune cells *in vitro*, including T and B cells. 1,25(OH)$_2$D$_3$ treatment of T cells decreased their activation and proliferation and altered cytokine expression profiles (67-70). 1,25(OH)$_2$D$_3$ also inhibited CD8+ T cell-mediated cytotoxicity (71). IFN-γ and IL-2 production was decreased by 1,25(OH)$_2$D$_3$ in CD4+T cells (72-74). 1,25(OH)$_2$D$_3$ regulation resulted from binding of VDR/RXR to a silencer region in the IFN-γ promoter and reduced IL-2 production was a result of delayed progression of transcription (73). The IL-2 promoter has a positive regulatory NFAT1 site where a complex containing T cell-specific transcription factors NFATp and AP1 can bind (75). 1,25(OH)$_2$D$_3$ inhibited IL-2 transcription by suppressing the formation of 1,25(OH)$_2$D$_3$/VDR/RXR with NFAT1/AP1. The inhibitory effects of 1,25(OH)$_2$D$_3$ on T cells resulted in part due to the reduced production of IL-2 (68). The major effects of 1,25(OH)$_2$D$_3$ on T cells were observed on memory T cells and it is consistent with the higher expression of the VDR in memory T cells compared with naïve T cells (76, 77). In addition, 1,25(OH)$_2$D$_3$ increased non-specific T cell suppressor activity as 1,25(OH)$_2$D$_3$ treated T cells had increased ability to inhibit primary mixed-lymphocyte reactions and cytotoxic T cell response (71). In contrast, 1,25(OH)$_2$D$_3$ promoted the development of Foxp3+ regulatory T cells and IL-10-producing T regulatory cells (78). In addition, 1,25(OH)$_2$D$_3$ promoted the development and function of regulatory iNKT cells and CD8αα T cells and these two subsets were reduced in VDR KO mice (79, 80). Together, 1,25(OH)$_2$D$_3$ is an immune-regulator that suppressed Th1 and Th17 cell responses and enhanced regulatory T cells and IL-10 production.

In addition to its inhibitory role on T cells, 1,25(OH)$_2$D$_3$ has been shown to induce apoptosis of activated B cells, inhibiting their ongoing proliferation without affecting their initial cell division (81). Although 1,25(OH)$_2$D$_3$ had modest effects on up-regulation of genes involved in B cell differentiation, it significantly inhibited generation of plasma cells and memory B cell (67, 81). It is suggested that the inhibitory role of 1,25(OH)$_2$D$_3$ in B cells is by up-regulation of p27 expression, a gene that controls cell cycle progression at G1 (81). There are conflicting reports on
the expression of the VDR by B cells (76, 81), it is therefore possible that the effects of 1,25(OH)_{2}D_{3} on B cells could be indirect effects on the B cells (82). Overall, 1,25(OH)_{2}D_{3} has an inhibitory effects on B cells.

**Vitamin D and IBD**

Vitamin D status is low in patients with IBD (10). It has been shown that vitamin D or VDR deficient mice developed more severe symptoms of autoimmunity than control mice in several different experimental animal models (IBD, multiple sclerosis, and type-1 diabetes, (83-85)). Supplementation with vitamin D or 1,25(OH)_{2}D_{3} to experimental animals attenuated the symptoms of disease (83-85). Autoimmune diseases including IBD are charachterized by pro-inflammatory Th1 and Th17 cells that produce IFN-γ and IL-17 respectively (72, 83). IL-10 plays an anti-inflammatory role in autoimmunity and IL-10 KO mice are able to develop IBD spontaneously (86). IL-10/VDR double KO mice have been shown to develop a fulminating form of colitis, which leads to the mortality of the mice by 8 weeks of age (16). In the T cell-transfer model of IBD, VDR KO derived CD4+CD45RB\textsuperscript{high} T cells induced more severe colitis than WT CD4+CD45RB\textsuperscript{high} T cells when transferred to Rag KO mice (16). Overall, these studies demonstrate that vitamin D deficiency correlates with the increased severity of IBD and 1,25(OH)_{2}D_{3} treatment suppressed the development of IBD where Th1 and Th17 cells were pathogenic.

This thesis probes the role of vitamin D and the VDR in the development of mucosal immune cells and experimental IBD. The first objective was to evaluate the effect of vitamin D/VDR on development and function of CD8+ T cell subsets that contribute to IBD (Chapter 2). The second objective was to determine the effect of the VDR on host resistant to *C. rodentium* infection and
the gastrointestinal microbiota (Chapter 3). Finally in Chapter 4, the role of vitamin D and 1,25(OH)$_2$D$_3$ on host immune system and resistance to primary and secondary infection with C. rodentium was determined.
Figure 1-1. Overview of vitamin D metabolism.

Vitamin D is produced from 7-dehydrocholesterol when exposed to sunlight, while a small amount is from the diet. To become biologically active, two hydroxylation steps are necessary. The first hydroxylation take place in the liver and the second in the kidney producing 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ respectively.

Adapted from Deeb, K.K. et al, Nat Rev Cancer. 2007 Sep; 7(9): 684-700.
Figure 1-2. Vitamin D effects on the immune system.

Systemic or locally produced 1,25(OH)₂D₃ exerts its effects on several immune-cell types, including macrophages, dendritic cells (DCs), T and B cells. The VDR expression is up-regulated following activation. The effects of 1,25(OH)₂D₃ on the immune cells can be inhibitory and stimulatory depending on different cell types. 1,25(OH)₂D₃ induced production of IL-1 and cathelicidin (an antimicrobial peptide) by macrophage, therefore contributing to innate immune responses to some bacteria. 1,25(OH)₂D₃ inhibited DC differentiation, maturation, inhibited the expression of MHC II molecules, CD40, CD80 and CD86. It inhibited IL-12 production and induced IL-10. 1,25(OH)₂D₃ decreased the production of IL-2, IL-17 and IFN-γ and inhibited the proliferation of T cells. 1,25(OH)₂D₃ might also promote the development of FoxP3+ regulatory T cells. 1,25(OH)₂D₃ inhibited B cell proliferation, plasma-cell differentiation and antibody production.

References


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Chapter 2

Vitamin D receptor expression controls proliferation of naïve CD8+ T cells and development of CD8 mediated gastrointestinal inflammation

Chapter adapted from the manuscript entitled:

“Vitamin D receptor expression controls proliferation of naïve CD8+ T cells and development of CD8 mediated gastrointestinal inflammation.”

Authors: Jing Chen, Danny Bruce, Margherita T. Cantorna

The data represented in Figure 2-1A, B are the contributions of Danny Bruce.
Abstract

Vitamin D receptor (VDR) deficiency contributes to the development of experimental inflammatory bowel disease (IBD) in several different models. T cells have been shown to express the VDR, and T cells are targets of vitamin D. In this article we determined the effects of VDR expression on CD8\(^+\) T cells. VDR KO CD8\(^+\) T cells, but not WT CD8\(^+\) T cells, induced colitis in Rag KO recipients. In addition, co-transfer of VDR KO CD8\(^+\) T cells with naïve CD4\(^+\) T cells accelerated colitis development. The more severe colitis was associated with rapidly proliferating naïve VDR KO CD8\(^+\) T cells and increased IFN-γ and IL-17 in the gut. VDR KO CD8\(^+\) T cells proliferated in vitro without antigen stimulation and did not downregulate CD62L and upregulate CD44 markers following proliferation that normally occurred in WT CD8\(^+\) T cells. The increased proliferation of VDR KO CD8\(^+\) cells was due in part to the higher production and response of the VDR KO cells to IL-2. Our data indicate that expression of the VDR is required to prevent replication of quiescent CD8\(^+\) T cells. The inability to signal through the VDR results in the generation of pathogenic CD8\(^+\) T cells from rapidly proliferating cells that contribute to the development of IBD.

Introduction

Inflammatory bowel diseases (IBD) are immune mediated diseases that result because of a complex interaction between genetics and the environment. Over 3.6 million people in the US and Europe suffer from IBD. Environmental factors that contribute to IBD development include the composition of the normal bacterial flora (1) and perhaps vitamin D. A major source of vitamin D results from its manufacture via a photolysis reaction in the skin and vitamin D available from sunlight exposure is significantly less in northern climates, and especially low
during the winter (2). In addition dietary intake of vitamin D is problematic since there are few foods, which are naturally rich in vitamin D. There is evidence for a link between vitamin D availability and the prevalence of immune mediated diseases in general and IBD in particular (3, 4). In addition, experimental models of IBD are more severe in vitamin D receptor (VDR) KO and vitamin D deficient models (5). Vitamin D is likely an environmental factor that affects the development of IBD.

T cells have been shown to express the VDR, and T cells are both direct and indirect targets of vitamin D. In addition, the active form of vitamin D (1,25(OH)₂D₃) suppresses the development of experimental models of T cell mediated diseases including IBD, multiple sclerosis and type-1 diabetes (6, 7). Conversely, vitamin D deficiency and/or VDR deficiency results in an exacerbation of experimental IBD (5). Expression of the VDR does not affect the development of normal numbers of CD4 and CD8αβ T cells in the thymus or in the periphery (5, 8, 9). VDR KO CD4⁺ T cells express more IL-17, and IFN-γ, proliferate more rapidly in a mixed lymphocyte reaction and induce more severe colitis than WT CD4 cells (5, 10). Mice with VDR specific KO in T cells have increased incidence of experimental autoimmune encephalomyelitis and VDR expression in T cells was shown to be essential for the effectiveness of 1,25(OH)₂D₃ for the suppression of disease (11). 1,25(OH)₂D₃ in vitro suppressed the proliferation of both CD4⁺ and CD8⁺ T cells and inhibited the production of IFN-γ, and IL-2 (12, 13). Vitamin D is required for the development of two regulatory cell populations: NKT cells and CD8αα expressing T cells (9, 14). In addition, 1,25(OH)₂D₃ induces CD4⁺ T regulatory cells in vitro and in vivo (15, 16).

Resting and activated CD8⁺ T cells have been shown to express higher levels of the VDR than CD4⁺ T cells; however, the physiological role of vitamin D and the VDR in regulating CD8⁺ T cell function has not been examined (17).

CD8⁺ T cells can be pathogenic, protective or tolerogenic. Pathogenic and protective CD8⁺ T cells share common features including IFN-γ and TNF-α production as well as cytotoxicity (18,
A pathogenic role for CD8+ T cells has been shown for IBD (18). Heat shock protein-specific CD8+ T cells transferred severe symptoms of IBD in immunodeficient mice due to the production of IFN-γ and TNF-α (18). Conversely, several CD8+ T cell subsets have been shown to act in a suppressive or regulatory manner. CD8+ T cells that do not express CD28 (CD8/CD28−), but produce IL-10 in human peripheral blood, suppressed cytotoxic activity and proliferation in vitro (20). In mice, IL-10 and TGF-β producing CD8/CD28− regulatory T cells inhibited experimental IBD development in the T cell transfer model of IBD (21). Human CD8/CXCR3+ cells are a second population of human regulatory CD8+ T cells (22). Like the CD8/CD28− T cells, the mouse homologs of the human CD8/CXCR3+ T cells (CD8/CD122+) suppressed the development of T cell-transfer colitis via the production of IL-10 (23). Lastly, CD8αα+TCRαβ+ cells found in the intraepithelial lymphocytes (IEL) of the small intestine (SI) have IL-10 mediated regulatory abilities and can suppress development of T cell-transfer colitis (24, 25). In experimental IBD CD8+ T cells can be either cause or protect from disease development.

Here we determined the effects of vitamin D on CD8αβ+ T cells. VDR KO mice had increased numbers of naïve CD8+ T cells that when purified and then transferred to Rag KO recipients induced colitis as determined by histological staining. In addition, co-transfer of VDR KO CD8+ T cells with naïve WT CD4+ cells accelerated the development of colitis in Rag KO recipients. The cause of the VDR KO CD8+ T cell pathogenicity was due to the uncontrolled proliferation of CD8+ T cells in general and naïve CD8+ T cells in particular. In addition, VDR KO CD8+ T cells overproduced IL-2. The rapidly dividing VDR KO CD8+ T cells accumulated in the gut where they induced IFN-γ and IL-17 producing cells.

Methods and Materials

Mice
WT, VDR KO, IL-10 KO, IL-10/VDR double (D) KO and Rag KO mice all on the C57BL/6 background were bred in the animal facilities at the Pennsylvania State University (University Park, PA). The original VDR KO breeders were a gift from Dr. Marie Demay (Harvard University, Boston MA) and the DKO mice were generated as previously described (5). All other breeders were originally from Jackson Laboratories (Bar Harbor, ME). Experimental procedures were approved by the Office of Research Protection Institutional Animal Care and Use Committee at the Pennsylvania State University.

**T cell isolation**

CD4⁺ or CD8⁺ T cells were purified from the spleen using the mouse CD4⁺ or CD8⁺ Recovery Column kit and the manufacturer’s instructions (Cedarlane, Burlington, NC). Column purification was followed by cell sorting (Cytome Influx, Seattle, WA) of CD4⁺, CD8⁺, CD4⁺CD45RB⁹⁹⁺, CD8⁺CD28⁻/⁺, CD8⁺CD122⁺ T cell subsets. Post-sorting confirmed that the purity of the T cells was >99% (Fig. 2-7). IELs were isolated for analyses by removing the Peyer's patches and splitting the SI lengthwise followed by cutting the SI into 0.5 cm pieces. The pieces were incubated twice in HBSS containing 0.15μg/ml dithiothreitol and 5%FBS (Sigma-Aldrich, St. Louis, MO) for 20 min at 37°C under 200 rpm rotation. The IEL were collected from the interface of 40/80% Percoll gradients (Sigma-Aldrich).

**Adoptive Transfer**

All transfers used T cells from the spleens of the donor mice. Groups of Rag KO mice were injected intraperitoneally (i.p.) with 1x10⁶ CD8⁺ cells or CD8⁺ T cell sorted subsets (CD28⁻, CD28⁺ or CD122⁺) from WT, VDR KO, IL-10 KO or IL-10/VDR DKO mice. Sorting purity and gating strategies are in Fig. 2-7. For the co-transfer experiments, Rag KO mice were injected i.p. with 1x10⁶ WT or VDR KO (CD45.2⁺) CD8⁺ T cells on day -1 and 4x10⁵ WT (CD45.1⁺) CD4⁺CD45RB⁹⁹⁺ cells on day 0. Both CD8 and CD4⁺CD45RB⁹⁹⁺ cells were resuspended in 200 μL PBS. The Rag KO recipients were weighed weekly and euthanized after 7 or 8 wks.
Colitis development

Colitis symptoms measured included: weight loss, colon/BW ratios, histopathology scores, diarrhea, rectal bleeding, and rectal prolapse exactly as described (5). 1 cm of the distal colon was fixed in formalin and sent to the Penn State University Animal Diagnostic Laboratories (University Park, PA) for sectioning and haematoxylin & eosin (H&E) staining. Inflammation and epithelial injury of the colons were scored blindly by two individuals for inflammation (0-4) and epithelial hyperplasia (0-4) (5). Total histopathology scores ranged from 0-8.

BrdU incorporation Assay

Rag KO mice were injected i.p. with 50μl of 25mg/ml BrdU dissolved in PBS every two days. The mice were euthanized at d3, d8 and d14 and the MLN and IEL were fixed with ice-cold 95% ethanol and paraformaldehyde digested with DNaseI solution (Sigma).

Flow Cytometry

Cells were stained with: FITC anti-CD8α, FITC anti-CD8β, FITC anti-CD45RB, PE anti-CD45.1, PE anti-CD28, PE anti-CD122, PE anti-CD4, PE anti-CD44, PE anti-CD25, PE-Texas Red (ECD) anti-CD4, PECy5 anti-CD62L, PECy5 anti-TCRβ and PECy7 anti-CD8α (BD Pharmingen, San Jose, CA) for surface markers. Gating strategies for FACS analysis are shown in Fig. 2-8. For BrdU incorporation assays, cells were stained with Biotin anti-BrdU, FITC streptavidin and Biotin Mouse IgG1, κ isotype control (Biolegend, San Diego, CA). For intracellular cytokine staining, IEL were stimulated with PMA (0.1μg/ml, Sigma), ionomycin (0.5μg/ml, Sigma) and Brefeldin A (10μg/ml, Sigma) for 6h, fixed with 4% paraformaldehyde (Sigma-Aldrich), permeabilized with 0.1% saponin (Sigma-Aldrich), and stained with FITC anti-IFNγ, PE anti-IL-17A, or the FITC/PE labeled Rat IgG1 isotype controls (BD Pharmingen). Flow cytometry was done on a FC500 bench top cytometer (Beckman Coulter, Brea, CA) and the data was analyzed with FlowJo 7.6.5 software (TreeStar, Ashland, OR).

Cell culture and CFSE staining
Purified CD8⁺ T cells were labeled with CFSE using Cell TRave CFSE Cell Proliferation Kit (Life Technologies, Grand Island, NY). The labeled CD8⁺ T cells (2x10⁶ cells/ml) were cultured with plate bound anti-CD3 (5μg/ml) and anti-CD28 (5μg/ml, BD Pharmingen) or 0.2μg/ml recombinant IL-2 with/without neutralizing IL-2 antibodies (50ng/ml, Clone: S4B6, BD Pharmingen). Supernatants were collected from 3d cultures of unstimulated or CD3/CD28 stimulated CD8⁺ T cells, and IL-2 in the supernatant was evaluated using an ELISA kit and the manufacturer’s instructions (BD Pharmingen).

**Quantitative real-time PCR**

Total RNA was isolated from small intestine and colon of Rag KO mice recipients of CD4+WTCD8 or CD4+KOCD8 following the manufacturer’s instructions (Qiagen, Valencia, CA). cDNA was synthesized by using the TaqMan reverse transcription reagents kit (Applied Biosystems, Carlsbad, CA) and was amplified for cytokines *Ifn-γ*, *Il-17A* and *Il-10* with SYBR green mix (BioRad, Hercules, CA) by MyiQ Single-Color Real-Time PCR machine (BioRad).

Expression levels of these cytokines were normalized by GAPDH and calculated by using ΔΔCt method. The primer sequences are *Ifn-γ* (forward 5’-TGCATCTTGCTTTGCAGCTCTCTTCTCATGGC-3’; reverse 5’-TGGACCTGTTGGTTGACCTCAAACCTTGGC-3’); *Il-17A* (forward 5’-CAGGGAGAGCTTACATCTGTGT-3’; reverse 5’-GCTGAGCTTTGAGGGATGAT-3’); *Il-10* (forward 5’-CAGAGCCACATGCTCCTAGA-3’; reverse 5’-TGTCAGCTTGGTCTTTGTT-3’)

**Statistics**

Statistical analyses were performed by GraphPad (PRISM software, La Jolla, CA). Data are presented as mean ± SEM values from two or three experiments or one representative of two or three individual experiments. Unpaired two-tailed Student’s t test, one-way ANOVA with Bonferroni post-hoc tests, and two-way ANOVA with Bonferroni post-hoc tests were used to calculate statistical significance via GraphPad Prism software. Values are significantly different
with $P$-values of $*P<0.05$, $**P<0.01$, and $***P<0.001$. Error bar represent standard error of the mean.

**Results**

**CD8$^+$ T cells from VDR KO mice transfer IBD to Rag KO recipients**

CD8$^+$ T cells from WT and IL-10 KO mice did not induce colitis when transferred to Rag KO recipients and histopathology of the colons from Rag KO mice that received $10^6$ WT or IL-10 KO CD8$^+$ T cells were normal (Fig.2-1A). Conversely, Rag KO mice that received $10^6$ VDR KO or IL-10/VDR double (D)KO CD8$^+$ T cells showed severe inflammation in the colon including hyperplasia and infiltration of immune cells (Fig.2-1A). The Rag KO mice that received CD8$^+$ T cells from DKO mice lost a significant amount of their starting body weight (BW) by 8wks (Fig. 2-1B). In fact the Rag KO recipients of DKO CD8$^+$ T cells developed a fulminating form of IBD that resembled the IBD that develops in the DKO mice (5). Rag KO mice that received no cells (CTRL) gained weight over the 8wk study and Rag KO mice that received WT, VDR KO and IL-10 KO CD8$^+$ T cells maintained their BW (Fig.2-1B). None of the over 20 Rag KO recipients of VDR KO CD8$^+$ T cells lost weight even though the mice had intense inflammation in both the colon and SI (data not shown and Fig.2-1A). The colon showed the greatest degree of inflammation following transfer of VDR KO CD8$^+$ T cells to Rag KO recipients. In order to determine what the differences were between VDR KO and WT CD8$^+$ T cells, the CD8$^+$ T cells were characterized from WT and VDR KO mice. There was a higher frequency of naïve (CD62L$^{high}$/CD44$^{low}$) and CD8/CD28$^-$ cells and a lower frequency of activated (CD62L$^{low}$/CD44$^{high}$) and CD8/CD122$^+$ cells in the spleen of VDR KO compared to WT mice (Fig.2-1C). CD25$^+$ expression on CD8$^+$ T cells was low and less than 2% of the CD8$^+$ T cells in VDR KO and WT mice expressed CD25 (Fig.2-7C). In addition, there was no difference
between expression of CD25 on VDR KO and WT CD8+ T cells (Fig.2-7C). There was no difference in the total number of splenocytes isolated from WT and VDR KO mice and therefore changes in frequency also resulted in changes in absolute numbers of cells. The frequency of granzyme B+ cells was not different in CD3/CD28 activated VDR KO and WT CD8+ T cells (data not shown). IL-17 and IL-10 were undetectable while IFN-γ was not different in the supernatants from CD3/CD28 activated VDR KO and WT CD8+ T cells (data not shown). The finding of more regulatory CD8/CD28− and less regulatory CD8/CD122+ from the VDR KO mice was followed up by doing additional transfers to Rag KO mice using purified populations of these regulatory cell types.

Rag KO recipients of 10^6 sorted WT CD8/CD28− or CD8/CD28+ T cells weighed the same as the CTRL Rag KO mice that did not receive any cells (Fig.2-1D). Conversely, Rag KO recipients of 10^6 sorted VDR KO CD8/CD28− or CD8/CD28+ T cells lost significantly more weight than the CTRL and WT CD8 T cell recipients (Fig.2-1D). Sorted 10^6 CD8/CD122+ T cell transfers from either WT or VDR KO mice did not induce weight loss or colitis in Rag KO recipients (data not shown). Histopathology of colonic tissue from Rag KO recipients of VDR KO CD8+ subsets (CD28+/−) showed significantly more inflammation and epithelial hyperplasia than CTRL (Fig.2-1E). Histopathology of colonic tissue from Rag KO recipients of WT CD8+ subsets (CD28+/−) was not different from CTRL (Fig. 2-1E). Increased colitis in Rag KO recipients of VDR KO CD8+ T cells is not a result of a difference in the CD28+/− subpopulations.

**VDR KO CD8+ T cells accelerate CD4/CD45RB^{high} cell-mediated colitis**

The effect of CD8+ T cells on CD4/CD45RB^{high} (naïve CD4+) T cell induced colitis in Rag KO recipients was examined. Rag KO mice were injected with 10^6 sorted CD8+ T cells from CD45.2+ VDR KO or CD45.2+ WT mice. The next day, the same Rag KO mice were injected with sorted 4x10^5 WT naïve CD45.1+/CD4 T cells to induce colitis. Rag KO recipients of only naïve CD4 T cells (CD4 only) lost 10% of their original BW, and weighed significantly less than the CTRL
mice at 7wks post-transfer (Fig. 2-2A). Weight loss in the Rag KO recipients of naïve WT CD4 cells plus WT CD8 T cells (CD4+WTCD8) was no different from the recipients of CD4 only T cells (Fig. 2-2A). Rag KO recipients of naïve WT CD4 plus VDR KO CD8 T cells (CD4+KOCD8) lost 20% of their original BW, which was significantly more weight loss than any other group (Fig. 2-2A). The colon/BW% was lowest in the CTRL, intermediate in the CD4 only and CD4+WTCD8 groups and highest in the CD4+KOCD8 group (Fig. 2-2B). The histopathology scores from Rag KO recipients of CD4+KOCD8 T cells were 6.5±0.4, which was significantly higher than the scores for all other groups (Fig. 2-2C and data not shown). CD8+ T cells from VDR KO mice produced twice as much IFN-γ and IL-17A than WT CD8+ T in the IEL of Rag KO recipients (Fig. 2-2D). In addition, the Rag KO recipients of CD4+KOCD8 T cells had significantly more total IFN-γ and IL-17A producing cells in the IEL than recipients of CD4+WTCD8 T cells (Fig. 2-2E). Rag KO recipients of CD8+ T cells from VDR KO mice had more IFN-γ and IL-17A that corresponded to the increased severity of naïve CD4+ T cell induced colitis.

**Increased CD8+, but reduced naïve CD4+ T cells in VDR KO cell recipients**

The MLN and IEL of the SI were used to determine the origin of T cells in the gastrointestinal tract of the Rag KO recipients. The MLN and IEL were used since these tissues normally have high frequencies of T cells and can demonstrate the extent of immune reconstitution of Rag KO mice following T cell transfer. 70±5% of the IEL in Rag KO recipients of only CD4 cells were the donor CD4/CD45.1+ T cells (Fig. 2-3A). The remaining cells in the IEL (30%) were the resident Rag KO IEL cells (innate immune cells) that did not express CD45.1 or CD8β. In the Rag KO recipients of CD4+WTCD8 cells, 36±5% of the IEL were CD4+ cells (CD45.1+) and 33±4% were CD8αβ (CD8β+/CD45.1−) T cells such that together 69% of the IEL in the recipient were T cells (Fig. 2-3A). The Rag KO recipients of the CD4+KOCD8 cells had fewer CD4+ cells (8 ± 1%) and more CD8αβ cells (64 ± 5%) than the other groups of mice (72% total T cells, Fig.
The reconstitution of the MLN mirrored the IEL in that the recipients of CD4+WTCD8 cells had approximately equal representation of CD4 and CD8αβ cells in the MLN while the mice that received CD4+KOCD8 cells had significantly more CD8+ than CD4+ T cells in the MLN (Fig. 2-3B). There were no differences in the total frequency of CD8αα+ expressing T cells (18-21% of the T cells) in the IEL of the Rag KO recipients of CD4+WTCD8 or CD4+KOCD8 T cells (Fig. 2-3C). Significantly more CD8+ T cells were recovered in the MLN and IEL of CD4+KOCD8 than CD4+WTCD8 T cell transferred Rag KO recipient mice.

**Increased proliferation of VDR KO CD8+ T cells in vivo**

Based on the finding of increased numbers (Fig. 2-3) of VDR KO CD8 cells in the IEL and MLN following co-transfer to Rag KO mice; additional experiments were done to determine whether VDR KO CD8 T cells proliferate more rapidly than WT CD8 T cells. Sorted CD8+ T cells were detectable in the MLN of Rag KO recipients on d3 following transfer (Fig. 2-4A). Conversely, there were no T cells found in the IEL after 3 days (Fig. 2-4A). At d3 in the MLN most of the CD8+ T cells from either WT or VDR KO donors were undergoing division (88 ± 2% incorporated BrdU). There were two distinct populations of BrdU+ cells in the MLN; one with low-mean fluorescence intensity (MFI, few rounds of proliferation) and the other one with high-MFI (Fig. 2-4A, multiple rounds of proliferation). 61±3% of VDR KO CD8+ T cells were of the BrdU high-MFI phenotype and only 41±2% of the WT CD8+ T cells were BrdU high-MFI (Fig. 2-4A). On d8 there were also more high-MFI profile VDR KO CD8+ T cells (51±4%) than WT CD8+ T cells (39±2%, Fig. 2-4A). By day 14 in the MLN there was a small population (16±3%) of BrdU very high-MFI VDR KO CD8+ T cells and significantly less of these cells in the WT CD8+ T recipients (4±1%, Fig. 4A). CD8+ T cells appeared in the IEL on d8 (Fig. 2-4A). Significantly fewer cells were BrdU high-MFI in the IEL of Rag KO recipients of WT CD8+ T cells (8±3%) than VDR KO CD8+ T cells (22±2%, Fig. 2-4A). There were also two distinct peaks of BrdU incorporation evident at d14 in the IEL samples and again the high-MFI peak was over
represented in the recipients of VDR KO CD8⁺ T cells (Fig. 2-4A). Total VDR KO CD8⁺ T cells were proliferating faster than WT CD8⁺ T cells \textit{in vivo}.

To identify which T cell subsets were undergoing rapid proliferation in the Rag KO recipients, the BrdU MFI-high cells from Fig. 2-4A were phenotyped. Naïve CD8⁺ T cells (CD62L\textsuperscript{high}CD44\textsuperscript{low}) from VDR KO mice proliferated more than their WT counterparts in the MLN and IEL on d3 and d8 post-transfer (Fig. 2-4B). Conversely, activated CD8⁺ T cells (CD62L\textsuperscript{low}CD44\textsuperscript{high}) from VDR KO mice proliferated at the same rate (differences were not significant) as activated cells from WT mice post-transfer (Fig. 2-4B). More naïve VDR KO CD8⁺ T cells than naïve WT CD8⁺ T cells were proliferating rapidly \textit{in vivo}.

**Increased proliferation of VDR KO CD8⁺ T cells \textit{in vitro}**

Proliferation of the CD8⁺ T cells from VDR KO and WT mice was also tested \textit{in vitro}. Even though there was very little proliferation detected in the CD3/CD28 stimulated cultures at d1, VDR KO CD8⁺ T cells had 2.4±0.2% of the cells proliferating while none of the WT CD8⁺ T cells were proliferating (Fig.2-5A). On d2 there were two rounds of proliferation evident in the WT CD8⁺ T cell cultures and three rounds of proliferation in the VDR KO CD8⁺ T cell cultures (Fig.2-5A). In addition, there were significantly more VDR KO CD8⁺ T cells proliferating (64±3%) compared to WT CD8⁺ T cells (51±2%) at d2. By d3 almost all of the cells in both cultures had proliferated and there were no longer significant differences in the frequency of CD8⁺ T cells that had undergone proliferation (Fig.2-5A). VDR KO CD8⁺ T cells were proliferating faster than WT CD8⁺ T cells \textit{in vitro}.

The CFSE stained cells in panel A were also stained to look at naïve and activated cells in the cultures. On day 2 significantly more of the naïve (CD62L\textsuperscript{high}CD44\textsuperscript{low}) CD8⁺ T cells from VDR KO mice had proliferated in response to CD3/CD28 stimulation than WT (Fig.2-5B). By day 3 the naïve VDR KO CD8⁺ T cells were proliferating rapidly in the presence and absence of CD3/CD28 stimulation while the naïve WT CD8⁺ T cells were not (Fig.2-5B). The naïve VDR
KO CD8+ cells were dividing but not downregulating CD62L and upregulating CD44 (Fig. 2-5B). CD8+ T cells with an activated phenotype (CD62LlowCD44high) proliferated strongly in both the WT and VDR KO cultures at both d2 and d3 post-stimulation and there were no differences between the proliferation of the activated VDR KO and WT CD8+ T cells (Fig. 2-5B). Phenotypically naïve VDR KO CD8+ T cells proliferated with or without stimulation but did not express activation markers as a result of proliferation.

**VDR KO CD8+ T cells over-produce and respond to IL-2 robustly**

The role of IL-2 in the increased proliferation of VDR KO CD8+ T cells was determined. CD25 expression was not different on VDR KO and WT CD8+ T cells (Fig. 2-7D). 3 days of culture were required in order to detect proliferation of CD8+ T cells in response to IL-2 (d2 data not shown, Fig. 2-6A). 24±3% of WT CD8 T cells and 50±4% of VDR KO CD8+ T cells divided at d3 following addition of IL-2 (Fig. 2-6A). Addition of IL-2 plus IL-2 neutralizing antibodies prevented all of the WT CD8+ T cell proliferation while VDR KO CD8+ T cells still had 6±2% undergoing proliferation in the presence of neutralizing antibodies to IL-2 (Fig. 2-6A). The supernatants from CD8+ T cells cultures stimulated with CD3 and CD28 antibodies were evaluated for the production of IL-2. Unstimulated VDR KO and WT CD8+ T cells did not produce detectable IL-2 after 3 days in culture (Fig. 2-6B). CD3/CD28 stimulated VDR KO CD8+ T cells produced 2-fold more IL-2 than WT CD8+ T cells (Fig. 2-6B). Overproduction of IL-2 contributes to the more rapid proliferation of CD3/CD28 activated VDR KO CD8+ T cells.

**Discussion**

VDR KO CD8+ T cells proliferated more rapidly both in vitro and in vivo. The increased rate of proliferation following activation was associated with the over production of IL-2 from the CD8+ T cells themselves. The rapidly proliferating naïve donor VDR KO CD8+ T cells found residence
in the MLN first and then in the IEL of the Rag KO mice where they out-competed the CD4\(^{+}\) T cells and contributed to colitis development by inducing IL-17A and IFN-\(\gamma\) production. In Rag KO recipients of WT CD8\(^{+}\) T cells endogenously produced 1,25(OH)\(_2\)D\(_3\) slows down the proliferation of the T cells and inhibits the ability of the CD8\(^{+}\) T cells to produce IL-17 and IFN-\(\gamma\). In VDR KO mice the naïve CD8\(^{+}\) T cells were still over-represented but their proliferation must have been controlled since VDR KO mice do not develop overt colitis symptoms (26). VDR KO mice have normal FoxP3\(^{+}\) T regulatory cells that may prevent the naïve CD8\(^{+}\) T cells from expanding and causing colitis (26). Naïve CD8\(^{+}\) VDR KO T cells divided in vitro even in the absence of stimulation (Fig.2-5B). This is unique to the VDR KO CD8\(^{+}\) T cells, since naïve CD4\(^{+}\) VDR KO T cells proliferated at the same rate as naïve CD4\(^{+}\) WT T cells (unpublished data and (26)). Proliferation was not associated with upregulation of activation markers since the proliferating CD8\(^{+}\) T cells were still CD62L\(^{hi}\)/CD44\(^{lo}\). VDR expression must be required for the quiescence of naïve CD8\(^{+}\) T cells.

Naïve and memory CD8\(^{+}\) T cells use different cytokines to regulate proliferation (27). The proliferation of naïve T cells requires IL-7 receptor (R) \(\alpha\), but not IL-15; conversely, memory T cells use either IL-7R\(\alpha\) or IL-15 for proliferation (27). It would be difficult to envision how this differential use of IL-7 versus IL-15 could account for the increased proliferation of predominately naïve CD8\(^{+}\) T cells. Instead it may be that developmentally the VDR is required to control the proliferation rate of the naïve CD8\(^{+}\) T cells but not the differentiated effector or memory cells. Somewhat surprisingly CD8\(^{+}\) T cells from VDR KO mice produced the same amounts of granzyme B and IFN-\(\gamma\) as WT CD8\(^{+}\) T cells (data not shown). Instead, CD8\(^{+}\) T cells from VDR KO mice overproduced IL-2 that may act in a paracrine manner on the CD8\(^{+}\) T cells themselves.

The original function of 1,25(OH)\(_2\)D\(_3\) in the immune system was shown to be as a suppressor of mitogen induced proliferation (28). Those early studies established that 1,25(OH)\(_2\)D\(_3\) suppressed
T cell proliferation in both CD4$^+$ and CD8$^+$ by blocking transition of the cycling cells from early G1 to late G1 (29, 30). In addition, IL-2 was shown to be a target of 1,25(OH)$_2$D$_3$ treatments and 1,25(OH)$_2$D$_3$ inhibited production of IL-2 (31). The inhibition of IL-2 expression was due to the direct interaction of the VDR/1,25(OH)$_2$D$_3$ complex that blocked the binding of the NFATp/AP1 complex to the IL-2 promoter (32). The effects of 1,25(OH)$_2$D$_3$ on IL-2 contributed to the suppression of T cell proliferation by 1,25(OH)$_2$D$_3$ but proliferation of 1,25(OH)$_2$D$_3$ treated T cells was only partially reversed by exogenous IL-2 addition (31). Administration of exogenous IL-2 can increase the frequency of CD8$^+$ memory cells (33, 34). Autocrine IL-2 secretion by memory CD8$^+$ T cells has been shown to be a critical mechanism to maintain the CD8$^+$ T cells (35). The data in the VDR KO CD8$^+$ T cells suggest that the VDR must participate as a negative co-regulator of proliferation and IL-2 production in CD8$^+$ T cells. The data further suggest that expression of the VDR may be important in regulation of the memory CD8$^+$ response.

Vitamin D serves as a regulator of proliferation across many different cell types. In addition to inhibiting the proliferation of T cells, 1,25(OH)$_2$D$_3$ inhibits the proliferation of keratinocytes, B cells, epithelial cells and several different types of cancer cells (36, 37). Topical 1,25(OH)$_2$D$_3$ inhibits proliferation and induces resolution of psoriasis (37). In addition, vitamin D deficiency is associated with an increase in several different types of cancer (37). VDR KO mice have increased epithelial cell proliferation in the gastrointestinal tract and skin (38). More recently we have shown that in the immune system control of homeostatic proliferation and apoptosis underlie the basic mechanisms whereby vitamin D regulates TCR$\alpha\beta$/CD8$\alpha\alpha$ and iNKT cell numbers (9, 39). Vitamin D and the VDR seem to be master regulators of proliferation across many cell types. In CD8$^+$ T cells there is a selective requirement for VDR signaling to control the numbers of naïve CD8$^+$ T cells in the periphery.

VDR KO CD8$^+$ T cells induce colitis in the Rag KO recipients. However, the Rag KO recipients of VDR KO CD8$^+$ T cells did not lose weight or develop overt symptoms of experimental IBD,
suggesting that there must be some regulatory cell or cytokine being produced to limit the pathogenicity of the cells. There did not appear to be any CD28− or CD122+ regulatory cell in the VDR KO CD8+ T cells. Confirming the literature the Rag KO recipients of IL-10 KO CD8+ T cells did not induce colitis (40). However, IL-10/VDR DKO CD8+ T cells induced a fulminating form of IBD including rectal bleeding, and significant weight loss. Therefore it seems that autocrine IL-10 production by CD8+ T cells prevents overt colitis induced by VDR KO CD8+ T cells.

The VDR is critical for controlling the rate of proliferation of naïve CD8+ T cells and the ability of the CD8+ T cells to both produce and respond to IL-2. In the absence of vitamin D signaling rapidly proliferating CD8+ T cells accumulate in the gut and contribute to the production of IL-17 and IFN-γ. Autocrine production of IL-10 limits VDR KO CD8+ T cell- induced colitis. In the T cell transfer model of IBD other regulatory cells are absent that might inhibit the proliferation of the VDR KO CD8+ T cells to control inflammation in the gastrointestinal tract. Expression of the VDR halts the nonspecific expansion of the naïve CD8+ T cells. Vitamin D control of the CD8+ T cell response contributes to the maintenance of gastrointestinal homeostasis.
References


Figure 2.1. CD8⁺ T cells from VDR KO mice induce colitis in Rag KO recipients.

Sorted CD8⁺ T cells from WT, IL-10 KO, VDR KO, and IL-10/VDR DKO mice were injected (10⁶ cells per mouse i.p.) into Rag KO recipients. CTRL had no cell transfer. (A) Representative colonic sections from the Rag KO recipients of CD8⁺ T cells 8 weeks post-transfer. Colonic samples were stained with H&E (scoring system in Methods) and were shown at 10x magnification; scale bar = 50 μm. Colon sections shown were rated: WT (score=0), IL-10 KO (score=0), VDR KO (score=7), and DKO (score=8). (B) The percentage change in BW of Rag KO recipients of CD8⁺ T cells from WT, IL-10 KO, VDR KO and DKO mice. (C) The phenotype of CD8⁺ T cells from the spleen of WT and VDR KO mice. The splenic lymphocytes were prepared and stained for TCRβ, CD8β, CD44, CD62L, CD28 and CD122 antibodies. The gating strategy is in Supplementary Fig. 2A. (D) The percentage change in BW of the Rag KO recipients following transfer of sorted CD8⁺/CD28⁺ or CD8⁺/CD28⁻ cells from WT or VDR KO mice (10⁶ cells per mouse i.p.). (E) Representative H&E stained colon sections from Rag KO recipients of CD8⁺/CD28⁺ or CD8⁺/CD28⁻ T cells at 8 weeks post-transfer. Colon sections shown were rated: CTRL (score=1), WT CD8⁺/CD28⁺ (score=3), WT CD8⁺/CD28⁻ (score=3), VDR KO CD8⁺/CD28⁺ (score=6), VDR KO CD8⁺/CD28⁻ (score=6). Values represent the mean ± SEM of 5-8 mice per group and one representative of three independent experiments (A-E). Two-way ANOVA with Bonferroni post-hoc tests (B,D) or Student’s t-tests (C). *P<0.05, **P<0.01.
Figure 2. VDR KO CD8\(^+\) T cells aggravate CD4/CD45RB\(^{\text{high}}\) cell-induced colitis.

Rag KO mice were injected i.p. with sorted 10\(^6\) WT or VDR KO (CD45.2\(^+\)) CD8\(^+\) T cells on day -1 and 4x10\(^5\) WT (CD45.1\(^+\)) CD4\(^+\)CD45RB\(^{\text{high}}\) cells on day 0. (A) The percentage change in original BW of Rag KO mice recipients of CTRL, or CD4/CD45RB\(^{\text{high}}\) (CD4 only), CD4/CD45RB\(^{\text{high}}\) plus WT CD8 (CD4 + WTC8), CD4/CD45RB\(^{\text{high}}\) plus VDR KO CD8 (CD4 + KOCD8) cells 7 weeks post-transfer. (B) The ratio of the colon/BW in the Rag KO recipients at week 7 post-transfer. (C) Representative sections of colonic tissue from CTRL (score=0), CD4 only (score=4), CD4 + WTC8 (score=6), and CD4 + KOCD8 (score=6). Colonic samples were stained with H&E and are shown at 10\(^\times\) magnification; scale bar = 50 \(\mu\)m. (D) The isotype controls and intracellular staining for IFN-\(\gamma\) and IL-17A in CD8\(^+\) T cells in the IEL from Rag KO mice recipients of CD4 + WTC8 or CD4 + KOCD8 T cells. (E) Total IFN-\(\gamma\) and IL-17A in cells from Rag KO recipients of CD4 + WTC8 or CD4 + KOCD8 T cells. Grey histograms are isotype controls. Data is from n=6-8 mice per group and the values represent the mean of three independent experiments ± SEM. Two-way ANOVA with Bonferroni post-hoc tests (A), one-way ANOVA with Bonferroni post-hoc tests (B, D) and two-tailed Student’s t-tests (E), *\(P<0.05\), **\(P<0.01\), ***\(P<0.001\).
Figure 2-3

(A) Dot plots show the frequencies of the reconstituted CD4/CD45.1+ and CD8β/CD45.1− T cells in the IEL at week 7 post-transfer for CD4 only, CD4 + WTCD8 and CD4 + KOCD8 Rag KO recipients (mice in Fig. 2-2). (B) Bar charts showing the frequency of the reconstituted CD4/CD45.1+ and CD8β/CD45.1− T cells in the MLN. (C) Dot plots showing the frequency of T cells in the Rag KO recipients that expressed CD8αα in the IEL. Data is from n=6-8 mice per group and the values represent the mean of three independent experiments ± SEM. One-way ANOVA with Bonferroni post-hoc tests (A, B), or Two-tailed Student’s t tests (C), *P<0.05, **P<0.01, ***P<0.001.
Figure 2-4

Figure 2-4. Proliferation of CD8+ T cells in vivo.

(A) Histograms showing BrdU incorporation in the MLN or IEL following transfer of sorted $10^6$ WT or VDR KO CD8+ T cells into Rag KO recipients from 3 to 14 days post transfer. Shaded histograms showed the BrdU isotype control staining, and open histograms were BrdU staining.

(B) Different CD8+ subsets were evaluated for BrdU incorporation and graphed at day 3 and day 8 post-transfer. Naïve CD8+ T cells are CD62LhighCD44low, and activated CD8+ T cells are CD62LlowCD44high. Data is from n=6-8 mice per group and the values represent the mean of two independent experiments ± SEM of percentages. Two-way ANOVA with Bonferroni post-hoc tests (A, B), *$P<0.05$, **$P<0.01$. 

(A) MLN

WT

VDR KO

WT

VDR KO

BrdU

naive CD8+

activated CD8+

0
20
40
60
80
100
% BrdU +

day 3

day 8

% BrdU +

day 3

day 8

WT MLN

VDR KO MLN

WT IEL

VDR KO IEL

*0±1

16±3*

60±3

76±4*

**39±2

51±4*

8±3

22±2*

41±2

81±3*
Figure 2-5

(A) Histograms show CFSE dilution induced following in vitro proliferation of sorted WT and VDR KO CD8+ T cells in response to CD3/CD28 stimulation. The shaded histograms show the CFSE stained T cells prior to stimulation with CD3/CD28, and open histograms are CFSE dilution following stimulation. Values are mean ± SEM of the frequency of T cells that have divided and diluted the CFSE label. (B) The CFSE labeled T cells in panel A were evaluated for CD8 subsets with or without (unstim) CD3/CD28 stimulation. Naïve CD8+ T cells are CD62L\textsuperscript{high}CD44\textsuperscript{low}, and activated CD8+ T cells are CD62L\textsuperscript{low}CD44\textsuperscript{high}. The shaded histograms are the CFSE staining at the beginning of the culture, and open histograms are CFSE dilution. Values are the mean of two independent experiments ± SEM. Two-way ANOVA with Bonferroni post-hoc tests (A, B), *P<0.05.

Figure 2-5. Proliferation of CD8+ T cells in vitro.

(A) Histograms show CFSE dilution induced following in vitro proliferation of sorted WT and VDR KO CD8+ T cells in response to CD3/CD28 stimulation. The shaded histograms show the CFSE stained T cells prior to stimulation with CD3/CD28, and open histograms are CFSE dilution following stimulation. Values are mean ± SEM of the frequency of T cells that have divided and diluted the CFSE label. (B) The CFSE labeled T cells in panel A were evaluated for CD8 subsets with or without (unstim) CD3/CD28 stimulation. Naïve CD8+ T cells are CD62L\textsuperscript{high}CD44\textsuperscript{low}, and activated CD8+ T cells are CD62L\textsuperscript{low}CD44\textsuperscript{high}. The shaded histograms are the CFSE staining at the beginning of the culture, and open histograms are CFSE dilution. Values are the mean of two independent experiments ± SEM. Two-way ANOVA with Bonferroni post-hoc tests (A, B), *P<0.05.
Figure 2-6. VDR KO CD8+ T cells produce more and proliferate more rapidly to IL-2.

(A) Histograms show CFSE dilution following *in vitro* proliferation of sorted WT and VDR KO CD8+ T cells in response to IL-2 stimulation or IL-2 stimulation with IL-2 neutralizing antibodies. The shaded histograms show the CFSE stained T cells prior to stimulation. (B) IL-2 production was measured from sorted CD8+ T cells with or without (unstim) CD3/CD28 stimulation. Supernatants were collected from 3d cultures of CD8+ T cells, and IL-2 in the supernatant was evaluated using ELISA. Values are the means from two independent experiments ± SEM. N.D., not detected. Two-way ANOVA with Bonferroni post-hoc tests (A, B), *P<0.05.
Figure 2-7

(A) CD8\(^+\) or CD4\(^+\) column purification was followed by cell sorting. Histograms show the frequencies of CD8\(^+\) T cell subsets before (pre-sort) and after (post-sort) cell sorting in splenocytes from WT and VDR KO mice. The bottom panel shows CD4\(^+\)CD45RB\(^{\text{high}}\) pre-sort and post-sort populations. The purity of the CD8\(^+\) and the CD4\(^+\)CD45RB\(^{\text{high}}\) T cells was >99\%. (B) Dot plots show the single staining for PE-conjugated CD45.1, FITC-conjugated CD8\(\beta\), and isotype controls for both PE and FITC staining in the IEL for the experiment shown in Fig. 3A. (C) Bar graphs shows the frequency of CD25\(^+\) T cells within the CD8\(^+\) population from WT and VDR KO mice. Splenocytes were prepared from WT and VDR KO mice and cells were stained for TCR\(\beta\), CD8\(\beta\) and CD25 antibodies. Lymphocytes were first gated on TCR\(\beta^+\) cells. Data is from n=8-10 mice per group and values are mean of three independent experiments ± SEM. Two-tailed Student’s t-tests, n.s., not significant.
Figure 2-8

(A) Forward and side scatter of splenic lymphocytes. CD8$^+$ cells were gated on and stained for CD28, CD122 and isotype controls. (B) Forward and side scatter for the IEL and MLN lymphocytes. (C) Sorted CD8 cells were cultured without stimulation or with CD3/CD28 for 3 days and stained for CD8β, CD44 and CD62L antibodies. CFSE staining was analyzed in the CD44$^{low}$/CD62L$^{high}$ (naive) and CD44$^{high}$/CD62L$^{low}$ (activated) populations.

Figure 2-8. The gating strategy for FACS analysis.
Figure 2-9

The mRNA expression of cytokines in the Rag KO recipients.

The mRNA expression of cytokines Ifn-γ, Il-17A and Il-10 in the small intestine (A) and colon (B) of Rag KO mice recipients of CD4/CD45RB$^{\text{high}}$ plus WT CD8 (CD4 + WTCD8), CD4/CD45RB$^{\text{high}}$ plus VDR KO CD8 (CD4 + KOCD8) cells 7 weeks post-transfer. Data is from n=6-8 mice per group and the values represent the mean of two independent experiments ± SEM. Two-tailed Student’s t tests (A,B), *P<0.05.
Chapter 3

Vitamin D receptor knockout mice are more susceptible to *Citrobacter rodentium* infection once colonization resistance is eliminated
Abstract

1,25-dihydroxyvitamin D3 [\(1,25(\text{OH}_2)\text{D}_3\)] and vitamin D receptor (VDR) are important regulators of the immune system. The effect of the VDR on the ability of mice to fight *Citrobacter rodentium* infection was determined. VDR knockout (KO) mice had fewer *C. rodentium* in the feces than wild-type (WT) mice and the kinetics of clearance was faster in VDR KO than WT mice. VDR KO mice had more IL-22 producing innate lymphoid cells, more RegIIIγ, Ang-4 and Muc2 than WT mice. In addition, VDR KO mice had different gut microbiota compared to WT. Disruption of the gut microbiota using antibiotics reversed colonization resistance to *C. rodentium* infection. Antibiotic treated VDR KO mice also had increased bacterial shedding and lethality following infection compared to antibiotic treated WT. VDR KO mice without T and B cells double VDR and recombinase activated gene (Rag) KO mice did not show colonization resistance. In fact, the VDR/Rag KO mice had significantly higher numbers of *C. rodentium* in the feces and died prematurely compared to single Rag KO mice. The data suggest that bacterial dysbiosis in VDR KO mice, but not double Rag/VDR KO mice, prevents colonization with *C. rodentium*. The bacterial dysbiosis in VDR KO mice coincides with increased numbers of IL-22+ innate lymphoid cells and overproduction of RegIIIγ. Once colonization resistance is overcome, VDR KO mice have increased susceptibility to *C. rodentium*. The VDR is required for successful clearance of *C. rodentium* by the acquired and innate immune system.

Introduction

Vitamin D and the vitamin D receptor (VDR) have been shown to play an important role in regulating the immune system. The effects of vitamin D can be found in myeloid and lymphoid cells that express the VDR, including dendritic cells (DCs), macrophages, T cells and B cells (1).
The active form of vitamin D, 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$) has been shown to inhibit the differentiation and maturation of dendritic cells (DCs) that prevent induction of alloreactive T-cells (2, 3). In addition, vitamin D receptor (VDR) knockout (KO) mice had increased frequency of mature DCs in the lymph nodes (4). 1,25(OH)$_2$D$_3$ promoted the production of IL-1β and antimicrobial peptide cathelicidin by monocytes and macrophages and 1,25(OH)$_2$D$_3$ enhanced the phagocytic and oxidative burst ability of macrophage (5, 6). Conversely, 1,25(OH)$_2$D$_3$ inhibited the pro-inflammatory cytokine production from macrophages and DCs, including IL-12, TNF-α, IL-1β and IL-6 (7-9). Besides innate immunity, 1,25(OH)$_2$D$_3$ has been shown to regulate the adaptive immune cells. 1,25(OH)$_2$D$_3$ suppressed T cell proliferation and inhibited the production of Th1 cytokines IFN-γ, IL-2 and TNF-α, resulting in a shift to a Th2 phenotype (10-13). 1,25(OH)$_2$D$_3$ suppressed the induction and secretion of IL-17 by Th17 cells (14). 1,25(OH)$_2$D$_3$ induced T regulatory cells, including regulatory Foxp3+ T cells, IL-10-producing cells, iNKT cells and CD8αα T cells (7, 15-18). In B cells 1,25(OH)$_2$D$_3$ suppressed proliferation and inhibited immunoglobulin secretion (19, 20). In sum, 1,25(OH)$_2$D$_3$ regulates the immune cells by suppressing pro-inflammatory responses and promoting regulatory T cells.

*In vivo*, vitamin D, has been shown to regulate the pathogenesis of Th1-driven experimental autoimmune diseases by inhibiting the pathogenic Th1 and Th17 cells and enhancing the T regulatory cells (multiple sclerosis, type-1 diabetes and inflammatory bowel disease (IBD)) (21, 22). Vitamin D deficiency has been shown in patients with IBD (23). Studies using experimental models of IBD have shown that deficiency of the VDR or vitamin D accelerates the development of IBD (8, 21, 24, 25). VDR KO mice were extremely susceptible to dextran sodium sulfate (DSS)-induced colitis, an animal model of IBD (24). IL-10 KO mice develop IBD spontaneously (25), and IL-10/VDR double KO mice had more severe IBD symptoms (24). Similarly, CD4+CD45RB$^{high}$ T cells from VDR KO mice induced more severe colitis in Rag KO recipients compared to WT T cells (24). Vitamin D or 1,25(OH)$_2$D$_3$ supplementation reduced the
symptoms of IBD in experimental animals (24). These studies indicate that vitamin D regulates the pathogenesis of experimental IBD in part by suppressing the pathogenic Th1 and Th17 cell responses.

The effect of vitamin D on host resistance to infection was studied over the years and the results were conflicting. Vitamin D supplementation during tuberculosis (TB) treatment was shown to be beneficial in some studies and not to have an effect in others (26-29). Experimentally in animals, the effect of 1,25(OH)₂D₃, vitamin D and the VDR may or may not control the bacterial burden during the infection (30). VDR KO mice infected with Schistosoma mansoni showed no effect on the worm burden, weight and fibrosis compared to WT mice (24). VDR KO mice had decreased Leishmania major parasite burdens compared to WT controls (31). Young VDR KO mice exhibited a delayed clearance of Listeria monocytogenes during primary challenge and produced higher levels of IFN-γ and IL-10 compared to WT mice (32). Other studies demonstrated that 1,25(OH)₂D₃ had no effect on host susceptibility to Herpes simplex or Candida albicans infection (33). In vitro, the bone marrow-derived macrophages from infected VDR KO mice were able to kill L. monocytogenes as well as WT (34). The effect of vitamin D on host immunity to infection may depend on the micro-organism and the location of the infection. At this time the role of vitamin D and the VDR in infectious immunity is not clear.

Citrobacter rodentium is a gram-negative murine pathogen that naturally colonizes and infects mice and forms attaching and effacing (A/E) lesions (35-38). C. rodentium shares important structural and functional similarities with human enteropathogenic E. coli that does not infect mice readily (39, 40). In WT mice, C. rodentium colonizes the cecum and colon transiently and is cleared over three weeks (41). At peak infection mice develop crypt hyperplasia, loss of goblet cells and mucosal infiltrations in the colon (35, 38, 41, 42). Th17 mediated immune responses are required for clearing the infection (43-45). T cell (αβ T cells and CD4+ T cells) or B cell KOs fail to clear a C. rodentium infection and develop systemic infections with the organism (37, 42, 46).
Innate immune cells also play a critical role in host defense against *C. rodentium*. In the gut innate lymphoid cells (ILC) that produce IL-22 and IL-17 are critical for early protection against *C. rodentium* infection (47, 48). ILCs are the major source of IL-22 during the first 6 days following infection (47). The adaptive and innate immune cells and cytokines in the host are required for the clearance of *C. rodentium*.

Here we determined the role of the VDR in host resistance to *C. rodentium* infection. Surprisingly, VDR KO mice were resistant to colonization with *C. rodentium*. VDR KO mice had increased IL-22-producing ILCs, higher expression of RegIIIγ, Ang-4 and Muc2 by epithelial cells and dysbiosis of the bacterial microbiota. Antibiotic (ABX) disruption of the gut microbiota reversed colonization resistance in VDR KO mice. ABX treated VDR KO mice were more susceptible than ABX treated WT mice to *C. rodentium* infection. Colonization resistance was not observed in VDR KO mice without T and B cells (VDR/Rag double (D) KO mice). Our data demonstrate that increased IL-22-producing ILCs contribute to dysbiosis and *C. rodentium* colonization resistance of VDR KO mice. ABX treated VDR KO mice and VDR/Rag DKO mice were extremely susceptible to *C. rodentium* infection. Taken together, our data suggest that the VDR regulates the gut microbiota, colonization and susceptibility to *C. rodentium*.

**Methods and Materials**

**Mice**

Age and sex matched WT, VDR KO, Rag KO and DKO mice on the C57BL/6 background were bred and housed in the animal facilities at the Pennsylvania State University (University Park, PA). Mice were fed on purified diets for 2 weeks prior and throughout the experiments as previously described (49, 50). Experimental procedures were approved by the Office of Research Protection, Institutional Animal Care and Use Committee at the Pennsylvania State University.
**Citrobacter rodentium infection**

The *C. rodentium* strain ICC169 was a kind gift of Gad Frankel (London School of Medicine and Dentistry, London UK). *C. rodentium* was cultured in Luria-Bertani (LB) broth containing 20 μg/ml nalidixic acid (EMD Chemicals) to stationary phage, centrifuged and resuspended in sterile PBS. Mice were infected by oral gavage with 200 μl of *C. rodentium* suspension, which contained $5 \times 10^9$ CFU. The concentration of the inoculum was confirmed by plating on LB agar plates containing 50 μg/ml nalidixic acid. Mice were placed in the cage without bedding to collect the feces left in the cages. The *C. rodentium* numbers in the feces were determined by homogenizing the feces in PBS, making serial dilutions and plating on LB agar plates with 50 μg/ml nalidixic acid. Bacterial colonies were counted the following day. Some mice were treated with the ABX vancomycin (20mg/ml) the day before infection. The BW, survival rate and bacterial numbers in the feces were measured post-infection.

**Cell isolation and flow cytometry**

Isolation of intestinal lamina propria (LP) lymphocytes were done as described previously (51). Small intestine (SI) or colon were collected and flushed with HBSS containing 5% FBS (Sigma-Aldrich, St. Louis, MO). Peyer's patches were removed and the tissue was opened lengthwise and cut into 0.5 cm pieces. The pieces were incubated twice in HBSS containing 5 mM EDTA, 0.15 μg/ml DTT and 5% FBS for 20 min at 37°C under 250 rpm rotation with stirring bar. The supernatant was discarded and the tissue was further incubated in RPMI-1640 containing 1 mg/ml collagenase type 1 (Worthington, Lakewood, NJ) and 10% FBS for 1.5 h at 37°C under 250 rpm rotation to obtain LP cells. The LP cells were collected from the interface of 40/80% Percoll gradients (Sigma-Aldrich). Cells were stained with FITC CD11b, FITC CD4, PE Gr-1, APC NKp46, PEcy5 F4/80, PECy7 CD3 (eBiosciences, San Diego, CA).

For intracellular cytokine IFN-γ and IL-17A staining, cells were stimulated in RPMI-1640 with PMA (0.1μg/ml, Sigma-Aldrich), ionomycin (0.5μg/ml, Sigma-Aldrich) for 5 h and for the final 3
h, Brefeldin A (10µg/ml, Sigma-Aldrich) was added to the culture medium. For the measurement of IL-22, cells were stimulated with mouse recombinant IL-23 (0.04µg/ml, R&D systems) for 5 h and for the final 3 h, Brefeldin A (10µg/ml, Sigma-Aldrich) was added to the culture medium. After surface staining, cells were fixed with 4% paraformadehyde (Sigma-Aldrich), permeabilized with 0.1% saponin (Sigma-Aldrich), and stained with FITC IFNγ, PE IL-17A, APC IL-22 or the FITC/PE/APC labeled isotype controls (eBiosciences). For RORγt staining was done using the transcription factor staining buffer kit and the manufacturer’s instructions (eBioscience). Cells were analyzed on BD Fortessa LSRII (BD Biosciences) and the data was analyzed with FlowJo 7.6.5 software (TreeStar, Ashland, OR).

**Quantitative real-time PCR**

Fecal DNA was isolated using QIAamp DNA stool minikit (Qiagen, Valencia, CA). Fecal DNA was amplified with universal 16S rDNA primers or specific primers for different bacterial phyla or genus using SYBR green mix (BioRad, Hercules, CA) by MyiQ Single-Color Real-Time PCR machine (BioRad). Relative 16S rDNA quantities were calculated using ΔΔCt method and were normalized by the amount of universal bacteria. Total RNA was isolated from colons of mice following the manufacturer’s instructions (Qiajen). cDNA was synthesized using the TaqMan reverse transcription reagents kit (Applied Biosystems, Carlsbad, CA) and was amplified for Ifn-γ, Il-17A, Il-22, RegIIIγ, RegIIIβ, Ang4, CRAMP, mBD-3 and Muc 1-4 with SYBR green mix (BioRad) by MyiQ Single-Color Real-Time PCR machine (BioRad). Expression levels of these molecules were normalized by GAPDH and calculated with the ΔΔCt method. The primer sequences are listed in Table 3-1.

**Denaturing gradient gel electrophoresis (DGGE)**

The 200 ng of fecal DNA was amplified with universal 16S rDNA primers that target the variable V3 region and conserved regions of the 16S rDNA in 30 µl PCR mixture (52). The primer sequences are shown in Table 3-1. The 25 µl per lane of PCR products were loaded onto a linear
30-60% denaturing gradient and run at 65V for 17V in DCode Universal Mutation Detection System (BioRad, Hercules, CA). DGGE banding patterns were normalized using DNA standards from purified cultures of *Clostridium propionicum* (ATCC strain 25522), *Lactobacillus murinus* (ATCC strain 35020) and *Parabacteroides distasonis* (ATCC strain 8503). The standards were used to compare the banding patterns between gels run on different days.

**Statistics**

Statistical analyses were performed using GraphPad software (PRISM software, La Jolla, CA). Two-tailed Student’s t tests were used to test differences between genotype (WT vs. KO). Two-way ANOVA with Bonferroni post-hoc tests were used to test the effects of experimental groups, time and their interactions. Log-rank tests were used to test the survival rate of experimental groups. The variances were unequal for bacterial shedding. These data were transformed (square root transformation) to eliminate unequal variances, followed by a repeated-measures (mix model) two-way ANOVA to test the effect of group by time on bacterial numbers. For all the analyses, *P*≤0.05 was used as the limit for significance. Values are shown as means ± SEMs of one or two of 2-3 independent experiments.

**Results**

**Reduced bacterial shedding and inflammatory response in VDR KO mice**

The amount of *C. rodentium* in the feces of VDR KO and WT mice were measured following infection (Fig. 3-1A). In WT mice, *C. rodentium* was detectable in the feces at d1 post-infection (Fig. 3-1A). The bacterial shedding increased through d7 when it peaked and then declined until d21 (Fig. 3-1A). The infection kinetics shown in Fig. 3-1A was similar to those published previously (53, 54). At d1 post-infection, VDR KO mice had 3 logs fewer bacteria in the feces than WT mice (Fig. 3-1A). The bacterial shedding in the feces was lower in VDR KO mice
compared to WT and the VDR KO mice cleared the infection by d18 (Fig. 3-1A). VDR KO mice were less susceptible to *C. rodentium* than WT mice (Fig. 3-1A).

The recruitment of inflammatory cells into the colonic LP was measured in VDR KO and WT mice. In uninfected (d0) WT and VDR KO mice, the frequencies of either inflammatory monocytes (CD11b⁺Gr-1<sup>high</sup>F4/80<sup>+</sup>, Fig. 3-1B) or neutrophils (CD11b⁺Gr-1<sup>high</sup>F4/80<sup>−</sup>, Fig. 3-1C) were low (0.1%). Frequencies of inflammatory monocytes and neutrophils increased at d10 and fell significantly at d21 in WT mice post-infection (Fig. 3-1B, C). Conversely, the frequencies of inflammatory monocytes remained low in VDR KO mice following infection (d10 and d21, Fig. 3-1B). The frequencies of neutrophils in VDR KO mice increased significantly at d10 and decreased at d21 post-infection (Fig. 3-1C). However, the neutrophils in VDR KO mice were significantly lower than WT mice at d10 post-infection (Fig. 3-1C). Infection increased the frequency of CD3⁺ T cells in the WT colonic LP at d10 and d21 post-infection (Fig. 3-1D). The increase in CD3 frequencies occurred after 21 days of infection in VDR KO mice (Fig. 3-1D). VDR KO mice had fewer CD3⁺ T cells in the colonic LP compared to WT mice at d10 post-infection (Fig. 3-1D). The total numbers of LP lymphocytes isolated from WT and VDR KO mice were not different, so changes in frequencies also reflected changes in absolute numbers of cells. The expression of *Ifn-γ*, *Il-17A* and *Il-6* were low in uninfected WT mice and increased significantly at d10 post-infection in WT mice (Fig. 3-1E-G). There was no increase in *Ifn-γ*, *Il-17A* and *Il-6* expression over the course of infection in VDR KO mice (Fig. 3-1E-G). In addition, VDR KO mice had significantly lower expression of *Ifn-γ*, *Il-17A* and *Il-6* than WT mice at d10 post-infection (Fig. 3-1E-G). Lower colonization of VDR KO mice with *C. rodentium* was associated with lower numbers of immune cell infiltrates and reduced cytokine expression in the colonic LP.

**Antibiotics eliminate *C. rodentium* colonization resistance in VDR KO mice**
The intestinal commensal bacteria compete with \textit{C. rodentium} for colonization (55). Previously we had shown that VDR KO and WT mice had different commensal bacteria in the feces (56). Here we used quantitative real-time PCR to determine the frequencies of bacterial phyla in the feces from mice used for these experiments (Fig. 3-2A). VDR KO mice had decreased quantities of bacteria from the Firmicutes phylum, and increased quantities of bacteria from the Bacteroidetes and the Proteobacteria phyla in the feces compared to WT mice (Fig. 3-2A). In addition, several bacterial genus members from these bacterial phyla were also different in VDR KO mice compared to WT, including \textit{Eubacterium}, \textit{Bacteroides} and \textit{Salmonella} (Fig. 3-6B-D). Similar phyla differences were also shown in the colon and SI from VDR KO and WT mice (Fig. 3-6E-J).

To determine the role of commensal bacteria in the colonization resistance of VDR KO mice to \textit{C. rodentium} infection, ABX was used to disrupt the bacteria. The one-dose ABX treatment significantly reduced the total amount of bacterial 16S rDNA in the feces of the ABX-treated WT and ABX-treated VDR KO mice compared to untreated control (CTRL) mice (Fig. 3-2B). In addition, ABX-treated WT and VDR KO mice had decreased numbers of DGGE bands as compared to untreated mice, indicating there was a decrease in the bacterial diversity with ABX treatment (Fig. 3-7). ABX treatment eliminated the bacterial differences in Firmicutes, Bacteroidetes or Proteobacteria phyla in feces, colon and SI (Fig. 3-6A, E-J). There was no effect of ABX on the WT clearance or susceptibility to \textit{C. rodentium} infection, and none of the ABX-treated WT mice died following infection (Fig. 3-2C, D). However, ABX VDR KO mice had significantly higher bacterial shedding starting at d2 post-infection and throughout the infection (Fig. 3-2C). In addition, the ABX VDR KO mice took longer to clear the infection than VDR KO, ABX WT or WT mice (Fig. 3-2C). ABX VDR KO mice were extremely susceptible to \textit{C. rodentium} infection and at peak infection 35\% of them died following infection (Fig. 3-2D). ABX
treatment reduced total bacterial numbers, changed the bacterial composition and removed *C. rodentium* colonization resistance in VDR KO mice.

**Changes in the expression of antimicrobial peptides and mucin in VDR KO mice**

To determine the cause of *C. rodentium* colonization resistance, the expression of mRNA for several antimicrobial peptides and mucin were measured in uninfected (d0) and infected (d10) WT and VDR KO mice. The expression of mRNA for RegIIIγ, angiogenin 4 (Ang 4), mucins (Muc 1-4), RegIIIβ, CRAMP and mouse β-defensin 3 (mBD-3) were not different in the colons of uninfected WT and VDR KO mice (Fig. 3-3A-C and Fig. 3-8). By d10 post-infection, the amount of RegIIIγ, Ang-4, RegIIIβ, mBD-3 were significantly increased in both infected WT and VDR KO mice compared to uninfected mice, while the amount of Muc2, CRAMP, Muc1 were only increased in infected VDR KO mice but not infected WT mice. The amount of Muc3 and Muc4 were not affected by infection or genotype (Fig. 3-3A-C and Fig. 3-8). At d10 post-infection, VDR KO mice had higher expression levels of RegIIIγ, Ang-4 and Muc 2, but had similar amount of RegIIIβ, CRAMP, mBD-3, Muc1, Muc3-4 compared to WT mice at d10 post-infection (Fig. 3-3A-C and Fig. 3-8). The colonic tissue from *C. rodentium*-infected ABX-treated mice in Figure 3-2D were also used to measure the expression of these antimicrobial peptides and mucins. At d10 post-infection, the difference in the expression of mRNA for RegIIIγ, Ang-4 and Muc2 between VDR KO and WT mice was eliminated with ABX treatment (Fig. 3-3D-F). VDR KO mice had increased expression of anti-microbial peptides compared to WT following *C. rodentium* infection and ABX treatment eliminated those changes.

**Increased IL-22+ ILCs in VDR KO mice**

IL-22 has been shown to induce epithelial cell production of antimicrobial peptides and the IL-22 receptor is expressed exclusively on epithelial cells (44, 57-60). RORγt+ ILCs have been identified to be the main source of IL-22 production during the early stage of *C. rodentium* infection (44). The RORγt+ ILCs are mainly localized in the intestinal LP (61). The frequencies
of ILCs were measured in VDR KO and WT mice. There were higher frequencies of ILCs in the SI of VDR KO mice than WT mice, but the amount of ILCs were not different in the colons of these mice (Fig. 3-4A). In addition, VDR KO mice had more IL-22-producing ILCs in the SI than WT mice (Fig. 3-4B). These cells were further stained with surface markers NKp46 and CD4. VDR KO mice had more NK-22 cells (CD3-RORγt+NKp46+) in the SI LP than WT mice, but not in the colonic LP (Fig. 3-4C). The frequencies of LTi4 (CD3-RORγt+NKp46-CD4+) cells and LTi0 (CD3-RORγt+NKp46-CD4-) cells were increased in both SI and colonic LP in VDR KO mice (Fig. 3-4D, E). The total cell numbers isolated from the SI or colonic LP were not different between WT and VDR KO mice (data not shown), so the changes in frequencies reflected the changes in absolute cell numbers. VDR KO mice had more IL-22-producing ILCs than WT mice.

**VDR/Rag DKO mice are more susceptible to *C. rodentium* infection**

RORγt+ ILCs are also present in Rag KO mice. The frequencies of ILCs (CD3-RORγt+) were also measured in SI and colon of Rag KO and DKO mice (Fig. 3-5A). Rag KO mice had 7.5% ILCs in the SI LP which is higher than those in WT mice (5%) (Fig. 3-5A, 3-4A). There were more ILCs present in the SI LP but not in the colonic LP of DKO mice compared with Rag KO mice (Fig. 3-5A). ILCs were further stained with surface markers NKp46 and CD4 to determine different subsets of ILCs. DKO mice had higher percentages of NK-22, LTi4 and LTi0 cells in the SI LP than Rag KO mice (Fig. 3-5B-D). There were not differences in NK-22, LTi4 or LTi0 cells in the colonic LP of Rag KO and DKO mice (Fig. 3-5B-D). The total cell numbers isolated from SI or colonic LP were not different between Rag KO and DKO mice (data not shown), so the changes in frequencies represented the changes in absolute cell numbers. DKO mice had higher frequencies of ILCs in the SI LP than Rag KO mice. In the absence of the VDR, ILCs accumulated in the SI.

Despite the differences in ILC numbers between DKO and Rag KO mice, there were no differences in the Firmicutes or Bacteroidetes phyla members in the feces from DKO and Rag
KO mice (Fig. 3-5E). There were increased numbers of the Proteobacteria phylum in the feces from DKO mice compared to Rag KO mice (Fig. 3-5E). To determine whether DKO mice would be colonization resistant to *C. rodentium*, DKO and Rag KO mice were orally inoculated with *C. rodentium*. The bacterial shedding in Rag KO mice increased by d7 post-infection and they failed to clear the infection during 21 days of study (Fig. 3-5F). Rag KO mice remained colonized with large numbers of *C. rodentium* but did not develop a fatal infection until d21 post-infection (Fig. 3-5G). DKO mice had significantly higher bacterial shedding in the feces at d2, d4 and d7 post-infection compared to Rag KO mice (Fig. 3-5F). In addition, all of the DKO mice died by d9 of infection following a systemic infection accompanied by significant weight loss (Fig. 3-5G and data not shown). DKO mice are significantly more susceptible to *C. rodentium* infection than Rag KO mice.

**Discussion**

Surprisingly, VDR KO mice were found to be significantly more resistant to *C. rodentium* infection than WT mice. The mechanisms of colonization resistance in VDR KO mice included dysbiosis in the bacterial microbiota found in the gut of the VDR KO mice. Consistent with previous work (56), VDR KO mice had increased bacterial members of the Bacteroidetes and Proteobacteria phyla but decreased Firmicutes phyla members compared to WT mice. It has been shown that the commensal bacteria especially Bacteroidetes are in competition with *C. rodentium* for structurally similar carbohydrates that are required for *C. rodentium* growth (55). Similarly, the bacteria from the Bacteroidetes phylum inhibit toxin production by enterohaemorrhagic *E. coli*, a human pathogen similar to *C. rodentium*, by secreting a soluble factor (62). Since commensal bacteria do not express the VDR, vitamin D must regulate the commensal microbiota indirectly by regulating the host immune system.
The data also show that VDR KO mice had increased amounts of IL-22-producing ILCs in the SI. This might lead to the increased production of IL-22 early following *C. rodentium* infection. The increased ILCs were also associated with increased amounts of RegIIIγ in the gut of VDR KO mice. The data further demonstrate that ABX disruption of the microbiota had no effect in WT mice but eliminated the colonization resistance of the VDR KO mice. Reduced IL-22 production may lead to the decreased epithelial production of antimicrobial peptides, which removed the colonization resistance in VDR KO mice. Together, the VDR may regulate the microbiome and IL-22 production by ILCs to maintain tolerance in the gastrointestinal tract.

VDR KO mice were extremely susceptible to *C. rodentium* infection after ABX treatment compared to those without ABX treatment. Similarly, germ-free mice infected with *C. rodentium* were unable to clear the infection, suggesting the critical role of commensal bacteria in eradicating *C. rodentium* in the gut (55). None of the germ-free mice died from systemic spread of *C. rodentium* despite high *C. rodentium* numbers in the gut (55). In contrast, our data showed that ABX VDR KO mice were unable to control the *C. rodentium* and 35% of them died within two weeks of infection, while none of ABX WT mice died. In addition, large numbers of *C. rodentium* in systemic organs of ABX VDR KO mice were detected, including the spleen, kidney and liver (data not shown), and the VDR is expressed in innate and adaptive immune cells (63, 64), suggesting that the VDR must be important to control *C. rodentium* infection and prevent systemic spread. T cells and B cells are important in preventing *C. rodentium* colonization in systemic locations. Mice infected with *C. rodentium* showed an increase in CD3+ T cell infiltrates in the colonic LP, most of which were CD4+ T cells (45), indicating a critical role of CD4+ T cells during *C. rodentium* infection. In addition, mice deficient in CD4+ T cells were unable to clear *C. rodentium* infection and developed severe systemic infections (46). In particular, Th17 cell responses have been shown to provide a protective immunity against *C. rodentium* (66). Immunoglobulins (Igs) secreted by B cells are also important in controlling
bacterial loads and systemic dissemination of *C. rodentium*. The VDR KO mice have T cells that are of an activated phenotype and that over produce IFN-γ and IL-17. As yet it is not clear why the VDR KO mice would be more susceptible to *C. rodentium* infection. Perhaps the T cell help for antibody responses is what is regulated by the VDR. Vitamin D has been shown to inhibit human B cell proliferation and antibody secretion (19, 70). B cells from patients with lupus produced reduced amount of antibodies when incubated with 1,25(OH)₂D₃ (71). How the VDR regulates acquired immunity against *C. rodentium* to prevent dissemination is as yet unclear. VDR/Rag DKO mice are more susceptible than VDR KO mice post-*C. rodentium* infection, showing that early innate immunity is also regulated by the VDR. Consistent to the previous study, Rag KO mice were unable to clear *C. rodentium* infection due to the lack of adaptive immune cells and the infection was fatal (72). 1,25(OH)₂D₃ induced production of cathelicidin (an antimicrobial peptide) by macrophage, therefore contributing to innate immune responses to pathogenic infection (73, 74). Previous work has shown that NK cells may directly or indirectly protect the host from systemic spread of *C. rodentium* as NK cell-deficient mice had higher numbers of *C. rodentium* in systemic organs during the peak infection (75). Although VDR KO mice had around 3.2% of NK cells in the SI IEL and the frequencies were not different from WT mice (17), vitamin D status has been shown to regulate NK cell activity (76). NKp46+RORγt+ ILCs were thought to be a new subset of classic NK cells (77, 78). These findings suggest that the VDR regulates *C. rodentium* colonization by regulating the innate immune response in the absence of adaptive immune cells. Without the VDR, mice were unable to control the systemic spread of *C. rodentium* during the early infection. A model of the effect of the VDR and vitamin D on the host resistance to *C. rodentium* infection is shown in Fig. 3-9.
References

8. Froicu, M., and M. T. Cantorna. 2007. Vitamin D and the vitamin D receptor are critical for control of the innate immune response to colonic injury. BMC immunology 8: 5.


Figure 3-1. The kinetics of *C. rodentium* infection in WT and VDR KO mice.

(A) *C. rodentium* numbers in the feces. (B-D) The frequencies of inflammatory monocytes (B), neutrophils (C) and T cells (D) in the colonic LP. (E-G) mRNA expression for *Ifn-γ* (E), *Il-17A* (F) and *Il-6* (G) in the colon. Data is from n=6-12 mice per group and the values represent the mean of three independent experiments ± SEM. Two-way ANOVA with Bonferroni post-hoc tests (A-G), ***P<0.001. Groups without a common letter differ at the indicated time point (B-G), P<0.05.
Figure 3-2

(A) The relative quantity of bacteria from the Firmicutes phylum, the Bacteroidetes phylum and the Proteobacteria phylum in the feces. The bacterial quantity was normalized to the expression of 16S rDNA universal bacteria.

(B) The relative amount of 16S rDNA in the feces of WT and VDR KO mice before and after ABX treatment. The values were normalized to the fecal weight.

(C) Shedding of C. rodentium in the feces of WT, VDR KO, ABX WT and ABX VDR KO mice.

(D) The survival rate of the WT, VDR KO, ABX WT and ABX VDR KO mice following infection with C. rodentium.

Means without a common letter are significantly different, *P < 0.05. (A) The relative quantity of bacteria from the Firmicutes phylum, the Bacteroidetes phylum and the Proteobacteria phylum in the feces. The bacterial quantity was normalized to the expression of 16S rDNA universal bacteria. (B) The relative amount of 16S rDNA in the feces of WT and VDR KO mice before and after ABX treatment. The values were normalized to the fecal weight. Means without a common letter are significantly different, *P < 0.05. (C) Shedding of C. rodentium in the feces of WT, VDR KO, ABX WT and ABX VDR KO mice. Groups without a common letter were significantly different, *P < 0.05. (D) The survival rate of the WT, VDR KO, ABX WT and ABX VDR KO mice following infection with C. rodentium. Data is from n=8-10 mice per group and the values represent the mean of two independent experiments ± SEM. Two-tailed student’s t tests (A), two-way ANOVA with Bonferroni post-hoc tests (B, C), and log-rank test (D), *P < 0.05.
Figure 3-3

(A-C) The relative amount of mRNA expression for RegIIIγ (A), Ang-4 (B) and Muc2 (C) in the colons. The values were normalized to the expression of GAPDH. Means without a common letter differ, $P<0.05$. (D-E) The relative amount of mRNA expression for RegIIIγ (D), Ang-4 (E) and Muc2 (F) in the colons of ABX WT and ABX VDR KO mice at d10 post-infection. Data is from n=6-8 mice per group and the values represent the mean of two independent experiments ± SEM. Two-way ANOVA with Bonferroni post-hoc tests (A-C), two-tailed Student’s t tests (D-F).

Figure 3-3. Expression of antimicrobial peptides and mucins in the colon of WT and VDR KO mice.
Figure 3-4

(A) The frequencies of CD3-RORγt+ ILCs in the SI or colonic LP of WT and VDR KO mice. (B) The frequencies of IL-22-producing ILCs in the SI LP of the uninfected WT and VDR KO mice. (C-E) The frequencies of NK-22 cells (CD3-RORγt+NKp46+), LTi4 cells (CD3-RORγt+NKp46-CD4+), and LTi0 cells (CD3-RORγt+NKp46-CD4-) in the SI and colonic LP of uninfected WT and VDR KO mice. Data shown is one representative of two independent experiments using n=6-8 mice/group. Two-tailed Student’s t tests (A-E), * P<0.05, **P<0.01.
Figure 3-5

(A) The frequencies of ILCs in the SI or colonic LP of Rag KO and DKO mice. (B-D) The frequencies of NK-22 cells (CD3-ROγT+NKp46+), LTIγ cells (CD3-ROγT+NKp46-CD4+), and LTIα cells (CD3-ROγγT+NKp46-CD4- γ) in the SI and colonic LP. (E) The relative quantities of bacteria from Firmicutes, Bacteroidetes and Proteobacteria phyla in the feces. The values were relative to the quantity of 16S rDNA universal bacteria. (F) The C. rodentium shedding in the feces from Rag KO and VDR/Rag DKO mice. (G) The survival rate of Rag KO and VDR/Rag DKO mice following infection with C. rodentium. Data shown is one representative of two independent experiments using n=4-10 mice/group. Two-tailed Student’s t tests (A-E), Two-way ANOVA with Bonferroni post-hoc tests (F), log-rank test (G), *P<0.05, ***P<0.001.
Figure 3-6

(A) The relative quantity of bacteria in the feces of ABX WT and ABX VDR KO mice. (B-D) The relative quantity of (B) Eubacterium and Clostridium perfringens genus, (C) Bacteroides genus and (D) Salmonella and Enterobacteriaceae genus present in the feces of WT and VDR KO mice. (E-G) The relative quantity of bacteria from (E) Firmicutes phylum, (F) Bacteroidetes phylum and (G) Proteobacteria phylum in the colon of untreated or ABX-treated WT and VDR KO mice. (H-J) The relative quantity of bacteria from Firmicutes phylum (H), Bacteroidetes phylum (I) and Proteobacteria phylum (J) in the SI of untreated or ABX-treated WT and VDR KO mice. Data shown is one representative of two independent experiments using n=4-8 mice/group. Values are the mean ± SEM. Means without a common letter differ, *P<0.05. Two-tailed Student’s t-tests (A-D), Two-way ANOVA with Bonferroni post-hoc tests (E-J), *P<0.05, **P<0.01.
Figure 3-7

(A) DGGE banding patterns of 16S rDNA of WT and VDR KO mice before and after ABX treatment. Numbers indicate individual mice before and after ABX treatment. (B) Cluster analysis showing the degree of similarity between the DGGE banding patterns shown in A. Mice treated with ABX had DGGE banding patterns that were more similar to each other than to the samples before ABX treatment. Data shown is one representative experiment of two independent experiments. F: females, M: males.
Figure 3-8

The relative amount of (A) RegIIIβ, (B) cathelicidin-related antimicrobial peptide (CRAMP), (C) β-defesin 3 (mBD-3), (D) mucin (Muc)1, (E) Muc3 and (F) Muc4 in the colon of WT and VDR KO mice. Values are the mean of two independent experiments ± SEM and are normalized to the expression of GAPDH. Two-way ANOVA with Bonferroni post-hoc tests (A-F), means without a common letter differ at the indicated time point, $P<0.05$. 
Figure 3-9. The role of vitamin D in *C. rodentium* infection.

1,25(OH)\(_2\)D\(_3\) regulates the function of T and B cells, which may change the composition of commensal bacteria, affecting the *C. rodentium* colonization in mice. Alterations in the commensal bacteria correlates with the IL-22 production by ROR\(\gamma\)t+ ILCs, which regulates the epithelial production of antimicrobial peptides, affecting the *C. rodentium* colonization. The role of 1,25(OH)\(_2\)D\(_3\) in regulating ROR\(\gamma\)t+ ILCs has not been determined.
Table 3-1

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Table 3-1. The primer sequence for bacterial, cytokines and antimicrobial peptides quantification.
Chapter 4

The role of vitamin D and 1,25-dihydroxyvitamin D3 in regulating plasma cells and *Citrobacter rodentium* susceptibility
Abstract

Vitamin D and the active form 1,25-dihydroxyvitamin D3 (1,25D3) are important regulators of the immune response. Vitamin D-sufficient (D+) and deficient (D-) wild-type (WT) or Cyp27B1 (enzyme that produces 1,25D3, Cyp) knockout (KO) mice were infected with Citrobacter rodentium. D+ WT and D+ Cyp KO mice cleared a primary infection with the same kinetics. D-WT mice cleared a primary infection with delayed kinetics and D- Cyp KO mice developed a severe infection that spread systemically and resulted in the premature lethality of 40% of the mice. At d14 of infection D- Cyp KO mice had increased Ifn-γ and Il-17A in the colon compared to the D- WT and the D+ mice. Decreased levels of antibody, CD4+ T cells, IgA+ plasma cells and IL-22-producing innate lymphoid cells in D- Cyp KO mice correlated with the increased susceptibility of these mice to C. rodentium infection. The response of the D+ WT and D+ Cyp KO mice to primary C. rodentium infection could not be differentiated. However the D+ Cyp KO mice showed reduced abilities to clear a secondary infection with C. rodentium compared to the D+ WT. Overall vitamin D deficiency resulted in the increased susceptibility to C. rodentium infection. There was no effect of 1,25D3 deficiency to a primary C. rodentium infection but 1,25D3 deficient mice did clear a secondary infection with C. rodentium with delayed kinetics.

Introduction

Vitamin D is a steroid hormone that is found in the diet or produced in the skin after sunlight exposure (1). Vitamin D is inactive and it is converted to 25-hydroxyvitamin D3 (25D3) by liver enzymes to form the major circulating form of vitamin D (2). The active form of vitamin D (1,25-dihydroxy vitamin D3, 1,25D3) is produced following additional hydroxylation of the 25D3 to
1,25D3 by the 1α-hydroxylase encoded by the CYP27B1 gene (2-5). 1,25D3 regulates the transcription of target genes by binding to the nuclear vitamin D receptor (VDR) (6). Immune cells express low levels of the VDR that are up-regulated following activation (7-10). 1,25D3 inhibited Th1 and Th17 cells directly and indirectly via regulation of dendritic cells and macrophages (11-13). In addition, 1,25D3 boosted the function of several types of T regulatory cells (12, 13). Vitamin D and 1,25D3 treatment have been shown to suppress Th1/Th17 mediated disease, including inflammatory bowel disease (IBD) (14, 15). Vitamin D and 1,25D3 are important regulators of immune cells, and in the absence of vitamin D or 1,25D3, mice are more susceptible to experimental IBD (16). Vitamin D and 1,25D3 treatment have been shown to inhibit immune mediated diseases where Th1 and Th17 cells are pathogenic.

Th1 and Th17 cell responses are critical for host defense against infection. The data with 1,25D3 inhibition of Th1 and Th17 cells would suggest that 1,25D3 might be harmful to the host response to infection. However, it has been shown that vitamin D induced the expression of antimicrobial peptides (17, 18), which would be beneficial for host resistance to infection. 1,25D3 promoted the antimicrobial activity of human monocytes/macrophages against *Mycobacteria tuberculosis* (19, 20). The activation of Toll-like receptor (TLR) 2/1 enhanced the expression of Cyp27B1 which led to the hydroxylation of 25D3 to 1,25D3 (21). Vitamin D deficiency has been correlated with increased rates of several bacterial infections, such as *Helicobacter, Salmonella* and *Bordetella* (22-25) and viral infections, such as influenza, human immunodeficiency virus and *Hepatitis C* (24, 26, 27). However, other studies showed that vitamin D had neither beneficial nor harmful effects on host immunity to *Listeria monocytogenes* (28, 29), *M. bovis* (30), *M. tuberculosis* (21), *Candida albicans* (31), *Herpes simplex* (31) and *Bordetella pertussis* (32) that requires Th1/Th17 immune responses. Mixed results from different studies showed that vitamin D may or may not play a role in host resistance to infectious diseases.
*Citrobacter rodentium* is a gram-negative murine bacterial pathogen, the colonization of which is limited to the cecum and colon (33). Infection with *C. rodentium* in mice is similar to infection with enteropathogenic *E.coli* (EPEC) in humans (34). Microscopic analysis of the colonic sections from infected mice showed crypt epithelial cell hyperplasia, inflammatory infiltrates and loss of goblet cells (33, 35-37). The adaptive immune system plays a critical role in clearance of *C. rodentium*. T cells (TCRβ+ and CD4+ T cells) were involved in clearance of *C. rodentium* as mice deficient in these cells were unable to clear the infection and died from systemic infection (38, 39). Immunoglobulins (Igs) produced by plasma cells were also involved in the clearance of *C. rodentium* (40, 41). Besides adaptive immune cells, IL-17 and IL-22 produced by innate lymphoid cells (ILCs) were critical in controlling bacterial burden during the early infection with *C. rodentium* (42-44). IL-17A/IL-17F double-KO mice or IL-22 KO mice had high bacterial burdens and increased mortality following infection with *C. rodentium* (45, 46). T cells, antibody producing B cells and IL-22/IL-17 by ILCs are protective following infection with *C. rodentium*. Here we determined the role of vitamin D and 1,25D3 in host resistance to *C. rodentium* infection. Vitamin D deficient (D-) and sufficient (D+) wild-type (WT) and Cyp27B1 (Cyp) KO mice were infected with *C. rodentium*. Cyp KO mice are unable to convert 25D3 to 1,25D3. Cyp KO mice accumulate 25(OH)D (47) and therefore to eliminate the possibility that high concentrations of 25D3 accumulated in Cyp KO mice, D- Cyp KO mice were generated by feeding them with D- diet throughout their lifetime. 25D3 was under the detection level in D- mice. Our data showed that there was no difference in the ability of D+ WT and D+ Cyp KO mice for clearance of a primary *C. rodentium* infection. D- WT mice cleared the infection with reduced kinetics. The D- Cyp KO mice developed a severe infection that resulted in the premature mortality of the mice by d14 post-infection. The increased susceptibility of D- Cyp KO mice to *C. rodentium* was associated with fewer CD4+ T cells, fewer IgA+ plasma cells, and lower IgA and IgG antibody production in the colonic LP of D- Cyp KO mice. Although there
was no difference in clearance of a primary infection, D+ Cyp KO mice cleared a secondary infection with *C. rodentium* with delayed kinetics compared to D+ WT mice, indicating a role for 1,25D3 for a memory response to *C. rodentium*. Our data demonstrate that vitamin D and 1,25D3 are required for host resistance to *C. rodentium* during primary and secondary infection. In the complete absence of vitamin D, mice were unable to control the bacteria in the gut and the mice died of systemic infection. In the absence of 1,25D3, mice showed a problem with the memory response. Vitamin D and 1,25D3 are critical regulators of mucosal immunity in the gut and protection from a gastrointestinal infection.

**Methods and Materials**

**Mice**

Age- and sex-matched C57BL/6 mice were produced and housed at the Pennsylvania State University (University Park, PA). Cyp KO breeders were a gift from Dr. Hector DeLuca (University of Wisconsin, Madison, WI). Heterozygous (Cyp ko/) mice were used as breeders so the WT and Cyp KO littermates were fed the same diets during the experiment. For D- mice, the heterozygous breeders were fed synthetic diets that do not contain vitamin D, as described previously (48). For D+ mice, they were fed synthetic diets containing vitamin D for 2 weeks prior to the experiment and continued throughout the experiment. All of the experimental procedures were approved by the Institutional Animal Care and Use Committee at the Pennsylvania State University.

**C. rodentium infection**

The *C. rodentium* strain ICC169 was a gift from Dr. Gad Frankel (London School of Medicine and Dentistry, London, UK). *C. rodentium* was cultured overnight in luria broth (LB) with 50 µg/ml nalidixic acid (EMD chemicals, Gibstown, NJ). Mice were orally gavaged with 5 x 10⁹
colony forming units (CFU) *C. rodentium* in PBS. *C. rodentium* shedding in the feces was measured by collecting the fecal samples, homogenizing in PBS (0.1g feces/1ml PBS). *C. rodentium* numbers in the distal colon, spleen, liver and peritoneal fluid were measured by collecting the tissues or fluid, weighing or measuring the total volume and homogenizing in PBS (0.1g tissue or 0.1ml fluid/1ml PBS). Serial dilutions were made and plated in triplicate on LB agar plates containing nalidixic acid, cultured overnight at 37°C, and counted colonies. Secondary infections were done 3 weeks after all the mice had cleared the primary infection with the same dose of *C. rodentium*. Histopathology of the distal colon of infected mice was scored on a scale from 0-8, as previously described (49).

**Cell isolation and flow cytometry**

Colonic lamina propria (LP) lymphocytes were isolated as described previously (50). The LP cells were collected from the interface of 40/80% Percoll gradients (Sigma-Aldrich, St. Louis, MO). Cells were stained with FITC CD8β, PE CD8α, PE-Texas Red CD4, PECyp5 TCR β, APC B220, PECyp7 CD3 (eBiosciences, San Diego, CA). For intracellular staining, cells were cultured with PMA (0.1 µg/ml, Sigma), ionomycin (0.5 µg/ml, Sigma) for 5 h and for the final 3 h, Brefeldin A (10µg/ml, Sigma-Aldrich) was added to the culture medium. For IL-22 measurement, cells were stimulated with mouse recombinant IL-23 (0.04µg/ml, R&D systems) for 5 h and for the final 3 h, Brefeldin A (10µg/ml, Sigma-Aldrich) was added to the culture medium. After surface staining, cells were fixed in 4% paraformaldehyde (Sigma-Aldrich), and permeabilized with 0.1% saponin (Sigma-Aldrich). Cells were stained with PE IL-17, APC IL-22 (eBiosciences) and PE IgA (Southern Biotech) or PE/APC IgG1 isotype controls or PE IgG1κ isotype control (eBioscience). For RORγt staining, transcription factor staining buffer set was used and manufacturer’s staining protocol was followed (eBioscience). Cells were analyzed on BD Fortessa LSRII (BD Biosciences) and the data was analyzed with FlowJo 7.6.5 software (TreeStar, Ashland, OR).
Antibody titers

Fecal samples and serum from infected mice were collected. Fecal samples were homogenized in PBS (0.1 g feces/1ml PBS). The samples were serially diluted and incubated in 96-well plated coated with 10 µg/ml sonicated-\textit{C. rodentium} protein. Plates were washed three times, incubated with HRP-conjugated anti-IgA, anti-IgG1 and anti-IgG2a (BD PharMingen, San Diego, CA) and incubated with 3.3’, 5.5’-tetramethylbenzidine substrate (BD Biosciences) and analyzed on a HTS 7000 BioAssay Reader (PerkinElmer, Norwalk, CT).

Quantitative real-time PCR

Total RNA was isolated from 1cm distal colons of mice following the manufacturer’s instructions (Qiagen, Valencia, CA). cDNA was synthesized by using the TaqMan reverse transcription reagents kit (Applied Biosystems, Carlsbad, CA) and was amplified for \textit{Ifn-γ}, \textit{Il-17A} with SYBR green mix (BioRad, Hercules, CA) by MyiQ Single-Color Real-Time PCR machine (BioRad). Expression levels of these molecules were normalized by GAPDH and calculated by using ΔΔCt method. The primer sequences are: \textit{Ifn-γ} (forward 5’-TGCATCTTGCTTTGCAGCTCTCTCTGC-3’; reverse 5’- TGGACCTGTGGGTTGTTGACCTC AAACCTTGGC-3’); \textit{Il-17A} (forward 5’- CAGGGAGAGCTTCATCTGTGT-3’; reverse 5’- GCTGAGCTTTGAGGGATG AT-3’).

Statistics

Statistical analyses were performed by GraphPad (PRISM software, La Jolla, CA). Unpaired two-tailed students t-tests, one-way ANOVA with Bonferroni post-hoc tests, two-way ANOVA with Bonferroni post-hoc tests and log-rank tests were used to calculate statistical significance by GraphPad Prism software. The variances for bacterial numbers were not equal, so these data were transformed (square root of the original data) to eliminate unequal variances. Repeated-measures (mixed model) two-way ANOVA were used to test the effect of group by time on bacterial
numbers. For all the analyses, P≤0.05 was used as the limit for significance. Values are shown as one representative experiment or means ± SEMs of 2-3 independent experiments.

Results

Characterization of immune cell populations in the colonic LP of D- and D+ WT and Cyp KO mice

The frequencies of T cells were measured. D+ WT mice had 50% of TCRβ+ cells in the colonic LP, which was similar to D+ Cyp KO, D- WT and D- Cyp KO mice (Fig. 4-1A). D+ WT mice had 30% of CD8αβ+ cells and 30% of CD8αα+ cells in the TCRβ+ cell population and the frequencies were not different compared to D+ Cyp KO, D- WT and D- Cyp KO mice (Fig. 4-1B, C). The frequencies of CD4+ cells in the TCRβ+ cell population were not different among D+ WT, D+ Cyp KO and D- WT, while D- Cyp KO mice had significantly lower numbers of CD4+ T cells in the colonic LP compared to the other three groups (Fig. 4-1D). D- Cyp KO mice had fewer CD4+ T cells in the colonic LP compared to D+ WT, D+ Cyp KO and D- WT mice.

The IL-22 and IL-17 cytokine production by RORγt+ innate lymphoid cells (ILCs) is important for host resistance to *C. rodentium* infection (44, 51, 52). D+ WT and D+ Cyp KO mice had similar percentage of ILCs (9%) in the colonic LP which were significantly higher than D- WT and D- Cyp KO mice (Fig. 4-1E). The frequencies of IL-22 producing ILCs were high in D+ WT, intermediate in D- WT mice and lowest in D- Cyp KO mice (Fig. 4-1F). D+ Cyp KO mice had the same frequencies of IL-22+ ILCs in the colonic LP as D+ WT and D- WT mice (Fig. 4-1F). The frequencies of IL-17-producing cells were not different among the four groups of mice (Fig. 4-1G). The frequency of IgA+ plasma cells in the colonic LP was similar in D+ WT, D+ Cyp KO and D- WT mice (Fig. 4-1H). D- Cyp KO mice had significantly lower frequencies of IgA+ plasma cells compared to the other three groups of mice (Fig. 4-1H). In the absence of vitamin D,
D- WT and D- Cyp KO mice had fewer total ILCs and IL-22-producing ILCs. In the absence of both vitamin D and 1,25D3, D- Cyp KO mice had fewer CD4+ T cells and IgA+ plasma cells in the colonic LP.

**Increased bacterial shedding in D- mice**

D+ WT, D- WT, D+ Cyp KO and D- Cyp KO mice were infected with *C. rodentium* (Fig. 4-2A-C). The *C. rodentium* shedding in the feces of D+ WT mice was detectable at d2, peaked between d7 and d14, and then fell significantly by d21 and was cleared at d35 post-infection (Fig. 4-2A). D+ Cyp KO and D+ WT mice had similar bacterial shedding in the feces (Fig. 4-2A). Early bacterial shedding was the same in D- WT and D+ WT mice (Fig. 4-2A). D- WT mice had significantly higher numbers of *C. rodentium* after d10 of infection in the feces than D+ WT and D+ Cyp KO mice and they took 56 days to clear the infection (Fig. 4-2A). D- Cyp KO mice had similar bacterial shedding by d10 post-infection but they had 3 logs higher bacterial numbers compared to the other three groups of mice at d14 post-infection (Fig. 4-2A). D+ WT mice gained weight following the infection (Fig. 4-2B). D+ Cyp KO and D- WT mice also gained weight and percentages of body weight change were not different from D+ WT mice (Fig. 4-2B). D- Cyp KO mice lost 10-20% of their original body weight following infection, which was significantly more than D+ WT, D+ Cyp KO and D- WT mice (Fig. 4-2B). All of the D+ WT, D+ Cyp KO and D- WT mice remained alive post-infection, while 40% of D- Cyp KO mice died by d10 following infection (Fig. 4-2C). The rest of the D- Cyp KO mice lost almost 20% of their original body weight at d14 post-infection and were sacrificed as a result (Fig. 4-2B, C). D- Cyp KO mice were extremely susceptible to *C. rodentium* infection. D- WT mice were also more susceptible to *C. rodentium* infection than the D+ mice.

Three weeks after the D+ WT and D+ Cyp KO mice cleared the infection, the mice were infected with a second dose of *C. rodentium* (Fig. 4-2D). As expected, the course of the secondary infection was shorter than the primary infection. Bacterial numbers in the feces of D+ WT mice
peaked at d2, then fell significantly and were cleared by d7 post-infection (Fig. 4-2D). D+ Cyp KO mice had higher bacterial numbers in the feces compared to D+ WT mice and they cleared the infection later than the D+ WT mice (Fig. 4-2D). D+ Cyp KO mice cleared a secondary infection with *C. rodentium* with delayed kinetics compared to D+ WT mice.

**Increased immune responses in the colon of D- mice post-*C. rodentium* infection**

The Th1 and Th17 immune responses were measured in the colon of D+ WT, D+ Cyp KO, D- WT and D- Cyp KO mice. The mRNA expression for Ifn-γ and Il-17 in the colons of mice were low in uninfected mice (Fig. 4-3A, B). At d14 post-infection, the expression of Ifn-γ was significantly increased in all groups of mice compared to uninfected mice (Fig. 4-3A). At d14 post-infection, D+ WT, D+ Cyp KO and D- WT mice had a 4-6 fold increase in the expression of Ifn-γ, while D- Cyp KO mice had an almost 8-fold increase, which was significantly more than D+ WT and D+ Cyp KO mice and similar to D- WT mice (Fig. 4-3A). The expression of Il-17A in the colon of D+ WT and D+ Cyp KO mice remained the same at d14 post-infection compared to uninfected mice (Fig. 4-3B). D- WT and D- Cyp KO mice expressed significantly more Il-17A in the colon at d14 post-infection than uninfected mice (Fig. 4-3B). At d14 post-infection, the expression of Il-17A was low in the colon of D+ WT or D+ Cyp KO mice, intermediate in D- WT mice and highest in D- Cyp KO mice (Fig. 4-3B). D- mice had increased expression of Ifn-γ and Il-17 in the colonic LP at the peak of *C. rodentium* infection.

The histopathology sections from D+ and D- WT and Cyp KO mice at d14 post-*C. rodentium* infection were evaluated. The histopathology scores in WT and Cyp KO mice were similar when fed with D+ diet or D- diet, so the scores of mice with the same diet were combined (Fig. 4-3D and data not shown). The microscopic analysis of colon tissue from D+ mice (both D+ WT and D+ Cyp KO) post-*C. rodentium* infection showed a mild inflammation in the colon, which included loss of goblet cells, the presence of a few cellular infiltrates and mild crypt epithelial cell hyperplasia, resulting in a low histopathology score (Fig. 4-3C, D). However, D- mice (both D-
WT and D-Cyp KO) showed severe inflammation in the colon, including damaged epithelium, greater loss of goblet cells, severe crypt epithelial cell hyperplasia and more infiltration of immune cells, which resulted in significantly higher histopathology scores than in D+ mice (Fig. 4-3C, D). D- mice had more severe colonic inflammation compared to D+ mice.

**Systemic spread of *C. rodentium* infection in D- mice**

To determine whether *C. rodentium* infection spread following oral infection, the colonization of *C. rodentium* was measured in different tissues of mice. There was no *C. rodentium* detected in the distal colon of uninfected mice (Fig. 4-4A). At d14 post-infection, large numbers of bacteria were found in the distal colon of D+ WT, D+ Cyp KO, D- WT and D- Cyp KO mice (Fig. 4-4A). D+ WT, D+ Cyp KO and D- WT mice had similar amount of *C. rodentium* in the distal colon, while D- Cyp KO mice had significantly higher bacterial numbers than the other three groups of mice (Fig. 4-4A). There was also no *C. rodentium* found in the spleen, liver and peritoneal fluid of uninfected mice (Fig. 4-4B-D). At d14 post-infection, D+ WT and D+ Cyp KO mice had no *C. rodentium* in the spleen, liver and peritoneal fluid (Fig. 4-4B-D). However, D- mice had *C. rodentium* in the spleen, liver and peritoneal fluid 14 days after oral infection (Fig. 4-4B-D). D- mice had increased systemic spread of *C. rodentium* following oral infection.

**Lower antigen-specific antibody responses following *C. rodentium* infection of D- mice**

To determine why D- mice were more susceptible to *C. rodentium* infection, the antigen-specific antibody responses were measured. In the uninfected mice, the relative amount of *C. rodentium*-specific fecal IgA, serum IgG1 and IgG2a were under detection level (data not shown). The amount of *C. rodentium*-specific fecal IgA, serum IgG1 and IgG2a in D+ WT and D+ Cyp KO mice were not different between each other at d14 and d28 post-*C. rodentium* infection (Fig. 4-5A-C). The production of *C. rodentium*-specific fecal IgA and the serum levels of *C. rodentium*-specific IgG1 and IgG2a from D+ WT and D+ Cyp KO mice increased by d14 to d28 post-infection. The amount of *C. rodentium*-specific IgA, IgG1 and IgG2a in D+ WT and D+ Cyp KO
mice was significantly higher at d28 than d14 post-infection (Fig. 4-5A-C). The amount of *C. rodentium*-specific fecal IgA, serum IgG1 and IgG2a in D- WT and D- Cyp KO mice were similar at d14 following infection (Fig. 4-5A-C). The amount of *C. rodentium*-specific fecal IgA, serum IgG1 and IgG2a in D- WT and D- Cyp KO mice was significantly increased at d14 post-infection compared to the uninfected mice, and the antibody levels remained the same in D- WT mice by d28 post-infection (Fig. 4-5A-C). The production of *C. rodentium*-specific fecal IgA was similar among the four groups of mice at d14 and D- WT mice had lower amount of IgA than D+ WT or D+ Cyp KO mice at d28 post-infection (Fig. 4-5A). The production of *C. rodentium*-specific serum IgG1 was lower in D- WT and D- Cyp KO mice at d14 compared to D+ WT and D+ Cyp KO mice, and D- WT mice had lower amount of IgG1 than D+ WT or D+ Cyp KO mice at d28 post-infection (Fig. 4-5B). The amount of *C. rodentium*-specific IgG2a in the serum of D+ WT mice was higher compared to D+ Cyp KO, D- WT and D- Cyp KO mice at d14, and D- WT mice had significantly lower level of *C. rodentium*-specific IgG2a than D+ WT or D+ Cyp KO mice at d28 post-infection (Fig. 4-5C).

The frequencies of IgA-secreting plasma cells were measured in the colonic LP of mice post-*C. rodentium* infection. In D+ WT mice, frequencies of IgA+ plasma cells in the colonic LP were increased from 5% to 11% at d14 post-infection compared to uninfected D+ WT mice (Fig. 4-5D and Fig. 4-1H). The frequencies of IgA+ plasma cells in D+ Cyp KO and D- WT mice were significantly increased at d14 post-infection compared to uninfected mice (Fig. 4-5D and Fig. 4-1H). Uninfected D- Cyp KO mice had low frequencies (1%) of IgA+ plasma cells that did not change following infection (Fig. 4-5D and Fig. 4-1H). The frequencies of IgA+ plasma cells in the colon LP of D+ Cyp KO and D- WT mice were not different from D+ WT mice or after infection (Fig. 4-5D and Fig. 4-1H). D- Cyp KO mice had the lowest frequencies of IgA+ plasma cells (Fig. 4-5D). The total cell numbers isolated from colonic LP were not different among the groups, so changes in frequencies represented the changes in cell numbers. Increased severity of
*C. rodentium* in D- mice was associated with lower antigen specific antibody production and lower numbers of IgA-producing cells. The phenotype was most pronounced in the D- Cyp KO mice.

**Discussion**

The data show that D- WT mice had a delay in the clearance of *C. rodentium* following primary challenge compared to D+ controls. The D- Cyp KO mice were more susceptible than the D- WT mice suggesting that in the absence of dietary vitamin D the D- Cyp KO had lower levels of all vitamin D metabolites than D- WT and/or D+ Cyp KO mice. Following *C. rodentium* infection the D- Cyp KO mice developed a systemic infection that resulted in the premature mortality of the mice. The data demonstrates a critical role for vitamin D in the clearance of a gastrointestinal infection. In addition, the D+ Cyp KO mice cleared a primary infection with *C. rodentium* with the same kinetics as the D+ WT suggesting that 25D3 was sufficient for the clearance of the infection and that the ability to produce 1,25D3 is not required for the primary response. For the secondary response the D+ Cyp KO mice had a delayed clearance of the infection suggesting that 1,25D3 is important for the memory response to *C. rodentium*. Others have shown that feeding Cyp KO mice vitamin D in excess results in high 25D3 levels that are adequate for calcium homeostasis and bone mineralization (47). It seems that the primary immune response to *C. rodentium* can largely be regulated by 25D3. It is interesting that the memory response seems to require 1,25D3. The immune system and T cells can make their own 1,25D3 and *in vitro* it has been shown that only activated T cells produce Cyp27B1 (53). The requirement of 1,25D3 for the memory response to *C. rodentium* may reflect the up-regulation of immune derived 1,25D3 production. Vitamin D is critical for host protection to *C. rodentium*. 
The role of vitamin D and the VDR in host resistance to infection has produced conflicting results (31, 54, 55). In contrast to the work presented here, injected 1,25D3 resulted in higher fecal shedding of *C. rodentium* (56). We did not use 1,25D3 to treat our mice and perhaps the discrepancy reflects the non-physiological dosing of mice with 1,25D3 and the effects of hypercalcemia that were not measured. During the early stage of *C. rodentium* infection, the infection kinetics of the mice were similar, indicating 25D3/1,25D3 did not regulate the host immune responses during the early infection. In addition, the number of CD4+ T cells was reduced in D- Cyp KO which corresponded to significantly higher bacterial numbers and increased mortality during peak infection, suggesting the critical role of CD4+ T cells in clearance of *C. rodentium*. The protective role of CD4+ T cells in host resistance to *C. rodentium* has been shown previously (39). The data suggest that 1,25D3 may regulate CD4+ T cells during the late stage of infection, which delayed clearance of a secondary *C. rodentium* infection.

The role of vitamin D and 1,25D3 as regulators of B cells has not been extensively studied. B cells expressed the VDR (57). 1,25D3 has been shown to inhibit the generation of plasma cells in cultures of human cells (57). Our data shows that D- mice have fewer IgA+ plasma cells, suggesting that vitamin D may be required for the development of B cells or the differentiation into plasma cells. Our work is consistent with work that showed that 1,25D3 increased the production of antigen-specific IgA (58, 59). IgA-deficient mice were capable of clearing a *C. rodentium* infection via the production of IgG antibodies (37). CD4+ T cells are required for B cells to undergo isotype switch. Our data showed that D- mice had decreased frequencies of CD4+ T cells and reduced antigen-specific IgA and IgG, indicating vitamin D may regulate CD4+ T cells or antibody responses which affect the *C. rodentium* susceptibility in mice. The D- mice had decreased production of IgG1 and IgG2a that corresponded with the increased susceptibility to *C. rodentium* infection. The data suggests that vitamin D is required for IgA,
IgG1 and IgG2a production and that the increased susceptibility of D- mice to *C. rodentium* infection may be due to defects in normal isotype switching and IgA plasma cell development. Our data indicate that the ability to produce 1,25D3 is not required for clearance of a primary infection with *C. rodentium*, while the ability to produce 1,25D3 is required for the secondary immune response. D- Cyp KO mice had fewer IL-22-producing ILCs, suggesting their protective role in the early stage of *C. rodentium* infection. Vitamin D is required for CD4+ T cells, IgA+ plasma cells and antibodies to generate a protective immune response that clears *C. rodentium*. Systemic spread of *C. rodentium* happened only in D- Cyp KO mice, demonstrating the critical role of vitamin D in maintaining gut homeostasis. Vitamin D is a critical regulator of host immunity and susceptibility to *C. rodentium*. 
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Figure 4-1

(A-E) Frequencies of TCRβ+ (A), CD8αβ+/TCRβ+ (B), CD8αα+/TCRβ+ (C) CD4+/TCRβ+ cells (D), and ILCs (innate lymphoid cells, CD3-RORγt+, E) in the colonic LP of D+ WT, D+ Cyp KO, D-WT and D- Cyp KO mice. The frequencies of TCRβ+ cells and ILCs were determined by gating on colonic LP. The frequencies of CD4+, CD8αβ+ and CD8αα+ cells were determined by gating on TCRβ+ cells. (F-G) The frequencies of the IL-22-producing ILCs (IL-22+CD3-RORγt+, F) and IL-17-producing ILCs (IL-17+CD3-RORγt+,G) in the colonic LP of D+ WT, D+ Cyp KO, D- WT and D- Cyp KO mice. The frequencies were determined by gating on total ILCs. (H) Frequencies of IgA-secreting plasma cells (IgA+B220-) in the colonic LP from uninfected mice. Values are mean ± SEM of 4 mice per group. Data shown is one representative of two independent experiments. One-way ANOVA with Bonferroni post-hoc tests (A-H). Means without a common letter are different between each other, P<0.05.
Figure 4-2. Susceptibility of D+ WT, D+ Cyp KO, D- WT and D- Cyp KO mice to primary and secondary infections with *C. rodentium*.

(A) Fecal shedding of *C. rodentium* following primary infection. (B) Percent original body weight (BW) change. Groups without a common letter differed, *P*<0.05. (C) The survival rate following infection. (D) Fecal shedding following secondary infection with *C. rodentium*. Groups without a common letter differed, *P*<0.05. Data is from n=8-10 mice per group and the values represent the mean of two independent experiments ± SEM. Two-way ANOVA with Bonferroni post-hoc tests (A, B, D), log-rank test (C).
Figure 4-3

Figure 4-3. The immune responses in D+ WT, D+ Cyp KO, D- WT and D- Cyp KO mice post-C. rodentium infection.

(A-B) The expression of mRNA for Ifn-γ (A) and Il-17A (B). The values were relative to the expression of GAPDH. Groups without a common letter differ at the indicated time point, \( P<0.05 \). (C) Histological scores of the distal colon of mice at d14 post-C. rodentium infection. (D) Representative sections of colonic tissue from D+ Cyp KO mice and D- Cyp KO mice at d14 post-C. rodentium infection. Colonic samples were stained with H&E (scoring system in Methods) and were shown at 10x magnification; scale bar = 50 μm. Representative colonic sections shown were rated: D+ Cyp KO (score = 2) and D- Cyp KO (score = 6). Data shown is one representative of two independent experiments using n=3-5 mice per group. Two-way ANOVA with Bonferroni post-hoc tests (A, B) and two-tailed Student’s t tests (C), **\( P<0.01 \).
Figure 4-4

(A-D) Colonization of *C. rodentium* in the distal colon (A), spleen (B), liver (C) and peritoneal fluid (D) from uninfected mice (d0) and infected (d14) mice. The values were normalized by the weight of tissue or the volume of fluid. Data is from one representative of two independent experiments using n=3-5 mice per group. Two-way ANOVA with Bonferroni post-hoc tests (A-D). Groups without a common letter differ at the indicated time point, *P* < 0.05.
Figure 4-5. The antibody responses in the feces, serum and colonic LP of D+ WT, D+ Cyp KO, D- WT and D- Cyp KO mice post-C. rodentium infection.

(A) The relative amount of C. rodentium-specific IgA in the feces. Values were normalized to D+ WT. (B-C) The relative amount of C. rodentium-specific IgG1 (B) and IgG2a (C) in the serum. Values were normalized to D+ WT. (D) Frequencies of IgA-secreting plasma cells (IgA+B220-) in the colonic LP of mice. Data is from n=6-8 mice per group and the values represent the mean of two independent experiments ± SEM. Two-way ANOVA with Bonferroni post-hoc tests (A-C) and one-way ANOVA with Bonferroni post-hoc tests (D). Groups without a common letter differ at the indicated time point, P<0.05.
Chapter 5

Summary and Conclusions
The role of vitamin D and the vitamin D receptor (VDR) in experimental inflammatory bowel disease (IBD) pathogenesis and host resistance to *C. rodentium* infection was investigated in this thesis. In Chapter 2, the effect of vitamin D on CD8\(^+\) T cell subsets was evaluated in the recombinase activated gene (Rag) transfer model of IBD. The microscopic evidence showed that VDR-deficiency results in the generation of pathogenic CD8\(^+\) T cells that contributes to the development of experimental IBD in Rag knockout (KO) recipients. But VDR KO CD8\(^+\) T cells did not induce severe symptoms of experimental IBD in Rag KO recipients, indicating some regulatory cells or cytokines were produced to limit the pathogenicity of the cells. However, CD8/CD28\(^-\) or CD8/CD122\(^+\) T regulatory cells in the VDR KO mice did not play an inhibitory role in experimental IBD. My data shows that the causes of the VDR KO CD8\(^+\) T cell induced IBD was the rapid proliferation of naïve CD8\(^+\) T cells, and the overproduction and response to IL-2. IL-2 has been shown to be a target of 1,25(OH)\(_2\)D\(_3\) treatments and 1,25(OH)\(_2\)D\(_3\) inhibited production of IL-2 (1). The effects of 1,25(OH)\(_2\)D\(_3\) on IL-2 contributed to the suppression of T cell proliferation by 1,25(OH)\(_2\)D\(_3\), but IL-2 addition can only partially reversed the proliferation of 1,25(OH)\(_2\)D\(_3\)-treated T cells (1). The data suggested that the VDR negatively regulates the proliferation and IL-2 production in CD8\(^+\) T cells. Together, these findings provide evidence of the role of the VDR in controlling homeostatic proliferation of CD8\(^+\) T cells and regulating the intestinal inflammation.

In Chapter 3, the effect of the VDR on the host resistant to *C. rodentium* infection was evaluated. Vitamin D has been shown to regulate experimental IBD (2), but what the role of vitamin D in host resistance to *C. rodentium* infection has not been studied. My study showed that VDR KO mice had reduced *C. rodentium* shedding in the feces and inflammatory responses in the colon compared to wild-type (WT) mice. The decreased colonization with *C. rodentium* in VDR KO mice was associated with alterations in gut microbiota composition. VDR KO mice had higher numbers of potentially pathogenic bacteria such as the Proteobacteria phylum and lower numbers
of beneficial bacteria such as Lachnospiraceae from the Firmicutes phylum in the gut compared to WT mice (3). Similarly, patients with IBD had increased numbers of Proteobacteria and decreased numbers of Lachnospiraceae from the Firmicutes phylum compared to the healthy controls (4). Many types of bacteria from the Proteobacteria phyla expressed tetrathionate reductase and tetrathionate respiration resulted in the outgrowth of pathogenic bacteria in the anaerobic environment of the gut during inflammation (5, 6), leading to changes in the bacterial composition. In addition, it has been shown that antibiotic disrupted the gut microbiota promoting commensal competition with C. rodentium for glycan (7). These findings suggest the association between vitamin D deficiency and human IBD pathogenesis, and the role of commensal bacteria on C. rodentium colonization. The VDR is not expressed by commensal bacteria, so the vitamin D/1,25(OH)_{2}D_{3} indirectly regulates the gut microbiota through the effect on host immune response. VDR KO mice had increased expression of antimicrobial peptides and mucin post-C. rodentium infection, and the frequencies of IL-22+ ILCs were increased as well, which may explain the decreased susceptibility of VDR KO mice to C. rodentium infection. The commensal bacteria regulated the IL-22 production by ILCs since germ-free mice were not able to produce as much IL-22 as conventional mice (8, 9). Collectively, these findings suggest that the VDR plays a role in regulating gut bacterial composition and host immune responses that affect C. rodentium colonization.

Finally, in Chapter 4, the role of vitamin D and 1,25(OH)_{2}D_{3} during C. rodentium infection was determined. Vitamin D-sufficient (D+) and vitamin D-deficient (D-) WT or Cyp27B1 (enzyme that produces 1,25(OH)_{2}D_{3}, Cyp) KO mice were used and infected with C. rodentium. Cyp KO mice are unable to convert 25(OH)D_{3} to 1,25(OH)_{2}D_{3}. D- Cyp KO mice were generated by feeding them with D- diet throughout their lifetime to eliminate 25(OH)D_{3} and 1,25(OH)_{2}D_{3}. My data shows that decreased production of antigen-specific antibodies and IgA+ plasma cells in D-Cyp KO mice correlated with the increased susceptibility of these mice to C. rodentium infection,
indicating the role of vitamin D in regulating the antibody responses. Consistent with our findings, 1,25(OH)₂D₃ has been shown to increase the production of antigen-specific IgA as a vaccine adjuvant at mucosal sites (10), suggesting a possible role of 1,25(OH)₂D₃ in IgA production during infection. IgG antibodies have been shown to indirectly provide immune protection against *C. rodentium* (11). IgA is more likely to play a critical role in host protection against *C. rodentium* in the gut compared to IgG, since IgA is secreted into the intestinal lumen by plasma cells (12, 13) and easily interacts with the pathogen. Together, these findings suggest that vitamin D affects host susceptibility to *C. rodentium* by regulating the antigen-specific antibody responses.

The work shown in this thesis highlights the critical role of vitamin D/VDR signaling in regulating gut homeostasis and experimental IBD susceptibility. Vitamin D regulates gastrointestinal homeostasis by controlling the composition of the intestinal bacterial and host immune responses. These findings highlight the potential of vitamin D supplementation as an alternative treatment for IBD and resistance to gastrointestinal infection. I showed that mice had increased number of IL-22-producing RORγt⁺ ILCs in the absence of the VDR, which correlated with the decreased susceptibility of VDR KO mice to *C. rodentium* infection. It is necessary to understand whether the VDR is able to influence the numbers of ILCs intrinsically or not. I showed that VDR KO mice had different composition of gut microbiota compared to WT mice, which caused the colonization resistance in VDR KO mice. It is important to determine whether gut microbiota from VDR KO mice are able to protect the WT mice from colonization with *C. rodentium*. Vitamin D supplementation could be used to improve gastrointestinal homeostasis and prevent severe gastrointestinal infection.
References

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