Journal Pre-proof

Diesel exhaust particle and dust mite induced airway inflammation is modified by cerium dioxide nanoparticles

Kirsty Meldrum, Sarah Robertson, Isabella Römer, Tim Marczylo, Timothy W. Gant, Rachel Smith, Teresa D. Tetley, Martin O. Leonard

PII: S1382-6689(19)30147-4

DOI: https://doi.org/10.1016/j.etap.2019.103273

Reference: ENVTOX 103273

To appear in: *Environmental Toxicology and Pharmacology*

Received Date: 26 May 2019

Revised Date: 4 October 2019

Accepted Date: 5 October 2019

Please cite this article as: Meldrum K, Robertson S, Römer I, Marczylo T, Gant TW, Smith R, Tetley TD, Leonard MO, Diesel exhaust particle and dust mite induced airway inflammation is modified by cerium dioxide nanoparticles, *Environmental Toxicology and Pharmacology* (2019), doi: https://doi.org/10.1016/j.etap.2019.103273

Diesel exhaust particle and dust mite induced airway inflammation is modified by cerium dioxide nanoparticles.

Kirsty Meldrum[a,c], Sarah Robertson[b], Isabella Römer[a], Tim Marczylo[a], Timothy W. Gant[a], Rachel Smith[a], Teresa D. Tetley[c], Martin O. Leonard[a,‡]

[a]Toxicology Department, Centre for Radiation, Chemical and Environmental Hazards, Public Health England, Chilton, Harwell Campus, OX11 0RQ, UK.

[b]Environmental Hazards and Emergencies Department, Centre for Radiation, Chemical and Environmental Hazards, Public Health England, Chilton, Harwell Campus, OX11 0RQ, UK.

[c]Lung Cell Biology, Airways Disease, National Heart & Lung Institute, Imperial College London, London, UK.

Author emails: KM; Kirsty.Meldrum@swansea.ac.uk SR; Sarah.Robertson3@phe.gov.uk, IR; isaromer@gmail.com, TM; Tim.Marczylo@phe.gov.uk, TWG; Tim.Gant@phe.gov.uk, RS; Rachel.Smith@phe.gov.uk, TDT; t.tetley@imperial.ac.uk

[‡]Address correspondence to:
Dr. Martin Leonard.
Toxicology Department,
Centre for Radiation, Chemical and Environmental Hazards,
Public Health England,
Chilton, Harwell Campus, OX11 0RQ.
U.K.
e-mail: Martin.Leonard@phe.gov.uk

Highlights

- Repeat CeO2NPs exposure with DEP+HDM enhanced type II and Th17 lung inflammation.
- Single exposure to CeO2NPs increased macrophage and IL-17A, while reducing CCL20.
- In the absence of HDM, CeO2NPs also modified DEP inflammation in the lung.

ABSTRACT

Cerium dioxide nanoparticles (CeO_2NPs) have been used as diesel fuel-borne catalysts for improved efficiency and pollutant emissions. Concerns that such material may influence diesel exhaust particle (DEP) effects within the lung we examined responses in mice with and without exposure to house dust mite (HDM). Repeated intranasal instillation of combined HDM and DEP increased airway mucin, eosinophils, lymphocytes, IL-5, IL-13, IL-17A and plasma IgE, which were further increased with CeO_2NPs co-exposure. A single co-exposure of CeO_2NPs and DEP after repeated HDM exposure increased macrophage and IL-17A levels above DEP induced levels. CeO_2NPs exposure in the absence of HDM also resulted in increased levels of plasma IgE and airway mucin staining, changes not observed with RPT DEP exposure alone. These observations indicate that CeO_2NPs can modify exhaust particulate and allergen induced inflammatory events in the lung with the potential to influence conditions such as allergic airway disease.

Keywords: Allergic airway disease, Lung, Nanoparticles, Particulate Pollutants.

1 INTRODUCTION

CeO_2NPs have been used in recent years across a number of applications where inadvertent human exposure may occur including as polishing materials (Kenneth Reed 2014), in oxide based fuel cells and as catalyst additives in diesel fuel (Dale et al. 2017). The transport sector is responsible for a large proportion of urban air pollution and fuel-borne catalyst products such as Eolys™, Platinum Plus™, and Envirox™ ,when added to fuel at the recommended concentrations of CeO_2NPs (9-10ppm) can increase fuel efficiency and reduce some gaseous and particulate emissions (Lung et al. 2014; Snow et al. 2014; Zhang et al. 2013; Zhang et al. 2016; Zhang and Balasubramanian 2017). However, increased emissions for other pollutants such as ultrafine particle numbers, nitric oxides and polycyclic aromatic hydrocarbons have led to some concerns on the potential impact of exhaust material on human health (Zhang et al. 2013; Zhang and Balasubramanian 2017). The prevalence of these commercially available products is unclear, but some estimate up to 4 million vehicles outside of the US (Erdakos et al. 2014). Regulatory control of metallic fuel additives (MFA) such as lead, and manganese exist to protect human health and the environment. While acknowledged within frameworks such as the EU fuel quality directive 98/70/EC, no requirements exist at the present time to regulate levels of other MFA such as cerium (Commission 2017).

No epidemiological studies have investigated the effects exhaust material derived cerium on human health with the majority of estimates for potential toxicity carried out through experimental testing. Inhalation exposure of atherosclerotic prone mice to exhaust material from diesel combustion caused a trend towards increased atherosclerotic plaque size and complexity, which was not present upon exposure to exhaust material from combustion of CeO_2NPs containing fuel (Cassee et al. 2012). In the same study however, there were increased inflammatory mediators within the brain and liver following inhalation of diesel fuel containing aqueous CeO_2NPs. Further work using this model by the same group revealed modifying effects on brain cortical AP-1 activation by diesel fuel exhaust inhalation, which was not observed on inhalation of exhaust material from fuel containing CeO_2NPs (Lung et al. 2014). Rats exposed to inhaled exhaust material from combustion of diesel fuel containing CeO_2NPs displayed increased lung inflammation compared to diesel fuel alone (Snow et al. 2014). This study also provided valuable *in vivo* evidence of extra-pulmonary translocation of the Ce after inhalation of diesel exhaust-containing CeO_2NPs.

In addition to speculation that changing emission characteristics may be responsible for altered biological effects observed for CeO_2NPs containing fuel, there is also speculation that CeO_2NPs themselves, as a component of particulate emissions have the potential to induce adverse effects. Indeed, as CeO_2NPs have been detected in ambient air in locations where fuel borne additives are used (Gantt et al. 2015; Park et al. 2008), it further highlights the concern for inhalation exposure and the effects on lung health. CeO_2NPs have unique redox properties and have been observed to possess anti-oxidant activity (Sims et al. 2017), a property which may protect against disease processes where

oxidant stress is a key factor. Indeed, analysis of diesel exhaust particulate (DEP) emissions from CeO_2NPs containing fuel displayed lower oxidative potential than standard fuel particulates (Zhang et al. 2016). However despite this, there is a substantial body of evidence that inhalation of CeO_2NPs can cause pulmonary inflammation (Gosens et al. 2014; Keller et al. 2014; Schwotzer et al. 2017). Moreover we have recently demonstrated that CeO_2NPs can exacerbate type II inflammation in a murine model of allergen induced asthma (Meldrum et al. 2018)

Individuals with susceptible lung conditions such as asthma comprise a particularly sensitive population group, with the potential to be impacted more greatly by pollutant exposures such as DEP than the general population (Brandt et al. 2015; Brandt et al. 2013; Codispoti et al. 2015; Diaz-Sanchez et al. 1999). Asthma is a chronic lung condition typified by reversible airway obstruction, airway hyperresponsiveness and excessive mucus production (Murphy and O'Byrne 2010). Pathological features common to most forms of the disease include goblet cell metaplasia, airway remodelling and chronic inflammation and can be captured using experimental models such as HDM allergen exposures (Gregory et al. 2009) used to assess the potential of different materials with the potential to impact disease. Importantly such models have allowed us to investigate the impact nanomaterials have on asthma and allergic airway disease (Meldrum et al. 2017b). Pollutant exposures such as DEP have also been tested and been observed to produce a mixed T helper (Th)2 / T_H17 type inflammation in a model of HDM allergen pulmonary exposure with increased mucin production (Brandt et al. 2015; Brandt et al. 2013). Through analysis of changes in lung function, mucus production, inflammatory cells such as neutrophils and eosinophils within the lung and meditators of allergic airway disease initiation and propagation including IL-33, CCL20, TSLP, IL-25, IL-4, IL-5, IL-13 and CCL11 as well as immunoglobulin E (IgE) and mast cell levels (Lambrecht and Hammad 2015), it is possible to use such models to assess the impact of pulmonary particle exposure on lung health including asthma.

In the absence of any toxicological information on how respiratory exposure to CeO_2NPs may modulate DEP particle effects on asthma and allergic airway disease, we set out to examine their effects using a model of HDM+DEP co-exposure. Exposure to DEP, CeO_2NPs and HDM was carried out across 9 separate intranasal exposures over 3 weeks and lung tissue was examined for alterations in inflammatory and mucin related changes as well as respiratory function.

2. METHODS

2.1 Instillation material preparation and characterisation

DEP were obtained from the US National Institute of Standards and Testing (Gaithersburg, Maryland, USA) (Cat # SRM2975). CeO_2NPs were obtained from Sigma Aldrich (Dorset, United Kingdom) (cat # 544841). DEP and CeO_2NPs were suspended in phosphate buffered saline (PBS) containing 0.05% Tween-80 with or without HDM (Greer Laboratories, Lenoir, cat# XPB82D3A25) for instillation procedures as well as for size and charge characterisation. Particles were dispersed by sonication (QSonica Sonicators, CT, USA) with 4.2x105kJ/m3. Particle size distribution was determined using nanoparticle tracking analysis (NanoSight LM10 instrument, NanoSight, Amesbury, UK). Measurements were assessed for a minimum of 60 seconds and processed using NTA 3.2 Analytical software. Zeta potential (particle charge) was determined by dynamic light scattering using a Zetasizer Nano (Malvern, Malvern United Kingdom). Size and zeta potential measurements were performed for 3 technical replicates with 3 independent samples.

2.2 Animal exposure and tissue collection

Female Balb/c mice (6-8 weeks) sourced locally (Envigo, UK) were anaesthetised with 5% isoflurane in oxygen using a precision vaporizer and intranasally instilled with 25 µl of combinations of DEP, CeO_2NPs and HDM as described in Figure 1. Specifically, mice were treated with HDM 8 times prior to a final instillation of either HDM+DEP (SGL - H+D), HDM+DEP+CeO_2NPs (2.5 µg/kg) (SGL - H+D+CeLD) or HDM+DEP+CeO_2NPs (75 µg/kg) (SGL - H+D+CeHD). Mice were also exposed to 9 repeat instillations of HDM+DEP (RPT - H+D), HDM+DEP+CeO_2NPs (2.5 µg/kg) (RPT - H+D+CeLD) or HDM+DEP+CeO_2NPs (75 µg/kg) (RPT - H+D+CeHD) also as described in Figure 1. Control animals (CTRL) were exposed to 9 repeat instillations of saline containing 0.05% Tween-80. After the treatment period, mice were euthanized using 0.1 mL sodium pentobarbital (200 µg/mL) by intraperitoneal injection and exsanguination by cardiac puncture. Blood was collected into EDTA coated tubes and plasma was isolated by centrifugation at 1500xg for 15 min at 4°C. Lung tissue was isolated after bronchoalveolar lavage (BAL), washed in sterile PBS and lobes either frozen or perfused with 10% formaldehyde solution. All procedures comply with ARRIVE guidelines and were performed in accordance with the Animals (Scientific Procedures) Act 1986 which complies with EU Directive 2010/63/EU. All experimental procedures were reviewed and approved by the local Animal Welfare and Ethical Review Body.

2.3 BAL cell analysis

Immediately after sacrifice, the trachea was exposed, cannulated and lavaged with 500 µl ice-cold sterile PBS. The lungs were aspirated, and the process repeated a further two times (1.5 ml total). BAL fluid was centrifuged at 1500xg for 10mins at 4°C and the supernatant from the first lavage was collected for subsequent enzyme-linked immunosorbent assay (ELISA) analysis. Total BAL cell counts were determined using a haemocytometer. Cell numbers were subsequently adjusted to a concentration of $1x10^6$ cells/ml and centrifuged using EZ single cytofunnels (Thermo Scientific, cat# 97200125) onto Superfrost™ Plus slides (Menzeil-Gläser Superfrost Plus, Thermo Scientific, cat# 10149870). Cells were then stained for differential immune cell counts using the Wright-Giemsa stain (Kwik Diff, Thermo Scientific, cat# 9990700). Slides were dried overnight before mounting and counting macrophages, lymphocytes, neutrophils and eosinophils across a minimum of 300-500 cells per sample. Cells were counted for each animal over three different slides.

2.4 ELISA

Lung tissue was homogenised using MagNa lyser beads (Roche, cat# 03358941001) in HBSS lysis buffer containing 2% Triton-x100 (Sigma Aldrich, cat# 93426-100ML) and protease inhibitor cocktail (Roche, cat# 05892970001). Lysates were then assessed for levels of the inflammatory mediators IL-4 (Cat# DY404), IL-5 (Cat# DY405), IL-13 (Cat# DY413), IL-33 (Cat# DY3626), TSLP (Cat# DY555), CCL20 (DY760), IL-1α (Cat# DY400) and IL-17A (Cat# DY421) using DuoSet kits from R&D systems (Abingdon, UK). For MCPT1, protein levels within lung lysates were determined as per manufacturer's instructions (Cat# 88-7503; Invitrogen, VWR Lutterworth, UK). Plasma IgE concentrations were also measured using a BD OptEIA ELISA kit (cat# 555248; BD Bioscience, Oxford, UK). Samples were analyzed in duplicate and diluted as appropriate in sample buffer. Absorbance was assessed at 450nm with background levels at 570nm using a plate reader (Bio-Tek Synergy HT).

2.5 ICP-MS measurements

Lung tissue was digested in 20% nitric acid using an UltraWAVE™ microwave digester. Total Cerium (Ce) content was measured using an iCAP Q ICP-MS (Thermo Fisher Scientific, Hemel Hempstead, UK). Samples were run in KED mode and the isotopes monitored was ^{140}Ce. Calibration standards (0 – 10µg/L) were prepared from a Spex CertPrep 50 µg/L stock solution and iridium was used as internal standard

2.6 RNA extraction and PCR analysis

Total RNA was isolated using the RNeasy spin column method (Qiagen, Valencia, CA). Lung tissue (post BAL procedure) was homogenised in RLT buffer using MagNA lyser bead disruption (Roche Diagnostics, West Sussex, UK) prior to RNeasy spin column-based RNA isolation. RNA quantity and purity were determined using nanodrop (Thermo Scientific) and cDNA synthesised using a random hexamer-based protocol and reverse transcriptase as per manufacturer's instructions (Cat# BIO-27036, Bioline Reagents Ltd, UK). SYBR green real time PCR (40 cycles) was carried out using the QuantStudio™ 6 Flex Real-Time PCR System. Primers were designed using Primer 3 software and supplied by Integrated DNA technologies, UK. Primer sequences are listed in supplementary figure 1. Quantification was assessed using the delta-delta Ct method and normalized to B-actin control.

2.7 Histochemical analysis

After tracheal perfusion with 10% formalin, the left lung was placed into cassettes (Tissue Tek Processing Embedding cassette, Tissue Tek, cat# 4185TT2), dehydrated and embedded in paraffin wax (Tissue-Tek, VIP Sakura). Lungs were then cut into 5µm serial sections (Accu-Cut SRM, Sakura) before drying overnight at 37°C. Periodic acid–Schiff (PAS) staining was used to determine airway epithelial cell mucin content. Sections were imaged using a Leica DM 2000 microscope and Leica DFC450C camera (Leica Microsystems Ltd). Mucin positive cells stained pink and were quantified in airways between 100-250µm in diameter. Scoring of airways for mucin positive cells was calculated as: <10% = 0 11-20% = 2 21-50% = 3 51-75% = 4 and >75% = 5. Counts were performed blinded and for 3 airways per individual animal.

2.8 Data handling and statistical analysis

Statistical significance between groups was carried out with Graphpad Prism Software using one-way ANOVA and Fisher's LSD Test subsequent to outlier removal using the ROUT method. Results are expressed as mean ± standard error of the mean (SEM) unless otherwise stated. Measurements were carried out on a minimum of 6 mice per treatment unless otherwise stated.

3. RESULTS

3.1 CeO_2NPs augment inflammatory responses to pulmonary HDM and DEP exposure.

Exposures were carried out according to two main exposure protocols (Figure 1). A single exposure of particle combinations was applied onto a background of repeated HDM exposure (SGL) to capture potential inflammatory exacerbation potential. Repeated particle exposures (RPT) given in combination with HDM were also carried out to examine potential effects on pulmonary tissue driven adaptive immune responses and type II inflammation.

We demonstrated both dose and repeat exposure dependent accumulation of elemental cerium using ICP-MS as a confirmation of particle exposure to the lower airways after intranasal administration. For the 2.5μg/kg administration, there was negligible accumulation on SGL exposure and 0.3μg/g tissue for RPT exposure. For the 75μg/kg administration there was 0.6μg/g and 1.5μg/g of Ce for SGL and RPT exposures respectively (Supplementary Figure 2B).

Total cell number was increased with HDM+DEP treatments (both SGL and RPT protocols) and was further modified by the higher dose of CeO_2NPs (CeHD) for the SGL protocol but not for any other cerium co-exposures (Figure 2A). Differential BAL cell analysis was also carried out (Figure 2B). The percentage of macrophages within the BAL decreased with both SGL and RPT HDM+DEP exposures, a likely consequence of the increased proportion of other immune cell types within the lungs of these animals. However, the proportion of macrophages increased with CeO_2NPs (significant for CeHD) SGL co-exposure, which was not observed with RPT exposures and indicates this cell type as prominent in the initial pulmonary responses to CeO_2NPs exposure. HDM+DEP exposures resulted in an increase in proportion of neutrophils, which was greater for SGL than RPT protocols. This increase in neutrophil numbers was reduced by the higher dose of CeO_2NPs for the SGL protocol only. Lymphocyte numbers were also increased with both SGL and RPT HDM+DEP exposures with cerium co-exposures attenuating SGL HDM+DEP levels, while further increasing RPT HDM+DEP induced levels. The RPT HDM+DEP but not the SGL exposure protocol resulted in increased levels of eosinophils, which were further increased with CeLD co-exposure (Figure 2B). Mast cell levels were also assessed through measurement of the mast cell protein MCPT1 within lung tissue. Protein levels were observed to increase over control levels with SGL and RPT HDM+DEP exposures, which was not statistically significant. There was however a significant increase in MCPT1 above HDM+DEP levels with CeO_2NPs co-exposures for the RPT protocol of treatments (Figure 2A). Total IgE protein levels within the plasma was observed to increase with RPT HDM+DEP exposures and was further increased with the lower dose of CeO_2NPs (CeLD) co-exposure (Figure 2A). CeO_2NPs co-exposure did not alter levels of MCPT1 or IgE when co-exposure with HDM+DEP was carried out according to the SGL protocol.

Goblet cell metaplasia was assessed through identification of mucin positive cells by PAS histochemical staining. These cells were examined within airways between 100-250μm diameter as airway epithelial

cells stained pink (Figure 2C). Quantification of mucin positive cells revealed a significant increase with RPT HDM+DEP exposures, which was further increased with CeO_2NPs co-exposure, which was statistically significant for the RPT protocol only (Figure 2D).

3.2 Changes in inflammatory mediators within the lung in response to CeO_2NPs, HDM and DEP

Increases in inflammatory components such as IL-33, IL-4, IL-5 and IL-13 within the lung indicate an activation of type II inflammation. We next measured protein levels of mediators of this type of inflammation within lung homogenates (Figure 3). HDM+DEP exposure for the SGL protocol resulted in increased levels of IL-4, IL-5, IL-13, IL-33, TSLP and CCL20. CeO_2NPs co-exposure for this protocol did not modify HDM+DEP levels for these cytokines except for CCL20, where there was a significant reduction at both dose levels. For the RPT protocol HDM+DEP caused an increase in IL-5, IL-13 and TSLP, which was further increased with CeO_2NPs co-exposure. While IL-33 levels were not significantly increased above CTRL with HDM+DEP RPT protocol, levels were increased with CeO_2NPs co-exposure. There were no significant differences in IL-1α levels with any exposure. IL-17A levels were also assessed as an indication of T_H17 type inflammation. IL-17A was not significantly altered with SGL-HDM+DEP treatment but was significantly enhanced above these levels with CeLD co-exposure. RPT protocol HDM+DEP exposure did increase IL-17A levels and this was further enhanced with both CeO_2NPs dose co-exposures.

As a next step to further understand the changes in inflammatory events within the lung and underlying mechanisms through which CeO_2NPs modify the responses to HDM and DEP exposures, we examined gene expression changes in whole lung tissue using PCR analysis of mRNA (Figure 4A). The greatest increases in gene expression for HDM+DEP exposures were observed for the RPT protocol with mucin related genes CLCA1, MUC5AC and SLC26A4 observed with the greatest magnitude of change. Of these genes CLCA1 and SLC26A4 were further enhanced by CeO_2NPs treatment (Figure 4B). CeO_2NPs were also observed to increase RPT HDM+DEP levels of other inflammatory mediators including CXCL5, CCL11, CCR7, IL-4, IL-17F, CCL17, CCL2, TSLP, IL-25 and IL-6 (Figure 4). For the SGL protocol of exposures, CCL8 was the only gene to display significant increases with HDM+DEP exposure and this was further enhanced by co-exposure with CeO_2NPs (Figure 4A). Significant reductions compared to CTRL levels were observed for IL1RL1, ACTA2, POSTN and PPARG with SGL HDM+DEP, which were further modified by CeO_2NPs for ACTA2, POSTN and PPARG.

3.3 CeO_2NPs and DEP effects in the absence of HDM co-exposure.

We next examined how CeO_2NPs modify the response to DEP pulmonary exposure in the absence of HDM to evaluate, whether allergen co-exposure is required for observed changes in inflammatory events. This was carried out according to the RPT protocol as described in Figure 5A using the same intranasal exposure method and doses as previously used with HDM allergen co-exposure. RPT DEP exposure did not result in any significant changes in plasma IgE or mucin positive cells with the airways. There was however a significant increase in the levels of both inflammatory endpoints with CeO_2NPs co-exposure at the higher dose (Figure 5B-C). We also examined BAL cell responses to particle exposure. Total cell counts/ml within the lung were increased with RPT DEP exposure but, although remaining significantly higher than control, fell significantly compared to DEP following CeHD co-exposure (Figure 5D). No statistically significant changes in differential counts of macrophages, neutrophils, lymphocytes or eosinophils were observed with any particle treatment (Figure 5E). However neutrophil counts can be observed to increase somewhat with RPT DEP and RPT DEP+CeLD, albeit not to a statistically significant level.

4. DISCUSSION

In this study we set out to examine whether CeO_2NPs can modify the impact DEP have on HDM induced inflammatory responses within the lung. RPT exposure to HDM+DEP resulted in increased levels of inflammatory cells including neutrophils, lymphocytes and eosinophils, which was also accompanied by increases in airway mucin production and the inflammatory mediators IL-5, IL-13 and IL-17A. These responses have been observed previously for DEP (Brandt et al. 2015; Brandt et al. 2013) and ambient particulate matter (Castaneda et al. 2018) upon co-exposure with HDM and describe elements of both type II and T_H17 type inflammation. RPT co-exposure also increased type II and T_H17 type inflammation within the airways, including increases in mucin production, lymphocytes, eosinophils, IL-5, IL-13 and IL-17A as well as gene expression changes for IL-4, IL-17F, CCL17 and CCL11. Increased IgE and mast cell levels (MCPT1), which are a major component of type II adaptive immune responses, were also induced by CeO_2NPs co-exposure. These results are consistent with our previous observations with CeO_2NPs (750µg/kg) in the absence of DEP, where we demonstrated an exacerbation of HDM induced type II inflammation using the same 3 week RPT protocol (Meldrum et al. 2018). Interestingly, one of the doses used in our previous study (75µg/kg) failed to produce any significant effects in the absence of DEP, while the same and lower doses (2.5 & 75µg/kg) when given in conjunction with DEP in our current study resulted in augmented HDM effects. This would indicate that DEP may act to facilitate cellular and tissue responses to CeO_2NPs effects. This suggestion is also supported by observations made for exposures in the absence of HDM, where CeO_2NPs increased IgE and PAS staining on co-exposure with DEP, observations which were not observed in the absence of DEP (Meldrum et al. 2018).

Single exposures of combined CeO_2NPs and DEP on a background of repeated HDM treatments were also examined (SGL protocol), both as a means to understand the mechanisms of RPT exposure effects and also to understand how established allergen induced disease may be impacted by pollutant exposures. SGL CeO_2NPs+DEP co-exposure resulted in an increase in total BAL cell counts above DEP induced levels that can likely be explained by the concomitant increase in the macrophage proportion of cells in the BAL. IgE and MCPT1 levels were not altered by particle treatment, which is consistent with the understanding that sensitisation processes require timeframes beyond 24hrs to develop (Lambrecht and Hammad 2015). Changes in type II inflammatory cytokines were observed with SGL DEP exposure as increased levels of IL-4, IL-5 and IL-13. An established source for these mediators is the T_H2 T-cell, which may have been recruited to the lung as a consequence of repeated HDM exposure. For IL-5 and IL-13, it should also be considered that resident innate lymphoid ILC2 cells may act as a source (Fahy 2015; Lambrecht and Hammad 2015). As these mediators are upregulated with RPT CeO_2NPs co-exposure but not SGL co-exposure, it could be argued that given the length of time necessary to mount a T_H2 adaptive immune response, it is the T-cell rather than the ILC2 cells that are responsible for mediator levels and subsequent type II inflammation. Alternatively, it could also be argued that cumulative particle dose and increased ILC2 recruitment could also play a role in RPT exposure effects. Interestingly IL-17A levels were increased with SGL CeO_2NPs co-exposure indicating

ILC3 and /or T_H17 cells (Kim et al. 2014) as potential primary responders with the potential to influence subsequent disease outcome. Further characterisation of the lymphocyte subtypes induced with RPT CeO_2NPs would help define the precise mechanisms of enhanced inflammatory effects.

Repeated treatment with CeO_2NPs resulted in increased levels of mucin positive epithelial cells above DEP induced levels within the airways. This was also paralleled by increased levels of gene expression for the epithelial expressed mucin regulating genes CLCA1 and SLC26A4, both of which have been associated with asthmatic disease and to be regulated by IL-13 (Brett 2015; Nakagami et al. 2008; Nakao et al. 2008). The innate cytokines IL-33, TSLP and IL-25 are released from the airway epithelium and activate dendritic cells, ILC2 cells and basophils to propagate allergen induced type II inflammation and sensitisation processes (Lambrecht and Hammad 2015) and were also increased with RPT CeO_2NPs exposure. These observations indicate that in addition to airway epithelial inflammation, that epithelial cells are likely active participants in directing type II inflammatory processes and associated induction of sensitisation in this study. This is further supported by the observation that CeO_2NPs increased levels of the lymph node homing chemokine receptor CCR7, which is typically found on activated dendritic cells.

Lymph nodes, as the site of B and T-cell differentiation are central to adaptive immune responses (Lambrecht and Hammad 2015). In addition to migrating antigen presenting dendritic cells, lung draining lymph nodes have also been observed as a site of nanoparticle accumulation after lung exposure (Meldrum et al. 2017b). Interestingly Ma and colleagues demonstrated that CeO_2NPs more readily accumulated in the rat lung draining lymph nodes after pulmonary administration than did DEP and that when combined, there appeared to be synergistic particle accumulation (Ma et al. 2014). This study was carried out in the absence of allergen and at doses up to 14 times higher than used in our own study and while they did not demonstrate any synergistic effect of CeO_2NPs and DEP on lung health, the observation of lymph node particle accumulation is intriguing and may reflect part of the mechanism through which CeO_2NPs influence adaptive immune responses in our own work.

It has been suggested that nanomaterials exert their adverse effects on the lungs through mechanisms involving the induction of oxidative stress (Nemmar et al. 2017). This mechanism of action is of particular interest given that CeO_2NPs possess unique redox properties that can potentially mitigate oxidative stress (Casals et al. 2017). Generally, studies have demonstrated antioxidant and protective effects of CeO_2NPs in conditions where oxidant injury is prominent (Manne et al. 2017) (Guo et al. 2015), while exposure in the absence of underlying disease results in inflammatory events (Demokritou et al. 2013; Keller et al. 2014). Interestingly in regards to the induction of sensitisation, it has been demonstrated that the ability of CeO_2NPs to direct dendritic cell function towards differentiation of T-cells to a T_H2 phenotype was related to their ability to reduce reactive oxygen species (Schanen et al. 2013). To investigate the role of oxidative stress, we previously examined anti-oxidant response gene expression in response to CeO_2NPs in the absence of DEP on a background of HDM co-exposure using

the same protocols in this study and did not find any change in NQO1 and HMOX1 (Meldrum et al. 2018). However, with DEP co-exposure in our current work we did find that CeO_2NPs induced the expression of NQO1. In the context of DEP co-exposure, Steiner and colleagues examined how exposure to pristine CeO_2NPs aerosol after diesel exhaust material exposure impacted in vitro airway inflammatory and oxidative stress endpoints. While little change in inflammatory markers was noted, CeO_2NPs did modify the effect of diesel exhaust on oxidative stress markers (Steiner et al. 2012). While the potential exists that oxidative stress acts as a mechanism through which CeO_2NPs enhance inflammatory events in our study, it is unclear at the moment how this may manifest at the molecular and cellular level.

Particle characteristics including size have been demonstrated to play an important part in how nanomaterials influence models of asthma and allergic airway disease (Meldrum et al. 2017b). We have previously demonstrated that the CeO_2NPs used in this current study have an average primary particle size of ~25nm and an agglomerate size of 166 nm (Meldrum et al. 2018). This is broadly within the size range of cerium particles found in diesel exhaust after use of CeO_2NP fuel additives (e.g. 50-300 nm (Dale et al. 2017) and ~ 75nm (Gantt et al. 2015). The size of DEP in our study were found to be ~135 nm (Supplementary Figure 2), which is not dissimilar to the accumulation mode size distribution typically found for DEP as part of exhaust material (peak accumulation mode (100-200nm)) (Kittelson et al. 2002). Addition of CeO_2NPs to DEP increased the mean particle agglomerate size to ~180 nm but as the SD was quite large, indicating a broad size distribution, the impact of the different particle size distributions as a contributing factor to the effects of CeO_2NPs above DEP induced effects (increased IgE and PAS staining in the absence of HDM) is unlikely but perhaps worthy of further consideration. However, the presence of HDM in the particle suspension further increased the particle mean size distribution, which was particularly pronounced for the HDM+DEP+CeO_2NPs combination (~250 nm). While not statistically different, it is interesting to speculate whether such a change in size could contribute in part to the way in which CeO_2NPs modify inflammatory events.

The translational context of doses used in our study to real world human exposure is important for how we should consider the impact of the findings in our model system. For DEP exposure, Li et al previously estimated for a polluted area in the US with air pollutant fine particulate matter ($PM_{2.5}$) levels of 7 9μg/m^3 that cumulative particle deposition within the human asthmatic lung at hot spots such as the carina was 2.3 μg/cm^2 over a period of 24hrs (Li et al. 2003). We have previously calculated that intranasal instillation of 2.5mg/kg DEP used in this study results in an approximate proximal lung airway deposition for 24 hrs of 4.2 μg/cm^2 (Meldrum et al. 2017a). The concentration range of CeO_2NPs as a proportion of DEP (0.1 & 3% (2.5 μg/ml and 75 μg/ml respectively)) used in this study was chosen to reflect the proportion of cerium found in exhaust PM (0.4-0.9%) when fuel borne catalysts such as Envirox™ are added to fuel at the recommended concentrations (9-10ppm) (Lung et al. 2014; Snow et al. 2014; Zhang et al. 2016). For both DEP and CeO_2NPs, while the doses used in our study do not exactly match real world human exposure estimates, we believe they are within a reasonable range to

have some translational significance. In addition to estimates of particulate deposition in polluted environments, it is also acknowledged that metal particulates from catalytic converters such as platinum (Pt) are present within vehicular exhaust material (Wiseman et al. 2016). How such materials may impact lung health is also worthy of investigation. As Pt nanoparticles possess superoxide anti-oxidant activity and the ability to scavenge reactive oxygen species similar to CeO_2NPs (Clark et al. 2011), it is interesting to speculate whether Pt nanoparticles within DEP exists at levels capable of modifying effects on lung health and inflammation.

Fuel borne catalysts directly influence particle and chemical exhaust characteristics as they change combustion properties within the engine (Zhang et al. 2013; Zhang and Balasubramanian 2017). This makes it difficult to disentangle the biological impact that may be attributed to the physical and chemical properties of the catalyst from the effects due to a changing pollutant mixture. The design of our experimental approach was therefore focussed on examining the direct effects of CeO_2NPs on a background of the same DEP. We do however acknowledge that exhaust particulate and chemical characteristics that change with fuel-borne catalyst use may influence biological response to inhaled material. We also acknowledge that the use of pristine nanoparticles in our study may not be entirely representative of the catalyst nanoparticle characteristics observed after combustion (Dale et al. 2017).

5. CONCLUSION

In summary we have demonstrated that relatively small proportional doses of CeO_2NPs relative to DEP can modify the immunological response within the lung towards type II inflammation. We believe our findings to be of significance and should be considered when assessing the potential hazard of fuel borne catalyst modified pollutant material in the environment especially for hazard potential in those individuals with disease conditions such as asthma and allergic airway disease.

Disclosure Statement

The authors report no conflicts of interests with the work in this article. The authors alone are responsible for the content and writing of the manuscript.

Funding and Acknowledgements

This research was funded through Public Health England's Asthma Program and PhD Studentship fund. The authors would like to thank Kevin Whitehill and Chris Ottaway for their help with animal studies.

Authors' contributions

KM and MOL designed the experiments and analysed the resulting data. KM, SR & MOL performed exposures and tissue collection. KM performed BAL cell, ELISA, histochemical analysis. KM & MOL performed PCR. KM, IR & TM performed nanomaterial characterisation and ICP-MS analysis. KM, MOL, RS, TWG & TDT all contributed to study design. MOL prepared the manuscript for publication. All authors read, edited and approved the final manuscript.

REFERENCES

Brandt EB, Biagini Myers JM, Acciani TH, et al. (2015) Exposure to allergen and diesel exhaust particles potentiates secondary allergen-specific memory responses, promoting asthma susceptibility. J Allergy Clin Immunol 136(2):295-303 e7 doi:10.1016/j.jaci.2014.11.043

Brandt EB, Kovacic MB, Lee GB, et al. (2013) Diesel exhaust particle induction of IL-17A contributes to severe asthma. J Allergy Clin Immunol 132(5):1194-1204 e2 doi:10.1016/j.jaci.2013.06.048

Brett TJ (2015) CLCA1 and TMEM16A: the link towards a potential cure for airway diseases. Expert Rev Respir Med 9(5):503-6 doi:10.1586/17476348.2015.1081064

Casals E, Gusta MF, Piella J, Casals G, Jimenez W, Puntes V (2017) Intrinsic and Extrinsic Properties Affecting Innate Immune Responses to Nanoparticles: The Case of Cerium Oxide. Front Immunol 8:970 doi:10.3389/fimmu.2017.00970

Cassee FR, Campbell A, Boere AJ, et al. (2012) The biological effects of subacute inhalation of diesel exhaust following addition of cerium oxide nanoparticles in atherosclerosis-prone mice. Environ Res 115:1-10 doi:10.1016/j.envres.2012.03.004

Castaneda AR, Vogel CFA, Bein KJ, Hughes HK, Smiley-Jewell S, Pinkerton KE (2018) Ambient particulate matter enhances the pulmonary allergic immune response to house dust mite in a BALB/c mouse model by augmenting Th2- and Th17-immune responses. Physiol Rep 6(18):e13827 doi:10.14814/phy2.13827

Clark A, Zhu A, Sun K, Petty HR (2011) Cerium oxide and platinum nanoparticles protect cells from oxidant-mediated apoptosis. J Nanopart Res 13(10):5547-5555 doi:10.1007/s11051-011-0544-3

Codispoti CD, LeMasters GK, Levin L, et al. (2015) Traffic pollution is associated with early childhood aeroallergen sensitization. Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology 114(2):126-33 doi:10.1016/j.anai.2014.10.020

Commission E (2017) REPORT FROM THE COMMISSION TO THE EUROPEAN PARLIAMENT AND THE COUNCIL; in accordance with Article 9 of Directive 98/70/EC relating to the quality of petrol and diesel fuels. https://ec.europa.eu/transport/sites/transport/files/com20170284-evaluationreportfuelqualitydirective.pdf

Dale JG, Cox SS, Vance ME, Marr LC, Hochella MF, Jr. (2017) Transformation of Cerium Oxide Nanoparticles from a Diesel Fuel Additive during Combustion in a Diesel Engine. Environ Sci Technol 51(4):1973-1980 doi:10.1021/acs.est.6b03173

Demokritou P, Gass S, Pyrgiotakis G, et al. (2013) An in vivo and in vitro toxicological characterisation of realistic nanoscale CeO(2) inhalation exposures. Nanotoxicology 7(8):1338-50 doi:10.3109/17435390.2012.739665

Diaz-Sanchez D, Garcia MP, Wang M, Jyrala M, Saxon A (1999) Nasal challenge with diesel exhaust particles can induce sensitization to a neoallergen in the human mucosa. The Journal of allergy and clinical immunology 104(6):1183-8

Erdakos GB, Bhave PV, Pouliot GA, Simon H, Mathur R (2014) Predicting the effects of nanoscale cerium additives in diesel fuel on regional-scale air quality. Environ Sci Technol 48(21):12775-82 doi:10.1021/es504050g

Fahy JV (2015) Type 2 inflammation in asthma--present in most, absent in many. Nat Rev Immunol 15(1):57-65 doi:10.1038/nri3786

Gantt B, Hoque S, Fahey KM, et al. (2015) Factors Affecting the Ambient Physicochemical Properties of Cerium-Containing Particles Generated by Nanoparticle Diesel Fuel Additive Use. Aerosol Science and Technology 49(6):371-380 doi:10.1080/02786826.2015.1027809

Gosens I, Mathijssen LE, Bokkers BG, Muijser H, Cassee FR (2014) Comparative hazard identification of nano- and micro-sized cerium oxide particles based on 28-day inhalation studies in rats. Nanotoxicology 8(6):643-53 doi:10.3109/17435390.2013.815814

Gregory LG, Causton B, Murdoch JR, et al. (2009) Inhaled house dust mite induces pulmonary T helper 2 cytokine production. Clin Exp Allergy 39(10):1597-610 doi:10.1111/j.1365-2222.2009.03302.x

Guo C, Smith R, Gant TW, Leonard MO (2015) Cerium dioxide nanoparticles protect against oxidative stress induced injury through modulation of TGF-β signalling. Toxicology Research 4(2):464-475 doi:10.1039/c4tx00210e

Keller J, Wohlleben W, Ma-Hock L, et al. (2014) Time course of lung retention and toxicity of inhaled particles: short-term exposure to nano-Ceria. Arch Toxicol 88(11):2033-59 doi:10.1007/s00204-014-1349-9

Kenneth Reed AC, Aniruddha Kulkarni, Mark Mayton, Dean Sayle, Fred Klaessig and Brad Stadler (2014) Exploring the properties and applications of nanoceria: is there still plenty of room at the bottom? *Environ Sci: Nano* 1:390-405 doi:10.1039/x0xx00000x

Kim HY, Lee HJ, Chang YJ, et al. (2014) Interleukin-17-producing innate lymphoid cells and the NLRP3 inflammasome facilitate obesity-associated airway hyperreactivity. Nat Med 20(1):54-61 doi:10.1038/nm.3423

Kittelson DB, Watts WF, Johnson J (2002) Diesel Aerosol Sampling Methodology - CRC E-43: Final Report. University of Minnesota, Report for the Coordinating Research Council

Lambrecht BN, Hammad H (2015) The immunology of asthma. Nat Immunol 16(1):45-56 doi:10.1038/ni.3049

Li N, Hao M, Phalen RF, Hinds WC, Nel AE (2003) Particulate air pollutants and asthma. A paradigm for the role of oxidative stress in PM-induced adverse health effects. Clin Immunol 109(3):250-65

Lung S, Cassee FR, Gosens I, Campbell A (2014) Brain suppression of AP-1 by inhaled diesel exhaust and reversal by cerium oxide nanoparticles. Inhal Toxicol 26(10):636-41 doi:10.3109/08958378.2014.948651

Ma JY, Young SH, Mercer RR, et al. (2014) Interactive effects of cerium oxide and diesel exhaust nanoparticles on inducing pulmonary fibrosis. Toxicol Appl Pharmacol 278(2):135-47 doi:10.1016/j.taap.2014.04.019

Manne N, Arvapalli R, Graffeo VA, et al. (2017) Prophylactic Treatment with Cerium Oxide Nanoparticles Attenuate Hepatic Ischemia Reperfusion Injury in Sprague Dawley Rats. Cell Physiol Biochem 42(5):1837-1846 doi:10.1159/000479540

Meldrum K, Gant TW, Leonard MO (2017a) Diesel exhaust particulate associated chemicals attenuate expression of CXCL10 in human primary bronchial epithelial cells. Toxicol In Vitro 45(Pt 3):409-416 doi:10.1016/j.tiv.2017.06.023

Meldrum K, Guo C, Marczylo EL, Gant TW, Smith R, Leonard MO (2017b) Mechanistic insight into the impact of nanomaterials on asthma and allergic airway disease. Part Fibre Toxicol 14(1):45 doi:10.1186/s12989-017-0228-y

Meldrum K, Robertson SB, Romer I, et al. (2018) Cerium dioxide nanoparticles exacerbate house dust mite induced type II airway inflammation. Part Fibre Toxicol 15(1):24 doi:10.1186/s12989-018-0261-5

Murphy DM, O'Byrne PM (2010) Recent advances in the pathophysiology of asthma. Chest 137(6):1417-26 doi:10.1378/chest.09-1895

Nakagami Y, Favoreto S, Jr., Zhen G, et al. (2008) The epithelial anion transporter pendrin is induced by allergy and rhinovirus infection, regulates airway surface liquid, and increases airway reactivity and inflammation in an asthma model. J Immunol 181(3):2203-10

Nakao I, Kanaji S, Ohta S, et al. (2008) Identification of pendrin as a common mediator for mucus production in bronchial asthma and chronic obstructive pulmonary disease. J Immunol 180(9):6262-9

Nemmar A, Yuvaraju P, Beegam S, Fahim MA, Ali BH (2017) Cerium Oxide Nanoparticles in Lung Acutely Induce Oxidative Stress, Inflammation, and DNA Damage in Various Organs of Mice. Oxidative Medicine and Cellular Longevity 2017:12 doi:10.1155/2017/9639035

Park B, Donaldson K, Duffin R, et al. (2008) Hazard and risk assessment of a nanoparticulate cerium oxide-based diesel fuel additive - a case study. Inhal Toxicol 20(6):547-66 doi:10.1080/08958370801915309

Schanen BC, Das S, Reilly CM, et al. (2013) Immunomodulation and T helper TH(1)/TH(2) response polarization by CeO(2) and TiO(2) nanoparticles. PLoS One 8(5):e62816 doi:10.1371/journal.pone.0062816

Schwotzer D, Ernst H, Schaudien D, et al. (2017) Effects from a 90-day inhalation toxicity study with cerium oxide and barium sulfate nanoparticles in rats. Part Fibre Toxicol 14(1):23 doi:10.1186/s12989-017-0204-6

Sims CM, Hanna SK, Heller DA, et al. (2017) Redox-active nanomaterials for nanomedicine applications. Nanoscale 9(40):15226-15251 doi:10.1039/c7nr05429g

Snow SJ, McGee J, Miller DB, et al. (2014) Inhaled diesel emissions generated with cerium oxide nanoparticle fuel additive induce adverse pulmonary and systemic effects. Toxicol Sci 142(2):403-17 doi:10.1093/toxsci/kfu187

Steiner S, Mueller L, Popovicheva OB, et al. (2012) Cerium dioxide nanoparticles can interfere with the associated cellular mechanistic response to diesel exhaust exposure. Toxicol Lett 214(2):218-25 doi:10.1016/j.toxlet.2012.08.026

Wiseman CL, Hassan Pour Z, Zereini F (2016) Platinum group element and cerium concentrations in roadside environments in Toronto, Canada. Chemosphere 145:61-7 doi:10.1016/j.chemosphere.2015.11.056

Zhang J, Nazarenko Y, Zhang L, et al. (2013) Impacts of a nanosized ceria additive on diesel engine emissions of particulate and gaseous pollutants. Environ Sci Technol 47(22):13077-85 doi:10.1021/es402140u

Zhang JJ, Lee KB, He L, et al. (2016) Effects of a nanoceria fuel additive on the physicochemical properties of diesel exhaust particles. Environ Sci Process Impacts 18(10):1333-1342 doi:10.1039/c6em00337k

Zhang ZH, Balasubramanian R (2017) Effects of Cerium Oxide and Ferrocene Nanoparticles Addition As Fuel-Borne Catalysts on Diesel Engine Particulate Emissions: Environmental and Health Implications. Environ Sci Technol 51(8):4248-4258 doi:10.1021/acs.est.7b00920

FIGURE LEGENDS

Figure 1. Experimental protocol for pulmonary exposure.

Mice were exposed through intranasal instillation to HDM (1.25 mg protein/kg) alone or in combination with DEP (2.5 mg/kg) and CeO_2NPs (2.5 µg/kg or 75 µg/kg). Three-week exposure protocols involved 9 individual instillations on the indicated days. Specific protocols involved either pre-treatment with HDM for the first 8 instillations followed by a combination of HDM + DEP+/- CeO_2NPs (SGL) or 9 repeat treatments of HDM + DEP+/- CeO_2NPs (RPT) as indicated. Sacrifice and collection of tissues was carried out on day 20.

Figure 1.

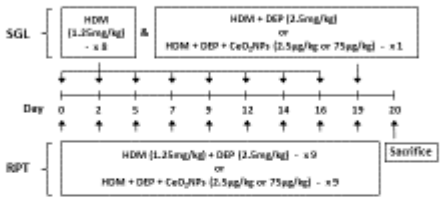

Figure 2. Inflammatory responses after exposure to HDM, DEP and CeO2NPs.

After instillation protocols in mice (n=5-6 per treatment group) , cells in bronchoalveolar lavage fluid were counted (A). Total blood plasma Immunoglobulin E (IgE) and lung homogenate levels for MCPT1 protein were also examined (A). Results were expressed as mean ± SEM fold over control (F.O.C.) and ng/ml levels respectively. Differential BAL cells were also examined (300-500 per animal) (B). In addition, lung tissue was fixed, and sections processed for PAS staining (scale bar of 100µm) (C).

Quantification of airway mucin positive cells is displayed as mean counts ± SEM PAS score (D). Statistical comparisons of SGL-H+D and RPT-H+D with CTRL levels are represented as * ($p<0.05$), with CeO_2NP treatments versus H+D controls represented as # ($p<0.05$).

Figure 2 .

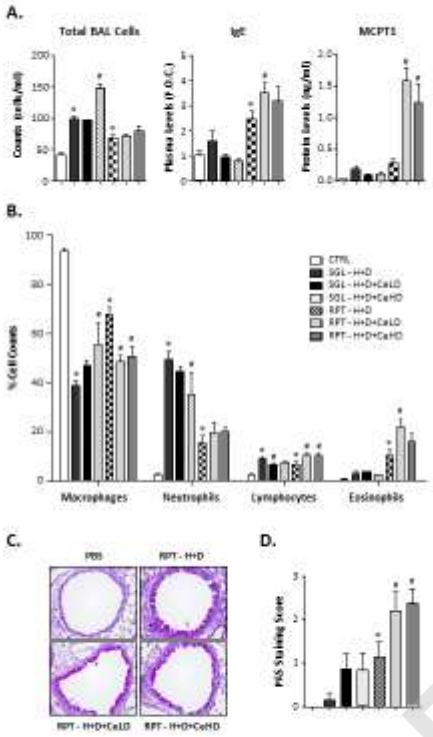

Figure 3. Inflammatory mediator expression after exposure to HDM, DEP and CeO2NPs.

After treatment, lung homogenates were analysed for protein levels. Results were expressed as mean ± SEM protein concentration (pg/ml). Statistical comparisons of SGL-H+D and RPT-H+D with CTRL levels are represented as * ($p<0.05$), with CeO_2NP treatments versus H+D controls represented as # ($p<0.05$).

Figure 3.

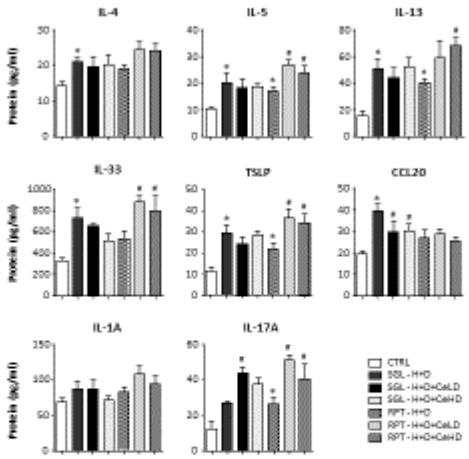

Figure 4. Lung gene expression analysis after exposure to HDM, DEP and CeO2NPs.

After instillation protocols, total lung gene expression was analysed. Results were expressed as Log2 fold over CTRL levels with green down and red upregulated expression, where the intensity of colour is proportional to magnitude of change (A). Selected gene expression was also displayed in more detail and expressed as mean ± SEM fold over control (F.O.C.) levels (B). Statistical comparisons of SGL-H+D and RPT-H+D with CTRL levels are represented as * ($p<0.05$), with CeO_2NP treatments versus H+D controls represented as # ($p<0.05$).

Figure 4.

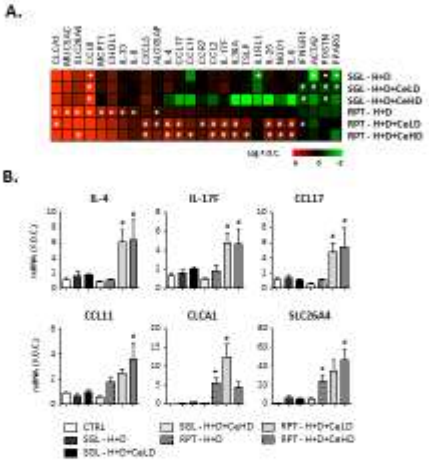

Figure 5. Inflammatory responses after exposure to DEP and CeO2NPs.

Mice (n=6) were exposed to 9 repeat instillations of diesel exhaust particles (DEP) (2.5mg/kg) and cerium dioxide nanoparticles (CeO2NPs) (2.5µg/kg or 75µg/kg) (A). After treatment, total blood plasma IgElevels were examined (B). Lung tissue was analysed for airway mucin positive cells (C). Cells in bronchoalveolar lavage fluid were counted with results expressed as mean ± SEM cells/ml of BAL fluid (D). BAL cells were also examined for differential immune cell content with results expressed as mean ± SEM % total cells counted (300-500 per animal) (E). Statistical comparison of RPT-D with CTRL represented as * ($p<0.05$), with CeO_2NPs treatments versus RPT-D represented as # ($p<0.05$).

Figure 5.