0888-8809/03/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 17(12):2386–2392

doi: 10.1210/me.2003-0281

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

MONICA FROICU, VERONIKA WEAVER, THOMAS A. WYNN, MARY ANN McDOWELL, JO ELLEN WELSH, AND MARGHERITA T. CANTORNA

Department of Nutrition (M.F., V.W., M.T.C.), University Park, Pennsylvania 16802; Immunopathogenesis Section (T.A.W.), National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892; and Department of Biology (M.A.M., J.E.W.), Notre Dame University, South Bend, Indiana 46556

The active form of vitamin D ($1,25D_3$) 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, $CD4^+/CD45RB^{high}$ T cells from VDR KO mice induced more severe colitis than wild-type $CD4^+/CD45RB^{high}$ 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 wk 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. (*Molecular Endocrinology* 17: 2386–2392, 2003)

THE ACTIVE METABOLITE of vitamin D (1,25 dihydroxycholecalciferol, $1,25D_3$) is an important modulator of the immune system. $1,25D_3$ treatment completely blocks the development of at least two different experimental autoimmune diseases (1, 2), and $1,25D_3$ therapy prolongs transplant graft survival for longer than the current drug of choice cyclosporine (3). Remarkably, $1,25D_3$ 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*, $1,25\text{-}(OH)_2D_3$ 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 $1,25\text{-}(OH)_2D_3$ 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 $1,25D_3$ inhibits IL-12 production by both macrophage and dendritic cells (9–11). Furthermore, $1,25D_3$ renders dendritic cells in a perpetual state of immaturity (9–11). 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 $1,25D_3$ 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 encephylomyelitis (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 response 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 de-

Abbreviations: BW, Body weight; $1,25D_3$, 1,25 dihydroxyvitamin D_3; DN, double negative; DP, double positive; EAE, experimental autoimmune encephalomyelitis; IBD, inflammatory bowel disease; IFN, interferon; KO, knockout; LI, large intestine; LN, lymph node; MLR, mixed lymphocyte reaction; OVA, ovalbumin; Rag, recombinase activated gene; SI, small intestine; VDR, vitamin D receptor; WT, wild-type.

velopment 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 1 wk 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 T cell mediated immunity measured. 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 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^{high} T cells develop a wasting disease and enterocolitis, whereas the transfer of unsorted CD4$^+$ T cells has no effect (16–21). The significance of CD45RB expression is unclear. It has been suggested that CD45RB^{high} T cells produce the inflammatory Th1 cytokines IFNγ, TNFα, and IL-2 and that CD45RB^{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^{low}, whereas naive Th precursor cells express CD45RB^{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^{high} T cell-induced IBD was significantly more severe when the CD45RB^{high} T cells came from VDR KO mice. Taken together, these results point to an essential role of vitamin D in restricting inflammation of the gastrointestinal tract and determining the severity of experimental IBD.

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 d 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 IFNγ than WT LN and spleen cells at 7 d post immunization (Fig. 1A); however, the difference disappeared by 14 d post immu-

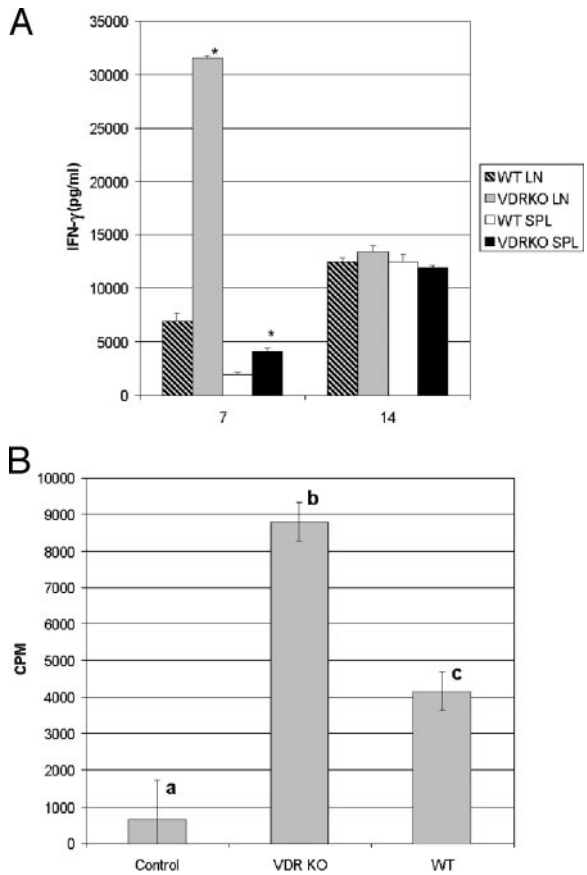

Fig. 1. Stronger IFNγ and MLR Responses in VDR KO Mice

A, Antigen-specific IFNγ secretion in cells from VDR KO and WT mice. A, IFNγ response at 7 and 14 d post immunization. The IFNγ response at 7 d post immunization was significantly ($P < 0.01$) higher in lymphocytes from the spleen and LN of VDR KO than the comparable lymphocytes from WT mice. At 14 d post immunization, the antigen-specific IFNγ 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 ($P < 0.03$) different from each other.

nization. Antigen naive (no immunization) IFNγ 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 *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 1 and data not shown), the size of granulomas in VDR KO mice were significantly larger than granulomas from control WT mice (Table 1). That is, in the absence of the VDR the granulomatous response was more vigorous. Interestingly, the calcium values in Table 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), cecum, 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. 2E). Five-week-old IL-10 KO, VDR KO, WT, and VDRKO/IL-10 +/− mice were also killed and IBD severity compared (Table 2 and Fig. 2). No symptoms of IBD were apparent in VDR KO or WT mice (Table 2 and Fig. 2B). Mild symptoms of IBD were present in VDR KO/IL-10 +/− and IL-10 KO mice (Table 2, Fig. 2, C 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

Table 1. VDR KO Mice Develop Larger Granualomas than WT Mice Following *S. mansoni* Infection

Mice (n)	Weight (g)	Calcium (mg/dl)	Granuloma volume
WT (18)	28.9 ± 1.2	7.8 ± 0.3	21.0 ± 1.1[a]
VDR KO (19)	28.2 ± 1.1	7.7 ± 0.3	27.7 ± 2.0

[a] The values in VDR KO mice are significantly ($P < 0.02$) larger than in WT mice.

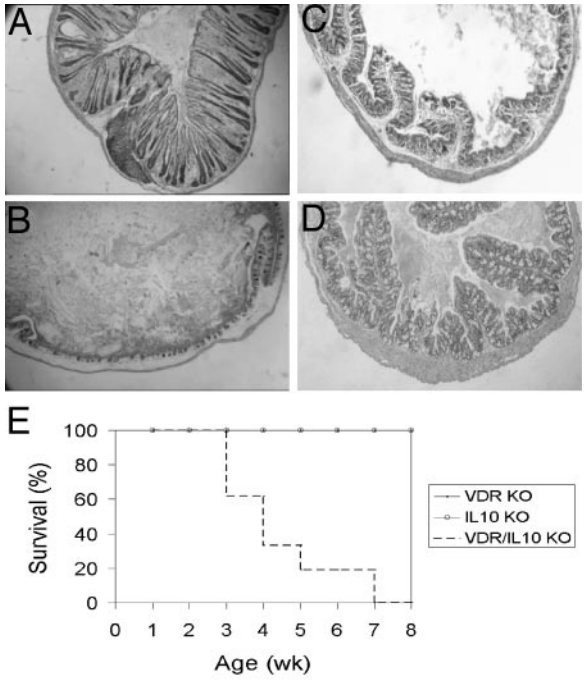

Fig. 2. Double IL-10/VDR KO Mice Develop Accelerated and Severe IBD by the Age of 5 Weeks

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).

Table 2. VDR/IL10 KO Mice Develop Severe IBD Symptoms

Genotype	Body Weight (g)	SI/BW (%)	LI/BW (%)	Histopathology Score
WT	17.9 ± 3.9[a]	5.6 ± 0.8[a]	3.5 ± 0.2[a]	0[a]
VDR KO	18.6 ± 3.6[a]	5.9 ± 1.7[a]	3.5 ± 0.3[a]	0[a]
IL10 KO	12.0 ± 0.9[b]	6.4 ± 0.7[a]	5.6 ± 0.5[b]	3.0 ± 0.3[b]
VDR KO/IL10 +/−	15.8 ± 1.1[a,b]	5.0 ± 0.2[a]	3.4 ± 0.2[a]	1.2 ± 0.2[c]
VDR/IL10 KO	8.1 ± 1.3[c]	7.7 ± 0.4[b]	7.8 ± 1.3[c]	7.2 ± 0.4[d]

All mice were 5 wk old and sex matched (to the VDR/IL10 KO) mice. % SI/BW, Small intestine weight/body weight × 100. % LI/BW, Large intestine weight/body weight × 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).

significantly different compared with all other strains. The double KO mice weighed the least but had the largest SI and LI (Table 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. 2A and Table 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. 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, $CD4^-8^-$) and single positive CD4 and CD8 thymocytes was increased in the VDR/IL-10 KO mice compared with all other mice (Fig. 3B). Concurrently, there was a significant decrease in the proportion of double positive (DP, $CD4^+8^+$) thymocytes in VDR/IL-10 KO mice (Fig. 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 (2.5×10^6 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 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 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.

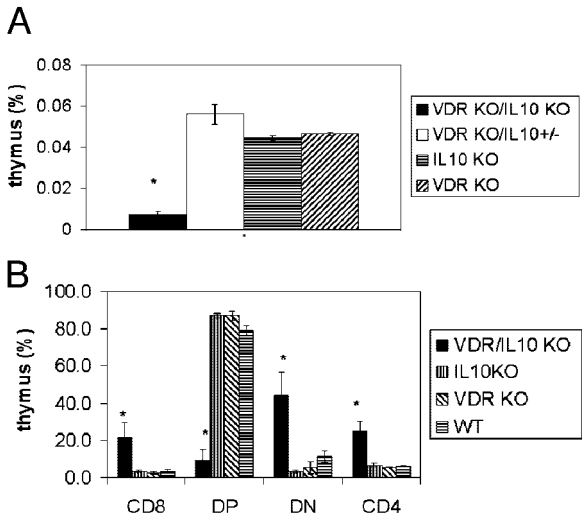

Fig. 3. Thymic Involution in Double IL-10 KO Mice

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.

$CD4^+$ $CD45RB^{high}$ 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 $CD4^+$ $CD45RB^{high}$ 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 $CD4^+$ $CD45RB^{high}$ T cells weighed the same, had larger SI/BW%, larger LI/BW %, and had more severe histopathology scores (Table 3). The data show that $CD4^+$ $CD45RB^{high}$ 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. $CD45RB^{high}$-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

Table 3. Colitis Symptoms in Rag KO Recipients of VDR/IL10 KO Spleens

Donor Cells	Body Weight (g)	SI/BW (%)	LI/BW (%)	Histopathology Score
VDR KO CD45RB^{high}	17.4 ± 0.6^a	11.0 ± 0.9^c	6.4 ± 1.0^b	5.8 ± 0.5^c
WT CD45RB^{high}	18.8 ± 0.8^a	6.8 ± 0.4^a	3.6 ± 0.6^a	2.9 ± 0.3^b
VDR/IL10 KO	18.6 ± 1.6^a	8.7 ± 0.3^b	7.5 ± 0.8^b	3.2 ± 0.7^b
WT	20.5 ± 2.4^a	5.9 ± 0.2^a	3.9 ± 0.1^a	1.1 ± 0.5^a

CD45RB^{high}: The spleens from VDR KO and WT mice were used to purify CD4+ T cells, which were sorted for CD4+ CD45RB^{high} 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.

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 $CD4^+$, $CD8^+$, 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 IFNγ 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. Hypocalcimia is likely up-regulating the 1-hydroxylase to form $1,25D_3$, which the VDR KO mice cannot use. It may be the $1,25D_3$ 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^{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.

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 liters) 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 899 bp 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 900 bp, 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 wk 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 d post immunization. The draining lymph nodes and spleens from the mice were collected, made into single cell suspensions and restimulated *ex vivo* with OVA (1 mg/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 25 pg/ml IL-2, 62 pg/ml IL-4, 312 pg/ml IL-5, and 1000 pg/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 $CD4^+$ $CD45RB^{high}$ cells were sorted using a FACstar (Becton Dickinson, San Jose, CA) cell sorter in the Pennsylvania State University Flow Cyotmetry Core Facility. The $CD4^+CD45RB^{high}$ T cells made up about 23% of all the sorted cells. 2.5×10^5 $CD4^+$ $CD45RB^{high}$ 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 hemotoxylin 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 $\pm$ 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).

Acknowledgments

Received July 17, 2003. Accepted September 10, 2003.

Address all correspondence and requests for reprints to: Dr. Margherita T. Cantorna, Department of Nutrition, 126 South Henderson Building, University Park, Pennsylvania 16802. E-mail: mxc69@psu.edu.

This work was supported by Crohn's and Colitis Foundation of America, Senior Research Award (to M.T.C.) and NIH-National Institute of Neurological Disorders and Stroke 1R01 NS38888-01A2 (to M.T.C.).

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