Vitamin D counteracts an IL-23-dependent IL-17A+IFNγ+ response driven by urban particulate matter.

Short title: Vitamin D opposes a UPM-driven Th17 response.

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Abstract word count: 238
Total text word count: 4015

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Acknowledgements: EHM, NCM, PEP and CMH designed the study and wrote the manuscript. EHM and TR performed experiments. IM and FJK provided advice throughout and kindly donated the pollution samples.
Abstract

Particulate matter (PM) air pollution and vitamin D deficiency are associated with poor outcomes in chronic airway diseases. We have previously shown that a reference source of urban PM (UPM) drives the maturation of CD1c+ DCs to promote IL-17A synthesis by memory CD4+ T cells. We now investigate whether UPM-driven IL-17A+ cells exhibit a pro-inflammatory Th17 phenotype, mechanisms for the observed effect and any evidence for cross-regulation by vitamin D. Myeloid (m)DCs were cultured overnight with or without a reference source of UPM and/or active vitamin D (1,25(OH)2D3) before co-culturing with autologous memory CD4+ T cells. Supernatants were harvested for cytokine analysis on day 5 of co-culture and intracellular cytokine staining performed on day 7. UPM-primed DCs increased the proportion of memory CD4+ T cells expressing Th17-associated cytokines IL-17A, IL-17F and IL-22 as well as IFNγ, GM-CSF and granzyme B. Notably, a large proportion of the UPM-driven IL-17A+ cells co-expressed these cytokines, but not IL-10, indicative of a pro-inflammatory Th17 profile. UPM-treated DCs expressed significantly elevated levels of il23 mRNA and increased production of IL-23p40. Neutralisation of IL-23 in co-culture reduced the frequency of IL-17A+IFNγ+ cells implicating a role for UPM-induced IL-23 in promoting pro-inflammatory Th17. 1,25(OH)2D3 inhibited the induction of IL-23 and the frequency of IL-17A+IFNγ+ cells. These data indicate that UPM may negatively impact inflammation by promoting a pro-inflammatory Th17 response. Restoring vitamin D sufficiency may counteract these UPM-driven effects without obliterating important homeostatic immune functions.

Key Words: Air pollution, vitamin D, Th17, IL-23, corticosteroids.
Introduction

Asthma is a complex and heterogeneous disease that is estimated to affect over 300 million people worldwide (1). It is typically well-controlled with β2-receptor agonists and corticosteroids. However 5-10% of asthmatics have particularly poorly controlled severe disease that is frequently associated with a neutrophilic infiltrate alongside elevated levels of IL-17A, the prototypical cytokine of Th17 cells, throughout the airways (2). Chronic obstructive pulmonary disease (COPD), another respiratory disease that is predicted to be the third leading cause of mortality by 2020, is also characterised by elevated levels of IL-17A in the bronchial submucosa and peripheral blood (3). Homeostatically Th17 cells are nevertheless essential for protecting against bacteria and fungi such as *Klebsiella pneumoniae* and *Candida albicans* at mucosal surfaces.

Th17 cells are not a homogenous population, and a subset of pro-inflammatory ‘Th17.1’ cells has been described that drives experimental autoimmune encephalomyelitis in mice (4, 5). Upon T cell receptor stimulation, Th17.1 cells differentiate in the presence of IL-23 to co-express the Th17-associated cytokines IL-17A, IL-17F and IL-22 with the Th1-associated IFNγ, but not the anti-inflammatory mediator IL-10. Using a *Candida albicans* model, Zielinski et al. similarly found that Th17 cell produced both IL-17A and IFNγ upon re-stimulation, but not IL-10 (6). Beyond Th1 and Th17 cytokines, the pathogenicity of IL-23-driven Th17 cells appears to be dependent upon GM-CSF, such that in the absence of GM-CSF autoimmune neuroinflammation does not develop in mice (7, 8). Ramesh et al. further phenotyped human pro-inflammatory Th17.1 cells by culturing CD4+ T cells in the presence of anti-CD3, anti-CD28 and IL-23 (9); these cells were identified as CCR6+CXCR3+CCR4+CCR10-CD161+, transiently expressing c-Kit and stably expressing multi-drug resistance type 1 (MDR1). This is of interest because levels of IL-17A and IFNγ are heightened in severe and steroid refractory asthma (10-12), and MDR1+ pro-inflammatory Th17 cells were found to be refractory to a range of corticosteroids (9).
The increase in the prevalence of chronic respiratory disease over recent decades highlights a key role for environmental factors in disease development and progression (1). In particular, there is a substantial body of epidemiological data that detrimentally link both poor air quality and vitamin D deficiency to asthma and COPD (13, 14). Exposure to elevated concentrations of ambient particulate matter (PM) has been associated with asthma exacerbations as well as increased hospitalisation and medication usage, with emerging evidence supporting a role in the initiation of asthma (15). Importantly within the epidemiological literature air pollution-triggered asthma exacerbations occur with a 2-5 day lag, suggesting that the role of the pollution itself may be indirect, possibly via perturbation of lymphocyte responses, rather than by the direct induction of bronchoconstriction or by triggering acute inflammation (16).

Consistent with this view, a reduction in PM-induced airway hyperresponsiveness and mucus cell hyperplasia has been reported in Rag1-/- mice that lack lymphocytes (17).

Vitamin D deficiency has also been associated with the increased incidence and severity of many respiratory diseases, including asthma, an observation that has been reviewed in detail elsewhere (14). The majority of vitamin D is generated when ultraviolet-B radiation photolyses 7-dehydrocholesterol in the skin into vitamin D₃. Vitamin D₃ can then be converted into the active form of vitamin D, namely 1,25(OH)₂D₃, by the enzyme CYP27B1 which is present in the kidneys and various peripheral immune cells. Whilst statistically significant associations have been reported between asthma and both air pollution and vitamin D deficiency, the underlying molecular mechanisms by which these environmental factors influence asthma pathology are not fully understood. Furthermore, although it has been proposed that vitamin D may counteract the detrimental effects of UPM, how it achieves this remains to be fully elucidated.
In vivo, the inhaled PM and desorbed components are presented to the immune system in part by interdigitating myeloid dendritic cells (mDCs) that line the airways and lung parenchyma. DCs can then traffic to the mesenteric lymph node and modulate effector T cell function, or can act more locally to promote airway inflammation (18). Whereas UPM has been reported to promote the maturation of DCs (18), vitamin D has independently been shown to have the opposite effect, acting to promote a tolerogenic DC phenotype (19). UPM-primed DCs reduce the generation of Th1 effector cells from naive CD4+ T cells in a mixed lymphocyte reaction (20), but have been reported to enhance production of IL-13 (21). However within the lung, memory CD4+ T cells are extremely abundant and therefore any modulation by UPM may play an important role in air pollution-induced disease exacerbations (22), something which the current study sought to address.

This study aimed to phenotype the cytokine profile of human memory CD4+IL-17A+ T cells generated following co-culture with UPM pre-treated CD1c+ mDCs, and specifically to identify whether UPM promoted a specifically pro-inflammatory Th17 phenotype, as well as the DC-derived signals that might drive such a response. Vitamin D has independently and repeatedly been shown to down-regulate Th17 responses both in vitro and in vivo (14). We therefore additionally addressed whether 1,25(OH)2D3 could counteract any effects of UPM in promoting a potentially pathogenic Th17 response.
Materials and Methods

Cell isolation

Peripheral venous blood from healthy donors (REC approval 14/LO/1699) was collected into sodium citrate-containing syringes (1:10) and PBMCs isolated as previously described (23). CD1c+ DCs were positively selected in accordance with the manufacturer’s instructions (Miltenyi Biotec; >98% CD11c+HLA-DR+). Unlabelled PBMCs were frozen overnight in RPMI containing 40% FCS and 10% DMSO then CD4+CD45RO+ cells isolated by negative selection (Miltenyi Biotec; >98.5% CD4+CD45RO+). Cells were re-suspended in RPMI-1640 (Invitrogen, Paisley, UK) supplemented with 10% filtered human AB serum, 2mM L-glutamine and 50mg/ml gentamycin. Nasal turbinates were surgically removed (REC 12/LO/1931) and CD1a+ DCs positively isolated as previously described (24) (>95% CD11c+HLA-DR+ after excluding debris). Cells were cultured as for peripheral DCs.

Cell culture

1x10⁴/ml DCs were cultured in a U-bottomed 96-well plate for 20 hours with 50ng/ml rhGM-CSF +/- 5μg/ml NIST unless stated (3% methanol vehicle control (VC)), 1-100nM 1,25(OH)₂D₃ (BIOMOL Research Labs; 0.01% DMSO VC), 100nM dexamethasone (Sigma Aldrich) and 2μg/ml anti-IL-23p19 or the relevant isotype control (goat IgG; R&D Systems). DC supernatants were harvested after 20 hours and where indicated 2x10⁵ autologous memory CD4+ T cells added (1:20). Five days later supernatants were harvested and cells transferred to a 48-well plate with 10IU/ml rhIL-2 (Eurocetus) for a further 2 days.

NIST refers to a standard reference source of total UPM (SRM-1648a) from the National Institute of Standards and Technology. SRM-1648a was prepared from UPM collected in St Louis, Missouri, over a 1-year period (1976-1977) and was re-suspended in 3% methanol to prevent the oxidation of aryl hydrocarbons. Particles ranged from 1.35 to 30.1 μm in diameter (mean 5.85 μm) and comprised a combination of polycyclic aromatic hydrocarbons,
polychlorinated biphenyl congeners, and chlorinated pesticides (reviewed in detail elsewhere (25)).

Surface staining
DCs were harvested using 2mM EDTA in PBS containing 2% FCS then incubated on ice with the following antibodies – CD40-FITC, ILT1-PE, ILT3-APC and CCR7-PE (BioLegend; 5C3, 24, ZM4.1 and G043H7 respectively), CD83-APC, HLA-DR-PerCP, ILT2-PECy7 and ILT4-AlexaFluor 647 (BD Biosciences; HB15e, L243, GHI/75 and 287219 respectively). DCs were washed and fluorescence assessed using an NxT Attune flow cytometer (Life Technologies).

Cell proliferation
Prior to culture memory CD4+ T cells were labeled with 5μM CellTrace Violet (Life Technologies). Cell proliferation was assessed on day 7 of co-culture by loss of fluorescence intensity on an NxT Attune.

Intracellular cytokine staining
Cells were stimulated at 37°C with 50ng/ml PMA (phorbol-12-myristate-13-acetate) and 500ng/ml Ionomycin (Sigma Aldrich) for 1 hour, adding 2μM Monensin for a further 4 hours. Cells were surface stained with CD4-PerCP (BD Biosciences; SK3) and aqua zombie (Biolegend) before fixing and permeabilising them using the BD Biosciences Fix/Perm kit. Cells were then stained for the following cytokines: IL-17A-APC and IL-22-eFluor450 (eBiosciences; eBio64DEC17 and 22URTI); IFNγ-FITC, GM-CSF-PE, IL-10-PE, IL-13-PE and Granzyme B-AlexaFluor700 (BD Biosciences; 4S.B3, BVD2-21C11, JES3-9D7, JES10-5A2 and GB-11); IL-17F-AlexaFluor488, TNFα-FITC (Biolegend; Poly5166 and MAb11).

Cytometric bead array
Cytometric bead array (CBA) was employed to measure the concentration of cytokines within cell culture supernatants in accordance with the manufacturer’s instructions (BD Biosciences).
RNA was isolated and cDNA generated as previously described (10). qRT-PCR was performed in triplicate using a ViiA7 (ThermoFisher Scientific), the endogenous control VIC-labelled 18s and FAM-labelled taq-man probes.

**Data analysis**

Flow cytometry data were analyzed using FlowJo (Treestar Inc. version 10). Cumulative data were analyzed using Graphpad Prism version 6.00 for Windows (Graphpad Software Inc.); after assessing for a Gaussian distribution, an appropriate statistical test was performed as outlined in the figure legends.
Results

1,25(OH)₂D₃ and dexamethasone counteract UPM-driven myeloid DC maturation

CD1c+ DCs are the precursors of CD11b- and CD11b+ DCs that line the airways and lung parenchyma respectively (26). Since both air pollution and vitamin D deficiency are associated with the incidence and severity of respiratory diseases, the impact of these factors on CD1c+ DC maturation and the downstream memory T cell responses was investigated. CD1c+ DCs were cultured for 20 hours in the presence of the indicated concentration of 1,25(OH)₂D₃ and/or a reference source of UPM (NIST SRM-1648a referred to as ‘NIST’), added at a concentration determined to consistently stimulate DC maturation (5 μg/ml) both here (Supplementary Figure 1) and previously (27). 50 ng/ml rhGM-CSF was added to all DC cultures as a substitute for that released by UPM-stimulated human bronchial epithelial cells (28, 29); this has previously been shown to enhance the CD1c+ DC maturation induced by NIST (20).

Figure 1A shows a magnified image of the DCs clumping around the NIST particle agglomerates after 20 hours in culture, in contrast to the resting cells cultured in the presence of the vehicle control (VC). DC surface staining was performed and both representative histograms (Figure 1B and Supplementary Figure 1Ai) and cumulative data (Figure 1C and Supplementary Figure 1Aii and 2A) are shown. The VC did not modulate expression of the surface markers assessed, but there was a 1,25(OH)₂D₃ dose-dependent downregulation in expression of CD40, CD83 and HLA-DR. This occurred alongside an upregulation in expression of the inhibitory receptor immunoglobulin-like transcript-3 (ILT3), as previously independently reported (30), but not of the ILT1, ILT2 or ILT4 molecules. In contrast, NIST dose-dependently increased expression of the maturation marker CD83, which was significantly upregulated relative to both the VC and 1,25(OH)₂D₃ condition (p<0.01), with a trend towards increased expression of the lymph node-homing receptor CCR7. Expression of HLA-DR, CD40 and ILT3 was not consistently modulated by NIST treatment. Treatment with
1,25(OH)$_2$D3 in combination with NIST significantly reduced expression of CD83 (p<0.01) and CD40 (p<0.05) relative to the NIST-only condition, whilst expression of ILT3 remained significantly elevated (p<0.05). Similarly levels of IL-6 (p<0.01) and TNFα (p=0.17) were elevated in NIST-treated DC culture supernatants after 20 hours (Supplementary Figure 2B). In support of this, mRNA expression of il6 was dose-dependently elevated in NIST-primed DCs (Supplementary Figure 1B) (add other cytokine qRT-PCR data).

Treatment of CD1a+ DCs derived from nasal turbinates with NIST modestly increased expression of CD40, CD83, HLA-DR and CCR7 in a small sample size, as well as elevated levels of IL-6 in cell culture supernatants (Supplementary Figure 2C-D). 1,25(OH)$_2$D3 alone had no effect, but appeared to counteract the elevated CD40 expression and levels of IL-6 when added in combination with NIST. Unfortunately due to a change in surgical technique this work was not pursued further.

**UPM-primed DCs drive a ‘Th17.1’-like phenotype which is opposed by 1,25(OH)$_2$D$_3$**

Considering the growing body of literature highlighting the existence of Th17 subsets and T-helper cell plasticity (9, 31), alongside studies showing a link between air pollution and IL-17A (27, 32), we aimed to phenotype NIST-driven IL-17A+ cells in greater detail. As well as significantly increasing the frequency of cells expressing IL-17A and IFNγ, co-culture of memory CD4+ T cells with NIST-primed CD1c+ DCs enhanced the proportion of cells expressing IL-17F, IL-22, GM-CSF and granzyme B (Figure 2A/B). Notably, the frequency of memory CD4+ T cells co-expressing IL-17A with IFNγ, GM-CSF or granzyme B was significantly elevated in the NIST condition as compared to the VC when shown as a percentage of all memory CD4+ T cells (Figure 2C), or as a percentage of total memory CD4+IL-17A+ T cells (Figure 2D). There was however no difference in the frequency of IL-17A+IL13+ cells or IL-17A+IL-10+ cells between the conditions and minimal IL-17A/IL-10 co-expression. Of note, levels of LPS were less than 1 pg per well and the NIST-driven
enhancement of IL-17A was double that induced by treating DCs with 10 μg/ml LPS (data not shown).

In contrast to NIST alone, addition of 1,25(OH)_{2}D_{3} +/- NIST reduced the frequency of IL-17A+ and IFN_{γ}+ cells as well as the percentage of IL-17A+IFN_{γ}+ cells (Figure 3A-B), a characteristic of pro-inflammatory 'Th17.1' cells. 1,25(OH)_{2}D_{3} similarly overcame the NIST-driven enhancement in expression of IL-17F, IL-22, GM-CSF and granzyme B (Supplementary Figure 3A). Whereas NIST-primed DCs significantly increased memory CD4+ T cell proliferation, as previously published (27), 1,25(OH)_{2}D_{3} appeared able to counteract this (p=0.05). There was not however any difference in the viability of memory CD4+ T cells co-cultured with DCs that had been pre-treated with 1,25(OH)_{2}D_{3} and/or NIST (data not shown).

Analysis of secreted cytokines present in culture supernatants reflected that of the intracellular cytokine staining (Figure 3C), with levels of IL-17A, IL-17F, IL-12/23p40 and IFN_{γ} consistently elevated in the NIST-treated condition and reduced by co-incubation of DCs with 1,25(OH)_{2}D_{3}. Levels of IL-13 and IL-10 were variable and not consistently modulated by either 1,25(OH)_{2}D_{3} and/or NIST. The mRNA expression of relevant Th17-associated transcription factors was additionally screened after 48 hours of co-culture and the data are shown in Supplementary Figure 3B; there was a trend towards increased expression of stat3, tbx21, mdr1 and irf4 in the NIST condition, with 1,25(OH)_{2}D_{3} treatment opposing this effect (p<0.05 for stat3, mdr1 and irf4; p=0.073 for tbx21).

Pre-treating CD1c+ DCs with the synthetic corticosteroid dexamethasone was similarly able to counteract the NIST-driven pro-inflammatory profile (Supplementary Figure 4); dexamethasone reduced expression of CD83 and CCR7 on CD1c+ DCs, and further enhanced the 1,25(OH)_{2}D3-mediated induction of ILT3, an effect opposed by NIST. Downstream, priming DCs with dexamethasone alone or in combination with 1,25(OH)_{2}D3
counteracted the ability of NIST treatment to promote autologous memory CD4+ T cell proliferation and reduced and levels of IL-17A, IFN\textsubscript{γ} and IL-23p40 within the cell culture supernatants.

**UPM upregulates a population of IL-17A+IFN\textsubscript{γ}+ cells in part via enhanced endogenous IL-23**

Since NIST pre-treatment of CD1a+ DC drove a phenotypically pro-inflammatory Th17.1-like profile with an increased frequency of IL-17A+IFN\textsubscript{γ}+ cells and levels of IL-12/23p40 were significantly upregulated in co-culture supernatants, a role for IL-23 as an intermediate was investigated. Significantly enhanced DC production of IL-23 was firstly confirmed at the mRNA levels over a NIST dose response (Figure 4A). A neutralizing antibody specific for IL-23p19, thereby inhibiting IL-23, but not IL-12, or a relevant isotype control, was then added throughout the culture period. As Figure 4B shows, NIST-primed DCs significantly increased expression of *il17a* and *mdr1* mRNA after 48 hours of co-culture in the isotype condition, but this was significantly reduced by addition of anti-IL-23 into culture. Similarly at the protein level, although anti-IL-23p19 had no effect upon NIST-driven cell division, it did significantly reduce the frequency specifically of IL-17A+IFN\textsubscript{γ}+ cells (Figure 4C/D).
The current study demonstrates that pre-treatment of human myeloid DCs with a common reference source of UPM (NIST) alters properties related to their antigen presenting function. Notably, this results in the expansion of a population of memory CD4+ T cells with a pro-inflammatory Th17.1-like phenotype; these cells are characterised by the co-expression of IL-17A with IFNγ, GM-CSF and Granzyme B, and are predicted to drive exacerbations of respiratory diseases. Our data indicate a role for NIST-induced IL-23 synthesis by myeloid DCs in driving this pro-inflammatory Th17.1-like phenotype. An additional novel feature of these data is the evidence that the reference source of UPM significantly increased expression of GM-CSF and the serine protease granzyme B, specifically enriching the proportion of cells co-expressing these cytokines with IL-17A. Granzyme B can be released from the granules of cytotoxic T cells and is traditionally thought of as mediating apoptosis of target cells, but it can also stimulate pro-inflammatory cytokine release and drive extracellular matrix remodelling (33). Co-expression of IL-17A and granzyme B by CD4+ T cells has, to date, been implicated predominantly in neuroinflammation (34), but elevated levels of granzyme B have been associated with various diseases including autoimmune conditions, type I diabetes and asthma.

In contrast to the effects of NIST, vitamin D reduced both CD1c+ DC priming and the subsequent pro-inflammatory memory T cell response when added alone and in combination with NIST, instead promoting a more tolerogenic environment. Whilst the individual effects of NIST (18) and 1,25(OH)2D3 (19) on DC maturation have previously been published, the capacity of 1,25(OH)2D3 to oppose certain pro-inflammatory properties of NIST when added in combination is novel and important given that these two environmental factors co-exist. The synthetic corticosteroid dexamethasone was similarly capable of reducing the expression of NIST-driven maturation markers on CD1c+ DCs, both alone and more prominently when added in combination with 1,25(OH)2D3. Most significantly 1,25(OH)2D3 counteracted the
induction of the maturation marker CD83 on CD1c+ DCs as well as the heightened frequency of IL-17A+IFNγ+ memory CD4+ T cells induced by NIST-primed CD1c+ DCs. 1,25(OH)2D3 also reduced expression of other inflammatory cytokines GM-CSF, granzyme B, IL-17F and IL-22 (Supplementary Figure 3A). It is properties such as this that might contribute to the fact that in one study, vitamin D insufficient children in Puerto Rico living close to a major road, and therefore traffic-related air pollution, had an elevated risk of severe asthma exacerbations (35). Notably, in the European Study of Cohorts for Air Pollution Effects (ESCAPE), the relative affect of air pollution on health outcomes differed between cohorts, with Scandinavian groups often being more sensitive despite lower levels of ambient PM (36). This may be due to geographical differences in PM composition and/or intrinsic population variation which warrant further study; for example, reduced UVR exposure in Scandinavian countries would lower circulating levels of vitamin D, which we show here to counteract certain potentially pathogenic properties of PM.

The fact that priming DCs with a source of urban particulate matter increased the frequency of IL-17A+ cells concurs with studies in mouse (32, 37) and man (32, 38) that identify links between air pollution and IL-17A, albeit in distinct experimental settings. However in this study, we extend this observation to show that NIST-primed DCs also enhanced the frequency of cells expressing Th17-associated cytokines IL-17F and IL-22 as well as IFNγ, GM-CSF and granzyme B. Critically a large proportion of the NIST-driven IL-17A+ cells in this study co-expressed IL-17F, IL-22, IFNγ, GM-CSF and granzyme B, but not immunoregulatory IL-10. This phenotype is indicative of an IL-23-driven, putatively pathogenic, pro-inflammatory Th17 cell (Th17.1) (4, 5, 7, 8). Although in mice this subset of Th17 cells has been shown to drive autoimmune conditions in a GM-CSF-dependent manner, their functional role and in vivo existence in humans is less clear. Ex vivo Th17/Th1 cells have nonetheless been identified in humans, predominantly in the periphery, inflamed joints and the gut, although functional analysis and detailed phenotyping of these cells has been understandably limited to date.
Production of both IL-17A and IFN\(_{\gamma}\) has however been shown to inversely correlate with lung function after corticosteroid therapy in steroid-refractory asthmatics (39), and GM-CSF was elevated even during the asymptomatic stage of asthma (40). Moreover, pro-inflammatory CCR6+MDR1+ human Th17 cells are reportedly resistant to a range of corticosteroids (9), a characteristic of severe asthma and COPD alongside various autoimmune conditions. What’s more, a recent study performed using the house dust mite mouse model of asthma found that both dexamethasone and anti-IL-17A was required to alleviate diesel exhaust particle (DEP)-induced corticosteroid-refractory asthma (41). We however observed that using the present in vitro co-culture system, priming of CD1c+ DCs from healthy donors in the presence of dexamethasone dampened the resultant NIST-driven proliferation and IL-17A/IFN\(_{\gamma}\) memory CD4+ T cell profile response, with no effect upon levels of IL-10. This conforms to data showing that dexamethasone-treated monocyte-derived DCs possessed a stable tolerogenic phenotype and promoted the differentiation of IL-10+ Tregs in co-culture (42). Nonetheless acting directly on human T cells in vitro or ex vivo, both we (10, 39) and others (43) have shown that corticosteroids failed to inhibit IL-17A production and enhanced levels of IL-10, highlighting the importance of the context in which corticosteroids are administered.

Considering the phenotype of the NIST-driven IL-17A+ cells and the fact that il23 DC mRNA expression was upregulated alongside enhanced levels of IL-12/23p40 in culture supernatants, it was hypothesised that NIST acted via enhanced IL-23 activity to promote the pro-inflammatory Th17 response. Indeed, neutralising the specific IL-23 subunit, namely IL-23p19, significantly impaired the NIST-driven IL-17A+IFN\(_{\gamma}\)+ response without having any effect upon cell proliferation. Importantly, as was true for vitamin D, anti-IL-23p19 did not obliterate all of the IL-17A+IFN\(_{\gamma}\)+ cells which concurs with another study in which only a minor subset of CCR6+CXCR3+ Th17/Th1 cells were IL-23 responsive (44). Although we identify a novel and important role for NIST-driven IL-23 in promoting a putatively pathogenic Th17 cell...
response, the molecular mechanisms by which NIST acts on the DCs was unfortunately beyond the scope of this current study. Previous work has however shown that NIST-primed DCs promote an effector memory CD4+ T cell response in a manner that is dependent upon HLA-DR but not IL-6 (27), and that the effects are not due to LPS contamination. It may well be that NIST can modify self-antigens that stimulate the DCs and/or that exogenous antigens such as viruses, bacteria or allergens are adsorbed to the NIST and drive the observed effects; such studies are on-going.

1,25(OH)_{2}D_{3} significantly reduced the concentration of IL-12/23p40 when added in combination with NIST as well as the resultant pro-inflammatory Th17.1 phenotype promoted by the NIST-pre-treated DCs. However addition of exogenous recombinant IL-23 was unable to overcome the effect (data not shown), suggesting that 1,25(OH)_{2}D_{3} is likely to act through other mechanisms to dampen Th17 responses (14). A reduction in IL-23 levels may nonetheless be a contributing factor that is masked due to redundancy in the present system. In vivo 1,25(OH)_{2}D_{3} is thought to act through several immunoregulatory mechanisms to dampen Th17 responses (14), which is likely to include a reduction of IL-23 availability as suggested by the current study. ILT3, which was shown here to be upregulated by 1,25(OH)_{2}D_{3}, may also play a role in dampening the pro-inflammatory response since it has been reported to reduce the synthesis of IL-17A and IFNγ in mice (45). In contrast interferon regulatory factor (IRF)-4, which contains vitamin D response elements (46), promotes Th17 cell differentiation in mice (47) and was downregulated by 1,25(OH)_{2}D_{3} at the mRNA level, with NIST having the reciprocal effect (Supplementary Figure 3B).

From a therapeutic perspective, we believe that the reported capacity of vitamin D to dampen, but not obliterate, adaptive Th17 responses is critical. It seems highly plausible that such effects act alongside the well-documented capacity of vitamin D to act on structural and innate cells to promote antimicrobial pathways (14). Vitamin D has also been shown to counteract other potentially detrimental properties of UPM beyond the data presented here. For example,
vitamin D can protect epithelial cells from oxidative stress (48), a major consequence of PM exposure that has been implicated in asthma, and oppose the induction of airway inflammation (49). Of particular relevance in the context of corticosteroid-refractory disease, vitamin D can overcome the oxidative stress-induced impairment in the nuclear translocation of ligand-bound receptors such as the glucocorticoid receptor (50).

This research further elucidates the mechanisms that are likely to contribute to the epidemiological associations between vitamin D deficiency, air pollution and respiratory diseases. Priming of CD1c+ DCs with NIST increased IL-23 synthesis, driving a phenotypically pro-inflammatory and potentially pathogenic Th17 profile. Addition of active vitamin D was however able to counteract some of the effects of NIST, supporting the notion that restoring vitamin D sufficiency may help to control inflammatory diseases and counteract the negative effects of air pollution. This may be particularly true in subgroups of individuals, such as those regularly exposed to high levels of air pollution and vitamin D deficient individuals.


cells, which is essential for the effector phase of autoimmune

8. Hirota K, Duarte JH, Veldhoen M, Hornsby E, Li Y, Cua DJ, Ahlfors H, Wilhelm C,
Tolaini M, Menzel U, Garefalaki A, Potocnik AJ, Stockinger B. Fate mapping
of IL-17-producing T cells in inflammatory responses. Nat Immunol 2011; 12:
255-263.

9. Ramesh R, Kozhaya L, McKeavit K, Djuretic IM, Carlson TJ, Quintero MA,
McCauley JL, Abreu MT, Unutmaz D, Sundrud MS. Pro-inflammatory human
Th17 cells selectively express P-glycoprotein and are refractory to

10. Nanzer AM, Chambers ES, Ryanna K, Richards DF, Black C, Timms PM,
Martineau AR, Griffiths CJ, Corrigan CJ, Hawrylowicz CM. Enhanced
production of IL-17A in patients with severe asthma is inhibited by 1α,25-
dihydroxyvitamin D3 in a glucocorticoid-independent fashion. J Allergy Clin
Immunol 2013.

Xystrakis E, Bush A, Saglani S, Hawrylowicz CM. Defective IL-10 expression
and in vitro steroid-induced IL-17A in paediatric severe therapy-resistant
asthma. Thorax 2013.

12. Agache I, Ciobanu C, Agache C, Anghel M. Increased serum IL-17 is an
independent risk factor for severe asthma. Respir Med 2010; 104: 1131-1137.

allergy : journal of the British Society for Allergy and Clinical Immunology
2011; 41: 1059-1071.


nonlymphoid tissue dendritic cell homeostasis but is dispensable for the

30. Penna G, Amuchastegui S, Giarratana N, Daniel KC, Vulcano M, Sozzani S,
Adorini L. 1,25-Dihydroxyvitamin D3 selectively modulates tolerogenic
properties in myeloid but not plasmacytoid dendritic cells. *J Immunol* 2007;

Pagani M, Abrignani S. Plasticity of human CD4 T cell subsets. *Front Immunol*
2014; 5: 630.

32. Brandt EB, Kovacic MB, Lee GB, Gibson AM, Acciani TH, Le Cras TD, Ryan PH,
Budelsky AL, Khurana Hershey GK. Diesel exhaust particle induction of IL-
17A contributes to severe asthma. *J Allergy Clin Immunol* 2013; 132: 1194-
1204.e1192.

33. Annoni R, Silva LF, Nussbaumer-Ochsner Y, van Schadewijk A, Mauad T,
Hiemstra PS, Rabe KF. Increased expression of granzymes A and B in fatal

34. Kebir H, Kreymborg K, Ifergan I, Dodelet-Devillers A, Cayrol R, Bernard M,
Giuliani F, Arbour N, Becher B, Prat A. Human TH17 lymphocytes promote
blood-brain barrier disruption and central nervous system inflammation. *Nat

35. Rosser F, Brehm JM, Forno E, Acosta-Perez E, Kurland K, Canino G, Celedon
JC. Proximity to a Major Road, Vitamin D Insufficiency, and Severe Asthma
Exacerbations in Puerto Rican Children. *Am J Respir Crit Care Med* 2014;
190: 1190-1192.


**Figures legends**

**Figure 1:** $1,25(\text{OH})_2\text{D}_3$ counteracts UPM-induced CD1c+ DC activation.

Peripheral CD1c+ DCs cultured with 50 ng/ml rhGM-CSF alone (-) or in the presence of $10^{-9}$, $10^{-7}$M $1,25(\text{OH})_2\text{D}_3$ (V*M), 1.25-20 μg/ml NIST and/or a vehicle control (VC) for 20 hours. A, light microscope images taken at 25-times magnification. Cell surface staining was performed and the MFI determined. Shown are representative histograms (B) and cumulative data (C; n=3/5). Data assessed by a repeated-measures one-way ANOVA with Holm-Sidak’s multiple comparisons test. * p ≤0.05, ** p ≤0.01.

**Figure 2:** UPM drives a putatively pathogenic Th17 cell phenotype in humans.

Peripheral CD1c+ DCs cultured with 50 ng/ml rhGM-CSF and either vehicle control (black) or 5 μg/ml NIST (white) for 20 hours. Autologous memory CD4+ T cells were co-cultured for a further 5 days followed by a 2 day expansion in the presence of 10 IU/ml IL-2. Cells were then stimulated for 5 hours with PMA and Ionomycin prior to assessing intracellular cytokine expression. A, representative plots from independent experiments. B-D, cumulative data for the total frequency of cytokine-expressing cells (B), the frequency of IL-17A co-expressing cells (C) and the percentage of IL-17A+ cells that co-expressed the indicated cytokine (D) (n=5-7 except for IL-17A and IFNγ (n=21 and 16 respectively)). Data assessed by paired-t-test comparing the vehicle and NIST; * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001.

**Figure 3:** Pre-treating CD1c+ DCs with $1,25(\text{OH})_2\text{D}_3$ counteracted the UPM-driven Th1/Th17 profile.

Peripheral CD1c+ DCs cultured with 50 ng/ml rhGM-CSF in the presence of $10^{-7}$M $1,25(\text{OH})_2\text{D}_3$, 5 μg/ml NIST and/or a vehicle control (VC) for 20 hours. Autologous CellTrace Violet-labelled memory CD4+ T cells were then added for a further 5 days after which supernatants were harvested before expanding the cells with 10 U/ml IL-2 for a further 2 days. Cells were stimulated for 5 hours with PMA and Ionomycin prior to assessing intracellular...
cytokine expression. Shown are representative dot plots (A) and cumulative data (B) for the percentage of cells divided and the frequency of cells expressing IL-17A and IFNγ (n=7-9; data assessed by repeated-measured one-way ANOVA with Tukey’s multiple comparison test). C, CBA was employed to assess the concentration of cytokines in supernatants harvested on day 5 of co-culture (n=6; data assessed by a Friedman test with Dunn’s multiple comparison test). * p ≤0.05, ** p ≤0.01, *** p ≤0.001.

Figure 4: UPM acts via IL-23 to drive the synthesis of IL-17A+IFNγ+ memory CD4+ T cells.

Peripheral CD1c+ DCs cultured with 50 ng/ml rhGM-CSF in the presence of 10⁻⁷M 1,25(OH)₂D₃, 5 μg/ml NIST and/or a vehicle control (VC) for 20 hours. A, DC pellets were harvested and RNA isolated for qRT-PCR; il23 gene expression is shown normalised to 18s endogenous control. B-D, Autologous CellTrace Violet-labelled memory CD4+ T cells were added after 20 hours DC priming; an isotype control or 2 μg/ml anti-IL-23p19 was added throughout the culture. A, after 48 hours of co-culture cells were harvested, RNA isolated and then qRT-PCR performed to assess mRNA expression relative to the isotype and VC condition (n=5; data assessed by a 2-way ANOVA with Sidak’s multiple comparisons test (* p<0.05 between VC and NIST; # p<0.05 between isotype and anti-IL-23). B/C, on day 5 of co-culture supernatants were harvested and the cells expanded with 10 U/ml IL-2 for a further 2 days before stimulating for 5 hours with PMA and Ionomycin prior to assessing intracellular cytokine expression. Shown are representative plots (B) and cumulative data (C; n=8; data assessed by a two-tailed paired t-test, *** p ≤ 0.001).

Supplementary Figure 1: Dose-dependent effects of NIST on CD1c+ DC maturation.

Peripheral CD1c+ DCs cultured with 50 ng/ml rhGM-CSF in the presence of a vehicle control (VC) or 1.25-20 μg/ml NIST for 20 hours. Surface staining was performed and DC pellets were harvested and RNA isolated for qRT-PCR; gene expression is shown normalised to 18s
endogenous control. A, mRNA (i) and surface expression (ii) of the maturation marker CD83.

B, mRNA expression of the indicated cytokines by NIST-primed CD1c+ DCs. Data assessed by a Friedman’s test with Dunn’s multiple comparisons test (n=4); * p ≤0.05, ** p ≤0.01.

**Supplementary Figure 2: The impact of UPM and 1,25(OH)₂D₃ on the profile of peripheral and tissue-derived myeloid DCs.**

A/B, peripheral CD1c+ DCs cultured with 50 ng/ml rhGM-CSF in the presence of 10⁻⁷M 1,25(OH)₂D₃, 5 µg/ml NIST and/or a vehicle control (VC) for 20 hours. A, cell surface staining was performed and the MFI determined. B, the concentration of cytokines in the supernatant was determined by CBA. C/D, CD1a+ DCs isolated from nasal turbinate tissue were cultured with 50 ng/ml rhGM-CSF in the presence of 10⁻⁷M 1,25(OH)₂D₃ and/or 5 µg/ml NIST for 20 hours. Cell surface flow cytometry staining was performed and shown are representative histograms (n=3). D, the concentration of IL-6 in 20-hour culture supernatants as determined by CBA show as raw data and fold change relative to the vehicle control (n=3). Data assessed by a Friedman’s test with Dunn’s multiple comparisons test (n=4); * p ≤0.05, ** p ≤0.01.

**Supplementary Figure 3: 1,25(OH)₂D₃-primed DCs oppose a UPM driven pro-inflammatory response.**

Peripheral CD1c+ DCs cultured with 50 ng/ml rhGM-CSF in the presence of 10⁻⁷M 1,25(OH)₂D₃, 5 µg/ml NIST and/or a vehicle control (VC) for 20 hours. Autologous memory CD4+ T cells were then added. A, on day 5 of the co-culture cells were expanded with 10 U/ml IL-2 for a further 2 days and then stimulated for 5 hours with PMA and Ionomycin prior to assessing intracellular cytokine expression. Shown are representative contour plots (n=3). B, after 48 hours of co-culture cells were harvested, RNA isolated and then qRT-PCR performed to assess mRNA expression relative to 18s endogenous control. Data assessed by one-way ANOVA with Tukey’s multiple comparisons.
Supplementary Figure 4: Pre-treating CD1c+ DCs with 1,25(OH)₂D₃ and dexamethasone countered the UPM-driven pro-inflammatory profile.

Peripheral CD1c+ DCs cultured with 50 ng/ml rhGM-CSF in the presence of 10⁻⁷M 1,25(OH)₂D₃, 10⁻⁷M dexamethasone and/or 5 μg/ml NIST for 20 hours. A, cell surface staining was performed and the MFI determined. Autologous CellTrace Violet-labelled memory CD4+ T cells added for a further 5 days and then supernatants harvested for CBA (C). B, after expanding the cells with 10 U/ml IL-2 for 2 days, cells were stimulated for 5 hours with PMA and Ionomycin prior to assessing intracellular cytokine expression. Data assessed by 2-way ANOVA with Sidak’s multiple comparisons test (n=4-6). * p ≤0.05, ** p ≤0.01, *** p ≤0.001, **** p ≤0.0001.
Figure 2

A

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B

Total frequency of cytokine expressing cells

C

Frequency of IL-17A co-expressing cells

D

Percentage of IL-17A+ cells co-expressing the indicated cytokine
Figure 3

A

Vehicle control | $\text{1,25(OH)}_2\text{D}_3$ | NIST | NIST + $\text{1,25(OH)}_2\text{D}_3$

IL-17A

CellTrace Violet

B

% Memory CD4+ T cells

IL-17A+

IFNy+

IL-17A+IFNy+

% Cells Divided

C

IL-17A

IL-17F

IL-23p40

IL-13

IL-10

pg/ml
Figure 4

A

DC - il23

(2^{-ΔΔCt} values) x 10^6

VC 5 μg/ml NIST

B

il17a rorc

Relative mRNA expression

Il23 mdr1

C

Isotype Anti-IL-23

IL-17A IFNγ

i)

2.2 5.4

2.05 3.67

27.8 23.7

ii)

NIST + iso NIST + aIL-23p19

D

Cells divided

% Memory CD4+ T cells

IL-17A+IFNγ-

IL-17A-IFNγ-

IL-17A-IFNγ+

0.0890

0.0920
Supplementary Figure 1

A (i) 

$\text{cd83}$

$\mu\text{g/ml NIST}$

(2-dCt values) $\times 10^6$

B

$i\text{I}1\text{b}$

(2-dCt values) $\times 10^6$

A (ii)

$i\text{I}2\text{b}$

$i\text{I}6$

$i\text{I}10$

$i\text{I}23$

CD83

Vehicle control
1.25 $\mu\text{g/ml NIST}$
2.5 $\mu\text{g/ml NIST}$
5 $\mu\text{g/ml NIST}$
10 $\mu\text{g/ml NIST}$
20 $\mu\text{g/ml NIST}$

MFI

$\mu\text{g/ml NIST}$

*
Supplementary Figure 2

A

Peripheral mDCs

ILT1

ILT2

ILT4

CCR7

B

Tissue mDCs

IL-6

TNFα

IL-12/23p40

C

D

TNFα and IL-12/23p40 undetectable in turbinate DC supernatants
Supplementary Figure 3

A

Vehicle Control 1,25(OH)\(_2\)D\(_3\) NIST 1,25(OH)\(_2\)D\(_3\) + NIST

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B

il-23r  stat3  rorc  tbx21 (tbet)

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Supplementary Figure 4

A

MFI

CD83

HLA-DR

CCR7

ILT3

Vehicle

Control

NIST

B(i)

Vitamin D

Dex

Vehicle

1,25(OH)₂D₃

Dexamethasone

NIST

CellTrace Violet

CellTrace Violet

Model

B(ii)

IL-17A+

IFNγ+

IL-17A+IFNγ+

% cells divided

Vehicle

Control

NIST

C

IL-17A

IFNγ

IL-12/23p40

IL-10

Vehicle

Control

NIST