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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

Urban particulate matter (UPM) air pollution and vitamin D deficiency are detrimentally associated with respiratory health; this is hypothesised to be due in part to regulation of IL-17A, which UPM is reported to promote. Here we use a myeloid (m)DC-memory CD4+ T cell co-culture system to characterize UPM-driven IL-17A+ cells, investigate the mechanism by which UPM-primed DCs promote this phenotype and address evidence for cross-regulation by vitamin D. CD1c+ mDCs were cultured overnight with or without a reference source of UPM and/or active vitamin D ($1,25(OH)_2D_3$) 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 elevated levels of *il23* mRNA and increased secretion of IL-23p40. Neutralisation of IL-23 in culture reduced the frequency of IL-17A+IFNγ+ cells without affecting cell proliferation. $1,25(OH)_2D_3$ counteracted the UPM-driven DC maturation and inhibited the frequency of IL-17A+IFNγ+ cells, most prominently when DCs were co-treated with the corticosteroid dexamethasone, whilst maintaining anti-inflammatory IL-10 synthesis. These data indicate that UPM might promote an inflammatory milieu in part by driving an IL-23-driven 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. 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 (1). 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 (2). 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 (3, 4). 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 Th1-associated IFNγ, but not the anti-inflammatory mediator IL-10. Zielinski *et al*. showed that human *Candida albicans*-specific Th17 cells produced both IL-17A and IFNγ, but not IL-10 upon re-stimulation (5). 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 (6, 7). 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 (8); these cells were identified as CCR6+CXCR3^{hi}CCR4^{lo}CCR10-CD161+, transiently expressing c-Kit and stably expressing multi-drug resistance type 1 (MDR1). This is of interest because levels of both IL-17A and IFNγ are heightened in severe and steroid refractory asthma (9-11), and MDR1+ pro-inflammatory Th17 cells were found to be refractory to a range of corticosteroids (8).

The increase in the prevalence of chronic respiratory diseases over recent decades highlights a key role for environmental factors in disease development and progression (12). 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 immediate triggering of innate immune responses (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, 18). The majority of vitamin D is generated when ultraviolet-B radiation photolyses 7-dehydrocholesterol in the skin into vitamin D_3. Vitamin D_3 can then be converted into the active form of vitamin D, namely $1,25(OH)_2D_3$, by the enzyme CYP27B1 which is present in the kidneys and various peripheral immune cells. Whilst statistically significant associations have been reported between respiratory diseases and both air pollution and vitamin D deficiency (discussed above), the underlying molecular mechanisms by which these environmental factors influence pathophysiology 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 (19). Whereas UPM has been reported to promote the maturation of DCs (19), vitamin D has independently been shown to have the opposite effect, acting to promote a tolerogenic DC phenotype (20). Within the lung, memory CD4+ T cells are extremely abundant and therefore any modulation of them by UPM-primed DCS may play an important role in air pollution-induced disease exacerbations (21), 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 further addressed whether $1,25(OH)_2D_3$ could counteract any effects of UPM in promoting a potentially pathogenic Th17 response.

Materials and Methods

Cell isolation

PBMCs were isolated from healthy donors by means of density centrifugation (Axis-Shield) (REC 14/LO/1699) and CD1c+ DCs positively selected (Miltenyi Biotec; >98% CD11c+HLA-DR+). Unlabelled PBMCs were frozen overnight in RPMI-1640 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 supplemented with 10% human AB serum, 2mM L-glutamine and 50mg/ml gentamycin. Inferior turbinate tissue was donated by patients undergoing turbinate resection (REC 12/LO/1931) and CD1a+ DCs positively isolated as previously described (22) (>95% CD11c+HLA-DR+ excluding debris).

Cell culture

$1x10^4$/ml DCs were cultured in a U-bottomed 96-well plate for 20 hours with 50ng/ml rhGM-CSF +/- 5µg/ml NIST (3% methanol vehicle control (VC)), 100nM $1,25(OH)_2D_3$ (BIOMOL Research Labs; 0.01% DMSO VC), 100nM dexamethasone (Sigma Aldrich) and/or 2µg/ml anti-IL-23p19 or the relevant isotype control (R&D Systems) unless stated. DC supernatants were harvested after 20 hours and $2x10^5$ autologous memory CD4+ T cells added. 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 here to a standard reference source of total UPM (SRM-1648a) from the National Institute of Standards and Technology. SRM-1648a was prepared from UPM previously collected in St Louis, Missouri, over a 1-year period and was re-suspended in 3% methanol to prevent the oxidation of aryl hydrocarbons. Particles ranged from 1.35 to 30.1µm in diameter (mean 5.85µm) and comprised a combination of polycyclic aromatic hydrocarbons, polychlorinated biphenyl congeners, and chlorinated pesticides (23).

Surface staining

DCs were harvested using 2mM EDTA in PBS containing 2% FCS then incubated on ice with different 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-AlexaFluor647 (BD Biosciences; HB15e, L243, GHI/75 and 287219 respectively). DCs were washed and fluorescence assessed using an NxT Attune (ThermoFisher).

Cell proliferation

Prior to culture T cells were labeled with 5μM CellTrace Violet (ThermoFisher). Cell proliferation was assessed on day 7 of co-culture by loss of fluorescence intensity.

Intracellular cytokine staining

Cells were stimulated at 37ºC with 50ng/ml PMA and 500ng/ml Ionomycin 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) +/- CD161-BV412 (BD Biosciences; DX12) and MDR1-PerCPeFluor710 (ebiosciences; UIC2) before fixing and permeabilising them (BD Biosciences Fix/Perm kit). Samples were then incubated with the following antibodies: IL-17A-APC and IL-22-eFluor450 (eBiosciences; eBio64DEC17 and 22URTI respectively); 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 respectively); IL-17F-AlexaFluor488, TNFα-FITC (Biolegend; Poly5166 and MAb11 respectively).

Cytometric bead array

Cytometric bead array (CBA) was employed to measure the concentration of cytokines within supernatants in accordance with the manufacturer's instructions (BD Biosciences).

qRT-PCR

RNA was isolated from Qiazol lysed cells using an miRNeasy Mini Kit (Qiagen). mRNA was converted to cDNA by using RevertAid Reverse Transcriptase and complementary reagents. qRT-PCR was performed in triplicate on a ViiA7 using taq-man probes (ThermoFisher).

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. After assessing for a Gaussian distribution, statistics were performed as outlined in figure legends.

Results

$1,25(OH)_2D_3$ counteracts UPM-driven myeloid DC maturation

CD1c+ DCs are the precursors of CD11b- and CD11b+ DCs that line the airways and lung parenchyma respectively (24). 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 $1,25(OH)_2D_3$ and/or a reference source of UPM (NIST SRM-1648a referred to as 'NIST'), added at a concentration (5 µg/ml) determined to consistently stimulate DC maturation both here (Supplementary Figure 1A) and previously (25)). 50 ng/ml rhGM-CSF was added to all DC cultures as a substitute for that released by UPM-stimulated human bronchial epithelial cells (26, 27); this has previously been shown to enhance the CD1c+ DC maturation induced by NIST (28).

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 3% methanol vehicle control (VC). DC surface staining was performed and both representative histograms and cumulative data are shown (Figure 1B/C). 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 (29), but not of the ILT1, ILT2 or ILT4 molecules (Supplementary Figure 2A).

In contrast, NIST dose-dependently increased expression of the maturation marker CD83 at the mRNA and protein level (Supplementary Figure 1A), such that expression was significantly enhanced relative to both the VC and $1,25(OH)_2D3$ condition ($p<0.01$) (Figure

1C). There was also a trend towards increased expression of the lymph node-homing receptor CCR7 on the NIST-treated DCs (Supplementary Figure 2A). Expression of HLA-DR, CD40 and ILT3 was not consistently modulated by NIST treatment. Addition of 1,25$(OH)_2$D3 to culture 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 ($p<0.05$) (Figure 1C). 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). Treatment of CD1a+ DCs derived from nasal turbinates with NIST modestly increased expression of CD40 and CCR7 in a small sample size, as well as a trend towards elevated levels of IL-6 in cell culture supernatants ($p=0.0531$) (Supplementary Figure 2C-D). 1,25$(OH)_2$D3 alone had no effect, but appeared to counteract the elevated expression of CD40 and CCR7 as well as the augmented levels of IL-6 when added in combination with NIST.

UPM-primed DCs drive a 'Th17.1'-like phenotype which is opposed by 1,25$(OH)_2D_3$

Considering the growing body of literature highlighting the existence of Th17 subsets and T-helper cell plasticity (8, 30), alongside studies showing a link between air pollution and IL-17A (25, 31), 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-

239 expression. Of note, levels of LPS were less than 1 pg per well and the NIST-driven
240 enhancement of IL-17A was double that induced by treating DCs with 10 µg/ml LPS (data
241 not shown).
242
243 In contrast to NIST alone, addition of $1,25(OH)_2D_3$ +/- NIST reduced the frequency of IL-
244 17A+ and IFNγ+ cells as well as the percentage of IL-17A+IFNγ+ cells (Figure 3A/B), a
245 characteristic of pro-inflammatory 'Th17.1' cells. $1,25(OH)_2D_3$ similarly overcame the NIST-
246 driven enhancement in expression of IL-17F, IL-22, GM-CSF and granzyme B
247 (Supplementary Figure 3A). Whereas NIST-primed DCs significantly increased memory
248 CD4+ T cell proliferation, as previously published (25), $1,25(OH)_2D_3$ appeared able to
249 counteract this (p=0.05). There was not however any difference in the viability of memory
250 CD4+ T cells co-cultured with DCs that had been pre-treated with $1,25(OH)_2D_3$ and/or NIST
251 (data not shown).
252
253 Analysis of secreted cytokines present in culture supernatants reflected that of the
254 intracellular cytokine staining (Figure 3C), with levels of IL-17A, IL-17F, IL-12/23p40 and
255 IFNγ consistently elevated in the NIST-treated condition and reduced by co-incubation of
256 DCs with $1,25(OH)_2D_3$. Levels of IL-13 and IL-10 were variable and not consistently
257 modulated by either $1,25(OH)_2D_3$ and/or NIST. Beyond cytokines, NIST-primed DCs also
258 increased T cell surface expression of the lectin-like receptor CD161 which was opposed by
259 $1,25(OH)_2D_3$, with a more modest effect on expression of MDR1 (Figure 3D), both markers
260 that have been associated with Th17.1 cells in humans (8). The mRNA expression of
261 relevant Th17-associated transcription factors was additionally screened after 48 hours of
262 co-culture and the data are shown in Supplementary Figure 3B; there was a trend towards
263 increased expression of *stat3, tbx21*, *mdr1* and *irf4* in the NIST condition, with $1,25(OH)_2D_3$
264 treatment opposing this effect ($p<0.05$ for *stat3*, *mdr1* and *irf4*; p=0.073 for *tbx21*).
265

Dexamethasone enhances the capacity of vitamin D to dampen the NIST-driven IL-17A+IFNγ+ T cell response

Given previous evidence of complimentary interactions between corticosteroids and vitamin D (14), we additionally examined the combination in the present cultures. Pre-treating CD1c+ DCs with the synthetic corticosteroid dexamethasone was, like $1,25(OH)_2D3$, able to counteract the NIST-driven pro-inflammatory profile (Figure 4). Dexamethasone reduced expression of CD83 and CCR7 on CD1c+ DCs (Figure 4A). Addition of vitamin D to dexamethasone-treated DCs further suppressed CD83 expression even in the presence of NIST. Dexamethasone also further enhanced the $1,25(OH)_2D3$-mediated induction of ILT3, an effect that was opposed by NIST. Downstream, priming DCs with dexamethasone alone or in combination with $1,25(OH)_2D3$ counteracted the ability of NIST treatment to promote autologous memory CD4+ T cell proliferation and reduced levels of IL-17A, IFNγ and IL-23p40 within the cell culture supernatants (Figure 4B/C). Notably the combination of vitamin D and dexamethasone suppressed the NIST-induced IL-17A+IFNγ+ memory CD4+ T cell response to the greatest extent.

UPM upregulates a population of IL-17A+IFNγ+ cells in part via enhanced endogenous IL-23

Since NIST pre-treatment of CD1c+ DC drove a phenotypically pro-inflammatory Th17.1-like profile with an increased frequency of IL-17A+IFNγ+ 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 5A). Of note, *il12* mRNA expression in DCs was undetectable by qRT-PCR (Supplementary Figure 1C). 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 5B shows, NIST-primed DCs significantly increased expression of *il17a* and *mdr1* mRNA after 48 hours of co-culture in

293 the isotype condition in agreement with protein data from Figure 3, but this was significantly
294 reduced by addition of anti-IL-23 into culture, whilst expression of *il-10* was unaffected.
295 Similarly at the protein level, although anti-IL-23p19 had no effect upon NIST-driven cell
296 division, it did significantly reduce the frequency specifically of IL-17A+IFNγ+ cells ($p<0.001$;
297 Figure 5C/D), with a more modest effect upon the IL-17A ($p=0.089$) and IFNγ ($p=0.092$)
298 single positive populations.

Discussion

The current study demonstrates that pre-treatment of human myeloid DCs with a common reference source of UPM (NIST) alters their maturation state resulting in the expansion of a population of memory CD4+ T cells possessing 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 central role for NIST-induced IL-23 synthesis by myeloid DC in driving this pro-inflammatory Th17 response. An additional novel feature of these data is the evidence that 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 (Figure 2). 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 (32). Co-expression of IL-17A and granzyme B by CD4+ T cells has, to date, been implicated predominantly in neuroinflammation (33), but elevated levels of granzyme B have been associated with various diseases including autoimmune conditions, type I diabetes and asthma. Collectively these data suggest that UPM, via actions on the antigen presenting cell compartment, promotes a Th17 population with a potentially pathogenic phenotype.

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 phenotype (Figure 1). Whilst the individual effects of NIST (19) and 1,25$(OH)_2D_3$ (20) on DC maturation have previously been published, the capacity of 1,25$(OH)_2D_3$ to oppose certain pro-inflammatory properties of NIST when added in combination is novel and important given that these two environmental factors co-exist. Furthermore, 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$(OH)_2$D3 (Figure 4). Most significantly 1,25$(OH)_2D_3$ 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$(OH)_2D_3$ also reduced expression of other inflammatory cytokines GM-CSF, granzyme B, IL-17F and IL-22 (Supplementary Figure 3). It is properties such as this that might help to explain evidence that, for example, 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 (34). Notably, in the European Study of Cohorts for Air Pollution Effects (ESCAPE), the relative effect of air pollution on health outcomes differed between cohorts, with Scandinavian groups often being more sensitive despite lower levels of ambient PM (35). 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 UPM increased the frequency of IL-17A+ cells concurs with studies in mouse (31, 36) and man (25, 31, 37) 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 (Figure 2), a phenotype that is indicative of a putatively pathogenic Th17 cell (Th17.1) (3, 4, 6, 7). Furthermore, NIST-primed DCs increased T cell expression of MDR1 and CD161 in co-culture (Figure 3D), both of which have been associated with a pro-inflammatory Th17 phenotype in humans (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. Th17/Th1 cells have nonetheless been identified in humans, predominantly in the periphery, inflamed joints and the gut (5, 8, 38), but functional analysis and detailed phenotyping of these cells has been understandably limited to date.

Production of both IL-17A and IFNγ 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 MDR1+ human Th17 cells are reportedly resistant to a range of corticosteroids (8), a characteristic of both severe asthma and COPD as well as various autoimmune conditions. Recently, a study performed using the murine house dust mite model of asthma found that both dexamethasone and anti-IL-17A were 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/IFNγ memory CD4+ T cell profile response, with no effect upon levels of IL-10 (Figure 4). This conforms to data showing that dexamethasone-treated monocyte-derived DCs possessed a stable tolerogenic phenotype (42). Nonetheless acting directly on human T cells both we (9, 39) and others (43) observed that corticosteroids failed to inhibit IL-17A production and enhanced levels of IL-10 using various *in vitro* and *ex vivo* experimental systems, 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* mRNA expression was significantly upregulated in DCs 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, specific neutralisation of IL-23

impaired the NIST-driven IL-17A and IFNγ response whilst maintaining levels of IL-10 and not affecting cell proliferation, most significantly targeting IL-17A+IFNγ+ cells (Figure 5). Importantly, as was true for vitamin D, anti-IL-23p19 did not obliterate all of the IL-17A+IFNγ+ cells which concurs with another study in which only a subset of CCR6+CXCR3+ Th17/Th1 cells were IL-23 responsive (44). We therefore identify a novel pathway wherein NIST drives enhanced IL-23 production by CD1c+ DCs which promotes a putatively pathogenic Th17 cell response in co-culture; the molecular mechanisms by which NIST acts on the DCs to induce IL-23 are not fully understood. 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 (25), but that the effects are not due to LPS contamination (data not shown). We speculate that NIST might modify self-antigens that stimulate the DCs and/or that exogenous antigens such as viruses, bacteria or allergens are adsorbed to the NIST to drive the observed effects; studies to address this important question is on-going.

$1,25(OH)_2D_3$ significantly reduced the concentration of IL-12/23p40 when added in combination with NIST as well as the resultant Th17 response promoted by the NIST-pre-treated DCs (Figure 3). However, addition of exogenous recombinant IL-23 was unable to overcome the effect (data not shown) suggesting that $1,25(OH)_2D_3$ is also likely to act through other mechanisms to dampen Th17 responses, although there may well be redundancy in the system. Indeed *in vivo*, $1,25(OH)_2D_3$ is thought to act through several immunoregulatory mechanisms to dampen Th17 responses (14): ILT3, which was shown here to be upregulated by $1,25(OH)_2D3$ (Supplementary Figure 3B), may 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 $1,25(OH)_2D_3$ 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, alone or in combination with corticosteroids, was however able to counteract some of the effects of NIST whilst maintaining levels of IL-10, supporting the notion that restoring vitamin D sufficiency may help to control inflammatory diseases and counteract certain negative effects of air pollution. This might be particularly true in subgroups of individuals, such as those regularly exposed to high levels of air pollution and vitamin D deficient individuals.

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Figures legends

Figure 1: 1,25(OH)$_2$D3 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(OH)$_2$D3 (V^{-x}M), 5 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 cytokine response.

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(OH)$_2$D3 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(OH)$_2$D3, 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

641 data (**B**) for the percentage of cells divided and the frequency of cells expressing IL-17A and
642 IFNγ (n=7-9; data assessed by repeated-measured one-way ANOVA with Tukey's multiple
643 comparison test). **C**, CBA was employed to assess the concentration of cytokines in
644 supernatants harvested on day 5 of co-culture (n=6; data assessed by a Friedman test with
645 Dunn's multiple comparison test). **D**, surface expression of CD161 and MDR1 assessed on
646 day 7 of co-culture (representative histogram n=2). * p ≤0.05, ** p ≤0.01, *** p ≤0.001.
647
648 **Figure 4: Pre-treating CD1c+ DCs with 1,25(OH)$_2$D3 and dexamethasone countered the**
649 **UPM-driven pro-inflammatory profile.**
650 Peripheral CD1c+ DCs cultured with 50 ng/ml rhGM-CSF in the presence of 10^{-7}M
651 1,25(OH)$_2$D3 (VitD; V), 10^{-7}M dexamethasone (Dex; D) and/or 5 μg/ml NIST for 20 hours. **A**,
652 cell surface staining was performed and the MFI determined (n=5). Autologous CellTrace
653 Violet-labelled memory CD4+ T cells added for a further 5 days and then supernatants
654 harvested for CBA (**C**; n=6). **B**, after expanding the cells with 10 U/ml IL-2 for 2 days, cells
655 were stimulated for 5 hours with PMA and Ionomycin prior to assessing intracellular cytokine
656 expression (n=5). Data assessed by 2-way ANOVA with Sidak's multiple comparisons test. *
657 p ≤0.05, ** p ≤0.01, *** p ≤0.001, **** p ≤0.0001.
658
659 **Figure 5: UPM acts via IL-23 to drive the synthesis of IL-17A+IFNγ+ memory CD4+ T**
660 **cells.**
661 Peripheral CD1c+ DCs cultured with 50 ng/ml rhGM-CSF in the presence of 10^{-7}M
662 1,25(OH)$_2$D3, 1.25-20 μg/ml NIST (5 μg/ml unless stated) and/or a vehicle control (VC) for
663 20 hours. **A**, DC pellets were harvested and RNA isolated for qRT-PCR; *il23* gene
664 expression is shown normalised to *18s* endogenous control (data assessed by Friedman's
665 test with Dunn's multiple comparisons test). **B-D**, Autologous CellTrace Violet-labelled
666 memory CD4+ T cells were added after 20 hours DC priming; an isotype control or 2 μg/ml
667 anti-IL-23p19 was added throughout the culture. **B**, after 48 hours of co-culture cells were
668 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). **C/D**, 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 (**C**) and cumulative data (**D;** 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. mRNA (**A**) and surface expression (**B**) of the maturation marker CD83. **C**, 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=3/5); * p ≤0.05, ** p ≤0.01.

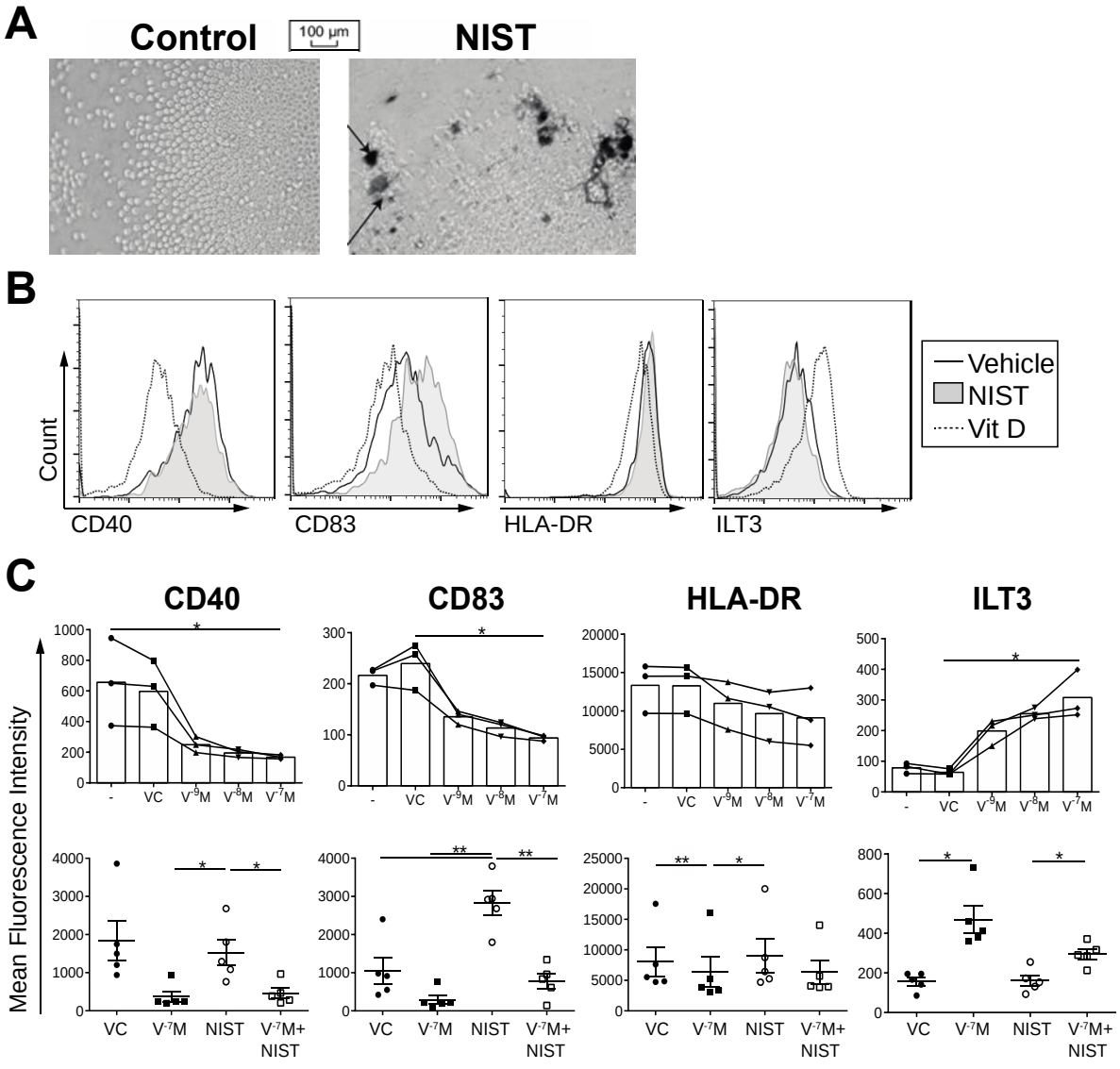
Supplementary Figure 2: The impact of UPM and 1,25(OH)$_2$D3 on the profile of peripheral and tissue-derived myeloid DCs.
Peripheral CD1c+ DCs (**A/B**) or CD1a+ DCs isolated from nasal turbinate tissue (**C/D**) were cultured with 50 ng/ml rhGM-CSF in the presence of 10^{-7}M 1,25(OH)$_2$D3, 5 µg/ml NIST and/or a vehicle control (VC) for 20 hours. **A**, cell surface staining was performed and the MFI determined (n=4). **B**, the concentration of cytokines in the supernatant was determined by CBA (n=4). **C**, Cell surface flow cytometry staining was performed and shown are representative histograms (**Ci**) alongside cumulative data (**Cii**; 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; * p ≤0.05, ** p ≤0.01.

Supplementary Figure 3: 1,25(OH)$_2$D3-primed DCs oppose a UPM driven pro-inflammatory response.

Peripheral CD1c+ DCs cultured with 50 ng/ml rhGM-CSF in the presence of 10^{-7}M 1,25(OH)$_2$D3 (V), 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; * $p \leq 0.05$, ** $p \leq 0.01$.

Figures

Figure 1:



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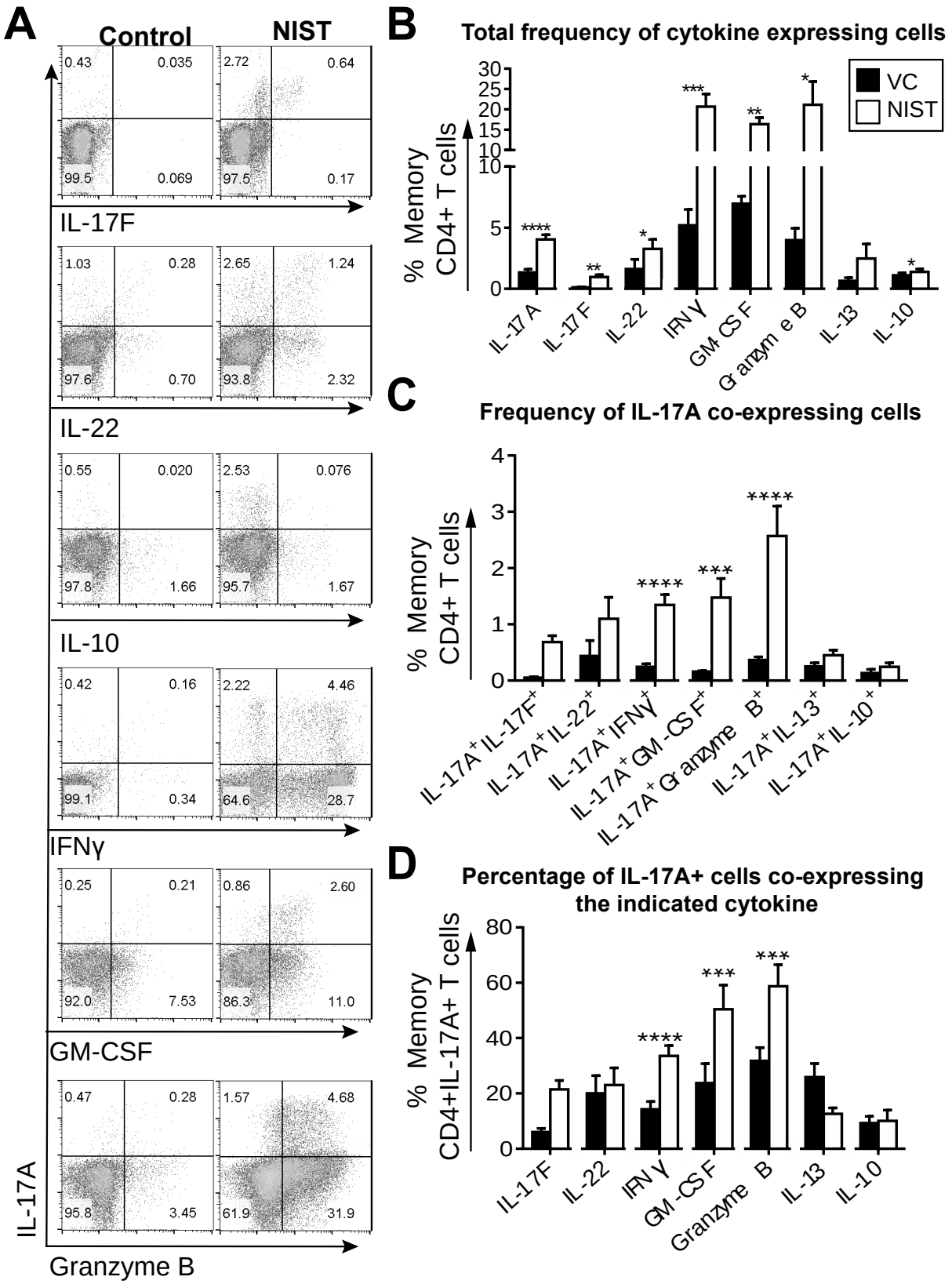

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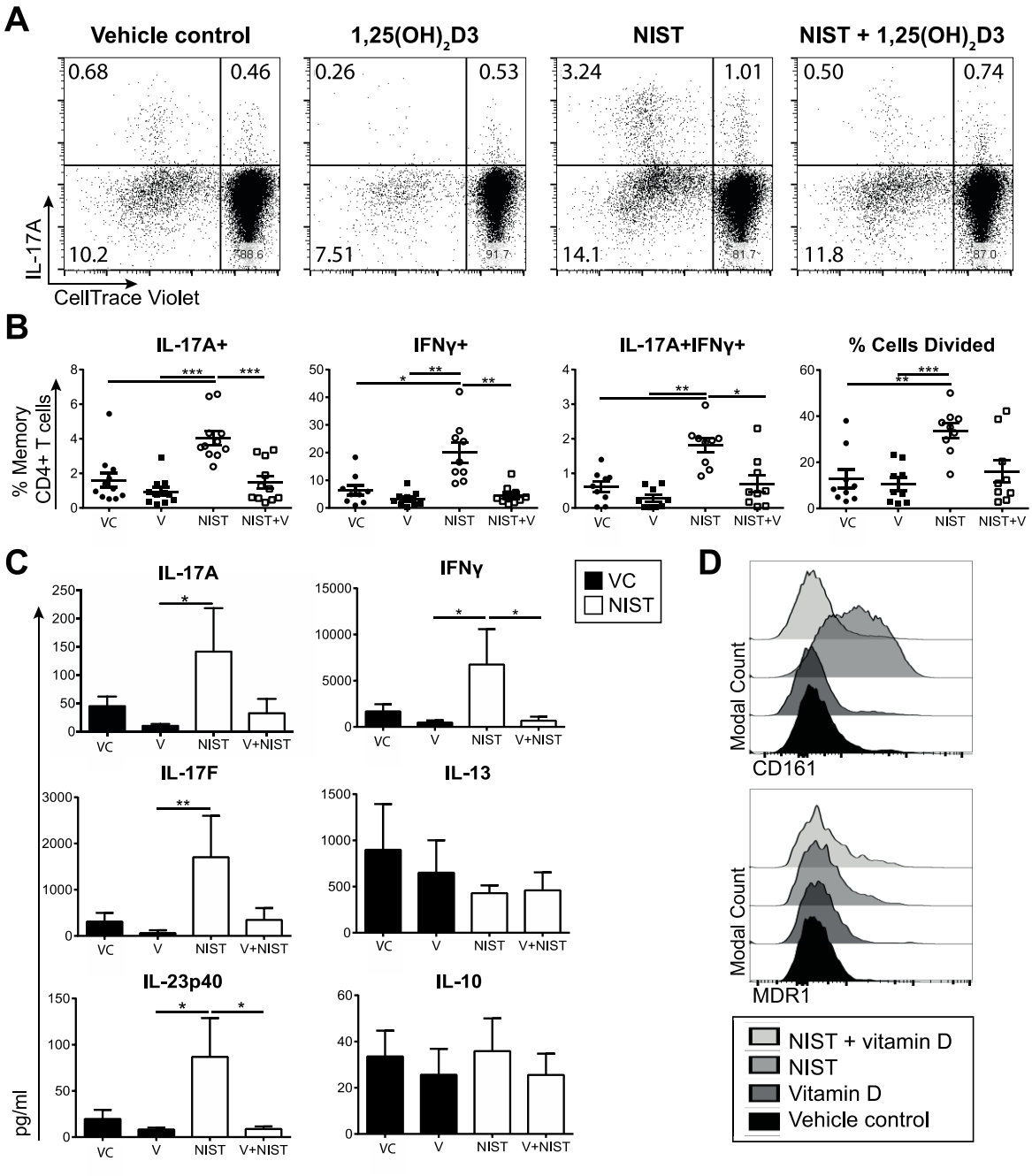

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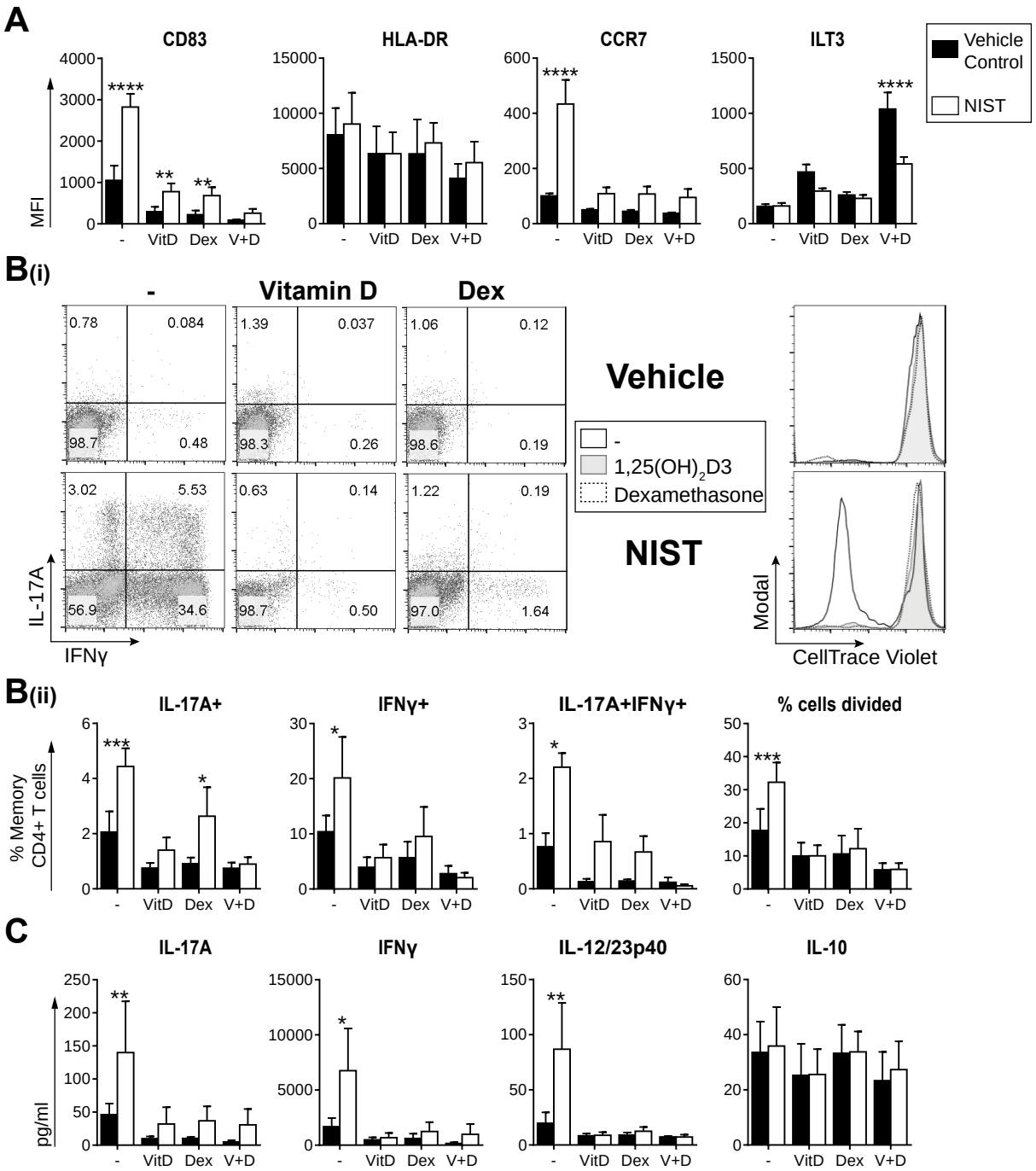

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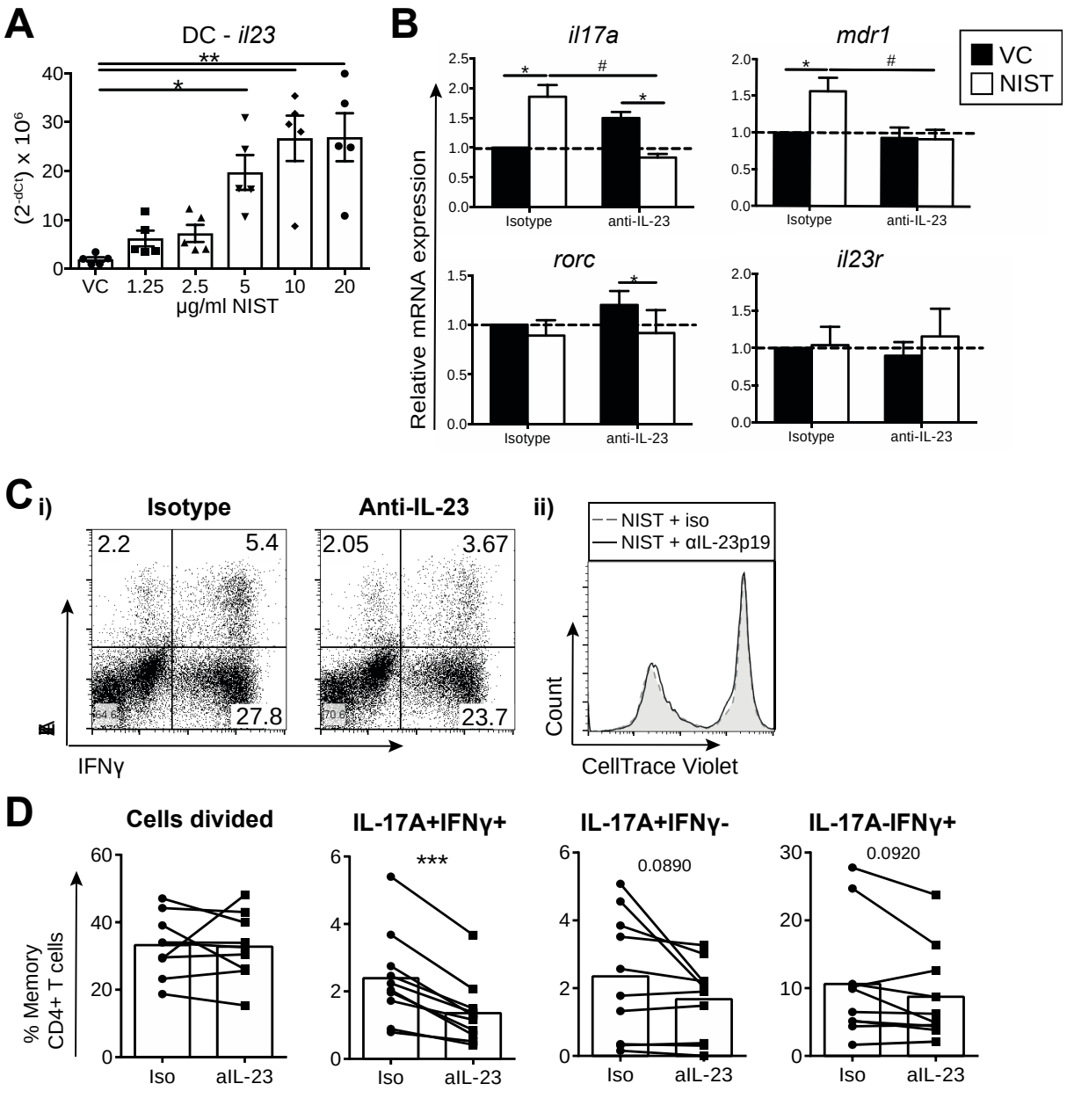

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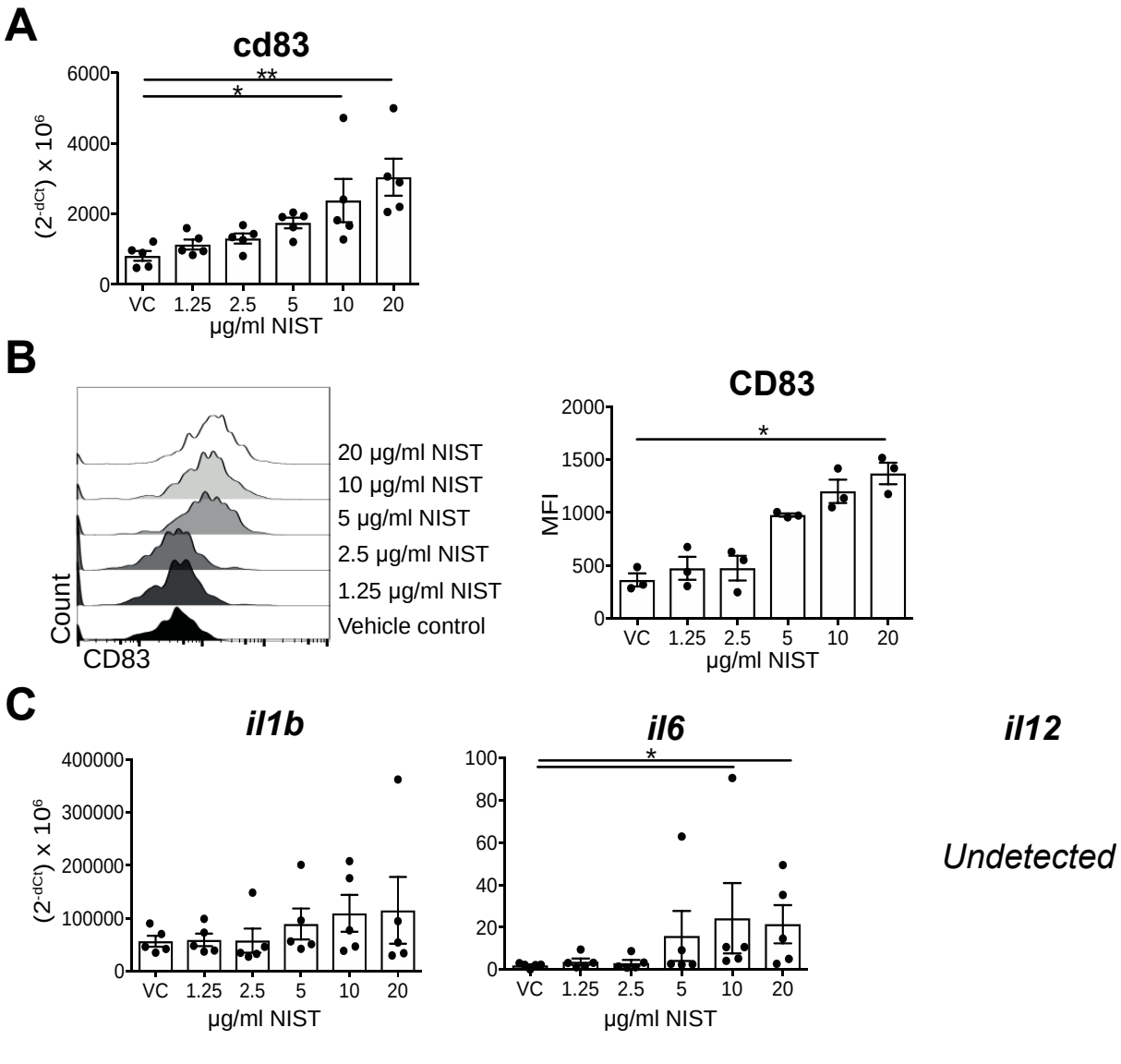
Supplementary Figure 1:



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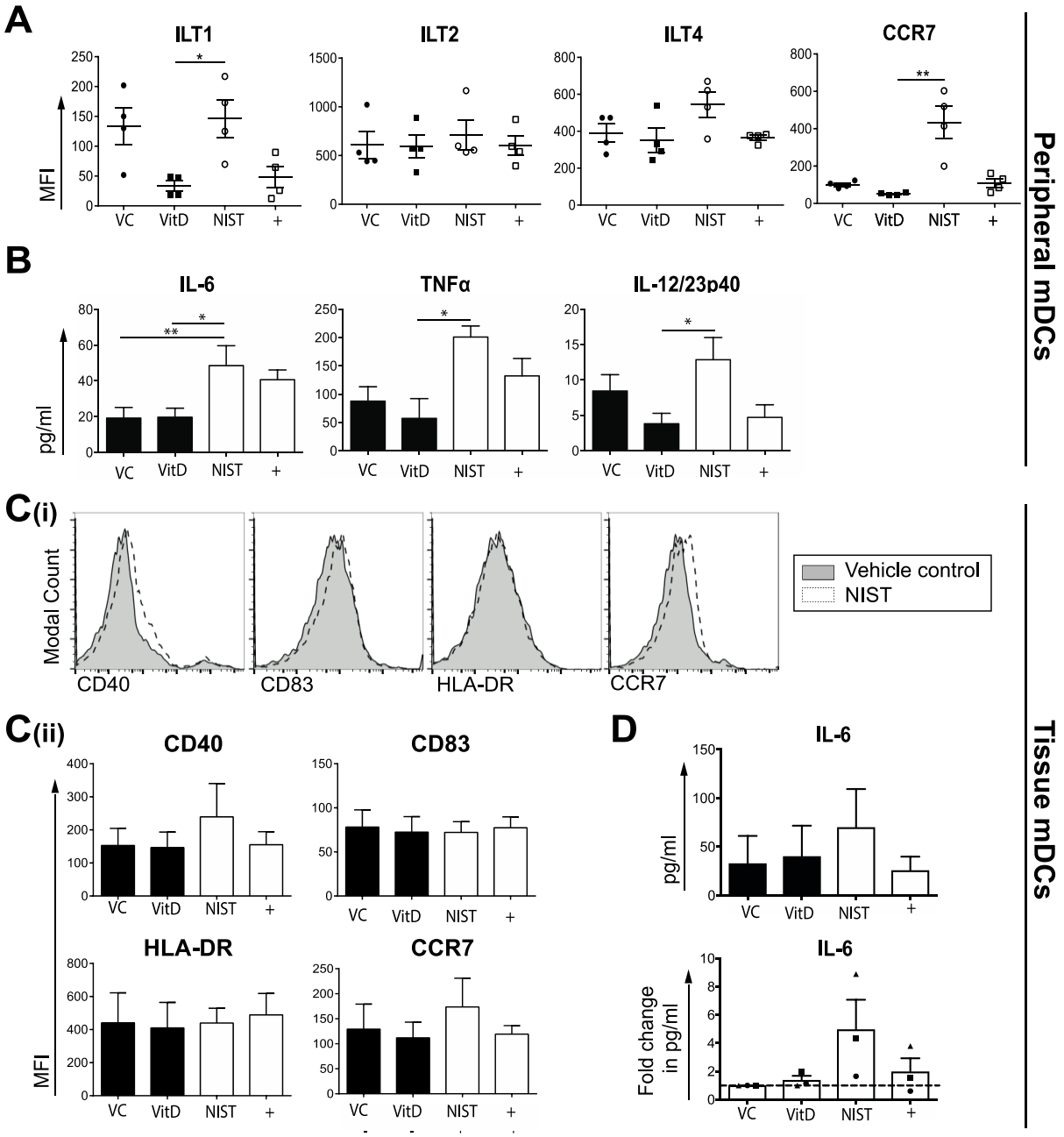

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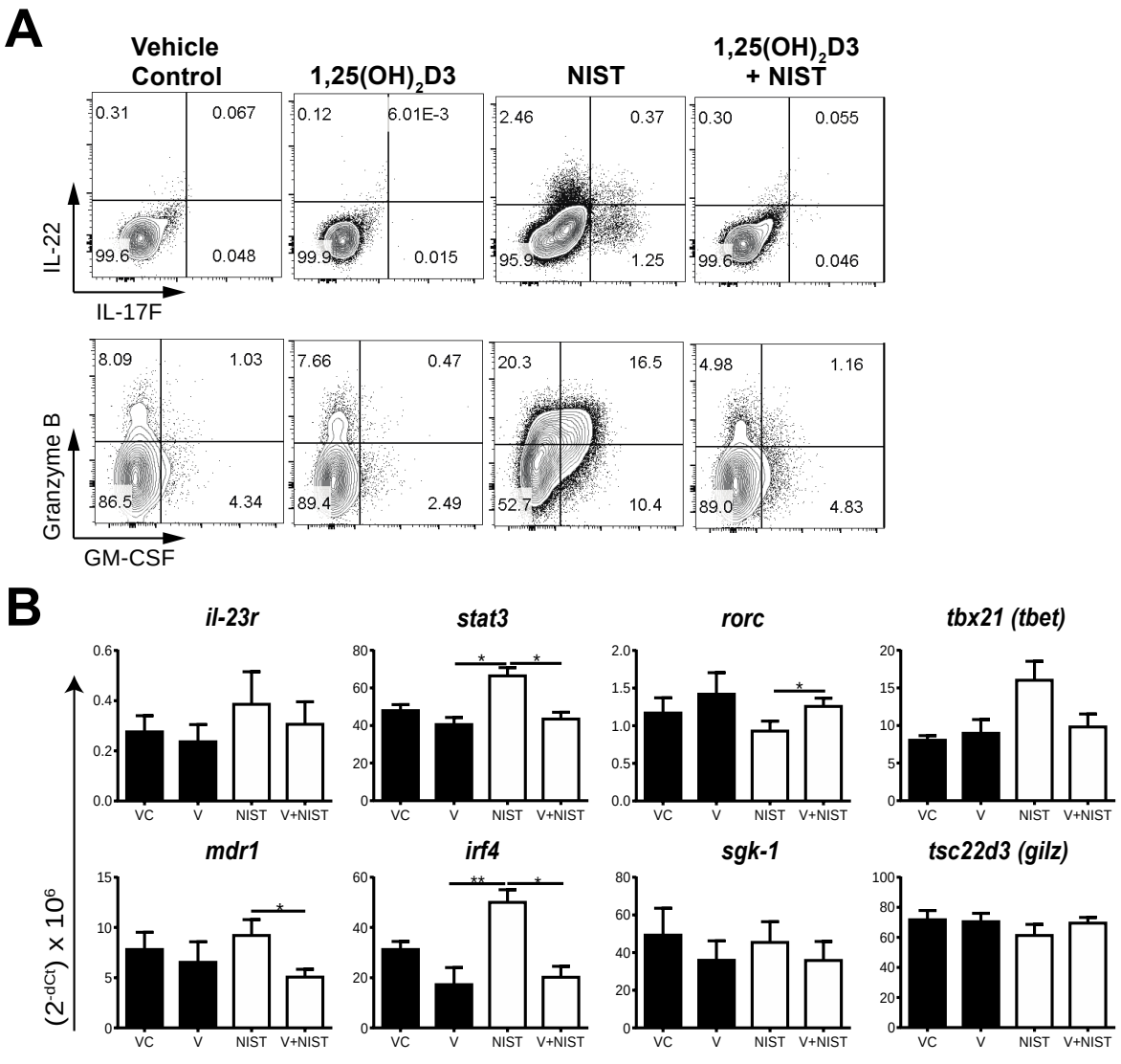

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