ORIGINAL RESEARCH

Particle depletion of diesel exhaust restores allergen-induced lung-protective surfactant protein D in human lungs

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ABSTRACT

Rationale Exposure to air pollution is linked with increased asthma morbidity and mortality. To understand pathological processes linking air pollution and allergen exposures to asthma pathophysiology, we investigated the effect of coexposure to diesel exhaust (DE) and aeroallergen on immune regulatory proteins in human airways.

Methods Fourteen allergen-sensitised participants completed this randomised, double-blinded, cross-over, controlled exposure study. Each participant underwent four exposures (allergen-alone exposure, DE and allergen coexposure, particle-depleted DE (PDDE) and allergen coexposure, and sham exposure) on different order-randomised dates, each separated by a 4-week washout. Serum and bronchoalveolar lavage (BAL) were assayed for pattern recognition molecules, cytokines, chemokines and inflammatory mediators.

Results In human airways, allergen-alone exposure led to accumulation of surfactant protein D (SPD; p=0.02). Coexposure to allergen and DE did not elicit the same increase of SPD as did allergen alone; diesel particulate reduction restored allergen-induced SPD accumulation. Soluble receptor for advanced glycation end products was higher with particle reduction than without it. In the systemic circulation, there was a transient increase in SPD and club cell protein 16 (CC16) 4 hours after allergen alone. CC16 was augmented by PDDE, but not DE. % eosinophils in BAL (p<0.005), eotaxin-3 (p<0.0001), interleukin 5 (IL-5; p<0.0001) and thymus and activation regulated chemokine (p=0.0001) were each increased in BAL by allergen. IL-5, SPD and % eosinophils in BAL were correlated with decreased FEV_1.

Conclusion Short-term coexposure to aeroallergen and DE alters immune regulatory proteins in lungs; surfactant levels are dependent on particle depletion.

Trial registration number NCT02017431.

Key messages

What is the key question?

► What is the impact of coexposure to traffic-related air pollution and allergen on immune mediators in allergen-sensitised airways?

What is the bottom line?

► We demonstrate that allergen exposure increases immune modulatory proteins such as surfactant protein D, eotaxin-3, and interleukin 5 and eosinophils in the airways; whole diesel exhaust (DE), but not particle-depleted DE, had reduced allergen-induced accumulation of surfactant protein D in the airways.

Why read on?

► We provide experimental evidence demonstrating short-term coexposure to aeroallergen and DE, particularly particles, alters immune regulatory proteins in lungs that may modulate asthma pathophysiology.

INTRODUCTION

Air pollution is the largest environmental cause of disease and premature death worldwide,[1] and short-term exposure to air pollution is associated with asthma-related emergency room visits[2 3] and asthma mortality.[4] Morbidity and mortality arising from air pollution in urban area are attributable to pollutants originating primarily from motorised traffic, such as particulate matter (PM) ≤2.5 µm in aerodynamic diameter ($PM_{2.5}$), nitrogen dioxide (NO_2) and ozone.[5 6] Understanding how air pollutants alter the innate and adaptive immune response to aeroallergens may reveal molecular mechanism linking air pollution exposures to asthma pathophysiology.

Inhaled gases and particles interact with the lung at the respiratory mucosa. The airway epithelium does not only serve as a physical barrier to environmental insults but also secrete protective proteins such as surfactant protein A (SPA) and D (SPD), club cell protein 16 (CC16) and soluble receptor for advanced glycation end products (sRAGE) that modulate the innate immune response of the respiratory mucosa. The airway epithelium, along with other resident immune cells and structural cells, orchestrates the production of chemokines, cytokines and inflammatory mediators in response to environmental insults to recruit immune cells to the airways and regulate the immune response. Therefore, we evaluated and detailed changes in the level of immune regulatory proteins in the airway on exposure to allergen and traffic-related air pollution (TRAP).

SPA, SPD and sRAGE are soluble pattern recognition receptors (sPRRs) that are largely secreted in the mucus lining of the airways by epithelial cells in response to pathogens and allergens.[7] SPA and SPD are dysregulated in both asthma and COPD, and

alveolar SPD level is decreased in disease and during acute cigarette smoke exposures.[8–10] CC16 is an anti-inflammatory protein produced by secretory club cells in the airway epithelium, and increase of serum CC16 is a marker of epithelial injury in response to allergen inhalation and other environmental irritants.[11] RAGE is a pattern-recognition receptor for pathogen-derived and host-derived endogenous ligands that initiates and amplifies innate immune responses to tissue injury, infection and inflammation; soluble RAGE proteins are decoy receptors that competitively inhibit RAGE signalling. Together, these four lung proteins are involved in maintaining lung homeostasis, and they have been studied extensively in the context of chronic respiratory diseases.

The first aim of this study was to provide insight on how these four protective lung proteins are impacted by coexposure to aeroallergens and diesel exhaust (DE), a model of real-world environmental exposure for asthmatics. We predicted that airways of allergen-sensitised individuals would respond to allergen inhalation with an accumulation of SPA, SPD, CC16 and sRAGE in bronchoalveolar lavage (BAL). Our prediction was based on the notion that secretion of sPRRs served to facilitate elimination of allergen and apoptotic leucocytes following allergic inflammation. We postulated that coexposure to DE and allergen would decrease the accumulation of these proteins, primarily due to the PM component of DE. We hypothesised this based on a notion that high PM exposure may be harmful to club cells and type II pneumocytes that secrete these protective proteins. Active smokers who have a high burden of PM in the airways were shown to have lower levels of SPD and SPA,[12 13] and tobacco smoke can decrease CC16 expression in the airways of non-human primates.[14] In addition to the first aim, we also investigated whether the four proteins were increased in peripheral blood at several intervals following allergen inhalation and whether the magnitude of change was different in either coexposure condition compared with allergen-alone exposure.

As a second aim, we investigated the impact of coexposure on a wide range of cytokines, chemokines, inflammatory mediators and % eosinophils in BAL. For this, we used a commercially available multiplex assay of 30 proteins that are known to regulate immune response. We performed flow cytometry analysis to determine % eosinophils in BAL. Finally, we performed correlation analysis to test whether immune mediator levels were correlated with declines in FEV_1.

METHODS

Study design

This was a randomised, double-blinded, controlled human exposure cross-over study. All participants gave written informed consent. Fourteen allergen-sensitised participants aged 23–50 (7 male; 7 female) completed the study. All participants were self-reported never smokers and tested negative for urinary cotinine (<12 ng/mL). All participants showed a positive skin prick test for one or more aeroallergen used in the protocol (house dust mite (HDM), birch or grass) and showed a 20% or greater decline in FEV_1 following allergen inhalation challenge during screening. Nine were hyper-responsive and five were normally responsive to methacholine challenge. Further details of participant characteristics are described in our previous publication.[15]

Air pollution exposures were carried out at the Air Pollution Exposure Laboratory in Vancouver, British Columbia, using the exposure system previously described[15 16] with a constant load of 2.5 kW applied to the diesel generator. Each participant was exposed to four coexposure conditions in random order, each separated by a 4-week washout period: (1) filtered air (FA) +saline (0.9% NaCl) (FA-S, negative control); (2) FA +allergen (FA-A); (3) DE diluted to 300 µg/m^3 of PM with aerodynamic diameter ≤2.5 µm ($PM_{2.5}$) +allergen (DE-A); and (4) particle-depleted DE +allergen (PDDE-A). For PDDE condition, PM was removed from DE using a combination of high-efficiency particulate air filtration and electrostatic precipitation (HE Plus 1400, Trion, Sanford, North Carolina, USA).[17] Exposures (FA, DE and PDDE) were 2 hours in duration. One hour after each exposure, we administered either saline or allergen inhalation challenge using an allergen concentration predetermined to cause a 20% drop in FEV_1 for each participant. For additional details, see the online supplemental methods.

DE exposure characteristics

We previously reported detailed characteristics of the DE and PDDE exposures.[15] In summary, the average concentration of $PM_{2.5}$ in DE (292.2 (95% CI 279.5 to 304.9) µg/m^3) was significantly higher ($p<0.0001$) than that of PDDE (18.9 (14.4 to 23.4) µg/m^3). The average concentrations of NO_2 in PDDE (150.3 (105.1 to 195.5) ppb) was significantly higher ($p<0.0001$) than with DE (52.56 (31.5 to 73.6) ppb), respectively. The concentration of total volatile organic compounds was higher ($p<0.001$) in DE (1932 ppb, 95% CI 1856 to 2008 ppb) compared with PDDE (1751 ppb, 95% CI 1672 to 1830 ppb). Concentrations of CO, CO_2 and NO wwere not statistically different between the DE and PDDE conditions.

Sample collection

Figure 1 summarises the exposure and inhalation conditions and sample collection timing. Serum samples were collected at baseline before exposure and at 4 hours, 24 hours and 48 hours post exposure. 48 hours post exposure and post allergen inhalation, each participant underwent a bronchoscopy where BAL was collected. Serum and BAL samples were centrifuged, processed, aliquoted and stored at −80°C, as described in the online supplemental methods.

Enzyme-Linked Immunosorbant Assay (ELISA)

All ELISAs were performed as per the manufacturer's protocol with some modifications to sample dilution factor, as detailed in the online supplemental methods. Soluble RAGE was assayed using the R&D Systems (Minneapolis, USA) Quantikine ELISA kit (Cat #DRG00). Human CC16 (Cat #RD191022200), human SPA (Cat #RD191139200R) and human SPD (Cat #RD194059101) ELISA kits were purchased from BioVendor (Brno, Czech Republic). Samples were run in duplicates, and the mean of the duplicates was used in the analysis. When the coefficient of variation of duplicates was greater than 20%, all samples from that participant were assayed again, and the values were excluded from the analysis if there was no consistent detection of the protein.

Electrochemiluminescent multiplex assay

The V-PLEX Human Cytokine 30-Plex Kit (Meso Scale Diagnostics, Rockville, Maryland, USA) was used to assay the following analytes in BAL: eotaxin, eotaxin-3, granulocyte-macrophage colony-stimulating factor, interferon gamma (IFN-γ), interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12/IL-23p40, IL-12p70, IL-13, IL-15, IL-16, IL-17A, IFN-γ-induced protein 10 kDa (IP-10), monocyte chemoattractant protein (MCP)-1, MCP-4, macrophage-derived chemokine (MDC), macrophage inflammatory protein (MIP)-1α, MIP-1β, thymus and activation regulated chemokine (TARC), tumour necrosis factor (TNF)-α, TNF-β and vascular endothelial growth

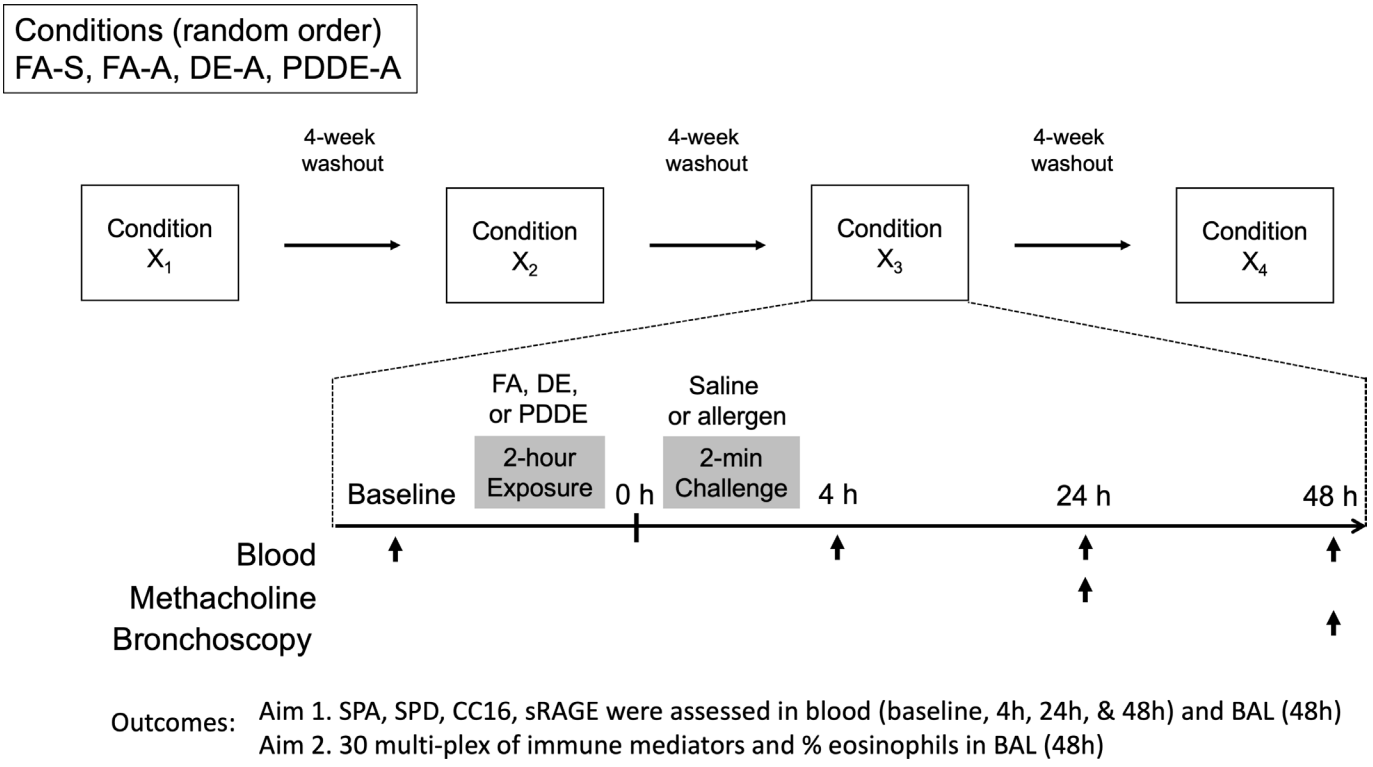

Figure 1 Schematic summary of the controlled human exposure study with exposure to diesel exhaust (DE) and allergen in allergen-sensitised research participants. Fourteen consenting research participants completed the study protocol. Conditions (X_{1-4}) were administered in randomised order: filtered air (FA), DE ([$PM_{2.5}$]=300 µg/m^3) or particle-depleted DE (PDDE) followed by 2 min tidal volume saline (control) or allergen inhalation at a dose predetermined to elicit a 20% drop in FEV_1. The four experimental conditions were: (1) filtered air +saline (FA-S), (2) filtered air +allergen (FA-A), (3) DE +allergen (DE-A) and (4) PDDE +allergen (PDDE-A). DE was diluted to maintain a concentration of $PM_{2.5}$ equal to 300 µg/m^3 during the 2 hours exposure. For the PDDE condition, DE particles were removed by a combination of high-efficiency particulateair filtration and electrostatic precipitation to mimic diesel particle filter system. Serum samples were collected at baseline, 4 hours, 24 hours and 48 hours post allergen exposure. Bronchoscopies were performed at 48 hours to collect bronchoalveolar lavage (BAL) samples. CC16, club cell protein 16; SPA, surfactant protein A; SPD, surfactant protein; sRAGE, soluble receptor for advanced glycation end products.

factor (VEGF)-A. The assay was performed per the manufacturer's protocol with twofold (cytokine panel 1 and proinflammatory panel 1) and fourfold (chemokine panel 1) dilution of BAL in assay diluent. The lower limit of detection (LLOD) was set at the signal intensity that was 2.5 SD above the background noise in the blank. For statistical comparisons, values below the LLOD were replaced with ½ of the respective LLOD value.

Flow cytometry

Within an hour after collection, BAL cells were stained for CD45, CD16, CD9, HLA-DR, CCR3 and CD69. Eosinophils were identified as CD45+, HLA-DR^{low}, CD9+ and CD16+. Percentage of eosinophils in BAL was determined by counting $CD45^+$ HLA-DR^{low} $CD9^+$ $CD16^+$ cells and CD45+ cells in 60 000 events via flow cytometry (BD FACS Canto II). Dilution factor and isotype information for specific markers and associated fluorophores are noted in online supplementary table S1.

Statistical analysis

Data are presented as mean and 95% CI. Effects of exposures on BAL and serum protein concentrations were assessed using linear mixed-effects models (nlme package V.3.1-140) in R (V.3.6.1). Initially, conditions (FA-S, FA-A, DE-A or PDDE-A) were used as the fixed effect and participant identifier (ID) as the random effect to assess the main effect of exposure on outcomes relative to FA-S.

To estimate the effect of DE-A and PDDE-A in comparison to allergen alone (FA-A), we first used conditions (FA-S, FA-A, DE-A or PDDE-A) as a fixed effect and participant ID as random effect. We then also estimated effects with a second model where conditions, type of allergen used (HDM, grass or birch) for inhalation challenge and airway hyper-responsivness (AHR) as measured by methacholine PC_{20} ≤8 mg/mL, were used as the fixed effects and participant ID as random effect. For this model, random slopes were introduced for each condition.

Data were log (base 10) transformed and results are presented as effect estimate based on $\log_{10}$ for the statistical comparison when necessary to normalise the data distribution. P values less than 0.05 were considered statistically significant.

To assess correlation between the immune mediators in BAL and change in FEV_1, repeated measures correlation was performed using the rmcorr package (V.0.3.0) in R.

RESULTS

Whole DE (but not PDDE) dampened airway allergen-induced surfactant protein accumulation

Results are presented as effect estimate based on $\log_{10}$ values unless noted otherwise. At 48 hours post allergen inhalation, the concentration of SPD in BAL was significantly higher for FA-A than FA-S (effect estimate:+0.253, 0.053 to 0.454, p=0.02; figure 2A). When participants underwent coexposure to DE and allergen, the concentration of SPD did not significantly increase from FA-S

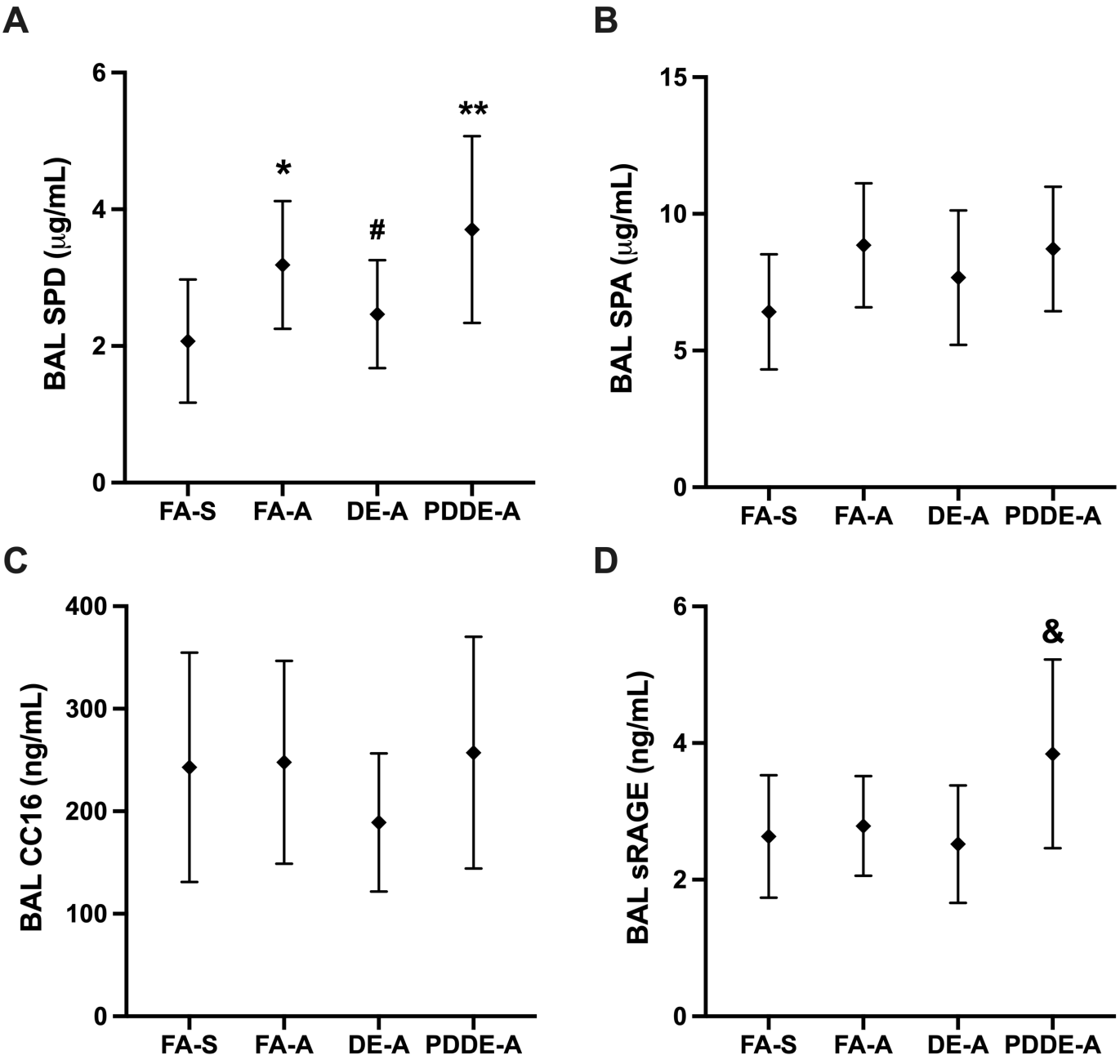

Figure 2 Effect of exposure to diesel exhaust (DE) and allergen inhalation on club cell protein 16 (CC16), surfactant protein A (SPA) and D (SPD), and soluble receptor of advanced glycation end products (sRAGE). Proteins were assayed from bronchoalveolar lavage (BAL) collected 48 hours post exposure. The four experimental conditions were: (1) filtered air +saline (FA-S), (2) filtered air +allergen (FA-A), (3) DE +allergen (DE-A) and (4) particle-depleted DE +allergen (PDDE-A). Values were expressed as mean±95% CI (n=14, except PDDE-A: n=13). Effects of exposures on the outcomes were assessed using linear mixed-effects models: *p<0.05 versus FA-S; #p<0.05 versus FA-A; and p<0.05 versus DE-A. Effect estimates, 95% CIs and p values are described in full in the main text.

(+0.136, –0.065 to 0.337, p=0.19), indicating that there was dampened allergen-induced accumulation of SPD in BAL. This dampening was absent when particle depletion was applied to DE; the concentration of SPD in PDDE-A was higher than FA-S (+0.299, 0.094 to 0.504, p=0.007; figure 2A). While there was no statistical difference between DE-A and FA-A in our primary model, there was a significant decrease in SPD in DE-A compared with FA-A (−0.117, –0.231 to −0.004, p=0.049) when including both allergen and each participant's AHR status as fixed effects (not as interaction terms) in the model. SPA increased marginally with allergen inhalation (FA-A vs FA-S: +0.191, 0 to 0.381, p=0.06; figure 2B). There was no detectable change in CC16 and sRAGE in BAL 48 hours post allergen inhalation (figure 2C&D). However, there was a significant increase in sRAGE concentration in BAL following PDDE-A compared with DE-A (+0.153, 0.014 to 0.291, p=0.04). We analysed for effect modification by allergen or AHR but did not observe significant condition-by-allergen or condition-by-AHR interaction in any of the four protein outcomes.

Inhaled allergen challenge increased serum SPD and CC16, but not SPA and sRAGE

At 4 hours post allergen inhalation, SPD in FA-A was higher than in FA-S (+0.051, 0.011 to 0.092, p=0.02) and CC16 in FA-A

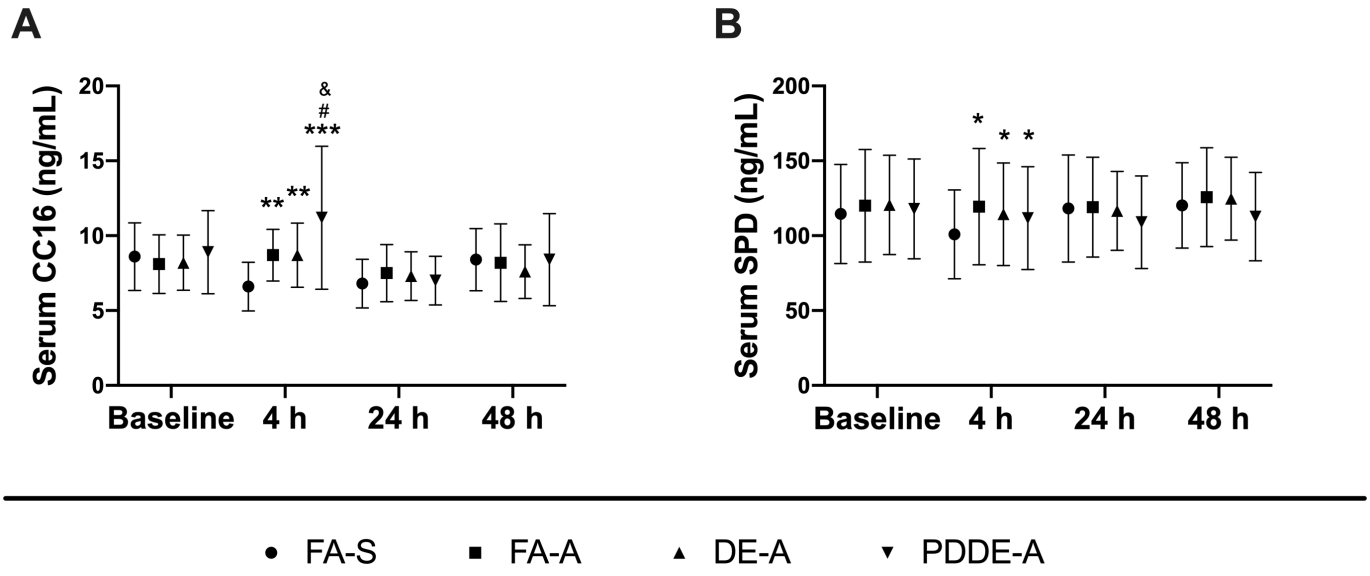

Figure 3 Allergen inhalation challenge increased serum club cell protein 16 (CC16) and surfactant protein D (SPD) at 4 hours post inhalation. Particle-depleted diesel exhaust (PDDE), but not diesel exhaust (DE), augmented the allergen-induced increase in CC16 in serum at 4 hours post exposure. Serum samples were taken at baseline (−4 hours), 4, 24 and 48 hours after DE exposure and allergen inhalation. The four experimental conditions were: (1) filtered air +saline (FA-S), (2) filtered air +allergen (FA-A), (3) DE +allergen (DE-A) and (4) PDDE +allergen (PDDE-A). Values were expressed as mean±95% CI (n=14). Effects of exposures on the outcomes were assessed using linear mixed-effects models: *p<0.05, **p<0.01, ***p<0.001 versus FA-S; #p<0.05 versus FA-A; &p<0.05 versus DE-A. Effect estimates, 95% CIs and exact p values are described in full in the main text.

was higher than in FA-S (+0.125, 0.049 to 0.202, p=0.003; figure 3). SPD in DE-A was higher than in FA-S (+0.053, 0.014 to 0.093, p=0.01), and CC16 in DE-A was higher than in FA-S (+0.129, 0.055 to 0.204, p=0.002). Serum concentration of CC16 in PDDE-A was higher than FA-A (+0.082, 0.005 to 0.158, p=0.04), indicating that PDDE exposure augmented the allergen-induced increase in serum CC16. At the same timepoint, neither DE nor PDDE exposure augmented the allergen-induced increase in serum SPD.

The allergen-induced increases in SPD and CC16 in serum returned to baseline by 24 hours post allergen inhalation (figure 3). SPA and sRAGE concentration in serum were assayed in the same samples, but there were no detectable differences between the four experimental conditions at any of the four timepoints measured (data not shown).

Effects of DE exposure on immune mediators in BAL

In human BAL collected 48 hours post exposure, eotaxin, IFN-γ, IL-4, IL-10, IL-12p70, IL-13, MCP-4, MDC, MIP-1α and TNF-β were not reliably detectable above the LLOD and were excluded from statistical analysis. Of the 19 detected proteins that were included in the analysis, eotaxin-3, IL-5 and TARC were significantly increased by allergen inhalation (table 1). % eosinophils were also significantly increased by allergen inhalation. Table 1 summarises immune mediators that were changed in our study. We did not observe any significant condition-by-AHR and condition-by-allergen interaction in these outcomes.

Relationship between immune modulator proteins in BAL and lung function

Table 2 summarises the correlation between change in mediators of lung immune response and change in FEV_1 across exposures. SPD (p=0.039), IL-5 (p=0.038) and % eosinophils in BAL (p=0.0001) were negatively correlated with changes in FEV_1 between baseline and 24 hours post exposure.

DISCUSSION

In this controlled human exposure study, we demonstrated that allergen inhalation increases the level of SPD in blood and BAL. This finding reaffirms previous observations that human airways respond to allergens with increased SPD in the airways.[18–20] Here, we used an inhalation allergen challenge model, which is more reflective of real-world inhalation than the segmental allergen challenge model we previously reported,[20] strengthening the evidence that SPD is involved in the host response to aeroallergens in allergen-sensitised individuals. Interestingly, we showed that exposure to whole DE before allergen challenge dampened the allergen-induced response in SPD. This dampening effect was not present in PDDE-A, suggesting that the particulate fraction of DE was responsible for the loss of surfactant protein D.

SPD, pattern recognition molecules of the collectin family of C-type lectins, are primarily produced in the distal airway epithelium to maintain homeostasis and regulate pulmonary innate immune systems.[8 21] SPD aggregates and aids in the removal of allergens and PM from the lungs and enhances the phagocytosis of pollen allergens by lung phagocytes.[22] SPD also promotes the elimination of apoptotic cells in the airways, which is vital in the resolution of inflammation following allergen inhalation.[8] SPD is lower in active smokers compared with non-smokers[12] and is even lower in patients with severe asthma.[23] Therefore, reduced SPD accumulation in airways may indicate impaired mechanism for eliminating aeroallergens and regulating the immune response to allergens. Because SPD is produced by bronchial epithelium and alveolar type II cells and its uptake is regulated by macrophages,[24] impaired accumulation of SPD may also indicate impaired production by the epithelium and/or increased uptake by macrophages.

Besides our finding of SPD in the airways, we demonstrated here that PDDE-A exposure resulted in an augmented increase of CC16 in serum at 4 hours post exposure (figure 3A),

Table 1 Summary of effects of exposures on immune mediators in bronchoalveolar lavage (BAL)

Protein	Contrast	Effect estimate	95% CI	P value
% Eosinophils	**FA-A versus FA-S**	**7.44**	**2.596 to 12.289**	**0.005**
	DE-A versus FA-S	**5.58**	**1.1 to 10.067**	**0.02**
	PDDE-A versus FA-S	**8.31**	**3.718 to 12.904**	**0.001**
	DE-A versus FA-A	−1.85	−6.598 to 2.88	0.45
	PDDE-A versus FA-A	0.868	−3.925 to 5.662	0.72
	PDDE-A versus DE-A	2.727	−1.761 to 7.216	0.24
Eotaxin-3	**FA-A versus FA-S**	**0.630**	**0.417 to 0.843**	**<0.0001**
	DE-A versus FA-S	**0.565**	**0.352 to 0.778**	**<0.0001**
	PDDE-A versus FA-S	**0.461**	**0.243 to 0.68**	**0.0002**
	DE-A versus FA-A	−0.065	−0.278 to 0.148	0.55
	PDDE-A versus FA-A	−0.168	−0.386 to 0.05	0.14
	PDDE-A versus DE-A	−0.103	−0.321 to 0.115	0.36
Interleukin 5	**FA-A versus FA-S**	**1.238**	**0.757 to 1.719**	**<0.0001**
	DE-A versus FA-S	**1.080**	**0.599 to 1.562**	**0.0001**
	PDDE-A versus FA-S	**1.169**	**0.676 to 1.661**	**<0.0001**
	DE-A versus FA-A	−0.158	−0.639 to 0.323	0.52
	PDDE-A versus FA-A	−0.070	−0.562 to 0.423	0.78
	PDDE-A versus DE-A	0.088	0.757 to 1.719	0.72
TARC	**FA-A versus FA-S**	**0.726**	**0.392 to 1.06**	**0.0001**
	DE-A versus FA-S	**0.45**	**0.116 to 0.784**	**0.01**
	PDDE-A versus FA-S	**0.478**	**0.136 to 0.82**	**0.009**
	DE-A versus FA-A	−0.276	−0.61 to 0.058	0.11
	PDDE-A versus FA-A	−0.249	−0.591 to 0.093	0.16
	PDDE-A versus DE-A	0.028	−0.314 to 0.37	0.87
VEGF-A	FA-A versus FA-S	−0.083	−0.225 to 0.06	0.26
	DE-A versus FA-S	−0.087	−0.229 to 0.056	0.24
	PDDE-A versus FA-S	0.06	−0.086 to 0.206	0.43
	DE-A versus FA-A	−0.004	−0.147 to 0.139	0.96
	PDDE-A versus FA-A	0.143	−0.003 to 0.289	0.06
	PDDE-A versus DE-A	**0.147**	**0 to 0.293**	**0.049**
Interleukin 15	FA-A versus FA-S	0.044	−0.104 to 0.193	0.56
	DE-A versus FA-S	−0.052	−0.2 to 0.097	0.50
	PDDE-A versus FA-S	0.140	−0.012 to 0.291	0.08
	DE-A versus FA-A	−0.096	−0.244 to 0.053	0.21
	PDDE-A versus FA-A	0.095	−0.056 to 0.247	0.22
	PDDE-A versus DE-A	**0.191**	**0.04 to 0.343**	**0.02**

Exposure effects were estimated using linear mixed-effects model with experimental condition as the fixed effect and subject ID as the random effect. Results are presented as effect estimate based on log10, except the % eosinophils result. BAL was assayed with 30-plex (V-Plex Human Cytokine, Meso Scale Discovery). BAL % eosinophils is reflective of proportion of cells HLA-DR^{Low} CD16^{+}CD9^{+} in CD45^{+} BAL cells measured using flow cytometry. Statistically significant values ($p<0.05$) are denoted in bold.
DE-A, diesel exhaust +allergen; FA-A, filtered air +allergen; FA-S, filtered air +saline; PDDE-A, particle-depleted diesel exhaust +allergen; TARC, thymus and activation regulated chemokine; VEGF-A, vascular endothelial growth factor A.

suggesting greater epithelial-blood barrier damage in the lungs by PDDE-A exposure than FA-A or DE-A. This observation may be attributable to a high level of NO_2 in PDDE exposure than FA or DE exposure. In our experimental setup, higher levels of NO_2 occurred in PDDE-A as a byproduct of using a particulate precipitator in removing PM from DE. An electrostatic precipitator positively charges particles and then removes those particles using ionizing-collecting cells. As a consequence of this process, there is a significant increase in NO_2 concentrations despite the reduction in the mass concentration of $PM_{2.5}$. This effectively recapitulates an undesirable effect of some particle-reducing technologies used in real-world settings.[25–27] For example, a diesel oxidation catalyst, one of the mainstream DE after-treatment technologies, increases NO_2:NO in order to increase the efficiency of a diesel particulate filter (DPF).[17] This can be remediated by selective catalyst reduction (SCR), but older engines often do not include an SCR. As such, a DPF may result in increased NO_2 concentrations, and DE subjected to a DPF has been shown in vitro to have increased cytotoxicity, ROS production and genotoxicity relative to unfiltered DE.[28] Besides

Table 2 Repeated measures correlation between change in mediators of lung immune response and change in FEV_1 across exposures

	FEV_1 % predicted (24 hours)			FEV_1 % predicted (48 hours)		
	r	95% CI	P value	r	95% CI	P value
SPD	**−0.323**	**−0.580 to −0.008**	**0.039**	−0.135	−0.432 to 0.188	0.399
SPA	−0.229	−0.508 to 0.093	0.151	−0.176	−0.166 to 0.147	0.270
sRAGE	−0.100	−0.403 to 0.222	0.533	−0.065	−0.373 to 0.255	0.684
CC16	0.148	−0.175 to 0.443	0.354	−0.162	−0.162 to 0.454	0.311
Eotaxin-3	−0.248	−0.523 to 0.073	0.117	0.035	−0.284 to 0.346	0.829
IL-5	**−0.325**	**−0.581 to −0.011**	**0.038**	−0.211	−0.494 to 0.112	0.185
TARC	−0.273	−0.542 to 0.046	0.084	−0.279	−0.546 to 0.040	0.077
BAL % eosinophils	**−0.595**	**−0.774 to −0.326**	**0.0001**	**−0.353**	**−0.614 to −0.023**	**0.032**

Immune regulatory mediators in BAL were correlated with the change in airflow as reflected by the change in FEV_1 % predicted. Columns labelled 'r' is correlation coefficient. BAL % eosinophils is reflective of proportion of cells HLA-DR^{Low} $CD16^+CD9^+$ in $CD45^+$ BAL cells. Statistically significant values ($p<0.05$) are denoted in bold.
BAL, bronchoalveolar lavage; CC16, club cell protein 16; IL-5, interleukin 5; SPA, surfactant protein A; SPD, surfactant protein D; sRAGE, soluble receptor for advanced glycation end products; TARC, thymus and activation regulated chemokine.

increasing NO_2 level, particle reduction technologies may change the physiochemical properties of ultrafine particles that may bypass the filtering mechanism. Therefore, reducing PM at the expense of increasing NO_2 or changing physiochemical properties of the particles may present a tradeoff with consequences on our respiratory system. Therefore, every particle reduction technology should be carefully evaluated for its effectiveness and impact on particle toxicity.

Beyond our findings with SPD and CC16 in this study, the BAL concentration of sRAGE was increased with PDDE-A compared with DE-A (figure 2D). RAGE is a pattern-recognition receptor for pathogen-derived and host-derived endogenous ligands that initiates and amplifies innate immune responses to tissue injury, infection, and inflammation; sRAGE proteins are decoy receptors that competitively inhibit RAGE signalling. RAGE promotes cellular repair and maintains epithelial barrier function,[29] so increased sRAGE in BAL may indicate that there was an augmented repair process in the airways following PDDE-A exposure. However, there may be alternative reason for the increased sRAGE in BAL; it may be that increased activity of matrix metalloproteinase (MMP)-9 and a disintegrin and metalloprotease 10 (ADAM10) in the airways led to increased sRAGE in BAL. Other studies have demonstrated that MMP-9 and ADAM10 can proteolytically cleave membrane RAGE to become sRAGE.[30 31] Future studies should investigate whether the gas fraction of DE, including NO_2, can increase the activities of MMP-9 and ADAM10, leading to increase of sRAGE in BAL. In addition to the impact on sRAGE, we observed a higher level of VEGF-A in BAL in PDDE-A compared with DE-A. Taken together with the augmented increase of serum CC16 following PDDE-A, these observations support a notion that PDDE-A exposure resulted in aggravated epithelial-endothelial barrier damage compared with DE-A.

Finally, as expected, our results show that aeroallergen inhalation caused increases of TARC, eotaxin-3 and IL-5 as well as an increase in BAL % eosinophils (table 1). Repeated measures correlation analysis revealed that SPD, IL-5 and % eosinophils in BAL were negatively correlated with the change in FEV_1 (% predicted) from baseline to 24 hours post exposure, indicating that participants who experienced a greater decrease in FEV_1 had a greater change of concentration of SPD and IL-5 and % in BAL elicited by the allergen and DE coexposure. In our previous report,[15] we showed a negative correlation between blood eosinophils and change in FEV_1. Combined with our finding in eotaxin-3, IL-5 and TARC in BAL, our data further support the notion that increased circulating eosinophils and eosinophil accumulation in the lung may have played a meaningful role in eliciting lung function decrements in the context of our exposures. Therefore, future studies should explore if interventions to reduce the recruitment or activation of eosinophils, through use of inhaled corticosteroids, for example, can prevent acute lung function decrements in the context of environmental exposures to TRAP and allergens.

There are some caveats that must be addressed in our study. First, the method of particle depletion did not completely remove all particles; while it decreased particles by more than 15-fold, the remaining particles certainly may have had some effect, especially as combined with allergen exposure. Moreover, even though our choice of particle reduction technology is representative of real-world technologies that are associated with increased NO_2, use of such technology made it so that we could not exactly compare the effects of particle removal with all other factors being equal. Having said this, it remains critical that studies aimed at elucidating the health effects from DE should include exposure to both particle filtered and whole DE, because different components in DE can have distinct and independent health effects.[32] Another limitation of our study was that it was not designed to study the activities of proteases in freshly collected samples, and our use of stored samples may have diminished the ability to see exposure-related changes.

In summary, our findings provide evidence that both gases and particles, combined with inhaled allergen, elicit acute damage and alteration to the mucosal barrier of the respiratory tract. Our results support the assertion that air pollution reduction strategies should aim to decrease both particulates and gases. We also showed that allergen-induced increases in eosinophils and IL-5 in the lungs are correlated with airflow limitation. Allergen-sensitised individuals should be guided to limit the exposure to allergen, while also minimising exposure to TRAP. Our model is limited to acute exposures, and an important question for further investigation is whether longer or repeated exposures to TRAP further compromise mucosal barrier function.

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