IMMUNOMODULATORY EFFECTS OF VITAMIN D IN EXPERIMENTAL INFLAMMATORY BOWEL DISEASES

A Thesis in
Pathobiology

by
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ABSTRACT

This work explored the role of vitamin D in the pathology of inflammatory bowel diseases (IBD) using murine models. In an acute or chronic model of experimental IBD, vitamin D or vitamin D deficiency led to a lethal form of intestinal inflammation. Each model used provided insight into the intricate mechanism of interplay between vitamin D and mucosal inflammation. At the level of the innate immune response in IBD, characterized broadly by the dextran sodium sulfate (DSS) model, vitamin D protected the epithelial barrier by increasing epithelial’ cells resistance to pathogenic aggressors and by decreasing the cytokine and chemokine production by innate immune cells in response to normally harmless antigens. At the level of adaptive the immune response, which is represented by the T cell transfer model in immunocompromised recipients and IL-10 KO mice, vitamin D decreased the pathogenic potential of CD4+ and CD8+ T cells in a manner that seemed to be independent of bacterial microflora. Lastly at the regulatory level, T cell transfer experiments revealed that vitamin D, rather than modulating regulatory T cell development, influenced their function during intestinal inflammation. While defects in one of the above mentioned compartments can disturb the fine immune balance and can lead to loss of tolerance for mucosal antigens and thus intestinal inflammation, vitamin D has modulatory capacities in more than one compartment. This thesis advocates for a protective role of vitamin D in establishing and maintaining immunological self tolerance in the intestinal tract.
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Chapter 1

Introduction
Autoimmunity

Autoimmune diseases are distinct clinical syndromes characterized by a breakdown of mechanisms assuring the recognition of self and non-self antigens that result in the damage and eventual dysfunction of the target organ, or the entire organism. Autoimmune diseases cause significant morbidity and disability and afflict 5% of the population in Western countries (1) with an incidence of 1 in 31 Americans (2). Incidence rates vary among sexes with estimated ranges from 65-80% of all autoimmune disease patients being young and middle-aged females in the United States (3). Furthermore it is estimated that approximately 1,186,015 new cases of these autoimmune diseases occur in the United States every 5 years, with women being at 2.7 times greater risk than men to acquire them (4). Young women have been shown to be approximately 10 times more susceptible than young men to developing autoimmune disease (5). It is considered that the younger the patient at disease presentation, the higher the familial risk. Although the exact cause or causes of the nearly 100 autoimmune disorders are not determined, there is a large body of evidence implicating genetic and environmental predisposing factors.

Genetic Factors in the Etiology of Autoimmune Diseases

Epidemiologic studies in both humans and in animal models of autoimmune disorders suggest that polymorphisms of multiple genes confer either protection from or predisposition to autoimmune diseases (6). Studies of concordance in monozygotic (genetically identical) and dizygotic twins (twins with 50% of genes in common) revealed that disease concordance in monozygotic twins is 4 times higher than in
dizygotic twins, but does not reach 100%. Family association studies that compare
disease incidence among first-degree relatives of patients with autoimmune disorders and
the general population also described strong familial association (odds ratio ranging from
5-10) for some of the autoimmune disorders such as multiple sclerosis (MS), type 1
diabetes, Graves’s disease, discoid lupus, and systemic lupus erythematosus (SLE) (6).
The co-association of multiple autoimmune diseases in individuals or families supports
the notion that there may be common genetic factors that predispose to autoimmunity.
Evidence for multiple susceptibility genes involving specific markers such as major
histocompatibility complex (MHC) and non-MHC loci came from genome-wide searches
in rheumatoid arthritis (RA), SLE, and MS (7-9). In experimental animal models there is
also significant overlapping of susceptibility loci for autoimmune diseases (10). Although
there is strong evidence for genetic factors contributing to the etiology of autoimmune
disease, genetics alone cannot account for the high incidence of these diseases in the
general population. Thus, it has been proposed that the immune response of a genetically
predisposed person is influenced by environmental factors that lead to the development of
autoimmune diseases.

Environmental Factors in the Etiology of Autoimmune Diseases

Even in a genetically predisposed person, an environmental exposure or a change in the
internal environment is usually essential for autoreactivity. Epidemiological studies of
genetically similar populations living in different conditions suggest the importance of
environmental triggers. For instance, the incidence of type 1 diabetes and MS in a
population changes as the members migrate to different regions (11, 12). Epidemiological
studies in emigrant populations along with the lower-than-expected rate of disease
concordance among monozygotic twins suggest that an environmental factor exposes an autoimmune diathesis (13). Environmental exposures could include infectious agents, chemicals or other compounds capable of modulating immune responses such as occupational/environmental pollutants or drugs, and behavioral factors such as diet and smoking (14). Clinical studies, in vitro research and animal models determined that environmental factors might be implicated not only in the induction, but also in the progression of the autoimmunity (15). There is evidence that the developing immune system can be permanently altered by the early environment. Early in life, the immune system learns to recognize and preserve self and recognize and destroy non-self without inducing autoimmune damage. Particularly important is the selection of T cells in the thymus leading to the deletion of auto-reactive clones (16).

**Infectious agents**

The study of patients and animal models has determined that bacterial, viral and parasitic infectious agents are involved in the induction and exacerbation of autoimmune diseases. It has been proposed that microbial antigens can initiate autoreactivity through three sets of mechanisms: polyclonal lymphocyte activation, molecular mimicry and the release of previously sequestered antigens (17). For example, viruses like hepatitis C virus have been involved in the etiology of myasthenia gravis (18), rheumatoid diseases (19) and autoimmune hepatitis (20), while Ebstain Barr Virus has been associated with chronic autoimmune diseases, like MS or SLE (21). Bacteria or bacterial products are also associated with autoimmunity. In inflammatory bowel disease (IBD) the normal mucosal microflora is required for the inflammatory process, apparently by providing antigens or
co-stimulatory factors that drive the autoreactivity in a genetically susceptible individuals (22). For example, infection with *Helicobacter pylori* in humans has been associated with autoimmune gastritis (23) whereas *Helicobacter hepaticus* increased the intestinal pathology in experimental models of IBD (24). Infectious agents may be protective for the development of autoimmunity as expressed by the hygiene hypothesis. According to this hypothesis, improved hygiene that lead to the reduced rate of infections in Western countries, is at the origin of increased incidence of allergic and autoimmune diseases (17). For example, injection with coxsackie viruses can enhance (25) or prevent disease in the NOD mouse (26), whereas microorganisms such as 'probiotics' are known to ameliorate, rather than persuade inflammation in IBD (27). Overall it is not clear whether microbial infections are inducers or protectors in autoimmune disorders. In order to find new therapeutic options, it is important to identify the genetic and environmental factors that determine the incidence and evolution and autoimmune diseases.

**Inflammatory Bowel Diseases Background:**

IBDs have increased in incidence in the last decade predominately in Westernized countries (17). The two forms of IBD, Crohn’s disease (CD) and ulcerative colitis (UC), are chronic and relapsing inflammatory disorders of unknown etiology characterized by a perturbed homeostasis between immune cell responses and harmless antigens of the gastrointestinal tract. Factors that predispose individuals to the development of IBD include genetic, microbial, immunological and poorly defined environmental factors (22). CD is a chronic granulomatous inflammation of the gastrointestinal tract that preferentially affects the terminal ileum, but might also affect the entire length of the small and large intestine. It is characterized by “skip” lesions, which are regions of the intestine not
affected by the disease, followed by regions severely affected by inflammation). UC is a
continuous non-granulomatous inflammatory lesion affecting the rectum and colon (28).
With an incidence between 3.1 and 14.6 cases per 100,000 persons/year and an overall
prevalence between 26.0 and 198.5 cases per 100,000 persons/year, CD is not considered a
“rare” disease (29). Actually, its incidence is rising in Westernized countries, and in the
United States there are approximately 1 million patients with CD. IBD incidence pecks
between 30-40 years of age and both genders are at equal risk. The etiology of IBD is very
complex and is a result of perturbations of both innate and adaptive immune responses to
the microflora in genetically susceptible individuals.

**Innate immune defects in IBD**

The immune system can be divided into innate and adaptive components. The innate
immune system is the first line of defense and relies on germline-encoded receptors, toll
like receptors (TLRs) that recognize highly conserved structures found exclusively in
microbial pathogens to control infection through elaboration of pro-inflammatory cytokines
and chemokines that recruit inflammatory cells at the site of injury. The innate immune
system is responsible for containing an infection and /or inflammation until the adaptive
immune system can orchestrate an antigen-specific immune response (30). The role of the
innate immune response in the pathogenesis of IBD emerged from the study of patients
with IBD and from mouse models with genetic abnormalities of components of the innate
immune response. Innate immune cells of the myeloid lineage (macrophages, monocytes,
and dendritic cells) and epithelial cells are clearly affected in IBD (31). For example,
targeted deletion of signal transducer and activator of transcription-3 (STAT-3), an
important transcription factor mediating the cell-signaling pathways of interleukin (IL)-10
and IL-6-related cytokines in macrophages, leads to severe enterocolitis, cachexia, and
death of the mice by 2 months of age (32). Furthermore, cells of innate immunity produce
cytokines such as IL-12 that activate more T cells and contribute to the continuity of the
disease. Immunohistological studies determined that in situ IL-12 is overproduced by
lamina propria mononuclear cells in Crohn's disease, but not in UC (33). Monocytes
isolated from intestinal biopsies of patients with CD produce increased amounts of IL-12 in
vitro, whereas macrophages that are isolated from patients with UC produce decreased
amounts of IL-12, compared with those from normal tissues (34). The importance of IL-12
in the pathogenesis of IBD is reinforced by the finding that treatment with an antibody
specific for the p40 chain of IL-12 leads to a significant amelioration of intestinal
inflammation in most patients (22). More recently, another cytokine IL-23 produced by
APCs was determined to be more important than IL-12 in sustaining inflammation in mice
(35) and humans (36) by continuously stimulating Th1 memory T cells.
Moreover, epithelial cell damage is another feature of IBD. During intestinal inflammation
epithelial proliferation and apoptosis are affected, which can lead to crypt hyperplasia, loss
of villi, ulcerations, and erosion of the epithelial layer (31). Epithelial damage can allow
increased contact between antigens from the intestinal tract and the immune cells in the
submucosa. For example, epithelial damage can be induced in mice via administration of
chemical irritants such as dextran sulfate sodium (DSS). Addition of 30–50 kDa DSS to the
drinking water at 3–10% causes colitis in a variety of animals, including hamsters, rats, and
mice (37). Colitis in these animals is manifested by bloody diarrhea, weight loss,
shortening of the colon and epithelial changes including fibrosis, crypt loss, goblet cell
hypoplasia, and focal ulceration (38).
Innate immune cells of the myeloid lineage function as antigen presenting cells in mucosal tissues and are key cells in the induction of both mucosal effector and regulatory cell responses. It is likely that defects of T cell responses arise either from defects in APC function or APC–T cell interactions (39). Alternatively, Malmstrom et al proposed that APCs are the target of regulatory cells (40). Overall the collaboration between innate and adaptive immune cells is critical in maintaining intestinal homeostasis.

Adaptive immune defects in IBD

Despite the wide variety of causes, effector and regulatory T cells play multiple roles in the pathology of IBD. CD4+ T cells represent the main cell populations that infiltrate mucosal tissues in all models of IBD studied and their deletion in vivo ameliorates inflammation (41). CD8+ T cells are also present in mucosal tissues and their deletion in vivo did not have a major effect on inflammation (42). CD4+ T cells can function in the various models as either Th1 or Th2 effector cells; they can also function as regulatory cells. Experimental models of intestinal inflammation demonstrate that the pathology is a result of either excessive effector T-cell function or deficient regulatory T-cell function. An excessive Th1 response that is associated with increased secretion of IL-12, IFN-γ , TNF-α and reduced secretion of IL-10 was documented in mouse models (43, 44) and patients with CD (45). T cells isolated from the affected tissues of patients with CD or clones that are derived from such cells produce markedly increased amounts of IFN-γ and low amounts of IL-4, compared with controls (46).

In UC, the inflammation is mostly under the influence of Th2 cells, although the influence of Th1 cells is also recognized, and under the influence of autoantibodies such as anti-neutrophil cytoplasmic and anti-tropomyosin antibody (47, 48). Moreover, increased IL-5,
another Th2 cytokine, but normal levels of IL-4, was associated with the evolution of ulcerative colitis (46).

**Regulatory T cells** (also known as suppressor T cells) are a specialized subpopulation of T cells that act to suppress activation of the immune system and thereby maintain immune system homeostasis and tolerance to self. They represent 1–10% of the CD4 T-cell population in healthy adult humans and mice and are characterized by constitutive expression of CD25, Fox P3 and glucocorticoid-induced TNF receptor-related protein (49). Regulatory T cells produce anti-inflammatory cytokines like IL-10 and TGF-β (50). The role of regulatory T cells in IBD was evaluated in experimental models of colitis that showed that transfer of CD4⁺CD25⁺ regulatory T cells are able not only to prevent the disease, but also to reverse an established colitis (51). Control of the proinflammatory function of both Th1 and Th2 cytokines in experimental models of IBD is dependent on the presence of the immunosuppressive cytokines IL-10 and TGF-β secreted by regulatory T cells (52). Regulatory T cells exert their immunosuppressive activities not only on other T cell populations but also on the innate immune system.

Overall there are several lines of defense in the intestine that may be affected during IBD. The first line is the innate immune response that is the main interface with the intestinal flora. Defects in innate immune response can result in IBD in mice even in the absence of the adaptive immune system (IBD in Rag KO or SCID mice that lack adaptive immunity). Moreover defects in antigen presenting cells may induce or accentuate more subtle defects in other compartments of the immune response such as abnormal activation of CD4+ T cells. The second level of immunity that is affected in IBD is represented by an excessive effector T cell response. Pathogenic effector T cells can continue to proliferate and induce
large amounts of cytokines that will perpetuate inflammation in the intestine and will ultimately lead to tissue destruction. The third component of immunity that is critical in IBD is the regulatory mechanism, which is able to suppress pathogenic activation of both innate cells and T cells.

**Vitamin D:**

Vitamin D has been reported to have *in vitro* potent anti-proliferative effects and to inhibit anti-inflammatory cytokine production by the cells that play a direct role in the pathogenesis of IBD. This evidence together with that generated by epidemiological studies previously presented suggested that vitamin D may be a potential candidate that influences the initiation and/or progression of IBD.

**Production and Metabolism of Vitamin D**

The vitamin D hormone, 1, 25-dihydroxyvitamin D3 is the natural form of vitamin D. It is produced from 7-dehydrocholesterol in the skin following exposure to ultraviolet light (270-300 nm range) to generate previtamin D3 which is rapidly isomerized to vitamin D3 (53). Vitamin D3 can also be acquired from dietary foods that naturally contain the vitamin such as liver, salmon, egg yolk and from fortified foods and beverages like milk, soy drinks, orange juice and margarine. Both forms of the vitamin D undergo 25-hydroxylation in the liver, followed by 1α-hydroxylation in the kidney, to make the biologically active compound 1,25-(OH)2D3 (54).

**Calcemic actions of Vitamin D**

Calcium levels are very tightly regulated in the plasma of animals and humans and its concentration is maintained constant (55). The classic function of vitamin D is to maintain calcium and phosphorus homeostasis by regulating calcium and phosphate
absorption in the intestine, by mobilizing calcium from bone when needed, by increasing osteoclastogenesis and the activity of osteoclasts (56) and in the kidney vitamin D is responsible for reabsorption of the last 1% of the filtered load of calcium (57).

However the calcium-sensing proteins that sense plasma calcium concentrations are found in the parathyroid gland (58). Thus, when calcium levels in plasma are lower than normal, the expression, production, and secretion of the parathyroid hormone is stimulated. In the kidney, parathyroid hormone increases tubular reabsorption of calcium, blocks the reabsorption of phosphate (59) and activates the 25-hydroxyvitamin D-1α-hydroxylase (25-OH-D₃-1α-OHase) that converts 25-hydroxyvitamin D₃ to the active hormone, 1,25-(OH)₂D₃. The 24-Hydroxylase is the enzyme responsible for the catabolism of 1,25(OH)₂D₃ that generates calcitroic acid and leads to the excretion of the vitamin (55). The 1α-hydroxylase and the 24-hydroxylase are very tightly regulated by 1,25(OH)₂D₃ itself and by the parathyroid hormone as well. While 1,25(OH)₂D₃ activates its own breakdown by inducing 24-hydroxylase expression and downregulating the 1α-hydroxylase, the parathyroid hormone induces the 1-hydroxylase and downregulates the 24-hydroxylase. These pathways regulated by 1,25(OH)₂D₃ and the parathyroid hormone act synergistically to preserve optimal levels of 1,25(OH)₂D₃ in the serum to maintain constant calcium levels (60).

**Molecular functions of Vitamin D**

One of the important findings about vitamin D was the discovery of its receptor, vitamin D receptor (VDR) in 1974 (61) in the nucleus. The target cells of vitamin D include enterocytes, osteoblasts, and kidney cells, but also parathyroid gland cells, colon cells, skin keratinocytes, promyelocytes, lymphocytes, pituitary gland cells, and ovarian cells
The VDR is a member of the class II steroid hormone superfamily that shares many features with other ligand-activated nuclear receptors such as retinoic acid receptor, thyroid hormone receptor and peroxisome proliferator activator receptor. The VDR contains a DNA-binding domain located towards the NH₂ terminus termed the C-domain, a ligand-binding domain called the E-domain, and an F-domain, which is one of the activating domains. The ligand-binding domain is accountable for high-affinity binding of the ligand, for dimerization with retinoid X receptor, and for binding to other transcription factors (62). The heterodimeric complex of VDR, 1,25(OH)₂D₃ and RXR binds to vitamin D responsive elements located within 1 kilobase of the start site of the target gene. The first insight into possible new functions of 1,25-(OH)₂D₃ beyond its actions in the regulation of calcium and phosphorus came with the discovery of VDR in lymphocytes and myeloid cells over 20 years ago.

**Vitamin D and the Immune system**

VDR expression was confirmed in several immune cells such as monocytes (63), monocyte-derived macrophages (64), dendritic cells (65) and activated T cells (63). The widespread distribution of VDR in the immune system suggested that the vitamin D endocrine system has properties extending well beyond its established role in calcium homeostasis. Indeed, the regulatory actions of vitamin D in immune cells were confirmed by numerous *in vivo* and *in vitro* studies that established 1,25-(OH)₂D₃ as a controller of the growth and differentiation of a broad range of immune cells.

**Effects on DCs and Macrophages**

Constitutive expression of high levels of the VDR in cells of the innate immune system makes them main targets of vitamin D. Indeed, it has been shown that 1,25-(OH)₂D₃ can
act directly to inhibit the differentiation of monocytes into DCs, and inhibit their maturation and function (66). In maturing DCs, 1,25-(OH)$_2$D$_3$ has been shown to inhibit IL-12 production while promoting IL-10 production and inducing spontaneous apoptosis (67). DC subsets that are induced from bone marrow cells have decreased expression levels of MHC class II, CD80, CD40 and leukocyte function-associated antigen-1 molecules following treatment with 1,25-(OH)$_2$D$_3$ and almost completely lost their immunostimulating activity for inducing Th1 immunity and cytotoxic T lymphocyte generation (68). Furthermore, vitamin D treatment significantly decreased the mRNA expression of IFN-γ and IFN-β that are essential for cytotoxic T lymphocyte induction (68). 1,25-(OH)$_2$D$_3$ abrogated the chemotactic response of DCs to CCL4 and CCL19 although the expression of CCR5 and CCR7 and the calcium fluxes triggered by CCL4 and CCL19 were not affected. These findings suggested that the suppressive effect of 1,25(OH)$_2$D$_3$ is also associated with a potent impairment of DC migration in response to inflammatory and lymph node-homing chemokines (69). The role for vitamin D in APC–T-cell interactions is further reinforced by the finding that the 1α-hydroxylase knockout mice have an increased number of mature DCs (70).

In macrophages 1,25(OH)$_2$D$_3$ inhibits IL-12 production by suppressing transcriptional activation of the p35 and p40 genes (71). IFN-γ–activated macrophages have suppressed listericidal activity, reduced phagocyte oxidase-mediated oxidative burst, and decreased a number of IFN-γ-inducible genes (72). Moreover 1,25(OH)$_2$D3 has been shown to down-regulate TLR2 and TLR4 expression on human monocytes at protein and mRNA level in a time- and concentration-dependent manner (73). 1,25(OH)$_2$D$_3$ inhibites DCs differentiation and maturation into potent antigen presenting cells, decreases the ability of
monocytes to senses microbes through downregulation of TLRs and inhibits the inflammatory capacities of macrophages.

An important aspect of the immune modulatory actions of 1,25(OH)_2D₃ appears to be mediated at an autocrine or paracrine level with APCs showing a high capacity for generating 1,25(OH)_2D₃ from its inactive form (65). As DCs differentiate toward a mature phenotype, they increased the expression of 1α-hydroxilase and consequently the synthesis of 1,25(OH)_2D₃, while mature DCs have a decline in expression of VDR leading to a paracrine mode of action for vitamin D on themselves (65). In this model 1,25(OH)_2D₃ produced by mature DCs acts in a paracrine fashion on less mature DCs, which have higher numbers of VDR and will suppress further DC development in a manner that is consistent with immune tolerance (65). Recent reports have highlighted the interaction between APCs and T-cells as the most likely target for vitamin D effects within the immune system.

**Effects on T cells**

Immunomodulatory effects of vitamin D on DC maturation (65, 74) has an indirect impact not only on suppression of T-cell proliferation but also has important consequences on T-cell phenotype, specifically the induction of regulatory T cells, which appears to be an essential component of immune tolerance (75). *In vitro* experiments demonstrated that 1,25(OH)_2D₃ inhibits the proliferation of activated T cells by blocking cell-cycle progression from G1a to G1b (76). The immunosuppressive effects of vitamin D are likely to be both direct, to inhibit CD4+ T cells, and indirect, by inhibiting APCs function and cytokine production. DCs matured in the presence of 1,25(OH)_2D₃ induced cocultured alloreactive CD4^+^ cells to secrete less IFN-γ upon restimulation, up-regulated
CD152, and down-regulated CD154 molecules leading to T cell hyporesponsiveness.

One major regulatory function of $1,25(\text{OH})_2\text{D}_3$ is to down-regulate cytokine synthesis IL-2 and IFN-γ by Th1 cells (77).

More recently it has been shown that vitamin D enhances and promotes the differentiation of Th2 T cells from CD4$^+$ T cells in the absence of APC and increases the production of IL-4, IL-5, and IL-10 while augmenting GATA-3 and c-\textit{maf} expression (78). Mahon et al. confirmed the ability of $1,25(\text{OH})_2\text{D}_3$ to decrease the proliferation of Th cells and decrease production of IFN-γ, IL-2, and IL-5 while increasing production of IL-4 by Th2 cells (79).

Recognition of a small population of T cells with regulatory proprieties resulted from experiments performed in the 1960s and 1970s which described the induction of suppressor T cells capable of down-regulating antigen-specific T-cell responses. The immunosuppressive activity of $1,25(\text{OH})_2\text{D}_3$ might be explained also by its ability to influence the production and/or function of regulatory T cells. The report of Penna et al. revealed that a combination of $1,25(\text{OH})_2\text{D}_3$ and dexamethasone lead to the development of a homogeneous population of IL-10–producing T regulatory cells in both murine and human CD4$^+$ T cells (75). Furthermore, $1,25(\text{OH})_2\text{D}_3$ and mycophenolate mofetil (an immunosuppressor substance) have been shown to induce donor-specific transplantation tolerance to islet allografts by enhancing the production of DCs with a tolerogenic phenotype and enhancing the frequency of CD4$^+$CD25$^+$ regulatory cells (80). The influence of vitamin D on \textit{in vitro} differentiation and function of multiple immune cells implicated in the etiology of IBD constituted the rationale for the research done to
elucidate its contribution in the prevention and/or treatment of these diseases using various animal models of intestinal inflammation.

**Vitamin D and Inflammatory Bowel Diseases**

**Vitamin D status in patients with IBD**

A north-south gradient in IBD incidence has been found in Europe and the United States. With a higher incidence and prevalence of IBD in northern latitudes such as the United Kingdom, Canada, North America and Europe, and a lower incidence in southern locations and nearer to the equator such as East Asia and India (29, 81). The diminished exposure to sun leads to reduced vitamin D synthesis in higher latitudes above and below 35 N and S (82). Furthermore, both UC and CD have a higher debut and relapses during winter when the production of vitamin D in the skin is the lowest.(83-85).

Deficiency and/or insufficiency of vitamin D and subsequent disturbances in calcium metabolism in IBD patients have been shown by many human studies. Sentongo et al. reported that hypovitaminosis D is common and was reported in 31% of IBD patients with the winter season and 56% with African American ethnicity that have markedly reduced cutaneous production of vitamin D (86). McCarthy et al. showed that serum 25-hydroxyvitamin D is significantly lower in CD patients than in control subjects during late-summer and late-winter (87). Gilman et al determined that from 58 Irish patients, 50% and 19% respectively had serum 25 hydroxyvitamin D levels <50 nmol/L during winter and summer (88).

Vitamin D concentrations in the serum have been correlated with disease activity. While undernourished Crohn's patients who have high levels of disease activity have lower circulating levels of vitamin D, those with inactive disease did not differ from the
controls(89). Tajika et al. confirmed this finding and showed that although 27.3% of the Japanese patients with CD were considered vitamin D deficient, the serum 25-hydroxyvitamin D levels were significantly related to disease duration (90).

Regarding vitamin D status in the two main forms of IBD, Jahnsen et al. reported that vitamin D deficiency (25-hydroxyvitamin D <30 nmol/L) was present in 27% of patients with CD and in 15% with UC (91). Vitamin D deficiency of up to 68% in patients with CD was reported by Vogelsang et al (92). In newly diagnosed Korean patients with CD the levels of vitamin D were much lower than in UC (93). In contrast with this study, Lamb et al. showed that although the levels of 25-hydroxyvitamin D were significantly lower in Crohn’s patients compared with the control group, levels did not differ between UC and CD patients (94). Finally genetic studies determined by linkage analysis that the VDR gene represents an important candidate susceptibility gene for IBD due to the fact that the VDR gene maps to a region on chromosome 12 that has been shown to be linked to IBD by genome screening techniques (95). This study was confirmed by Dresner-Pollak et at. (96). Although these observations provide strong evidence that establishes vitamin D as a physiologic regulator of IBD, the mechanism of this regulation remains to be investigated.
REFERENCES


Chapter 2

A Crucial Role for the Vitamin D Receptor in Experimental Inflammatory Bowel Diseases

Chapter adapted from the manuscript entitled:

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Authors: Monica Froicu, Veronika Weaver, Thomas A. Wynn, Mary Ann McDowell, Jo Ellen Welsh and Margherita T. Cantorna

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ABSTRACT

The active form of vitamin D (1,25D₃) suppressed the development of animal models of human autoimmune diseases including experimental inflammatory bowel disease (IBD). The vitamin D receptor (VDR) is required for all known biologic effects of vitamin D. Here we show that VDR deficiency (knockout, KO) resulted in severe inflammation of the gastrointestinal tract in two different experimental models of IBD. In the CD45RB transfer model of IBD, CD4⁺/CD45RB<sup>high</sup> T cells from VDR KO mice induced more severe colitis than wild-type CD4⁺/CD45RB<sup>high</sup> T cells. The second model of IBD used was the spontaneous colitis that develops in IL-10 KO mice. VDR/IL-10 double KO mice developed accelerated IBD and 100% mortality by 8 weeks of age. At 8 wk of age, all of the VDR and IL-10 single KO mice were healthy. Rectal bleeding was observed in every VDR/IL-10 KO mouse. Splenocytes from the VDR/IL-10 double KO mice cells transferred IBD symptoms. The severe IBD in VDR/IL-10 double KO mice is a result of the immune system and not a result of altered calcium homeostasis, or gastrointestinal tract function. The data establishes an essential role for VDR signaling in the regulation of inflammation in the gastrointestinal tract.
INTRODUCTION

The active metabolite of vitamin D (1,25 dihydroxycholecalciferol, 1,25D₃) is an important modulator of the immune system. 1,25D₃ treatment completely blocks the development of at least two different experimental autoimmune diseases (1, 2), and 1,25D₃ therapy prolongs transplant graft survival for longer than the current drug of choice cyclosporine (3). Remarkably, 1,25D₃ selectively regulates these immune responses without compromising the host’s ability to fight infection (4).

The identification of the vitamin D receptor (VDR) in peripheral blood mononuclear cells sparked the early interest in vitamin D as an immune system regulator (5, 6). In vitro, 1,25(OH)₂D₃ has been shown to inhibit T cell proliferation and to decrease the production of the Th1 cytokines IL-2, interferon IFN-γ, and TNF-α (7). Conversely, the production of IL-4 by Th2 cells increases after 1,25(OH)₂D₃ stimulation (8). In vitro, the targets of vitamin D in CD4⁺ T cells have been shown to depend on the activation and differentiation status of the T cell (8). In addition to T cells, cells of the myeloid lineage are also targets of vitamin D. In vitro experiments have demonstrated that 1,25D₃ inhibits IL-12 production by both macrophage and dendritic cells (9, 10, 11). Furthermore, 1,25D₃ renders dendritic cells in a perpetual state of immaturity (9, 10, 11). The nature of an immune response is dependent upon the interaction of multiple cell types that differ in their relative maturational and activation states. This complexity, coupled with the fact that vitamin D treatment has divergent effects depending on activation and differentiation states, indicates that the ultimate effects of vitamin D status on immune responses will vary depending upon the phenotype of a particular immune response.
Not only does 1,25D₃ regulate immunity, but vitamin D deficiency also has profound effects on the immune system. Vitamin D deficiency increases the severity of several autoimmune diseases including inflammatory bowel disease (IBD) and experimental autoimmune encephalomyelitis (EAE). However, mice that are VDR deficient develop less severe EAE than their wild-type (WT) counterparts (12). In support of these data, early work demonstrated that vitamin D deficiency decreased in vivo delayed type hypersensitivity responses (13); the same type of reaction responsible for the pathology associated with EAE. The explanation of the conflicting effects of vitamin D deficiency and the results for EAE in the VDR knockout (KO) mouse may depend on the relative roles of the vitamin D ligand and/or VDR in regulating immune responsiveness.

The experiments described here were performed to determine the impact of VDR expression on the development of the immune response in experimental IBD. The primary immune response to ovalbumin (OVA) immunization was similar in VDR-KO and WT mice, except for an increased burst of IFN-γ secretion from VDR KO splenocytes 1wk after antigen exposure. T cells from VDR KO mice exhibited a greater proliferative response to alloantigens in a mixed lymphocyte reaction (MLR) and increased granulomatous inflammation during Schistosoma mansoni infection as compared with WT mice. Overall VDR deficiency resulted in minor changes in the measured T cell mediated immunity. Next we tested the effect of VDR deficiency on experimental IBD in two different models.

The IBD models chosen were the prototypic IL-10 KO model and the CD45RB^{high} transfer model. The enterocolitis, which develops in IL-10 KO mice, is due to an uncontrolled immune response to conventional microflora because germ-free IL-10 KO
mice do not develop the disease. In addition, mice raised in specific pathogen-free facilities develop milder disease, which does not result in the death of the mice (14). The pathology in the IL-10 KO mice is associated with inflammation of all parts of the small intestine and colon (14, 15). Immunodeficient mice that receive CD4$^+$ CD45RB$^{\text{high}}$ T cells develop a wasting disease and enterocolitis, whereas the transfer of unsorted CD4$^+$ T cells has no effect (16, 17, 18, 19, 20, 21). The significance of CD45RB expression is unclear. It has been suggested that CD45RB$^{\text{high}}$ T cells produce the inflammatory Th1 cytokines IFN-γ, TNF-α, and IL-2 and that CD45RB$^{\text{low}}$ T cells preferentially produce the Th2 type cytokines (16, 21, 22). There are some problems with this hypothesis because Th1 cells that cause type 1 diabetes have been shown to express CD45RB$^{\text{low}}$, whereas naive Th precursor cells express CD45RB$^{\text{high}}$ (18).

VDR/IL-10 KO mice developed a rapid onset of severe colitis, resulting in epithelial hyperplasia and significant weight loss. Spleens from the double KO mice transferred IBD to Rag KO recipients. In addition, there was significant thymic atrophy associated with the IBD in the double KO mice. CD45RB$^{\text{high}}$ T cell-induced IBD was significantly more severe when the CD45RB$^{\text{high}}$ T cells came from VDR KO mice. Taken together, these results point to the essential role vitamin D has in restricting inflammation of the gastrointestinal tract and determining the severity of experimental IBD.

MATERIALS and METHODS

Mice

Age- and sex-matched C57BL/6 (IL-10 KO, Rag KO, VDR KO and WT) mice, and Balb/c mice were produced in the Pennsylvania State University (University Park, PA) breeding colony. The breeding pairs for the Balb/c and IL-10 KO mice were obtained
from The Jackson Laboratory (Bar Harbor, ME). The breeding pairs for the VDR KO and WT mice were originally provided by Dr. Marie DeMay (28) (Harvard University, Boston, MA). To generate the IL-10, VDR double KO mice, female VDR KO mice were bred to male IL-10 KO mice and the double heterozygote mice (F1) were bred back to the VDR KO females (F2). VDR KO/IL-10 heterozygotes were identified and maintained as the breeders for this strain. Genotyping (F2 and future litters) was by PCR. The protocols used were approved by The Pennsylvania State University Animal Care and Use Committee, Protocol No. A314 41-01.

**Genotyping**

Genomic DNA was isolated from tail clippings, and PCRs were performed to determine the genotype of the VDR KO/IL-10 KO mice. IL-10 WT and IL-10 KO/+ mice were identified by PCR products, which were 899bp long. Primers were generated by Invitrogen Corp. (Chicago, IL). PCR with the IL-10 forward primer and the neomycin reverse primer yielded a product of approximately 900bp, which was present only in IL-10 KO/+ or IL-10 KO mice. Genomic DNA from C57BL/6 WT, VDR KO, and IL-10 KO mice served as the controls. Breeders were maintained VDR KO/IL-10 KO/+.

**Diets**

Breeding IL-10 KO and WT mice were fed commercial mouse diets (no. 5105; Ralston Purina, Richmond, IN), and breeding VDR KO mice were fed commercial mouse diets (Harlan Teklad, Madison, WI) high in lactose (20%) and calcium (2%), which have been shown to be required for optimal breeding (2, 28, 29). The experimental diets for all groups were identical and included lactose and high levels of calcium to ensure that the VDR KO mice would remain healthy.
**S. mansoni infection**

Infection of mice with *S. mansoni* was done exactly as described (30). Briefly, groups of 10 VDR KO and WT mice were infected percutaneously with 25–30 *S. mansoni* cercariae, and animals were killed 9 weeks later to determine the size of liver granulomas.

**Immunization**

VDR KO and WT mice were immunized with OVA (Sigma Aldrich, St. Louis, MO) in complete Freund’s adjuvant (Difco Laboratories, Detroit, MI) intradermally. Mice were killed at 7 and 14 days post immunization. The draining lymph nodes and spleens from the mice were collected, made into single cell suspensions and restimulated *ex vivo* with OVA (1mg/ml).

**Proliferation and cytokine production**

Lymphocytes from OVA-immunized mice were cultured for proliferation and cytokine production exactly as described (8). MLR proliferation was done using CD4+ T cells isolated using Cell Select Columns (Cedarlane, Hornnby, Canada) from Balb/c, VDR KO, and WT mice as the responder cells and 10^5 mitomycin C (Sigma, St. Louis, MO)-treated Balb/c splenocytes as the stimulator cells. Unimmunized control mice did not produce OVA-specific cytokines. Mouse IL-2, IL-4, IL-5, and IFN-γ production were detected using ELISA kits from PharMingen (San Diego, CA), and the instructions provided. Detection limits were 25pg/ml IL-2, 62pg/ml IL-4, 312pg/ml IL-5, and 1000pg/ml IFN-γ.

**CD45RB^{high} transfer IBD**

Groups of six C57BL/6 VDR KO and WT mice were killed and the spleens collected. CD4+ cells were enriched using Collect Plus columns (Biotex, Edmonton, Alberta,
Canada) according to the manufacturer’s protocols. The CD4$^+$ T cells were stained with Perkin-Elmer (Foster City, CA) conjugated anti-CD4 antibodies and fluorescein isothiocyanate-conjugated anti-CD45RB antibodies (both from Pharmingen, San Diego, CA). Appropriate isotype control antibodies and single staining controls were run. The CD4$^+$ CD45RB$^{\text{high}}$ cells were sorted using a FACstar (Becton Dickinson, San Jose, CA) cell sorter in the Pennsylvania State University Flow Cytometry Core Facility. The CD4$^+$CD45RB$^{\text{high}}$ T cells made up about 23% of all the sorted cells. $2.5 \times 10^5$ CD4$^+$CD45RB$^{\text{high}}$ T cells were injected into each C57BL/6 Rag KO mice. Twelve weeks after cell transfer, the Rag KO recipients were killed and sections of the SI, cecum, and colon were stained with hematoxylin and eosin at the Penn State Diagnostic Laboratory.

**IBD severity**

Single cell suspensions of the thymus were stained with Perkin-Elmer-conjugated CD4 antibodies and fluorescein isothiocyanate-conjugated CD8 antibodies (Pharmingen). Cells were analyzed on a Coulter (Miami, FL) XL-MCL tabletop cytometer. Tissue were fixed in formalin and sent to the Penn State University Animal Diagnostic Laboratories for sectioning and staining with hematoxylin and eosin. Sections were scored blindly by two observers on a scale of 0 to 4 for inflammation and 0–4 for epithelial thickening.

**Inflammation:**

0, no inflammation; 1, increased number of leukocytes in the mucosa; 2, multiple loci of inflammation, leukocyte infiltration of mucosa and submucosa; 3, extensive leukocytic infiltrate in mucosa, submucosa, ulceration, depletion of mucin-secreting goblet cells; 4, extensive transmural leukocytic infiltrate, crypt abscesses.

**Epithelial thickening:**

0, normal; 1, slight epithelial cell hyperplasia; 2, pronounced epithelial cell hyperplasia (2- to 3-fold increase in crypts); 3, marked epithelial cell
hyperplasia (3- to 10-fold increase in crypts); 4, marked epithelial cell hyperplasia (crypts were more than 10-fold greater). Total histopathology score ranged from 0–8. The ratio of the SI weight to the BW and the LI weight to the BW has been previously been shown to be an objective indicator of inflammation in the gastrointestinal tract (2). The results are presented as means ± SEMs.

Statistics
The data were analyzed by ANOVA, with genotype as a between subject factor. Fisher’s protected least significant difference test post hoc analysis was used to determine significance. The level of significance was set at $P < 0.05$. Data were analyzed using StatView (SAS Institute Inc., Cary, NC)

RESULTS

Lymphocytes from VDR KO mice are of an inflammatory phenotype
This series of experiments used VDR KO and WT mice. All of the mice were fed the same high calcium- and lactose-containing diets. Serum calcium values for VDR KO and WT mice averaged 9.4 and 9.5 mg %, respectively. The expression of CD4$^+$, CD8$^+$, CD45RB, and other T cell markers were similar in the thymus and spleen of WT and VDR KO mice (data not shown). VDR KO and WT mice were immunized with OVA in complete Freund’s adjuvant, and the antigen-specific response was evaluated at 7 and 14 days post immunization. There was no difference in antigen-specific proliferation, and IL-5 and IL-2 secretion from VDR and WT lymphocytes and splenocytes (data not shown). IL-4 was undetectable in supernatants from either the VDR KO or WT mice. Interestingly, VDR KO cells secreted significantly more IFN-$\gamma$ than WT LN and spleen cells at 7 days post immunization (Fig. 2-1A); however, the difference disappeared by 14
days post immunization. Antigen naive (no immunization) IFN-\(\gamma\) secretion was identical from VDR KO and WT T cells stimulated with CD3 and CD28 antibodies (data not shown). The mixed lymphocyte reaction using CD4\(^+\) T cells from VDR KO mice was twice that of CD4\(^+\) T cells from WT mice (Fig. 1B). Lastly, VDR KO and WT mice were infected with \textit{S. mansoni}, and various parameters of the severity of infection were measured. Although there were no differences in weight, calcium, worm burden, and fibrosis in VDR KO and WT mice (Table 2-1 and data not shown), the size of granulomas in VDR KO mice were significantly larger than granulomas from control WT mice (Table 2-1). That is, in the absence of the VDR the granulomatous response was more vigorous. Interestingly, the calcium values in Table 2-1 were significantly lower than calcium values in other experiments (7.7 vs. 9.5)

**Severe and accelerated IBD in VDR/IL-10 double KO mice**

The experimental diets for all groups were identical and included lactose and high levels of calcium to ensure that the VDR KO mice would remain healthy. Serum calcium values for the VDR/IL-10 KO mice were 9.6 ± 0.5 and were not different from IL-10 KO, VDR KO, or WT mice on the same diets. VDR/IL-10 double KO mice developed severe colitis (rectal bleeding) beginning as early as 3 wk of age. By 5 wk of age, the double KO mice weighed 50% of the weight of their littermates and 67% the weight of age matched IL-10 KO mice (Table 2). Macroscopically, the small intestine (SI), ceccum, and large intestine (LI), of VDR/IL-10 KO mice were enlarged compared with VDR KO/IL10 +/- littermates. All of the VDR/IL-10 KO mice were dead by 8 wk of age compared with none of the VDR KO or IL-10 KO mice (Fig. 2-2E). Five-week-old IL-10 KO, VDR KO, WT, and VDRKO/IL-10 +/- mice were also killed and IBD severity compared (Table 2-2
and Fig. 2-2). No symptoms of IBD were apparent in VDR KO or WT mice (Table 2-2 and Fig. 2-2B). Mild symptoms of IBD were present in VDR KO/IL-10 +/- and IL-10 KO mice (Table 2-2, Fig. 2-2C and D). The body weight, the ratio of the SI/body weight (BW), the ratio of the LI/BW, and histopathology scores of the double KO mice were significantly different compared with all other strains. The double KO mice weighed the least but had the largest SI and LI (Table 2-2). Rectal bleeding was apparent in all of the double KO mice and histopathology sections of the colon showed severe inflammation and epithelia hyperplasia (Fig. 2-2A and Table 2-2).

Severe IBD in the double KO mice was associated with a marked involution of the thymus. The thymuses from age and sex matched WT, IL-10 KO, VDR KO, and VDR KO/IL-10 +/- mice were examined as controls for the VDR/IL-10 double KO mice. Thymuses from double KO mice weighed 85% less than the thymuses from any of the single KO or VDR KO/IL-10 +/- littermates (Fig. 2-3A). The total thymocytes recoverable from VDR/IL-10 KO mice were significantly lower (90% less) than all other mice (data not shown), and the proportion of double negative (DN, CD4⁻CD8⁻) and single positive CD4 and CD8 thymocytes was increased in the VDR/IL-10 KO mice compared with all other mice (Fig. 2-3B). Concurrently, there was a significant decrease in the proportion of double positive (DP, CD4⁺CD8⁺) thymocytes in VDR/IL-10 KO mice (Fig. 2-3B).

**VDR/IL-10 double KO splenocytes transfer IBD**

Splenocytes from VDR/IL-10 KO (n=6) or WT (n=3) mice were isolated and injected into recombinase activated gene 2 (Rag) KO recipients (2.5 x 10⁶ cells/mouse). Rag KO mice do not contain T or B lymphocytes, and immune reconstitution of these mice is
possible. The Rag KO recipients were monitored for 12 wk. By 12 wk, all of the VDR/IL-10 KO recipients had diarrhea, and four of the six recipients had prolapsed colons or were bleeding rectally. None of the Rag KO mice, which received WT (Table 2-3), or IL-10 and VDR single KO (data not shown) splenocytes, developed any outward symptoms of colitis. VDR/IL-10 KO splenocytes transferred IBD to Rag KO recipients (Table 2-3). Histopathology scores were as follows: duodenum 4.3 ± 0.6, ileum 1.9 ± 0.5, ascending colon 3.2 ± 0.7, and descending colon 3.5 ± 1.0. WT splenocyte recipients had only mild colitis (scores <2 for all four sections). Interestingly, the inflammation in these Rag KO recipients seems to be most severe in the duodenum of the SI and the colon. The severe IBD, which develops in the VDR/IL-10 KO mice, can be transferred to Rag KO mice using mixed populations of spleen cells.

**CD4\(^+ \) CD45RB\(^{\text{high}} \) induced IBD is more severe when VDR KO cells are used**

The relative ability of VDR KO (vs. WT) CD4\(^+ \) T cells to transfer IBD into the Rag KO mice was assessed. Equal numbers of CD4\(^+ \) CD45RB\(^{\text{high}} \) T cells from either VDR KO or WT mice were injected into Rag KO mice and the mice were kept for 12 wk and IBD severity determined. The Rag KO mice that received the VDR KO CD4\(^+ \) CD45RB\(^{\text{high}} \) T cells weighed the same, had larger SI/BW\%, larger LI/BW \%, and had more severe histopathology scores (Table 2-3). The data show that CD4\(^+ \) CD45RB\(^{\text{high}} \) T cells from VDR KO mice increased the severity of IBD in the recipient Rag KO mice compared with similar cells from WT mice.

**DISCUSSION**

Two different experimental IBD models were made more severe by VDR deficiency. CD45RB\(^{\text{high}} \)-induced IBD is more severe when the T cells are from VDR KO mice. In
addition, VDR/IL-10 double KO mice develop severe and accelerated IBD symptoms beginning at 3 wk of age as compared with 9 or more wk for the single IL-10 KO mice. Transferring whole splenocytes from VDR/IL-10 KO mice to Rag KO recipients resulted in the transfer of IBD. This is important because it shows that the IBD in VDR/IL-10 KO mice comes as a result of the immune system, ruling out an essential role of the gut mucosa or epithelial cells as a cause of the severe IBD in the double KO mice. More severe IBD in these two models validates earlier data, which showed that vitamin D deficiency accelerated the development of IBD in IL-10 KO mice (2).

Paradoxically, EAE severity is less in VDR KO mice, whereas dietary vitamin D deficiency has been shown to accelerate the development of EAE (1, 12). Therefore, autoimmunity in the gastrointestinal tract increases in the absence of vitamin D and in the absence of its receptor, whereas autoimmunity in the central nervous system increases in the absence of the vitamin D ligand and is reduced in the absence of the VDR. The interesting differences in the effect of VDR deficiency in IBD and EAE suggest that there must be fundamental differences in the mechanisms by which vitamin D regulates autoimmunity in the gastrointestinal tract and central nervous system, respectively.

The onset of severe IBD in the VDR/IL-10 KO mice was associated with the involution of the thymus. There was a corresponding 10-fold decrease in the total number of thymocytes isolated from the VDR/IL-10 KO mice compared with either of the single VDR KO, or IL-10 KO mice. Of the remaining thymocytes present in the VDR/IL-10 KO mice, there were increased proportions of CD4+8+, CD8+, and DN (CD4−8−) thymocytes and decreased proportions of DP (CD4+8+) thymocytes. Thymic atrophy has been observed in several model systems, including graft-vs.-host disease (23), aging (24), and tumor
development (25). We hypothesize that in the absence of IL-10, VDR signaling provides a survival signal to DP thymocytes. The VDR/IL-10 KO mice do not have the survival signal and the thymic cells undergo a rapid maturation and death. Further experimentation will be necessary to determine the mechanisms involved in the rapid thymic atrophy, and severe IBD that develops in VDR/IL-10 KO mice.

The role of vitamin D in the regulation of the immune system depends on the nature of the immune response studied. Except for early, high levels of IFN-γ secretion, other cytokine responses in VDR KO mice were similar to the WT mice. VDR KO mice developed larger granulomas after S. mansoni infection. VDR KO and WT mice infected with S. mansoni had low serum calcium values suggesting that maybe the infection resulted in decreased serum calcium. Hypocalcemia is likely up-regulating the 1-hydroxylase to form 1,25D₃, which the VDR KO mice cannot use. It may be the 1,25D₃ that is reducing granuloma volume in the WT mice. It would be of interest to follow up this finding. In a MLR, VDR KO mice had twice the alloreactive T cells compared with WT controls. These similarities and differences in the VDR KO and WT responses suggest that vitamin D plays different roles depending on the immune response and system studied. However, it is clear that irrespective of the system studied, VDR KO mice have a stronger inflammatory phenotype. Because serum calcium was normalized (with the exception of the S. mansoni WT and VDR KO mice), the effects noted must be due to the absence of the VDR and not an affect of calcium homeostasis.

VDR/IL-10 double KO mice developed a severe and accelerated form of IBD. The sensitivity of this autoimmune disease to vitamin D status is likely to be a result of the regulation of the immune system as well as regulation of the epithelial cells and other cell
types in the gastrointestinal tract. However, we have shown that double KO splenocytes are sufficient to transfer IBD symptoms. A second model of IBD (CD45RB$^{\text{high}}$ transfer) was also more severe when induced using VDR KO T cells. Vitamin D deficiency is common in patients with IBD even when the disease is in remission (26, 27). It is unclear why vitamin D deficiency occurs more frequently in IBD. It is probably due to the combined effects of low vitamin D intake, malabsorption of many nutrients including vitamin D, and decreased outdoor activities in climates that are not optimal for vitamin D synthesis in the skin. Although the cause of vitamin D deficiency in IBD may be unknown, the data presented here point to a crucial role of vitamin D and other vitamin D-regulated processes in IBD.
Figure 2-1: **Stronger IFN-γ and MLR Responses in VDR KO Mice**

**A**, Antigen-specific IFN-γ secretion in cells from VDR KO and WT mice. IFN-γ response at 7 and 14 days post immunization. The IFN-γ response at 7 days post immunization was significantly higher in lymphocytes from the spleen and LN of VDR KO than the comparable lymphocytes from WT mice. At 14 days post immunization, the antigen-specific IFN-γ response was similar in spleens and lymph nodes from VDR KO and WT mice. One representative experiment of two. Values are means ± SD of individual mice (n = 6 per group) for the spleen and means ± SD of triplicates wells of pooled LN cells. **B**, T cells from VDR KO and WT mice were tested in a MLR assay. T cells from VDR KO (C57BL/6) mice responded twice as well to Balb/c splenocytes than T cells from WT (C57BL/6) mice (significantly different, P = 0.01). As expected Balb/c T cells didn’t proliferate to syngeneic-Balb/c splenocytes (control). Values are the mean from triplicate experiments ± SEM. Values with different letters are significantly different (P < 0.03) from each other.
Tab. 2-1

Table 2-1: **VDR KO Mice Develop Larger Granulomas than WT Mice Following *S. mansoni* Infection**

<table>
<thead>
<tr>
<th>Mice (n)</th>
<th>Weight (g)</th>
<th>Calcium (mg/dl)</th>
<th>Granuloma volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (18)</td>
<td>28.9 ± 1.2</td>
<td>7.8 ± 0.3</td>
<td>21.0 ± 1.1</td>
</tr>
<tr>
<td>VDR KO (19)</td>
<td>28.2 ± 1.1</td>
<td>7.7 ± 0.3</td>
<td>27.7 ± 2.0</td>
</tr>
</tbody>
</table>

1 The values in VDR KO mice are significantly (*P* < 0.02) larger than in WT mice.
Tab. 2-2

Table 2-2: VDR/IL10 KO Mice Develop Severe IBD Symptoms

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Body Weight (g)</th>
<th>SI/BW (%)</th>
<th>LI/BW (%)</th>
<th>Histopathology Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>17.9 ± 3.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.6 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.5 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>VDR KO</td>
<td>18.6 ± 3.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.9 ± 1.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.5 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL10 KO</td>
<td>12.0 ± 0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.4 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.6 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.0 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>VDR KO/IL10 +/-</td>
<td>15.8 ± 1.1&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>5.0 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.4 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.2 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>VDR/IL10 KO</td>
<td>8.1 ± 1.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.7 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.8 ± 1.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.2 ± 0.4&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All mice were 5 wk old and sex matched (to the VDR/IL10 KO) mice. % SI/BW, Small intestine weight/body weight x 100. % LI/BW, Large intestine weight/body weight x 100. Histopathology scores, The sum of the inflammation score (0–4) and the epithelial hyperplasia score (0–4: total of 0–8, see Materials and Methods). Values are the mean ± SEM of five to eight mice per group. Values with different letters are significantly (P < 0.05) different from each other. The ratio of the SI weight to the BW and the LI weight to the BW has been previously shown to be an objective indicator of inflammation in the gastrointestinal tract (2).
Figure 2-2: **Double IL-10/VDR KO Mice Develop Accelerated and Severe IBD by the Age of 5 Weeks**

Histopathology sections from the colons of double KO mice (panel A, severity score of 6), the colons of VDR KO mice (panel B, severity score of 0), the colon of VDR KO/IL-10 +/- mice (panel C, severity score of 1), and the colon of IL-10 KO mice (panel D, severity score of 2). E, Mortality curve of VDR/IL-10 KO, VDR KO, and IL-10 KO mice. One hundred percent of the VDR/IL-10 KO mice died by 8 wk of age (n = 21) compared with none of the IL-10 KO (n =30) or VDR KO (n =30).
Fig. 2-3: Thymic Involution in Double IL-10 KO Mice

A. Severe IBD is associated with thymic involution in the double VDR/IL-10 KO mice. B. The proportion of CD4⁺, CD8⁺, DP, and DN thymocytes is significantly altered in VDR/IL-10 KO mice. Values are means ± SEM of four to six mice. *, Values from the VDR/IL-10 KO mice were significantly ($P < 0.05$) different from all other groups.
### Tab. 2-3

Table 2-3: **Colitis Symptoms in Rag KO Recipients of VDR/IL10 KO Spleens**

<table>
<thead>
<tr>
<th>Donor Cells</th>
<th>Body Weight (g)</th>
<th>SI/BW (%)</th>
<th>LI/BW (%)</th>
<th>Histopathology Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>VDR KO CD45RB&lt;sup&gt;high&lt;/sup&gt;</td>
<td>17.4 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.0 ± 0.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.4 ± 1.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.8 ± 0.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>WT CD45RB&lt;sup&gt;high&lt;/sup&gt;</td>
<td>18.8 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.8 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.6 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.9 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>VDR/IL10 KO</td>
<td>18.6 ± 1.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.7 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.5 ± 0.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.2 ± 0.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>WT</td>
<td>20.5 ± 2.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.9 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.9 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.1 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
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CD45RB<sup>high</sup>: The spleens from VDR KO and WT mice were used to purify CD4+ T cells, which were sorted for CD4+ CD45RB<sup>high</sup> cells. Two of seven VDR KO recipients and one of seven WT recipients died due to severe colitis before the end of 12 wk. Data are mean ± SEM of five to six remaining mice.

VDR/IL10 KO: The spleens from VDR/IL10 KO and WT mice were injected without further manipulation into Rag KO recipients. By 12 wk, all of the VDR/IL10 KO recipients had diarrhea, and four of the six recipients had prolapsed colons or were bleeding rectally. None of the Rag KO mice, which received WT splenocytes, developed any outward symptoms of colitis.

The ratio of the SI weight to the BW and the LI weight to the BW has been previously been shown to be an objective indicator of inflammation in the gastrointestinal tract (2). Values with different letters are significantly ($P < 0.05$) different from each other.
REFERENCES:

12. Meehan TF, DeLuca HF 2002 The vitamin D receptor is necessary for 1α, 25-dihydroxyvitamin D(3) to suppress experimental autoimmune encephalomyelitis in mice. Arch Biochem Biophys 408:200–204


Chapter 3

Vitamin D receptor is required to control gastrointestinal immunity in IL-10 knockout mice

Chapter adapted from the manuscript entitled:

“Vitamin D receptor is required to control gastrointestinal immunity in IL-10 knockout mice.”

Authors: Monica Froicu, Zhu Yan and Margherita T. Cantorna

ABSTRACT

The vitamin D receptor (VDR) is a nuclear receptor expressed in a number of different cells of the immune system. This study was performed to determine the effect of VDR deficiency on immune function and inflammation of the gastrointestinal tract in a model of inflammatory bowel disease, namely interleukin-10 (IL-10) knockout mice. IL-10 knockout mice were generated which either could or could not respond to vitamin D (double IL-10/VDR knockout; DKO). The distribution and function of lymphocytes in both the primary and secondary lymphoid organs were compared and determined as a function of the severity of intestinal inflammation. DKO mice had normal thymic development and peripheral T-cell numbers at 3 weeks of age, but a week after intestinal disease was detected the thymus was dysplastic with a reduction in cellularity. The atrophy was coupled with increased apoptosis. The spleen weight of DKO mice increased as a result of the accumulation of red blood cells; however, there was a 50% reduction in the numbers of T and B cells. Conversely, the mesenteric lymph nodes were enlarged and contained increased numbers of lymphocytes. The T cells from DKO mice were of a memory phenotype and were hyporesponsive to T-cell receptor stimulation. Colitis in the DKO mice was associated with local and high expression of IL-2, interferon-γ, IL-1β, tumor necrosis factor-α and IL-12. The primary and secondary lymphoid organs in DKO mice are profoundly altered as a consequence of the fulminating inflammation in the gastrointestinal tract. VDR expression is required for the T cells and other immune cells to control inflammation in the IL-10 KO mice.
INTRODUCTION

Crohn's disease is a chronic intestinal inflammatory disorder of unknown etiology. The pathogenesis of this disease is dictated by environmental factors that lead to an inappropriate and exaggerated mucosal immune response to gastrointestinal antigens in genetically susceptible individuals (1). Mouse models of colitis offer new opportunities for identifying inflammatory bowel disease (IBD) genes or pathways that may lead to the development of new therapies for this disease. Mice carrying targeted mutations in a variety of mouse genes, such as interleukin-2 knockout (IL-2 KO), T-cell receptor-β KO and IL-10 KO mice (2) spontaneously develop IBD symptoms resembling certain aspects of human ulcerative colitis or Crohn's disease.

A growing body of evidence suggests that vitamin D is an environmental factor that influences the course and severity of IBD (3). Low vitamin D status has been reported in patients with IBD (4) and, in IL-10 KO mice, vitamin D deficiency accelerates the development of experimental IBD (5). *In vivo* treatment of IL-10 KO mice with active vitamin D [1,25(OH)₂D₃] blocked the progression of disease and prevented their death. In addition, the vitamin D receptor (VDR) gene maps to a region on chromosome 12 that has been linked to IBD by genome screening techniques, and polymorphisms in the VDR gene are associated with susceptibility to IBD (6). There are data in human and animal models of IBD suggesting that vitamin D status and VDR signal transduction are important in determining IBD susceptibility and severity.

The discovery of the VDR in cells of the immune system and the presence of the 1α-hydroxylase in dendritic cells and macrophages (7) suggest that locally produced 1,25(OH)₂D₃ has regulatory autocrine and paracrine properties at the site of
inflammation. Synthesis of active vitamin D requires the presence of 1α-hydroxylase, which catalyses the conversion of 25(OH)D₃ to 1,25(OH)₂D₃. The pleiotropic actions of 1,25(OH)₂D₃ are mediated by its binding to the VDR, which acts as a transcription factor to modulate the expression of specific genes in a tissue-specific manner. The VDR is constitutively expressed in a variety of immune cells (8). Resting T cells (both CD4⁺ and CD8⁺) express low levels of VDR, which are up-regulated following activation. Although a membrane form of the VDR has been described, its role has not been demonstrated in the regulation of the immune system (9,10).

The role of vitamin D in immunity is complex. *In vitro*, 1,25(OH)₂D₃ inhibits the proliferation of activated T cells by blocking cell-cycle progression from G1a to G1b (11) and suppresses the production of IL-2, tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) (12). Monocytes and dendritic cells are also targets of VDR ligands. Dendritic cell maturation is arrested in the presence of 1,25(OH)₂D₃, while in macrophages antigen-presenting capacity, phagocytic activity, and production of granulocyte–macrophage colony-stimulating factor, IL-1, TNF-α and IL-12 are inhibited (13). The *in vitro* effects of 1,25(OH)₂D₃ suggest that vitamin D may preferentially suppress T helper type 1 (Th1) -driven immune responses.

Vitamin D and VDR-mediated regulation of the immune system has been well described *in vitro*; however, there has been little research to assess the effects of the VDR signalling pathway during intestinal inflammation *in vivo*. We have recently reported that lymphocytes from VDR KO mice are of an inflammatory phenotype, produce more IFN-γ upon stimulation and have a stronger response in a mixed lymphocyte reaction compared to wild-type (WT) cells (14). Moreover, CD4⁺ CD45RB<sup>high</sup> T cells from VDR
KO mice transfer more severe colitis than CD4⁺ CD45RB亮眼 T cells from WT mice when injected into leukopenic hosts (recombinase-activated gene KO mice). In addition, we have shown that the VDR/IL-10 double KO (DKO) mouse develops a fulminating form of colitis, leading to premature death at 3–5 weeks of age (14).

Here we characterize the immune changes that contribute to the severe IBD found in the DKO mice. We show that the VDR is highly expressed in the thymus and colon of WT and IL-10 KO mice and that VDR deficiency alters lymphocyte homing and function in both the primary and secondary lymphoid organs during intestinal inflammation. As the DKO mice develop colitis symptoms, the thymus undergoes rapid atrophy, which corresponds to the relative absence of the double-positive CD4⁺ CD8⁺ (DP) T-cell subtype. T cells in the periphery are fewer in number, non-responsive to stimulation and of a memory phenotype. Activated lymphocytes are found in the colon where they overproduce a number of inflammatory proteins including IFN-γ, TNF-α and IL-12. The fulminating colitis in the DKO mice is accompanied by alterations in all of the lymphoid compartments.

MATERIAL and METHODS

Animals

All of the mice were from a C57BL/6 background and housed in conventional animal facilities. IL-10 KO (The Jackson Laboratory, Bar Harbor, ME), VDR KO, WT mice (gift from M. Demay, Harvard University, Cambridge, MA) and DKO mice [previously described (14)] were bred and maintained at The Pennsylvania State University. All VDR KO mice (DKO and single VDR KO) mice were fed diets high in calcium that were
sufficient to maintain serum at a normal level (8.5–9mg/dl) and at the same level as found in WT and IL-10 KO mice. All procedures were reviewed and approved by the Pennsylvania State University Institutional Animal Care and Use Committee.

**Histopathology**

The thymus, spleen, small intestine and colon were removed from the mice, fixed in formalin and sent to the Penn State University Animal Diagnostic Laboratories (University Park, PA) for hematoxylin & eosin (H&E) staining. The inflammation and epithelial injury of the colon were scored blindly on a scale from 0 to 4 using previously described criteria (14,15).

**Reverse transcription-polymerase chain reaction (RT-PCR)**

One-centimeter segments of ascending colon were harvested immediately upon euthanasia and snap-frozen in liquid nitrogen. Colonic mRNA was extracted using RNeasy Midi kit (Qiagen, Valencia, CA) and 5μg total RNA were reverse-transcribed (Promega, Madison, WI). The PCR primer sequences were designed to cross intron/exon borders and were as follows: VDR [129 base pairs (bp)], 5'-TTCATCATGCCAATGCCAATGTCCAC-3' and 5'-GTTCAACCTGCCCCTTCAAT-3'; IL-2 (413bp), 5'-TTCAAGCTCCACTTCAAGCTCTACAGCGGAAG-3' and 5'-GACAGAAGGCTATCCATCTCAGAAAGTCC-3'; IFN-γ (365bp), 5'-TGCATCTTGCTTTGCAGCTCTCTCCTCATGGC-3' and 5'-TGGACCTGTGGGTGGTTGACCTCAAACTTGGC-3'; IL-12p40 (550bp), 5'-CTCACATCTGCTGCTCCACAA-3' and 5'-CTCCTTCATCTTTTCTTTTCTT-3'; IL-12p35 (850bp), 5'-TTGCCCTCCTAAACCACCTCA-3', 5'-CTTGCTCTTCTGCTAAACAT-3'; IL-1β (563bp), 5'-
ATGGCAACTGTTCCTGAACTCAACT-3' and 5'-
CAGGACAGGTATAGATTCTTTTCCTTT-3'; TNF-α (354bp) 5'-
TTCTGTCTACTGAACCTCGGGGTGATCGGTCC-3' and 5'-
GTATGAGATAGCAATCGGCTGACGTTGTTG-3'. PCR cycling conditions were as follows: denaturation at 94° for 1 min, annealing at 59° for 1 min, and extension at 72° for 1 min. The number of cycles was adjusted (30–35) based on the titration of cDNA (for each gene) required to ensure that the PCR reaction was run during the exponential phase of the reaction. PCR products were analyzed on 1.5% agarose gels and the intensity of the bands was determined using densitometry scanning software (Scion Image for Windows, Frederick, MD) and corrected for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) expression.

**Flow cytometry and apoptosis assays**

Spleen cells (10^6) were stained for flow cytometry. The antibodies were phycoerythrin (PE)-conjugated anti-mouse CD4 (L3T4), CD44 (IM7), CD69 (H1.2F3), immunoglobulin M (IgM; AF6-78) (BD Pharmingen, San Diego, CA), F4/80 (BM8) (Bioscience, San Diego, CA), fluorescein isothiocyanate (FITC) -conjugated anti-mouse CD8 (53-6.7), CD62L (MEL-14), B220 (RA3-6B2), CD25 (7D4) (BD Pharmingen) and CD11b (M1/70) (Bioscience).

To analyze apoptosis freshly isolated thymocytes (10^6) were resuspended in sterile saline and fixed by the drop-wise addition of 2ml ice-cold 70% ethanol. The cells were held on ice for 1 hr and then resuspended in 500μl of propidium iodide (PI)/RNase A solution (BD Pharmingen) and incubated at room temperature for 1 hr in the dark. Annexin V staining alone was performed according to the manufacturer's instructions (Apoptosis
Detection Kit, BD Pharmingen). Double staining with Annexin V and PI was carried out using Annexin V staining followed by the incubation of the cells with 50μg/ml PI. Flow cytometry analysis was performed on an XL-MCL benchtop cytometer (Beckman Coulter, Miami, FL).

**Lymphocyte proliferation assay**

CD4$^+$ and CD8$^+$ T cells were isolated from the spleen and mesenteric lymph nodes (MLN) using Cell Select Columns (Cedarlane, Hornby, Canada). The purity of the T cells was at least 95%. For stimulation, cells were seeded at $5 \times 10^5$ cells/ml on plastic dishes coated with 10μg/ml anti-CD3 monoclonal antibody. Then, 0.4 μCi [$^3$H]thymidine (ICN, Costa Mesa, CA) was added to each well (96-well plate, Costar, Corning, NY) at 48 hr and the cells were incubated for an additional 24 hr. Radioactive thymidine incorporation was determined by liquid scintillation using a beta plate counter.

**Blood analysis**

Blood was collected by cardiac puncture in tubes coated with ethylenediaminetetraacetic acid (Becton Dickinson, Franklin Lakes, NJ) and analyzed using an ADVIA 120 Hematology System (Bayer Diagnostic, Tarrytown, NY).

**Intracellular Ca$^{2+}$ measurement**

Splenocytes were collected and the red blood cells were lysed using hypotonic buffer. Cells were loaded with Fluo-4 (Invitrogen, Eugene, OR; 10mg/ml) at 37° for 45 min at a concentration of 3–4mg/ml. The cells were then washed twice and the mean fluorescence intensity vs. time was measured. Gating was carried out using the forward and right-angle light scatter after the exclusion of dead cells.
Data analysis

Results are expressed as the mean ± SE. Statistical analysis was performed using the unpaired t-test and analysis of variance (ANOVA; StatView, SAS Institute, Cary, NC). A value of $P \leq 0.05$ was considered statistically significant.

RESULTS

VDR distribution in colon, primary lymphoid and secondary lymphoid organs

VDR expression was measured by RT-PCR in the thymus, spleen, MLN and colon of IL-10 KO and WT mice. VDR mRNA expression levels were comparable in the thymus, spleen and MLN of IL-10 KO and WT mice (Fig. 3-1). Within the thymus the VDR was expressed constitutively in DP as well as CD4/CD8 double-negative thymocytes (data not shown). VDR was also highly expressed in the colon, including high VDR expression in colons from young IL-10 KO mice (Fig. 3-1). The ratios of VDR to G3PDH × 100 were 81 ± 10 in old IL-10 KO mice that developed colonic prolapse and 118 ± 5 in 5-week-old and healthy IL-10 KO mice without symptoms. There was no change in colonic VDR expression in young versus old WT mice (VDR/G3PDH × 100 ratios were ~ 120 for both). Interestingly, the development of IBD symptoms in IL-10 KO mice was associated with a 31% reduction in VDR expression in the colon.

Over-expression of inflammatory cytokines in the colons of DKO mice during severe IBD

DKO mice developed severe IBD that involved the whole intestinal tract and necropsies of the mice showed the most dramatic changes in the gross anatomy and inflammation of the colon (Fig. 3-2a), with the majority of the DKO mice developing rectal prolapse
Cytokine expression in the colon was measured by RT-PCR using mice that were age-matched (5 weeks old) to the DKO mice. Consistent with their non-inflammatory status, WT mice did not express any cytokines in the colon (Fig. 3-2c). In contrast, VDR KO mice expressed IL-1β in the colon as well as faint TNF-α bands (Fig. 3-2c). Old VDR KO mice (9 months) had 10-fold higher IL-1β expression and 20-fold more TNF-α in the colon (data not shown). IL-10 KO mice with minor symptoms of IBD expressed TNF-α, IL-2 and IFN-γ but not IL-1β in the colon (Fig. 3-2c). The DKO mice expressed two- to three-fold higher levels of IL-1β, IL-2, IFN-γ and TNF-α mRNA in their colons compared with the colons of single KO control mice (Fig. 3-2c). Interestingly, only the DKO mice expressed IL-12p35 and p40 in the colon (Fig. 3-2c). Th2 genes (IL-4 and IL-5) were undetectable in mRNA from the colons of WT, VDR KO, IL-10 KO and DKO mice.

**Accelerated thymic involution in mice with severe IBD**

Thymic size and cellularity was normal in young DKO mice (3–4 weeks old), which had not developed IBD [low large intestine (LI)/body weight (BW)% ratio]. We have shown previously that the LI/BW ratios are an objective measure of the severity of IBD in IL-10 KO mice (15). The DKO thymus weight and cellularity declined rapidly as the mice developed IBD (increase in LI/BW percentage; Fig. 3-3a), whereas the size and cellularity of the thymus in single KO and WT mice increased over the ensuing weeks (data not shown). The total thymocyte number in the thymus of DKO mice at the peak of IBD disease was less than 10–20% of that of the single KO mice (Fig. 3-3a). Histological staining revealed pronounced structural changes in the thymus of DKO mice at 5 weeks of age (Fig. 3-3b). DKO mice with progressive disease had thymuses with no
clear corticomedullary demarcation, scattered cortical cells and an accumulation of thymocytes in the medulla (Fig. 3-3b). The thymuses of VDR KO and IL-10 KO mice at 5 weeks of age were no different from those of WT mice (Fig. 3-3b).

**DKO thymocytes are hypersensitive to apoptosis**

DP cells are the major cell type that undergo apoptosis during normal thymic development (16) and are extremely sensitive to systemic mediators of apoptosis (17). Because the DP population is severely reduced during the progression of the disease, we investigated whether increased apoptosis was the mechanism responsible (Fig. 3-4). Ten per cent of DKO thymocytes were PI-positive compared with only 1% in single KO mice; therefore, more apoptotic events had occurred in the DKO thymus. In addition, evidence of early apoptosis was found in the DKO thymocytes which showed higher levels of Annexin V staining, 25%, compared to 14% in control thymocytes. Double staining with Annexin V and PI was used to discriminate between early stage (annexin V\(^+\) PI) and late-stage (annexin V\(^+\) PI\(^+\)) apoptotic cells. A similar percentage of cells were in the early stage of apoptosis in all groups (Fig. 3-4). However, the DKO thymuses had significantly more thymocytes in the late-stage of apoptosis (20%) compared with VDR KO, IL-10 KO and WT mice (7%, Fig. 3-4).

**Alterations in the peripheral lymphoid compartments**

Severe IBD symptoms (bleeding, weight loss and rectal prolapse, Fig. 3-2b) in DKO mice were associated with four-fold increases in blood neutrophil counts and two-fold decreases in lymphocyte cell counts \((P > 0.05, \text{Fig. 3-5})\). In addition, the decreased number of erythrocytes and lower hemoglobin concentrations in the DKO mice (Fig. 3-5) suggested that the DKO mice were anemic.
The spleens from DKO mice were two-fold larger than single KO and WT mice. Histological sections of the spleen from DKO mice (Fig. 3-6a) revealed an almost complete absence of the white pulp and an expanded red pulp that had a congestive aspect because of the accumulation of red blood cells. The spleens from both single KO and control mice were normal showing the obvious presence of germinal centers and good demarcation of the red and white pulp (Fig. 3-6a). The overall cellularity of the spleen from DKO mice was reduced, with a decrease in the percentage of CD4\(^+\), CD8\(^+\) and B cells to approximately half the levels found in VDR KO, IL-10 KO or WT mice (Fig. 3-6b). Conversely, the F4/80/CD11b macrophages were higher in the spleen of DKO mice than in other strains of mice (Fig. 3-6b).

The MLN from DKO mice were three-fold to four-fold enlarged and had increased cell numbers relative to other strains of mice (data not shown). The percentages of CD4\(^+\) and CD8\(^+\) T cells in the MLN were the same in the DKO, VDR KO, IL-10 KO and WT mice, but the absolute number of CD4\(^+\) and CD8\(^+\) T cells were three-fold higher in MLN from DKO mice.

The DKO-derived CD4\(^+\) T cells were large as measured by forward light scatter, but showed normal expression of the activation markers CD25 and CD69 (data not shown). The percentage of CD4\(^+\) CD25\(^+\) T cells was not different in the spleen, thymus or MLN of DKO, VDR KO, IL-10 KO and WT mice. The DKO CD4\(^+\) T cells had a memory phenotype characterized by high expression of CD44, and low expression of CD62L (data not shown). Conversely, the CD4\(^+\) T cells from the single KO and WT mice were of a naive phenotype and had low expression of CD44 and high expression of CD62 ligand.
To determine if the cells were functional, the CD4^+ and CD8^+ T cells from DKO mice were stimulated with anti-CD3 antibodies. Interestingly, both the CD4^+ and CD8^+ DKO T cells showed reduced proliferative capacity compared with cells from single KO and WT mice (Fig. 3-7a). In addition, intracellular free calcium concentrations [Ca^{2+}], which are essential for T-cell proliferation, differentiation and apoptosis, were determined. Ca^{2+} concentrations were significantly lower in unstimulated DKO lymphocytes (mean fluorescence intensity 3.3; Fig. 3-7b) compared to lymphocytes from single KO and WT mice (mean fluorescence intensity from 7.9 to 8.3, Fig. 3-7b).

**DISCUSSION**

VDR/IL-10 DKO mice spontaneously develop a fulminating form of IBD involving all areas of the small intestine and colon. Because the inflammatory process occurs throughout the small intestine and colon, the disease in DKO mice is most similar to Crohn's disease in humans. Additionally, their thymuses undergo rapid atrophy, while thymocytes and lymphocytes in peripheral organs undergo phenotypic and functional changes. The net result of the immune system alterations in the DKO mice is a severe form of IBD and over-expression of numerous inflammatory cytokines in the colon. In contrast, the disease in IL-10 KO mice with functional VDRs is less pronounced (milder, affected less mice, and took many months to develop), suggesting that VDR deficiency exacerbates IBD severity. The rapid kinetics of the immune alterations in DKO mice argues that VDR signalling is an additional factor controlling disease susceptibility in IL-10 KO mice. It is noted that neither the VDR KO nor IL-10 KO mice developed such an
aggressive form of IBD. Therefore, this accelerated and increased severity of disease was a cumulative effect of both VDR and IL-10 deficiencies.

As expected, WT mice did not express inflammatory cytokines in the colon. Age-matched IL-10 KO mice developed minor colitis, and expressed TNF-α, IFN-γ and IL-2 in the colon, but not IL-1β, or IL-12. VDR KO mice with macroscopically and microscopically normal gastrointestinal tracts had high IL-1β expression in the colon and detectable TNF-α expression. Interestingly, older VDR KO mice express more IL-1β and TNF-α in the colon than younger VDR KO mice. Surprisingly, only DKO mice showed up-regulation of IL-12 p35 and p40 in the colon, suggesting that their immune response was strongly Th1 predisposed.

Colitis in IL-10 KO mice is a Th1-mediated disease and Th1 cytokines, especially IFN-γ, IL-12 and TNF-α, have been shown to play an important role in its pathogenesis. TNF-α blockade resulted in clinical improvement of experimental IBD (18,19). The inhibition of IBD symptoms by 1,25(OH)₂D₃ treatment of IL-10 KO mice has been shown to be partly the result of the inhibition of the TNF-α pathway (20). Administration of neutralizing antibodies specific for IL-12 or IFN-γ prevented colitis in young IL-10 KO mice (21). Like the VDR KO and DKO mice, biopsies from Crohn's disease patients show increased IL-1β expression in both the inflamed and non-inflamed areas (22). In another model of Th1 autoimmune disease (type 1 diabetes), vitamin D deficiency is associated with higher IL-1β expression at the site of inflammation (23). In addition, 1,25(OH)₂D₃ has been shown to suppress macrophage production of IL-1β in vitro (24). It will be interesting to evaluate the colon of VDR KO mice in aged animals to determine if VDR deficiency alone predisposes them to IBD symptoms. However, 12-week-old VDR KO mice did not
have inflammatory cells in the colon (data not shown). The severe IBD and high expression of IL-1β, IL-2, IL-12, IFN-γ and TNF-α in the colons of DKO mice suggest that VDR signalling protected IL-10 KO mice from an early burst of IL-12 and overproduction of IL-1β in the colon, which induces Th1 cells and colitis as early as 3–6 weeks of age.

Approximately 90% of the DKO mice became anemic at 5 weeks of age with decreased numbers of red blood cells and hemoglobin concentrations, whereas none of the IL-10 KO mice showed hematological abnormalities. The 5-week-old IL-10 KO mice in our colony did show minor microscopic evidence of intestinal pathology; however, they did not develop outward symptoms of disease, or show hematological abnormalities. In some mouse colonies, IL-10 KO mice have been reported to develop anemia associated with the IBD symptoms, but not before 7–11 weeks of age (25). The abrupt debut of anemia in 3- to 5-week-old DKO mice must be a consequence of the IBD symptoms, which included intestinal bleeding. In what may be an attempt to bolster the red blood cell number, the spleens from DKO mice increased approximately three-fold in size because of hyperplasia of the red pulp, indicating that extensive extramedullary hematopoiesis occurred to attempt to compensate for the anemia.

The thymuses of DKO mice with IBD were 10–20% less in both size and cellularity compared to age-matched IL-10 KO, VDR KO, or WT mice. This thymic atrophy might be a consequence of several mechanisms: blockade of thymic development, decreased proliferation, increased apoptosis, or elevation of serum corticosteroids. The number of thymocytes and the thymic mass were normal in young DKO mice but immediately after the onset of disease, acute thymic atrophy was detected. DP cells are the major cell type
undergoing apoptosis during normal thymic development (26) and are extremely sensitive to systemic mediators of apoptosis such as the corticosteroids (27). Increased numbers of DKO thymocytes were in the early and late stages of apoptosis compared to the single KO and WT thymocytes. However, DKO thymocytes proliferated normally when stimulated with concanavalin A and IL-2 (data not shown). Only 2–3 days separated the onset of macroscopically detectable intestinal symptoms and the drastic reduction of the thymus in DKO mice. The rapidity of the thymic atrophy, which follows the development of a severe form of IBD, argues that corticosteroid production induced by the severe IBD may be the underlying cause of thymic atrophy. It seems likely that during the severe intestinal stress in DKO mice, pro-inflammatory cytokines such as IL-1β and TNF-α stimulated production of corticosteroids, which resulted in the rapid apoptosis of the DP thymocytes. Endotoxic shock (driven by IL-1β and TNF-α) has been shown to upregulate corticosteroid production and to result in rapid atrophy of the thymus (28). Our results suggest a scenario in which VDR signalling promotes thymocyte survival and during systemic stress protects thymus integrity and function by controlling the level of the systemic inflammatory response.

DKO mice with colitis had large, but lymphopenic, spleens with a 50% reduction in the number of CD4+ and CD8+ T cells and B cells. In addition DKO mice had a 50% reduction in lymphocytes in the blood. Conversely, the MLN were enlarged, and had a two- to three-fold higher number of CD4+ and CD8+ T cells. The data suggest that the lymphocytes may have relocated from the spleen and peripheral blood in closer proximity to the site of active inflammation. Both CD4+ and CD8+ T cells from DKO mice had a markedly decreased proliferative response compared with WT and single KO T cells, suggesting
that peripheral T cells from DKO mice were anergic. The hyporesponsiveness of DKO T cells might be a result of defective T-cell signalling at the membrane receptor level or downstream of the TCR. Nevertheless, the mitogenic response of lymphocytes is dependent on increases in intracellular free Ca$^{2+}$, which activates calcineurin, dephosphorylates nuclear factor-AT and induces transcription of IL-2 resulting in proliferation (29). Concentrations of Ca$^{2+}$ were significantly lower in resting DKO lymphocytes compared to single KO and WT lymphocytes, suggesting that the DKO lymphocytes failed to adequately accumulate intracellular Ca$^{2+}$. These results suggest that VDR signalling was involved in intracellular calcium regulation and may account for the lymphocyte hyporesponsiveness observed in DKO mice with colitis. Interestingly, serum Ca$^{2+}$ from DKO mice was not different from the other strains of mice (data not shown).

Younger DKO mice, which did not show outward signs of colitis, had normal spleens, MLN and lymphocyte function.

Together our data in this murine model of experimental colitis define VDR signalling as a potent anti-inflammatory mechanism \textit{in vivo}. VDR deficiency accelerated the development of experimental IBD in IL-10 KO mice by inducing phenotypic and functional changes in T-cell responsiveness. The combined VDR and IL-10 deficiency resulted in acceleration, 100% incidence and the increased severity of IBD symptoms. Since all of the immune changes (thymus, spleen and MLN) only occur in mice with IBD symptoms it is difficult to determine what effect VDR deficiency has on the immune system to cause the accelerated and fulminant IBD. It is clear that VDR expression is essential for the IL-10 KO mouse to maintain the level of lymphocyte homeostasis required to control the gastrointestinal immune response to commensal bacteria.
**Fig. 3-1**

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Figure 3-1: **Constitutive expression of the VDR in thymus, spleen, MLN and colon of 5-week-old IL-10 KO and WT mice.** RNA was isolated, reverse transcribed and then amplified using PCR with primers specific for the VDR and G3PDH. The G3PDH bands indicate that equivalent amounts of mRNA were analyzed in each sample. One representative experiment of five is shown.
Figure 3-2: **Fulminant colitis and cytokine expression in the colons of DKO mice.** (a) The cecum and colon of DKO, IL-10 KO, VDR KO and WT mice. The DKO colon is enlarged compared to the colons of age-matched single KO or WT mice. (b) Rectal prolapse is common in DKO mice at 5 weeks of age. Age-matched IL-10 KO, VDR KO and WT (CTR-control) mice did not develop colonic prolapse. (c) Cytokine expression in the colon of DKO mice. RT-PCR amplification of G3PDH, TNF-α, IL-2, IFN-γ, IL-1β, IL-12 p35 and IL-12 p40 mRNA extracted from colonic tissue of mice at 5 weeks of age. The PCR products were run on a 1.5% agarose gel and stained with ethidium bromide.
Figure 3-3: **Thymic involution parallels colitis development in DKO mice.** (a) Cell number in the thymus of DKO mice as a function of the ratio of the LI/BW%. (b) H&E staining of the thymus.
Figure 3-4: **Increased thymocyte susceptibility to apoptosis.** Thymocytes were stained with annexin V and PI. The percentage of cells that were in early apoptosis (annexin V$^+$ PI$^-$) or late apoptosis (annexin V$^+$ PI$^+$) were determined. The results are representative of experiments with six mice per group. DKO values for late apoptosis were significantly different from single KO and WT values. $P < 0.05$. 
Figure 3-5: **Anemia and alterations in neutrophil and lymphocyte subpopulations in the peripheral blood of DKO mice.** Blood was analyzed using the Advia system and the percentages of neutrophils (Neut), lymphocytes (Lymph), red blood cells (RBC) and hemoglobin (Hgb) in the blood of each strain were determined. *Data are expressed as mean % ± SE of six mice per group. DKO values were significantly different than those from VDR KO, IL-10 KO and WT mice. P < 0.05.
Figure 3-6: The spleen of DKO mice is larger and contains more macrophages but fewer T and B cells than the spleens of single KO or WT controls. (a) H&E staining of the whole spleen sections. (b) Single cell suspensions of spleens from DKO, IL-10 KO, VDR KO and WT mice were analyzed by flow cytometry for the expression of CD4, CD8, B220/IgM, and F4/80 CD11b. Reduced CD4, CD8 and, B220/IgM proportions and increased F4/80 CD11b proportions in splenocytes from DKO mice. *DKO values were significantly different than the corresponding VDR KO, IL-10 KO and WT values. $P < 0.05$ n = 6 to n = 12.
Figure 3-7: **Altered proliferation of DKO T cells.** (A) Purified CD4⁺ and CD8⁺ were isolated from DKO, VDR KO, IL-10 KO and WT mice and stimulated for 72 hr with plate-bound CD3 antibodies. The CD4⁺ and CD8⁺ T cells from DKO mice were relatively unresponsive to CD3 stimulation compared to the same cells from VDR KO, IL-10 KO and WT mice. Bars represent means ± SE (n = 5 or n = 6). *P < 0.05. (B) Single cell suspensions of splenocytes from DKO mice were compared to single KO and WT mice for the resting levels of intracellular Ca²⁺. DKO splenocytes had lower levels of intracellular Ca²⁺ (dark band at lower fluorescence intensity) compared to VDR KO, IL-10 KO, and WT splenocytes (control, CTR).
REFERENCES:

Chapter 4

Vitamin D receptor expression prevents gut autoimmunity independent of disease causing bacterial micro-flora
ABSTRACT

Disease inducing bacteria have been identified as important determinants of intestinal pathology in all experimental models of inflammatory bowel disease (IBD). Our aim was to determine the role of disease causing micro-flora in the severe form of IBD which develops in the interleukin-10 (IL-10)/vitamin D receptor (VDR) knockout (KO) mouse. Colitis severity was evaluated in wild-type, VDR KO, IL-10 KO and double VDR/IL-10 KO (DKO) mice in the presence or absence of disease inducing bacteria. In addition, the ability of purified CD4+ and CD8+ T cells from these mice to transfer colitis to leukopenic mice and for CD4+T cells to inhibit IBD was determined. We report that VDR deficiency results in a form of IBD that was independent of disease causing micro-organisms. IBD in the IL-10 KO only occurred with *H. hepaticus* infection, whereas DKO mice developed fulminating IBD in the presence and absence of *H. hepaticus*. Reconstitution of DKO mice with wild-type CD4+T cells inhibited IBD, while VDR KO CD4+ T cells accelerated disease development. Furthermore, CD4+T cells from DKO mice transfer disease to leukopenic mice regardless of *H. hepaticus* infection. CD8+T cells from DKO mice also transfer disease but only if the mice are *H. hepaticus* infected. The data suggests that signaling through the VDR is one of the factors that regulate T cell responses and controls immunity, inflammation and homeostasis in the gastrointestinal tract.
INTRODUCTION

Inflammatory bowel diseases (IBD) that encompass Crohn’s disease (CD) and ulcerative colitis (UC) are chronic inflammatory conditions of the gastrointestinal tract. Factors that predispose individuals to the development of IBD include genetic, microbial, immunological and poorly defined environmental factors [1]. The high prevalence of IBD in North America and Europe where the availability of vitamin D from sun exposure is low, especially during the winter months suggests that vitamin D status may be an environmental factor that contributes to IBD development [2].

The discovery of the vitamin D receptor (VDR) in cells of the immune system and the presence of the $1\alpha$ hydroxylase in dendritic cells and macrophages [3] suggests that locally-produced $1,25(OH)_2D_3$ has regulatory autocrine and paracrine properties at the site of inflammation. Synthesis of active vitamin D requires the $1\alpha$ hydroxylase, which catalyzes the conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$. The pleiotropic actions of $1,25(OH)_2D_3$ are mediated by its binding to the VDR, which acts as a transcription factor to modulate the expression of specific genes in a tissue-specific manner. The VDR is a member of the steroid/hormone superfamily of nuclear transcription factors and is constitutively expressed in a variety of immune cells [4]. Resting T cells (both CD4+ and CD8+) express low levels of VDR, which are upregulated following activation.

The active form of vitamin D ($1,25(OH)_2D_3$) has been recognized as an immunosuppressive agent that ameliorates the pathogenesis of Th1-autoimmune diseases including IBD [5-8]. Furthermore, vitamin D deficiency and VDR deficiency have been shown to exacerbate experimental IBD in the IL-10 KO mouse and the CD4/CD45RB$^{high}$ transfer models [5, 9, 10]. In addition, genome screening techniques suggest that VDR
polymorphisms are associated with increased susceptibility to both CD [11] and UC [12] in humans.

Current evidence from both the clinic and animal models highlight a role for the commensal flora as the antigenic stimulus for gut inflammation in IBD [13-16]. As a result, broad spectrum antibiotic are being proposed as treatments for IBD and have been shown to reduce the intestinal bacterial load and ameliorate the inflammatory condition in experimental models of IBD [17, 18]. Intestinal inflammation in multiple rodent models requires luminal bacteria given that the disease develops only in conventional facilities, is moderate in specific pathogen-free (SPF) and fails to develop in germfree conditions (GF) [19]. In IL-10 KO mice the disease is driven by gut flora and Helicobacter hepaticus is one of the pathogens involved in disease initiation [14]. Mice lacking VDRs do not develop overt symptoms or present histological evidence of IBD when housed in H. hepaticus infected conventional facilities, although increased expression of IL-1β and TNF-α are detected in the colon of young (5 wk old) and old (9 month) VDR KO mice when compared to age matched WT mice [9, 20]. In addition, T cells from VDR KO mice have been shown to express an inflammatory phenotype, respond 2-times higher in a mixed lymphocyte response, and induce more severe CD4/CD45RB\textsuperscript{high} transferred experimental IBD than their WT counterparts [9]. VDR KO mice have heightened immune responses, which suggest that the absence of signaling through the VDR might predispose susceptible animals to develop IBD.
MATERIALS and METHODS

Animals

All of the mice were on the C57BL/6 background. IL-10 KO, Rag KO (The Jackson Laboratory, Bar Harbor, ME), VDR KO (gift from M. Demay, Harvard University, Cambridge, MA), WT and DKO mice (previously described) were bred and maintained in either SPF or conventional animal facilities at The Pennsylvania State University. Conventional animals tested positive for Helicobacter hepaticus, but negative for murine viruses, other Helicobacter species and all other bacterial pathogens. All mice (SPF and conventional) were confirmed negative for H. bilis, H. rodentium, H. trogontum and H. typhlonius species (data not shown). SPF mice differed from conventional mice only in H. hepaticus status. SPF mice were housed in autoclaved, microisolator cages which where changed under a laminar flow hood to maintain sterility (specific pathogen free conditions, SPF). These conditions have been adequate to maintain and breed healthy IL-10 KO and Rag KO mice over the last 3 years. All procedures were reviewed and approved by the Pennsylvania State University Institutional Animal Care and Use Committee.

Antibiotic therapy

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

Cell purifications and adaptive transfer of CD4+ and CD8+ T cells

CD4+ and CD8+ T cells were isolated from the spleen and mesenteric lymph nodes of mice using Cell Select Columns (Cedarlane, Hornnby, Canada). FACS analysis
confirmed that the purity of the T cells was at least 95%. A total of $2.5 \times 10^6$ cells were injected intraperitoneally into synegeic Rag KO. DKO mice were injected with $10^6$ purified CD4+ cells. Purified CD4+ T cells from WT mice were stained with PE-conjugated anti-mouse CD4 (L3T4) and FITC-conjugated anti-mouse CD25(7D4) (BD Pharmingen) and were sorted using a FACstar (Becton Dickinson, San Jose, CA) cell sorter (Pennsylvania State University Flow Cytometry Core Facility). The purity of CD4+CD25+ and CD4+CD25- T cells was more than 98%.

**Real-time PCR**

CD4+CD25+ and CD4+CD25- T cells were used for mRNA extraction using RNeasy Mini kit (Qiagen, Valencia, CA) and 1 µg of total RNA was reverse-transcribed (Promega, Madison, WI). Real-time PCR was performed using standard procedures. Thermal cycling parameters for primer optimization were as follows: activation at 95 °C for 10 min followed by 40 cycles 95 °C for 15 s and 60 °C for 1 min. The mRNA level for each sample was normalized against G3PDH house keeping gene.

**IBD disease severity**

Colitis development was monitored by weight curves, observation of stool consistency, small intestine over body weight ratios (SI/BW), large intestine over body weight ratios (LI/BW), histopathology scores, anal bleeding, colonic prolapse, and death. Macroscopic markers of intestinal pathology such as the ratios of the SI/BW and LI/BW have previously been shown to be objective measures of IBD severity [5]. Mice were sacrificed before they lost 20% of their body weight. The small intestine and colon were removed, fixed in formalin and sent to the Penn State University Animal Diagnostic Laboratories for paraffin embedding and H&E staining. Sections were scored blindly by
two observers on a scale of 0 to 4 for inflammation and 0-4 for epithelial thickening. Inflammation: 0- no inflammation, 1- increased number of leukocytes in the mucosa, 2- multiple loci of inflammation, leukocyte infiltration of mucosa and submucosa, 3- extensive leukocytic infiltrate in mucosa, submucosa, ulceration, depletion of mucin secreting goblet cells, 4- extensive transmural leukocytic infiltrate, crypt abscesses. Epithelial thickening: 0- normal, 1- slight epithelial cell hyperplasia, 2- pronounced epithelial cell hyperplasia (2-3 fold increase in crypts), 3- marked epithelial cell hyperplasia (3-10 fold increase in crypts), 4- marked epithelial cell hyperplasia (crypts were more than 10-fold greater). Total histopathology score ranged from 0-8. For cell transfer experiments mice were characterized as Normal - indistinguishable from uninjected control Rag KO mice (histopathology score=1-2); Mild- slight epithelial hyperplasia and increased number of leukocytes in the mucosa (histopathology score= 3-5); or Severe- marked epithelial cell hyperplasia and extensive transmural leukocytic infiltrate, crypt abscesses (histopathology score=6-8).

**Data analysis**

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

**RESULTS**

**SPF DKO mice develop fulminating IBD**

In order to determine the role of *H hepaticus* in initiation and perpetuation of intestinal inflammation in DKO mice, SPF mice were compared to *H. hepaticus* infected mice for
IBD development (Table 1). We determined that 100% of the SPF DKO mice developed fulminating IBD and the severity (SI/BW and LI/BW) of IBD was the same compared to *H. hepaticus* infected DKO mice (Table 1). Conversely, *H. hepaticus* infected IL-10 KO mice developed IBD while SPF IL-10 KO mice were free from symptoms (Table 1). It should be noted that *H. hepaticus* infected DKO mice had accelerated disease compared to SPF DKO mice (Fig. 1 and Table 1). All SPF DKO mice showed outward signs of IBD, including prolapsed colons, and rectal bleeding by 16 weeks of life, while SPF IL-10 KO mice do not develop outward signs of IBD (Table 1).

**Antibiotic treatment in SPF DKO mice is ineffective**

No specific group of microorganisms has been conclusively identified as causing IBD. Ciprofloxacin administration to IL-10 KO mice has been shown to markedly reduce bacteria levels and prevent IBD development [17, 18]. Thus, ciprofloxacin was administered to SPF DKO mice from birth via breast milk to weaning, followed by administration in drinking water [17, 18]. Fifty percent of the antibiotic treated DKO mice manifested colonic prolapse and cachexia at 12 wks of age, whereas the rest of the mice exhibited rectal bleeding and were sacrificed at 16 wks of age because they had lost more than 20% of their body weight (BW). We concluded that the antibiotic treatment (that is known to significantly reduce the bacterial microflora) was ineffective for preventing IBD in the SPF DKO mice (Table 1) and had no effect on disease severity. Microscopic evaluation of the gastrointestinal tract of SPF DKO mice showed severe inflammation and hyperplasia in the SI (histology score 5.0 ± 0.1) and colon (histology score 6.5 ± 0.3). Macroscopically the SI/BW and LI/BW ratios in antibiotic treated SPF DKO mice did not change as a result of the treatment (Table 1). Since SPF IL-10 KO
mice did not develop IBD in our colony it was not unexpected that antibiotic treatment of SPF IL-10 KO mice had no effect on IBD disease development (Table 1). In fact the antibiotic treated SPF IL-10 KO SI/BW and LI/BW ratios (Table 1) resembled those previously shown to be that of normal WT mice [5] suggesting a complete suppression of all IBD symptoms in these mice.

**CD4+ T cells from *H. hepaticus* infected DKO mice transfer IBD**

We show here (Fig 2) and others have shown [21, 22] that CD4+ T cells from conventional IL-10 KO mice induce intestinal inflammation when transferred to conventional Rag KO recipients. The CD4+ donors and Rag KO recipient mice were *H. hepaticus* infected.

*H. hepaticus* infected Rag KO mice did not show significant intestinal inflammation in the absence of T cell reconstitution during the duration of the study. Rag KO mice receiving CD4+ T cells from DKO mice developed chronic inflammation, manifested by weight lost (Fig 2A), bloody diarrhea, high ratios of SI/BW (10.4 ±0.4) and LI/BW (7.1 ± 0.4), colonic prolapse (approximately 40%) and death in 8-9 wks. Histological analysis revealed inflammatory infiltrates in all the intestinal layers, severe epithelial hyperplasia, and hemorrhagic mucosal ulcerations of the small and large intestine (Fig 2B). Rag KO recipients of CD4+ DKO T cells exhibited significantly more severe disease than the recipients of IL-10 KO CD4+ T cells (Fig. 2B,C). These data demonstrates that VDR deficiency increases the pathogenic potential of IL-10 deficient CD4+ T cells.

Interestingly, Rag KO recipients of VDR KO CD4+ T cells had microscopic evidence of inflammation and hyperplasia in the small intestine (but not the LI) that was similar in severity to that of the Rag KO recipients of DKO CD4+ T cells (Fig. 2C). These findings
suggest that VDR expression might play an IL-10-independent role in regulation of the CD4+ mediated response in the gut.

**CD8+ T cells from conventional DKO mice transfer IBD**

Although the involvement of CD4+ T cells in IBD has been clearly demonstrated, the role of CD8+ T cells in intestinal inflammation is less defined. The CD4+ donors and Rag KO recipient mice were *H. hepaticus* infected. CD8+ T cells from conventional DKO, IL-10 KO, VDR KO and WT mice were isolated and transferred into conventional Rag KO mice. The Rag KO mice that received CD8+ T cells from DKO mice lost weight by 8 wks (Fig 3A) and had significantly higher ratios of SI/BW (8.5 ± 0.6) and LI/BW (7.0 ± 0.7) than the recipients of CD8+ T cells from IL-10 KO, VDR KO or WT mice. Microscopic analysis of the gastrointestinal tract of recipient mice showed severe changes in both the small and large intestine after transfer of CD8+ T cells from DKO and VDR KO donor mice (Fig 3B). However, it should be noted that the Rag KO recipients of VDR KO CD8+ T cells did not develop outward signs (wasting, bleeding or colon prolapse) of IBD.

**CD4+ but not CD8+ T cells from SPF DKO mice transfer IBD**

To determine whether the pathogenicity of the T cells was exclusively *H hepaticus* dependent, we repeated the reconstitution experiments using SPF donors and recipients in clean rooms. The SPF DKO CD4+ T cells transferred colitis to SPF Rag KO mice as was evident by a drop in the body weight of Rag KO mice (Fig 4A). Conversely, Rag KO recipients injected with CD4+ T cells from VDR KO and IL-10 KO mice increased their BW by approximately 10 to 15% (Fig 4A) and looked healthy for 12 wks post-transfer. In contrast, the DKO CD8+ driven colitis shown in Fig. 3B did not develop when the
CD8+ T cells were derived from SPF DKO mice (Fig. 4B,D). The Rag KO recipients of SPF DKO CD4+ T cells developed diarrhea, and 20% of the mice showed rectal prolapse. In SPF conditions the CD4+ DKO T cells still induced pathology when transferred to Rag KO recipients; however the IBD symptoms took longer to develop (only a 10% drop in BW by 12 weeks) than when the CD4+ transfer was done in conventional mice. Histopathology scores from the Rag KO recipients of the SPF DKO CD4+ T cells were 6.8 ± 0.5 in the small intestine and 6.0 ± 0.4 in the colon and confirmed the development of IBD (Fig. 4C). In contrast, the DKO CD8+ driven colitis shown in Fig. 3B did not develop when the CD8+ T cells were derived from SPF DKO mice (Fig. 4B,D).

**CD4+ T cells from WT mice prevent IBD in DKO mice**

Regulatory CD4+CD25+ T cells have been shown to suppress experimental IBD. The expression of VDR is 2 fold higher in resting CD4+CD25+ T cells compared with CD4+CD25- T cells (Fig 5A). It should be noted however that DKO, and VDR KO mice have normal numbers of CD4+ CD25+ T cells [9]. Next, we investigated whether we could prevent the disease development in DKO mice by transfer of WT or VDR KO CD4+ T cells. Purified WT or VDR KO CD4+ T cells containing regulatory T cells were injected into DKO mice either before or after colitis symptoms had developed. Figure 5B shows that WT CD4+ T cells suppressed IBD in DKO mice (increase in BW) if the mice did not show obvious symptoms of intestinal inflammation prior to injection. Interestingly, injection of VDR KO CD4+ T cells into symptom free DKO mice had an accelerating effect on intestinal pathology and lead to the early death of the mice (Fig. 5B).

Histopathological analysis of the DKO mice reconstituted with WT CD4+ T cells
confirmed attenuated inflammation when compared with untreated or VDR KO CD4+ injected DKO mice (data not shown). Injection of WT or VDR KO CD4+ T cells to DKO mice with diarrhea had no effect on BW or other measures of IBD severity (Fig 5B). The data show that the spontaneous IBD that develops in the DKO mice can be suppressed but that there is a lack of regulatory activity in the VDR KO CD4+ T cell compartment.

DISCUSSION

Deletion of the VDR in IL-10 KO mice results in a form of experimental IBD that is of a similar severity in conventional, SPF conditions and after antibiotic treatment. Conversely, the VDR expressing IL-10 KO mice show IBD symptoms in conventional conditions, minimal symptoms (slightly enlarged SI/BW and LI/BW) in SPF conditions and are completely disease free when treated with ciprofloxacin. The data suggests that DKO mice develop IBD independent of the microbial flora in the colony. The other possible explanation of the data would be that VDR deficiency renders the IL-10 KO mice hypersensitive to antibiotic resistant commensal flora. The definitive experiment would be to generate germfree DKO mice that as yet has not been done. We show here that the ability of CD4+ T cells from DKO mice to transfer intestinal pathology to Rag KO mice is similar in conventional and SPF conditions. Our findings argue that VDR expression in CD4+T cells is a protective factor that controls bacterial antigen dependent and independent immune responses in the gut and suggests that vitamin D status and signaling through the VDR is crucial for controlling inflammation in the gastrointestinal tract.
Regulatory CD4+ T (T reg) cells main function appears to be the maintenance of self-tolerance. Data by others provide a strong case for the commensal bacterial antigen specificity of the CD4+ T reg cells [22]. Interestingly, we describe a population of CD4+ T cells from our DKO mice that transfer IBD in the absence of disease inducing bacteria. Vitamin D has been shown to be important in the induction of T reg cells and we show here for the first time that CD25+ T regs express 2-fold more VDR than their CD25- counterparts. Furthermore, we have shown previously that inhibition of IBD symptoms by 1,25(OH)₂D₃ is ineffective in IL-2 KO mice and these mice do not make CD4+ CD25+ regulatory T cells [23, 24]. In vivo-1,25(OH)₂D₃ treatment of experimental autoimmune diabetes induces a population of CD4+ CD25+ regulatory T cells that correlates with protection of the mice from diabetes [25]. Barrat et al. [26] have shown that a combination of 1,25(OH)₂D₃ and dexamethasone induces IL-10-producing regulatory T cells in human and mouse CD4+ T cells. Our results indicate that WT CD4+ T cells, but not VDR KO CD4+ T cells, contain regulatory T cells that are able to suppress IBD development in DKO mice. The data support a scenario whereby vitamin D status and signaling is required for the development or function of a CD4+ T reg cell population that is crucial to maintain tolerance in the GI tract.

CD8+ T cells can be protective (viral infections), or pathogenic (autoimmune diseases), and CD8+ T cells can also mediate tolerance. Our data show a critical role for the VDR in CD8+ T cell regulation of immunity to the bacterial flora in the GI tract. Others have shown [21] and we confirm that CD8+T cells from IL-10 KO mice are not pathogenic when transferred to Rag KO mice. VDR deficiency renders the IL-10 KO CD8+ T cells competent to transfer IBD in conventional facilities (Fig. 3). A pathogenic role for
CD8+ T cells has been shown for IBD [27] and other autoimmune diseases like experimental models of MS (EAE) and diabetes [28, 29]. Subclasses of CD8+ T cells have also been described that are suppressive/regulatory. Regulatory CD8+ T cells in humans are CD8+ and CD28- and their suppressive function has been shown to be both antigen-specific and antigen non-specific [30]. In mice CD8+ T cells have been show to be protective in EAE, and can be induced by oral tolerance [31]. Deletion of CD8+ T cells in CD28 KO mice reverts EAE resistant mice to EAE susceptible mice suggesting that CD8+ CD28- Ts cells may be protective in animal models of autoimmunity [31]. However, it should be noted that peripheral CD8+ T cells with suppressive activity have not been described in experimental IBD. Poussier et al have described a population of self-specific T cells within the CD4-CD8αβ- intraepithelial lymphocytes (IEL) subset that are able to protect the intestinal mucosa against chronic inflammation [32]. Furthermore Das et al have shown that subpopulations of CD4-CD8αα+ IEL also possess regulatory function and inhibit Th1-induced intestinal inflammation [33]. It seems possible but at this time speculative that VDR expression might be important for the development of a suppressive or regulatory CD8+ T cell in the gut. However, we do show that VDR deficiency results in a CD8+ T cell compartment that is pathogenic in experimental IBD. The data suggests that vitamin D receptor expression is an important modulator of the CD8+ T cell compartment and that in the absence of the VDR peripheral CD8+ T cells develop which induce gastrointestinal inflammation.

The incidence of CD and ulcerative colitis is highest in northern Europe with excessive cases in urban compared with rural environments. There is no dispute that both genetics and the environment contribute to the etiology of IBD. Concordance rates between
identical twins are only 50% for CD and about 18% for ulcerative colitis, which highlights the importance of environmental effects on IBD development. We show evidence in an experimental model of IBD that VDR expression is an independent risk factor for disease development. In addition we show evidence that expression of the VDR is required within both the CD4+ and CD8+ T cell compartment to prevent gastrointestinal inflammation. We propose a new model whereby the amount of vitamin D available in the environment (from either the diet or sunshine) signals through the VDR and shapes the T cell response and that an undefined threshold of vitamin D is required to maintain tolerance and to prevent bacterial driven inflammation in the gut.
Figure 4-1: Mortality of DKO mice, regardless of whether they are housed in conventional or SPF conditions. Conventionally (H. hepaticus infected) housed DKO mice (n=45) had 100% mortality by 10 wks of age. None of the identically housed IL-10 KO mice (n=33) died in this time frame. SPF DKO mice (n=28) had 100% mortality by 16 wks of age. None of the identically housed SPF IL-10 KO mice (n=20) died within the first year of life.
**Figure 4-2:** CD4+ T cell transfers from VDR KO, IL-10 KO and DKO mice induce IBD symptoms in Rag KO mice. 

(A) CD4+ T cells were sorted from spleen and MLN of age and sex-matched DKO, IL-10 KO, VDR KO and WT mice and 2.5x10^6 were injected ip into Rag KO mice. The purity of the cells was 95-98% as assessed by flow cytometry. Recipient mice were weighed on the day of transfer and weekly thereafter. The percentage change in body weight (g) over time is plotted ± SEM for each group of recipients. Recipients of WT CD4+ T cells gained weight in the 8 week time period. Conversely, Rag KO recipients of IL-10 KO or VDR KO CD4+ T cells lost about 5% of their BW in 8 weeks. Rag KO recipients of DKO CD4+ T cells lost >20% of their BW and succumbed to a fulminating form of IBD. 

(B) Representative colonic sections stained with H&E. Sections were scored blindly for pathology (see methods)[9]. Colon sections shown were rated: DKO-severe, IL-10 KO- severe, VDR KO- normal, WT-normal (C) The percentage of mice that showed normal, mild or severe symptoms of IBD are recorded for each group of Rag KO mice receiving CD4+ T cells from DKO, IL-10 KO, VDR KO and WT mice.
Figure 4-3: CD8+ T cells from VDR KO or DKO mice induce colitis in Rag KO recipients. (A) CD8+ T cells were isolated as described in Fig. 2 and ip. injected in conventionally housed *H. hepaticus* infected Rag KO recipients. Rag KO recipients of IL-10 KO or WT CD8+ T cells maintained their body weight over the 8 week time frame. Rag KO recipients of VDRKO CD8+ T cells lost about 5% of their body weight while DKO CD8+ recipients lost 13% of their body weight in 8 weeks. (B) Representative colonic sections stained with H&E and characterized. Colon sections shown were rated: DKO-severe, IL-10 KO- normal, VDR KO-severe, WT-normal. (C) The percentage of mice that showed normal, mild or severe symptoms of IBD are recorded for each group of Rag KO mice receiving CD8+ T cells from DKO, IL-10 KO, VDR KO and WT mice.
Fig. 4-4

Figure 4-4: **CD4+, but not CD8+ T cells from SPF DKO mice transfer IBD to SPF Rag KO mice.** (A) Body weights of Rag KO recipients following CD4+ T cells or (B) CD8+T cells transfers as described in Fig. 2 and 3. All donors and recipients are SPF. Rag KO recipients of CD4+ T cells from IL-10 KO, and VDR KO, or CD8+ T cells from IL-10 KO, VDR KO, and DKO mice gained weight following reconstitution. Conversely, DKO CD4+ T cells induced an 8 % drop in the BW of Rag KO recipients. (C) Representative colonic sections stained with H&E and characterized. Colon sections shown were rated: DKO-mild, IL-10 KO-normal, VDR KO-normal. (D) The percentage of mice that showed normal, mild or severe symptoms of IBD are recorded for each group of Rag KO mice receiving CD4+ or CD8+ T cells from DKO, IL-10 KO, and VDR KO mice.
Figure 4-5: **T regs are vitamin D targets.** (A) Real-time PCR analysis of VDR expression in purified CD4+CD25+ and CD4+CD25- T regs from WT mice. Values are corrected for G3PDH expression. *Significantly higher expression compared to the value from CD4+CD25- T cells; P<0.05. (B) CD4+T cells were purified from WT or VDR KO mice and 10⁶ cells were injected into DKO recipients that showed symptoms of IBD (WT □, VDR KO ○, n=5-6) or that were symptom free (WT ■, VDR KO ●, n=3-5). The values for BW represent the time of reconstitution of the sick DKO mice (4 wks of age) and 2nd wk after reconstitution (done at 2 weeks of age) of symptom free DKO mice. WT CD4+ T cells injections suppressed IBD in DKO mice that were symptom free prior to the transfer. VDR KO CD4+ T cells accelerated IBD development in the symptom free DKO mice.
**Tab. 4-1**

Table 4-1: The commensal flora has no effect on the severity of IBD in DKO mice.

<table>
<thead>
<tr>
<th>Genotype (n)</th>
<th>Facilities</th>
<th>IBD incidence</th>
<th>SI/BW (%)</th>
<th>LI/BW (%)</th>
<th>Mortality (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10 KO</td>
<td>Conventional</td>
<td>33/33 (100)¹</td>
<td>7.5 ± 0.5</td>
<td>4.9 ± 0.4</td>
<td>&gt;190²</td>
</tr>
<tr>
<td>IL-10 KO</td>
<td>SPF</td>
<td>0/20 (0)</td>
<td>6.3 ± 0.4</td>
<td>4.1 ± 0.4</td>
<td>&gt;365³</td>
</tr>
<tr>
<td>IL-10 KO</td>
<td>SPF/antibiotic</td>
<td>0/4 (0)</td>
<td>6.3 ± 0.2</td>
<td>3.9 ± 0.1</td>
<td>&gt;112</td>
</tr>
<tr>
<td>DKO</td>
<td>Conventional</td>
<td>45/45 (100)</td>
<td>8.5 ± 0.4*</td>
<td>7.7 ± 0.5*</td>
<td>41 ± 3*</td>
</tr>
<tr>
<td>DKO</td>
<td>SPF</td>
<td>28/28 (100)</td>
<td>8.3 ± 0.9*</td>
<td>6.9 ± 0.6*</td>
<td>80 ± 5*</td>
</tr>
<tr>
<td>DKO</td>
<td>SPF/antibiotic</td>
<td>11/11 (100)</td>
<td>8.8 ± 0.1*</td>
<td>6.5 ± 0.3*</td>
<td>112 ± 3*</td>
</tr>
</tbody>
</table>

¹IBD incidence was defined as mice showing either a decrease in body weight of ≥10%, rectal bleeding, or colon prolapse.
²IL-10 KO mice in conventional rooms with *H. hepaticus* were killed at 190 days of age. At that time 3 of the 16 mice died due to severe IBD (dead after 103 d, 135d, 187d).
³IL-10 KO mice in SPF rooms are breeders, which have to date not shown external evidence (decrease in body weight of ≥10%, rectal bleeding, or colon prolapse) of IBD.
*Values from DKO mice were significantly different than the values from IL-10 KO mice housed under similar conditions (P<0.05).
REFERENCES:


Chapter 5

Vitamin D receptor protects the intestinal tract against chemical induced inflammation by modulating the innate immune response
ABSTRACT

The vitamin D receptor (VDR) is a member of the steroid hormone nuclear receptors and is a ligand inducible transcription factor. The role of VDR and its ligand, 1,25(OH)₂D₃ in the innate immune response during colonic injury was probed using the acute model of IBD: dextran sodium sulfate (DSS) induced colitis. VDR KO mice were extremely sensitive to DSS and there was 80% lethality even at 2.5% DSS that only caused a mild form of colitis in the wildtype (WT) mice. DSS colitis in the VDR KO mice was accompanied by increased local colonic mRNA expression of TNF-α, IL-1α, IL-1β, IL-12, IFN-γ, IL-10, MIP-1α and KC when compared to WT mice. The intestinal wall of VDR KO mice was extremely sensitive to chemical injury and DSS concentrations as low as 0.5% was enough to induce bleeding, ulceration and weight loss followed by a delayed recovery phase compared to WT mice that had no damage induced by this minimal dose. The increased mortality of VDR KO with severe colitis may be a result of systemic endotoxaemia as VDR KO mice succumbed significantly faster to intravenous administration of LPS compared with WT mice. Administration of 1,25(OH)₂D₃ in the diet or intrarectally improved the severity and the extent of DSS-induced inflammation in WT mice, as assessed by clinical condition and macroscopic appearance and histology of the colon. Overall the results provide a direct support for the fact that vitamin D receptor and its ligand deficiency profoundly affects the early innate immune response during intestinal inflammation.
INTRODUCTION

Crohn’s disease and ulcerative colitis, the two forms of inflammatory bowel disease (IBD) in humans, are characterized by acute and chronic intestinal inflammation in genetically susceptible subjects in response to luminal bacteria (1). Crohn's disease can affect both the small and large intestine and the inflammation extends deep into all layers of the intestinal wall, whereas ulcerative colitis is continuous and is confined to the mucosal lining of the colon and rectum causing bloody diarrhea and inflammation (1). While both diseases are characterized by chronic inflammatory events, the initiation of the inflammation and clinically significant reactivations of the disease are associated with augmentation of the innate immune response and progressive upregulation of IL-12, IFN-γ, IL-1β, TNF-α in the intestine (2). Most of the research done so far focused on the role of the adaptive immune response in the development of intestinal inflammation. It is probably equally important to determine how the innate immune response that is involved in the early phases of the colitis and in reactivation events is controlled. Thus, short-term administration of dextran sodium sulfate (DSS) to mice was used to induce acute experimental inflammation in the gut. DSS is an agent that initiates mucosal epithelial cell damage and disrupts the barrier function, leading to ulceration, bleeding and a relatively slow mucosal repair process upon its withdrawal (3). When administered for a short time, DSS causes a self-limiting colitis microscopically characterized by infiltration of inflammatory cells into the lamina propria, focal crypt damage, and epithelial ulceration. Acute DSS colitis depends on innate immunity and not on acquired immunity because it also occurs in T and B cell-deficient animals such as SCID mice (4). In SCID mice depleted of NK cells the disease is characterized by macrophage-derived cytokines
such as IL-1β, IL-6, TNF-α and granulocyte-macrophage colony-stimulating factor and not by T-cell cytokines IL-2 or IFN-γ (5). Overall dysfunction of the mucosal innate immune response triggers an inflammatory cascade in the intestine via production of inflammatory mediators that leads to destruction of the intestinal wall. Identifying immunomodulatory substances that target early events in the development of mucosal and systemic inflammation and relapses may open exciting perspectives in prevention and/or treatment of IBD recurrences.

1,25(OH)₂D₃ experts its effects through binding of the vitamin D receptor, a nuclear receptor expressed in antigen-presenting cells, activated T cells and epithelial cells(6). 1,25(OH)₂D₃ inhibits dendritic cells differentiation and maturation in both human and murine systems, impairs their normal turnover in tissues (7) and inhibits the antigen presenting capacity and phagocytic activity of macrophages (8). Moreover, 1,25(OH)₂D₃ has been shown to inhibit IL-12 production by both macrophages and dendritic cells(9).

In chronic models of intestinal inflammation driven by the adaptive immune response, such as IL-10 KO mice (10) and the CD4+CD45RB<sup>high</sup> transfer model of colitis (11) there are clear evidence that the vitamin D receptor is an important protective factor that regulates the in gut homeostasis. The study here proposed to elucidate the role of VDR and its ligand 1,25(OH)₂D₃ in early events following intestinal epithelia damage, induction of inflammation and recovery after injury.

**Materials and methods**

**Animals**

C57BL/6 (Jackson Laboratory, Bar Harbor, ME) WT and VDR KO (M. Demay, Harvard University, Cambridge, MA) mice that were 10-12 wks old and weighed 20-25 g were
used in all the experiments. All procedures were reviewed and approved by the Pennsylvania State University Institutional Animal Care and Use Committee.

**Induction of colitis**

Mice were administered 0.5%-3.5% DSS (MW= 40 kDa; ICN Biomedicals, Aurora, OH) dissolved in filter-purified and sterilized water ad libitum for 5 days, followed by 5 days of water alone. Control mice were treated with regular drinking water. Animals were weighed every day and monitored clinically for rectal bleeding, diarrhea, and general signs of morbidity.

**Antibiotic treatment**

Neomycin sulfate (Sigma, St. Louis, MO), (1.5 g/L) and metronidazole (MP Biomedicals, Solon, OH),(1.5g/L) were administrated to the mice in drinking water simultaneously with DSS as described by Maeda et al (12).

**Colonic bleeding score**

The gross colonic blood scoring system as previously described by Siegmund et al.(13) was used. Briefly scoring was as follows: 0- no visible blood in the entire colon ; 1- gross blood detected in less than 1/3 of the colon; 2- gross blood detected in less than 2/3 of the colon; and 3- gross blood in the entire colon.

**Pheripheral blood analysis**

Blood was collected by cardiac puncture into tubes coated with EDTA (Becton Dickinson Vacutainer System, NJ) and analyzed using an ADVIA 120 Hematology System (Bayer Diagnostic, NY). In endotoxemia experiments a total of 300–400 μl of blood was collected by retroorbital sinus bleeding at the indicated time points by using heparinized microcapillary pipettes.
**Colonic measurements**

The entire colon (from ceacum to anus) was removed and the length was determined as described (3). The distal colon was removed from the mice, fixed in 10% formalin and sent to the Penn State University Animal Diagnostic Laboratories (University Park, PA) for processing and H&E staining. Histological analysis was performed blinded by 2 independent investigators on a scale from 0 to 40 as follows: the severity of inflammation (0– none, 1- slight, 2- moderate, 3- severe), extent of injury (0– none, 1- mucosal, 2- mucosal and submucosal, 3- transmural), and crypt damage (0- none, 1- basal 1/3 damaged, 2- basal 2/3 damaged, 3- only surface epithelium intact, 4- entire crypt and epithelium lost). Each score was then multiplied by a factor equivalent with the percentage of tissue involvement (x1: 0–25%, x2: 26–50%, x3: 51–75%, x4: 76–100%). This is the method described by Vowinkel et al (14).

**Colonic homogenate cytokines**

The last part of the distal colon and rectum were weighed and the same amount of tissue was removed from each mouse and cut open and washed in 1XPBS containing penicillin (100 U/ml) and streptomycin (100µg/ml). The tissue was then dissociated in 1ml PBS using a razor blade. The homogenized colon tissue was centrifuged at 10,000 g at 4°C for 10 min and approximately 500-750 μl supernatant was collected for each sample.

**ELISA**

The whole colonic homogenate supernatant was assayed for mouse TNF-α, IL-12p70, IFN-γ, IL-1α, IL-1β, IL-10 production using the Ab pairs and standards provided in the OPTI- ELISA kit (BD PharMingen, San Diego, CA) according to the manufacturer’s instructions. For KC and MIP-1α the ELISA kids were from R&D Systems (Minneapolis,
Detection limits were 31.25 pg/ml for TNF-α, 125 pg/ml for IL-12p70, 125 pg/ml for IFN-γ, 31.25 pg/ml for IL-1α and β, 7.8 pg/ml for MIP-1α and 15.6 pg/ml for KC.

RT-PCR

One centimeter segments of descending colon were harvested immediately upon euthanasia and snap frozen in liquid nitrogen. Colonic mRNA was extracted using RNeasy Midi kit (Qiagen, Valencia, CA) and 2 µg of total RNA was reverse-transcribed (Promega, Madison, WI). The PCR primer sequences were designed to cross intron/exon borders and were as follows: IL-1β and TNF-α as previously reported (10) and ICAM1 forward: 5'-TTACTTGGCTCCCTTCCGAC-3'; ICAM-1 reverse: 5'-GAG CCT CCG GAC TTT CGA TCT-3' (370 bp product). MIP-2 forward: 5'-GAA CAA AGG CAA GGC TAA CTG A-3 and reverse 5'-AAC ATA ACA ACA TCT GGG CAA T-3' (240 bp product). The number of cycles was adjusted (30–35) based on the titration of cDNA for each gene and the PCR products were run on agarose gel and density of the bands was determined as previously described(10).

Endotoxic Shock

C57BL/6 mice were injected intravenously or intraperitoneally with LPS from Escherichia coli 0111:B4 (Sigma, St Louis, MO) at a dose of 10 mg/kg body weight. Mice were monitored 3-4 times daily during the experiment, and moribund animals were sacrificed.

1,25(OH)2D3 treatment

Each mouse received 50 ng of 1,25(OH)2D3 in the diet/day as previously described (15) beginning 1 week prior and throughout DSS administration. For local treatment, 50 ng of 1,25(OH)2D3 was suspended in 20 μL corn oil and was administered intrarectally 1 day
prior to DSS administration and every other day thereafter for the duration of the experiment. Control mice received the corresponding amount of ethanol (1,25(OH)₂D₃ carrier) diluted in corn oil.

**Statistical analysis**

Statistical analysis was performed using the paired Student's t test test and ANOVAs (StatView; SAS Institute, Cary, NC). The log-rank test was used to compare Kaplan-Meier survival curves. P values < 0.05 were considered significant. Error bars represent means +/- SEM.

**Results**

**Increased mortality following DSS induced colitis in VDR KO mice**

Acute DSS was induced in WT and VDR KO mice by oral administration of 3.5% DSS. At this concentration of DSS there was a rapid mortality of the VDR KO mice and by 8 days there was a 100% lethality of VDR KO mice (Fig. 1A). The mortality was preceded by severe diarrhea, bleeding and the loss of more than 25% of the body weight (BW) (Fig. 1B). Due to 100% lethality of VDR KO mice at 3.5% DSS, the dosage was reduced to 2.5%. The mortality of the VDR KO (n=58) mice was 80% by day 12 post-induction of DDS colitis (Fig 3A) when the mice lost 25% of their BW (Fig 3B). At doses of either 2.5% or 3.5% DSS the WT (n=36) mice did not die and lost only 10% of their BW while showing only mild diarrhea and bleeding.

In this DSS model of intestinal inflammation there is a recovery phase which follows the withdrawal of DSS in the water (16). WT mice recovered both BW (3 weeks, Fig. 1B) and colonic length (Fig. 1C) as expected. The recovery phase was delayed in surviving VDR KO mice when compared to WT mice as evident by the delay and ultimate inability
of VDR KO mice to completely regain BW even 30 days after treatment (Fig. 1B). In addition, by day 10 post DSS the WT colons had recovered completely (Fig. 1C) while the VDR KO colons were still significantly shortened and not different than 5 days post-DSS (Fig. 1C). Even at lower doses of DSS (1.5%) the recovery period was significantly different: for VDR KO mice was 33 +/- 3.5 days while for WT was only 12 +/- 1.5 days (data not shown).

**VDR KO mice are sensitive to very low doses of DSS**

The increased mortality of VDR KO mice following DSS administration suggested that the intestinal wall of VDR KO mice is prone to develop inflammation. Very low doses of 0.5%-2% DSS were tested and produced little to no harm to the WT intestinal mucosa. Doses of 0.5%-1% DSS induced a loss of 15%-18% of the initial BW of the VDR KO mice and did not affect the BW of WT mice (Fig 1D). A dose of 0.5% DSS in VDR KO mice induced the same decrease in BW as 2.5% DSS in the WT mice (Fig. 1D).

**Hematological and histological alterations in DSS-treated VDR KO mice**

Beginning with day 1 post-DSS administration blood was detected in the feces of VDR KO mice and by day 5 the mice were moribund. Multiple bleeding ulcerations of the intestinal mucosa occurred early and with greater severity in VDR KO mice following DSS administration (Fig 2). Consequently VDR KO mice became severely anemic as determined by the measurement of red blood cells and hematocrit concentrations (data not shown) in circulating blood immediately after the start of DSS. In addition to reduced red cells the blood of VDR KO mice had increased white blood cells, neutrophils and lymepocytes (Fig 2B). It is likely that VDR KO mice were dying of severe bleeding and possible endotoxemia following perforation of the bowel induced by DSS. Blinded
histological injury scores were quantified in the distal colon from 2.5% DSS-treated WT and VDR KO mice at 5 and 10 days (Fig. 3). Control animals that received regular drinking water showed no signs of inflammation (Fig 3A, and 3B). DSS administration induced focal inflammation in WT mice (Fig 3 C,E) and small discrete ulcers (Fig 3G). Colons from DSS-treated VDR KO mice showed extensive tissue destruction following 3.5% DSS with complete ablation of intestinal vili (Fig 3D) and cellular infiltrate, crypt damage, submucosal edema (* Fig 3F), and large areas of epithelial erosion (Fig 3F, H, arrows). The overall histopathology score as well as specific parameters (inflammation, extent, and crypt damage) were significantly higher in VDR KO mice regardless of the dose of DSS administration compared to WT mice (Fig 5).

**Colonic cytokines and chemokine production from DSS-treated mice**

Increased susceptibility to intestinal injury may be due to an increased production of pro-inflammatory cytokines, chemokines or defective production of cytoprotective and/or reparative factors. As shown in Fig 5A the highest release of TNFα, IL-12, IL-10, IL-1α and IL-1β was detected in the DSS-exposed VDR KO group at 5 days and the levels decreased by day 10, but were still significantly higher than in control WT mice. IFN-γ showed an increased trend from day 5 to day 10 post DSS- treatment both in WT and VDR KO mice, but was significantly higher in VDR KO mice (Fig 5). The protein production in colonic homogenates for inflammatory cytokines was confirmed at mRNA level using RT-PCR. Fig 5B shows that IL-1β and TNF-α mRNA expression followed the trend observed in the protein production such as both cytokines were highly expressed in the colonic tissue of VDR KO mice at 5 and 10 days post-DSS administration. Furthermore chemokines such as KC-1 and MIP-1α produced by the
colons of mice before administration of DSS and after intestinal injury by DSS were determined by ELISA. The colons of WT and VDR KO mice produced little levels of these chemokines prior to DSS administration (data not shown) and the production of KC-1 and MIP-1 was significantly upregulated at 5 days after the administration of DSS (Fig 5A). Concordant with the severe injury, VDR KO mice produced significantly higher levels of these chemokines compared with WT mice. At mRNA level MIP-2 was also higher in the colonic tissue of VDR KO mice at 5 days and continued to rise at 10 days post-DSS when compared to WT mice. The mRNA expression of ICAM-1 was not different between VDR KO and WT mice at 5 days, but was higher at 10 days in VDR KO mice. Overall a number of cytokines and chemokine that are associated with pathological changes in IBD were over expressed in VDR KO mice suggesting that at least in part they are the mediators of the severe colitis in these mice.

**Lethal endotoxemia in VDR KO mice**

The increased lethality of VDR KO mice following DSS-administration may be a result of endotoxemia following perforation of the large intestine. VDR KO mice (n=11) and WT (n=10) were injected with the bacterial outer wall component LPS and survival was assessed daily for 7 days (Fig 6). Eighty percent of VDR KO mice died during the first 4 days post-injection and only 20% survived to day 7 (Fig 6). In contrast, 80% of the WT mice were alive by the end of the experiment (Fig 6, \( p < 0.05 \)). Shortly after intravenous administration of LPS VDR KO mice became extremely ill, experiencing diarrhea, hunched posture, shaking, lethargy and finally succumbed to severe sepsis at a faster rate than the WT mice. PBS-injected VDR KO (n=3) and WT (n=3) mice remained healthy throughout the 7-day study (not shown). The increased sensitivity of VDR KO mice to
septic shock does not depend on the route of administration. When 10mg/kg LPS were injected ip. VDR KO mice died with slightly delayed kinetics with the ones injected IV (data not shown).

1,25 (OH)$_2$D$_3$ ameliorates DSS colitis

Oral administration of 1,25(OH)$_2$D$_3$ did not significantly influence the BW (Fig 7A) of the mice or the colonic length when compared with controls on regular diet (data not shown). The 1,25(OH)$_2$D$_3$ feeding significantly up-regulated IL-10 production in colonic homogenate at 14 days post-DSS induction (Fig 7B) and this was associated with decreased total histopathological score (Fig 7C). There was no difference in all the other cytokines measured in the colonic homogenate between 1,25(OH)$_2$D$_3$-fed and control mice (data not shown). When administrated at the site of injury (intra-rectal) 1,25(OH)$_2$D$_3$-treatment resulted in the reduced loss of BW (Fig 7D) and decreased histological severity scores compared with control mice (data not shown). The calcium level in the blood of 1,25(OH)$_2$D$_3$ fed or treated mice and control was not different at the end of the experiment (data not shown).

DISCUSSION

VDR and its ligand, 1,25(OH)$_2$D$_3$ are protective factors in the early steps of intestinal injury. In an acute model of intestinal inflammation that is driven by the innate immune response, VDR deficiency lead to severe intestinal inflammation and lethality following 3.5% or 2.5% DSS administration that caused only moderate symptoms in WT mice. C57BL/6 mice have been shown to displayed no symptoms of colitis after the removal of 2.5% DSS from drinking water and their BW returned to normal levels in 5-7 days (17). The recovery phase in VDR KO mice was delayed in the surviving mice as apparent from
their inability to regain weight and significantly shorter colonic length. As evident from
the histopathological sections, the mucosal barrier was severely injured in VDR KO mice
with large ulcerations extending throughout 75% of the colonic mucosa, crypt loss and
goblet cell hypoplasia. Studies in humans and experimental models of acute intestinal
inflammation have implicated the impaired mucosal barrier function, defective epithelial
reparatory processes and exacerbation or defective innate immune response in the
pathogenesis of acute DSS-induced colitis(1).

DSS induced colitis is a direct result of damaged epithelium due to DSS toxicity (18)
with subsequent recruitment and activation of inflammatory cells and upregulation of
inflammatory mediators (19). During injury or inflammation, intestinal epithelial cells
are rapidly proliferating and this process of mucosal repair and regeneration is critical for
gut homeostasis (20). Thus we assume that VDR signaling might be involved at the
intestinal epithelial level in either transepithelial resistance or restitution, the process
through which epithelial continuity is reestablished that is central to healing after any
form of injury (21). VDR was reported to be highly expressed in human (22) and mouse
(10) colonic mucosa and intestinal epithelial cells constitutively express 1α-hydroxylase
that converts circulating 25-OH D₃ to active form 1,25-(OH)₂ D₃ in intestinal epithelial
cells (23). The intestinal epithelial resistance is profoundly altered in the absence of
VDR signaling as minimal amounts of only 0.5% DSS induced 15% weight loss and
microscopic evidence of injury in the VDR KO mice but no signs of pathology in WT
mice. The fact that epithelial barrier is defective is further confirmed by our observations
that one year old VDR KO mice develop intestinal inflammation in normal animal
facilities.
A previous report has shown that 1,25 (OH)2D3 controls normal villus and crypt development by regulating proliferation and differentiation of intestinal cells (24). Furthermore 1,25 (OH)2D3 has been shown to inhibit cell proliferation and/or stimulate cell differentiation in many tissues (25). Intestinal wound repair mechanism was indirectly evaluated by recovery experiments. VDR KO mice took more than 1 month to recover their BW following 1.5% DSS administration where as only 12 days were necessary for the full recovery of WT mice. In DSS colitis most of the pathological changes are localized in the distal colon that was found in humans and mice to be a site of low proliferation of epithelial cells (25). As a subsequent loss of growth control by 1,25-(OH)2D3, the baseline proliferative state of colonic crypts in VDR KO mice was reported to be elevated with increased expression of markers of cycling cells, such as proliferating cell nuclear antigen (PCNA) and cyclin D1 when compared with WT mice (25).

Although increased proliferation in the colon of VDR KO mice might be seen as beneficial, it has been shown that crypts with increased numbers of epithelial cells in cell cycle are more susceptible to radiation-induced injury as determined by their inability to repopulate the crypt (26). Similar with VDR KO mice, MyD88 KO mice are extremely sensitive to DSS administration, display a high level of epithelial proliferation at baseline but have severe radiation-induced epithelial damage (27).

Once the mucosal barrier is breached, the submucosa is exposed to a vast pool of luminal antigens, including foods and bacteria, and the cells involved in innate immune response are activated and produce large amounts of cytokines. In addition to homeostatic imbalance, increased susceptibility to intestinal injury may be mediated by excessive production of pro-inflammatory cytokines. Analysis of cytokine production by colonic
homogenates revealed significant elevation of TNF-α, IL-1α, IL-1β, IL-12p70, IFN-γ and IL-10 at 5 days post-DSS treatment in VDR KO mice when compared with WT mice. It is possible that the high levels of the same cytokines in the recovery phase might perpetuate intestinal inflammation and interfere with the healing of mucosa. Human and animal studies support the idea that TNF-α and IFN-γ are major players in IBD (28). In humans with IBD approximately two thirds of the patients responded to treatment with a single infusion of anti-TNF-α monoclonal antibody (29), whereas in mice the intestinal inflammation was significantly attenuated by anti–IFN-γ and/or anti–TNF-α monoclonal antibodies (30). The production of IFN-γ, TNF-α, IL-1α and β in the colonic homogenates of VDR KO mice was substantially higher at 10 days post-DSS than in WT mice consistent with the observed delay in recovery from inflammation in these mice. Indeed, IFN-γ alone or in combination with TNF-α have been shown to have antiproliferative effects on epithelial cells (31, 32). Furthermore, TNF-α has inhibitory effects on TGF-β, a key player in regulating intestinal epithelial cell migration and wound repair, and in this way might contribute to the progression of the inflammatory response and compromise repair processes (33). Fernandez-Martin et al. have reported that TNF-α inhibits 1,25(OH)2D3 action by decreasing the binding of VDR and RXR to the vitamin D response element (VDRE) in target genes (34). Not only inflammatory cytokines influences the responsiveness of intestinal wall to 1,25(OH)2D3, but also antigens present in the intestinal lumen. Pramanik et al have shown that LPS from gram negative bacteria impairs 1,25(OH)2D3 functions by decreasing the VDR protein levels in human blood monocytes and in this way may negatively affect the ability of 1,25(OH)2D3 to induce myeloid differentiation into monocytes/macrophages (35). The observation relevance to
the gut may be inferred, since peripheral blood monocytes are precursors of macrophages that are recruited to the lamina propria by chemokines and bacterial products during inflammation and are producing most of the inflammatory cytokines involved in the acute colitis (36).

A number of recent studies revealed that during experimental acute colitis members of the α-chemokine family, which includes human IL-8, human GROα, rat KC, murine KC and MIP-2 are involved in the development of intestinal inflammation. Ajuebor et al have shown that in experimental IBD colonic KC expression was increased and lead to leucocyte recruitment and increased MIP-1α expression was associated with both leucocyte recruitment and the onset of ulcerative lesions (37). Furthermore studies by Banks et al 2003 showed that the expression of MIP-1α correlated with the severity of colonic inflammation in patients with IBD (38). Additionally, MIP-2, a chemotactic factor for neutrophils, is only expressed in intestinal epithelial cells after stimulation with lipopolysaccharide or proinflammatory cytokines (39, 40). The significantly higher levels of KC and MIP-1α in the colonic homogenates of VDR KO mice at 5 and 10 days post-DSS administration when compared with WT mice suggest that VDR may be involved in the control of inflammatory cells recruitment of the site of injury, and thus perpetuation of inflammation. Moreover it is considered that one of the earliest changes in DSS-treated mice is the expression of ICAM-1 in the vasculature of the submucosa in the distal large intestine (41). ICAM has reported to play a role in recruiting effector immune cells to the mucosa, in establishing the inflammatory process and preceding surface ulceration (42, 43). The increased expression of ICAM-1 at 10 days post-DSS in the colonic tissue
of VDR KO mice compared with WT mice it can be another factor that mediates the
defective reparatory process in these mice.

It is conceivable that a breach in the intestinal mucosa increased the entry of bacterial cell
wall antigens into the blood steam leading to an excessive, uncontrolled, systemic
inflammation including, at the extreme, septic shock. Masubuchi et al have shown that
endotoxin levels detected in the portal blood of rats treated with DSS was higher than
those in control rats (44), whereas in mice endotoxin concentrations correlated with
macroscopic inflammation score and cytokine production (45). In humans, systemic
endotoxemia has been described in ulcerative colitis (46, 47) and Crohn’s disease patients
and shown to correlate positively with disease activity, pro-inflammatory cytokine
production and the extent of intestinal ulceration (48-53). VDR deficient mice are
extremely sensitive to intravenous or intraperitoneal administration of LPS, supporting
our assumption that VDR KO mice with colitis might die due to endotoxemia.

Interestingly 80% of the VDR KO mice injected with LPS presented blood in the small
intestine at the necropsy, whereas none of the WT mice sacrificed at the same time with
VDR KO mice presented internal bleeding. It have been shown that TNF-α when
administered in quantities similar to those produced endogenously in response to
endotoxin, causes ischemic and hemorrhagic lesions of the gastrointestinal tract (54).
Thus it is possible that the large amounts of TNF-α detected in the colonic homogenates
of DSS- treated VDR KO mice may be responsible for the death of the mice following
DSS administration.

Exogenous administration of 1,25-(OH)₂D₃ in the food or locally intrarectal ameliorates
colitis induced by low doses DSS in WT mice mainly by increasing local production of
an anti-inflammatory cytokine IL-10. 1,25-(OH)_2 D_3 was previously reported to block the progression and ameliorate the symptoms of IBD in a model of chronic colitis (15). Furthermore 1,25(OH)_2 D_3 has the ability to directly induce antimicrobial gene expression and activity of the antimicrobial peptide CAMP and defensin β2 genes (55). CAMP is a potent antisepsis agent that blocks macrophage induction, enhances the survival of mice treated with lethal doses of LPS (56) and accelerates epithelial wound healing (57). The induction of CAMP and other antimicrobial genes suggested that 1,25(OH)_2 D_3 might be protective against sepsis after injury and might accelerate epithelial wound healing (55). The active vitamin D converted in the kidney has systemic actions, whereas the extrarenal converted 1,25-(OH)_2 D_3 in colorectal epithelia appears to act in an autocrine or paracrine fashion by modulating cell proliferation, differentiation and function at a local level (58). While 1α-hydroxylase was found to be highly expressed in colonic biopsies from patients with CD or UC, a reduced expression of VDR was detected in the same patients when compared with normal colonic biopsies (59). We have previously shown that VDR expression is decreased in the colonic tissue of IL-10 KO mice that manifested severe intestinal inflammation (10). Overall the present study reveals that 1,25-(OH)_2 D_3 produced or administered locally in the intestine increase epithelial cells resistance to pathogenic aggressors and works to suppress innate immune response to luminal antigens through VDR signaling.
Fig 5-1

Increased mortality of VDR KO mice following DSS administration. A) Survival following induction of colitis with 3.5% DSS. WT (n=10) and VDR KO (n=14) mice received 3.5% DSS and WT (n=36) and VDR KO (n=58) mice were exposed to 2.5% DSS. The difference in mortality between 3.5% or 2.5% DSS-treated WT mice and VDR KO mice was significant (P<0.0001). B) BW loss as a function of time post-DSS. Percent weight change of animals following 2.5% or 3.5% DSS administration and the recovery phase after cessation of DSS intake was monitored for 30 days. C) Colon length at day 5 and day 10 following of DSS-exposure in WT and VDR KO mice and controls (controls- mice that received water) was measured. D) VDR KO mice were administered 0.5%-2% and WT mice received 1-2% DSS (n=5/group). BW changes were monitored, and the values are expressed as percentage of the original BW.
Figure 5-2: Hematological changes and colonic bleeding in VDR KO mice. A) Colonic blood score as a function of time post-DSS. Two WT and 2 VDR KO mice were sacrificed each day after 2.5% DSS administration and their colon was analyzed for the presence of blood as described in Material and Methods. B) Hematological changes in WT and VDR KO mice following 2.5% DSS administration. *$P < 0.05$
Figure 5-3: **Colitis development following DSS administration.**
Histological assessment of colitis using H&E staining of colonic samples from A) WT and B) VDR KO mice receiving water; C) WT and D) VDR KO mice at 5 days after receiving 3.5% DSS; E) WT and F) VDR KO mice at 5 days after 2.5% DSS; G) WT and H) VDR KO mice at 10 days after 2.5% DSS. Edema (asterisk), cellular inflammation in all layers (arrows).
Figure 5-4: **Histology scores in mice treated with DSS.**
Colonic injury score in WT and VDR KO mice that received 2.5% DSS at 5 or 10 days post-administration. Scores were determined as described in Material and Methods. *$P < 0.05$ – significantly different than the WT counterparts at 5 days post-DSS. **$P < 0.05$ – significantly different than the WT counterparts at 10 days post-DSS.
Figure 5-5: Cytokine and chemokine production in the colon after DSS-induced colitis.

A) TNF-α, IL-12, IFN-γ, IL-1α, IL-1β, IL-10, MIP-1α, KC protein expression in the colonic homogenates. B) Cytokine and chemokine mRNA expression in DSS-treated murine colons. *P < 0.05 – significantly different than the WT counterparts at 5 days post-DSS. **P < 0.05 - significantly different than the WT counterparts at 10 days post-DSS.
Figure 5-6: Increased sensitivity of VDR KO mice to systemic LPS administration. VDR KO (n=11) and WT (n=10) were injected i.v. with 10mg/kg LPS and the survival monitored for 7 days.
Figure 5-7: \textbf{1,25(OH)\textsubscript{2}D\textsubscript{3} ameliorates DSS colitis.}

\textbf{A)} Time course of BW loss following DSS administration. Percent weight change of WT mice fed with control diet (n=10) or diet supplemented with 50 ng\textsubscript{1,25-(OH)\textsubscript{2}D\textsubscript{3}}/mouse/day (1,25D3)(n=10).

\textbf{B)} IL-10 production from the colonic homogenates of control or 1,25D3 treated mice. *P < 0.05.

\textbf{C)} Total histological score was determined for control and 1,25D3 treated mice. Histological scores were significantly lower in 1,25-(OH)\textsubscript{2}D\textsubscript{3} treated mice at 14 days post-DSS. *P < 0.05.

\textbf{D)} Percent weight change of WT mice treated intrarectally every other day with control oil (n=6) or oil supplemented with 50 ng\textsubscript{1,25-(OH)\textsubscript{2}D\textsubscript{3}} (1,25D3) (n=10). *P < 0.05.

\textbf{E)} Colonic injury score in mice that received intrarectal oil or oil supplemented with 50 ng\textsubscript{1,25-(OH)\textsubscript{2}D\textsubscript{3}} at day 10 post-DSS administration.
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Chapter 6

Summary and conclusions
The immune system evolved to respond selectively and specifically to a myriad of potential threats in the gastrointestinal tract. In healthy individuals, inflammatory signals are downregulated by a complex relationship between the innate and adaptive immune response in order to coexist with gut microflora. The delicate balance that exists between responsiveness and non-responsiveness is directly influenced by genetic variability and the environment. The incidence of IBD is rising in Westernized countries with approximately one million patients only in the Unites States (1). So far the therapies for IBD use either non-specific agents such as glucocorticoids and 5-aminosalicylates, or biologic agents that target specific aspects of the immune response such as inhibitors of TNF-α. Thus the study of environmental and genetic factors is an important area of research that will advance our understanding of the immune defects in IBD and will undoubtedly improve the spectrum of agents available, while permitting individualized therapy.

Over the past couple of years researchers and clinicians have been making the case that vitamin D deficiency is an “unrecognized epidemic” in the United States as well as world-wide that often occurs in African Americans and Caucasian children and the elderly (2). It is clear that vitamin D status and vitamin D receptor (VDR) signals impact the development of the immune system in general and Th1 driven autoimmunity specifically (3). With a receptor constitutively expressed in the cells of the innate and adaptive immune system, vitamin D is being recognized as an immunomodulator of numerous and complex aspects of the immune response in various diseases. Evidence from humans and animal models revealed that the cellular and molecular mechanisms that lead to IBD have several components: defects in the adaptive immune response
represented by excessive effector T-cell function or deficient regulatory T-cell function and/or defects in adaptive immune response represented by defects in myeloid and epithelial cell. Initial studies demonstrated that vitamin D is critical for the T cell compartment in two different animal models of mucosal inflammation. First I showed that naïve T cells represented by CD4+/CD45RB\textsuperscript{high} population of T cells are one of the targets of vitamin D regulatory system. Transfer of CD4+/CD45RB\textsuperscript{high} T cells from VDR KO mice into immunocompromised mice induced more severe colitis than wild-type CD4+/CD45RB\textsuperscript{high} T cells, arguing for a protective role of VDR signaling in naïve T cells and their ability to induce inflammation when transferred to an immune deficient setting. Previous work using this model found that the inflammation was due to a Th1-mediated T cell response driven by IL-12 and mediated by IFN-\(\gamma\) (4). The data presented show that the antigen-specific IFN-\(\gamma\) production was significantly higher in VDR-deficient animals. The increased pathological potential of VDR-deficient naïve T cells and the increased capacity of VDR-deficient T cells in the periphery to produce IFN-\(\gamma\) suggests that vitamin D may act on the IFN-\(\gamma\) pathway to suppress mucosal inflammation.

Another classical setting of chronic mucosal inflammation, IL-10 KO mice that developed mucosal hyperplasia and transmural inflammation in response to conventional microflora at 7-8 weeks of age (5, 6), was used to analyze other aspects of vitamin D’s effects on T cell-mediated mucosal inflammation. VDR deficiency exacerbates the symptoms of enterocolitis in IL-10 KO mice as VDR/IL-10 double KO (DKO) mice rapidly developed wasting and began dying at 3 weeks of age due to a severe form of intestinal inflammation, rectal prolapse and bleeding. Although it has been shown that
CD45RB^lo^ T cells (memory T cells) obtained from IL-10–deficient mice do not prevent colitis when administered with CD45RB^hi^ T cells (7), total splenocytes that contain both naïve and memory T cells isolated from IL-10 KO mice from our colony did not induce outward symptoms of colitis when transferred to immunocompromised hosts at 12 weeks post-reconstitution. Instead the immunocompromised recipients of DKO splenocytes developed diarrhea, prolapsed colons and were bleeding rectally at 12 weeks. The aggravated phenotype of the disease induced by DKO splenocytes may support our previous conclusions and be due to a more pathogenic potential of naïve T cells induced by VDR deficiency, and/or might came as a consequence of diminished regulatory capacities of memory T cells. Either way, vitamin D status does have a protective imprint on naïve and/or regulatory T cells behavioral in intestinal inflammation.

VDR deficiency dramatically increased the pro-inflammatory cytokine expression in the colonic tissue at a very early age. Five week old DKO mice expressed high levels of IL-12, IL-1β, IL-2, IFN-γ and TNF-α mRNA in their colons whereas age-matched IL-10 KO mice started to show minor expression of some of these cytokines. The data imply that vitamin D might have a beneficial effect in colitis by targeting directly the proinflammatory cytokine production. Whether 1,25(OH)^2^D\textsubscript{3} acts specifically on only one cytokine and disrupts the inflammatory cascade that eventually leads to the damage of the targeted organ (in this case IL-12) or has a cumulative effect on diverse cytokine repertoire is not clearly elucidated yet.

Intestinal inflammation does not develop in IL-10 KO mice raised under germ-free conditions (8) but is enhanced by specific pathogens such as Helicobacter hepaticus (9) and thus the pathological process is believed to be driven by antigens in the gut
microflora. Conversely, neither specific pathogen free (SPF) \((H. \text{hepaticus})\) free, or antibiotic treatment rescued DKO mice from the development of lethal colitis. Furthermore, transfer experiments showed that DKO CD4\(^+\) T cells induced intestinal pathology in immunocompromised hosts in conventional and SPF conditions. Although CD8\(^+\) T cells from IL-10 KO mice are not pathogenic when transferred to Rag KO mice (10), DKO CD8\(^+\) T cells became pathogenic in immunocompromised hosts when isolated from and transferred in mice maintained in conventional facilities. Thus it is reasonable to suggest that vitamin D might modulate the CD4\(^+\) T cell compartment independent of gut microflora, whereas might control only antigen dependent responses in CD8\(^+\) T cells during intestinal inflammation. Finally rescue experiments using CD4\(^+\) T cells from WT or VDR KO mice containing both naïve and regulatory T cells were injected into DKO mice before or after colitis symptoms had developed. Only WT CD4\(^+\) T cells ameliorated DKO disease development when administrated before the onset of the symptoms. In opposition, VDR KO CD4\(^+\) T cells seemed to be more detrimental than beneficial to DKO mice. It is possible that while the regulatory T cell population contained in the WT CD4\(^+\) T cell pool performed their function and inhibited the inflammatory process in DKO mice, regulatory T cells from VDR KO animals are defective and thus lacked the suppressive capacities. Additionally, CD4\(^+\)CD25\(^+\) regulatory T cells expressed higher levels of VDR when compared with naïve CD4\(^+\)CD25\(^-\) T cells. At this stage, without any functional assay performed on VDR KO regulatory T cells, the findings together with the aforementioned ones emphasize a modulatory/ enhancing role of vitamin D on regulatory T cells function.
Finally the role of vitamin D on the innate immune component during intestinal inflammation was evaluated using a murine model induced by the physical agent, dextran sulfate sodium. Since WT mice developed moderate intestinal lesions at low doses of 2.5% DSS, while VDR KO mice died due to severe ulcerations in the colon and had a 3 times longer recovery phase, it is likely that vitamin D might be involved in the maintenance of epithelial barrier function and/or in mucosal repair and regeneration in the intestinal tract. Increased epithelial cell permeability exposed the innate immune cells to a vast pool of antigens and lead to their activation. Subsequently DSS treatment elicited a nonhomeostatic immune response characterized by the secretion of large amounts of TNF-α, IL-1α, IL-1β, IL-12p70, IFN-γ and IL-10 in VDR KO mice while only small to moderate amounts of these cytokines were detected in colonic homogenates of WT mice. The mechanism by which pro-inflammatory cytokines regulate the pathological process in IBD is complex and multifactorial: antiproliferative effects on epithelial cells, increased expression of inflammatory associated chemokines, increased vascular permeability, decreased anti-inflammatory cytokine production [11]. Accordingly, VDR signaling is protective in early stages of intestinal aggression and in the recovery phase through modulation of one or multiple cytokines.

Consistent with our previous findings that showed reduced progression of colitis in IL-10 KO following dietary supplementation with 1,25(OH)2D3 [12], 1,25(OH)2D3 was beneficial in intestinal inflammation in a DSS colitis model. Despite a significant effect on pro-inflammatory cytokine production in the colon of WT DSS-treated mice, mice receiving food supplemented with vitamin D up-regulated IL-10 production. Furthermore, according to macroscopic and histological indices, administration of
vitamin D at the site of inflammation, intrarectally, has even better consequences. Thus it is conceivable that vitamin D is responsible for limiting DSS-induced pathology by contributing to increased innate IL-10 levels. Given the potent anti-inflammatory qualities of this cytokine, its up-regulation may explain in part, the lower inflammation observed in vitamin D-treated mice with colitis. Further elucidation of the molecular mechanisms that modulate the anti-inflammatory signaling pathways of vitamin D in intestinal inflammation needs to be addressed.

Taken together, these data presented in this thesis provide ample reasons to postulate that vitamin D is a crucial mediator in the dialog of host innate, adaptive and regulatory immune response with microflora in experimental IBD. While defects in one of the above mentioned compartments can disturb the fine immune balance and can lead to loss of tolerance for mucosal antigens and thus intestinal inflammation, vitamin D has modulatory capacities in more then one component. The intricacies of vitamin D regulation of each aspect of the intestinal immune response allowed me to draw a broad picture of the events that ultimately lead to intestinal injury (Fig 1).

Overall, my research calls the attention of the scientists on the importance of one of the modifiable environmental factors, vitamin D, and shows that it has a significant impact on the dynamics of immunological events to an extent that should indeed prompt further exploration of its potential in the development of therapies for human IBD.
Figure 6-1: VDR signaling as a mediator in the dialog of host innate, adaptive and regulatory immune response with microflora in experimental IBD.

Arrow pointing up or down are representative of upregulation or downregulation of the specified factors by Vitamin D.
REFERENCES


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Publications
• Froicu M, Zhu Y, Cantorna MT. Vitamin D receptor is required to control gastrointestinal immunity in IL-10 knockout mice. Immunology, 2006 Mar; 117(3):310-8.