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

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Non-standard abbreviations:

1 α,25-dihydroxyvitamin D3 (1,25$(OH)_2$D3); 25-hydroxyvitaminD, 25(OH)D; Steroid Sensitive, SS; Steroid Resistant, SR.

Key Words:

Asthma; Vitamin D; IL-17A; Steroid Resistant; Steroid Sensitive; Th17; Glucocorticoids

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Summary:

PBMC cultures from asthma patients synthesised high levels of IL-17A compared to non-asthmatic controls, most significantly in steroid refractory patients. Glucocorticoids could enhance IL-17A, but 1,25$(OH)_2$D3 inhibited this response in a glucocorticoid-independent manner.

Abstract

Background: Th17 cells are proposed to play a role in the pathology of asthma, including steroid resistant disease. We previously identified a steroid-enhancing function of vitamin D in steroid resistant asthma, in restoring the impaired response to steroids for production of the anti-inflammatory cytokine IL-10.

Objective: To investigate the production of the Th17-associated cytokines IL-17A and IL-22 in culture in moderate-severe asthma patients 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, $1,25(OH)_2D3$.

Methods: PBMC were stimulated in culture with or without dexamethasone and $1,25(OH)_2D3$. Cytometric bead array, ELISA and intracellular cytokine staining were used to assess cytokine production. The role of CD39 in inhibition of the Th17 response was studied by qRT-PCR, flow cytometry and addition of the antagonist, POM-1, to culture.

Results: Asthma patients synthesised much higher levels of IL-17A and IL-22 than non-asthmatic control subjects, with steroid resistant asthmatics 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. An antagonist of the ectonucleotidase CD39 reversed $1,25(OH)_2D3$ mediated inhibition of the IL-17A response.

Conclusion: Severe asthmatics exhibit elevated levels of Th17 cytokines, which is not inhibited by steroids. $1,25(OH)_2D3$ inhibits Th17 cytokine production in all patients studied, irrespective of their clinical responsiveness to steroids, identifying novel steroid-enhancing properties of vitamin D in asthma.

Introduction

Asthma is broadly defined as bronchial hyperresponsiveness with reversible expiratory airflow limitation. Historically asthma has been described as having an underlying Th2-mediated inflammatory profile, however there is increasing evidence for heterogeneity in asthma phenotypes with a significant proportion of asthma patients demonstrating a low or non-Th2 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 (reviewed in (7-10)), including genetic susceptibility, defects in glucocorticoid receptor (GR) binding, increased expression of the functionally inactive GR-β, activation of transcription factors such as AP-1 (activator protein 1) or decreased synthesis of immunoregulatory cytokines like 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, difficult to control disease.

Th17 cells are critical for defence 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).

A body of circumstantial evidence consistent with the hypothesis that IL-17A exacerbates asthma and/or reduces patients' responsiveness to therapy already exists. Thus IL-17A expression in sputum or bronchial mucosal biopsies has been correlated with bronchial hyperresponsiveness (16),

granulocyte infiltration (17) and production of fibrogenic mediators by bronchial fibroblasts (18). Furthermore, elevation of serum IL-17A concentrations appears to be both a marker (19) and an independent risk factor for severe asthma (16-18, 20, 21). Involvement of Th17 cells in severe steroid resistant asthma is proposed from murine and human studies (22, 23). In addition to IL-17A, IL-22 is commonly produced by Th17 cells. In various animal models of disease IL-22 can elicit damaging or protective responses possibly dependent on the location, cellular sources, 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 immunological basis of this relationship, we have shown that 1 α,25-dihydroxyvitamin D3 ($1,25(OH)_2D3$) exerts immunomodulatory effects on T cells from patients with SR asthma patients, enhancing the impaired steroid-induced IL-10 response in these individuals (31), while others have highlighted the capacity of this molecule to inhibit Th17 responses (32-35).

Building on these studies we hypothesised that $1,25(OH)_2D3$ inhibits elevated IL-17A production by blood Th17 cells from severe asthmatics independently of glucorticoids *in vitro* and independently of their clinical responsiveness to glucorticoid therapy *in vivo*. To address the hypothesis we studied the effects of $1,25(OH)_2D3$ and the glucocorticoid dexamethasone on the production of IL-17A and its common co-product IL-22, by peripheral blood CD4+ T cells from a cohort of severe asthmatics, carefully characterised for their clinical glucocorticoid responsiveness, and non-asthmatic controls. 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, elevated expression of which has previously been reported to be associated with inhibition of IL-17A production in autoimmune disease (36). We hypothesised 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.

Materials and Methods

Subjects

Healthy adults (study approved by the local research ethics committee, 09/H0804/77) and asthma patients (08/H0804/84) were recruited. All healthy controls and patients gave written informed consent. Asthma patients had moderate to severe asthma for at least 6 months on therapy step 3 or 4 of the British Thoracic Society (BTS) guidelines on management of asthma, diagnosed by a specialist physician. The average inhaled corticosteroid dose in beclomethasone diproprionate equivalent was 1177mcg for SR patients and 1180mcg for SS patients. All patients had a pre-bronchodilator FEV_1 of < 80% predicted, documented airway variability of > 12% following 400mcg of short-acting bronchodilator and had undergone detailed assessment to exclude a diagnosis other than asthma and co-morbidities affecting asthma control. Patients were not on oral corticosteroids for 4 weeks prior to the study. Steroid sensitivity was defined by an increase in FEV_1 > 10% from baseline following a 2 week course of Prednisolone of 40mg/1.73m^2 body surface area. Steroid resistance was defined by a <10% increase in FEV_1 from baseline following a 2 week course of Prednisolone of 40mg/1.73m^2 body surface area. Compliance with Prednisolone during the study was assessed with Cortisol serum levels pre- and post-steroids. Patients on immunotherapy, smokers or patients who suffered from a respiratory tract infection or asthma exacerbation during or 4 weeks prior to enrolling for the study were excluded. 18 steroid resistant (SR) and 10 steroid sensitive (SS) patients were assessed with a mean age of 54 (SR) and 50 years (SS). Mean pre-bronchodilator FEV_1 before and after a course of oral corticosteroids was 1.99L (64.56%) and 1.96L (64.05%) in the SR patients and 1.72 (57.1%) and 2.19L (73.2%) in the SS patients (post steroid FEV_1 SR vs SS $p<0.001$, ANCOVA), 10 healthy controls were assessed for comparison, mean age 41.

Cell isolation and Culture

Human PBMC were isolated as previously described (31). Briefly, CD8-depleted PBMCs were obtained by negative selection using CD8+ Dynalbeads (Invitrogen, Paisley, U.K.). CD4+ T cells were purified by positive selection using Dynalbeads. Cells ($1x10^6$ cells/ml) were cultured in RPMI

(containing 10% FCS, 2mM L-glutamine and 50 μg/ml gentamycin), and stimulated with plate-bound anti-CD3 (1μg/ml; OKT-3) plus 50U/ml recombinant hIL-2 (Eurocetus, Harefield U.K.), in the presence or absence of Dexamethasone (Sigma-Aldrich, Gillingham, U.K.) and 1 α,25-dihydroxyvitamin D3 ($1,25(OH)_2D3$; BIOMOL Research Labs, Exeter U.K.) 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 re-cultured at the same density of $1x10^6$/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, U.K.), and CD39 (ebioA1; eBiosciences, Hatfield, U.K.). For *ex vivo* staining, red blood cells were lysed using Facs Lysing solution (working dilution 1:10; BD Biosciences), then were further stained for intranuclear Foxp3 (PCH101; ebiosciences) using the Foxp3 staining kit as per the manufacturer's instructions (ebiosciences).

For intracellular cytokine staining on day 7, cells were restimulated for 4 hours with 5 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml ionomycin, with 2 μM monensin (Sigma-Aldrich) added for the final 2 hours. Cells were washed, fixed, and permeabilised using Cytofix/Cytoperm kit (BD Biosciences) and were stained with fluorescently labeled monoclonal antibodies to IL-22 and IL-17A (22URTI and eBio64CAP17; ebiosciences). Dead cells (7-aminoactinomycin D [7-AAD] positive; Sigma-Aldrich) were gated out. Analysis was performed using a FACSCalibur flow cytometer (BD Biosciences).

qRT-PCR

RNA was extracted from cell pellets using the RNeasy Mini kit (Qiagen, Crawley U.K.) according to the manufacturer's instructions. The RNA was quantified using Nanodrop ND-1000 spectrophotometer (ThermoScientific, Wilmington U.S.A.) 250ng of RNA was reverse transcribed

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

Cytokine Analysis

IL-22 was measured using ELISA and matched antibody pairs (BD Biosciences). The lower limit of detection for IL-22 was 100 pg/ml. IL-17A, IL-10, IFNγ and IL-5 were measured by Cytometric Bead Array (BD Biosciences) according to the manufacturers protocol, the lower limit of detection was 1.5 pg/ml.

Statistics

Data are presented as the mean ± standard error of the mean (SEM), unless indicated. Data were assessed for equivalence to a Gaussian distribution and equality variance after which the appropriate parametric or non-parametric 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 Steroid Sensitive (SS) and Refractory (SR) asthma patients

Details of the two cohorts of moderate to severe asthma patients, defined as SS or SR on the basis of changes in their lung function following a 2-week course of oral prednisolone, and of healthy non-asthmatic control subjects studied, are provided in Materials and Methods. Notably these two 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 (BDP) equivalence).

PBMCs from the asthma patients were evaluated for their capacity to synthesise the Th17-associated cytokines IL-17A and IL-22 in culture, and comparisons made with control subjects. PBMCs depleted of CD8+ T cells were stimulated *in vitro* with anti-CD3 and IL-2 for seven days and then stained for intracellular cytokine expression. Significantly greater percentages of IL-17A+ and IL-22+ expressing cells were observed in cultures from asthma patients, as compared to healthy control subjects (Figure 1A & B). When patients were subdivided based on clinical responsiveness to glucocorticoids only SR patients showed a significant difference in IL-17A immunoreactivity (SR $p=0.003$; SS $p=0.142$) and IL-22 immunoreactivity (SR $p=0.037$; SS $p=0.701$), compared to control subjects (Figure 1B). Cells co-expressing IL-17A and IL-22, are proposed to represent the most pathogenic population, and were significantly increased in all asthmatics and SR asthmatics compared to healthy control subjects (all asthmatics $p=0.029$; SR $p=0.009$). At seven days cells were also harvested and re-cultured at equal cell densities for 48h with anti-CD3 and IL-2 alone. Culture supernatants from asthma patients contained significantly higher quantities of secreted IL-17A and IL-22 as compared to healthy controls (Figure 1C). Production of IL-17A and IL-22 was significantly increased in SR compared to controls (IL-17A: $p=0.009$; IL-22: $p=0.036$). Supernatants from SR had significantly more IL-17A as compared to SS ($p=0.016$), whereas culture supernatants from SS and SR patients contained comparable levels of IL-22 (Supplementary Figure 1A).

Glucocorticoids fail to 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}M dexamethasone to cultures from healthy controls significantly increased the mean percentage of cells expressing IL-17A, but did not alter the low percentage of cells expressing IL-22 (Figure 2A). IL-17A and IL-22 secretion were increased in a concentration-dependent manner by dexamethasone in cultures from healthy donors (Figures 2B, 2C). 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). The effects of dexamethasone were therefore assessed in the presence or absence of anti-CD3 and/or IL-2 on IL-17A and a range of additional cytokines (Supplementary Figure 2). 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 IFNγ, IL-13, and IL-5 although the inhibition of IFNγ in particular was less marked when anti CD3 plus IL-2, as compared to anti CD3 or IL-2 alone, was present in these cultures.

The effects of dexamethasone in asthmatic patient cultures was less marked, where notably there was a more than sevenfold higher basal level of IL-17A synthesis compared to non-asthmatic cultures (Figure 2B). A trend towards increased IL-17A secretion (p=0.102; Fig 2B), and a significant increase in the frequency of IL-17A positive cells in patient cultures containing dexamethasone 10^{-7}M was observed (Figure 3A). An effect observed in both SS and SR subgroups was partial inhibition by dexamethasone of IL-22 secretion (Figure 2C and Supplementary Figure 1).

1 α,25-dihydroxyvitamin D 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 immunoreactive for IL-17A or IL-22 in culture (Figure 3A, 3B). Although dexamethasone failed to reduce IL-22 or IL-17A immunoreactivity compared to

control cultures, the combination of dexamethasone and 1,25$(OH)_2$D3 led to a marked reduction in the frequency of cytokine positive cells that was not significantly different from cultures with 1,25$(OH)_2$D3 alone (Figure 3A, 3B). Protein expression in culture supernatants following 1,25$(OH)_2$D3 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$(OH)_2$D3, or 1,25$(OH)_2$D3 plus dexamethasone (Figure 3B, 3C). The effects of 1,25$(OH)_2$D3 on IL-17A and IL-22 synthesis were comparable in the SS and SR asthmatics (Supplementary Figure 1B). The reduction of IL-17A production by 1,25$(OH)_2$D3 is unlikely to be explained by inhibition of proliferation (Supplementary Figure 3) or decreased viability (data not shown) as 1,25$(OH)_2$D3 at the concentration used did not inhibit either as we recently reported (38). These data highlight the capacity of 1,25$(OH)_2$D3 to counteract any detrimental or lack of effect of dexamethasone on Th17-associated cytokine synthesis in cell cultures from asthma 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+ T regulatory cells (Treg) 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 as compared to severe asthmatics ($p=0.003$). CD39 can be expressed on both CD4+ memory and Foxp3+ T cells (36, 39), so we further analysed 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 non-asthmatic donors as compared to the severe asthmatics (Figure 4A), in contrast to the higher IL-17A synthesis observed in the patients.

The impact of $1,25(OH)_2D3$ and dexamethasone on CD39 expression, was next investigated. CD4+ T cells cultured in the presence of $1,25(OH)_2D3$ demonstrated a significantly increased gene expression of CD39 (Figure 4B). When surface expression of CD39 was assessed by flow cytometry there was a modest but significant increase in the level of expression of CD39 as determined by Mean Fluorescence Intensity (MFI), 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 MFI (Figure 4C and D). To investigate the role of CD39 in $1,25(OH)_2D3$ inhibition of IL-17A production we employed POM-1, a known inhibitor of CD39 ectonucleotidase activity. POM-1 partially abrogated the inhibition of IL-17A synthesis effected by $1,25(OH)_2D3$ in cultured T cells. This effect was specific for IL-17A since the production of IL-10 and other Th1 and Th2 cytokines was not affected (Figure 4E).

Discussion

Our data demonstrate that the production of IL-17A and IL-22 by human peripheral blood CD4+ T cells is elevated in severe asthma. Notably, IL-17A, but not IL-22 was approximately 5-fold greater in SR as compared to SS asthmatics. Strikingly the glucocorticoid dexamethasone significantly enhanced the frequency of IL-17A positive cells in culture in both non-asthmatic control and asthmatic subjects, although IL-17A secretion was only significantly increased by dexamethasone in control cultures. In contrast the active form of vitamin D reduced both cytokines directly and also when dexamethasone was present in culture. Importantly this *in vitro* effect was equivalent in clinically glucocorticoid resistant and sensitive asthma patients, with greater than 80% inhibition of the Th17 response in both patient cohorts. The data support a potential role for the ectonucleotidase CD39 in $1,25(OH)_2D3$ mediated inhibition of the IL-17A response. Together our data are consistent with the hypothesis that $1,25(OH)_2D3$, through inhibition of IL-17A, improves disease control in asthmatics independently of their glucocorticoid responsiveness.

The present study extends earlier observations showing an association between elevated IL-17A production and severe asthma by investigating two asthma patient cohorts with clearly defined clinical responsiveness or non-responsiveness to steroids at the time of study, but comparable disease severity. While blood T cells from all asthmatics demonstrated elevated IL-17A and IL-22 synthesis, the most striking differences from the control group were observed in the SR asthma patients. Analysis of cytokine production from SS patients alone demonstrated a non-significant trend for enhanced IL-17A and IL-22 production in comparison to healthy control subjects. In comparisons between SS versus SR asthmatics, 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 pro-inflammatory properties of IL-17A (32, 41).

Independent studies have suggested that Th17 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 elevated IL-17A production is associated with inhaled corticosteroid administration. Although dexamethasone increased the frequency of IL-17A positive cells in both non-asthmatic and asthmatic cultures, the levels secreted were only significant in non-asthmatic cultures, although a trend for increase was observed ($p=0.102$). A major difference between these two subject groups was the baseline level of IL-17A synthesis, which was more than 7-fold lower in the non-asthmatic cultures as compared to the asthmatic cultures, and may contribute to the capacity to visualise the enhancement by dexamethasone. Furthermore, in a retrospective analysis of our data we observed an association between inhaled corticosteroid dosages (beclometasone equivalent prior to the trial of oral prednisolone therapy) and the amounts of blood PBMC IL-17A released *in vitro* (Pearson correlation $r=0.459$; $p=0.014$), supporting the possibility that at least some of the IL-17A produced by PBMCs in asthmatics 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 Th17 cells was not inhibited by dexamethasone, in contrast to that mediated by the adoptive transfer of Th2 cells (22). IL-17A may 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 remodelling shown in some studies to correlate with asthma severity (45).

IL-17A has been proposed to affect global corticosteroid responsiveness through effects on the glucocorticoid receptor (GR). GR-α is ubiquitously expressed and is responsible for the induction and

repression of target genes, while GR-ß is expressed at much lower levels and is a dominant negative inhibitor of GR-α. Increased expression of GR-ß has been linked with severe asthma in some studies (42, 46), and a recent study suggested that IL-17A and IL-17F increase the expression of GR-ß, an effect that was more prominent in asthmatics than in healthy controls (23). These data imply a complex and potentially detrimental relationship between IL-17A, steroid treatment and responsiveness in severe asthma, on which vitamin D may have a positive impact.

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+ regulatory T 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, while 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 asthma and other respiratory conditions is currently highly topical. This arises in part from studies demonstrating that patients with poorly controlled asthma and/or poor responsiveness to corticosteroid have significantly reduced vitamin D, 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 remodelling as is subepithelial fibrosis, and IL-17A is a key mediator implicated in lung structural changes with pro-fibrotic activity within the bronchial epithelium (45). Understanding the various mechanisms by 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 $1,25(OH)_2D3$ down regulating pro-inflammatory cytokines such as IL-17A and IL-22, its capacity to enhance anti-microbial pathways, regulatory T cells and other homeostatic mechanisms such as CD200 (49), are all likely to contribute to promoting homeostasis in the airway and lung health.

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Figure 1: Severe asthma patients express higher levels of IL-17A and IL-22

A-C. Control and asthmatic cultures were assessed for IL-17A and IL-22 production by intracellular flow cytometry and antibody capture assay. **A,** Representative dot plots. **B,** Cumulative data of cytokine positive cells (healthy n=8; SS n=8 and SR n=14) and **C,** secreted cytokines (Healthy n=10; SS n=10 and SR n=14). Analysis by students' t-test, * $p<0.05$; ** $p<0.001$. Open circles SS; closed squares SR; and closed triangles healthy controls.

Figure 2: Dexamethasone does not inhibit IL-17A production.

A, Cumulative data of effects dexamethasone on the frequency of IL-17A and IL-22 positive cells in non-asthmatic cultures (Healthy n=8), and **B-C,** IL-17A and IL-22 secretion in control (left) and asthmatic cultures (right) (Healthy n=10; Asthmatic n=28). **A,** Paired t-test and **B-C,** one-way ANOVA with Tukeys post test. * $p<0.05$; ** $p<0.01$; *** $p<0.001$. D^x, Dexamethasone at 10^{-x}M.

Figure 3: $1,25(OH)_2D3$ inhibits IL-17A and IL-22 in asthmatic patient cultures.

A, Representative dot plots, **B,** Cumulative % cytokine positive cells (n=22) or cytokine secretion (n=28). $1,25(OH)_2D3$ reduced IL-17A and IL-22 in asthmatic cultures with or without dexamethasone. Statistical significance by one-way ANOVA with Tukeys post test. ** $p<0.01$; *** $p<0.001$ ns = non-significant. D^x, V^x, Dexamethasone and $1,25(OH)_2D3$ respectively, at 10^{-x}M.

Figure 4: $1,25(OH)_2D3$ increases CD39 expression by CD4+ T cells.

A, The frequency of CD39+ (i) CD3+ T cells (ii) Foxp3+ and Foxp3- T cells in the peripheral blood of Healthy donors (n=8) and Severe asthma patients (n=19). **B-D**, Effects of treatment with dexamethasone or 1,25$(OH)_2$D3 in culture on healthy non-asthmatic donors **B,** Relative CD39 mRNA (n=5), **C,** Representative histograms, and **D,** Cumulative data CD39 expression (n=6). **E,** cumulative data (n=8) effects POM-1 (10µM; CD39 antagonist) on 1,25$(OH)_2$D3-modulation of IL-17A, IFNγ, IL-10 and IL-5, expressed as fold change from No drug condition. **B and D** one-way ANOVA with Tukeys post test, **E,** Wilcox-matched pairs signed rank test. ** $p<0.01$; *** $p<0.001$.

Supplementary Figure 1. 1,25$(OH)_2$D3 inhibits Th17-associated cytokines in SS and SR severe asthmatics.

A, Cumulative data showing higher IL-17A expression in SR, and comparable IL-22 in SS and SR cultures (SS n=10; SR n=18). **B,** Secreted IL-17A and IL-22 from SS (left) and SR (right) cultures containing dexamethasone and 1,25$(OH)_2$D3 (SS n=10; SR n=18). **A,** Unpaired t-test and **B,** one-way ANOVA with Tukeys post test. * $p<0.05$; ** $p<0.01$; *** $p<0.0001$. D^x, V^x Dexamethasone and 1,25$(OH)_2$D3 respectively, at 10^{-7}M.

Supplementary Figure 2. The effect of IL-2 in culture on Dexamethasone modulation of IL-10, IFNγ and IL-5.

A, Secreted IFNγ, IL-10, IL-13, IL-5 and IL-17A under different stimulation conditions in healthy controls (n=6). D^x Dexamethasone, at 10^{-7}M.

Supplementary Figure 3. 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 controls (n=4). **C,** Cumulative data cytokine secretion data of IFNγ, IL-10, and IL-5 secretion in healthy donors (n=8). **B, and C,** one-way

ANOVA with Tukeys post test. * $p<0.05$; ** $p<0.01$;. D^x, V^x Dexamethasone and $1,25(OH)_2D3$ respectively, at 10^{-x}M.

Figure 1:

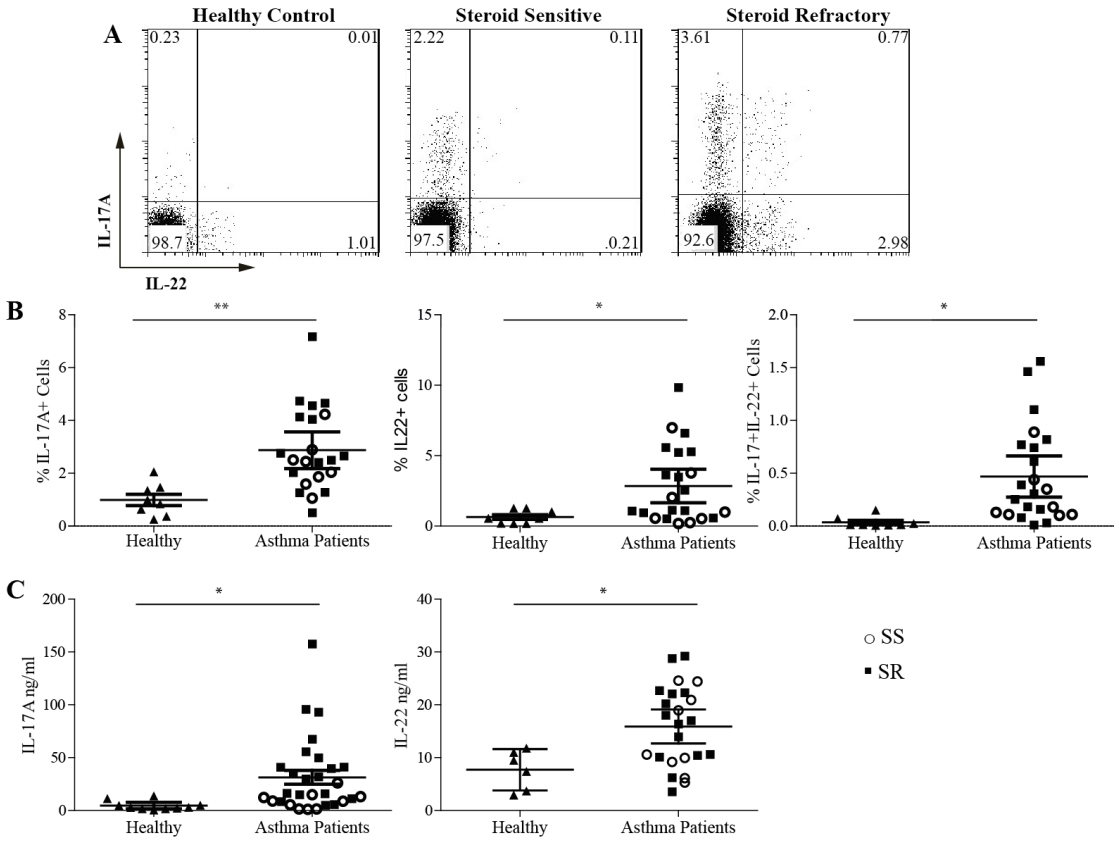

A
Healthy Control
Steroid Sensitive
Steroid Refractory
0.23
0.01
98.7
1.01
2.22
0.11
97.5
.0.21
3.61
0.77
92.6
2.98
IL-17A
IL-22
B
% IL-17A+ Cells
% IL22+ cells
% IL-17+IL-22+ Cells
Healthy
Asthma Patients
C
IL-17A ng/ml
IL-22 ng/ml
SS
SR

Figure 2

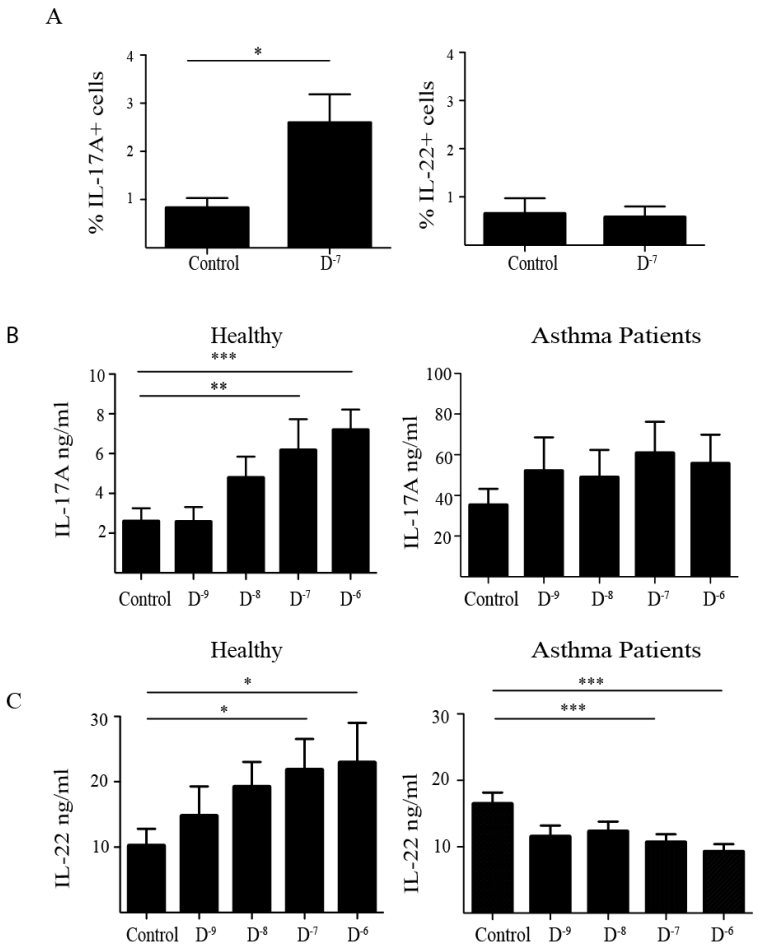

A
% IL-17A+ cells
% IL-22+ cells
Control
D-7
B
Healthy
Asthma Patients
IL-17A ng/ml
Control
D-9
D-8
D-7
D-6
C
IL-22 ng/ml

Figure 3

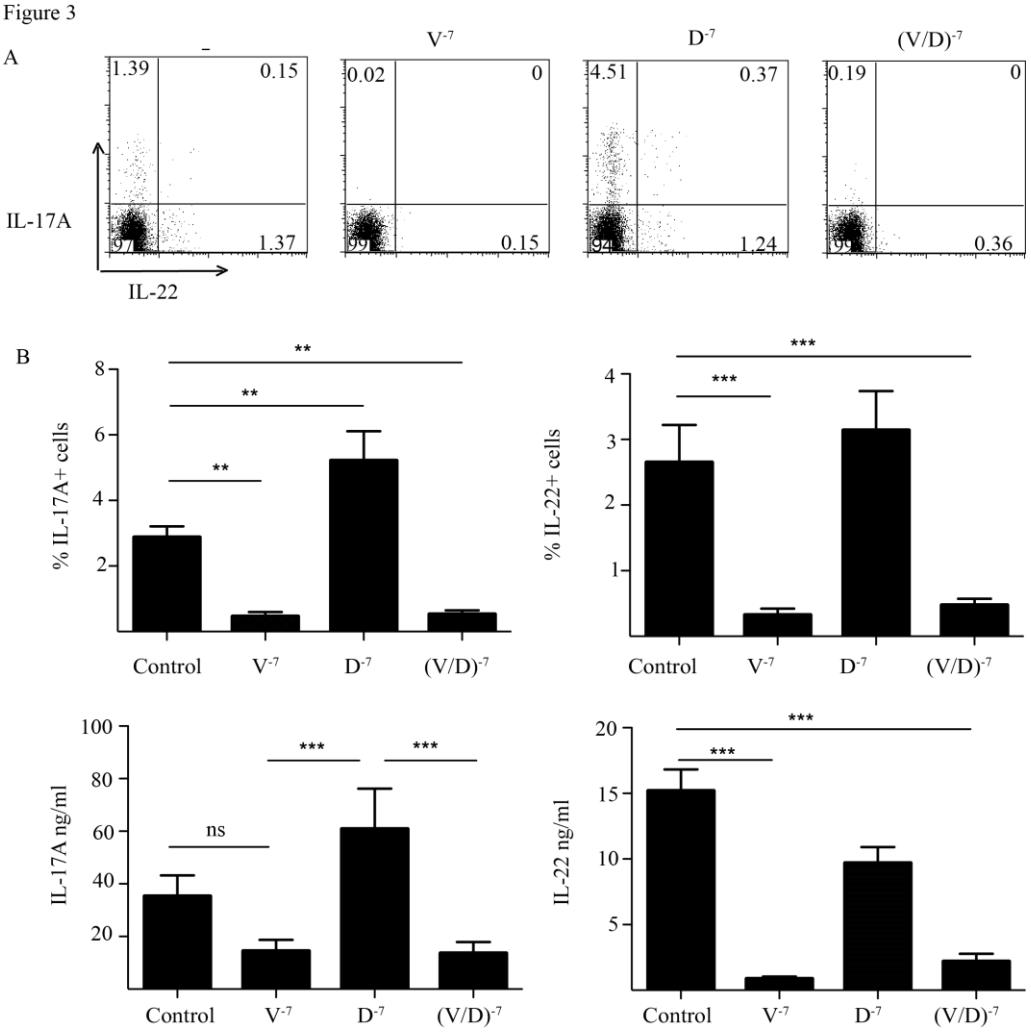
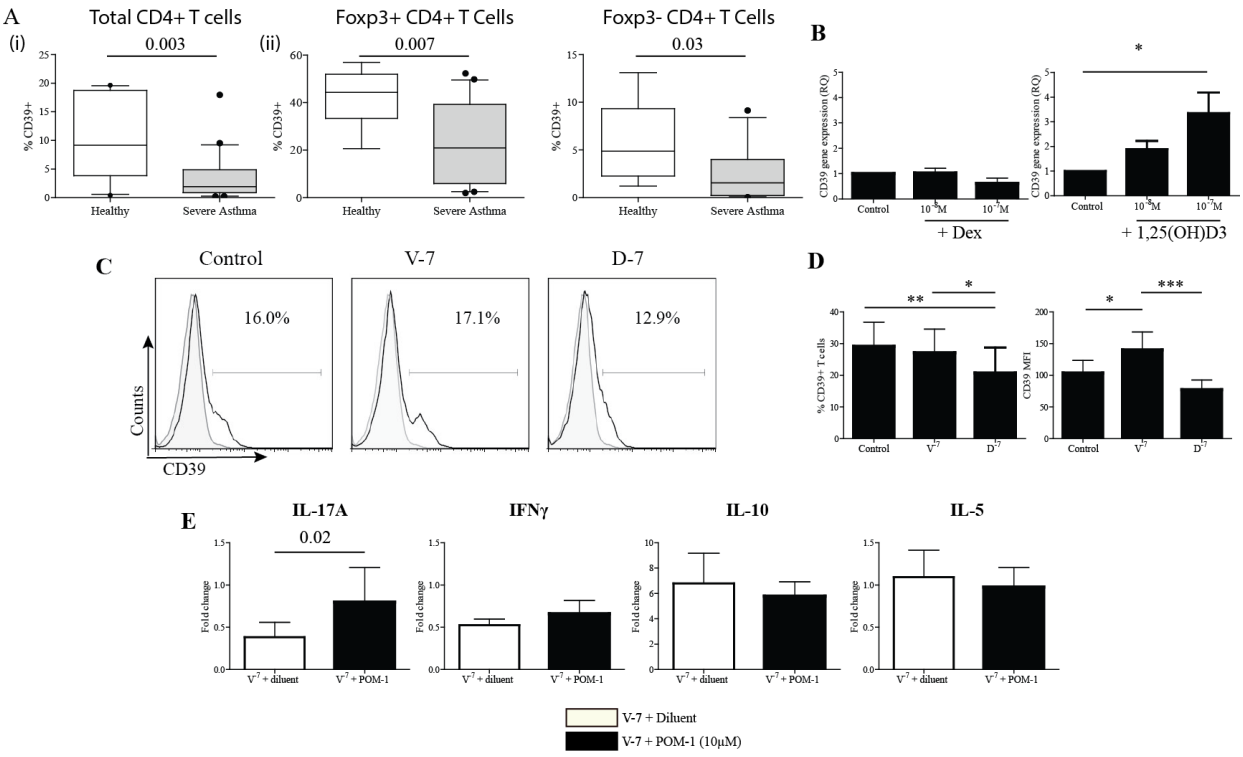
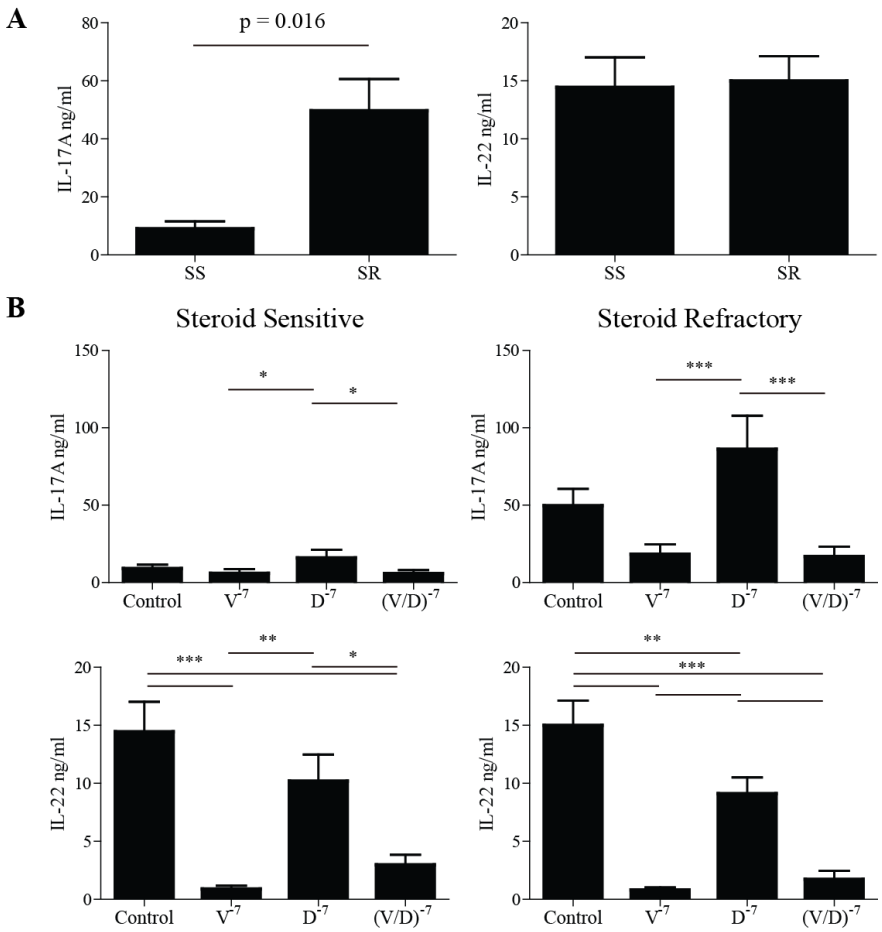

A
–
V-7
D-7
(V/D)-7
1.39
0.15
97
1.37
0.02
0
99
0.15
4.51
0.37
94
1.24
0.19
0
0.36
IL-17A
IL-22
B
% IL-17A+ cells
% IL-22+ cells
IL-17A ng/ml
IL-22 ng/ml
Control
V-7
D-7
(V/D)-7
**
***
ns

Figure 4


A
Total CD4+ T cells
Foxp3+ CD4+ T Cells
Foxp3- CD4+ T Cells
(i)
(ii)
0.003
0.007
0.03
% CD39+
Healthy
Severe Asthma
B
CD39 gene expression (RQ)
Control
+ Dex
+ 1,25(OH)D3
*
C
Control
V-7
D-7
16.0%
17.1%
12.9%
Counts
CD39
D
% CD39+ T cells
CD39 MFI
**
*
***
E
IL-17A
IFNγ
IL-10
IL-5
0.02
Fold change
V-7 + diluent
V-7 + POM-1
V-7 + Diluent
V-7 + POM-1 (10μM)

Supplementary Figure 1:



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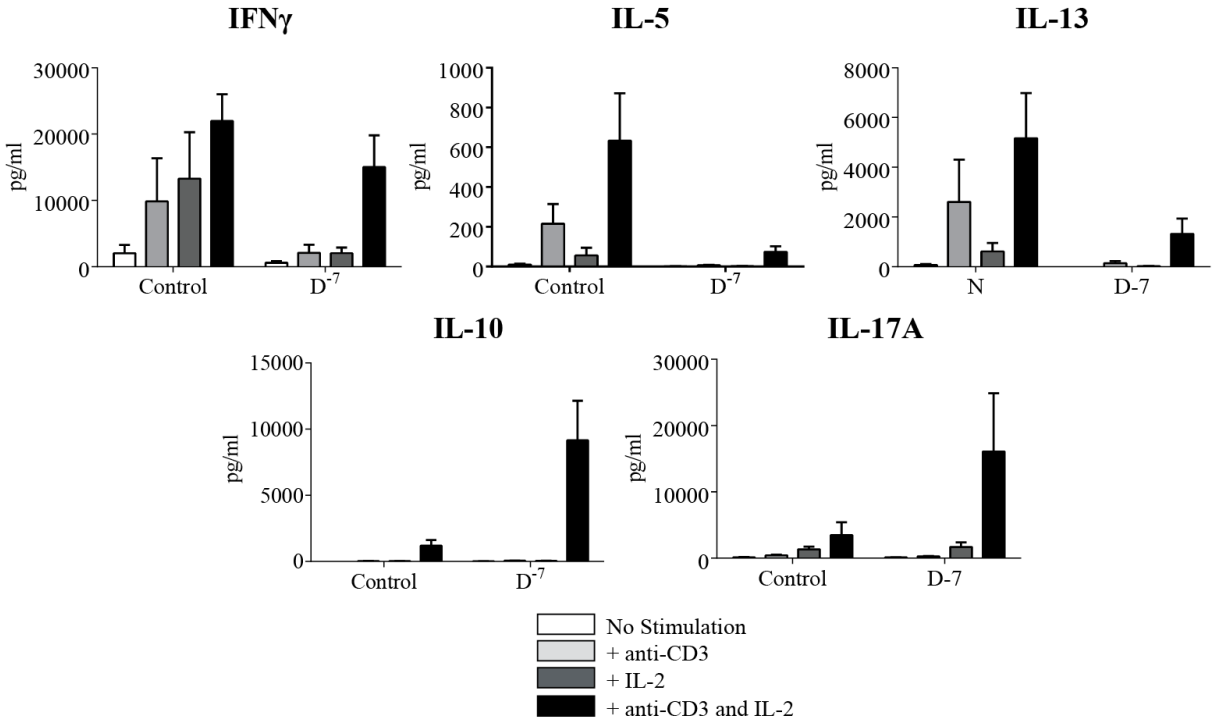

Supplementary Figure 3: