1   # ***Effects of vitamin D on inflammatory and oxidative stress***

2   # ***responses of human bronchial epithelial cells exposed to***

3   # ***particulate matter***

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5   **Running Title:** Vitamin D alters responses to urban particulate matter

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2

Abstract

Background: Particulate matter (PM) pollutant exposure, which induces oxidative stress and inflammation, and vitamin D insufficiency, which compromises immune regulation, are detrimental in asthma.

Objectives: Mechanistic cell culture experiments were undertaken to ascertain whether vitamin D abrogates PM-induced inflammatory responses of human bronchial epithelial cells (HBECs) through enhancement of antioxidant pathways.

Methods: Transcriptome analysis, PCR and ELISA were undertaken to delineate markers of inflammation and oxidative stress; with comparison of expression in primary HBECs from healthy and asthmatic donors cultured with reference urban PM in the presence/absence of vitamin D.

Results: Transcriptome analysis identified over 500 genes significantly perturbed by PM-stimulation, including multiple pro-inflammatory cytokines. Vitamin D altered expression of a subset of these PM-induced genes, including suppressing *IL6*. Addition of vitamin D suppressed PM-stimulated IL-6 production, although to significantly greater extent in healthy versus asthmatic donor cultures. Vitamin D also differentially affected PM-stimulated GM-CSF, with suppression in healthy HBECs and enhancement in asthmatic cultures. Vitamin D increased HBEC expression of the antioxidant pathway gene *G6PD*, increased the ratio of reduced to oxidised glutathione, and in PM-stimulated cultures decreased the formation of 8-isoprostane. Pre-treatment with vitamin D decreased CXCL8 and further decreased IL-6 production in PM-stimulated cultures, an effect abrogated by inhibition of G6PD with DHEA, supporting a role for this pathway in the anti-inflammatory actions of vitamin D.

Conclusions: In a study using HBECs from 18 donors, vitamin D enhanced HBEC antioxidant responses and modulated the immune response to PM, suggesting that vitamin D may protect the airways from pathological pollution-induced inflammation.

Introduction

Asthma is the most common chronic lung disease with globally increasing prevalence, implying the importance of environmental factors in its aetiology [1]. Vitamin D insufficiency/deficiency and ambient air pollution are two major environmental factors that appear to influence the pathogenesis and stability of asthma [2] [3] [4] [5], as well as other respiratory diseases [6] [7]. However there remains debate resulting from the heterogeneity of findings relating to the effects of these environmental factors on airway pathology [5] [8] [9] [10]. For example, European studies have shown heterogeneity between different cities in the magnitude of the effects of pollution on health outcomes such as hospital admissions for respiratory diseases [9] and asthma incidence [11], despite using standardised analyses. Environment-environment interactions are a major possible explanation for inconsistent results between different patient cohorts but have been little studied, particularly at the mechanistic level. In a recent meta-analysis, latitude of study location influenced associations between air pollutants and severe asthma exacerbations, and latitude is also known to affect sunlight-derived vitamin D production, although this association is complicated by other factors such as hours of daily skin exposure to sunlight [5]. In the urban environment Rosser and colleagues have shown that vitamin D insufficient children, but not those vitamin D sufficient, living close to major roads show an elevated risk of severe asthma exacerbations [12], although the mechanisms by which vitamin D may protect against pollution toxicity remain unclear and the interaction likely complex.

A growing body of research highlights the importance of epithelial immunology in asthma [13]. Evidence shows that inhaled ambient particulate matter (PM) adversely affects the bronchial epithelium through various mechanisms including the imposition of oxidative stress, which stimulates redox sensitive signalling pathways and drives the transcription of pro-inflammatory mediators relevant to asthma and other inflammatory lung diseases [14]. There is evidence that in asthma this pro-oxidant/pro-inflammatory action is superimposed

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92   on a background of oxidative stress. For example, Teng and colleagues have shown

93   concentrations of H_2O_2 are elevated in exhaled breath condensate of asthmatics compared

94   with controls, with concentrations increasing with asthma severity [15]. Mak and colleagues

95   have reported elevated plasma concentrations of 8-isoprostane, a lipid peroxide marker of

96   oxidative stress, during asthma exacerbations [16]. Indeed oxidative stress has been

97   implicated in many of the key pathophysiological features of asthma, including airways

98   hyper-responsiveness and mucus hypersecretion (reviewed by Zuo *et al.* [17], and Li *et al.*

99   [18]).

100

101   There is a large volume of research highlighting the anti-inflammatory effects of

102   vitamin D on the adaptive immune system [19] [20], but limited research as to the effects of

103   vitamin D on the human bronchial epithelium. Hansdottir and colleagues have shown vitamin

104   D to decrease production of pro-inflammatory cytokines by virally infected primary human

105   bronchial epithelial cells (HBECs) [21], but the capacity of vitamin D to affect the epithelial

106   response to urban particulate matter, consistent with it acting as a modifier of PM-induced

107   respiratory effects, to our knowledge has never been examined.

108

109   In view of this, we set out to examine whether vitamin D could abrogate urban PM-

110   induced pro-inflammatory responses in primary human bronchial epithelial cells. We elected

111   to commence with an unbiased, transcriptomic analysis with the objective of identifying PM-

112   induced pro-inflammatory cytokines that show distinct patterns of alteration by vitamin D *in*

113   *vitro*. We studied the epithelial response to both active 1α,25-dihydroxyvitamin D3

114   (1,25(OH)$_2$D3) but also to vitamin D in its circulating precursor form as 25-hydroxyvitamin D3

115   (25(OH)D3). To examine the effects in a broader human context we studied epithelial cells

116   from both patients with diagnosed asthma and those without. We hypothesised that HBECs

117   from asthmatic, as compared with healthy control subjects, would display an enhanced pro-

118   inflammatory cytokine response to ambient PM exposure. We further hypothesised that the

119   epithelial response to PM exposure could be favourably modified by vitamin D through

120   induction of antioxidant defences.

121

Materials and Methods

123

Materials

125   NIST SRM1648a Urban Particulate Matter (National Institute of Standards &

126   Technology, USA) was suspended at 500μg/ml in 5% methanol vehicle. SRM1648a is an

127   urban total particulate matter reference material with mean particle diameter 5.85μm that

128   was collected in the USA [22]. NIST SRM1648a in methanol vehicle, hereafter referred to as

129   NIST, was prepared as follows. Appropriate weights of dry powder particulate matter were

130   placed in a 50ml falcon tube to which 5% methanol (HPLC-grade) in Chelex-100 resin-

131   treated water was added. Following re-suspension and sonication on ice at an amplitude of

132   15 microns for 30 seconds using a Soniprep 150 plus probe sonicator (MES (UK) Ltd, UK),

133   the resultant suspension was then separated into 1ml aliquots with resuspension during the

134   aliquoting procedure to avoid PM sedimentation. The aliquots were stored at -70°C.

135   Separate aliquots of the 5% methanol vehicle alone were also used as a vehicle control

136   (VC). A NIST concentration of 50 μg/ml in primary HBEC cultures corresponded to a

137   theoretical surface deposition of 11 μg/ cm^2.

138   Ultra-high purity 1,25(OH)$_2$D3 (1α,25-dihydroxyvitamin D3; Enzo Life Sciences, UK)

139   was aliquoted dissolved in DMSO (Sigma-Aldrich, UK) at 100μM. Ultra-high purity 25(OH)D3

140   (25-hydroxyvitamin D3; Enzo Life Sciences, UK) was aliquoted dissolved in sterile absolute

141   ethanol at 1mM. Both were prepared in low light conditions and stored at -80°C prior to use.

142   Dehydroepiandrosterone (DHEA; Sigma-Aldrich, UK) was dissolved in absolute ethanol at

143   100mM. Poly(I:C) (Invivogen, USA) was dissolved in sterile H$_2$O at 1 mg/ml. Sulforaphane

144   (Sigma-Aldrich, UK) was dissolved in DMSO at 300μM.

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Primary human bronchial epithelial cell (HBEC) culture

Primary human bronchial epithelial cells (HBECs) were acquired from Lonza, Switzerland, and locally from endobronchial brushings / biopsies obtained at fibreoptic bronchoscopy with written informed consent of volunteers (Guy's Research Ethics Committee, South London REC Office 3, REC approval number 09/H0804/108, 16/03/2010) (Table 1).

Table 1. Characteristics of donors of HBECs cultured in this study.

Characteristic	Healthy (n=10)	Asthmatic (n=8)
from Lonza (n)	2	2
from local bronchoscopy (n)	8	6
Gender	6 male, 4 female	3 male, 5 female
Age Mean in years (Range)	30.4 (19 – 60)	34.9 (24 – 52)
% taking ICS *	-	83%
% taking LABA *	-	50%
% taking LTRA *	-	33%
Daily ICS Dose * Mean (Range) Beclomethasone equivalent	-	350 (0 – 1000) micrograms
Post-BD FEV1 % predicted * Mean (Range)	105% (97% - 113%)	111% (86% - 144%)

BD, bronchodilator. ICS, inhaled corticosteroid. LABA, inhaled long-acting beta-2 agonist. LTRA, leukotriene receptor antagonist. * % data only for donors enrolled locally (Lonza HBEC donors, for whom these data are not available, excluded).

157

158   For locally collected samples, non-smoking volunteers were phenotyped as atopic or

159   non-atopic based upon skin-prick test results to a standard panel of aeroallergens, and as

160   healthy or asthmatic, the latter based on clinical history and confirmation by lung function

161   testing (documented variability in PEF/FEV$_1$ of 12% or more in past year, or positive

162   metacholine / mannitol challenge if diagnosis uncertain).

163   Cells cultures were incubated in flasks in Bronchial Epithelial Cell Growth Medium

164   (BEGM) – constituted by Bronchial Epithelial Basal Medium (BEBM; Lonza, Switzerland) with

165   SingleQuot Supplements (Lonza, Switzerland) of Bovine Pituitary Extract, Insulin,

166   Hydrocortisone, Gentamicin and Amphotericin-B, Retinoic Acid, Transferrin, Triiodothyronine,

167   Epinephrine, and human Epidermal Growth Factor. In later experiments 1% Penicillin-

168   Streptomycin solution (Sigma-Aldrich, UK) and 1% Nystatin suspension (Sigma-Aldrich, UK)

169   were added to passage 0 and passage 1 cultures. Medium was changed every 2 to 3 days.

170   For samples grown from brushings: at bronchoscopy multiple brushings with an

171   endobronchial brush were made to collect epithelial cells from 10 areas of the bronchial

172   mucosa of normal appearance. The detached cells were washed with warmed BEGM and

173   re-suspended in flasks of warmed BEGM then incubated at 37°C with 5% CO_2. The medium

174   was changed on the next day and the passage 0 cultures were then subcultured as

175   described.

176   For samples grown from biopsies: small fragments of endobronchial biopsy with any

177   visible smooth muscle removed by micro-dissection were placed in a universal tube

178   containing 6ml warmed BEGM cell culture medium. Samples were treated with Liberase TL

179   Research Grade (Roche, USA) collagenase (final concentration 62.5 µg/ml) for one hour at

180   37°C then centrifuged twice in BEGM medium to wash off collagenase before transfer to a

181   cell culture flask and incubation at 37°C with 5% CO_2. The medium was changed on the

182   next day and the passage 0 cultures were then subcultured as described.

8

183   Cell cultures once near-confluent were passaged by detachment of cells using the

184   recommended Trypsin Subculture ReagentPack (Lonza, Switzerland), centrifugation and

185   then re-suspension in fresh BEGM in flasks for further passage or flat-bottomed cell culture

186   plates for experiments.

187   Flasks and culture plates for primary HBECs obtained locally at bronchoscopy were

188   collagen coated before use – collagen solution was added to each flask / well base and

189   incubated for 2 hours before washing 3 times with sterile H_2O. Collagen solution comprised

190   of 20µl type 1 calf skin collagen reagent (Sigma-Aldrich, UK) per ml 0.02M acetic acid in

191   sterile H_2O.

192   For experiments, once cultures were near-confluent medium was changed to BEGM

193   containing all SingleQuot Supplements except Bovine Pituitary Extract, Retinoic Acid and in

194   later experiments also excluding Hydrocortisone. Bovine Pituitary Extract was removed as it

195   has been found to contain proteins that can provide exogenous protection against oxidative

196   stress [23]. Bovine Pituitary Extract can also contain low concentrations of vitamin D [24].

197   Retinoic acid was removed as there is some evidence that it may compete with / antagonise

198   vitamin D [25]. For experiments examining effect of vitamin D pre-treatment, 25(OH)D3 was

199   added at 100nM final concentration with/without 100µM DHEA to appropriate wells. After a

200   further 24 hours, cell cultures were stimulated using fresh BEGM (excluding relevant

201   SingleQuot Supplements as above) with stimulation (50µg/ml NIST, 1µg/ml Poly(I:C)

202   with/without 100nM 1,25(OH)D$_2$3, 100nM 25(OH)D3 and/or 100µM DHEA) as appropriate.

203   Culture supernatants and lysed cell monolayers were harvested or other assays conducted 4

204   hours or 24 hours after stimulation of cell cultures. Cell culture experiments were conducted

205   with triplicate wells for each condition in each experiment to allow for variation in plating

206   density with primary cells. For experiments epithelial cells were used between passage 3 and

207   passage 5.

208

Gene transcription microarray

210   Total RNA was extracted from HBECs cultured for 24 hours stimulated with 50 μg/ml

211   NIST and unstimulated, in the presence and absence of 100nM 1,25(OH)$_2$D3, from four

212   different donors (two healthy and two asthmatic). Cell culture monolayers were lysed with

213   Qiazol reagent (QIAGEN, USA) then homogenised with QIAshredder columns before storage

214   at -80°C pending extraction of total RNA using a miRNeasy Mini Kit (QIAGEN, USA)

215   according to an adapted manufacturer's protocol with an off-column DNA digest with

216   TurboDNase (Ambion, USA). mRNA was quantified using a Qubit Fluorimeter (Invitrogen,

217   USA) and quality controlled using an Agilent 2100 Bioanalyser (Agilent Technologies, USA).

218   Samples were prepared for array analysis using SuperScript III Reverse Transcriptase

219   (Invitrogen, USA) and TargetAmp Nano-g Biotin-aRNA Labelling Kit (Epicentre, Illumina,

220   USA). The microarray was conducted on Illumina HT-12v4 Expression BeadChips (Illumina,

221   USA) on an iScan platform (Illumina, USA). Raw array signal intensities were processed in

222   GenomeStudio (Illumina, USA) with a quantile normalisation before export for analysis in

223   GenomicsSuite (Partek, USA). ANOVA analysis was conducted of the normalised data in

224   GenomicsSuite (Partek, USA).

225

226 Real-time quantitative Polymerase Chain Reaction (qPCR) for

227 measurement of gene mRNA expression

228   Total RNA was extracted as above and quantified using a NanoDrop ND-1000

229   Spectrophotometer (Thermo Scientific, USA) and then reverse transcribed to cDNA using

230   RevertAid Reverse Transcriptase and complementary reagents (Fermentas, Thermo

231   Scientific, USA). Relative Quantification (RQ) of target genes relative to *18S* rRNA house-

232   keeping gene was conducted in triplicates by real-time quantitative polymerase chain

233   reaction (qPCR) using Taqman Universal PCR MasterMix (Applied Biosystems, USA), and

234   an Applied Biosystems Viia 7 real-time thermal cycler. Results were analysed using Viia 7

235   software (Applied Biosystems, USA). Taqman primers were purchased from Applied

236   Biosystems, USA (S1 Table). Relative expression of mRNA was corrected for efficiency of

237 amplification using the Pfaffl method [26]. In experiments comparing different culture

238 stimulations expression of genes was analysed relative to both *18S* and the unstimulated

239 control cultures (RQ$_{Unstimulated}$).

240

Cytokine protein measurement

242 Culture supernatants from individual wells were stored at -20°C pending

243 measurement of secreted proteins from individual samples. Concentrations of cytokines

244 were measured in culture supernatants by Cytometric Bead Array (CBA; BD Biosciences,

245 USA). Supernatants were incubated for 3 hours with CBA capture beads before beads were

246 washed and then incubated for 2 hours with CBA detection reagent followed by bead sample

247 analysis as per manufacturer's instructions. Cytokine levels were measured separately for

248 each of the triplicate wells in primary HBEC cultures and then mean averaged before further

249 analysis.

250

Oxidative stress assays

252 8-Isoprostane ELISA: Cell culture supernatant, pooled from triplicate wells, was

253 collected into tubes containing desferrioxamine (DFO) and butylated hydroxytoluene (BHT)

254 and stored at -80°C pending assay. 8-isoprostane was assayed using a commercial ELISA

255 kit (Cayman Chemical, Michigan, USA) according to the manufacturers protocol.

256 Glutathione Assay: Intracellular total and oxidized glutathione were assessed using

257 the GSH-GSSG-Glo Assay Kit (Promega, Wisconsin, USA) using an adapted protocol. The

258 protocol was adapted as the un-adapted protocol resulted in readings outside the range of

259 the luminometer standard curve. Near-confluent HBECs were cultured for 24 hours with

260 100nM 25(OH)D3, with 6 wells per condition per experiment, in phenol-red free Airway

261 Epithelial Cell Medium (Promocell, Germany) with all supplements except bovine pituitary

262 extract, retinoic acid and hydrocortisone. After removal of supernatant, one triplicate of wells

263 for each condition was lysed with kit passive lysis buffer (for assay of total glutathione) and

11

264   the second triplicate for each condition was lysed with kit lysis buffer containing N-

265   ethylmaleimide (for assay of oxidized glutathione). The 50µl lysate in each well was then

266   diluted by addition of 150µl dH$_2$O, mixed on an orbital shaker, before 40µl of diluted lysate

267   was transferred to white-sided (flat transparent-bottom) 96 well assay plates. A glutathione

268   standard curve in lysis buffer was prepared according to kit protocol and 40µl added to

269   appropriate wells. 10µl of prepared Luciferin-NT reagent was then added to each well and

270   the plate incubated on an orbital shaker for 5 minutes. The assay was then completed

271   according to the manufacturer's protocol. A solution of glutathione-S-transferase,

272   dithiothreitol (DTT) and buffer was added to each well and the plate incubated for 30

273   minutes. Finally, a luciferase containing reagent solution was then added to each well and

274   the plate incubated for 20 minutes prior to reading well luminescence with a GloMax plate

275   luminometer (Promega, Wisconsin, USA). For each condition in each experiment, the

276   concentration of reduced glutathione was calculated by subtracting the mean concentration

277   in the oxidized glutathione triplicate from the mean concentration in the total glutathione

278   triplicate.

279

280   **Statistical Analysis**

281   GraphPad Prism 6.0 (GraphPad Software, USA) was used for all statistical tests

282   except for the microarray data. Unless otherwise stated in figure legends, data are presented

283   as mean +/- standard deviation (SD) for normally-distributed data and as box-and-whisker

284   plots showing median, inter-quartile and absolute ranges for non-parametric data. Unless

285   otherwise stated, non-parametric data were analysed using Friedman Tests with Dunn's

286   multiple-comparisons test and parametric data by repeated-measures ANOVA with

287   Bonferroni corrected post-tests. Significant results shown in figures as follows: *, $p \leq 0.05$;

288   **, $p \leq 0.01$; ***, $p \leq 0.001$. In some experiments results from HBEC cultures from healthy

289   (both atopic and non-atopic) and asthmatic subjects were combined to better analyse the

290   effects of PM and vitamin D across a larger and broader population of individuals.

12

291

Results

293

Effects of vitamin D on expression of pro-inflammatory cytokine genes by HBECs exposed to particulate matter

296   In initial studies to investigate vitamin D mediated inhibition of the pro-inflammatory

297   effects of particulate matter, a gene transcription microarray was conducted on HBECs

298   stimulated with PM for 24 hours, using cultures from four adult donors. Expression of 510

299   genes was altered (fold-change ≥ ± 1.4) upon stimulation with 50 µg/ml NIST SRM1648a, a

300   reference preparation of urban PM, hereafter referred to as NIST (full microarray data

301   available in S1 Data Appendix). This concentration of NIST had previously been ascertained

302   to induce significantly increased production of pro-inflammatory cytokines after 24 hour cell

303   culture. Of these genes, 49 also showed evidence of regulation (fold-change ≥ ± 1.4) in the

304   presence of the active form of vitamin D, $1,25(OH)_2D3$, at a concentration of 100nM (Fig 1A,

305   S2 Table). Expression of the pro-inflammatory cytokine genes *IL6*, *CXCL10* and *IL24* was

306   induced by NIST, but suppressed by the addition of vitamin D (Fig 1B). Expression of other

307   pro-inflammatory cytokine genes, for example *IL8*, was induced by NIST, but not altered by

308   addition of vitamin D. In contrast, NIST suppressed and vitamin D increased the expression

309   of *TGFB2*. As previously reported [27], vitamin D induced expression of *IL1RL1* but

310   interestingly this was also induced by NIST stimulation.

311

312   **Fig 1. Transcription microarray of 24 hour primary human bronchial epithelial cell**

313   **(HBEC) cultures stimulated with NIST in the presence/absence of vitamin D.**

314   (A)   Volcano plot of the 510 genes with ≥ 1.4 fold differential expression comparing

315   50µg/ml NIST stimulated to unstimulated 24 hour cultures (n=4), showing fold-change in

316   gene expression in NIST stimulated cultures in the presence vs absence of 100nM

317   1,25(OH)$_2$D3 (horizontal axis) plotted again probability of statistical significance for that fold-

318   change (vertical axis).

319   (B)   Plot showing microarray results for fold-change in expression of all cytokine genes

320   upon stimulation of HBECs with 50μg/ml NIST (horizontal axis) and fold-change in gene

321   expression upon addition of 100nM 1,25(OH)$_2$D3 to NIST-stimulated cultures (vertical axis).

322

323   The microarray findings were validated by quantitative real-time PCR using HBEC

324   cultures from a larger number of donors with expression of cytokine genes of interest studied

325   in both 4 hour and 24 hour cultures (Fig 2). Exposure of HBECs to NIST *in vitro* significantly

326   upregulated expression of mRNA encoding *IL6, IL8, IL24* and *CXCL10* after 4 hours and 24

327   hours of culture. Expression of *CSF2* was significantly increased by NIST stimulation at 24

328   hours but not significantly at 4 hours. Co-culture with 100 nM 1,25(OH)$_2$D3 reduced *IL6,*

329   *IL24* and *CXCL10* expression at both time points although the effect of vitamin D on *CXCL10*

330   expression at 24 hours did not attain statistical significance. NIST-induced *IL8* expression

331   was not significantly altered by vitamin D at 4 hours but was decreased by 1,25(OH)$_2$D3 at

332   24 hours.

333

334   **Fig 2. Confirmation by qPCR of the effect of NIST and vitamin D on expression of**

335   **cytokine genes in HBEC cultures.**

336   (A)   Gene mRNA expression at 4 hours relative to *18S* and the unstimulated Control

337   condition in HBEC cultures stimulated with 50μg/ml NIST in the presence/absence of 100nM

338   1,25(OH)$_2$D3. *IL6*, n=7; *IL8*, n=7; *IL24*, n=6; *CFS2*, n=8; *CXCL10*, n=6; *TGFB2*, n=6.

339   (B)   mRNA expression at 24 hours. *IL6*, n=9; *IL8*, n=7; *IL24*, n=6; *CFS2*, n=8; *CXCL10*,

340   n=6 (one outlying replicate excluded); *TGFB2*, n=6. All repeated-measures ANOVAs and

341   post-tests with Bonferroni corrections as shown. VC; vehicle control for NIST. Statistical

342   significance as follows: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.

343

Comparison of the effects of vitamin D on PM-stimulated epithelial cytokine production by HBECs from healthy and asthmatic individuals *in vitro*

Production by HBECs of the pro-inflammatory cytokines IL-6, CXCL8 and GM-CSF (encoded by the genes *IL6, IL8* and *CSF2* respectively) was chosen for further scrutiny given that all three mediators are featured in asthma pathophysiology and their expression was induced by NIST-stimulation, but differently affected by the additional presence of vitamin D. Culture of primary HBECs from 14-17 donors per experiment (approximately equal numbers of healthy and asthmatic subjects) for 24 hours in the presence of 50 µg/ml NIST significantly increased the supernatant concentrations of IL-6, CXCL8 and GM-CSF as compared with vehicle control, consistent with the gene expression data (Fig 3A). In the additional presence of 100nM $1,25(OH)_2D3$ throughout the 24 hour stimulation, production of IL-6 was significantly inhibited, but that of CXCL8 or GM-CSF not significantly altered (Fig 3A).

Fig 3. Effects of $1,25(OH)_2D3$ on production of IL-6, CXCL8 and GM-CSF by NIST stimulated HBEC cultures.

(A)   Addition of 100nM $1,25(OH)_2D3$ reduced production of IL-6 by primary HBEC cultures stimulated for 24 hours with 50µg/ml NIST, but not CXCL8 or GM-CSF. VC: NIST vehicle control. n=14-17.

(B)   Fold-increase in production of IL-6, CXCL8, and GM-CSF above that in unstimulated cultures upon stimulation with 50µg/ml NIST in the presence/absence of $1,25(OH)_2D3$, by disease status. Two-way ANOVAs with Bonferroni-corrected post-tests. n=8-9 healthy, n=7-8 asthmatic.

(C)   Percentage suppression by $1,25(OH)_2D3$ of cytokine production in PM-stimulated cultures of HBECs from healthy donors compared to asthmatic donors. Un-paired t-tests. n=8-9 healthy, n=7-8 asthmatic. Statistical significance as follows: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$, ****, $p \leq 0.0001$.

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371

372   Spontaneous production of all three of these cytokines by HBECs from both the non-

373   diseased and asthmatic donors varied considerably between individuals, with no significant

374   difference between the asthmatic group and healthy group (S1 Fig). To allow for this inter-

375   individual variation, NIST responses were also examined in terms of fold-changes above

376   spontaneous production in unstimulated cultures. Contrary to our hypothesis, NIST-induced

377   fold-increases in cytokine production were not significantly greater in HBECs from asthmatic

378   compared to healthy subjects in the absence of vitamin D (Fig 3B).   Nevertheless,

379     $1,25(OH)_2D3$ exhibited a significantly greater capacity to suppress NIST-stimulated IL-6

380   production by HBECs from the non-diseased, compared with the asthmatic donors (Fig 3C).

381   There was also a significant difference in the effect of $1,25(OH)_2D3$ on NIST-induced GM-

382   CSF production, with suppression in cell cultures from the non-diseased donors, but

383   enhancement in those from the asthmatic donors. As a result, in NIST-stimulated vitamin D

384   treated cultures induction of IL-6 and GM-CSF production was significantly greater in

385   asthmatic than in healthy HBECs (Fig 3B). There was no significant difference in the effect

386   of vitamin D on CXCL8 production by HBECs from the non-diseased and asthmatic donors.

387

388   We examined the possibility that these differences in the effects of $1,25(OH)_2D3$ on

389   IL-6 and GM-CSF protein production reflected differences in early induction of cytokine gene

390   expression. No significant differences were evident between cultures of HBECs from healthy

391   and asthmatic donors in NIST-stimulated induction of *IL6* or *CSF2* at 4 hours and 24 hours,

392   compared in the presence and absence of $1,25(OH)_2D3$ (two-way ANOVA analyses, S2 Fig).

393   Additionally, gene expression was compared with the capacity of vitamin D to suppress

394   cytokine production. A proportion of HBEC donor cultures showed greater induction of *CSF2*

395   at 4 hours with less suppression of GM-CSF production at 24 hours by vitamin D (S2 Fig),

396   and notably these were from asthmatic donors. A similar pattern was not evident for IL-6 (S2

397   Fig).

398

Comparison of capacity for 25(OH)D3 and 1,25(OH)$_2$D3 to modulate HBEC cytokine responses

401   Exposure of HBECs to NIST (50 µg/ml, 24 hours) significantly increased the

402   expression of the gene *CYP27B1* (S3 Fig), encoding the cytochrome P450 enzyme that

403   converts 25(OH)D3 to active 1,25(OH)$_2$D3.   However, the magnitude of induction of

404   *CYP27B1* with NIST exposure was significantly lower than that observed with the established

405   TLR3 agonist Poly(I:C) (mean (SD) 1.2 (0.22) fold induction with NIST compared to 5.4 (3.3)

406   fold induction with TLR3 agonist) (S3 Fig).   Similarly, exposure to NIST modestly, but

407   significantly, increased expression of *VDR*, encoding the vitamin D receptor (S3 Fig).

408   Concordantly, both 25(OH)D3 and 1,25(OH)$_2$D3 at 100nM concentrations exerted similar

409   effects on cytokine expression by NIST-stimulated HBECs (S3 Fig).

410

411   Given the distinct effects of vitamin D on cytokine gene expression in HBECs from

412   healthy compared to asthmatic donors, we were interested to investigate whether this

413   reflected differences in their capacity to respond to vitamin D.   We therefore assessed

414   expression of *CAMP*, the gene encoding cathelicidin, and *CYP24A1;* two genes known to be

415   strongly induced by 1,25(OH)$_2$D3 (Fig 4).   No significant differences were apparent between

416   cells from healthy and asthmatic donors in the capacity of 100nm 1,25(OH)D$_2$3, in the

417   presence of NIST, to induce increased expression of *CYP24A1* or *CAMP* relative to

418   unstimulated controls (Fig 4A,C).   Similarly expression of the target genes relative to *18S* in

419   the stimulated condition were not significantly different between cultures from healthy and

420   asthmatic donors (Figs 4B,D).

421

422   **Fig 4. Comparison of expression of vitamin D axis genes between HBECs from healthy**

423   **and asthmatic donors.**

424   (A) Induction of *CYP24A1* by NIST with 100nM 1,25(OH)$_2$D3, relative to the unstimulated

425   control condition, in 4 hour and 24 hour HBEC cultures. H, Healthy donor HBECs; A,

426   Asthmatic donor HBEC cultures. 4hr healthy, n=5; 4hr asthmatic, n=5; 24hr healthy, n=7;

427   24hr asthmatic, n=6.

428   (B) Expression of *CYP24A1* as measured by qPCR relative to *18S* in 4 hour and 24 hour

429   HBEC cultures stimulated with 50µg/ml NIST and 100nM 1,25(OH)$_2$D3. 4hr healthy, n=5; 4hr

430   asthmatic, n=5; 24hr healthy, n=7-8; 24hr asthmatic, n=6.

431   (C) Induction of *CAMP* by NIST with 100nM 1,25(OH)$_2$D3, relative to the unstimulated control

432   condition, in 4 hour and 24 hour HBEC cultures. 4hr healthy, n=5; 4hr asthmatic, n=5; 24hr

433   healthy, n=5; 24hr asthmatic, n=6.

434   (D) Expression of *CAMP* as measured by qPCR relative to *18S* in 4 hour and 24 hour HBEC

435   cultures stimulated with 50µg/ml NIST and 100nM 1,25(OH)$_2$D3. 4hr healthy, n=5; 4hr

436   asthmatic, n=5; 24hr healthy, n=7; 24hr asthmatic, n=6.

437

438   **Effects of vitamin D on HBEC oxidative stress and antioxidant**

439   **responses**

440   We next sought to establish whether the anti-inflammatory effects of vitamin D

441   following PM challenge were related to its upregulation of antioxidant pathways. Culture of

442   HBECs with 100nM 25(OH)D3 for 24 hours significantly increased the intracellular ratio of

443   reduced to oxidised glutathione concentrations (GSH:GSSG; Fig 5A), and in NIST-stimulated

444   cultures 100nM 25(OH)D3 significantly decreased the production of the lipid oxidation

445   product 8-isoprostane (Fig 5B). In further support of an antioxidant action of vitamin D, the

446   antioxidant enzyme-inducing compound sulforaphane similarly suppressed production of IL-

447   6, but not CXCL8 or GM-CSF (Fig 6) in NIST-stimulated cultures.

448

449   **Fig 5. Effect of 25(OH)D3 on antioxidant responses in primary HBEC cultures.**

450   (A)   Ratio of reduced (GSH) to oxidised (GSSG) glutathione in 24 hour cultures of HBECs

451   treated with 100nM 25(OH)D3; n=6.

452   (B)   Fold-increase in 8-isoprostane levels in culture supernatants from primary HBECs

453   cultured with 50µg/ml NIST with/without 100nM 25(OH)D3 for 24 hours, compared to VC

454   control cultures; ratio paired t-test, n=8. Statistical significance as follows: *, $p \leq 0.05$.

455

456   **Fig 6. Effect of antioxidant enzyme-inducing sulforaphane on production of IL-6,**

457   **CXCL8 and GM-CSF by NIST-stimulation HBECs.**

458   Cytokines produced by primary HBECs in submerged cultures for 24 hours stimulated with

459   NIST at 50µg/ml and/or sulforaphane at 3µM. Friedman's tests with Dunn's multiple

460   comparisons tests; n=4.   Statistical significance as follows: **, $p \leq 0.01$.

461

462   The transcriptomic analysis described above revealed that expression of the gene

463   *G6PD*, which encodes the key antioxidant enzyme glucose-6-phosphate dehydrogenase

464   (G6PD) [28], was significantly upregulated by vitamin D (1.82 fold-increase for NIST +

465     $1,25(OH)_2D3$ compared to NIST alone; Fig 1).   The capacity of $1,25(OH)_2D3$ to elevate

466   expression of *G6PD* was confirmed by qPCR in HBEC cultures treated with 100 nM

467     $1,25(OH)_2D3$ for 4 hours and 24 hours (both $p<0.001$; Fig 7A and B).   25(OH)D3 similarly

468   enhanced *G6PD* expression in a concentration-dependent manner (Fig 7B).   There were no

469   significant differences between asthmatic and healthy donor cultures in spontaneous

470   expression of *G6PD* in unstimulated cultures at 4 hours and 24 hours (Fig 7C).   Additionally

471   there were no significant differences in the capacity of NIST with/without $1,25(OH)_2D3$ to

472   induce expression of *G6PD* above that in unstimulated cultures (Fig 7D) in HBECs from

473   asthmatics compared to healthy donors.

474

475   **Fig 7: Capacity of vitamin D to enhance primary HBEC expression of *G6PD*.**

476   (A)   Expression of *G6PD* in 4 hour cultures of primary HBECs stimulated with 50µg/ml

477   NIST with/without 100nM $1,25(OH)_2D3$ (n=6).

19

478   (B)   Expression of *G6PD* in 24 hour cultures of primary HBECs stimulated with 50ug/ml

479   NIST with/without 100nM 1,25(OH)$_2$D3 (n=6) or a concentration series of 25(OH)D3, n=5.

480   (C)   Expression of *G6PD* as measured by qPCR relative to *18S* in unstimulated 4 hour

481   and 24 hour HBEC cultures. H, Healthy donor HBECs; A, Asthmatic donor HBEC cultures.

482   4hr healthy, n=5; 4hr asthmatic, n=5; 24hr healthy, n=8; 24hr asthmatic, n=6.

483   (D)   Induction of *G6PD* by 50μg/ml NIST in the presence / absence of 100nM

484   1,25(OH)$_2$D3 in 4 hour and 24 hour cultures of HBECs from healthy and asthmatic donors.

485   4hr healthy, n=5; 4hr asthmatic, n=5; 24hr healthy, n=6; 24hr asthmatic, n=6. Statistical

486   significance as follows: *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001; ****, p ≤ 0.0001.

487

Assessing the capacity of vitamin D pre-treatment to suppress pro-inflammatory cytokine production by NIST-stimulated HBECs

490   Since greater induction by vitamin D of *G6PD* was evident in the HBECs at 24 hours

491   compared with 4 hours in the preceding experiments, we finally investigated whether 24

492   hours pre-treatment of HBEC cultures with vitamin D prior to particulate challenge would

493   further augment its capacity to decrease production of pro-inflammatory cytokines.

494   Accordingly, we compared the effects of concurrent treatment with 100 nM 25(OH)D3 (as in

495   the previous experiments above) with those of additional treatment of the HBECs with 100

496   nM 25(OH)D3 for 24 hours prior to exposure to NIST-stimulation (Fig 8A). Additional vitamin

497   D pre-treatment of NIST-stimulated HBECs significantly increased the percentage

498   suppression of IL-6 production (Fig 8A). Vitamin D pre-treatment also suppressed NIST-

499   induced CXCL8 production, a phenomenon not observed when vitamin D was added only

500   concurrently with NIST. However, suppression of IL-6 production (44.78%, 18.45% - 71.10%

501   [mean, 95% confidence interval]) was greater than that of CXCL8 (22.36%, -1.42% -

502   46.14%). In contrast, there was no significant effect on NIST-induced GM-CSF production.

503

504   The possible contribution of enhanced expression of G6PD to this phenomenon was

505   assessed using the G6PD inhibitor dehydroepiandrosterone (DHEA).   DHEA at a

506   concentration of 100 μM significantly abrogated the ability of 100nM 25(OH)D3, applied for

507   24 hours in advance and with NIST stimulation, to suppress CXCL8 production in NIST-

508   stimulated HBEC cultures with evidence of a trend towards decreasing suppression of IL-6

509   (Fig 8B).

510

511   **Fig 8: Effect of vitamin D pre-treatment of HBEC cultures on suppression of NIST**

512   **induced IL-6 and CXCL8.**

513   (A)   Percentage suppression of cytokine production in 50μg/ml NIST stimulated 24 hour

514   cultures by concurrent and additional 24 hour pre-treatment with 100nM 25(OH)D3

515   compared to concurrent only; n=7-9 (3-4 Healthy, 4-5 Asthmatic). Two-tailed paired t-tests.

516   (B)   Percentage suppression of cytokine production by 100nM 25(OH)D3 concurrent and

517   pre-treatment in 50μg/ml NIST stimulated HBEC cultures, in the absence or presence of the

518   G6PD inhibitor DHEA at 100μM; n=7-9 (2-3 Healthy, 5-6 Asthmatic). Two-tailed paired t-

519   tests. Statistical significance as follows: *, $p \leq 0.05$; **, $p \leq 0.01$.

520

521   # Discussion

522   Particulate matter air pollution and vitamin D deficiency are environmental factors that

523   have both been implicated in the current 'epidemic' of asthma [1] [7] [19]. There is increasing

524   appreciation of the importance of the interaction between multiple environmental factors,

525   defining an individual's total exposome, in determining health effects, but to date the

526   interactive effect of vitamin D on responses to air pollution has received little attention [5] [29]

527   [30]. In the present study we demonstrate that vitamin D abrogates pro-inflammatory effects

528   of urban PM on primary human bronchial epithelial cells, suppressing production of key PM-

529   stimulated pro-inflammatory cytokines, such as IL-6, through a mechanism at least in part

530   dependent on enhancement of antioxidant pathways by vitamin D. Although epithelial cells

21

531   from both healthy and asthmatic donors were responsive to vitamin D, the capacity for

532   vitamin D to suppress PM-induced production of pro-inflammatory cytokines was impaired in

533   cells from patients with asthma compared to healthy subjects in this study. A microarray was

534   employed to generate an unbiased depiction of the effects of these environmental factors on

535   epithelial cytokine responses and additionally identified further vitamin D promoted genes

536   such as *G6PD* and *TGFB2*.

537

538   Multiple pro-inflammatory cytokines were induced by PM-stimulation, however, the

539   effect of vitamin D on cytokine responses was not uniform with *IL6*, *CXCL10* and *IL24*

540   transcripts suppressed by vitamin D, whereas others such as *IL8* and *CSF2* were not

541   similarly inhibited. We subsequently focused on IL-6, CXCL8 and GM-CSF as these

542   cytokines are well-established to be up-regulated by PM exposure, as well as being

543   implicated in the pathogenesis of asthma, with elevated concentrations seen in lungs of

544   asthmatic individuals [31] [32] [33] [34]. IL-6 is implicated in systemic inflammation [35] [36]

545   [37]. CXCL8 (IL-8) has a major role in neutrophil chemotaxis and activation, with also

546   evidence for a role in eosinophil chemotaxis [38]. GM-CSF is a known HBEC-derived

547   mediator that enhances the pro-inflammatory effects of PM on dendritic cells (DCs) [39] and

548   promotes eosinophil responses [40], but may protect against oxidative injury under a variety

549   of situations [41] [42].

550

551   Vitamin D suppressed PM-induced IL-6 production to a greater extent in cultured

552   epithelial cells from healthy as compared with asthmatic donors. In contrast, in cultures from

553   asthmatic donors vitamin D modestly increased GM-CSF production. Why healthy and

554   asthmatic HBECs in this study responded differently to vitamin D is unclear, and future

555   research is needed to address this issue and verify findings in a larger cohort.

556   Nevertheless, this novel finding suggests that future research studying the role of vitamin D

557   in disease pathology should be conducted using primary cells from individuals with those

558   diseases given the important differences apparent between the vitamin D responses of cells

559   from healthy and diseased donors.

560

561   Vitamin D exhibits both inhibitory and inductive effects of importance in chronic

562   airway diseases and is therefore likely to influence the response of the lung to pollution

563   exposure. For example, any action of vitamin D to decrease PM-stimulated IL-6 production

564   is likely to be clinically relevant and beneficial. Higher concentrations of IL-6 have been

565   shown in induced sputum from asthmatic patients compared to controls [31], in the plasma of

566   asthmatic patients [43], and a genome-wide association study has previously highlighted the

567   IL-6 receptor gene locus as a possible risk locus for asthma [44]. *In vivo* human exposure to

568   PM has been shown to increase both airway and systemic concentrations of IL-6, with

569   associated acute neutrophilic inflammation [35] [45]. IL-6 can stimulate proliferation of

570   lymphocytes [46], inhibit the action of regulatory T lymphocytes (Tregs) [37] and conversely

571   enhance Th17/IL-17 synthesis [47], which is associated with neutrophilic inflammation and

572   steroid insensitivity in asthma [48]. Elevated IL-17A has been reported with both *in vitro* and

573   *in vivo* particulate matter exposure [49] [50]. Furthermore, the benefit of reducing pollution-

574   induced IL-6 is likely broader than its effect on airway inflammation, since pollution-induced

575   vascular dysfunction and increased blood coagulation have been shown to be IL-6

576   dependent in murine models [35] [36].

577   In contrast vitamin D up-regulated expression of *TGFB2*, a cytokine with the capacity

578   to both inhibit inflammation and promote wound healing/fibrosis, with PM having the

579   reciprocal effect. Whilst PM-stimulation creates an immune microenvironment that promotes

580   pro-inflammatory leukocyte responses, vitamin D appears to have the capacity to regulate

581   the production by epithelial cells of multiple mediators that act on the adaptive immune

582   system, such as the cytokines above and as we recently described sST2 [27], to produce a

583   less inflammatory microenvironment.

584

585   Vitamin D enhanced expression of the gene *G6PD* encoding glucose-6-phosphate

586   dehydrogenase, the rate-limiting step in the generation of NADPH, necessary for the action

587   of critical antioxidant enzymes including those involved in the production of reduced

588   glutathione [28]. In keeping with this action to enhance antioxidant pathways, vitamin D

589   increased the ratio of intracellular reduced to oxidized glutathione and decreased the

590   production of 8-isoprostane. This action of vitamin D is consistent with previous evidence

591   that it can protect various other types of epithelial cell from oxidative stress *in vitro*, including

592   H_2O_2-treated prostatic and breast epithelial cells [51] [52] and $CoCl_2$-treated trophoblasts

593   [53]. Similarly, using immortalised epithelial cell lines, notwithstanding that immortalised cell

594   lines are known to manifest distinctly different responses to vitamin D [54], it has been shown

595   that vitamin D can abrogate impairment caused by oxidative stress of nuclear translocation of

596   the ligand bound glucocorticoid receptor [55].

597   This action of vitamin D to enhance antioxidant pathways and mitigate against

598   oxidative stress likely contributes to its capacity to suppress IL-6 production by PM-

599   stimulated HBECs. Consistent with this are our observed effects of sulforaphane in cell

600   culture and the findings of Sienra-Monge *et al.* that antioxidant supplementation of children

601   significantly decreases nasal lavage concentrations of IL-6 but not CXCL8 [56]. Oxidative

602   stress has also been shown to contribute to corticosteroid resistance, a major feature of

603   severe asthma [57], and we have previously shown that vitamin D exerts a steroid-sensitising

604   action in severe asthma, restoring dexamethasone-induced IL-10 production by T cells [58].

605   However the capacity of vitamin D to suppress production of cytokines such as IL-6 is

606   likely multifactorial since vitamin D also has the propensity to inhibit other signalling

607   pathways, such as those involving nuclear factor kappa-B (NFκB) [21] and mitogen-activated

608   protein kinase (MAPK) [59]. The differing effects of vitamin D on IL-6, CXCL8 and GM-CSF

609   reveals the complex actions of vitamin D on the multiple intracellular signalling pathways in

610   the induction of each cytokine. The suppression of IL-6 by vitamin D in our *in vitro* cultures is

611   consistent with the previous finding of Codoner-Franch *et al.* that systemic concentrations of

24

612 IL-6 in obese children were significantly higher in those with lower serum vitamin D

613 concentrations [60].

614

615   A limitation of this research is that the detailed series of mechanistic experiments

616 could only be conducted with epithelial cell cultures from a limited number of healthy and

617 asthmatic donors. Furthermore the asthmatic donors were predominantly of mild severity

618 and atopic; whether cells isolated from more severe and non-atopic asthmatics would

619 respond similarly is an important consideration. The impact of asthma medications on these

620 responses is also an important question but could not be analysed with this sample

621 population. It is therefore reassuring that our findings are consistent with *in vivo* cytokine

622 responses as discussed above [56] [60]. Furthermore our results are consistent with the

623 epidemiological study of Rosser and colleagues that showed vitamin D insufficient children

624 living close to major roads have an increased risk of severe asthma exacerbations [12].

625 Importantly the concentrations of vitamin D and particulate matter used in this research are

626 representative of the concentrations evident in the real world. Li *et al.* have reconciled

627 theoretical and experimental particulate airway deposition, allowing for increased deposition

628 at airway bifurcation points with uneven air flow in disease, and shown $PM_{2.5}$ deposition

629 concentrations of 0.2–20 µg/cm^2 are representative for real-world exposure [18] with higher

630 deposition concentrations for total particulate matter. Circulating serum levels of 25-

631 hydroxylated vitamin D are in the range 75nmol – 150nmol in sufficient individuals, and

632 therefore an *in vitro* concentration of 100nmol 25(OH)D3 is representative of real-world

633 vitamin D sufficiency. With respect to 1,25(OH)$_2$D3 there is greater uncertainty as to *in vivo*

634 tissue concentrations but the *in vitro* effects of 100nm 1,25(OH)$_2$D3 on immunological

635 markers mirror *ex vivo* correlates supporting this concentration being representative [19].

636   Although there are very few studies examining a beneficial role for vitamin D in

637 alleviating adverse effects of pollution, there is a larger volume of evidence examining

638 antioxidant supplementation as a strategy to reduce the harm to human health from air

25

639 pollution. For example multiple studies have investigated the capacity of antioxidant

640 supplements (such as vitamin C and vitamin E) to abrogate any deleterious effect of air

641 pollution on lung function and many of these have shown significant benefit [61]. Similarly

642 there is suggestive evidence that supplementation of vitamin C and E may improve athletic

643 performance in ozone-exposed runners [62], and that B vitamin supplementation can

644 alleviate particulate exposure associated cardiac autonomic dysfunction [63], amongst other

645 outcome measures studied. The major difference between vitamin D and other antioxidant

646 vitamins (and minerals) is that the major natural source for vitamin D in humans is through

647 sunlight exposure not diet. Vitamin D insufficiency / deficiency are extremely common in the

648 developed world [64] and a healthy diet alone rarely contains adequate vitamin D to achieve

649 sufficient status. Although ultraviolet (UV) light supplementation might be an option [65],

650 population-level oral vitamin D supplementation may be necessary to alleviate the epidemic

651 of vitamin D deficiency and reduce harm from air pollution.

652   Our study provides mechanistic evidence to support larger trials of vitamin D

653 supplementation to alleviate the harmful impact of air pollution, similar to those conducted

654 with other antioxidant vitamins. Our conclusions are based on samples from a limited

655 number of subjects and next need to be followed up in larger translational studies. In the first

656 instance exposure chamber studies in vitamin D deficient individuals randomised to vitamin

657 D supplementation / placebo prior to exposure would provide the opportunity to confirm

658 whether *in vivo* vitamin D supplementation beneficially impacts on the pulmonary response to

659 ambient pollutants. Clinical endpoints and biomarkers could be studied in a larger sample of

660 participants including healthy controls, a broader range of asthmatic patients and patients

661 with other respiratory diseases.

662

663   This research focused on the effects of vitamin D on pollution-induced pro-

664 inflammatory cytokine production by human bronchial epithelial cells, through the

665 upregulation of antioxidant defences. Transcriptomics was used to identify cytokines of

26

666   interest, with the advantage of revealing differentially modulated cytokines in an unbiased

667   manner. The array also identified multiple non-cytokine disease-associated genes regulated

668   by PM-stimulation and vitamin D - such as *CLDN7*, *SERPINB1*, *COL1A1*, *SLPI* and *MMP9* -

669   that were beyond the scope of this research. Many of these have been implicated in airways

670   pathology and deserve future research.

671

Conclusions

672

673

674   In summary, vitamin D decreased oxidative stress and the particulate matter-induced IL-6

675   response in HBEC cultures; with vitamin D pre-treatment suppressing the IL-6 response

676   consistently in cultures from both healthy and asthmatic donors. Therefore vitamin D

677   sufficiency is likely beneficial in protecting against pollution-induced inflammation in asthma,

678   as well as in other pollution-associated diseases related to the induction of airway and

679   systemic inflammation. Vitamin D supplementation has been shown to be safe and effective

680   in preventing severe asthma exacerbations and acute respiratory tract infections when given

681   to appropriate target groups [66] [67]. This research mechanistically supports a strategy of

682   public health intervention to optimise the body's own defences against pollution (for example

683   supplementation with vitamin D [12] or other vitamins [63]) to reduce the burden of pollution-

684   associated disease.

685

27

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Supporting Information Captions

S1 Table. Taqman primer probesets.

S2 Table. Genes showing differential regulation by both NIST stimulation and vitamin D in the transcription microarray.

Gene expression fold-changes in a transcription microarray of HBECs cultured for 24 hours with/without 50µg/ml NIST stimulation in the presence/absence of 100nM 1,25(OH)2D3. Short-list of genes showing both differential expression with NIST stimulation (fold change ≥ ± 1.4) and differential expression with vitamin D treatment in presence of NIST stimulation (fold change ≥ ± 1.4).

S1 Fig: Cytokine production by HBEC cultures from healthy and asthmatic donors stimulated by NIST PM with / without 1,25(OH)$_2$D3.

903   Primary HBECs stimulated for 24 hours with 50µg/ml NIST in the presence/absence of

904   100nM 1,25(OH)$_2$D3. VC; vehicle control for NIST. Healthy donor cultures: n=8 for IL-6, n=9

905   for CXCL8, n=8 for GM-CSF. Asthmatic donor cultures: n=7 for IL-6, n=8 for CXCL8, n=6 for

906   GM-CSF. Friedman's tests with Dunn's multiple-comparisons tests. Statistical significance: *,

907    $p \leq 0.05$.

908

909   **S2 Fig: Induction of cytokine gene expression in HBEC cultures from healthy and**

910   **asthmatic donors stimulated by NIST PM with / without 1,25(OH)$_2$D3.**

911   (A)   Induction of *IL6* and *CSF2* by 50µg/ml NIST PM stimulation in the presence / absence

912   of 100nM 1,25(OH)$_2$D3 in 4 hour and 24 hour cultures of HBECs from healthy and asthmatic

913   donors. H, Healthy donor HBECs; A, Asthmatic donor HBEC cultures. Tie-bars show paired

914   results from the same donor cultures. 4hr healthy, n=5; 4hr asthmatic, n=5; 24hr healthy,

915   n=6-7; 24hr asthmatic, n=6.

916   (C)   Induction of *IL6* and *CSF2* by NIST at 4 hours compared to suppression by 100nM

917   1,25(OH)$_2$D3 at 24 hours of IL-6 and GM-CSF production respectively in NIST PM stimulated

918   HBEC cultures. n=8 (4 healthy and 4 asthmatic).

919

920   **S3 Fig: Effect of NIST stimulation on expression of the vitamin D axis genes *CYP27B1***

921   **and *VDR*, and comparison of response to 25(OH)D3 and 1,25(OH)$_2$D3.**

922   (A)   Expression of *CYP27B1* mRNA in 24 hour HBEC cultures stimulated with 50µg/ml

923   NIST PM with/without 100nM 1,25(OH)$_2$D3 (n=9), or with 1µg/ml Poly(I:C) (paired t-test,

924   n=6). Gene expression measured by qPCR relative to the house-keeping gene *18S*, and

925   shown relative to the control condition. Statistical significance as follows: *, $p \leq 0.05$; **, $p \leq$

926   0.01; ***, $p \leq 0.001$.

927   (B)   Expression of *VDR* in 24 hour cultures stimulated with PM with/without 1,25(OH)$_2$D3

928   (n=7), or with 1µg/ml Poly(I:C) (paired t-test, n=4).
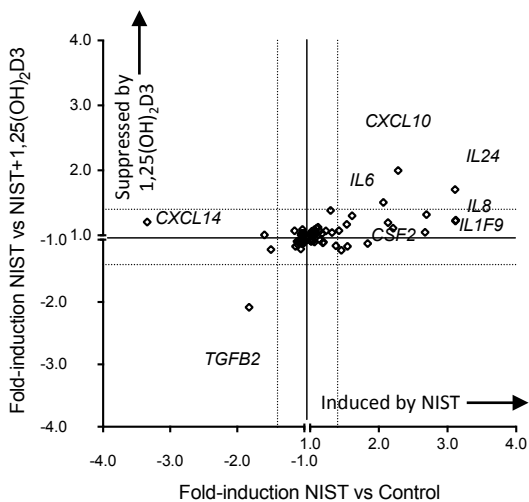
929   (C)   Percentage suppression of cytokine-production in 24 hour cultures of PM-stimulated

930   HBECs upon concurrent addition of 1,25(OH)$_2$D3 or 25(OH)D3. Cultures from healthy donors

931   shown as open circles and those from asthmatic donors as filled squares. IL-6, n=7 healthy,

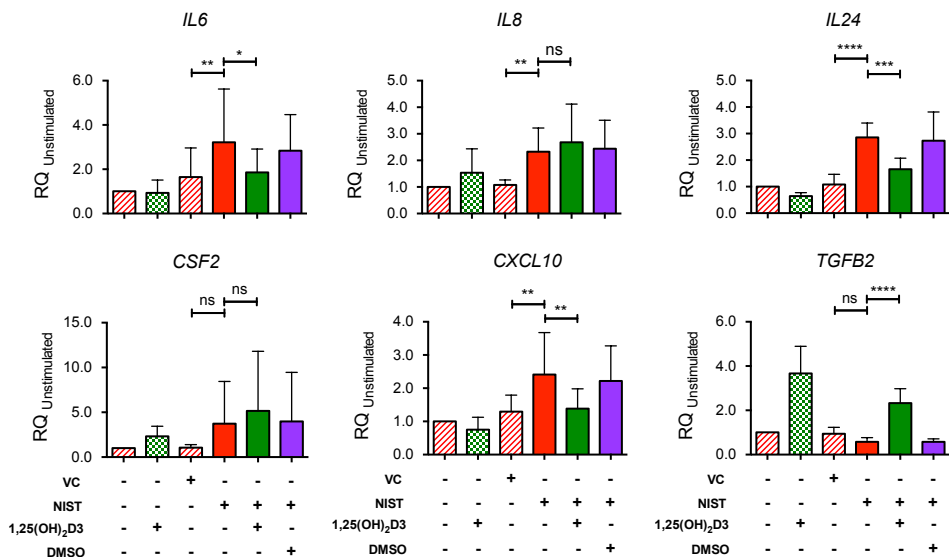932   6 asthmatic; CXCL8, n=8 healthy, 7 asthmatic; GM-CSF, n=6-7 healthy, 6 asthmatic.

933

934   **S1 Data Appendix. Transcription microarray results after quantile normalisation and**
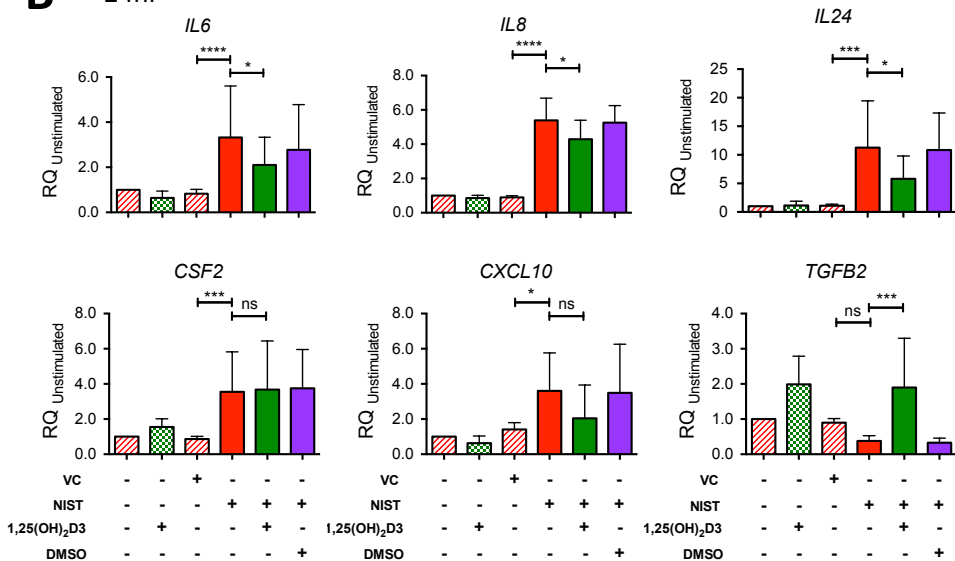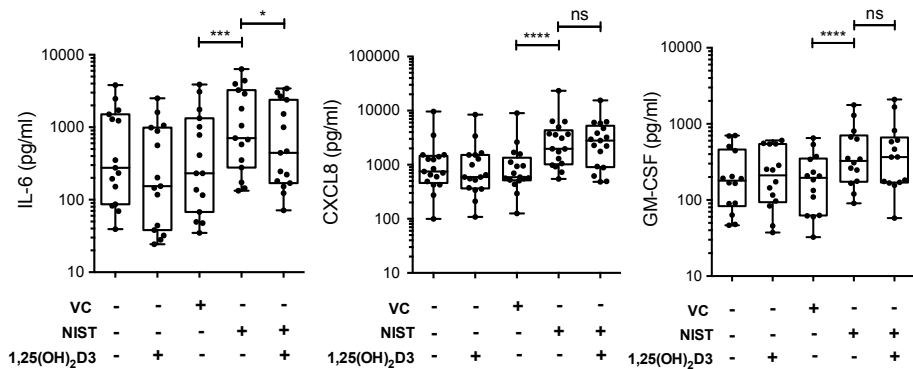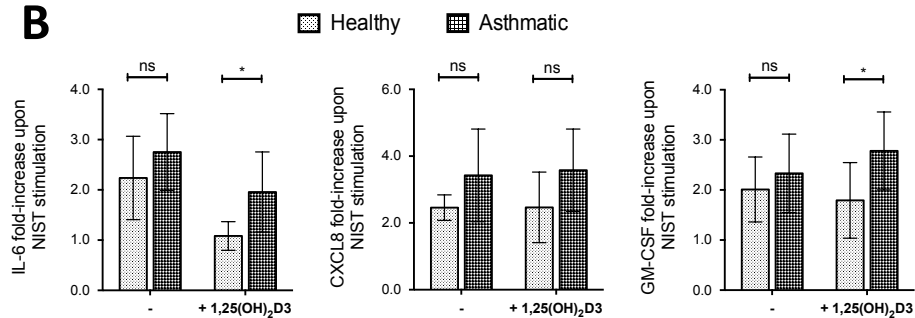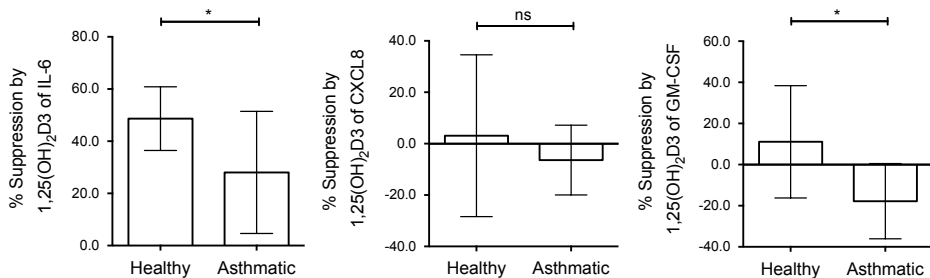
935   **ANOVA analysis (full gene list)**.

A

higher in NIST+1,25(OH)₂D3 condition

higher in NIST condition

Fold-change NIST vs NIST+1,25(OH)₂D3

B

Fold-induction NIST vs Control

A 4hr

IL6

IL8

IL24

CSF2

CXCL10

TGFB2

VC	-	-	+	-	-	-
NIST	-	-	-	+	+	+
1,25(OH)$_2$D3	-	+	-	-	+	-
DMSO	-	-	-	-	-	+

B 24hr

IL6

IL8

IL24

CSF2

CXCL10

TGFB2

VC	-	-	+	-	-	-
NIST	-	-	-	+	+	+
1,25(OH)$_2$D3	-	+	-	-	+	-
DMSO	-	-	-	-	-	+

A

IL-6 (pg/ml)

VC	-	-	+	-	-
NIST	-	-	-	+	+
1,25(OH)$_2$D3	-	+	-	-	+

CXCL8 (pg/ml)

VC	-	-	+	-	-
NIST	-	-	-	+	+
1,25(OH)$_2$D3	-	+	-	-	+

GM-CSF (pg/ml)

VC	-	-	+	-	-
NIST	-	-	-	+	+
1,25(OH)$_2$D3	-	+	-	-	+

B

Healthy   Asthmatic

IL-6 fold-increase upon NIST stimulation

CXCL8 fold-increase upon NIST stimulation

GM-CSF fold-increase upon NIST stimulation

C

% Suppression by 1,25(OH)$_2$D3 of IL-6

% Suppression by 1,25(OH)$_2$D3 of CXCL8

% Suppression by 1,25(OH)$_2$D3 of GM-CSF

Healthy   Asthmatic

A

RQ $_{Unstimulated}$ *CYP24A1*

B

NIST + 1,25(OH)$_2$D3

Copies *CYP24A1* : 1x10^6 *18S*

○ Healthy
■ Asthmatic

C

RQ $_{Unstimulated}$ *CAMP*

D

NIST + 1,25(OH)$_2$D3

Copies *CAMP* : 1x10^6 *18S*

A

GSH:GSSG ratio

50 —
40 —
30 —
20 —
10 —
0 —

*

25(OH)D3   -   100nM

B

8-Isoprostane
(fold-change compared to VC)

4.0 —
3.0 —
2.0 —
1.0 —
0.0 —

*

NIST   +   +
25(OH)D3   -   +

A

A   4hr *G6PD*

B   24hr *G6PD*

C

D   *G6PD*

A

B