Vitamin D Receptor-Deficient Mice Fail to Develop Experimental Allergic Asthma[1]

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The active metabolite of vitamin D (1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$)) is known to modulate the immune response in Th1 cell-directed diseases. To investigate the role of vitamin D in Th2 cell-directed diseases, experimental allergic asthma was induced in vitamin D receptor (VDR) knockout and in wild-type (WT) mice. As expected, WT mice developed symptoms of airway inflammation with an influx of eosinophils, elevated Th2 cytokine levels, mucous production, and airway hyperresponsiveness. The administration of $1,25(OH)_2D_3$ had no effect on asthma severity. The only discernable effect of $1,25(OH)_2D_3$ on experimental allergic asthma in WT mice was an increased expression of two Th2-related genes (soluble CD23 and GATA-3) in lungs of BALB/c mice exposed to Ag through the nasal route only. By contrast, asthma-induced VDR knockout mice failed to develop airway inflammation, eosinophilia, or airway hyperresponsiveness, despite high IgE concentrations and elevated Th2 cytokines. The data suggest that although $1,25(OH)_2D_3$ induced these Th2-type genes, the treatment failed to have any affect on experimental asthma severity. However, VDR-deficient mice failed to develop experimental allergic asthma, suggesting an important role for the vitamin D endocrine system in the generation of Th2-driven inflammation in the lung. ***The Journal of Immunology*, 2004, 173: 3432–3436.**

Asthma is a chronic lung disease characterized by inflammation of the airways, increased mucous production, and airway hyperresponsiveness (AHR).[3] The prevalence of asthma has increased since the early 1980s across all age, sex, and racial groups, especially in children. Current pharmaceutical treatments for asthma include the corticosteroids cyclosporin A, FK506, and rapamycin, all of which nonspecifically inhibit the immune system. Long-term use of these drugs has been associated with side effects including osteoporosis, high blood pressure, and increased susceptibility to infection.

In response to airborne allergens, the immune system of an asthmatic generates a strong cell response in the lung airways. Th2 cell cytokine production, inflammation, and eosinophil infiltration result in the increased production of mucous, and epithelial cell thickening, which results in AHR. The Th2 cell products IL-4, IL-5, and IL-13 have been associated with disease severity in experimental allergic asthma (1). These cytokines are involved in the stimulation of B cells to produce IgE and in promoting the infiltration of target tissues by mast cells and eosinophils (1–4). Introduction of Ag-specific Th2 cells alone, or IL-4 and IL-13 alone, results in AHR, and blockade of these cytokines prevents the development of AHR in mice (2–4). In humans, blockade of the Th2 cytokine IL-4 can relieve some of the symptoms of asthma (5).

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[3] Abbreviations used in this paper: AHR, airway hyperresponsiveness; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; VDR, vitamin D receptor; KO, knockout; WT, wild type; Penh, enhanced pause; sCD23, soluble CD23.

The active metabolite of vitamin D (1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$)) is a potent regulator of the immune response. $1,25(OH)_2D_3$ treatment has been shown to suppress many experimental autoimmune diseases, which are characterized by Th1 cells and the production of Th1 cytokines IL-2, TNF-α, and IFN-γ (6–8). $1,25(OH)_2D_3$ decreases the production of the Th1 cytokines IFN-γ and IL-2. In Th2 cells, $1,25(OH)_2D_3$ increased the production of IL-4 and up-regulated the expression of the Th2 transcription factor GATA-3 (9). In addition, the expression of the low affinity IgE-binding factor (soluble CD23 (sCD23)) was shown to be up-regulated by $1,25(OH)_2D_3$ addition to Th cells (9). In vivo, the inhibitory effect of $1,25(OH)_2D_3$ on experimental multiple sclerosis was shown to depend on the ability of the host to produce IL-4 (10). The data suggest that the inhibitory effects of $1,25(OH)_2D_3$ on Th1-driven diseases occurs in part due to a shift in the Th cell compartment, which inhibits Th1 cells and up-regulates Th2 cells. The effect of $1,25(OH)_2D_3$ or vitamin D status on Th2-driven diseases has not been thoroughly investigated.

Vitamin D is a member of the steroid thyroid superfamily of nuclear receptors. $1,25(OH)_2D_3$ functions by binding to the vitamin D receptor (VDR). Together with a number of other transcription factors, the $1,25(OH)_2D_3$/VDR complex regulates the transcription of targeted genes. Mice lacking the VDR show impaired bone formation (11), but revealed no abnormalities in the numbers and types of cells present in the spleen and thymus (12). More recently, VDR knockout (KO) mice have been shown to be more susceptible to inflammatory bowel disease (13). In addition, $CD4^+$ T cells from the VDR KO mice expressed increased levels of IFN-γ, and had stronger mixed lymphocyte reactions (13). The effect of VDR deficiency has not been examined in a Th2-driven disease.

To investigate the effect of $1,25(OH)_2D_3$ in a Th2-driven disease, the standard experimental asthma model (OVA-induced) was chosen. The VDR is required for all known biologic effects of vitamin D and we show here that VDR KO mice failed to develop symptoms of experimental asthma. However, $1,25(OH)_2D_3$ treatment of both BALB/c and C57BL/6 wild-type (WT) mice had no

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effect on asthma severity. The data suggest that signaling through the VDR is required for the generation of this Th2-driven disease.

Materials and Methods

Mice and diets

BALB/c mice (The Jackson Laboratory, Bar Harbor, ME), VDR KO, and WT C57BL/6 mice (gift from M. Demay, Harvard University, Cambridge, MA) were bred and maintained at the Pennsylvania State University (University Park, PA). On day 0, all mice were fed synthetic diets (8), replaced every 2–3 days during the 18 days of the experiment. The vitamin D-treated mice received 0.2 μg of $1,25(OH)_2D_3$ per day in the diet (8). All experimental procedures were approved by the Office of Research Protection's Institutional Animal Care and Use Committee (Pennsylvania State University).

Immunization and challenge protocol

Mice were injected i.p. with OVA (0.1 mg of Grade III; Sigma-Aldrich, St. Louis, MO) adsorbed in aluminum hydroxide (1 mg; Pierce, Rockford, IL) on days 5 and 10 (primed group). Subsequently, mice were challenged intranasally with OVA (0.04 mg) on days 15 through 17. All mice were sacrificed on day 18. In a second protocol, mice did not receive the i.p. OVA/aluminum hydroxide injections but were challenged intranasally (unprimed group) with OVA.

Measurements of serum IgE and calcium levels

Blood was collected for serum isolation. Calcium measurements were done using a kit from Sigma-Aldrich (587-A) and the manufacturer's instructions. Total IgE and OVA-specific IgE serum levels were measured using ELISA (BD Pharmingen, San Diego, CA). The detection limit for total IgE was 6.25 ng/ml. For OVA-specific IgE, the plates were coated with OVA (20 mg/ml) and levels were compared with values of untreated mice as described (14).

Cell culture and cytokine analysis

OVA-specific cytokine secretion was determined from splenic cultures. Seventy-two hours after OVA (1 mg/ml) restimulation, supernatants from triplicate wells were removed. Cytokine levels were measured using ELISA OptEIA Mouse Sets (BD Pharmingen) for IL-2, IL-4, IL-5, IFN-γ, and R&D Systems (Minneapolis, MN) for IL-13. The detection limits were 12.5 pg/ml for IL-2, 31.25 pg/ml for IL-4, 62.5 pg/ml IL-5, 25 pg/ml for IFN-γ, and 78 pg/ml for IL-13.

Quantitative mRNA analysis

mRNA was isolated from lung tissues using the RNeasy Mini kit (Qiagen, Valencia, CA) and reverse-transcribed (Promega, Madison, WI). Real-time quantitative PCR using dual-labeled fluorgenic probe (TaqMan Probe) PCR product accumulation was analyzed (15) (Nucleic Acid Facility, Pennsylvania State University). The expression levels for sCD23 and GATA-3 were determined using primers described earlier (9).

Histopathology

Lungs were fixed in formalin, sectioned, and stained with H&E at the Animal Diagnostic Laboratory (University Park, PA). The sections were scored blindly by two different individuals on a scale of 0–4 for inflammation and 0–4 for epithelial thickening. Inflammation: 0-no inflammation, 1-inflammatory cells present, 2-a few (<3) loci of inflammation, 3-multiple (≥3) loci of inflammation, 4-inflammatory cells throughout the lung. Epithelial thickening: 0, normal; 1, epithelial thickening visible; 2, loci of thickened airway; 3, airways nearly completely obstructed by epithelial thickening; 4, normal structure not present, little to no obstructed airway. The scores for inflammation and epithelial thickening were added together and divided by 2 (0, no disease to 4, maximal disease). The results are presented as means ± SE. In addition, lung sections were stained with periodic acid-Schiff reagent to stain mucous-secreting cells. Mucous scores were: 0-no mucous, 1-a few cells secreting mucous, 2-many cells secreting mucous, and 3-extensive mucous production.

Airway hyperresponsiveness

Respiratory function was measured using a whole body plethysmography (Department of Veterinary Science, Pennsylvania State University). Mice were placed in a chamber and, following acclimatization, were exposed first to PBS and then to increasing concentrations (3.12, 6.25, 12.5, 25, and 50 mg/ml) of nebulized methacholine. Readings were taken for 5 min after nebulization. The enhanced pause (Penh) (Penh = pause × (peak inspiratory box flow/peak expiratory box flow)) values for each methacholine dose were determined. Results are reported as the percent control relative Penh value for each methacholine concentration compared with baseline values.

Lung cell isolation and flow cytometric analysis

Whole lungs were digested with collagenase A (Roche Diagnostics, Indianapolis, IN) and layered onto Percoll gradients as described elsewhere (16). For flow cytometry, 10^6 cells were stained with PE anti-mouse CD4 and FITC anti-mouse CD8 Abs (BD Pharmingen) and analyzed using a XL-MCL benchtop cytometer (Beckman Coulter, Miami, FL).

Data analysis

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

Results

VDR KO mice failed to develop experimental allergic asthma

VDR KO, WT, and $1,25(OH)_2D_3$ treated WT mice on the C57BL/6 background were primed and challenged with OVA to induce experimental asthma. WT mice developed severe symptoms of inflammation in the lungs without any effect of $1,25(OH)_2D_3$ treatment on the asthma severity (Fig. 1, *B* and *C*). The influx of inflammatory cells occurred around the bronchi and bronchioles in the lung and revealed a high percentage (70–80%) of eosinophils. VDR KO mice had little inflammation and few eosinophils in the lungs of primed mice, which resembled those of WT mice without any Ag exposure (Fig. 1, *A* and *D*). The lung histopathology scores from primed VDR KO mice were significantly less than in WT mice ($p = 0.039$) or $1,25(OH)_2D_3$-treated

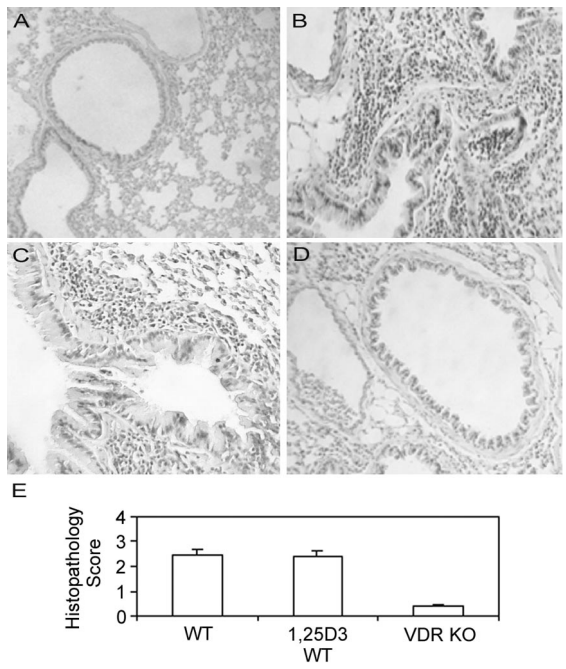

FIGURE 1. Reduced airway inflammation in VDR KO mice following asthma induction. Mice were primed and challenged with OVA. Lungs were analyzed by H&E stain and scored for pathology as described in *Materials and Methods*. The data is from at least 9 mice and as many as 12 mice in each group. *A*, Unsensitized WT lung (score-0); *B*, primed WT lung (score-3); *C*, primed $1,25(OH)_2D_3$ (1,25D3) WT lung (score-3); *D*, primed VDR KO lung (score-0); and *E*, mean histopathology scores. Values from VDR KO mice are significantly different from WT and 1,25D3 WT mice ($p = 0.039$ and $p = 0.037$, respectively).

WT mice ($p = 0.037$) (Fig. 1*E*). Consistent with the lack of inflammation, VDR KO mice did not produce mucous in their lungs (Fig. 2*D*). As expected WT and $1,25(OH)_2D_3$ WT mice produced mucous in their lungs (Fig. 2, *B* and *C*).

The endpoint of these pathological changes is the development of AHR. Therefore, we exposed similarly treated mice to varying concentrations of methacholine and determined Penh to measure AHR. Fig. 3 shows that the primed WT mice had higher mean Penh responses than unsensitized WT mice. In addition, these mice experienced distress (Penh ≥10) at methacholine concentrations of 25 mg/ml. The AHR response of the primed WT mice was significantly higher than all other mice at 12 mg/ml methacholine. By contrast, primed VDR KO mice had responses similar to that seen in unsensitized WT mice (Fig. 3). Interestingly, unsensitized VDR KO mice had reproducibly ($n = 14$) increased Penh values but only at the lower concentrations of methacholine compared with all other mice including the primed VDR KO mice. At higher concentrations, the unsensitized VDR KO responses were similar to unsensitized WT mice (Fig. 3).

Primed VDR KO mice express a peripheral Th2 cell response with elevated IgE levels

We next determined whether mice lacking VDR generated an Ag-specific response to OVA priming. Splenocytes from primed or control mice were restimulated with OVA in vitro and cytokine levels determined. We found that OVA-stimulated splenocytes from both primed VDR KO and WT mice responded by producing similar levels of IFN-γ, IL-5, and IL-13 (data not shown). However, only splenocytes from primed WT mice produced detectable IL-4 (37 pg/ml) when stimulated ex vivo with OVA (data not shown). We also determined whether there was any difference in the recruitment of T cells into the lungs of these mice following prime and challenge. The results showed that lung mononuclear cells from primed WT mice contained 1.6 ± 1.0% $CD4^+$ and 2.4 ± 0.6% $CD8^+$ cells. By contrast, the percentage of T cells in the VDR KO lungs were approximately one-third that of WT lungs, with 0.6 ± 0.2% $CD4^+$ and 0.9 ± 0.1% $CD8^+$ T cells. Analysis of serum IgE, another marker of Th2 response, showed that primed VDR KO mice had the highest serum total IgE response of all groups. More interestingly, unsensitized VDR KO mice had higher total IgE in the serum compared with unsensitized WT mice (Fig. 4). OVA-specific IgE responses also resembled the total IgE levels with VDR KO mice expressing higher amounts than WT mice (data not shown).

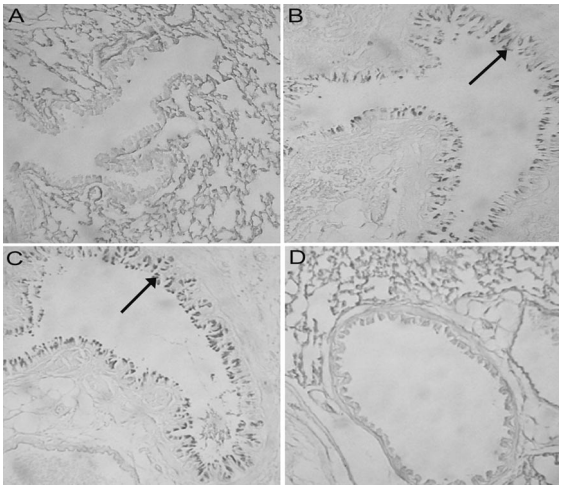

FIGURE 2. Reduced mucous production in VDR KO mice following asthma induction. Mice were primed and challenged with OVA as described in Fig. 1 and lungs were analyzed by periodic acid-Schiff stain for mucous production and scored as described in *Materials and Methods*. Arrows indicate the darkly stained mucous-secreting cells. *A*, Unsensitized WT lung (score-0); *B*, primed WT lung (score-2); *C*, primed 1,25D3 WT lung (score-2); and *D*, primed VDR KO lung (score-0).

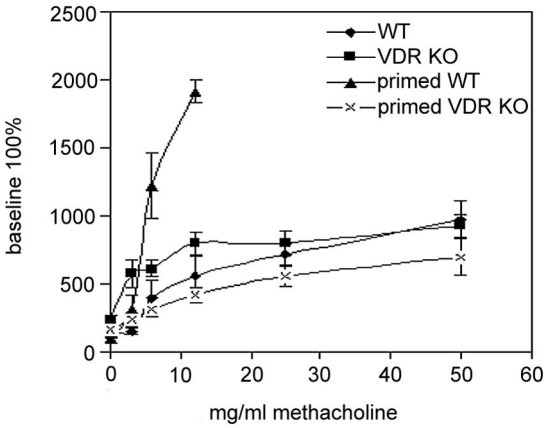

FIGURE 3. Development of in vivo AHR in primed VDR KO and WT mice. Mice were primed and challenged with OVA as described in Fig. 1, and AHR analyzed using a Buxco Plethysmograph (Buxco Electronics, Wilmington, NC), represented as the percent increase over baseline. WT (unsensitized, $n = 4$); VDR KO (unsensitized, $n = 14$); primed WT ($n = 5$); primed VDR KO ($n = 7$). Primed WT mice had significantly higher AHR at 12 mg/ml methacholine than all other groups. Note that AHR was elevated (significant, $p = 0.007$) in primed WT mice compared with primed VDR KO mice.

sCD23 and GATA-3 are up-regulated by $1,25(OH)_2D_3$ treatment in unprimed BALB/c mice

The experiments above show that the VDR may regulate the development of lung pathology and AHR during induction of allergic asthma. These data suggest that $1,25(OH)_2D_3$ may regulate these responses by reducing Th1 activation and cytokine production. To determine whether this is the case, experimental asthma was induced in BALB/c mice treated or not with $1,25(OH)_2D_3$. As expected, OVA-primed mice had more prominent symptoms of allergic asthma than unprimed BALB/c mice (Fig. 5*A*). There was no effect of $1,25(OH)_2D_3$ treatment on the lung pathology (histopathology scores) in either the primed or unprimed BALB/c mice (Fig. 5*A*). However, examination of genes potentially involved in Th2 cell function showed that $1,25(OH)_2D_3$ treatment of unprimed BALB/c mice resulted in increased expression of sCD23 (Fig. 5*B*, $p = 0.05$) and GATA-3 (Fig. 5*C*, $p = 0.03$) mRNA in the lungs. This effect of $1,25(OH)_2D_3$ on sCD23 and GATA-3 expression was not evident if the BALB/c mice were primed. No significant difference was observed in the expression of sCD23 or GATA-3 in VDR KO and WT C57BL/6 mice (data not shown).

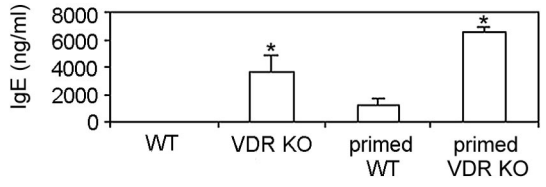

FIGURE 4. IgE production in VDR KO and WT mice. Total serum IgE analyzed from mice primed and challenged with OVA as described in Fig. 1. ∗, The VDR KO response was significantly different from the WT response ($p = 0.01$).

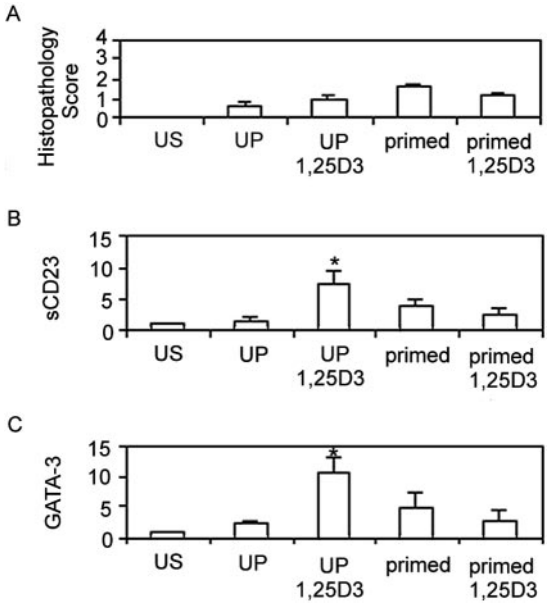

FIGURE 5. sCD23 and GATA-3 expression is up-regulated in the lungs of unprimed BALB/c mice treated with $1,25(OH)_2D_3$. *A*, Mice were primed and challenged with OVA and lung histopathology scores of primed and unprimed BALB/c mice determined as in Fig. 1. US, unsensitized; UP, unprimed $1,25(OH)_2D_3$ treated; UP 1,25D3, primed, and primed 1,25D3 treated. The level of mRNA induction for (*B*) sCD23 and (*C*) GATA-3 in lung tissue of BALB/c mice was also determined. ∗, Values from unprimed $1,25(OH)_2D_3$-treated mice were significantly higher than controls. Result is one representative experiment of three experiments.

Discussion

Vitamin D is known for its beneficial effects in diseases with strong Th1 responses, perhaps by altering Th1/Th2 balance in vivo (6, 8, 17). It has been reported that $1,25(OH)_2D_3$ treatments reduced airway eosinophilia using a similar model of allergic asthma (18). We found no evidence to support such an effect, suggesting that $1,25(OH)_2D_3$ has no beneficial effect on experimental asthma in mice. Matheu et al. (18) used a different strain of mouse (B10.RIII) and there were other differences in the experimental protocol including injecting $1,25(OH)_2D_3$ every other day instead of feeding the mice continuously. More recently, Topilski et al. (19) reported that $1,25(OH)_2D_3$ suppressed eosinophilia, and lung inflammation scores in what may be the same model of allergic asthma induced in either BALB/c or C57BL/6 mice. Unfortunately, the experimental details of the Topilski et al. experiments are not clearly stated. There could be differences in the OVA immunization, OVA dose, and what type of mice were used (BALB/c or C57BL/6, and it is unclear whether intranasal or aerosolized exposure of the mice was done (19). In addition, $1,25(OH)_2D_3$ was injected and not fed (19). It is likely that these experimental details explain the discrepancy. However, they also observed that $1,25(OH)_2D_3$ treatment inhibited chemokine-induced migration of T cells (19). In our experiments, WT mice developed severe symptoms of airway inflammation, while $1,25(OH)_2D_3$ treatment failed to have an effect on the severity of lung pathology in BALB/c and C57BL/6 mice. However, the VDR is involved in the allergic asthma response in mice because in our experiments, VDR KO mice showed mild symptoms of airway inflammation and failed to develop experimental asthma. Paradoxically, VDR KO mice produced a strong Th2 response, characterized by IL-5, IL-13, and increased IgE production, but failed to develop AHR, lung inflammation, and other markers of allergic asthma, suggesting that the VDR may affect the development of this disease by affecting both the Th2 response as well as perhaps the response to Th2 cytokines.

As previously reported and in association with epithelial layer thickening and the presence of inflammatory cells in the lungs, primed WT mice were hyperresponsive to methacholine exposure and showed signs of restriction in breathing capacity. Conversely, in agreement with the lung pathology, primed VDR KO mice resembled unsensitized WT mice and had normal AHR responses. The AHR results confirmed the histopathology scores, which showed few infiltrating inflammatory cells in the lungs of VDR KO mice primed to develop asthma. $1,25(OH)_2D_3$ has been hypothesized to be important in lung development, and VDR KO mice have been reported to have normal pulmonary function (20). However, AHR measurements have never previously been done on VDR KO mice. We show that unsensitized VDR KO mice are responsive to low doses of methacholine (Fig. 3). The VDR KO mice have trouble maintaining calcium homeostasis because they cannot respond to vitamin D. Although the VDR KO mice had normal serum calcium values (on the diets fed in these experiments), it is possible that smooth muscle contractions are more sensitive to calcium perturbations. The more puzzling finding is that VDR KO mice primed to develop experimental asthma do not show the elevated airway response to low doses of methacholine seen in the unsensitized VDR KO mice. Perhaps the prior OVA sensitization allowed the lungs to adapt, reducing responses to the lower doses of methacholine. The AHR measurements in naive or primed VDR KO mice were not significantly different from the unsensitized WT AHR responses, while primed WT mice had significantly elevated AHR responses compared with the VDR KO (primed and unsensitized) and unsensitized WT mice.

Along with a strong Th2 response upon priming, unsensitized VDR KO mice also had elevated total IgE levels, in contrast to WT mice, suggesting that the VDR negatively regulates Th2 responses. Ag priming and challenge increased total IgE and OVA-specific IgE levels in VDR KO mice. These mice had the highest IgE production and the lowest production of mucous, inflammation, and AHR. Although pulmonary inflammation in the allergic mouse is characterized by elevated levels of IgE in the serum (21, 22), a specific role for IgE in this model is controversial and in the case of robust eosinophilic airway inflammation, IgE is not essential for airway inflammation and AHR (23). Clearly, the high IgE levels observed in the VDR null mice were not associated with airway inflammation and the lungs of Ag-primed and -challenged VDR KO mice had very few eosinophils. Altogether, the data suggest that signaling through the VDR is required for controlling IgE responses.

As observed with the increased IgE levels, the failure of the VDR KO mouse to develop experimental asthma was not a result of the failure to generate a Th2 response, because splenocytes from primed VDR KO and WT mice produced a robust Th2 cell response characterized by both IL-5 and IL-13 production. These cytokines have been shown to be central to eosinophilic airway inflammation and AHR (24–26). By contrast, IL-4 levels were lower in primed VDR KO mice than WT mice. Although IL-4 contributes to IgE production (27), airway inflammation can be reconstituted in IL-4-deficient mice by IL-5 administration (26). Thus, the VDR KO mouse develops a robust Th2 cell response, characterized by IL-5 and IL-13, along with elevated IgE responses, but is resistant to the development of experimental asthma symptoms in the lungs. These data suggest a role for the VDR in controlling other responses that lead to pathological and physiological symptoms of allergic asthma.

Trafficking of Th2 cells during allergic lung inflammation depends on signaling through chemoattractant receptors (28). Release of chemokines can cause cellular activation and other inflammatory mediators to be released by basophils and eosinophils (29). Few eosinophils and T cells were detectable in lungs of primed VDR KO mice, and perhaps the activation and/or recruitment of these cells may be affected in the VDR null animals. The recruitment of inflammatory cells is complex and it is well established that interactions among mast cells, lymphocytes, eosinophils, and macrophages are important in the development of asthma (30). It has been reported that vitamin D-deprived animals have decreased macrophage chemotaxis and more recently, $1,25(OH)_2D_3$-treated T cells have been shown to exhibit decreased chemokine-induced migration (Wientroub et al. (31); Topilski et al. (19)). In addition to the failure to recruit T cells and eosinophils, the lack of the VDR might inhibit downstream events, which regulate gene expression. Although Th2 cells develop in VDR KO mice, inflammatory lymphocytes and eosinophils fail to accumulate in the lungs. The data suggest that the VDR may also be important for the trafficking of immune cells to the lung.

The transcription factor GATA-3 and the low affinity IgE receptor sCD23 are in vitro targets of $1,25(OH)_2D_3$ in Th0 and Th2 cells (9). $1,25(OH)_2D_3$ treatment of unprimed, but not primed, WT mice led to increases in the message for GATA-3 and sCD23 in the lung. GATA-3 is of interest as an important transcription factor that regulates Th2 cell development and is known to be involved in airway inflammation (32). Expression of GATA-3 is associated with asthma (33) and blocking GATA-3 expression has been shown to abrogate lung inflammation (34). In addition, CD23 deficiency in mice results in elevated IgE levels, with airway eosinophilia and AHR (35). The increased expression of GATA-3 and sCD23 in the lungs following $1,25(OH)_2D_3$ treatment and 3 days of Ag exposure, suggest a potential for increased airway inflammation via $1,25(OH)_2D_3$ in unprimed mice. However, it should be noted that we were unable to measure any differences in the asthma symptom severity between $1,25(OH)_2D_3$ and control treated unprimed mice. Our data, however, show that GATA-3 and sCD23 are in vitro and in vivo $1,25(OH)_2D_3$ targets. Our data also support an important role for the VDR in the development of Th2-driven immune responses, and suggest that VDR deficiency may have a protective role in a Th2 cell-directed disease.

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