Enhanced production of IL-17A in patients with severe asthma is inhibited by 1α,25-dihydroxyvitamin D3 in a glucocorticoid-independent fashion

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Background: T_H17 cells are proposed to play a role in the pathology of asthma, including steroid-resistant (SR) disease. We previously identified a steroid-enhancing function of vitamin D in patients with SR asthma in restoring the impaired response to steroids for production of the anti-inflammatory cytokine IL-10.

Objective: We sought to investigate the production of the T_H17-associated cytokines IL-17A and IL-22 in culture in patients with moderate-to-severe asthma defined on the basis of their clinical response to steroids and the susceptibility of this response to inhibition by steroids and the active form of vitamin D, 1α,25-dihydroxyvitamin D3 ($1,25[OH]_2D3$).

Methods: PBMCs were stimulated in culture with or without dexamethasone and $1,25(OH)_2D3$. A cytometric bead array, ELISA, and intracellular cytokine staining were used to assess cytokine production. The role of CD39 in inhibition of the T_H17 response was studied by using quantitative real-time PCR, flow cytometry, and addition of the antagonist POM-1 to culture.

Results: Asthmatic patients synthesized much higher levels of IL-17A and IL-22 than nonasthmatic control subjects, with patients with SR asthma expressing the highest levels of IL-17A. Glucocorticoids did not inhibit IL-17A cytokine expression in patients and enhanced production in cultures from control subjects. Treatment with $1,25(OH)_2D3$ with or without dexamethasone significantly reduced both IL-17A and IL-22 levels. An antagonist of the ectonucleotidase CD39 reversed $1,25(OH)_2D3$-mediated inhibition of the IL-17A response.

Conclusion: Patients with severe asthma exhibit increased levels of T_H17 cytokines, which are not inhibited by steroids. $1,25(OH)_2D3$ inhibits T_H17 cytokine production in all patients studied, irrespective of their clinical responsiveness to steroids, identifying novel steroid-enhancing properties of vitamin D in asthmatic patients. (J Allergy Clin Immunol 2013;■■■:■■■-■■■.)

Key words: *Asthma, vitamin D, IL-17A, steroid resistant, steroid sensitive, T_H17, glucocorticoids*

Asthma is broadly defined as bronchial hyperresponsiveness with reversible expiratory airflow limitation. Historically, asthma has been described as having an underlying T_H2-mediated inflammatory profile; however, there is increasing evidence for heterogeneity in asthma phenotypes, with a significant proportion of asthmatic patients demonstrating a low or non–T_H2-mediated phenotype that appears less sensitive to control by glucocorticoids.[1,2] Although glucocorticoids are currently the most effective treatment for asthma, steroid-resistant (SR) asthma is a significant clinical problem. These patients generally require prolonged systemic treatment, their asthma is less stable and more difficult to control, and they are subject to higher morbidity and mortality.[3-6] The precise cause or causes of SR asthma are unknown, but several mechanisms have been proposed to account for it,[7-10] including genetic susceptibility, defects in glucocorticoid receptor (GR) binding, increased expression of the functionally inactive GR-β, activation of transcription factors (eg, activator protein 1), or decreased synthesis of immunoregulatory cytokines, such as IL-10. Although it is difficult to investigate SR asthma in patients with milder disease because its definition is based on improvement in originally abnormal lung function, it is reasonable to suppose that SR asthma is likely to be associated with severe and difficult-to-control disease.

T_H17 cells are critical for defense against bacterial and fungal infections.[11] Mouse models demonstrate that animals deficient in IL-17A or IL-17RA are highly susceptible to infection with mucosal pathogens, such as *Klebsiella pneumoniae* and *Candida albicans*.[12,13] IL-17A–mediated activation of the innate immune system and neutrophil influx can be protective against infection but can also lead to damage of the surrounding tissues associated with immune pathology.[14,15]

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Abbreviations used
Foxp3: Forkhead box protein 3
GR: Glucocorticoid receptor
$1,25(OH)_2D3$: 1α,25-Dihydroxyvitamin D3
SR: Steroid resistant
SS: Steroid sensitive
Treg: Regulatory T

A body of circumstantial evidence consistent with the hypothesis that IL-17A exacerbates asthma, reduces patients' responsiveness to therapy, or both already exists. Thus IL-17A expression in sputum or bronchial mucosal biopsy specimens has been correlated with bronchial hyperresponsiveness,[16] granulocyte infiltration,[17] and production of fibrogenic mediators by bronchial fibroblasts.[18] Furthermore, increased serum IL-17A appears to be both a marker[19] and an independent risk factor for severe asthma.[16-18,20,21] Involvement of T_H17 cells in patients with severe SR asthma is proposed from murine and human studies.[22,23] In addition to IL-17A, IL-22 is commonly produced by T_H17 cells. In various animal models of disease, IL-22 can elicit damaging or protective responses, possibly dependent on the location, cellular source, cytokine milieu, and timing of its expression.[24-26]

There is great interest in the therapeutic potential of vitamin D to regulate the severity of asthma and to reduce the amount of anti-inflammatory medication required for disease control. Several studies have pointed out striking links in populations between vitamin D deficiency and asthma severity or requirements for anti-inflammatory medication.[27-30] In previous studies investigating the immunologic basis of this relationship, we have shown that 1α,25-dihydroxyvitamin D3 ($1,25[OH]_2D3$) exerts immunomodulatory effects on T cells from patients with SR asthma, enhancing the impaired steroid-induced IL-10 response in these subjects,[31] whereas others have highlighted the capacity of this molecule to inhibit T_H17 responses.[32-35]

Building on these studies, we hypothesized that $1,25(OH)_2D3$ inhibits increased IL-17A production by blood T_H17 cells from patients with severe asthma independently of glucocorticoids *in vitro* and independently of their clinical responsiveness to glucocorticoid therapy *in vivo*. To address this hypothesis, we studied the effects of $1,25(OH)_2D3$ and the glucocorticoid dexamethasone on the production of IL-17A and its common coproduct, IL-22, by peripheral blood $CD4^+$ T cells from a cohort of patients with severe asthma who were carefully characterized for their clinical glucocorticoid responsiveness and nonasthmatic control subjects. Finally, in an attempt to elucidate possible mechanisms of the effect of $1,25(OH)_2D3$ in altering IL-17A production, we measured its effects on the expression of the ectonucleotidase CD39, increased expression of which has previously been reported to be associated with inhibition of IL-17A production in patients with autoimmune disease.[36] We hypothesized that dexamethasone, but not $1,25(OH)_2D3$, reduces CD39 expression on blood T cells and that inhibition of its ectonucleotidase activity abolishes any effects of $1,25(OH)_2D3$ on IL-17A expression.

METHODS

Subjects

Healthy adults (study approved by the local research ethics committee, 09/H0804/77) and asthmatic patients (08/H0804/84) were recruited. All healthy control subjects and patients provided written informed consent. Asthmatic patients had moderate-to-severe asthma for at least 6 months on therapy step 3 or 4 of the British Thoracic Society guidelines on the management of asthma diagnosed by a specialist physician. The average inhaled corticosteroid dose in beclomethasone diproprionate equivalents was 1177 μg for patients with SR asthma and 1180 μg for patients with steroid-sensitive (SS) asthma. All patients had a prebronchodilator FEV_1 of less than 80% of predicted value, had documented airway variability of greater than 12% after 400 μg of short-acting bronchodilator, and had undergone detailed assessment to exclude a diagnosis other than asthma and comorbidities affecting asthma control. Patients were not taking oral corticosteroids for 4 weeks before the study. Steroid sensitivity was defined by an increase in FEV_1 of greater than 10% from baseline after a 2-week course of prednisolone at 40 mg/1.73 m^2 body surface area. Steroid resistance was defined by a less than 10% increase in FEV_1 from baseline after a 2-week course of prednisolone at 40 mg/1.73 m^2 body surface area. Compliance with prednisolone during the study was assessed with cortisol serum levels before and after steroids. Patients undergoing immunotherapy, smokers, or patients who had a respiratory tract infection or asthma exacerbation during or 4 weeks before enrollment into the study were excluded.

Eighteen patients with SR asthma and 10 patients with SS asthma were assessed with a mean age of 54 (SR) and 50 (SS) years. Mean prebronchodilator FEV_1 before and after a course of oral corticosteroids was 1.99 L (64.56%) and 1.96 L (64.05%) in the patients with SR asthma and 1.72 L (57.1%) and 2.19 L (73.2%) in the patients with SS asthma (poststeroid FEV_1, patients with SR vs patients with SS asthma: $P < .001$, analysis of covariance). Ten healthy control subjects were assessed for comparison (mean age, 41 years).

Cell isolation and culture

Human PBMCs were isolated, as previously described.[31] Briefly, CD8-depleted PBMCs were obtained by means of negative selection with $CD8^+$ Dynalbeads (Invitrogen, Paisley, United Kingdom). $CD4^+$ T cells were purified by means of positive selection with Dynalbeads. Cells (1×10^6 cells/mL) were cultured in RPMI (containing 10% FCS, 2 mmol/L L-glutamine, and 50 μg/mL gentamicin) and stimulated with plate-bound anti-CD3 (1 μg/mL, OKT-3) plus 50 U/mL recombinant human IL-2 (Eurocetus, Harefield, United Kingdom) in the presence or absence of dexamethasone (Sigma-Aldrich, Gillingham, United Kingdom) and $1,25(OH)_2D3$ (BIOMOL Research Labs, Exeter, United Kingdom) at the indicated concentrations in a 24-well plate for 7 days. There was no significant difference between viability between the different conditions (data not shown). Where indicated, cells were recultured at the same density of 1×10^6/mL after 7 days with plate-bound anti-CD3 and IL-2 alone in a 48-well plate, and supernatants were harvested at 48 hours for cytokine analysis.

Flow cytometry

The following antibodies were used for cell-surface phenotyping: CD3 (SK7; BD Biosciences, Oxford, United Kingdom) and CD39 (ebioA1; eBioscience, Hatfield, United Kingdom). For *ex vivo* staining, red blood cells were lysed with FACS Lysing Solution (working dilution, 1:10; BD Biosciences) and then further stained for intranuclear forkhead box protein 3 (Foxp3; PCH101, eBioscience) by using the Foxp3 staining kit per the manufacturer's instructions (eBioscience).

For intracellular cytokine staining on day 7, cells were restimulated for 4 hours with 5 ng/mL phorbol 12-myristate 13-acetate and 500 ng/mL ionomycin, with 2 μmol/L monensin (Sigma-Aldrich) added for the final 2 hours. Cells were washed, fixed, and permeabilized with the Cytofix/Cytoperm kit (BD Biosciences) and stained with the fluorescently labeled mAbs to IL-22 and IL-17A (22URTI and eBio64CAP17, eBioscience). Dead cells (7-aminoactinomycin D positive, Sigma-Aldrich) were gated out. Analysis was performed with a FACSCalibur flow cytometer (BD Biosciences).

Quantitative real-time PCR

RNA was extracted from cell pellets with the RNeasy Mini kit (Qiagen, Crawley, United Kingdom), according to the manufacturer's instructions. The RNA was quantified with a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, Del), and 250 ng of RNA was reverse transcribed into

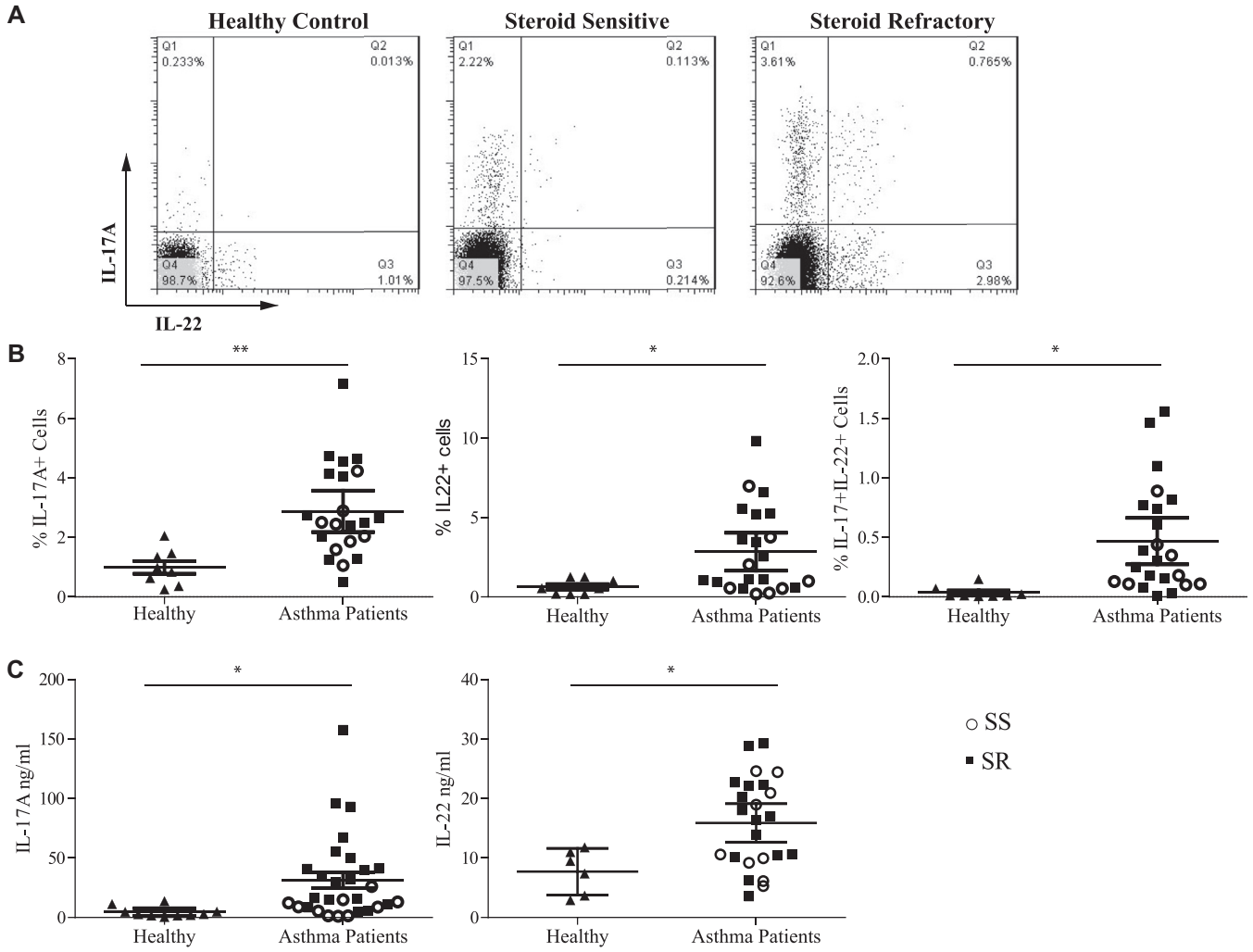


FIG 1. Patients with severe asthma express higher IL-17A and IL-22 levels. Control and asthmatic cultures were assessed for IL-17A and IL-22 production by using intracellular flow cytometry and an antibody capture assay. **A,** Representative dot plots. **B** and **C,** Cumulative data of cytokine-positive cells (healthy subjects, n = 8; patients with SS asthma, n = 8; and patients with SR asthma, n = 14; Fig 1, *B*) and secreted cytokines (healthy subjects, n = 10; patients with SS asthma, n = 10; and patients with SR asthma, n = 14; Fig 1, *C*). **P* < .05 and ***P* < .001, Student *t* test. *Open circles*, Patients with SS asthma; *solid squares*, patients with SR asthma; and *solid triangles*, healthy control subjects.

cDNA. Quantitative real-time PCR was performed in triplicate by using an Applied Biosystems 7900 HT system and FAM-labeled Assay-on-Demand reagent sets for *CD39* (Hs00969559_m1). Quantitative real-time PCR reactions were multiplexed with VIC-labeled 18S primers and probes (Hs99999901_s1) as an endogenous control and analyzed with SDS software, version 2.1 (Applied Biosystems, Foster City, Calif), according to the $2^{-\Delta\Delta Ct}$ method.

Cytokine analysis

IL-22 levels were measured by using ELISA and matched antibody pairs (BD Biosciences). The lower limit of detection for IL-22 was 100 pg/mL. IL-17A, IL-10, IFN-γ, and IL-5 levels were measured with the Cytometric Bead Array (BD Biosciences), according to the manufacturer's protocol; the lower limit of detection was 1.5 pg/mL.

Statistics

Data are presented as means ± SEMs unless indicated. Data were assessed for equivalence to a Gaussian distribution and equality variance, after which the appropriate parametric or nonparametric test was performed (see individual figure legends). Differences were considered significant at the 95% confidence level.

RESULTS

Comparison of IL-17A and IL-22 production by PBMCs in culture from patients with SS and patients with SR asthma

Details of the 2 cohorts of patients with moderate-to-severe asthma, defined as SS or SR asthma on the basis of changes in lung function after a 2-week course of oral prednisolone, and of healthy nonasthmatic control subjects are provided in the Methods section. Notably, these 2 patient groups exhibited comparable disease severity based on impairment of lung function and were taking comparable doses of inhaled corticosteroids (total dosages based on beclomethasone dipropionate equivalence).

PBMCs from the asthmatic patients were evaluated for their capacity to synthesize the T_H17-associated cytokines IL-17A and

IL-22 in culture, and comparisons were made with control subjects. PBMCs depleted of $CD8^+$ T cells were stimulated *in vitro* with anti-CD3 and IL-2 for 7 days and then stained for intracellular cytokine expression. Significantly greater percentages of $IL\text{-}17A^+$ and $IL\text{-}22^+$ expressing cells were observed in cultures from asthmatic patients compared with healthy control subjects (Fig 1, *A* and *B*). When patients were subdivided based on clinical responsiveness to glucocorticoids, only patients with SR asthma showed a significant difference in IL-17A immunoreactivity (SR asthma, $P = .003$; SS asthma, $P = .142$) and IL-22 immunoreactivity (SR asthma, $P = .037$; SS asthma, $P = .701$) compared with control subjects (Fig 1, *B*). Cells coexpressing IL-17A and IL-22 are proposed to represent the most pathogenic population, and their numbers were significantly increased in all asthmatic patients and patients with SR asthma compared with healthy control subjects (all asthma, $P = .029$; SR asthma, $P = .009$). At day 7, cells were also harvested and recultured at equal cell densities for 48 hours with anti-CD3 and IL-2 alone. Culture supernatants from asthmatic patients contained significantly higher quantities of secreted IL-17A and IL-22 compared with those from healthy control subjects (Fig 1, *C*). Production of IL-17A and IL-22 was significantly increased in patients with SR asthma compared with that seen in control subjects (IL-17A, $P = .009$; IL-22, $P = .036$). Supernatants from patients with SR asthma had significantly more IL-17A compared with those from patients with SS asthma ($P = .016$), whereas culture supernatants from patients with SS and patients with SR asthma contained comparable levels of IL-22 (see Fig E1, *A*, in this article's Online Repository at www.jacionline.org).

Glucocorticoids do not inhibit IL-17A and IL-22 synthesis in culture

The capacity of glucocorticoids to alter production of IL-17A and IL-22 was next examined. Addition of 10^{-7} mol/L dexamethasone to cultures from healthy control subjects significantly increased the mean percentage of cells expressing IL-17A but did not alter the low percentage of cells expressing IL-22 (Fig 2, *A*). IL-17A and IL-22 secretion was increased in a concentration-dependent manner by dexamethasone in cultures from healthy donors (Fig 2, *B* and *C*). IL-2 is present in the stimulation cultures, and addition of IL-2 plus IL-4 has previously been described to promote steroid insensitivity in culture.[37] Therefore the effects of dexamethasone were assessed in the presence or absence of anti-CD3, IL-2, or both on IL-17A and a range of additional cytokines (see Fig E2 in this article's Online Repository at www.jacionline.org). Notably, IL-17A (and IL-10) enhancement by dexamethasone only occurred when the combination of anti-CD3 and IL-2 was present. Dexamethasone inhibited the production of IFN-γ, IL-13, and IL-5, although the inhibition of IFN-γ in particular was less marked when anti-CD3 plus IL-2 compared with anti-CD3 or IL-2 alone was present in these cultures.

The effects of dexamethasone in asthmatic patient cultures were less marked, where notably there was a greater than 7-fold higher basal level of IL-17A synthesis compared with that seen in nonasthmatic cultures (Fig 2, *B*). A trend toward increased IL-17A secretion ($P = .102$; Fig 2, *B*) and a significant increase in the frequency of $IL\text{-}17A^+$ cells in patient cultures containing 10^{-7} mol/L dexamethasone was observed (Fig 3, *A*). An effect observed in both the SS asthma and SR asthma subgroups was

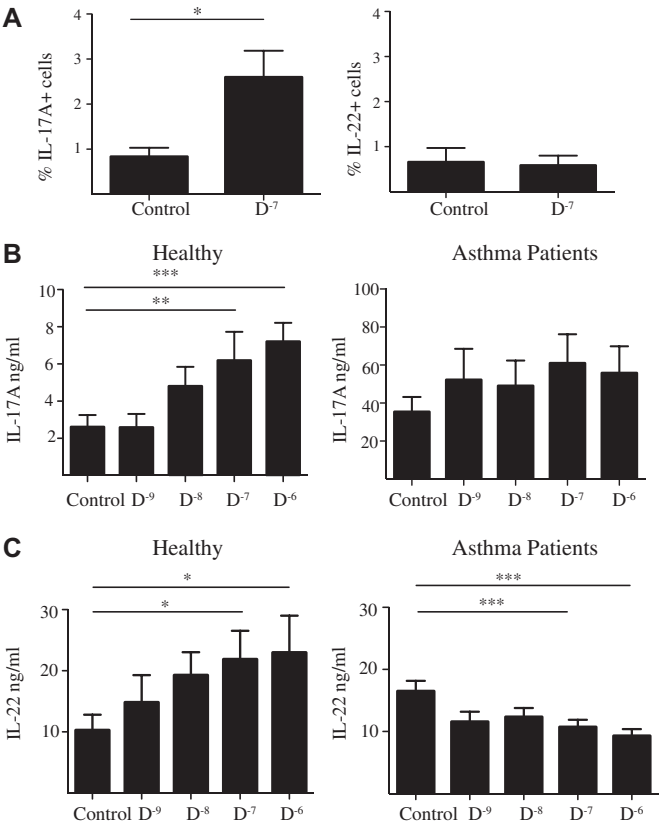


FIG 2. Dexamethasone does not inhibit IL-17A production. **A,** Cumulative data of the effects of dexamethasone on the frequency of $IL\text{-}17A^+$ and $IL\text{-}22^+$ cells in nonasthmatic cultures (healthy subjects, n = 8). **B** and **C,** IL-17A and IL-22 secretion in control *(left)* and asthmatic *(right)* cultures (healthy subjects, n = 10; asthmatic patients, n = 28). $*P < .05$, $**P < .01$, and $***P < .001$; Fig 2, *A*, paired *t* test, and Fig 2, *B* and *C*, 1-way ANOVA with the Tukey post test. D^{-x}, Dexamethasone at 10^{-x} mol/L.

partial inhibition by dexamethasone of IL-22 secretion (Fig 2, *C*, and see Fig E1).

1α,25-dihydroxyvitamin D3 inhibits IL-17A and IL-22 expression

The capacity of $1,25(OH)_2D3$ to inhibit IL-17A production alone and in combination with dexamethasone was next investigated in cultures from asthmatic patients. $1,25(OH)_2D3$ significantly decreased the mean percentages of cells that were immunoreactive for IL-17A or IL-22 in culture (Fig 3). Although dexamethasone did not reduce IL-22 or IL-17A immunoreactivity compared with control cultures, the combination of dexamethasone and $1,25(OH)_2D3$ led to a marked reduction in the frequency of cytokine-positive cells that was not significantly different from cultures with $1,25(OH)_2D3$ alone (Fig 3). Protein expression in culture supernatants after $1,25(OH)_2D3$ treatment alone or in combination with dexamethasone demonstrated a comparable trend, with no significant difference in the mean concentration of IL-17A or IL-22 between cultures containing $1,25(OH)_2D3$ or $1,25(OH)_2D3$ plus dexamethasone (Fig 3). The effects of $1,25(OH)_2D3$ on IL-17A and IL-22 synthesis were comparable in the patients with SS asthma and those with SR asthma (see Fig E1, *B*). The reduction in IL-17A production by $1,25(OH)_2D3$ is unlikely to be explained by inhibition of proliferation (see Fig E3 in this article's Online Repository at www.jacionline.org) or

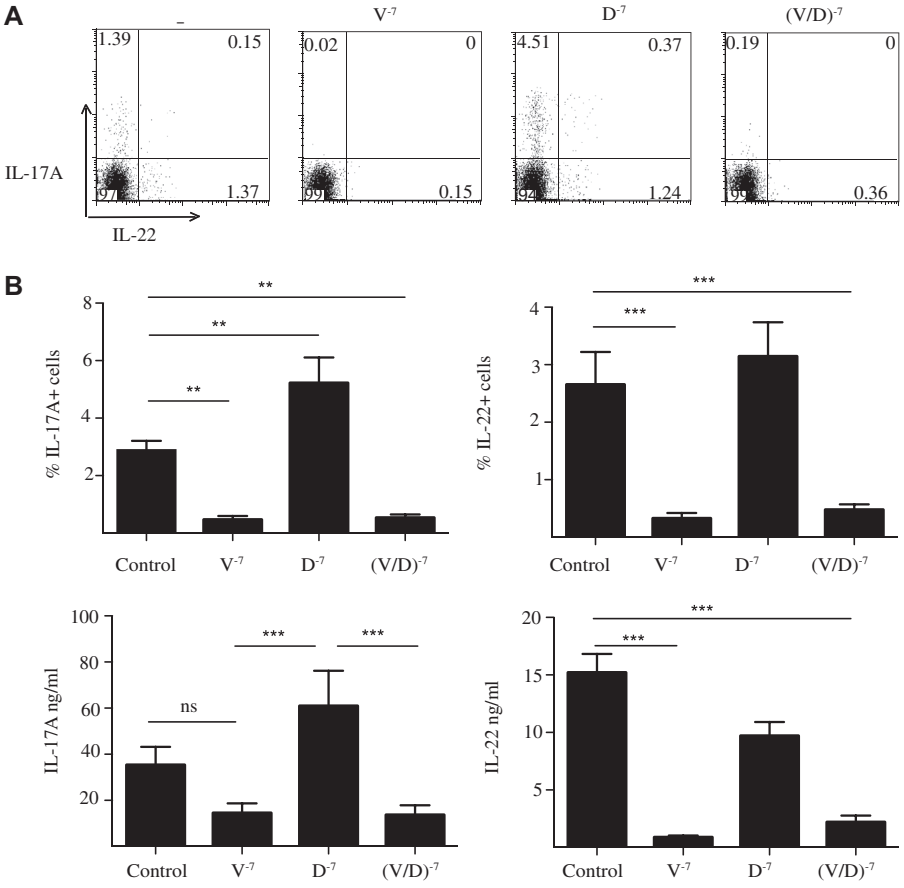


FIG 3. 1,25(OH)$_2$D3 inhibits IL-17A and IL-22 in cultures from asthmatic patients. **A,** Representative dot plots. **B,** Cumulative percentage of cytokine-positive cells (n = 22) or cytokine secretion (n = 28). 1,25(OH)$_2$D3 reduced IL-17A and IL-22 levels in asthmatic cultures with or without dexamethasone. $^{**}P < .01$ and $^{***}P < .001$, 1-way ANOVA with the Tukey post test. *ns,* Not significant; D^{-x} and V^{-x}, dexamethasone and 1,25(OH)$_2$D3 respectively, at 10^{-x} mol/L.

decreased viability (data not shown) because 1,25(OH)$_2$D3 at the concentration used did not inhibit either, as recently reported.[38] These data highlight the capacity of 1,25(OH)$_2$D3 to counteract any detrimental effect or lack of effect of dexamethasone on T_H17-associated cytokine synthesis in cell cultures from asthmatic patients. 1,25(OH)$_2$D3 effectively inhibited IL-17A production in cultures from healthy control subjects (data not shown), as previously reported by others.[33-35]

1,25(OH)$_2$D3 enhances expression of CD39$^+$ T cells, a potential mechanism for IL-17A inhibition

Expression of the ectonucleotidase CD39 by Foxp3$^+$ regulatory T (Treg) cells has previously been reported to be associated with inhibition of IL-17A production.[36,39,40] Analysis of e*x vivo* expression of CD39 demonstrated a significantly higher frequency of CD3$^+$CD39$^+$ T cells in the peripheral blood of healthy donors compared with that of patients with severe asthma ($P = .003$). CD39 can be expressed on both CD4$^+$ memory and Foxp3$^+$ T cells,[36,39] and therefore we further analyzed expression on Foxp3$^+$ and Foxp3$^-$ populations. A significantly higher frequency of CD39-expressing cells was detected in both CD3$^+$Foxp3$^-$ and CD3$^+$Foxp3$^+$ T cells in the nonasthmatic donors compared with the patients with severe asthma (Fig 4, *A*) in contrast to the higher IL-17A synthesis observed in the patients.

Next, the effect of 1,25(OH)$_2$D3 and dexamethasone on CD39 expression was investigated. CD4$^+$ T cells cultured in the presence of 1,25(OH)$_2$D3 demonstrated a significantly increased gene expression of CD39 (Fig 4, *B*). When surface expression of CD39 was assessed by using flow cytometry, there was a modest but significant increase in the expression level of CD39, as determined based on mean fluorescence intensity, but there were no differences in the overall frequency of CD39$^+$ T cells. In comparison, addition of dexamethasone reduced the percentage of CD39$^+$ T cells and mean fluorescence intensity (Fig 4, *C* and *D*). To investigate the role of CD39 in 1,25(OH)$_2$D3 inhibition of IL-17A production, we used POM-1, a known inhibitor of CD39 ectonucleotidase activity. POM-1 partially abrogated the inhibition of IL-17A synthesis effected by 1,25(OH)$_2$D3 in cultured T cells. This effect was specific for IL-17A because the production of IL-10 and other T_H1 and T_H2 cytokines was not affected (Fig 4, *E*).

DISCUSSION

Our data demonstrate that the production of IL-17A and IL-22 by human peripheral blood CD4$^+$ T cells is increased in patients with severe asthma. Notably, IL-17A levels, but not IL-22 levels, were approximately 5-fold greater in patients with SR asthma compared with those seen in patients with SS asthma. Strikingly,

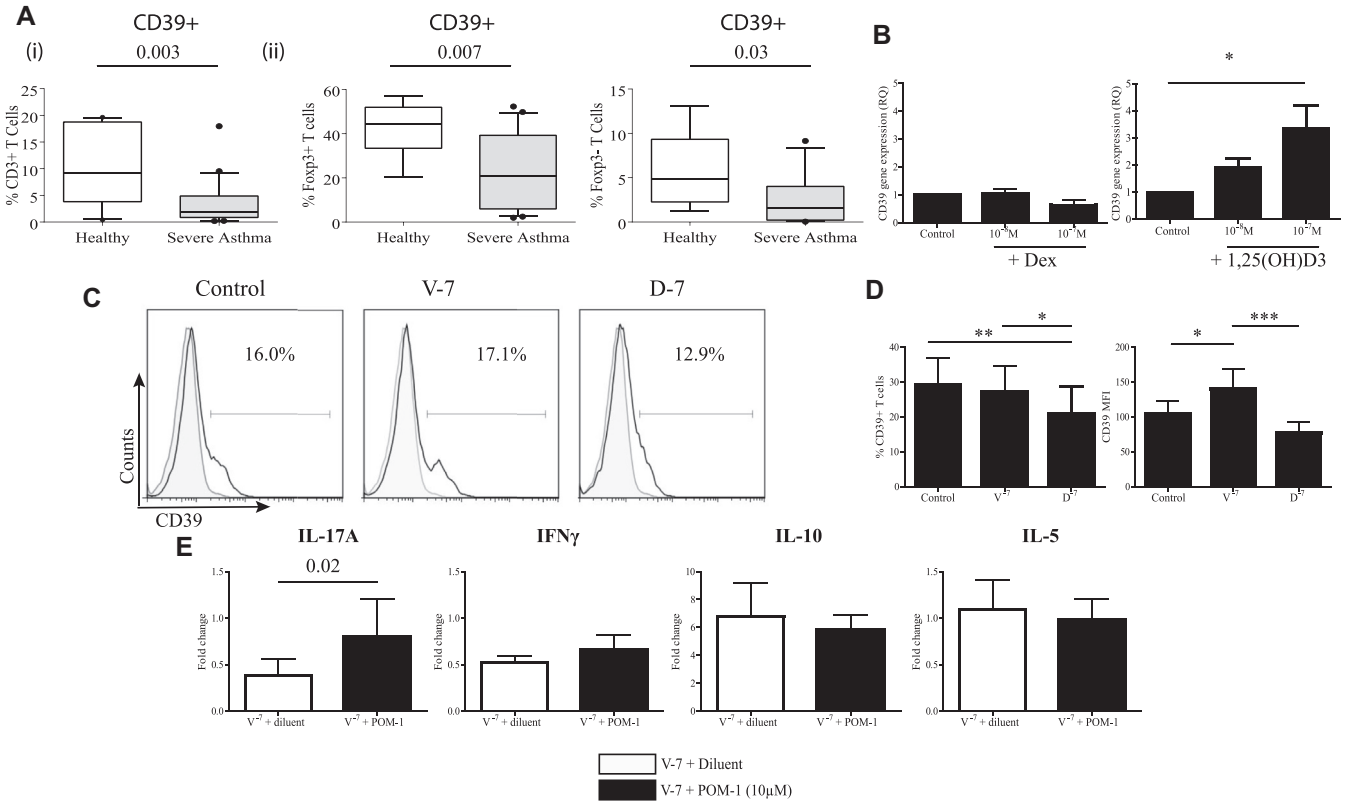


FIG 4. 1,25(OH)$_2$D3 increases CD39 expression by CD4$^+$ T cells. **A,** The frequency of CD39$^+$ and CD3$^+$ T cells *(i)* and CD39$^+$ and Foxp3$^+$ and Foxp3$^-$ T cells *(ii)* in the peripheral blood of healthy donors (n = 8) and patients with severe asthma (n = 19). **B-D,** Effects of treatment with dexamethasone or 1,25(OH)$_2$D3 in culture on healthy nonasthmatic donors. Fig 4, *B*, Relative CD39 mRNA (n = 5). Fig 4, *C*, Representative histograms. Fig 4, *D*, Cumulative data on CD39 expression (n = 6). **E,** Cumulative data (n = 8) on effects of POM-1 (10 μmol/L, CD39 antagonist) on 1,25(OH)$_2$D3-modulation of IL-17A, IFN-γ, IL-10, and IL-5 expressed as fold change from the no-drug condition. **P* < .05, ***P* < .01, and ****P* < .001; Fig 4, *B* and *D*, 1-way ANOVA with the Tukey post test, and Fig 4, *E*, Wilcox matched pairs signed-rank test.

the glucocorticoid dexamethasone significantly enhanced the frequency of IL-17A$^+$ cells in culture in both nonasthmatic control subjects and asthmatic patients, although IL-17A secretion was only significantly increased by dexamethasone in control cultures. In contrast, the active form of vitamin D reduced levels of both cytokines directly and also when dexamethasone was present in culture. Importantly, this *in vitro* effect was equivalent in clinically glucocorticoid-resistant and glucocorticoid-sensitive asthmatic patients, with greater than 80% inhibition of the T_H17 response in both patient cohorts. The data support a potential role for the ectonucleotidase CD39 in 1,25(OH)$_2$D3-mediated inhibition of the IL-17A response. Together, our data are consistent with the hypothesis that 1,25(OH)$_2$D3, through inhibition of IL-17A, improves disease control in asthmatic patients independently of their glucocorticoid responsiveness.

The present study extends earlier observations showing an association between increased IL-17A production and severe asthma by investigating 2 asthmatic patient cohorts with clearly defined clinical responsiveness or nonresponsiveness to steroids at the time of study but comparable disease severity. Although blood T cells from all asthmatic patients demonstrated increased IL-17A and IL-22 synthesis, the most striking differences from the control group were observed in the patients with SR asthma. Analysis of cytokine production from patients with SS asthma alone demonstrated a nonsignificant trend for enhanced IL-17A and IL-22 production in comparison with that seen in healthy control subjects. In comparisons between patients with SS asthma versus patients with SR asthma, the most notable differences were in the frequency of the levels of secreted IL-17A and IL-17A$^+$IL-22$^+$ double-positive cells. Double-positive conventional T cells are proposed to represent the more pathogenic population, with studies showing that IL-22 enhances the proinflammatory properties of IL-17A.[32,41]

Independent studies have suggested that T_H17 cytokine production plays a mechanistic role in increasing asthma severity and reducing corticosteroid sensitivity.[22,42] Nevertheless, we cannot completely exclude from the present data the possibility that increased IL-17A production is associated with inhaled corticosteroid administration. Although dexamethasone increased the frequency of IL-17A$^+$ cells in both nonasthmatic and asthmatic cultures, the levels secreted were only significant in nonasthmatic cultures, although a trend for an increase was observed ($P = .102$). A major difference between these 2 subject groups was the baseline level of IL-17A synthesis, which was more than 7-fold lower in the nonasthmatic cultures compared with the asthmatic cultures and might contribute to the capacity to visualize the enhancement by dexamethasone. Furthermore, in a retrospective analysis of our data, we observed an association between inhaled corticosteroid dosages (beclomethasone equivalent before the trial of oral

prednisolone therapy) and the amounts of blood PBMC IL-17A released *in vitro* (Pearson correlation $r = 0.459$, $P = .014$), supporting the possibility that at least some of the IL-17A produced by PBMCs in asthmatic patients results from corticosteroid exposure.

Our data are also broadly complementary of studies in an animal model, demonstrating that IL-17A production by PBMCs was not inhibited by dexamethasone in culture and that airways inflammation induced in mice by adoptive transfer of T_H17 cells was not inhibited by dexamethasone, which is in contrast to that mediated by the adoptive transfer of T_H2 cells.[22] IL-17A might contribute to asthma pathogenesis through a number of different mechanisms, such as induction of neutrophilia and stimulation of lung innate and structural cells, to secrete proinflammatory cytokines.[15,43,44] Also worthy of note is the association of IL-17A with subepithelial fibrosis, a feature of airway remodeling shown in some studies to correlate with asthma severity.[45]

IL-17A has been proposed to affect global corticosteroid responsiveness through effects on the GR. GR-α is ubiquitously expressed and responsible for the induction and repression of target genes, whereas GR-β is expressed at much lower levels and is a dominant negative inhibitor of GR-α. Increased expression of GR-β has been linked to severe asthma in some studies,[42,46] and a recent study suggested that IL-17A and IL-17F increase the expression of GR-β, an effect that was more prominent in asthmatic patients than in healthy control subjects.[23] These data imply a complex and potentially detrimental relationship between IL-17A, steroid treatment, and responsiveness in patients with severe asthma, on which vitamin D might have a positive effect.

We investigated a possible mechanism for the inhibition of T-cell IL-17A synthesis by $1,25(OH)_2D3$. The ectonucleotidase CD39, which is expressed by memory T cells, as well as $Foxp3^+$ Treg cells, has previously been reported to inhibit IL-17A synthesis.[36,39,40] In the present study treatment with $1,25(OH)_2D3$ increased the mean intensity of CD39 expression by human $CD4^+$ T cells, whereas the CD39 antagonist POM-1 partially abrogated $1,25(OH)_2D3$-mediated inhibition of the IL-17A production, supporting a mechanistic role for this pathway but not excluding others. In contrast, dexamethasone tended to reduce CD39 expression.

Manipulation of vitamin D status for therapeutic benefit in asthmatic patients and patients with other respiratory conditions is currently highly topical. This arises in part from studies demonstrating that patients with poorly controlled asthma, poor responsiveness to corticosteroids, or both have significantly reduced vitamin D levels, at least as assessed by the circulating precursor 25-hydroxyvitamin D3.[27-30,47] We and others have previously demonstrated an association with $25(OH)_2D3$ status and airways smooth muscle mass.[27,28,48] Bronchial smooth muscle hypertrophy and hyperplasia are prominent features of airway remodeling, as is subepithelial fibrosis, and IL-17A is a key mediator implicated in lung structural changes with profibrotic activity within the bronchial epithelium.[45] Understanding the various mechanisms through which vitamin D controls respiratory health and steroid responsiveness is central in targeting this pathway therapeutically, and our results provide support for an additional beneficial effect. We believe that evidence of $1,25(OH)_2D3$ downregulating proinflammatory cytokines, such as IL-17A and IL-22, and its capacity to enhance antimicrobial pathways, Treg cells, and other homeostatic mechanisms, such as CD200,[49] are all likely to contribute to promoting homeostasis in the airway and lung health.

Key messages

- **PBMC cultures from steroid refractory asthma patients synthesize significantly higher levels of IL-17A than steroid sensitive patients and nonasthmatic control cultures.**
- **Glucocorticoids do not inhibit IL-17A synthesis in patient cultures and enhance synthesis in nonasthmatic cultures.**
- **$1,25(OH)_2D3$ inhibits the IL-17A response in a glucocorticoid-independent manner, suggesting additional benefits of vitamin D to control severe asthma.**

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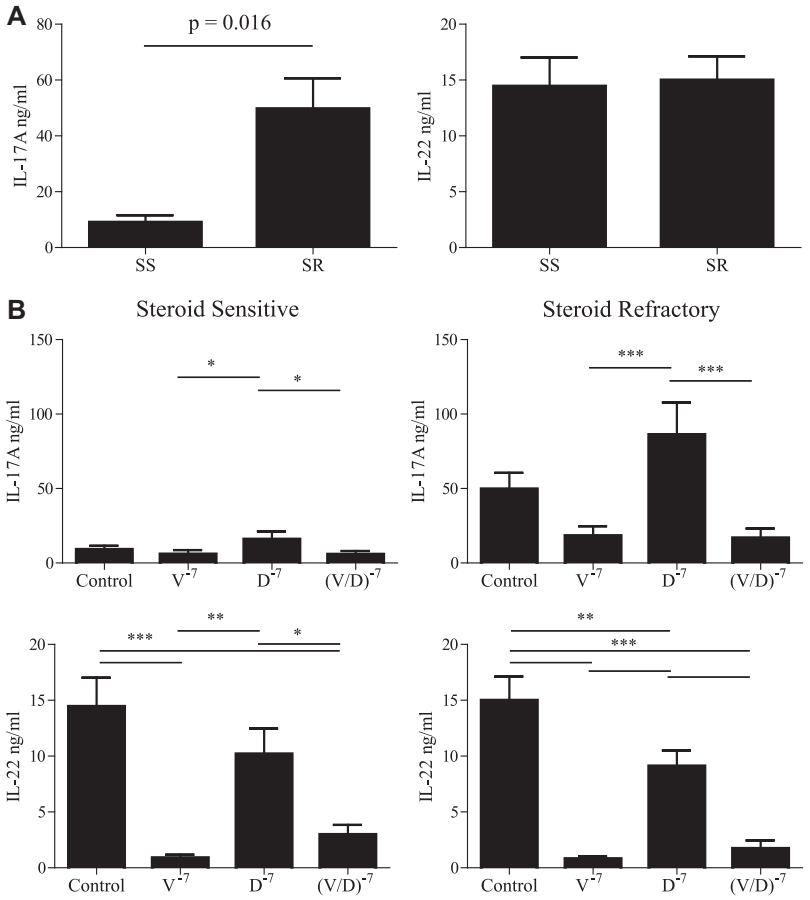


FIG E1. 1,25(OH)$_2$D3 inhibits T_H17-associated cytokines in patients with SS severe asthma and patients with SR severe asthma. **A,** Cumulative data showing higher IL-17A expression in patients with SR asthma and comparable IL-22 levels in cultures from patients with SS asthma and patients with SR asthma (SS asthma, n = 10; asthma, n = 18). **B,** Secreted IL-17A and IL-22 from cultures of patients with SS asthma *(left)* and patients with SR asthma *(right)* containing dexamethasone and 1,25(OH)$_2$D3 (SS asthma, n = 10; SR asthma, n = 18). **P* < .05, ***P* < .01, and ****P* < .0001; Fig E1, *A*, unpaired *t* test, and Fig E1, *B*, 1-way ANOVA with the Tukey post test. D^{-x} and V^{-x}, Dexamethasone and 1,25(OH)$_2$D3, respectively, at 10^{-7} mol/L.

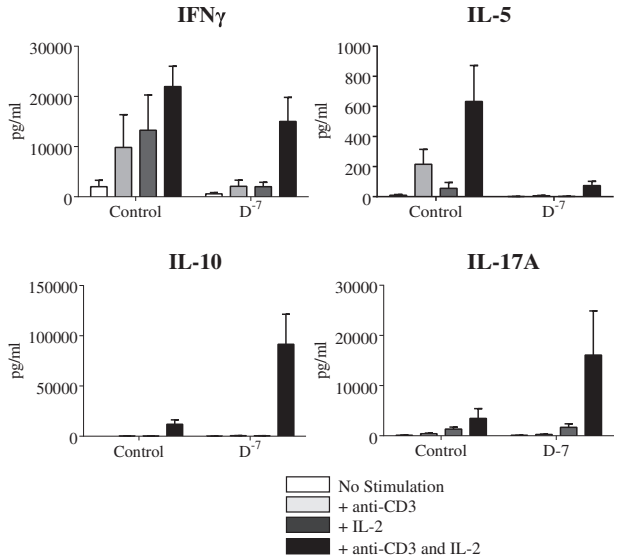


FIG E2. Effect of IL-2 in culture on dexamethasone modulation of IL-10, IFN-γ, and IL-5. Levels of secreted IFN-γ, IL-10, IL-13, IL-5, and IL-17A under different stimulation conditions in healthy control subjects are shown (n = 6). D^x, Dexamethasone at 10^{-7} mol/L.

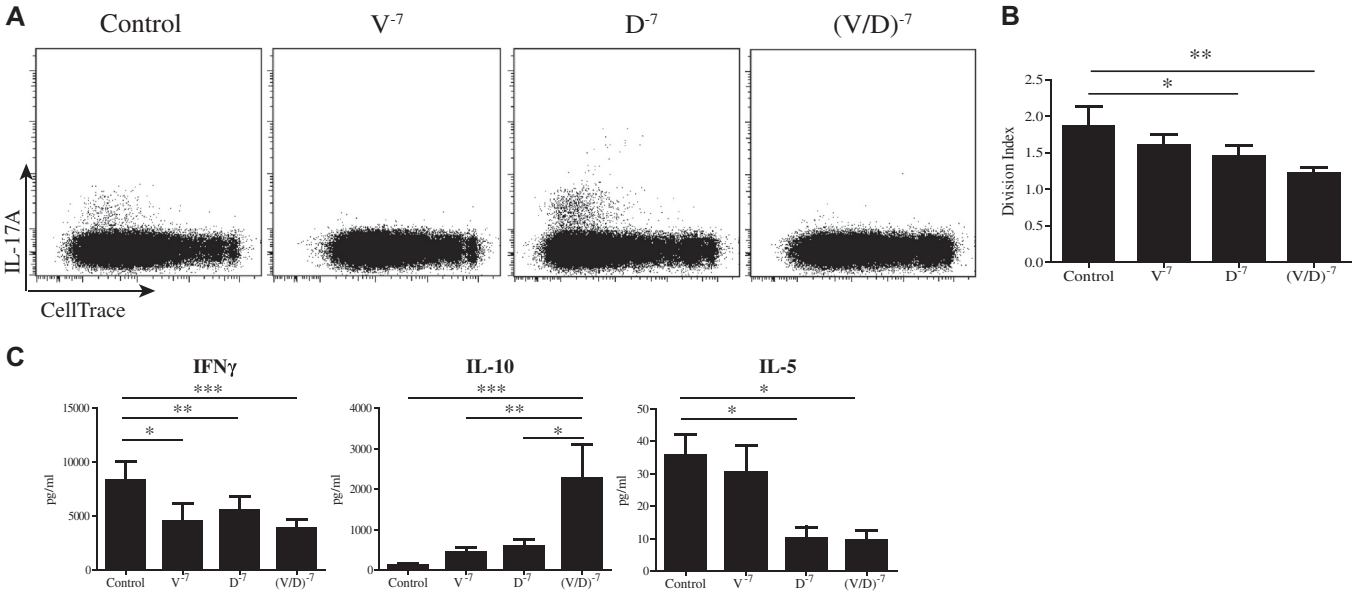


FIG E3. Dexamethasone, but not 1,25(OH)$_2$D3, inhibits PBMC proliferation. **A,** Representative dot plots. **B,** Cumulative data showing proliferation of PBMCs in the presence or absence of dexamethasone and 1,25(OH)$_2$D3 in healthy control subjects (n = 4). **C,** Cumulative data cytokine secretion data of IFN-γ, IL-10, and IL-5 secretion in healthy donors (n = 8). **P* < .05, ***P* < .01, and ****P* < .001; Fig E3, *B* and *C*, 1-way ANOVA with the Tukey post test. D^x and V^x, Dexamethasone and 1,25(OH)$_2$D3, respectively, at 10^{-x} mol/L.