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Research Article

Pulmonology

Allergic bronchopulmonary aspergillosis (ABPA) is caused by a dominant Th2 immune response to antigens derived from the opportunistic mold *Aspergillus*, most commonly *Aspergillus fumigatus*. It occurs in 4%–15% of patients with cystic fibrosis (CF); however, not all patients with CF infected with *A. fumigatus* develop ABPA. Therefore, we compared cohorts of *A. fumigatus*-colonized CF patients with and without ABPA to identify factors mediating tolerance versus sensitization. We found that the costimulatory molecule OX40 ligand (OX40L) was critical in driving Th2 responses to *A. fumigatus* in peripheral CD4<sup>+</sup> T cells isolated from patients with ABPA. In contrast, CD4<sup>+</sup> T cells from the non-ABPA cohort did not mount enhanced Th2 responses in vitro and contained a higher frequency of TGF-β-expressing regulatory T cells. Heightened Th2 reactivity in the ABPA cohort correlated with lower mean serum vitamin D levels. Further, in vitro addition of 1,25 OH-vitamin D<sub>3</sub> substantially reduced DC expression of OX40L and increased DC expression of TGF-β. This in vitro treatment also resulted in increased Treg TGF-β expression and reduced Th2 responses by CD4<sup>+</sup> T cells from patients with ABPA. These data provide rationale for a therapeutic trial of vitamin D to prevent or treat ABPA in patients with CF.

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# Vitamin D<sub>3</sub> attenuates Th2 responses to *Aspergillus fumigatus* mounted by CD4<sup>+</sup> T cells from cystic fibrosis patients with allergic bronchopulmonary aspergillosis

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**Allergic bronchopulmonary aspergillosis (ABPA) is caused by a dominant Th2 immune response to antigens derived from the opportunistic mold *Aspergillus*, most commonly *Aspergillus fumigatus*. It occurs in 4%–15% of patients with cystic fibrosis (CF); however, not all patients with CF infected with *A. fumigatus* develop ABPA. Therefore, we compared cohorts of *A. fumigatus*-colonized CF patients with and without ABPA to identify factors mediating tolerance versus sensitization. We found that the costimulatory molecule OX40 ligand (OX40L) was critical in driving Th2 responses to *A. fumigatus* in peripheral CD4<sup>+</sup> T cells isolated from patients with ABPA. In contrast, CD4<sup>+</sup> T cells from the non-ABPA cohort did not mount enhanced Th2 responses *in vitro* and contained a higher frequency of TGF- $\beta$ -expressing regulatory T cells. Heightened Th2 reactivity in the ABPA cohort correlated with lower mean serum vitamin D levels. Further, *in vitro* addition of 1,25 OH-vitamin D<sub>3</sub> substantially reduced DC expression of OX40L and increased DC expression of TGF- $\beta$ . This *in vitro* treatment also resulted in increased Treg TGF- $\beta$  expression and reduced Th2 responses by CD4<sup>+</sup> T cells from patients with ABPA. These data provide rationale for a therapeutic trial of vitamin D to prevent or treat ABPA in patients with CF.**

## Introduction

The development of Th2 responses, as in asthma and allergic bronchopulmonary aspergillosis (ABPA), is driven by both genetic and environmental factors. Mechanistically, inhaled allergens are presented by lung DCs to naive T cells, which leads to induction of allergen-specific Th2 cells (1–3). In mouse models of experimental asthma, T cell anergy and allergen tolerance have been shown to be critical to prevent the development of Th2 responses. Recently, our group has shown that CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs that express membrane TGF- $\beta$  are critical to the development of allergen tolerance in the lung (1), and inhibition of these cells augments antigen-induced Th2 responses in the lung (4). Conversely, it has been demonstrated that cytokine products of the airway epithelium such as thymic stromal lymphopoietin (TSLP) or IL-25 can augment Th2 differentiation (5–9). Notably, 90% of children who have similar exposures to environmental allergens fail to develop Th2 sensitization or clinical asthma, indicating robust mechanisms of immune tolerance in the lung.

One example of failure of immune tolerance in the lungs is the development of ABPA in cystic fibrosis (CF) patients. CF is the most common severely life-shortening genetic disease among people of mixed European descent and has a smaller but significant

prevalence in Hispanics, African Americans, and Asians, affecting approximately 30,000 people in the United States (10, 11) and another 70,000 people worldwide. CF results from mutations in CFTR, an anion channel found in the apical plasma membrane of epithelial cells throughout the body. Lack of CFTR function in airway epithelia leads to impaired mucociliary clearance, allowing for altered microbial colonization of the lungs of CF patients with bacterial species, especially *Pseudomonas aeruginosa*, and in up to 50% of patients with fungi (12). Among fungal organisms that colonize the respiratory tracts of patients with CF, the ubiquitous environmental mold *Aspergillus fumigatus* is the most prevalent. In fact, in one study, up to 80% of children with CF demonstrate IgG antibody to Asp f1, an immunodominant *Aspergillus* peptide antigen, by an early age (13). The presence of *A. fumigatus* in a patient's sputum and immune recognition may or may not manifest in overt clinical disease. However, when *A. fumigatus* does cause clinical symptoms, they are most often along the spectrum of ABPA, which occurs in 4%–15% of all CF patients (14) and is characterized clinically by wheezing, pulmonary infiltrates, bronchiectasis, and parenchymal fibrosis. Because of the high prevalence of *A. fumigatus* colonization but relatively low prevalence of ABPA, we hypothesized that factors other than CFTR dysfunction would contribute to development of ABPA in CF patients.

In patients with ABPA, immunological responses to a variety of *A. fumigatus* antigens result in a heightened Th2 response and an elevated IgE level (15, 16). However, what controls Th2 versus

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**Table 1**  
Patient characteristics

	ABPA-positive patients	ABPA-negative patients	P value
FEV1 (l)	2.755 ± 1.511 (0.96–5.71)	2.120 ± 1.187 (0.72–4.89)	0.1463
FEV1 (% predicted)	69.28 ± 5.288 (31–113)	62.60 ± 5.689 (26–98)	0.6053
BMI	21.92 ± 2.993 (17.06–27.68)	21.96 ± 4.571 (17.19–32.24)	0.6283
IgE (IU/ml)	461.3 ± 105.3 (2–2,000)	151.8 ± 53.49 (7–666)	0.0088
<i>A. fumigatus</i> -specific IgE (kUA/l)	14.02 ± 3.323 (0.35–62.00)	5.168 ± 2.35 (0.35–25.70)	0.0024
Age (yr)	26.08 ± 2.203 (16–56)	36.63 ± 3.304 (19–62)	0.004
Sex	16 males, 9 females	9 males, 7 females	NS
Genotype	14 homozygous ΔF508; 2 ΔF508/2789+5G-A; 1 homozygous 711ΔT; 1 ΔF508/621+1G-T; 2 ΔF508/R533X; 3 ΔF508/unknown allele; 1 G542X/unknown allele; 1 unreported	6 homozygous ΔF508; 1 ΔF508/R347H; 1 ΔF508/2789+5G-A; 3 ΔF508/unknown allele; 1 G542X/unknown allele; 1 621+1GT/unknown allele; 2 unreported	NS
Steroid use	4/25	4/16	NS
Ursodiol use	7/25	3/16	NS

Numbers in parentheses represent the range of the cohort.

Treg lineage choices and, therefore, what controls tolerance versus allergy in patients remains unclear. In particular, the contribution of signals from the lung epithelium versus nonepithelial-derived signals to DCs remains to be defined.

To identify factors mediating Th2 sensitization versus tolerance, we studied 2 groups of CF patients (ABPA patients versus *A. fumigatus*-exposed patients without ABPA [non-ABPA patients]) to test the hypothesis that Th2 sensitization may be controlled by epithelial TSLP. Moreover, as TSLP can induce OX40 ligand (OX40L) on DCs and OX40L is a critical factor for Th2 inflammation in the lung (17) and can break immune tolerance (18), we also investigated the role of OX40L in *A. fumigatus* Th2 responses in patients with ABPA. Last, we hypothesized that *A. fumigatus*-colonized patients without ABPA would have higher percentages of Foxp3<sup>+</sup>CD4<sup>+</sup> T cells in response to *A. fumigatus* stimulation and that these cells suppress Th2 responses in these individuals.

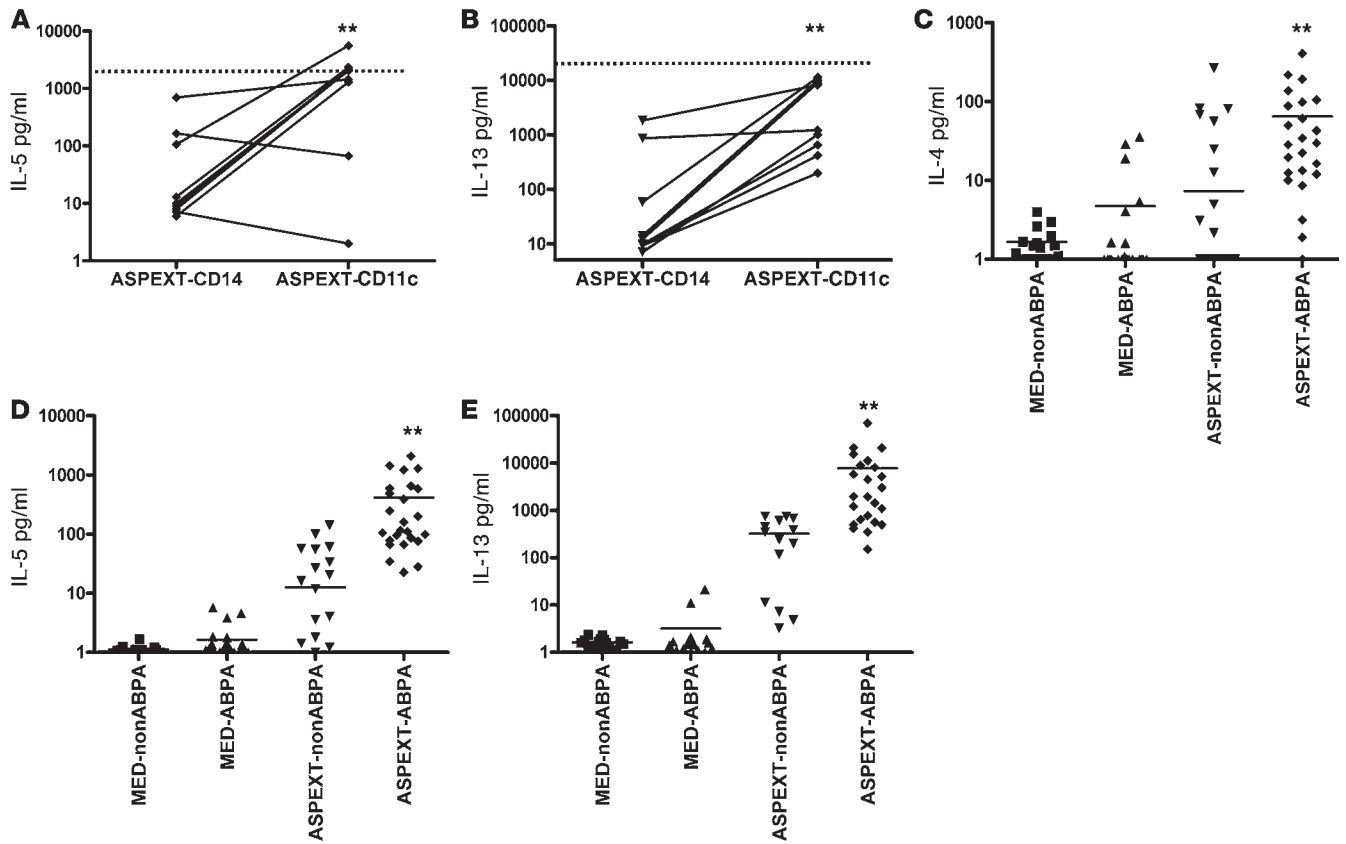
Here, we show that TSLP induced potent Th2-skewing activity by CD11c<sup>+</sup> DCs from ABPA patients, which was dependent on OX40L. Furthermore, we discovered that ABPA correlated with vitamin D deficiency, and supplementation with vitamin D potentiated Treg-mediated regulation of Th2 reactivity. These data support the development of a clinical trial of vitamin D to prevent or treat ABPA in CF and other Th2-related diseases.

## Results

**Patient demographics.** All patients were accrued from the CF Center at the Children's Hospital of Pittsburgh and the University of Pittsburgh, at which *A. fumigatus* exposure is defined by at least one positive sputum culture in the year prior to enrollment. *A. fumigatus* exposure exceeds 50% in our subjects in the CF registry of approximately 450 human subjects, and of these colonized patients, 15.5% meet diagnostic criteria for ABPA (the ABPA cohort) (14). The remaining 84.5% did not have elevated IgE levels or clinical ABPA yet had cultured *A. fumigatus* on at least one occasion (the non-ABPA cohort).

We enrolled a total of 25 CF patients with ABPA and 16 CF patients with *A. fumigatus* exposure, defined by a positive sputum culture for *A. fumigatus* within one year of enrollment into the study but no immunological evidence of ABPA as defined by consensus criteria (14). The patient characteristics are listed in Table 1. There was no difference in sex, forced expiratory volume in 1 second (FEV1), or CF genotype between the ABPA<sup>+</sup> cohort and the *A. fumigatus*-colonized non-ABPA cohort. The patients with ABPA were significantly younger (Table 1). The difference in age may be attributable to the fact that some patients with ABPA were consented at the time of hospitalization for a clinically suspected ABPA exacerbation, as younger patients were more likely to be hospitalized than older adult CF patients. As expected serum total and *A. fumigatus*-specific IgE levels were significantly higher in the ABPA cohort at the time of enrollment. As we did not exclude patients with a previous history of ABPA who were controlled with less than 0.5 mg/kg of prednisone per day, there was a wide range of total IgE levels ranging from 2 to 2,000 IU/ml. Thus, it is important to note that these values represent the IgE level at the time of enrollment, and we permitted patients on low-dose prednisone to participate in this study. However, there was no difference in steroid use or use of ursodiol between the 2 groups (Table 1).

*CD14<sup>+</sup>-derived DCs are poor inducers of A. fumigatus-specific Th2 responses in patients with ABPA, while CD11c<sup>+</sup> DCs are potent inducers.* To better understand the role of DCs in the development of ABPA, we initially examined monocyte-derived DCs from CD14<sup>+</sup> cells obtained from 9 patients with confirmed ABPA. CD14<sup>+</sup> DCs were purified from peripheral blood and were grown in recombinant human GM-CSF and IL-4 for 6 days. At the end of the culture, DCs were more than 90% positive for CD80, CD86, and class II MHC. DCs were pulsed with zymosan, heat-killed swollen conidia (HKSC) (19), or *A. fumigatus* extract (ASPEXT) for 1 hour, after which autologous CD4<sup>+</sup> T cells were added to the culture for 96 hours. As a positive control CD4<sup>+</sup> T cells were stimulated with CD3/CD28 beads. As shown in Supplemental Figure 1 (supplemental material



**Figure 1**

CD11c<sup>+</sup> DCs elicit stronger Th2 responses in patients with ABPA. CD14<sup>+</sup> DCs (*n* = 9) grown in GM-CSF and IL-4 for 6 days or CD11c<sup>+</sup> DCs from patients with confirmed ABPA were pulsed with media (data not shown) or ASPEXT. CD4<sup>+</sup> T cells were added to DCs for 96 hours. Supernatants were harvested and analyzed using Luminex for (A) IL-5 and (B) IL-13 production. The dashed line represents the mean CD3/CD28-stimulated response. Next CD11c<sup>+</sup> DCs (*n* = 9) from sex-matched patients with documented *A. fumigatus* colonization without ABPA (non-ABPA) or CD11c<sup>+</sup> DCs (*n* = 19) from patients with confirmed ABPA were pulsed with media (MED) (data not shown) or ASPEXT. CD4<sup>+</sup> T cells were added to DCs for 96 hours. Supernatants were harvested and analyzed using Luminex for (C) IL-4, (D) IL-5, or (E) IL-13 production. Horizontal bars indicate the mean. \*\**P* < 0.01 by Mann-Whitney.

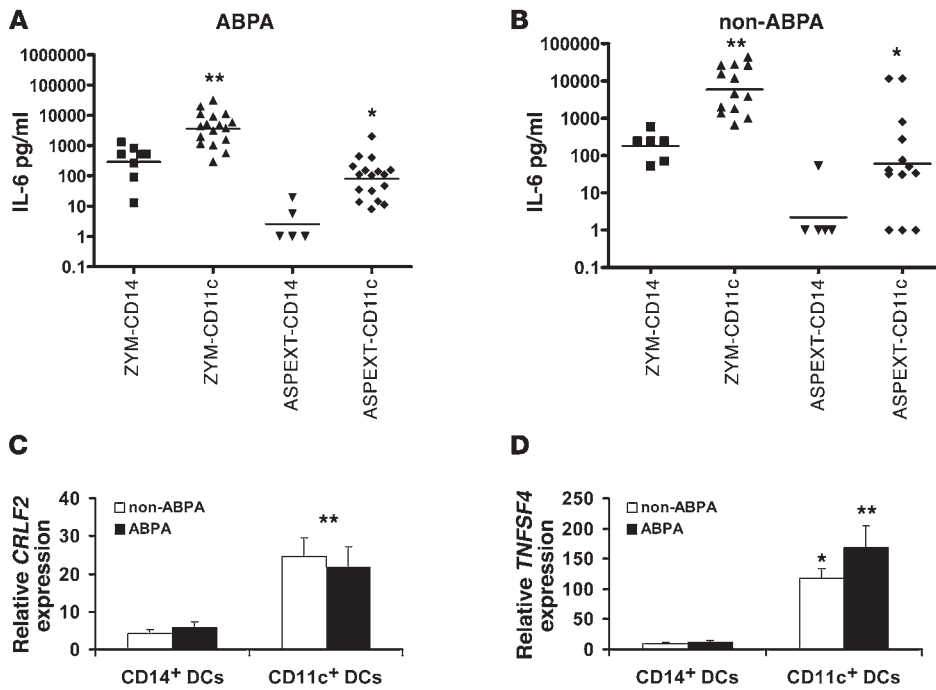
available online with this article; doi:10.1172/JCI42388DS1), DCs pulsed with zymosan (a particle derived from *S. cerevisiae* enriched for β1,3 glucan), HKSC (also shown to express β1,3 glucan), or ASPEXT resulted in minimal production of the Th2 cytokines IL-4, IL-5, and IL-13 (Supplemental Figure 1). In contrast, CD3/CD28 stimulation resulted in significant induction of IL-4, IL-5, and IL-13 in T cells from all subjects. Both zymosan and HKSC resulted in a significant increase in the expression of CD86 and class II MHC, and thus the lack of T cell response was not due to a lack of expression of these molecules (Supplemental Figure 2).

Based on the negative results with CD14<sup>+</sup>-derived DCs, we re-enrolled these same subjects to compare CD14<sup>+</sup>-derived DCs with CD11c<sup>+</sup> DCs, because CD11c<sup>+</sup> cells have been shown to elicit better Th2 immune responses (20). Compared with CD14<sup>+</sup> DCs, CD11c<sup>+</sup> DCs pulsed with ASPEXT, followed by the addition of autologous CD4<sup>+</sup> T cells, elicited significantly greater IL-5 responses in patients with ABPA (Figure 1A). In fact, 5 out of 8 patients with ABPA had IL-5 levels that exceeded the mean CD3/CD28-stimulated IL-5 response (depicted as a black dashed line), whereas CD14<sup>+</sup> DCs from the same patients did not elicit this response. Similar to IL-5 responses, IL-13 responses were significantly greater with ASPEXT-pulsed CD11c<sup>+</sup>

DCs compared with CD14<sup>+</sup> DCs (Figure 1B). Unpulsed DCs (either CD14 or CD11c) elicited IL-5 or IL-13 from autologous CD4<sup>+</sup> T cells that were below or near the limit of detection (data not shown).

We next asked whether Th2 responses elicited by ASPEXT-pulsed CD11c<sup>+</sup> DCs could discriminate between patients with ABPA versus sex-matched non-ABPA control CF patients. CD11c<sup>+</sup> DCs from ABPA patients pulsed with ASPEXT elicited significantly greater production of IL-4 (Figure 1C) and IL-5 (Figure 1D) from cocultured CD4<sup>+</sup> T cells than did cells from non-ABPA patients. Similar to IL-5, ASPEXT elicited substantially greater IL-13 production in CD4<sup>+</sup> T cells from ABPA patients compared with those from patients with *A. fumigatus* exposure (non-ABPA). In fact, cells from 18 out of 24 subjects with ABPA exceeded 1,000 pg/ml of IL-13 in this coculture assay, whereas no patients in the non-ABPA cohort showed this response. In contrast to Th2 responses, there were no differences between ABPA and non-ABPA patients in IL-17, IL-10, or IFN-γ responses to ASPEXT (Supplemental Figure 3).

As Th2 cytokine responses were greater with CD11c<sup>+</sup> cells pulsed with *A. fumigatus* compared with CD14<sup>+</sup> DCs, we assessed expression of CD80, CD86, and of cytokines that may induce Th2 responses in both subsets of DCs. Treatment of CD14<sup>+</sup> or CD11c<sup>+</sup> DCs with LPS



**Figure 2**

CD11c<sup>+</sup> DCs produce more IL-6 in response to zymosan and *Aspergillus* extract compared with CD14<sup>+</sup> DCs. (A) CD14<sup>+</sup> DCs (*n* = 5–7) grown in GM-CSF and IL-4 for 6 days or CD11c<sup>+</sup> DCs (*n* = 19) from patients with confirmed ABPA pulsed with media (data not shown), zymosan (ZYM), or ASPEXT for 24 hours. Supernatants were harvested and analyzed using Luminex for IL-6. (B) CD14<sup>+</sup> DCs (*n* = 5–7) grown in GM-CSF and IL-4 for 6 days or CD11c<sup>+</sup> DCs (*n* = 19) from patients with *A. fumigatus* colonization (non-ABPA) were pulsed with media (data not shown), zymosan, or ASPEXT for 24 hours. Supernatants were harvested and analyzed using Luminex for IL-6. Horizontal bars indicate the mean. Relative transcript expression (compared with 18s rRNA) of (C) TSLPR (*CRLF2*) and (D) OX40L (*TNFSF4*) in CD11c<sup>+</sup> DCs compared with that of CD14<sup>+</sup> DCs in patients with ABPA (*n* = 7 for CD14<sup>+</sup> DCs and *n* = 8–10 for CD11c<sup>+</sup> DCs in each group). \**P* < 0.05, \*\**P* < 0.01 by Mann-Whitney.

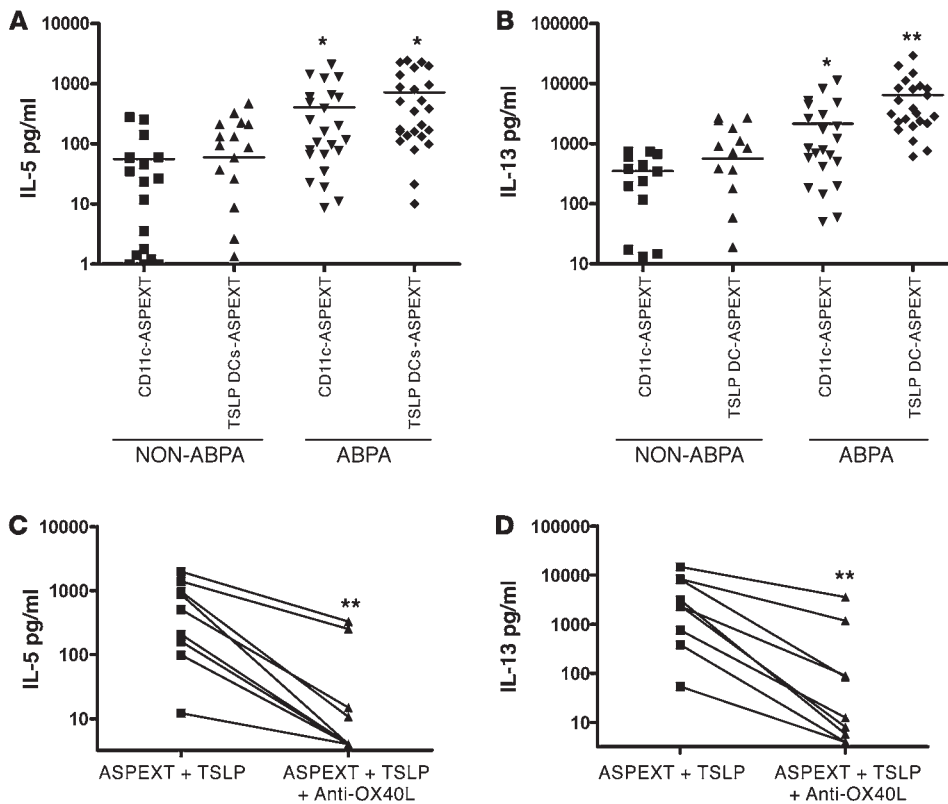
resulted in equivalent increases in the percentage of cells positive for CD86 (gated on HLA-DR positive cells). Moreover, there were no differences in the MFI of CD86, regardless of treatment conditions (Supplemental Figure 2). There were no significant differences in IL-12p40 (a component of IL-23) induction between CD14<sup>+</sup> or CD11c<sup>+</sup> cells pulsed with zymosan (to stimulate β-glucan signaling) or ASPEXT (data not shown). Zymosan induced IL-12p70 responses were less than 50 pg/ml. In contrast, IL-6 was induced to a much greater extent in CD11c<sup>+</sup> cells than in CD14<sup>+</sup> DCs by zymosan or ASPEXT in patients, regardless of if they were diagnosed with ABPA or if they were classified as non-ABPA (Figure 2, A and B). In contrast to IL-6, there was no difference in the induction of TNF-α by zymosan or ASPEXT between CD11c<sup>+</sup> and CD14<sup>+</sup> DCs, regardless of ABPA status (Supplemental Figure 4). Taken together, these data demonstrate that CD11c<sup>+</sup> cells can elicit more robust Th2 responses than CD14<sup>+</sup> DCs; however, these differences in eliciting T cell responses could not be fully explained by differences in induction of CD80, CD86, or IL-6.

*TSLP-DCs from ABPA patients elicit more potent Th2 responses via an OX40L-dependent pathway.* Several investigators have shown that the TSLP receptor (TSLPR, encoded by *CRLF2*) is expressed on CD11c<sup>+</sup> DCs but not CD14<sup>+</sup>-derived DCs (5, 20). We confirmed similar increases in *CRLF2* mRNA by real-time PCR in CD11c<sup>+</sup> DCs obtained from patients with CF in either the ABPA or non-ABPA cohort (Figure 2C). TSLP has been shown to prime Th2 responses through the upregulation of OX40L (5, 20). Based on this, we examined OX40L (*TNFSF4*) mRNA levels in CD14<sup>+</sup>-derived DCs versus CD11c<sup>+</sup> DCs from patients with CF with non-ABPA or ABPA (Figure 2D). Similar to TSLPR, CD11c<sup>+</sup> DCs had substantially higher expression of OX40L transcripts compared with CD14<sup>+</sup>-derived DCs (Figure 2D). CD11c<sup>+</sup> DCs from ABPA patients had higher levels of OX40L expression compared with those with non-ABPA (Figure 2D).

Based on these data, we examined whether CD11c<sup>+</sup> DCs exposed to TSLP (TSLP-DCs) and OX40L were required for *A. fumigatus*-specific Th2 responses in CF patients with ABPA.

TSLP-DCs pulsed with ASPEXT elicited nearly a half log greater IL-5 response (Figure 3A) in patients with ABPA. Moreover, TSLP (TSLP-DCs) resulted in significantly greater IL-13 responses in patients with ABPA and significantly reduced the standard error compared with CD11c<sup>+</sup> DCs alone (Figure 3B). TSLP-DCs did not elicit nonspecific increases in IL-5 or IL-13 responses in non-ABPA patients (Figure 3, A and B). To investigate whether these responses were dependent on OX40L, we blocked OX40L in the pulsed DC cultures prior to the addition of CD4<sup>+</sup> T cells. The addition of anti-OX40L to the DC/T cell cocultures significantly blocked the IL-13 and IL-5 response to ASPEXT in TSLP-DCs compared with that of an isotype control antibody (Figure 3, C and D). We also observed that the lower amount of Th2 responses to ASPEXT in non-ABPA patients was also dependent on OX40L (Supplemental Figure 5). These data strongly implicate a critical role of TSLP and OX40L in the Th2 response that is critical in the pathogenesis of ABPA.

Cytokines produced by lung epithelium such as TSLP and IL-25 have been shown to regulate the ability of DCs to prime Th2 immune responses (5–9). To determine whether the increased prevalence of ABPA in CF patients versus non-CF asthmatics is due to epithelial factors such as TSLP or IL-25, we examined whether *A. fumigatus* conidia would differentially induce TSLP or IL-25 in CF versus non-CF bronchial epithelial cells in vitro. For these studies, we apically applied resting conidia (RC) or HKSC, which express more β1,3 glucan (19), to the apical surface of non-CF human bronchial epithelial (HBE) cells or cells from patients homozygous for the ΔF508 CF mutation (Figure 4). RC, HKSC, and the positive control polyinosinic-polycytidylic acid (poly I:C) (21) induced TSLP production by HBE cells derived from non-CF and CF donors (Figure 4A) to similar extents. In contrast to RC and HKSC, only poly I:C resulted in induction of IL-25 production by epithelial cells, and again there was no difference between HBE cells grown from non-CF

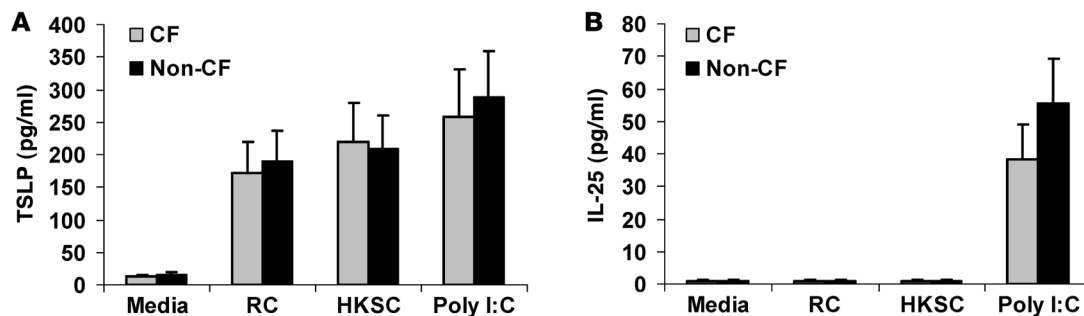


**Figure 3** TSLP-DCs elicit stronger Th2 responses in ABPA and require OX40L. CD11c<sup>+</sup> DCs with ( $n = 19$ ) from patients with confirmed ABPA were treated with media or TSLP (5 ng/ml) and then pulsed with zymosan or ASPEXT. Purified CD4<sup>+</sup> T cells were added for 96 hours. Supernatants were harvested and analyzed using Luminex for (A) IL-5 and (B) IL-13 production. Horizontal bars indicate the mean. \* $P < 0.05$ , \*\* $P < 0.01$  by Mann-Whitney. In a subgroup of ABPA patients, TSLP-DCs were pulsed with ASPEXT and then treated with anti-OX40L (10 ng/ml, BD PharMingen) or isotype control prior to the addition of CD4<sup>+</sup> T cells for 96 hours. (C) IL-5 and (D) IL-13 were measured at 96 hours in cell supernatants using Luminex. \*\* $P < 0.01$  by paired  $t$  test.

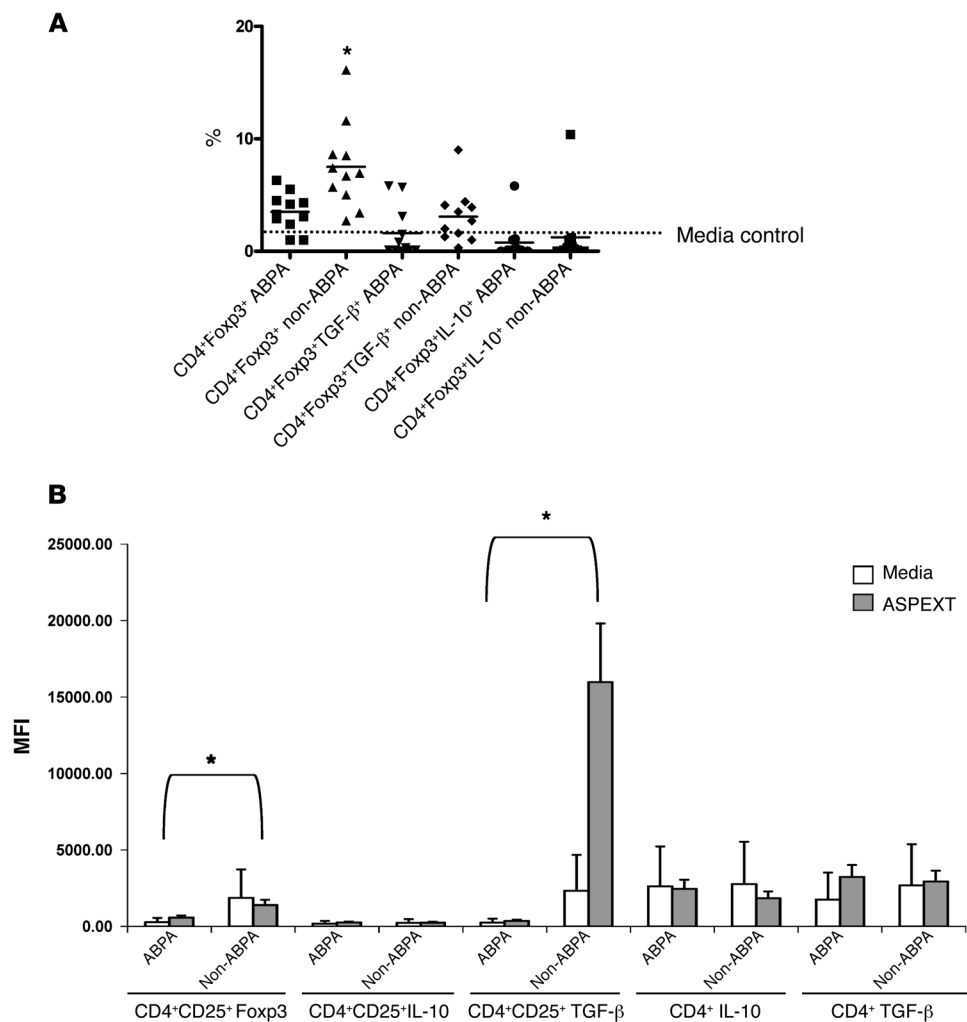
donors or patients with homozygous  $\Delta F508$  mutations (Figure 4B). These data suggest that differential production of epithelial cell TSLP or IL-25 or DC maturation do not explain the relative high prevalence of ABPA in CF.

*A. fumigatus*-colonized CF patients without ABPA have differences in Treg populations compared with CF patients with ABPA. As we observed no differences in DC maturation or epithelial TSLP production, we examined whether there were differences in putative Treg populations in patients with *A. fumigatus* colonization (non-ABPA) compared with ABPA patients. After a 4-day incubation of peripheral blood CD4<sup>+</sup> cells with media or ASPEXT, CD4<sup>+</sup> cells were stained for CD4, CD25, intracellular Foxp3, surface TGF- $\beta$ , and IL-10 and assayed by multicolor flow cytometry. Patients without ABPA had higher percentages of CD4<sup>+</sup>Foxp3<sup>+</sup> cells compared with those

patients with ABPA (Figure 5A). There was also a trend toward higher percentages of CD4<sup>+</sup>, Foxp3<sup>+</sup>TGF- $\beta$ <sup>+</sup>, and CD4<sup>+</sup>Foxp3<sup>+</sup>IL-10<sup>+</sup> cells after stimulation with ASPEXT, but due to variability among subjects these differences were not statistically significant (Figure 5A). Because we have recently shown that allergen-induced tolerance in mice can be mediated by TGF- $\beta$ <sup>+</sup> Tregs (22), we specifically examined the MFI of surface TGF- $\beta$ . After 4 days of stimulation with ASPEXT, there was significantly higher expression of Foxp3 and surface TGF- $\beta$  in CD4<sup>+</sup>CD25<sup>+</sup> cells (Figure 5B). We did not observe an increase in the amount of intracellular IL-10 or the MFI of IL-10 or TGF- $\beta$  when simply gated on bulk CD4<sup>+</sup> T cells (Figure 5B). To determine whether these CD4<sup>+</sup>CD25<sup>+</sup> cells were functional suppressor cells in *A. fumigatus*-colonized patients, we depleted CD4<sup>+</sup>CD25<sup>+</sup> cells from total CD4<sup>+</sup> cells and then added



**Figure 4** Induction of TSLP by *Aspergillus* in HBE cells. Homozygous  $\Delta F508$  CF or non-CF HBE cells were grown at ALI, followed by the apical application of  $10^6$  RC,  $10^6$  HKSC, or polyinosinic-polycytidylic acid (25  $\mu$ g/ml) for 24 hours. Basolateral supernatants were harvested at 24 hours, and (A) TSLP or (B) IL-25 were measured by ELISA.

**Figure 5**

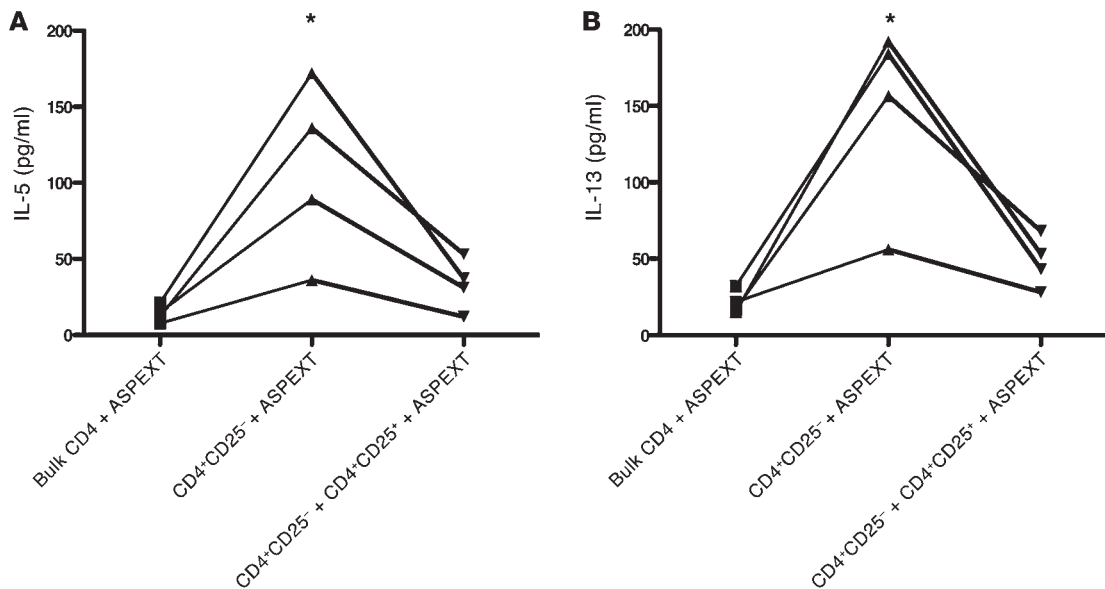
CD4<sup>+</sup>Foxp3<sup>+</sup> subsets in ABPA versus non-ABPA. *A. fumigatus*-colonized patients. CD11c<sup>+</sup> DCs were pulsed with media or ASPEXT, followed by the addition of autologous CD4<sup>+</sup> T cells from patients with ABPA ( $n = 9$ ) or non-ABPA ( $n = 11$ ). Cells were incubated for 96 hours followed by staining for CD4, intracellular CD25, Foxp3, IL-10 and TGF- $\beta$ , followed by analysis on a FACSaria. (A) Data are plotted as the percentage of Foxp3<sup>+</sup>, TGF- $\beta$ <sup>+</sup>, Foxp3<sup>+</sup>, or IL-10<sup>+</sup>/Foxp3<sup>+</sup> cells gated on CD4 for each subject. The dashed line indicates the average CD4<sup>+</sup>FoxP3 percentage of the ABPA and non-ABPA cohort in unstimulated cultures. Horizontal bars indicate the mean. (B) Data are plotted as the MFI of CD25, Foxp3, IL-10, or TGF- $\beta$  on CD4<sup>+</sup>CD25<sup>+</sup> cells. \* $P < 0.05$  by Mann-Whitney.

the remaining CD4<sup>+</sup> cells to ASPEXT-pulsed CD11c<sup>+</sup> DCs in a subset of 4 non-ABPA (*A. fumigatus*-colonized) patients. Depletion of CD4<sup>+</sup>CD25<sup>+</sup> cells was confirmed by FACS and resulted in significantly higher induction of IL-5 and IL-13 in response to ASPEXT (Figure 6), demonstrating that the CD4<sup>+</sup>CD25<sup>+</sup> cell population contained suppressor activity.

**Vitamin D deficiency and ABPA.** Vitamin D has been implicated in Treg development (23, 24), and vitamin D treatment along with dexamethasone can induce Tregs as well as IL-10 production (24). As up to 80% of CF patients have exocrine pancreatic insufficiency and therefore may have malabsorption of fat-soluble vitamins, even in the presence of exogenous pancreatic enzymes, we assessed serum levels of vitamin A, D, and E in our cohort (Table 2). Patients with ABPA had significantly lower 25-OH vitamin D (the major circulating form of vitamin D<sub>3</sub>) levels compared with non-ABPA controls (Table 2), and the mean level was significantly below the recommended level of 30 ng/ml in CF (25). Notably, there was no significant difference in BMI (Table 1) or vitamin A and E levels between ABPA and non-ABPA patients, suggesting that the relative vitamin D deficiency was not associated with relative malnutrition of this cohort of patients. To exclude potential environmental/geographical differences in our cohort, we used geographical information systems (GIS) mapping to determine whether there was geographi-

cal clustering of ABPA patients versus non-ABPA patients; however, this analysis did not identify significant clustering in western or northern Pennsylvania (data not shown). To exclude age as a covariant, we analyzed vitamin D levels in only the patients accrued as outpatients, where age was no longer significantly different in the ABPA versus non-ABPA cohort. In this subset of patients as in the overall cohort, 25-OH vitamin D levels remained statistically different between the 2 cohorts (Supplemental Table 1). Finally, there was no significant difference in terms of month of accrual in the study between the ABPA or non-ABPA cohort that would potentially account for differences in vitamin D status (Supplemental Figure 7). Taken together, these findings strongly suggest that the relative 25-OH vitamin D deficiency observed in ABPA patients was not a surrogate marker of another variable but rather may be a causal factor in the development of ABPA.

Therefore, we next examined whether addition of 1,25 OH-vitamin D<sub>3</sub> could reduce Th2 cytokine responses in CD4<sup>+</sup> cells from patients with ABPA. Patients with confirmed ABPA were reenrolled, and we again restimulated CD4<sup>+</sup> cells with ASPEXT-pulsed CD11c<sup>+</sup> DCs in the presence and absence of TSLP. Addition of 0.1  $\mu$ M 1,25 OH-vitamin D<sub>3</sub> significantly attenuated *A. fumigatus*-induced increases in IL-5 and IL-13 (Figure 7, A and B). Moreover, the addition of TSLP had no effect on the ability of 1,25



**Figure 6** Depletion of CD25<sup>+</sup> cells in non-ABPA patients exacerbates *Aspergillus*-induced Th2 responses. CD11c<sup>+</sup> DCs from patients with non-ABPA (*n* = 4) were pulsed with ASPEXT followed by addition of autologous bulk CD4<sup>+</sup> T cells or CD4<sup>+</sup> T cells depleted of CD25<sup>+</sup> T cells. Cells were incubated for 96 hours and (A) IL-5 or (B) IL-13 was measured in cell supernatants using Luminex. \**P* < 0.05 by paired *t* test.

OH-vitamin D<sub>3</sub> to reduce *A. fumigatus*-induced IL-5 and IL-13 production (Figure 7, C and D). In contrast to its effects on Th2 cytokine production, vitamin D had no effect on IL-10 production in the culture, whether experiments were performed with CD11c<sup>+</sup> DCs (Figure 7E) or TSLP-DCs (Figure 7F), suggesting that IL-10 production alone cannot account for the vitamin D-dependent reductions in IL-5 and IL-13. Moreover, the addition of 1,25 OH-vitamin D<sub>3</sub> reduced IFN-γ response to ASPEXT, but this difference was not significant (Supplemental Figure 6).

Incubation with 1,25 OH-vitamin D<sub>3</sub> was associated with an increase in the number of CD4<sup>+</sup>CD25<sup>+</sup>TGF-β<sup>+</sup> cells from a mean of 1.24% to over 3% (Figure 8A), although this difference was not statistically significant. However, we observed a significant increase in the MFI of TGF-β on CD4<sup>+</sup>CD25<sup>+</sup> cells treated with 1,25 OH-vitamin D<sub>3</sub> (Figure 8B). A representative dot plot is shown in Figure 8C (without 1,25 OH-vitamin D<sub>3</sub>) and Figure 8D (with 1,25 OH-vitamin D<sub>3</sub>). The shift in intensity of TGF-β staining was assessed using the patient as their own control in the presence of vitamin D, and the approximate 3-fold increase in TGF-β staining was consistent and statistically significant compared with the absence of vitamin D. The increase in TGF-β1 and Foxp3 expression by vitamin D in DCs pulsed with

ASPEXT was also confirmed by real-time PCR analysis. There was also a trend toward greater amounts of secreted TGF-β in cultures treated with 1,25 OH-vitamin D<sub>3</sub>, but this difference was not statistically significant (Supplemental Figure 6). Treatment with 1,25 OH-vitamin D<sub>3</sub> in vitro also significantly reduced OX40L expression in these cultures (Figure 8E). IL-10 expression was not significantly altered by 1,25 OH-vitamin D<sub>3</sub> treatment in vitro.

To determine whether TGF-β was a functional suppressor of Th2 responses, we studied a subgroup of enrolled CF patients with ABPA. CD11c<sup>+</sup> DCs were stimulated with or without TSLP and pulsed with ASPEXT, followed by the addition of autologous CD4<sup>+</sup> T cells. DC/T cell cocultures were then incubated with 1 μM 1,25 OH-vitamin D<sub>3</sub> alone or in the presence of anti-IL-10 or sTGF-βRII/Fc to antagonize IL-10 or TGF-β activity, respectively. Incubation of DC/T cell cocultures with sTGF-βRII significantly reversed the suppressive effects of 1,25 OH-vitamin D<sub>3</sub> on *Aspergillus*-stimulated IL-5 (Figure 9A) and IL-13 levels (Figure 9B). In contrast, the addition of anti-IL-10 had no effect on IL-5 or IL-13 responses (Figure 9, C and D).

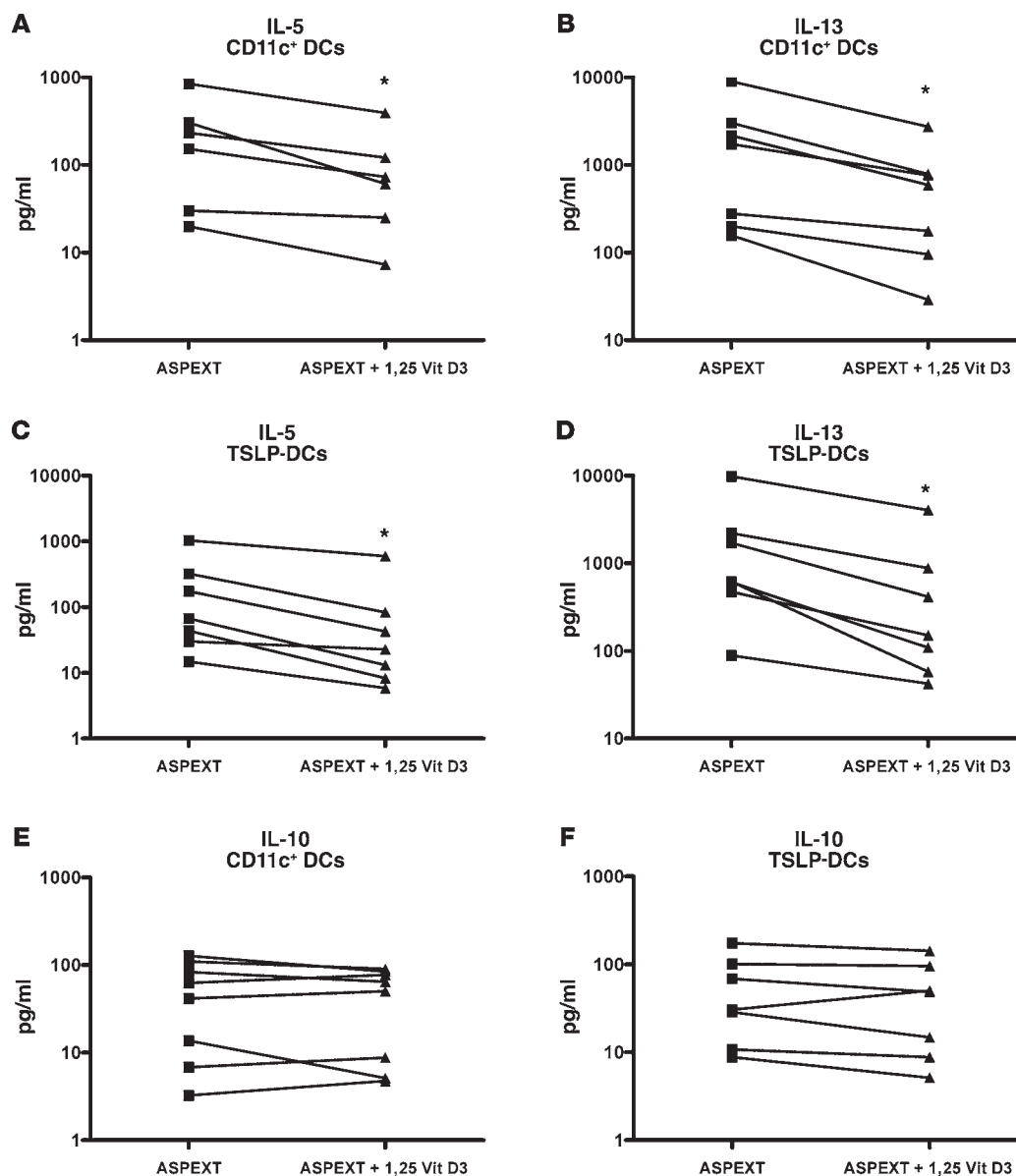
*Vitamin D regulates the expression of TGF-β and OX40L in murine CD11c<sup>+</sup> DCs.* To examine whether nutritional vitamin D deficiency could regulate DC function, we generated vitamin D-defi-

**Table 2** Serum levels of vitamin A, D, and E

	ABPA-positive patients	ABPA-negative patients	P value
Vitamin D (ng/ml)	22.04 ± 1.999	36.56 ± 5.021	0.0201
Vitamin D with IL-5 concentration >1,300 pg/ml	18.22 ± 2.160	ND	ND
Vitamin D with IL-5 concentration <1,300 pg/ml	22.45 ± 2.175	ND	ND
Vitamin A (μg/dl)	50.93 ± 2.955	53.27 ± 4.188	0.989
Vitamin E (μg/ml)	9.754 ± 0.9482	10.13 ± 1.092	0.73

Vitamin A, D, and E levels in ABPA-positive versus ABPA-negative patients are shown as well as Vitamin D levels in the ABPA cohort with high levels of *A. fumigatus*-induced IL-5 (>1,300 pg/ml) versus those with low IL-5 responses (<1,300 pg/ml).

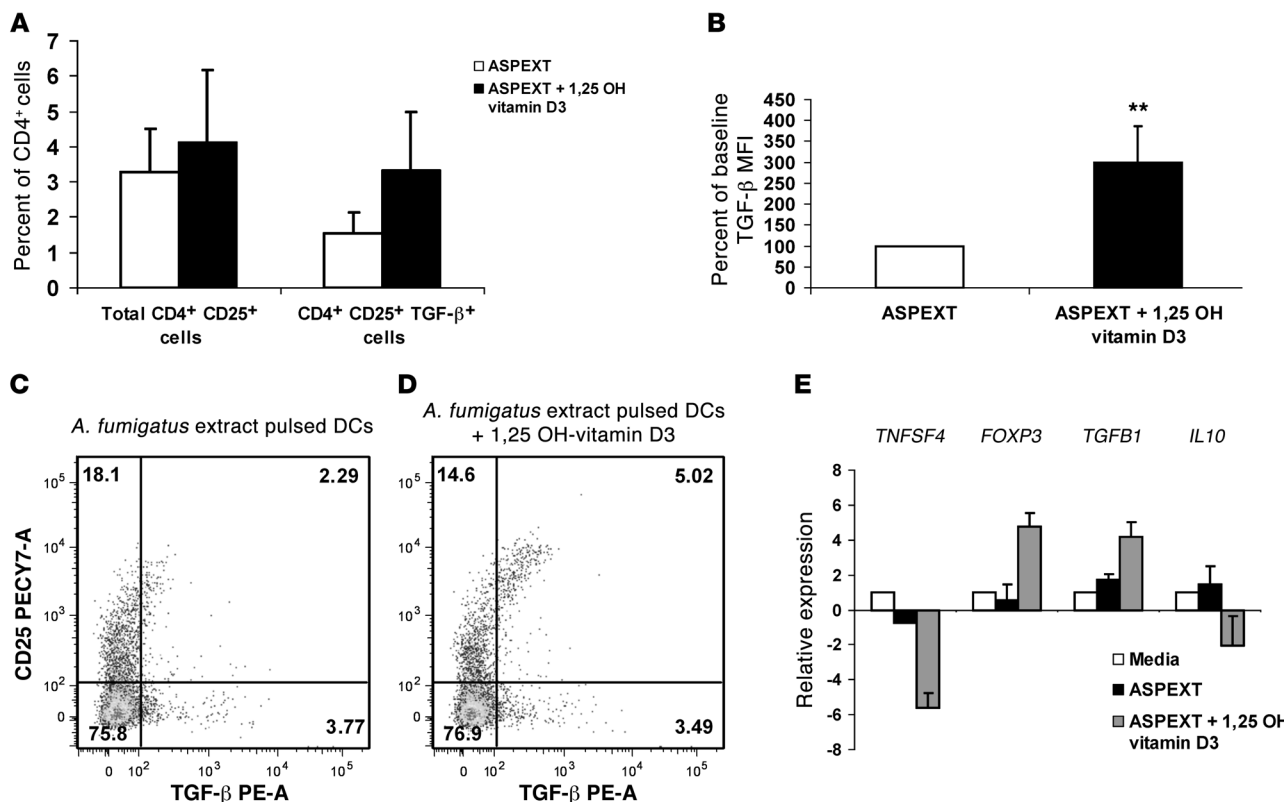


**Figure 7**

1,25 OH-vitamin D<sub>3</sub> suppresses *Aspergillus*-induced Th2 responses in CD4<sup>+</sup> T cells from patients with ABPA. CD11c<sup>+</sup> DCs or TSLP-DCs from patients with ABPA ( $n = 6$ ) were pulsed with ASPEXT, followed by addition of autologous bulk CD4<sup>+</sup> T cells, followed by addition of 1,25 OH-vitamin D<sub>3</sub> (1,25 Vit D3) or vehicle. Cells were incubated for 96 hours, and IL-5 (A and C), IL-13 (B and D), and IL-10 (E and F) were measured in cell supernatants using Luminex. \* $P < 0.05$  by paired  $t$  test.

cient mice, as previously described (26). After 4 weeks of being fed a vitamin D-deficient diet, mice had a mean serum 25-OH vitamin D<sub>3</sub> level of  $10.3 \pm 1.9$  ng/ml versus  $31.6 \pm 2.9$  ng/ml for pair-fed controls. After 4 weeks, we obtained splenic CD11c<sup>+</sup> DCs and assayed their capacity to induce Th2 differentiation to OVA peptide using naive CD4<sup>+</sup> T cells from DO11.10 mice, as previously described (27). CD11c<sup>+</sup> cells from vitamin D-deficient mice primed significantly greater IL-5 and IL-13 responses in vitro when cells were restimulated with antigen compared with control vitamin D-sufficient DCs (Figure 10A). Addition of 0.1  $\mu$ M 1,25 OH-vitamin D<sub>3</sub> to the culture had no effect on the Th2 priming of CD11c<sup>+</sup> DCs from vitamin D-sufficient mice but signifi-

cantly reduced the enhanced Th2 differentiation observed with CD11c DCs from vitamin D-deficient mice (Figure 10A). As we had observed that OX40L is critical in Th2 priming, we examined the level of transcripts for TGF- $\beta$  and OX40L in CD11c<sup>+</sup> DCs from vitamin D-deficient versus -sufficient mice. Similar to the human DCs, CD11c<sup>+</sup> DCs from vitamin D-deficient mice had significantly lower levels of *Tgfb* mRNA and higher levels of OX40L expression (Figure 10B). Treatment of these DCs with 0.1  $\mu$ M 1,25 OH-vitamin D<sub>3</sub> significantly increased TGF- $\beta$  expression and reduced OX40L expression (Figure 10B). As TGF- $\beta$  and OX40L have been implicated in Treg and Th2 differentiation respectively, we examined whether vitamin D affected subsequent Foxp3



**Figure 8**

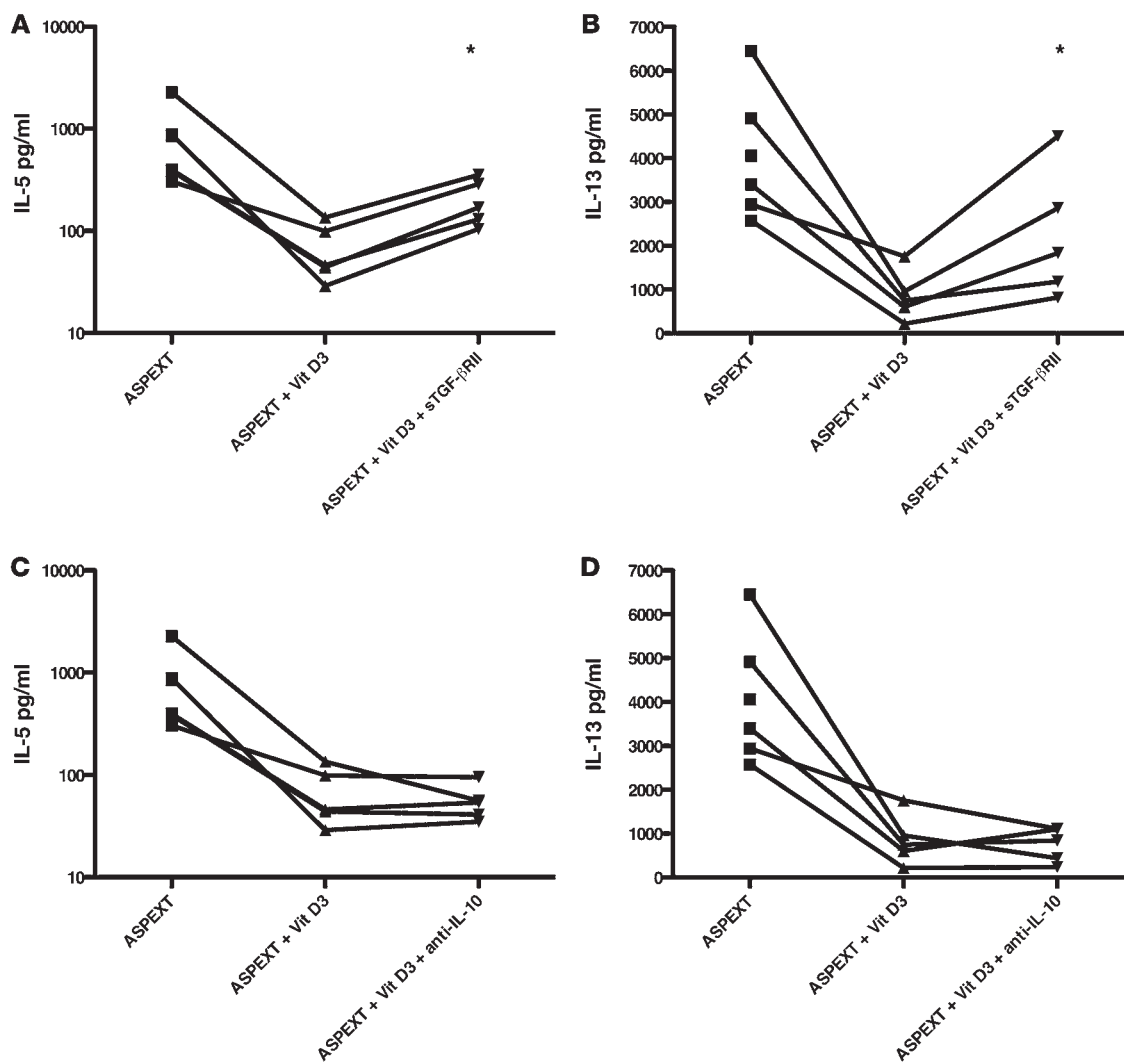
1,25 OH-vitamin D<sub>3</sub> increases the level of cell-associated TGF-β in patients with ABPA. CD11c<sup>+</sup> DCs from patients with ABPA (n = 6) were pulsed with ASPEXT, followed by the addition of autologous bulk CD4<sup>+</sup> T cells, followed by addition of 1,25 OH-vitamin D<sub>3</sub> or vehicle. Cells were incubated for 96 hours, followed by staining for CD4, CD25, and TGF-β, followed by analysis on a FACSaria. (A) Data are plotted as the percentage of CD4<sup>+</sup>CD25<sup>+</sup> and the percentage of CD4<sup>+</sup>CD25<sup>+</sup>TGF-β<sup>+</sup> cells. (B) Relative MFI of TGF-β on CD4<sup>+</sup>CD25<sup>+</sup> cells, before and after the addition of 1,25 OH-vitamin D<sub>3</sub>. (C and D) Representative dot plot of TGF-β<sup>+</sup> on CD4<sup>+</sup>CD25<sup>+</sup> cells exposed to *A. fumigatus*-pulsed DCs, with or without 1,25 OH-vitamin D<sub>3</sub>. (E) Relative gene expression of *TNFSF4*, *FOXP3*, *TGFB1*, and *IL10* in DC/T cell cocultures at the end of the 96-hour incubation. \*\*P < 0.01 by Mann-Whitney.

and IL-13 expression in the Th2 differentiation assay. Similar to what we observed at the protein level (Figure 10A), vitamin D-deficient DCs induced significantly higher IL-13 expression in T cells undergoing Th2 differentiation compared with DCs from vitamin D-sufficient mice (Figure 10C). This enhanced IL-13 response in Th2 cells stimulated with DCs from vitamin D-deficient mice was associated with significantly reduced expression of *Foxp3* mRNA (Figure 10C). No IL-13 or *Foxp3* expression was observed in cultures that lacked the addition of naive CD4<sup>+</sup> T cells (data not shown). Furthermore, addition of 1,25 OH-vitamin D<sub>3</sub> to Th2 cultures primed with DCs from vitamin D-deficient mice significantly reduced IL-13 induction and increased the induction of *Foxp3* expression in the cells (Figure 10C). These data suggest that 1,25 OH-vitamin D<sub>3</sub> increases the ratio of TGF-β to OX40L in the cell favoring induction of Tregs versus Th2 cells. To test this possibility, we examined whether the addition of soluble OX40L (sOX40L) could reverse the effect of exogenous 1,25 OH-vitamin D<sub>3</sub> on vitamin D-deficient DCs. As observed on Figure 10A, the addition of 1,25 OH-vitamin D<sub>3</sub> reduced the ability of DCs from vitamin D-deficient mice to induce IL-5 and IL-13 production in cells undergoing Th2 differentiation; however, the addition of sOX40L restored this response nearly to the level of that of untreated DCs (Figure 10D). Addition of sOX40L to

cultures of vitamin D-deficient DCs not treated with exogenous 1,25 OH-vitamin D<sub>3</sub> had no effect on Th2 cytokine production, suggesting that OX40L expression was already maximal in this condition (Figure 10D).

**Discussion**

Tolerance to many inhaled antigens is the normal immunological response in the lung and is mediated by both anergy and Tregs (1-3). When tolerance is not established, sensitization to inhaled antigens can result in asthma, hypersensitivity pneumonitis, and ABPA. ABPA is rare in the non-CF population, though seen in 0.1% to 0.5% of asthmatic patients. In patients with CF, ABPA prevalence ranges from 4% to 15%. Several risk factors have been identified to date. The most studied risk factor is the class II MHC haplotype of the affected patient. Alleles encoding HLA-DR2 and -DR5 confer susceptibility, whereas HLA-DQ2 has shown protection (28, 29). However, experience in other disease models suggests there are likely risk factors other than the MHC haplotype that are critical to development of ABPA (30). For example, in another chronic inflammatory disease, Crohn disease, MHC is a relatively minor allele compared with polymorphism in the IL-23 receptor or other loci (31). Thus, other factors beyond MHC are likely critical for the development of ABPA.

**Figure 9**

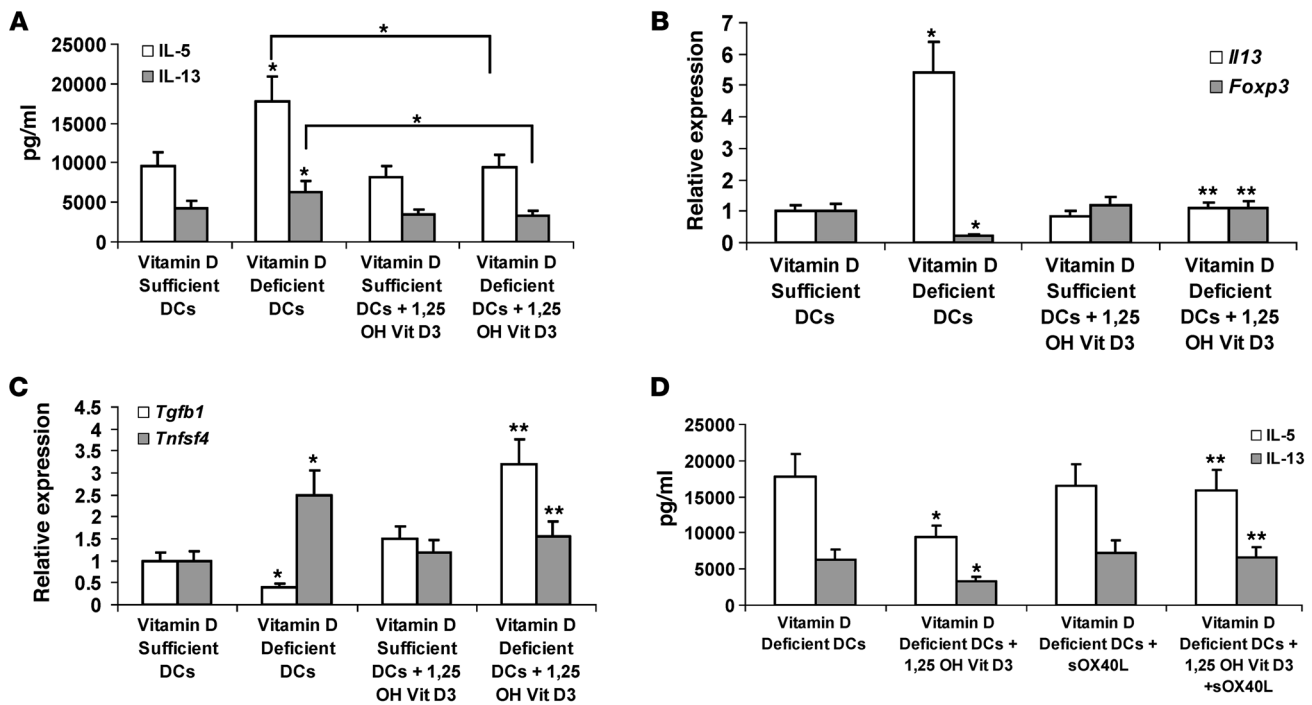
Blockade of TGF- $\beta$  but not IL-10 antagonizes the 1,25 OH-vitamin D<sub>3</sub> suppression of *Aspergillus*-induced Th2 responses in CD4<sup>+</sup> T cells from patients with ABPA. CD11c<sup>+</sup> DCs or TSLP-DCs from patients with ABPA ( $n = 5$ ) were pulsed with ASPEXT, followed by the addition of autologous bulk CD4<sup>+</sup> T cells, followed by addition of 1,25 OH-vitamin D<sub>3</sub>, with or without recombinant human TGF- $\beta$ RII Fc chimera (A and B) or anti-IL-10 (C and D). Cells were incubated for 96 hours, and IL-5 and IL-13 were measured in cell supernatants using Luminex. \* $P < 0.05$  by paired  $t$  test.

There are several advantages to studying ABPA in CF as a model to better understand mechanisms of immune tolerance. First, its prevalence in CF is high enough to be studied. Second, most centers screen for ABPA at least annually, and most CF subjects in North America and Europe are already participating in disease registries. Third, as opposed to allergen-induced asthma, the inciting allergen is known in ABPA, as the vast majority of patients have disease due to *A. fumigatus* sensitization (15). Therefore, we sought to define T cell subsets in these patients to better elucidate mechanisms of immune tolerance versus sensitization in a cohort of ABPA patients versus non-ABPA patients that had documented colonization with *A. fumigatus*.

In this study, we found that CD11c<sup>+</sup> DCs and TSLP-DCs elicited more robust Th2 cytokine production from CD4<sup>+</sup> T cells in an OX40L-dependent fashion after pulsing with *A. fumigatus* antigens compared with similarly treated CD14<sup>+</sup> DCs. We hypothesized that CF epithelium may produce more TSLP or IL-25 in response to either *Aspergillus* or to poly I:C, but this was not the case, as CF HBE

cells produced similar amounts of TSLP and IL-25 compared with non-CF HBE cells. These data therefore do not favor a hypothesis that CF-specific epithelial factors are responsible for the high prevalence of ABPA in CF.

As mentioned above, tolerance may be mediated by anergy or by the induction of Tregs. In *A. fumigatus*-colonized individuals without ABPA, we observed greater percentages of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells and particularly a higher surface TGF- $\beta$  expression in CD4<sup>+</sup>CD25<sup>+</sup> cells. Evidence in a subgroup of ABPA supports the contention that these cells are suppressor cells, as depletion of the CD25<sup>+</sup> subset enhanced Th2 responses to *A. fumigatus* antigens. Due to the fact that this was a subgroup analysis, we cannot exclude a role of anergy in some patients that failed to develop ABPA despite *A. fumigatus* colonization. GIS mapping of patients did not reveal environmental clues to why some patients develop ABPA, and thus we turned our attention to dietary factors. In particular, we investigated vitamin D, because patients with CF are at greater risk of



**Figure 10**

Vitamin D deficiency increases Th2 priming of CD11c<sup>+</sup> DCs. (A) Ova-specific IL-5 and IL-13 response in Th2-polarized cells differentiated with splenic CD11c<sup>+</sup> DCs from vitamin D-sufficient or -deficient mice ( $n = 6-8$ ). (B) Relative gene expression of *Tgfb1* and *Tnfsf4* in splenic CD11c<sup>+</sup> DCs from vitamin D-deficient or -sufficient mice, before and after treatment with 1,25 OH-vitamin D<sub>3</sub>. (C) Relative gene expression of *Il13* and *Foxp3* in DC/T cell cultures, in which CD11c<sup>+</sup> DCs were from vitamin D-deficient or -sufficient mice, before and after treatment with vehicle or 1,25 OH-vitamin D<sub>3</sub>. (B and C) \* $P < 0.05$  by Mann-Whitney, compared with the vitamin D-sufficient group; \*\* $P < 0.05$  by Mann-Whitney compared with the vitamin D-deficient group. (D) Effect of sOX40L on Th2 differentiation in the presence or absence of 1,25 OH-vitamin D<sub>3</sub>. \* $P < 0.05$  by Mann-Whitney, compared with the vitamin D-deficient group; \*\* $P < 0.05$  by Mann-Whitney compared with the vitamin D-deficient group treated with 1,25 OH-vitamin D<sub>3</sub>.

vitamin D deficiency and vitamin D has been implicated in Treg development (23, 24, 32). Moreover, vitamin D receptor knockout mice show exacerbated experimental allergic asthma compared with sensitized and challenged control mice (33). We observed that the ABPA cohort had significantly lower levels of serum vitamin D compared with the non-ABPA cohort. Despite this finding, there was no difference in BMI or vitamin A or E levels, suggesting that the vitamin D deficiency is not a marker of a more global nutritional defect in the patients studied. Treatment of CD4<sup>+</sup> T cells in vitro with 1,25 OH-vitamin D<sub>3</sub> significantly reduced *A. fumigatus*-induced increases in IL-5 and IL-13, regardless of whether the T cells were primed with CD11c<sup>+</sup> DCs or TSLP-DCs. This suppression of Th2 responses is mediated by an increase in TGF- $\beta$  expression in CD4<sup>+</sup>CD25<sup>+</sup> cells. This is supported by the fact that treatment with 1,25 OH-vitamin D<sub>3</sub> increases the MFI of TGF- $\beta$  on CD4<sup>+</sup>CD25<sup>+</sup> cells and the expression of TGF- $\beta$ 1 transcripts as well as the fact that blockade of TGF- $\beta$  signaling in vitro attenuates the suppressive affects of 1,25 OH-vitamin D<sub>3</sub> on Th2 cytokine responses to *Aspergillus* antigens. For these studies, we used 10% autologous AB serum and the measured final 1,25 vitamin D<sub>3</sub> in the media was 30.16 pg/ml, and thus the responses we observed were unlikely affected by active vitamin D in the media. This appears to be due to the effect of 1,25 OH-vitamin D<sub>3</sub> on the DCs and not T cells. This is supported by the fact that in vitro treatment with 1,25 OH-vitamin D<sub>3</sub> suppressed OX40L expression in human DC/T cell cocultures. Also, CD11c<sup>+</sup> DCs from vitamin D-deficient mice had

higher levels of OX40L and primed stronger Th2 responses. Again, in vitro 1,25 OH-vitamin D<sub>3</sub> treatment reduced OX40L expression and reduced Th2 priming. Th2 priming could be restored with the addition of sOX40L. Lastly, 1,25 OH-vitamin D<sub>3</sub> only reduced the IL-5 and IL-13 response to ASPEXT-pulsed DCs and not to CD3/CD28 stimulation (data not shown), further suggesting that the affect of 1,25 OH-vitamin D<sub>3</sub> is through the DC.

Additionally, 1,25 OH-vitamin D<sub>3</sub> has been shown to also inhibit relB (34) and DC maturation (35, 36) in vitro, which may inhibit Th2 T cell development. In this study, we found a specific effect on OX40L expression. In fact, what may be critical is the relative expression of OX40L and TGF- $\beta$ 1, as OX40L has been shown recently to block the induction of Foxp3 in T cells (18). LPS has been shown to antagonize airway tolerance to antigen and is mediated by OX40L induction on both DCs and B cells. The upregulation of OX40L increased both Th2 and Th1 responses to antigen. In addition to altering OX40L expression, vitamin D treatment of DCs has been shown to lead to apoptosis of alloreactive T cells.

There is a great deal of interest in vitamin D and asthma, another Th2-driven disease (37-39). In a cohort of children with asthma from Costa Rica, Brehm et al. found that vitamin D deficiency was significantly and inversely associated with total IgE and eosinophil counts (38). In a recent study, vitamin D deficiency was also associated with increased use of inhaled corticosteroids (40). Additionally, in vitro 1,25 OH-vitamin D<sub>3</sub> synergized to increase IL-10 levels in Tregs (24), an affect not seen in our DC/T cell coculture system.



It has recently been shown that 1,25 OH-vitamin D<sub>3</sub> can increase TLR9 expression in inducible Tregs and that activation via TLR9 can reduce their regulatory function (41). These data suggest that vitamin D has profound effects on both DCs and iTregs. It is also possible that this TLR9 pathway may be another pathway in which iTregs lack efficacy in a chronic infectious condition such as CF.

The basis for vitamin D<sub>3</sub> deficiency in our cohort remains unclear at the present but does not appear to be a direct effect of geographical location. There was no difference in steroid or ursodiol use (the latter, a crude measure of biliary disease) in our cohort, and all patients were prescribed a combination of vitamin A, D, E, and K. The fact that vitamin A and E levels were not different suggests that nonadherence was unlikely a factor. One possible factor is genetic polymorphisms in the vitamin D pathway. In a recent study, SNPs were assessed to determine relative risk in an asthma cohort in the vitamin D receptor (VDR) as well as CYP2R1 (the 25 hydroxylase) and CYP24A1, which mediates the clearance of 1,25 OH-vitamin D<sub>3</sub> (37). SNPs in CYP2R1 and CYP24A1 were associated with asthma, and in a 2-gene model, SNPs in VDR and IL10 were also associated. We are currently performing exon sequencing of these genes in our cohort, as SNPs in this pathway could clearly determine efficacy of vitamin D treatment in this cohort. Taken together, our data strongly implicate vitamin D deficiency as a risk factor for ABPA and lay the ground work for clinical trials of enhanced vitamin D supplementation to prevent or treat ABPA. Moreover, our data suggest that enumeration of OX40L expression on DCs and Treg frequency may be useful biomarkers to follow response to vitamin D augmentation.

## Methods

**HBE cultures.** Primary HBE cells were provided by the Tissue Core Laboratory at the University of Pittsburgh or purchased from Cambrex (Lonza). Primary cells were grown as polarized air-liquid interface (ALI) cultures, as previously described (42). HBE cells were stimulated apically with RC, HKSC (19), or poly I:C for 24 hours, and TSLP was measured by ELISA.

**Analytical assays.** TSLP was measured by sandwich ELISA as previously described (7). IL-4, IL-5, IL-10, IL-13, and IL-17 were measured using Luminex (Millipore).

**Human subjects, cell harvest, and sorting.** Accrual of all study subjects occurred after approval of the research protocol by the Institutional Review Board at the University of Pittsburgh. Human CD4<sup>+</sup>, CD11c<sup>+</sup>, and CD14<sup>+</sup> cells were obtained from whole blood from CF donors who gave their written informed consent. The blood was then ficollized in Vacutainer CPT tubes (BD Pharmingen) and then resuspended in cell buffer composed of EDTA (Gibco), BSA (Sigma-Aldrich), and 1X PBS (Fisher). All cells were isolated by magnetic bead-activated sorting using microbeads and MidiMacs (Miltenyi). The CD4<sup>+</sup> cells were first isolated by positive separation in MS columns using CD4 Microbeads (Miltenyi). CD11c<sup>+</sup> cells were subsequently isolated from the negative fraction of the CD4<sup>+</sup> isolation using CD11c-APC microbeads and then anti-APC microbeads for the final magnetic separation. CD14<sup>+</sup> DCs were selected by CD14<sup>+</sup> microbeads (Miltenyi) and cultured in human recombinant GM-CSF (50 ng/ml; PeproTech) and IL-4 (20 ng/ml; PeproTech) for 6 days.

**Robotic plate set up.** DCs were then plated in flat bottom 96-well plates at a density of  $5 \times 10^5$  and  $5 \times 10^4$  DCs per well in medium containing RPMI, L-glutamine, penicillin/streptomycin (pen/strep), FBS (Gibco), and Human AB serum (Atlanta Biologicals). Cells in the plate were then stimulated with the following stimulators: TSLP (5 ng/ml; R&D Systems), Zymosan (50 mg/ml; Molecular Probes Inc.), or ASPEXT (1 µg/ml; Hollister-stier). A fourth condition included both ASPEXT (1 µg/ml) and TSLP (5 ng/ml). One well in the

plate was left unstimulated as a control, and then  $5 \times 10^5$  CD4<sup>+</sup> cells were added. Control wells received CD4<sup>+</sup> T cells that were cultured in media or stimulated with CD3/CD28 beads (Dyna, Invitrogen). Additionally, recombinant IL-2 was added to all wells on the plate (7.5 ng/ml). Medium containing RPMI, 5% L-glutamine, 5% pen/strep, 10% FBS (Gibco), and 5% Human AB serum (Atlanta Biologicals) was then added to each well to bring up to final volume. All cells were then incubated at 37°C and 5% CO<sub>2</sub> for 96 hours.

In experiments with 1,25 OH-vitamin D<sub>3</sub>, the conditions mentioned above were plated in duplicate, in which one set was additionally incubated with 1α, 25-OH-dihydroxyvitamin D<sub>3</sub> (Biomol) at a concentration of 0.1 µM per condition versus an ethanol control. In some experiments, anti-human IL-10 (1 µg/ml final concentration) or recombinant human TGF-β sRII Fc chimera (10 µg/ml final concentration) were added.

**Cell collection and FACS analysis.** Cells in the plate were collected after 96-hour incubation, after which the release of cytokines was stopped by administering Golgi Plug (BD Pharmingen) for the last 6 hours of stimulation. The cells from each well were then harvested for flow cytometry, and supernatants were assessed for cytokine contents using Luminex (Lincoplex). The cells harvested for flow cytometry were stained with a cocktail containing Pacific Blue-conjugated CD4 and PECy7-conjugated CD25 (BD Pharmingen). After initial staining, the cells were then fixed and permeabilized using a FOPX3 Staining Buffer Set (eBioscience) and subsequently stained with another cocktail containing the following: FITC-conjugated Foxp3 (eBioscience), PE-conjugated TGF-β (R&D Systems), and additional Pacific Blue-conjugated CD4 (BD Pharmingen). All cells were analyzed on a FACSARIA.

**Vitamin D-deficient diet.** All mouse experiments were approved by the IACUC at LSUHSC. Six-week-old female Balb/c mice (Charles River) 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 ELISA (IDS) to ensure vitamin D deficiency. Control mice were kept on regular mouse chow, supplied by the Division of Animal Care at LSUHSC (Harlan Teklad 2019S). Splenic CD11c<sup>+</sup> cells were purified from the spleen using CD11c<sup>+</sup>-coated magnetic beads (Miltenyi) and used for Th2 differentiation assays as previously described (27). In certain experiments sOX40L (R&D Systems) was added.

**Statistics.** All data are presented as the mean ± SEM. For paired experiments, statistical significance was determined by a 2-tailed, paired *t* test. For comparisons of data from 2 independent groups, we used the nonparametric 2-tailed Mann-Whitney test, since the data often appeared inconsistent with an assumption of normality. A *P* value of less than 0.05 was considered significant.

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