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Vitamin D Regulation of OX40 Ligand in Immune Responses to *Aspergillus fumigatus*

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OX40 ligand (OX40L) is a costimulatory molecule involved in Th2 allergic responses. It has been shown that vitamin D deficiency is associated with increased OX40L expression in peripheral CD11c⁺ cells and controls Th2 responses to *Aspergillus fumigatus* *in vitro* in cystic fibrosis (CF) patients with allergic bronchopulmonary aspergillosis (ABPA). To investigate if vitamin D deficiency regulated OX40L and Th2 responses *in vivo*, we examined the effect of nutritional vitamin D deficiency on costimulatory molecules in CD11c⁺ cells and *A. fumigatus*-induced Th2 responses. Vitamin D-deficient mice showed increased expression of OX40L on lung CD11c⁺ cells, and OX40L was critical for enhanced Th2 responses to *A. fumigatus* *in vivo*. In *in vitro* assays, vitamin D treatment led to vitamin D receptor (VDR) binding in the promoter region of OX40L and significantly decreased the promoter activity of the OX40L promoter. In addition, vitamin D altered NF-κB p50 binding in the OX40L promoter that may be responsible for repression of OX40L expression. These data show that vitamin D can act directly on OX40L, which impacts Th2 responses and supports the therapeutic use of vitamin D in diseases regulated by OX40L.

Dendritic cells (DCs) are potent antigen-presenting cells (APCs) that are responsible for activating T cells (1) and promoting the proliferation of distinct T helper (Th) cell subsets (2). CD11c⁺ is a transmembrane protein found on the surface of DCs. CD11c⁺ DCs are a critical DC subpopulation responsible for antigen-specific T cell activation (3). Human CD11c⁺ cells in peripheral blood express receptors for thymic stromal lymphopoietin (TSLP), an epithelial cell cytokine that can drive Th2 differentiation (4). Expression of TSLP is increased in antigen-induced allergen models (5), and Th2 responses downstream of TSLP are necessary for the development of inflammatory allergic responses (6). TSLP potently activates CD11c⁺ cells and primes naïve T cells to produce the Th2 cytokines interleukin-4 (IL-4), IL-5, and IL-13 (4). Further, TSLP-activated DCs express the costimulatory molecule OX40 ligand (OX40L), which is responsible for triggering Th2 polarization (7), and blocking OX40L inhibits antigen-specific Th2 inflammation (8). Recently, it has been shown that TSLP induces OX40L in DCs through the activation of NF-κB components which can bind to NF-κB-like binding sites in the OX40L promoter to induce production of OX40L (9).

We have recently shown that the active form of vitamin D (1,25-dihydroxyvitamin D₃) can suppress *Aspergillus fumigatus*-specific Th2 responses in peripheral CD4⁺ T cells in patients with cystic fibrosis (CF) and allergic bronchopulmonary aspergillosis (ABPA) (10). This suppression of Th2 development was associated with an increase in transforming growth factor β-positive (TGF-β⁺) regulatory T cells as well as suppression of OX40L, a costimulatory molecule regulated by TSLP *in vitro* (7, 10). These data are consistent with prior data showing that vitamin D can decrease the maturation of DCs (11) and decrease DC capacity to activate alloreactive T cells (12).

In order to study the role of vitamin D in *in vivo* Th2 responses to *A. fumigatus*, we developed a model of vitamin D sufficiency versus deficiency and investigated the responses to intrapulmonary *A. fumigatus* challenge. Specifically, we hypothesized that vitamin D deficiency would exacerbate Th2 responses to *A. fumigatus* and that vitamin D can directly regulate OX40L expression to

regulate Th2 development *in vivo*. Here, we show that vitamin D deficiency increases expression of several costimulatory molecules on CD11c⁺ cells, specifically, OX40L, CD80, CD86, and inducible T-cell costimulator ligand (ICOS-L). Moreover, vitamin D deficiency led to an increased Th2 response to *A. fumigatus*, which was attenuated with neutralization of OX40L *in vivo*, suggesting that OX40L is required for enhanced Th2 responses in this model. Furthermore, we show that vitamin D treatment leads to vitamin D receptor (VDR) binding to the OX40L promoter and represses OX40L promoter activity *in vitro*. We discovered that VDR, NF-κB p50, and NF-κB p65 bind to the promoter region of OX40L, which can regulate the expression of OX40L. These data demonstrate that vitamin D directly regulates the expression of OX40L, which may explain the increased immune response in vitamin D deficiency seen in Th2-mediated diseases such as ABPA.

MATERIALS AND METHODS

Animals. We purchased 4- to 6-week-old female BALB/c mice (from the Jackson Laboratory). Mice were housed in specific-pathogen-free rooms within animal care facilities at the Louisiana State University Health Sciences Center (LSUHSC) and the Children's Hospital of Pittsburgh of UPMC. All mouse experiments were approved by the Institutional Animal Care and Use Committee at LSUHSC and the Children's Hospital of Pittsburgh of UPMC under university-approved protocols. Mice were provided with food and water *ad libitum* and housed under 12-h light-

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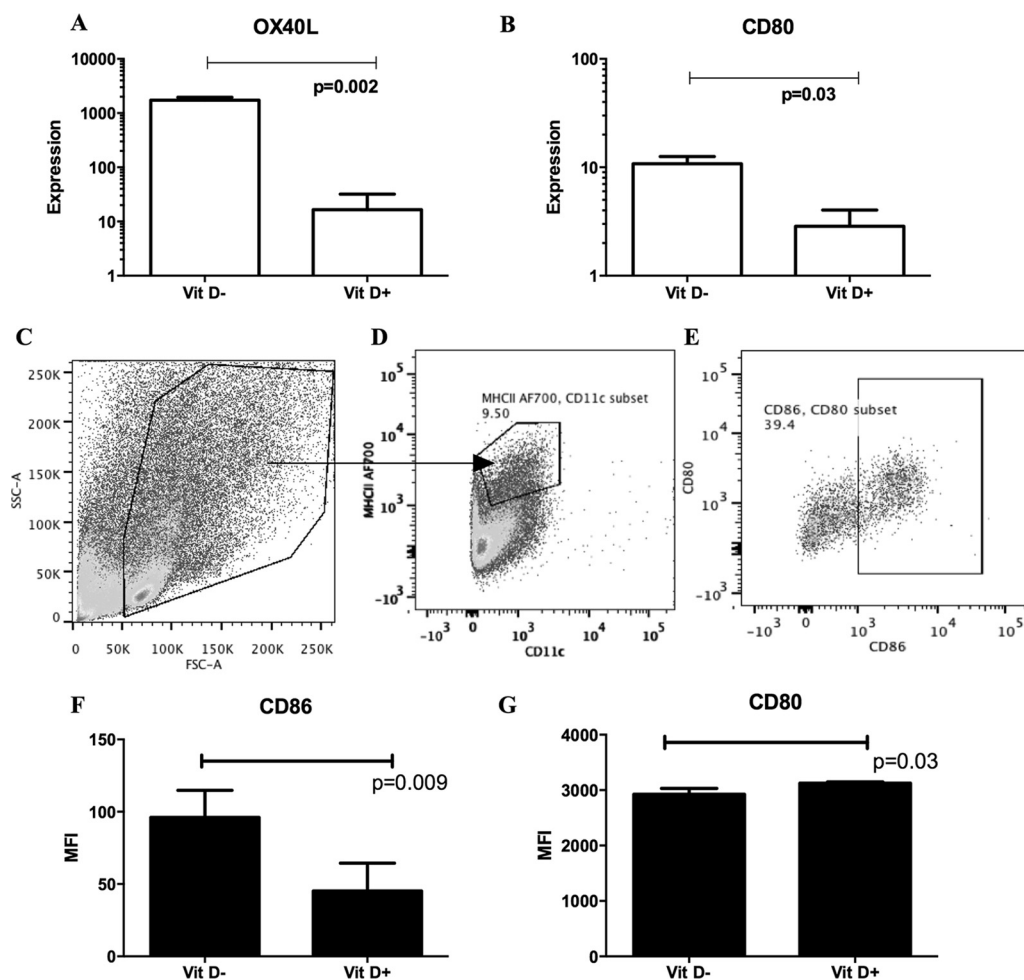


FIG 1 Vitamin D deficiency increases OX40L expression. CD11c cells from vitamin D-deficient (Vit D⁻) or vitamin D-sufficient (Vit D⁺) mice were isolated from splenocytes by magnetic separation, and gene expression was analyzed with the Illumina Expression BeadChip assay. (A and B) CD11c cells from vitamin D-deficient mice have increased expression of OX40L (A) and CD80 (B) compared to CD11c cells from vitamin D-sufficient mice. (C to E) CD11c cells from Vit D⁻ and Vit D⁺ mice were dendritic cells expressing CD11c and MHCII (C and D) in addition to CD80 and CD86 (E). (F and G) Vit D⁻ cells had a higher mean fluorescence intensity (MFI) of costimulatory molecule CD86. Representative data from three independent experiments with $n = 3$ per group are shown. P values of <0.05 are significant by a paired Student t test.

dark cycles. For infection models, mice were anesthetized with isoflurane (IsoFlo; Abbott Laboratories) and given 50 μ l of *A. fumigatus* conidia at 3.5×10^8 conidia/ml intratracheally. For anti-OX40L experiments, mice were given 50 μ g anti-mouse OX40L monoclonal antibody (MAB) (RML134; rat IgG2b/ κ ; BioLegend) intratracheally 4 h before challenge with *A. fumigatus* conidia.

Vitamin D-deficient diet. Four- to 6-week-old female BALB/c mice (The Jackson Laboratory) were placed on a vitamin D-deficient diet (Bio-Serv AIN-93G) and maintained on the diet for at least 4 weeks. Serum 25-OH vitamin D levels were measured by enzyme-linked immunosorbent assay (ELISA) (IDS) to ensure vitamin D deficiency. Control mice were kept on regular mouse chow (AIN-93G), supplied by the Division of Animal Care at LSUHSC or the Division of Laboratory Animal Resources at the Children's Hospital of Pittsburgh of UPMC (Harlan Teklad 2019S).

Preparation of *Aspergillus fumigatus*. *Aspergillus fumigatus* strain 42202 (ATCC) was spread onto potato dextrose agar (BD Biosciences) plated in a 260-ml tissue culture flask with a membrane filter lid and incubated at 37°C for 5 days. Spores were dislodged by adding a few glass beads to a flask with gentle shaking with 8 ml of phosphate-buffered saline (PBS) (Gibco) containing 0.1% Tween 20 (resuspension buffer). *A. fumigatus* conidia were counted on a hemocytometer and stored at 4°C.

Immediately before *in vivo* administration, *A. fumigatus* was spun out of resuspension buffer and resuspended in sterile PBS (Gibco) at a concentration of 3.5×10^8 conidia/ml.

Cell culture. For CD11c⁺ isolation, splenic CD11c⁺ dendritic cells were purified from the spleen using CD11c⁺-coated magnetic beads (Miltenyi) and used for microarray analysis (13). Lung CD11c⁺ cells were purified from lungs of vitamin D-deficient (Vit D⁻) and vitamin D-sufficient (Vit D⁺) BALB/c mice. Before lung harvest, lungs were perfused via the left ventricle with 10 to 15 ml of PBS with heparin (20 U/ml) to remove blood cells. Lungs were harvested, minced, and digested with 1.66 mg/ml collagenase (Sigma) at 37°C for 1 h. After digest, lung tissue was passed through a sterile 70- μ m filter (BD Falcon) to obtain a single-cell suspension. After cells were washed, CD11c⁺ cells were purified using CD11c⁺-coated magnetic beads (Miltenyi) for gene expression analysis.

Microarray. Microarray analysis was performed using the mouse WG-6 v2.0 Expression BeadChip platform (Illumina) by the Louisiana State University Health Sciences Center Microarray and Genome Bioinformatics Core. We analyzed the data with GeneSifter microarray analysis software.

Lung and mediastinal lymph node tissue collection. Lungs were harvested, and the right lung was homogenized in 1 ml of PBS with Complete

protease inhibitor (Roche). Homogenate was centrifuged at $12,000 \times g$ for 10 min, and supernatant was harvested and stored at -80°C for later cytokine analysis. Left lungs were harvested and homogenized in 1 ml of Trizol. RNA was isolated with a Trizol protocol.

Isolation of spleen single cells for intracellular cytokine staining (ICS). Spleens were isolated from vitamin D-deficient and vitamin D-sufficient mice and crushed with the end of a 5-ml syringe. Tissue was passed through a sterile 70- μm filter (BD Falcon) to get a single-cell suspension. After washing, the cells were ready for flow cytometry.

Flow cytometry analysis. Single cells from spleen were surface stained for 15 to 30 min at 4°C with anti-mouse major histocompatibility complex class II (MHCII), anti-CD11c, anti-CD80, and anti-CD86 (eBioscience) in PBS supplemented with 1% bovine serum albumin (BSA) and 0.2% sodium azide. Samples were acquired on a LSR-II flow cytometer, and data analysis was conducted with FlowJo software (Treestar).

Cytokine analysis. Lung homogenate was analyzed for protein levels of IL-4, IL-5, and IL-13 using a Luminex multiplex suspension cytokine array (Millipore) according to the manufacturer's instructions. The data were analyzed with Bio-Plex Manager software (Bio-Rad).

Real-time PCR. RNA was isolated from lung tissue or cultured cells, and a real-time PCR detection system (Bio-Rad CFX96) was used to detect genes of interest. Gene-specific primers and probes were purchased for IL-4, IL-5, IL-13, FoxP3, OX40L, and *A. fumigatus*-specific 18S rRNA (Applied Biosystems). Data are expressed using the threshold cycle ($\Delta\Delta C_T$) method and normalized to the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT) (Applied Biosystems).

Luciferase reporter assay. Plasmids were constructed by standard molecular biology techniques. U937 cells were cultured in RPMI medium (Gibco) with 10% fetal bovine serum (FBS). Cells were transiently transfected with a 1,000-bp OX40L promoter-inserted firefly luciferase reporter plasmid (pGL4.10; Promega; generously given by Yong Jun-Liu) (9) and a constitutive simian virus 40 (SV40) *Renilla* luciferase expression vector (Promega) with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Transfected cells were treated with vitamin D (0.1 μM) and/or tumor necrosis factor alpha (TNF- α) (10 ng/ml) for 24 h. Promoter activity was measured using the DLR assay kit (Promega) on a luminometer (Bio-Tek). Promoter activity was expressed as arbitrary units (firefly luciferase activity normalized to *Renilla* luciferase activity).

ChIP. Chromatin immunoprecipitation (ChIP) assays were performed with the MagnaA ChIP kit (Millipore) according to the manufacturer's instructions. Antibodies against NF- κB p50 (C-19) and p65 (C-20) (Santa Cruz) and vitamin D receptor (Abcam) were used for immunoprecipitations. Anti-H3K4 (Millipore) and anti-rabbit IgG (Millipore) were used as positive and negative controls, respectively. Precipitated DNA was amplified by PCR using the following primers: OX40L promoter region, 5'-AGT GCC AGG CTC ATG TGA TGT ACT-3' and 5'-GCG ATT GAA AGA GCA AAG CGG ACT-3'.

Statistics. All paired sample statistical analyses were performed using the two-tailed unpaired *t* test or the Mann-Whitney test for nonparametric data. For experiments with more than 2 conditions, means were compared using one-way analysis of variance (ANOVA) with a *post hoc* Tukey test. A *P* value of less than 0.05 was considered statistically significant. All statistical analyses were performed with Prism software (GraphPad).

RESULTS

CD11c⁺ cells from vitamin D-deficient mice have increased costimulatory expression. We have previously shown that CD11c⁺ cells from cystic fibrosis patients with ABPA are potent inducers of *A. fumigatus*-specific Th2 responses (10). To investigate the effects of vitamin D deficiency on CD11c⁺ cell function *in vivo*, we generated BALB/c mice that were nutritionally deficient in vitamin D as previously described (14). After 4 weeks on the diet, vitamin D-deficient mice had 25-(OH) D_3 levels of 10.13 ± 0.611 ng/ml compared to vitamin D-sufficient mice with 25-(OH) D_3 levels of 52.67 ± 6.429 ng/ml ($P < 0.01$). After vitamin D deficiency was

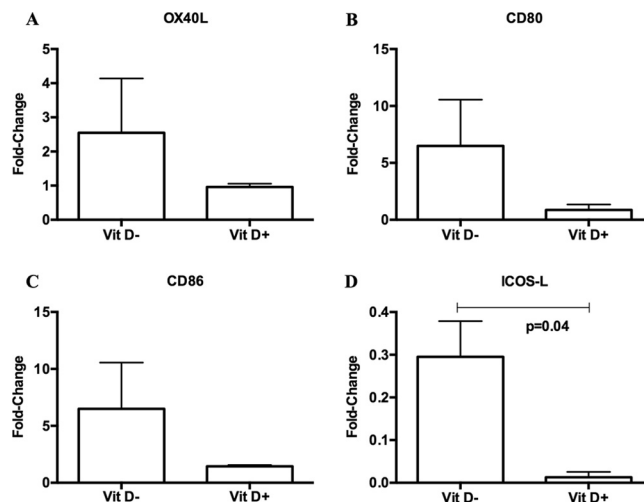


FIG 2 Vitamin D deficiency changes costimulatory expression in lungs. CD11c⁺ cells isolated from lungs of vitamin D-deficient mice had a higher expression of costimulatory molecules OX40L (A), CD80 (B), CD86 (C), and ICOS-L (D) than did vitamin D-sufficient mouse lung-derived CD11c⁺ cells. Gene expression is normalized to HPRT. Representative data from three independent experiments are shown with *n* = 3 per group. *P* values of <0.05 are considered significant by a paired Student *t* test.

established, CD11c⁺ cells were isolated from splenocytes from vitamin D-deficient and vitamin D-sufficient mice. Gene expression from RNA from vitamin D-deficient (Vit D⁻) and vitamin D-sufficient (Vit D⁺) CD11c⁺ cells was analyzed, and vitamin D-deficient CD11c⁺ cells showed significantly increased expression of the costimulatory molecule OX40L (Fig. 1A). Expression of OX40L in vitamin D-deficient CD11c⁺ cells was 105.19-fold higher than that in vitamin D-sufficient CD11c⁺ cells (Fig. 1A). In addition to OX40L, the costimulatory molecule CD80 showed increased expression in vitamin D-deficient CD11c⁺ cells (Fig. 1B) with a 3.76-fold increase over the level in vitamin D-sufficient CD11c⁺ cells (Fig. 1B). By microarray, CD86 did not show increased expression in Vit D⁻ CD11c⁺ cells (data not shown).

Further, splenocytes from vitamin D-deficient and vitamin D-sufficient mice were prepared in a single-cell suspension and stained for surface molecules. The gating strategy is shown in Fig. 1C. Splenocytes used for microarray analysis stained positive for dendritic cell markers MHCII and CD11c (Fig. 1D), staining which was increased when cells were stimulated with heat, killed swollen conidia, or lipopolysaccharide (LPS) (data not shown). In addition, cells were stained for expression of CD80 and CD86 (Fig. 1E). Surface staining showed increased mean fluorescence intensity of CD86 (Fig. 1F) but not CD80 (Fig. 1G) in vitamin D-deficient mice compared to that in vitamin D-sufficient mice. While microarray analysis showed increased expression of OX40L in vitamin D-deficient mice, OX40L antibodies for flow cytometry are unreliable and did not show increased staining over isotype control (data not shown). Taken together with microarray data, this suggests that CD11c cells from vitamin D-deficient mice have increased costimulatory expression compared to those from vitamin D-sufficient mice.

Vitamin D-deficient lung CD11c cells have increased expression of OX40L. As we observed an increased Th2 response to *A. fumigatus* in vitamin D-deficient mice and an increase in costimulatory molecules OX40L and CD80 in spleen-derived CD11c⁺

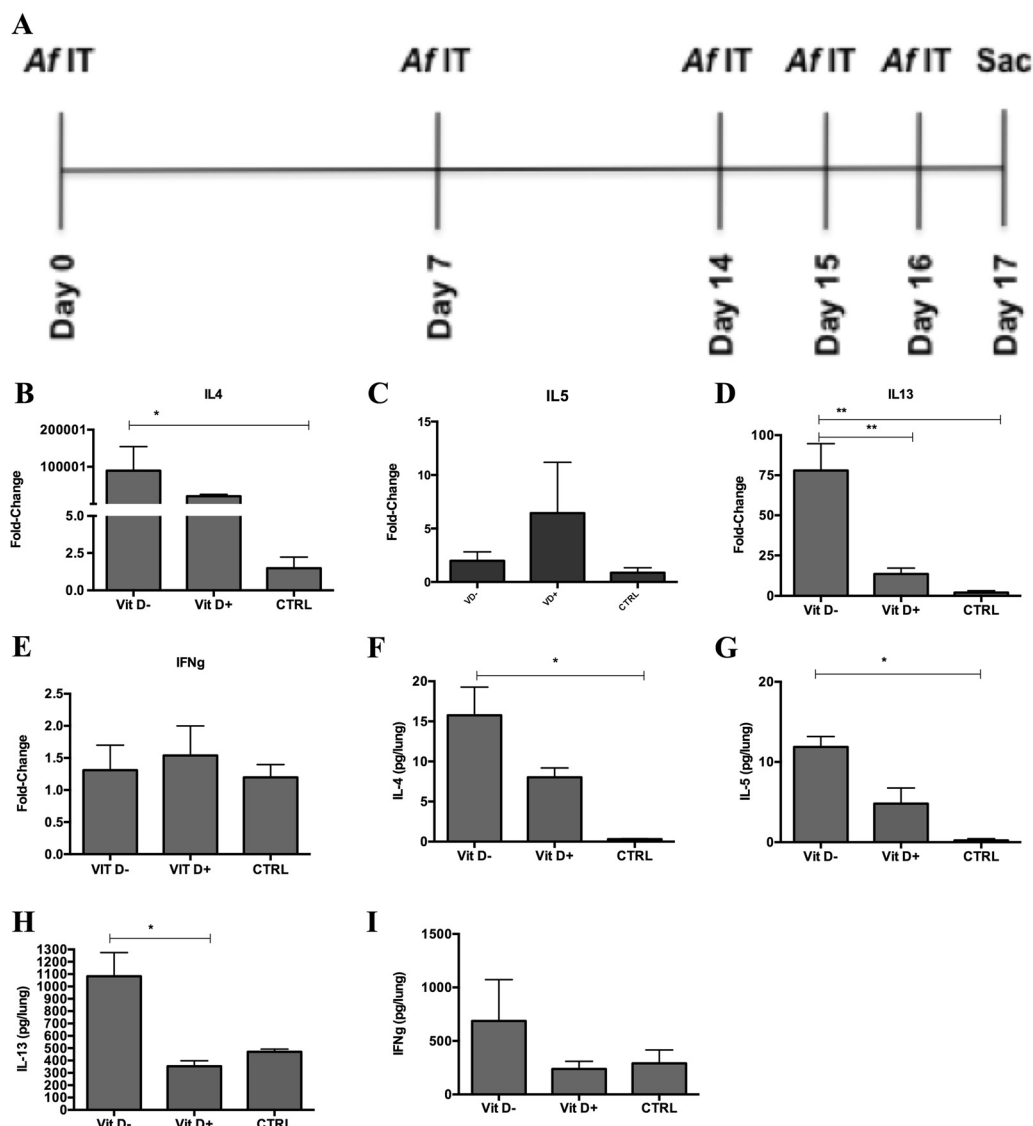


FIG 3 Vitamin D deficiency increases Th2 response to *A. fumigatus* in vivo. (A) Schematic of *Aspergillus* model. Mice were infected with *Aspergillus fumigatus* conidia intratracheally at the indicated time points and sacrificed 24 h after the final challenge. Serum vitamin D was measured to ensure vitamin D deficiency; vitamin D-deficient mice had serum 25-OH D₃ levels of 7.3 ng/ml, and vitamin D-sufficient mice had 25-OH D₃ levels of 26.0 ng/ml. (B to I) Vitamin D-deficient mice show increased Th2 responses to *A. fumigatus*. Vitamin D-deficient mice had significantly higher expression of Th2 cytokines IL-4 (B) and IL-13 (D) but no difference in IL-5 (C) or IFN-γ (E) and higher production at the protein level of Th2 cytokines IL-4 (F), IL-5 (G), and IL-13 (H) but no difference in IFN-γ cytokine production (I). (B to E) Gene expression is relative to HPRT. Representative data from three independent experiments are shown. *, $P < 0.05$ by one-way ANOVA and *post hoc* Tukey test.

cells, we next examined whether there were differences in CD11c⁺ cells in the lungs of vitamin D-deficient and vitamin D-sufficient mice. CD11c⁺ cells were isolated from BALB/c vitamin D-deficient or vitamin D-sufficient mice, and expression of costimulatory molecules was analyzed by real-time PCR. Similarly to gene expression from spleen-derived CD11c⁺ cells, lung-derived CD11c⁺ cells from vitamin D-deficient mice had increased expression of OX40L (*TNFSF4*) (Fig. 2A) and CD80 (Fig. 2B). Further, CD11c⁺ cells derived from vitamin D-deficient mice had increased expression of the other costimulatory molecules CD86 (Fig. 2C) and ICOS ligand (*ICOSLG*) (inducible T-cell costimulator ligand) (Fig. 2D), findings which together suggest that increased expression of costimulatory molecules in vitamin D-defi-

cient mice is responsible for the increased Th2 response to *A. fumigatus* in vivo.

Vitamin D-deficient mice have increased Th2 responses to *Aspergillus fumigatus* infection that are dependent on OX40L. To determine if vitamin D status alters the immune response to *A. fumigatus* in vivo, vitamin D-deficient and vitamin D-sufficient BALB/c mice were sensitized twice and challenged for three consecutive days a week later with *A. fumigatus* conidia intratracheally (Fig. 3A) (15). After sensitization and challenge with *A. fumigatus*, vitamin D-deficient mice had substantially higher Th2 responses than did vitamin D-sufficient mice and uninfected controls. Vitamin D-deficient mice (Vit D⁻) had higher Th2 cytokine expression in the lung than did vitamin D-sufficient mice (Vit D⁺),

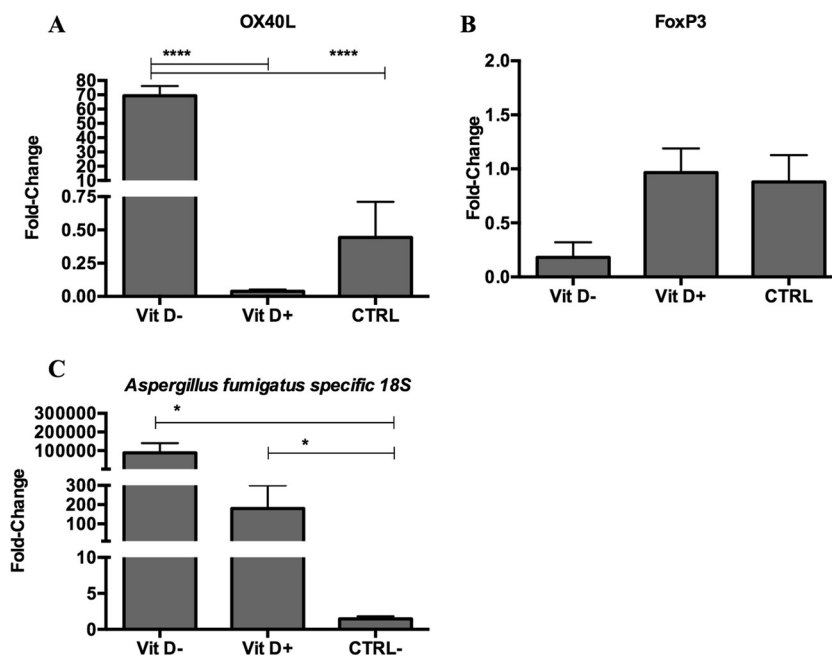


FIG 4 Vitamin D deficiency modifies the immune response to *A. fumigatus*. Vitamin D-deficient and vitamin D-sufficient mice were infected as described in the Fig. 3A legend. (A and B) Vitamin D-deficient mice had significantly higher expression of costimulatory molecule OX40L (A) and lower expression of Treg transcription factor FoxP3 (B), which has been shown to promote tolerance to *A. fumigatus*. (C) Vitamin D-deficient mice had a significantly higher fungal burden measured by *Aspergillus fumigatus* 18S rRNA than did vitamin D-sufficient mice or uninfected controls. Representative data from three independent experiments are shown. ****, $P < 0.0001$, and *, $P < 0.05$, with one-way ANOVA and *post hoc* Tukey test.

measured by quantitative real-time PCR, for IL-4 (Fig. 3B) and IL-13 (Fig. 3D) but not IL-5 (Fig. 3C). Further, there were no differences detected in Th1 responses measured by gamma interferon (IFN- γ) gene expression (Fig. 3E) between the vitamin D-deficient and vitamin D-sufficient cohorts. Similarly to what was seen at the gene expression level, increased levels of Th2 cytokines IL-4 (Fig. 3F), IL-5 (Fig. 3G), and IL-13 (Fig. 3H) were also seen at the protein level in lung homogenate in vitamin D-deficient mice compared to that in uninfected control mice. Of infected mice, vitamin D-deficient mice had significantly higher IL-13 levels (Fig. 3C) than did vitamin D-sufficient mice. Levels of the Th1 cytokine IFN- γ (Fig. 3I) were not significantly different between the groups, corresponding to reverse transcription-PCR (RT-PCR) data. This enhanced Th2 response in vitamin D-deficient mice was also associated with a significant increase in the expression in OX40L (*TNFSF4*) transcripts in the lung (Fig. 4A). This increased OX40L expression was significant and increased only in vitamin D-deficient mice and not vitamin D-sufficient mice. While it was not significant, vitamin D-sufficient mice tended to have higher levels of the Treg transcription factor gene *Foxp3* (Fig. 4B). The increase in OX40L and Th2 cytokines in the lung also correlated with increased fungal burden in the lung measured by *A. fumigatus*-specific 18S rRNA quantitative real-time PCR (16) (Fig. 4C). Taken together, these data suggest that vitamin D-deficient mice have enhanced Th2 responses to *A. fumigatus*, which is associated with an increase in OX40L expression. Further, increased expression of FoxP3 is associated with a decreased Th2 response in vitamin D-sufficient mice.

It has been shown that giving mice anti-OX40L antibody decreases inflammatory response in Th2-mediated diseases marked by decreased Th2 cytokine production (17, 18). Since we observed

an increased Th2 response to *A. fumigatus* in vitamin D-deficient mice that was associated with increased OX40L, we next examined if neutralizing OX40L in the lung in vitamin D-deficient and vitamin D-sufficient mice before challenge with *A. fumigatus* would decrease Th2 response. Vitamin D-deficient and vitamin D-sufficient BALB/c mice were infected as shown in Fig. 3A with the exception of being administered anti-OX40L 4 h before challenges with *A. fumigatus* conidia. Vitamin D-deficient and vitamin D-sufficient mice had increased Th2 responses compared to those of uninfected control mice, as we had observed previously. When treated with neutralizing antibody to OX40L before challenge with *A. fumigatus*, both vitamin D-deficient and vitamin D-sufficient mice had decreased Th2 cytokine production in the lung as measured by levels of IL-4 (Fig. 5A) and IL-5 (Fig. 5B). Only vitamin D-deficient mice had a significant decrease in the production of IL-13 (Fig. 5C) measured in lung homogenate after neutralization of OX40L. In addition, neutralization of OX40L before *A. fumigatus* challenge decreased gene expression of *A. fumigatus*-specific 18S rRNA in the lung (Fig. 5D). Taken together with our previous data, this indicates that OX40L is critical for the enhanced Th2 response to *A. fumigatus* seen in vitamin D-deficient animals.

Vitamin D decreases OX40L promoter activity. Since we observed increased expression of OX40L in vitamin D-deficient mice, we next analyzed if vitamin D regulates the promoter activity of OX40L (19). U937 cells were transiently transfected with human OX40L promoter and treated with 1,25-dihydroxyvitamin D₃ (0.1 μ M) and TNF- α (0 or 10 ng/ml) (20), and promoter activity was measured 24 h later by luciferase activity. Transfected cells treated with TNF- α had significantly increased promoter activity over that in unstimulated control cells (Fig. 6A), which was

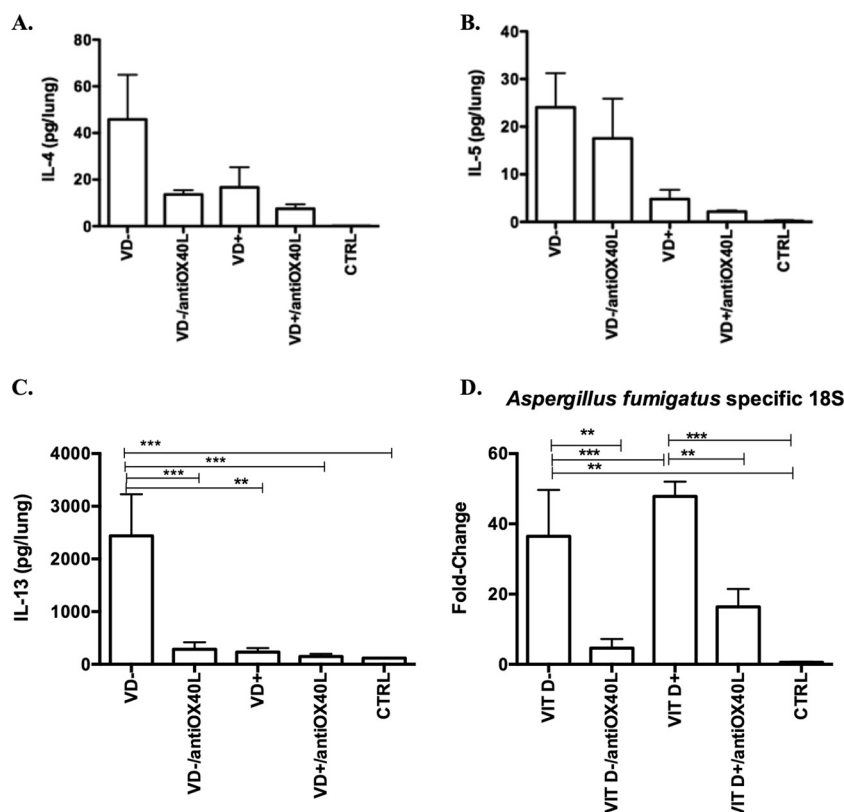


FIG 5 Neutralization of OX40L decreases Th2 response to *A. fumigatus* in vivo. Mice were infected with *Aspergillus fumigatus* conidia (3.5×10^8 conidia/ml in 50 μ l PBS) intratracheally as shown in Fig. 3A and sacrificed 24 h after the final challenge. At 4 h before challenges, mice received 50 μ g anti-OX40L (RM134L) intratracheally. (A and B) Vitamin D-deficient mice had significantly higher Th2 cytokine expression, which was decreased by neutralizing OX40L in IL-4 (A) and IL-5 (B). (C) Vitamin D-deficient mice had significantly decreased IL-13 response in the lung when given anti-OX40L before challenge with *A. fumigatus*. (D) Neutralization of OX40L before *A. fumigatus* challenge decreases fungal burden in the lung measured by *A. fumigatus*-specific 18S rRNA RT-PCR. Data are normalized to HPRT. Representative data from three independent experiments with $n = 5$ are shown. ***, $P < 0.0005$, and **, $P < 0.01$, with one-way ANOVA and *post hoc* Tukey test.

anticipated since previous findings have shown that the OX40L promoter contains NF- κ B-like binding sites (9). Treatment with vitamin D significantly decreased OX40L promoter activity compared to TNF- α treatment, and OX40L promoter activity was decreased compared to that in control treated cells (Fig. 6A). Treatment of cells with vitamin D and TNF- α together inhibited promoter activity, indicating that vitamin D could suppress TNF- α -induced OX40L promoter activity (Fig. 6A). While treating cells with TSLP alone did not induce OX40L promoter activity, treatment of cells with TSLP and TNF- α induced OX40L promoter activity (Fig. 6B). In addition, vitamin D was able to decrease OX40L promoter activity induced by TSLP and TNF- α treatment (Fig. 6B). The ability of vitamin D to regulate OX40L promoter activity could be responsible for the vitamin D-mediated Th2 response to *A. fumigatus* seen in vivo.

Vitamin D receptor binds to the promoter region of OX40L. It is known that TSLP induces OX40L on DCs, which triggers Th2 polarization and Th2-related inflammation (7, 21). More recently, it was shown that there are two distinct NF- κ B-like binding sites in the promoter region of OX40L and that OX40L could be activated by NF- κ B proteins p50 and RelB, a transcription factor that enhances promoter activity paired with NF- κ B p50 (9). Since vitamin D deficiency increases expression of OX40L and vitamin D treatment decreases OX40L promoter activity, we hypothesized

that there are direct interactions with vitamin D receptor and DNA binding sites in the promoter region of OX40L that are responsible for regulating expression of OX40L. Further, a recent ChIP sequencing (ChIP-seq) paper identified VDR binding sites near and within the OX40L gene in lymphoblastoid cell lines (19). Since TSLP and NF- κ B have been shown to induce OX40L on DCs, we treated U937 cells with vitamin D, TNF- α , TSLP, or a combination for 1 h. Chromatin immunoprecipitation (ChIP) assays with VDR antibodies also showed VDR binding in the OX40L promoter region upon treatment with vitamin D (Fig. 7A). In addition, stimulation of U937 cells with TNF- α , TSLP, and vitamin D also showed VDR binding (Fig. 7B), indicating that there are VDR binding sites within the OX40L promoter region.

ChIP assays with NF- κ B p50 and NF- κ B p65 antibodies revealed binding of NF- κ B p65, but there was no detectable NF- κ B p50 binding in the promoter region of OX40L in unstimulated cells (Fig. 8A) and cells treated with TNF- α and TSLP (Fig. 8B). Interestingly, treating cells with vitamin D showed binding of NF- κ B p50 in the OX40L promoter when treated with the combination of TNF- α , TSLP, and vitamin D (Fig. 8B). Quantitative RT-PCR of immunoprecipitated DNA showed a significant increase of NF- κ B p50 and NF- κ B p65 binding in the presence of vitamin D or TNF- α , TSLP, and vitamin D (Fig. 8C). Fold enrich-

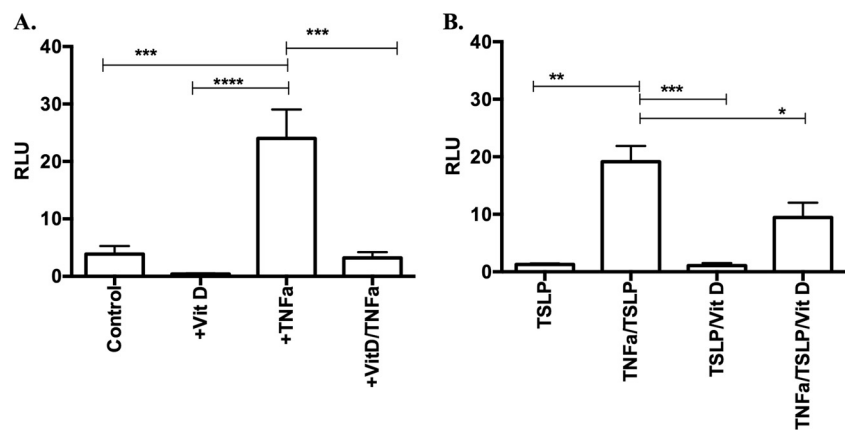


FIG 6 Vitamin D regulates promoter activity of OX40L. (A) Vitamin D treatment (0.1 μ M) of U937 cells transfected with 1,000-bp OX40L promoter-inserted firefly luciferase reporter plasmid significantly attenuates OX40L promoter activity, and treating cells with TNF- α (10 ng/ml) significantly increases OX40L promoter activity. (B) Treatment of transfected cells with TSLP (5 ng/ml) and TNF- α (10 ng/ml) significantly increases OX40L promoter activity, and vitamin D treatment attenuates OX40L promoter activity. Representative data from three independent experiments with $n = 5$ to 6 are shown as luciferase activity normalized to *Renilla* luciferase activity $\pm$ standard errors of the means. Cells were treated for 24 h before luciferase activity was measured. RLU, relative light units. ****, $P < 0.0001$; ***, $P < 0.005$; **, $P < 0.01$; *, $P < 0.05$.

ment of NF- κ B p50 was higher than fold enrichment of NF- κ B p65 under all conditions (Fig. 8C). Taken together, these results show that in addition to regulation of vitamin D binding in the promoter region, OX40L can be regulated by NF- κ B in the promoter region.

DISCUSSION

We have shown that vitamin D can regulate Th2 immune responses to *A. fumigatus* in peripheral blood in part through the

regulation of the costimulatory molecule OX40L (10). Moreover, we have shown here that CD11c⁺ cells derived from nutritionally vitamin D-deficient BALB/c mice also have increased expression of OX40L compared to that in vitamin D-sufficient mice. Previously, *in vitro* work has shown that vitamin D-deficient mouse-derived CD11⁺ cells prime increased Th2 proliferation (10), so we sought to determine the mechanism for this response. It is well publicized that infection with *A. fumigatus* induces a Th2 response. Exposure to *A. fumigatus* in mouse models causes an in-

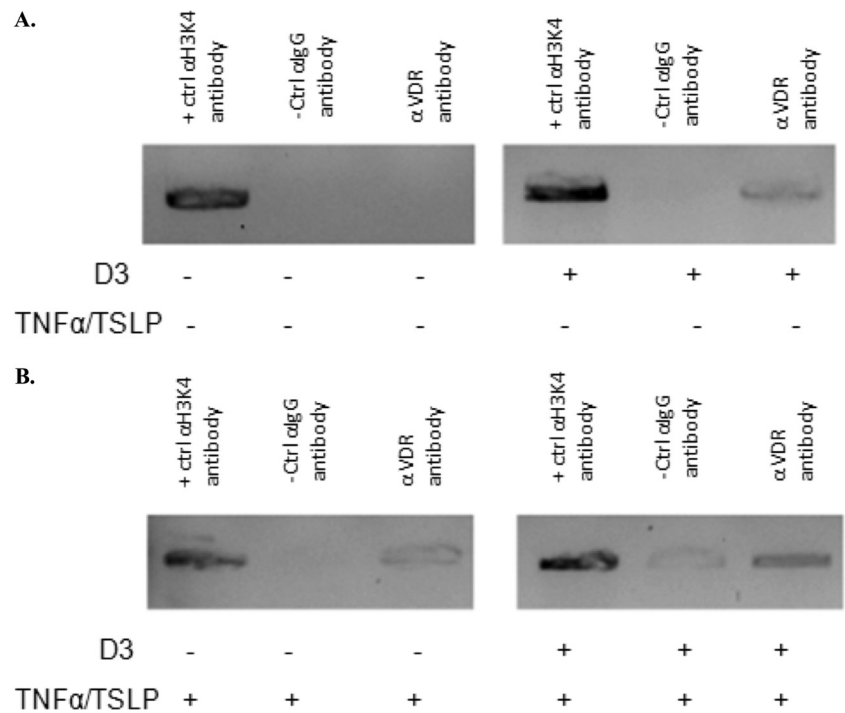


FIG 7 Vitamin D regulates expression of OX40L. U937 cells were treated with vitamin D (0.1 μ M), TNF- α (10 ng/ml), and/or TSLP (5 ng/ml). ChIP assays show binding of VDR in the promoter region of OX40L when cells are stimulated with vitamin D alone (A) and with TNF- α , TSLP, and vitamin D (B). The experiment was repeated three times, and representative data from one experiment are shown.

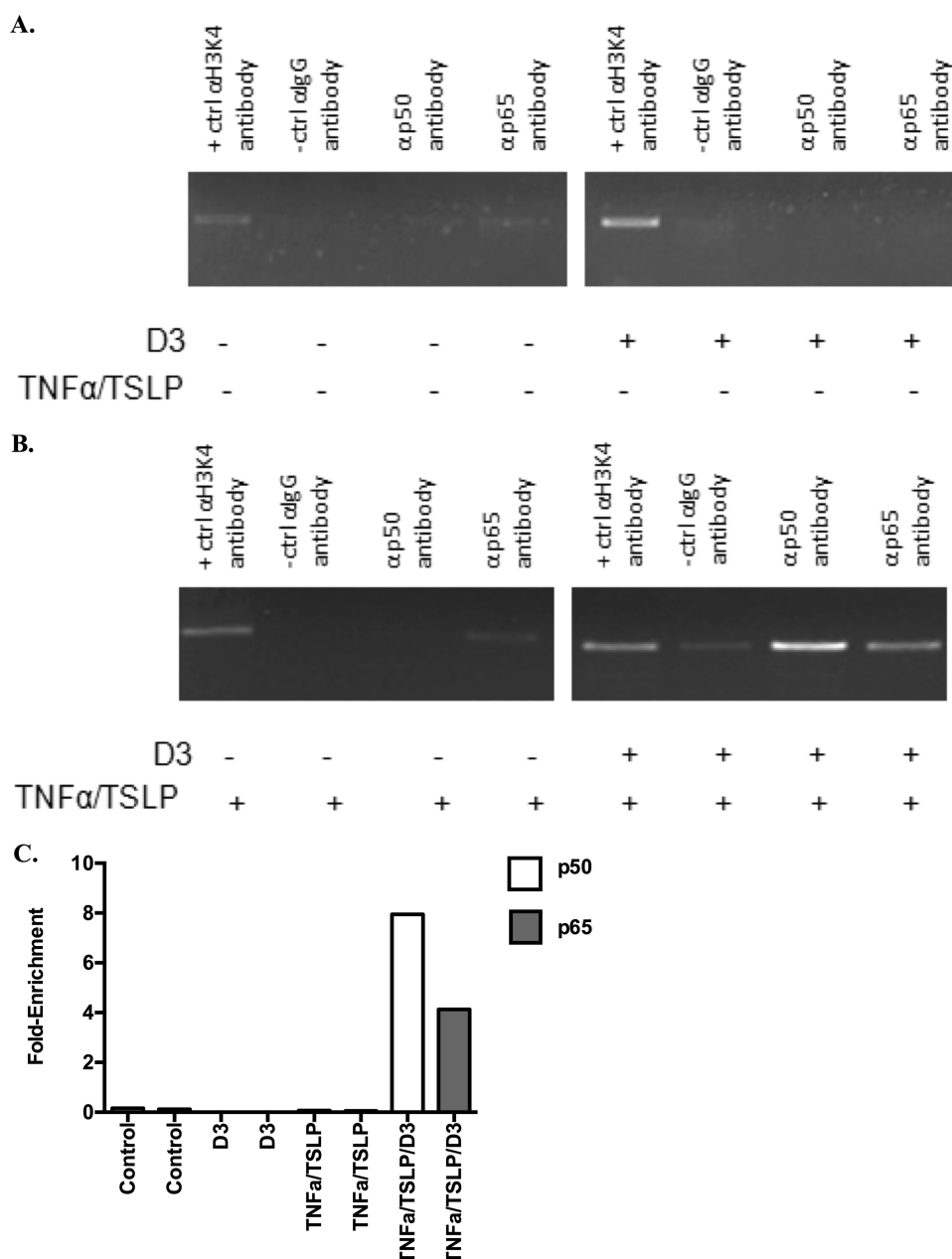


FIG 8 NF- κ B molecules regulate expression of OX40L. U937 cells were treated with vitamin D (0.1 μ M), TNF- α (10 ng/ml), and/or TSLP (5 ng/ml). (A) ChIP assays show no NF- κ B p50 or p65 binding in unstimulated cells. (B) ChIP assays show binding of NF- κ B p65 in the promoter region of OX40L when cells are stimulated with TNF- α and TSLP. Treating cells with TNF- α , TSLP, and vitamin D shows binding of NF- κ B p65 and NF- κ B p50. (C) Vitamin D treatment and TNF- α -TSLP treatment slightly increase fold enrichment of p50 and p65, whereas combined TNF- α , TSLP, and vitamin D treatment largely increases p50 and p65 enrichment. Fold enrichment is normalized to negative-control rabbit IgG. Representative images from ChIP experiments are shown.

crease in IL-4- and IL-5-positive cells in addition to increases in IgE (22, 23). Similarly to the increased Th2 response to *A. fumigatus* seen in vitamin D-deficient cystic fibrosis (CF) patients with ABPA, BALB/c mice that are deficient in vitamin D have an increased Th2 response to *A. fumigatus* compared to that in vitamin D-sufficient mice. More specifically, *A. fumigatus*-induced IL-13 levels are significantly increased in vitamin D-deficient mice compared to those in vitamin D-sufficient mice. These results correlate with human studies showing that CF patients with ABPA have higher *A. fumigatus*-induced responses to IL-13 than to other Th2

cytokines, IL-4 and IL-5 (10). There are data that suggest that the vitamin D receptor (VDR) is important to generate Th2-driven inflammation in the lung (24), since VDR-knockout (KO) mice do not develop lung inflammation, airway hyperresponsiveness, and eosinophilia in a Th2-driven experimental asthma model. Similarly to what we observed in splenic CD11c⁺ cells, we also observed an increase in OX40L expression in the lungs of vitamin D-deficient mice undergoing sensitization and challenge with *A. fumigatus*. In addition to OX40L, we also observed an increase in other costimulatory molecules in the lungs of vitamin D-deficient

mice, including CD80, CD86, and ICOS-L. Vitamin D has also been shown to inhibit the expression of CD86 in a dose-dependent manner that may be responsible for the vitamin D inhibition of antigen-presenting cell (APC)-dependent T cell activation (25). Recently, it has also been shown that in patients with Th2-mediated rhinosinusitis, vitamin D levels were inversely correlated with the percentage of circulating CD86⁺ cells (26). It has been shown that neutralizing OX40L during sensitization of Th2 asthma models decreases asthmatic responses measured by Th2 cytokines, airway hyperresponsiveness, and eosinophilia (17). However, there has not been any study on the effect of neutralizing OX40L in fungal allergy. Neutralizing OX40L in the lungs of vitamin D-deficient mice and vitamin D-sufficient mice before challenge with *A. fumigatus* decreased Th2 cytokine production. We saw a significant decrease in IL-13 only in vitamin D-deficient mice. Taken together with our data, this shows a correlation between vitamin D deficiency and increased expression of costimulatory molecules, which can lead to increased Th2 responses. Further, neutralization of OX40L is able to attenuate the Th2 response in *A. fumigatus*-susceptible vitamin D-deficient mice. Again, the most dramatic changes in Th2 cytokine levels were seen for IL-13, not IL-4 or IL-5. This correlates with human data from CF patients with ABPA, where the most dramatic Th2 responses are seen in the cytokine IL-13 (17). Taken together with our *in vivo* mouse data, this suggests that increased Th2 responses to *A. fumigatus* in vitamin D-deficient systems are due to increases in the cytokine IL-13. Furthermore, IL-13 is a critical driver of the allergic response in the lung, which mediates both goblet cell metaplasia and airway hyperresponsiveness. It has recently been shown that anti-IL-13 can improve lung function in patients with moderate asthma, particularly in the subset who have high levels of periostin, a biomarker regulated by IL-13 (27). In addition to anti-IL-13, there is a human clinical trial using human monoclonal antibody to OX40L to treat asthma, another Th2-mediated disease. Perhaps, in combination with vitamin D supplementation, treatment with anti-OX40L or anti-IL-13 may decrease Th2-mediated ABPA exacerbations.

It has been recently shown that the human OX40L promoter region contains NF- κ B-like binding sites and that binding of an NF- κ B p50 and RelB complex drives DCs to produce OX40L and promote Th2 differentiation (9). In addition to a genome-wide study that showed VDR binding sites near the OX40L gene (19), transcription factor binding site prediction software shows putative VDR binding sites in the OX40L promoter (data not shown). We found that vitamin D inhibited the promoter activity of OX40L. Further, vitamin D treatment was able to inhibit OX40L promoter activity that was induced by TNF- α . While there have been data suggesting that vitamin D can act on CD4⁺ T cells to modulate immune response (28–30), there is less evidence showing that vitamin D can affect DC function. It has been shown that vitamin D inhibits DC maturation (31, 32) and decreases expression of costimulatory molecules CD40, CD80, and CD86, which leads to a decreased capacity to activate T cells (12). Our data here show that vitamin D can act directly on the promoter region of OX40L, present on DCs, to inhibit its expression and thereby decrease Th2 proliferation. These data are also consistent with recent CHIP-seq data in human lymphoblastoid cell lines (19). Here, we observed VDR binding in the OX40L promoter region that contains 1,000 bp upstream of the transcription start site. Interestingly, vitamin D treatment of cells showed increased NF- κ B p50

binding in the presence or absence of TNF- α and TSLP, known activators of the NF- κ B (33) pathway in DCs (34). NF- κ B p50 homodimers are abundant in cells that are tolerant to lipopolysaccharide (LPS) (35) in addition to CD4⁺ T cells (36). In cells tolerant to LPS, there is increased p50-p50 homodimer binding, which results in decreased LPS-induced TNF production (35). In tolerant CD4⁺ T cells, p50-p50 homodimer binding can occur within the IL-2 promoter, and this is correlated with repression of NF- κ B-driven transcription (36). One possibility is that vitamin D may regulate p50 homodimer binding in the OX40L promoter. This postulate is the subject of current study.

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