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Invariant NKT Cell Defects in Vitamin D Receptor Knockout Mice Prevents Experimental Lung Inflammation

Sanhong Yu,¹ Jun Zhao, and Margherita T. Cantorna

Vitamin D receptor (VDR) deficiency (knockout [KO]) results in a failure of mice to generate an airway hyperreactivity (AHR) response on both the BALB/c and C57BL/6 background. The cause of the failed AHR response is the defective population of invariant NKT (iNKT) cells in the VDR KO mice because wild-type (WT) iNKT cells rescued the AHR response. VDR KO mice had significantly fewer iNKT cells and normal numbers of T cells in the spleen compared with WT mice. In BALB/c VDR KO mice, the reduced frequencies of iNKT cells were not apparent in the liver or thymus. VDR KO and WT Th2 cells produced similar levels of IFN- γ and IL-5. On the BALB/c background, Th2 cells from VDR KO mice produced less IL-13, whereas on the C57BL/6 background, Th2 cells from VDR KO mice produced less IL-4. Conversely, VDR KO iNKT cells were defective for the production of multiple cytokines (BALB/c: IL-4, IL-5, and IL-13; C57BL/6: IL-4 and IL-17). Despite relatively normal Th2 responses, BALB/c and C57BL/6 VDR KO mice failed to develop AHR responses. The defect in iNKT cells as a result of the VDR KO was more important than the highly susceptible Th2 background of the BALB/c mice. Defective iNKT cell responses in the absence of the VDR result in the failure to generate AHR responses in the lung. The implication of these mechanistic findings for human asthma requires further investigation. *The Journal of Immunology*, 2011, 187: 000–000.

Asthma is an immunologic disease characterized by airway inflammation, increased production of mucus, and airway hyperreactivity (AHR) (1). The symptoms of asthma include recurrent wheezing, coughing, and shortness of breath (1). There are different forms of asthma in the clinic including asthma associated with allergy, infection, air pollution, and exercise that involve distinct pathways. The most common form of asthma is allergic asthma, which is dependent on CD4⁺ T cells and associated with Th2-driven inflammatory responses in the lung (2). In experimental allergic asthma, mice that are predisposed to Th2 responses (BALB/c) are more susceptible to disease than mice that are Th1 predisposed (C57BL/6). The mechanisms by which asthma is controlled are still not fully understood.

Invariant NKT (iNKT) cells have been shown to be required for the development of AHR by several groups (3). iNKT cells are a unique subset of lymphocytes that express markers of both $\alpha\beta$ TCR⁺ T cells and NK cells. iNKT cells express a conserved TCR that recognizes glycolipid Ags. The sponge-derived ligand α -galactosylceramide (α -GalCer) has been particularly useful in the characterization of iNKT cells because of the high specificity

for CD1d and iNKT cells (3). iNKT cells act very early in an immune response and are responsible for the rapid production of large amounts of cytokines including IL-4, IL-13, and IFN- γ upon TCR stimulation (3). iNKT cell-deficient mice (both CD1d^{-/-} and α 18^{-/-}) fail to develop AHR and have greatly reduced eosinophilia after sensitization and challenge with allergen, ozone, or virus (4–6). The requirement of iNKT cells for AHR development was specific because adoptive transfer of wild-type (WT) iNKT cells into α 18^{-/-} mice reconstituted the development of AHR (4, 5). Activation of pulmonary iNKT cells can directly initiate the development of AHR as intranasal (i.n.) administration of α -GalCer or glycolipids from *Sphingomonas* bacteria activated iNKT cells, resulting in AHR and airway inflammation (7). The development of AHR in different models of asthma requires distinct subsets of iNKT cells. CD4⁺ iNKT cells produce both IL-13 and IL-4 and were required for allergen-induced AHR (4), whereas CD4⁻ iNKT cells make IL-13 only and were involved in virus-induced AHR (4). iNKT cells participate in the development of AHR in the lungs and are therefore potential targets for regulation in asthma and allergy.

1 α ,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], the active form of vitamin D, is a potent regulator of immune responses. 1,25(OH)₂D₃ binds to the vitamin D receptor (VDR), which is a ligand inducible transcription factor. All cells of the immune system tested express the VDR, and 1,25(OH)₂D₃ has been shown to be an important regulator of T cell function (8, 9). 1,25(OH)₂D₃ treatment has been shown to suppress animal models of Th1/Th17-type autoimmune diseases (10, 11). Furthermore, in vitro studies showed that 1,25(OH)₂D₃ treatment increased the production of IL-4 by Th2 cells and decreased the production of IFN- γ by Th1 cells (12, 13). Mice lacking the VDR have been shown to be more susceptible to autoimmunity and in particular inflammatory bowel disease (14). Th17 cells are targets of vitamin D because 1,25(OH)₂D₃ suppresses IL-17 production, and regulatory T (Treg) cells are induced by 1,25(OH)₂D₃ in vitro and in vivo (15). Two regulatory cells require the VDR for normal development and function, CD8 α ⁺/TCR α β ⁺ T cells and iNKT cells (16, 17). C57BL/6 VDR knockout (KO) mice failed to de-

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Abbreviations used in this article: AHR, airway hyperreactivity; Alum, aluminum hydroxide; BALF, bronchoalveolar lavage fluid; α -GalCer, α -galactosylceramide; i.n., intranasal; iNKT, invariant NKT; KO, knockout; 1,25(OH)₂D₃, 1 α ,25-dihydroxyvitamin D₃; Treg, regulatory T; VDR, vitamin D receptor; WT, wild-type.

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velop AHR following induction of experimental allergic asthma using OVA and aluminum hydroxide (Alum) (18). The C57BL/6 VDR KO mice have very few iNKT cells (16). To determine whether a requirement of the VDR for Th2 and/or iNKT cells was responsible for the failure of C57BL/6 VDR KO mice to develop experimental asthma, experiments were done to evaluate the roles of these two cell types.

C57BL/6 VDR KO mice were backcrossed onto the Th2-biased and experimental allergic asthma-susceptible BALB/c background. Consistent with the finding from the C57BL/6 VDR KO mice, BALB/c VDR KO mice failed to develop OVA-induced experimental allergic asthma. AHR responses were low in VDR KO mice in the OVA model and also when α -GalCer was administered i.n. to either the BALB/c or C57BL/6 VDR KO mice. In vitro, VDR KO and WT Th2 cell responses were very similar to each other, whereas VDR KO iNKT cell responses were lower than WT for several different cytokines. The ability of iNKT cells to produce IL-4, IL-5, and IL-13 in response to α -GalCer was 2–19-fold less in VDR KO than WT cells. iNKT cells from C57BL/6 WT mice produced high amounts of IL-17, and VDR deficiency reduced iNKT cell production of IL-17. The failure of VDR KO mice to develop AHR was due to impaired function of iNKT cells because adoptive transfer with WT iNKT cells reconstituted AHR development in VDR KO mice. The ability of VDR KO CD4⁺ T cells to become Th2 cells was largely intact. Conversely, VDR KO iNKT cells produced less of the asthma-inducing cytokines compared with WT iNKT cells. The data demonstrate that the failure of VDR KO mice to develop AHR is not strain specific and due to impaired iNKT cell function in the mice.

Materials and Methods

Mice

The C57BL/6 VDR KO mice were backcrossed onto the BALB/c background (seven generations, 99.2% BALB/c). The mice were a gift of Dr. Mary Ann McDowell (University of Notre Dame, South Bend, IN). For experiments, age- and sex-matched VDR KO and WT mice on C57BL/6 and BALB/c were produced at The Pennsylvania State University (University Park, PA). Experimental procedures received approval from the Office of Research Protection Institutional Animal Care and Use Committee at The Pennsylvania State University.

Flow cytometry

Single-cell suspensions of thymus, spleen, and liver were isolated. Mononuclear cells from liver were prepared as described previously (17). Cells were stained with PE-labeled CD1d-PBS57 tetramers (PBS57 is a synthetic iNKT cell agonist; gift of the National Institutes of Health Tetramer Facility, Atlanta, GA), CD4, CD8, and PE-Cy5-labeled anti-TCR β (H57-597) (BD Pharmingen, San Diego, CA). Empty tetramers (National Institutes of Health Tetramer Facility) and isotype control Abs (BD Pharmingen) for each cell-surface marker and cytochrome were used to establish specificity. For sorting, liver mononuclear cells were stained with PE-labeled CD1d-PBS57 tetramer PE-Cy5-labeled anti-TCR β . T cells (TCR β ⁺tetramer⁻) and iNKT cells (TCR β ⁺tetramer⁺) were then sorted by Cytopeia influx (BD Biosciences). Purity of both T cells and iNKT cells was >98%.

Cell culture and cytokine analysis

WT and VDR KO splenocytes were isolated and cultured in RPMI 1640. Cells were cultured in RPMI 1640 supplemented with 10% FCS (Hyclone, Logan, UT), 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 5 mM 2-ME (Invitrogen, Carlsbad, CA). For Th2 induction, 2×10^6 splenocytes was stimulated with 5 μ g/ml anti-CD3 (2C11; BD Pharmingen) for 3 d in six-well plates in the presence of IL-4 (5000 IU/ml; PeproTech, Rocky Hill, NJ), and 1 μ g/ml anti-IFN- γ neutralizing Abs (BD Pharmingen). Three milliliters new media with 20 IU/ml IL-2 (PeproTech) was added into each well at day 3. At day 4, cells were collected, washed twice, and restimulated with 10 ng/ml PMA and 2.5 μ g/ml ionomycin for another 72 h (Sigma-Aldrich, St. Louis, MO) when the supernatants were collected (total of 7 d in culture). For iNKT cell cultures, 2×10^6 splenocytes were stimulated with 100 nM α -GalCer for a total of 72 h.

Lungs were lavaged via the tracheal tube with 1 ml PBS at room temperature. Total leukocyte, lymph, and basophil numbers were measured using a Coulter Counter (Coulter, Hialeah, FL). Cytokines in bronchoalveolar lavage fluid (BALF) were analyzed by ELISA using the standards and kits as provided (BD Pharmingen). Supernatants were analyzed for levels of IL-4, IL-5, IL-17, IL-13, and IFN- γ by a Luminex multiplex bead system kit (Lincplex, Billerica, MA) on a Bioplex system (Bio-Rad, Hercules, CA). For some experiments, IFN- γ values were obtained by ELISA kits (BD Pharmingen).

OVA-induced allergic asthma model

Mice were immunized i.p. on days 0 and 5 with 50 μ g/ml OVA (Sigma-Aldrich) complexed with Alum (10 μ g OVA/1 mg Alum; Thermo Fisher Scientific, Rockford, IL). Beginning 12 d after immunization, mice were exposed to 60 μ g OVA i.n. for 4 consecutive d, and sacrificed 24 h after the last exposure or on day 15. For some experiments, 2 μ g α -GalCer or PBS was administered to the mice at day 0. VDR KO mice also received purified iNKT cells (1.5×10^6) or T cells (1.5×10^6) i.p. from WT mice on day 0.

α -GalCer-induced lung inflammation

α -GalCer (Axxora, San Diego, CA) was dissolved in PBS containing 0.5% Tween 20, heated to 80°C for 10 min, and sonicated for 5 min on ice. Two micrograms α -GalCer was administered i.n. to mice anesthetized with isoflurane. Glycolipid vehicles were administered as controls. Twenty-four hours after the α -GalCer administration, mice were analyzed for AHR, and sections of the lungs were stained for lung inflammation.

AHR and lung histopathology

AHR was determined using a Flexivent mechanical ventilator (SciReq, Chandler, AZ). Mice were anesthetized and a cannula placed in the trachea so that the lungs were ventilated at a rate of 120 breaths/min; tidal volume = 0.2 ml, flow rate 1.5 ml/s at 2 to 3 cm H₂O positive end-expiratory pressure. Airway pressure in response to methacholine (0–100 mg/ml) was determined using a differential pressure transducer. Following the AHR measurements, lungs were fixed, sectioned (Veterinary Diagnostic Laboratories, The Pennsylvania State University), and scored for the severity of the inflammation using a range of 0, no disease, to 4, maximal disease and exactly as previously described (18, 19).

Results

BALB/c VDR KO mice fail to develop experimental allergic asthma

VDR KO and WT mice on the BALB/c background were immunized with OVA and Alum to induce experimental allergic asthma exactly as described (18). Twenty-four hours after the last i.n. challenge, mice were subjected to mechanical ventilation for analysis of AHR. As expected, immunized WT BALB/c mice showed an increase in AHR with increasing dose of methacholine (Fig. 1A). Conversely immunized VDR KO mice did not respond to increasing doses of methacholine (Fig. 1A). In fact, the AHR response of immunized VDR KO mice was the same as mock PBS-treated WT mice (WT naive, Fig. 1A). Histopathology sections from immunized WT mice showed increased leukocyte accumulation around the bronchi and bronchioles in the lungs (Fig. 1B). Immunized VDR KO mice had little inflammation, similar to what was found in the sections from naive WT mice (Fig. 1B). The lung histopathology scores from immunized VDR KO mice were significantly less than those from WT mice (Fig. 1C). The histopathology scores from primed WT mice were 3.2 ± 0.5 , whereas primed VDR KO were 0.5 ± 0.1 (mean $\pm$ SEM). BALB/c VDR KO mice are resistant to the development of experimental allergic asthma induced by OVA/Alum immunization.

VDR KO mice are hyporesponsive to α -GalCer

To determine the role of iNKT cells in the development of airway inflammation in VDR KO mice, α -GalCer was administered i.n. to WT and VDR KO mice on the C57BL/6 and BALB/c backgrounds. As expected, exposure to α -GalCer 24 h prior to methacholine challenge resulted in increased airway resistance in both

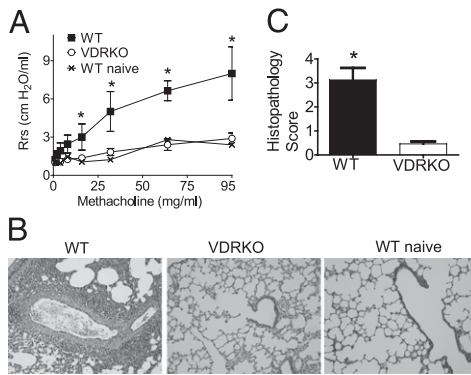


FIGURE 1. BALB/c VDR KO mice fail to develop experimental allergic asthma. BALB/c WT and VDR KO mice were induced to develop allergic asthma. **A**, AHR was measured in naive WT (WT + PBS) and OVA/Alum-immunized and OVA-challenged WT and VDR KO mice. Immunized WT mice developed AHR following increasing doses of methacholine. Conversely, immunized VDR KO mice failed to respond, and the AHR response was similar to the WT + PBS naive mice. $n = 4$ mice per group and one representative experiment of four. The WT AHR response was significantly higher than VDRKO or WT + PBS. **B**, The histopathology section (original magnification $\times 100$) from one representative lung from each group of mice in **A** is shown. **C**, The mean histopathology scores from all of the sensitized mice in the group. Values from VDR KO mice are significantly different from WT mice ($n = 12$ mice per group, mean $\pm$ SEM). $*p < 0.05$.

C57BL/6 and BALB/c WT mice (Fig. 2A). Conversely, α -GalCer treatment resulted in VDR KO mice nonresponsive to methacholine on both the C57BL/6 and BALB/c background (Fig. 2A, Table I). In fact, there was no increase in AHR responses with α -GalCer treatment of VDR KO mice over that with the PBS controls. Airway resistance of WT mice was significantly higher than that of VDR KO or WT challenged with PBS at doses of methacholine >25 mg/ml (Fig. 2A). Histopathology sections of the lungs showed that eosinophils and lymphocytes infiltrated into peribronchial spaces of the WT lungs exposed to α -GalCer (Fig. 2B). The histopathology sections of VDR KO mice exposed to α -GalCer were more similar to the unsensitized (PBS) mice than the α -GalCer-exposed WT (Fig. 2B and WT PBS data not shown). The BALF from C57BL/6 WT mice exposed to α -GalCer had high levels of IL-4 (Fig. 2C). Consistent with the lack of AHR response, the C57BL/6 VDR KO mice exposed to α -GalCer had no detectable IL-4 in the BALF, and the IL-4 response was similar to the unsensitized PBS-treated controls (Fig. 2C). The data suggest that activation of pulmonary iNKT cells fails in the VDR KO mice regardless of the genetic background of the mice (Table I).

BALB/c VDR KO mice had reduced numbers of iNKT cells

Previously, it has been shown that the frequencies of CD4 and CD8 T cells in the spleen, liver, and thymus of C57BL/6 VDR KO and WT are similar (17). The BALF from the lungs of C57BL/6 VDR KO mice contained normal frequencies of CD4⁺ and CD8⁺ T cells compared with WT (Fig. 3C). The frequencies of CD4⁺ T cells were the same in VDR KO and WT BALB/c mice in the spleen, whereas the BALB/c VDR KO BALF had significantly fewer CD8⁺ T cells compared with WT mice (Fig. 3B, 3D). CD4 numbers and frequencies are the same in the spleen and lung of BALB/c VDR KO and WT mice (Fig. 3A, 3D). There were no differences in the numbers of lymphocytes collected from the spleens and BALF of VDR KO and WT mice, and therefore the total CD4⁺ and CD8⁺ frequencies reflect the actual numbers of cells in the VDR KO and WT mice (Fig. 3) (17). The percentages of iNKT cells (defined as TCR β and CD1d-PBS57 tetramer

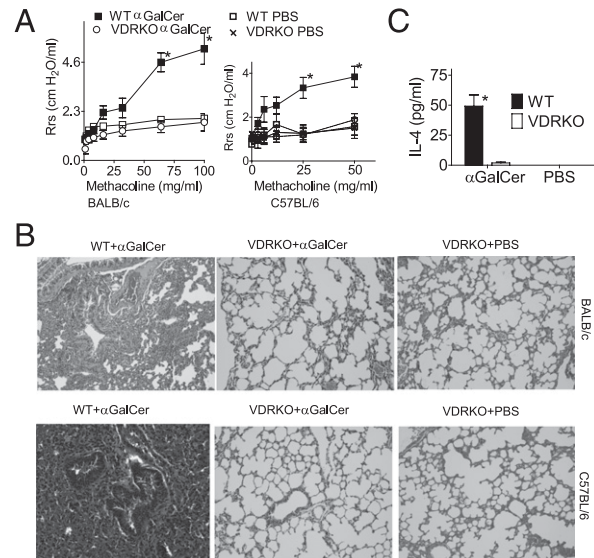


FIGURE 2. α -GalCer-induced AHR fails in VDR KO mice on the BALB/c and C57BL/6 background. WT and VDRKO mice were challenged i.n. with α -GalCer and analyzed 24 h later. **A**, α -GalCer-induced sensitivity to methacholine in BALB/c and C57BL/6 WT mice. α -GalCer treatment of either BALB/c or C57BL/6 VDR KO mice failed to induce an increase in air resistance. WT α -GalCer AHR values are significantly higher than all other groups. $*p < 0.05$ (one representative of two experiments; $n = 3$ mice per group per experiment). **B**, The histopathology section (original magnification $\times 100$) from one representative lung from each group of mice in **A** is shown. **C**, BALF from WT and VDRKO mice on the C57BL/6 mice were collected to determine the IL-4 level. IL-4 was detectable only in the WT BALF from mice exposed to α -GalCer. WT α -GalCer IL-4 values were significantly higher than all other groups. $*p < 0.05$ ($n = 6$ mice per group; error bars are $\pm$ SEM).

positive) were significantly lower in the BALB/c VDR KO spleen but not different in the BALF compared with BALB/c WT mice (Fig. 3B, 3D). The frequencies of iNKT cells in the thymus and liver of BALB/c VDR KO mice were not significantly different from the BALB/c WT mice (Fig. 3B). This is in sharp contrast to the significant and 4–10-fold lower numbers of iNKT cells in the C57BL/6 VDR KO mice (17). In the spleen, BALB/c and C57BL/6 KO mice had half as many iNKT cells as their respective WT counterparts (0.8–1% WT and 0.5–0.3% VDR KO). The BALF of both the BALB/c and C57BL/6 VDR KO lung contained similar frequencies and total numbers of iNKT cells compared with WT mice (Fig. 3C–E). The data show that VDR KO mice on the

Table I. VDR KO versus WT response on the BALB/c and C57BL/6 backgrounds

	IL-4	IL-5	IL-13	IL-17	IFN- γ	AHR	Lung Histopathology
BALB/c							
Th2 ^a	–	–	↓	–	–	↓	↓
iNKT ^b	↓	↓	↓	–	–	↓	↓
C57BL/6							
Th2 ^a	↓	–	–	–	–	↓	↓
iNKT ^b	↓	–	–	↓	–	↓	↓

^aFor cytokines, indicates a summary of the results from Th2 cultures of the spleen (Fig. 4); for AHR and lung histopathology, indicates a summary of the results from OVA/Alum-induced experimental asthma.

^bFor cytokines, indicates a summary of the results from iNKT cell cultures of the spleen (Fig. 4); for AHR and lung histopathology, indicates a summary of the results from α -GalCer-induced lung inflammation.

↓, VDR KO response is significantly less than WT; –, VDR KO and WT responses are not different.

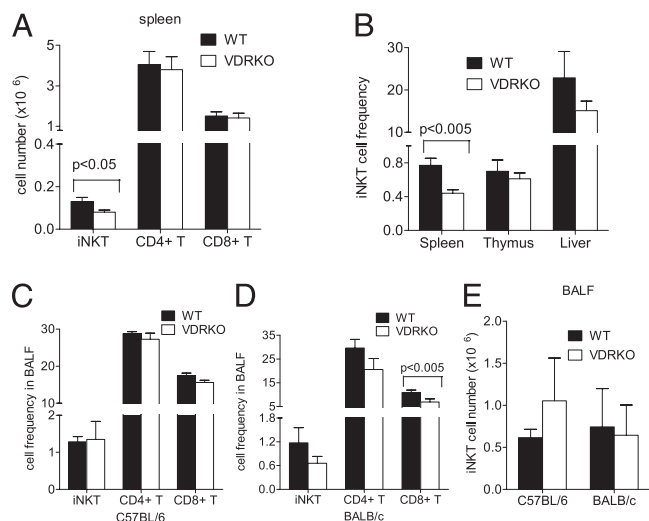


FIGURE 3. iNKT, CD4⁺ T, and CD8⁺ T cell numbers in the BALB/c VDR KO and WT mice. **A**, The total number of iNKT, CD4⁺ T, and CD8⁺ T cells isolated from the spleen of BALB/c mice. **B**, The frequency of iNKT cells (CD1d tetramer/TCR β ⁺ T cells) in BALB/c WT and VDR KO spleen, thymus, and liver. VDR KO mice had significantly fewer iNKT cells than WT in the spleen ($n = 4-6$ mice per group). The frequency of iNKT, CD4⁺ T, and CD8⁺ T cells isolated from the BALF of C57BL/6 (**C**) and BALB/c VDR KO and WT mice (**D**) ($n = 6$ to 7 mice/group). **E**, The total number of iNKT cells in the BALF of the C57BL/6 and BALB/c mice.

BALB/c background have reduced frequencies and numbers of iNKT cells but only in the spleen.

Th2 and iNKT cell responses in VDR KO mice

T cells are recruited to the lung during chronic asthmatic responses, producing cytokines such as IL-4, IL-5, and IL-13 that are associated with the pathology of asthma and allergy (20–22). The underlying cause of the observed hyporesponsiveness of VDR KO mice to either OVA and Alum- or α -GalCer-induced lung inflammation could be as a result of reduced Th2 cells, reduced iNKT cells, or both. Splenocyte cultures were used to induce Th2 and/or iNKT cells in vitro and the profile of cytokine secretion evaluated. The Th2 cultures from VDR KO mice had comparable levels of IL-5, IL-17, and IFN- γ as the WT Th2 cell cultures (Fig. 4A, 4B). IL-13 was lower in the Th2 cell cultures from BALB/c VDR KO, and IL-4 was lower in the C57BL/6 VDR KO Th2 cell

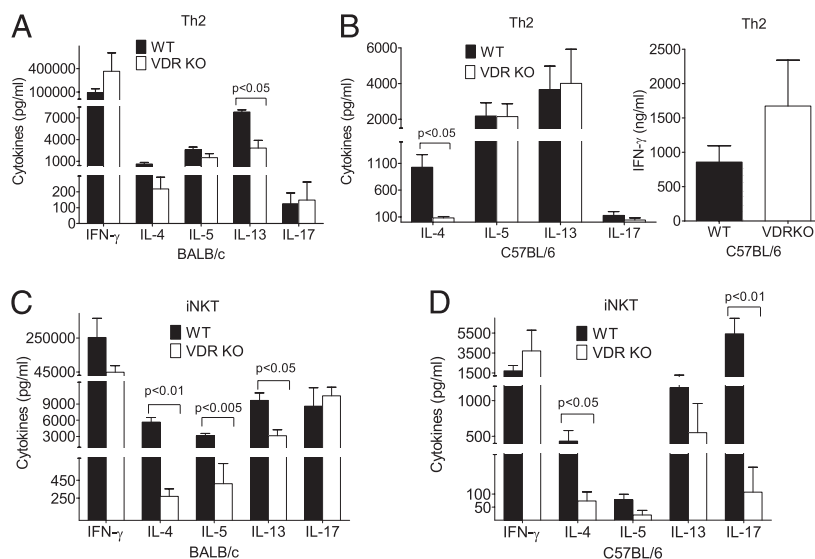
cultures than WT. There was no effect on the Th2 cell production of IL-5, IL-17, and IFN- γ in VDR KO mice (Table I).

iNKT stimulation of splenocytes using α -GalCer (72 h) showed that VDR KO splenocytes secreted significantly less IL-4 than similarly treated WT cells (Fig. 4C). Even though there are significantly fewer iNKT cells in the spleen of VDR KO mice, IFN- γ secretion in response to α -GalCer was not different between VDR KO and WT mice (Fig. 4C, 4D). IL-17 production by C57BL/6 VDR KO splenocytes was 51-fold lower than WT (Fig. 4D). VDR KO α -GalCer-stimulated splenocytes secreted 6–19-fold less IL-4, 4–8-fold less IL-5, and 2- to 3-fold less IL-13 (Fig. 4C, 4D). Only in the BALB/c VDR KO iNKT cell cultures did the IL-5 and IL-13 reduction reach significance (Fig. 4C, 4D). VDR deficiency has a significant effect on iNKT cell production of several key asthma-inducing cytokines (Table I).

Transfer of WT iNKT cells to VDR KO mice rescues lung inflammation

iNKT cells or T cells were purified from WT mice and injected into VDR KO C57BL/6 recipients. Some of the mice were injected with α -GalCer prior to being induced to develop experimental allergic asthma using OVA/Alum. AHR was highest in WT mice that had received α -GalCer prior to OVA immunization (WT iNKT, Fig. 5A). AHR was lowest in VDR KO mice that received T cells plus α -GalCer (VDR KO T cells, Fig. 5A). AHR responses of VDR KO mice that had received WT iNKT cells and α -GalCer (VDR KO iNKT) approached those of the WT mice and were significantly higher than the VDR KO mice that received T cells at 95 mg/ml methacholine (Fig. 5A). The AHR of VDR KO mice with WT iNKT cells was not different from WT at 64 mg/ml methacholine (Fig. 5A). The composition of BALF was analyzed following mechanic ventilation in the mice shown in Fig. 5A. Consistent with the elevated AHR measurements, WT mice with α -GalCer and OVA immunization had more WBCs and lymphocytes (Lymph) than all other groups (Fig. 5B). Eosinophils were not detected in either the VDR KO or WT BALF. The numbers of WBCs were not different between VDR KO mice with WT iNKT cells, α -GalCer, and OVA immunization and WT mice with PBS and OVA immunization (Fig. 5B). The WBC counts in VDR KO with PBS and OVA immunization had slightly lower but not significantly lower WBC numbers than the WT PBS and VDR KO iNKT values (Fig. 5B). WT transfer of iNKT cells rescues AHR development in VDR KO mice immunized with OVA/Alum.

FIGURE 4. Th2- and iNKT-generated cytokine secretion from VDR KO and WT mice. Splenocytes from WT and VDR KO mice were stimulated under Th2 or iNKT cell conditions and the ability to produce IL-4, IL-5, IL-13, IL-17, and IFN- γ was evaluated. Th2 cell cultures of BALB/c (**A**) or C57BL/6 (**B**) spleens. iNKT cell cultures generated by α -GalCer treatment of BALB/c (**C**) or C57BL/6 (**D**) splenocytes. Data shown are the mean $\pm$ SEM of three to four individual mice and one representative of three experiments.



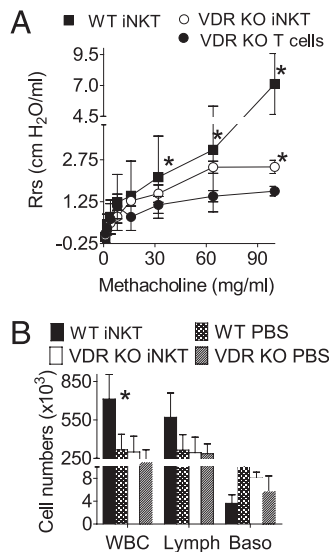


FIGURE 5. WT iNKT cells rescued AHR in VDR KO mice. Mice were injected with α -GalCer and immunized/challenged with OVA. VDR KO mice were given WT iNKT cells (VDR KO iNKT) or WT T cells depleted of iNKT cells (VDR KO T cells). **A**, WT mice developed AHR following exposure to methacholine, and the AHR response was significantly higher than VDR KO mice that received T cells (at doses of methacholine >16 mg/ml; $n = 5$ mice per group). VDR KO mice that received iNKT cells had higher AHR responses than VDR KO mice that received T cells, and the difference was significant at 95 mg/ml methacholine ($n = 5$ mice/group). **B**, Cellular influx in the BALF of WT and VDR KO mice 24 h after last OVA i.n. challenge. The values from WT α -GalCer mice were significantly higher than all other groups ($n = 5$ mice/group). * $p < 0.05$. VDR KO iNKT, α -GalCer/iNKT cell transfer and OVA-sensitized; VDR KO PBS, PBS and OVA-sensitized; WT iNKT, α -GalCer and OVA-sensitized; WT PBS, PBS and OVA-sensitized.

Discussion

The inability of VDR KO mice to develop AHR is a result of impaired iNKT cell development and not Th2 cell defects. Reconstitution of iNKT cells and not T cells was able to recover AHR responses in VDR KO mice. Interestingly, even in Th2-biased BALB/c mice, the iNKT cell defect overcomes the Th2 allergic asthma bias of the mice, and VDR KO BALB/c mice fail to develop allergic asthma. The role of iNKT cells in experimental allergic airway disease has been demonstrated in several models. The production of IL-13, IL-4, and IL-5 by iNKT cells is thought to promote AHR, eosinophilia, and IgE class switching (23, 24). In addition, iNKT cells that produce IL-17 result in the increased presence of neutrophils in the lungs (23, 24). Vitamin D has been shown to be important in the development of the neonatal lung (25). Data using bone marrow transplantation also suggest that in addition to the immune-mediated effects of vitamin D in experimental asthma, the lung may also require vitamin D (19). The data presented in this study support a critical role for vitamin D in iNKT cell development and function in the lung. Previously, it has been shown that disease-causing Th2 cells develop in the VDR KO host (19), and the data presented in this study support the finding that vitamin D is less critical for the regulation and development of Th2 cell responses. It is clear that iNKT cells can participate in asthma development and that vitamin D is an important iNKT cell regulator. The question that remains is whether iNKT cells and vitamin D participate in human disease.

Surprisingly, BALB/c VDR KO mice had fewer iNKT cells but only in the spleen. This is in contrast to the result in C57BL/6 VDR KO mice that have fewer iNKT cells in all tissues examined but the lung (17). VDR expression is also needed for maturation of

iNKT cells, and VDR KO iNKT cells are defective for the production of cytokines (17). The data show that i.n. exposure to α -GalCer failed to induce inflammation in the VDR KO lung regardless of the genetic background of the mice (Table I). VDR expression is required for several aspects of iNKT cell development and function. The VDR-mediated effects on iNKT cell frequency in the thymus and liver are different in BALB/c and C57BL/6 mice, whereas other functional aspects are similar including the inability to induce AHR and inflammation in the lung following α -GalCer administration.

The effect of vitamin D on OVA-induced experimental allergic asthma has been studied by several groups. The active form of vitamin D [1,25(OH)₂D₃] has been shown to have no effect, beneficial effects, and detrimental effects on various symptoms of experimental asthma (reviewed in Ref. 26). 1,25(OH)₂D₃ has been shown to induce IL-4 and IL-13 secretion (20) and inhibit IL-4 secretion by Th2 cells in another study (21). This seemingly disparate result might reflect the population of cells present in the cultures. Beneficial effects of vitamin D might include the induction of Treg cells that have not only been shown to be induced but also to protect against experimental allergic asthma (22, 27). Other benefits of 1,25(OH)₂D₃ treatment might be the inhibition of IL-17 production (23, 24, 26). VDR KO mice have normal numbers of functional Treg cells (16). CD4⁺ T cells from VDR KO mice have more activated and memory cells that readily develop into Th17 and Th1 cells (14, 28). Th2 development is largely intact in the VDR KO mice (19). In the OVA/Alum models, the critical T cell types for disease development include the Treg, Th2, and iNKT cells. In VDR KO mice, the inability of the iNKT cells to induce a robust Th2 response with the presence of normal functional Treg cells prevents the induction of AHR and development of other symptoms of allergic asthma.

There has been extensive speculation that vitamin D deficiency may be associated with several chronic inflammatory diseases including asthma (26). These association studies lack the ability to understand the mechanisms by which a nutrient like vitamin D could impact chronic inflammatory diseases like asthma or inflammatory bowel disease. The epidemiological data in humans would seem to undermine the major conclusions from these more mechanistic studies in mice. One explanation for this is that vitamin D does not induce and regulate human iNKT cells. Another possibility is that iNKT cells are not important in the development of asthma in humans. For vitamin D and asthma, the data from humans are not without controversy because there is at least one group that has shown that increased vitamin D supplementation during childhood is associated with increased wheezing (29). The role of iNKT cells in human asthma is also a controversial topic, with different groups reporting disparate results and finding either very low frequencies (30) or high frequencies [60% of the T cells in the lung (31)] of iNKT cells. There are numerous explanations for the discrepancies in the data, and it seems possible that even low frequencies of iNKT cells could have a significant impact on asthma development. The connections among vitamin D status, iNKT cells, and asthma in humans require further investigation.

Vitamin D is an important regulator of iNKT cells in the lung. In the absence of the VDR, iNKT cells fail to induce Th2 cells and AHR in the lungs. Even in BALB/c mice that are very susceptible to experimental asthma, the iNKT cell defect in VDR KO mice results in the failure to generate AHR. WT iNKT cells rescue AHR and asthma development when transferred to VDR KO mice. The iNKT cell data in isolation suggest that asthma might be made worse by increasing vitamin D status in humans. However, it is clear that vitamin D regulates several different pathways in the lung, and therefore careful determinations of all mechanisms at

play are required. A better understanding of the mechanisms by which vitamin D regulates both the lung and immune function is needed to be able to predict what effects increasing vitamin D status might have on human asthma. Several ongoing or planned human clinical trials are underway to look at the role of vitamin D in preventing and ameliorating asthma development so, hopefully, answers are forthcoming.

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