

Ambient particulate matter induces an exacerbation of airway inflammation in experimental asthma: role of interleukin-33

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Abstract:

High levels of ambient environmental particulate matter (PM₁₀ i.e. <10 µm median aerodynamic diameter) have been linked to acute exacerbations of asthma. We examined the effects of delivering a single dose of Sydney PM₁₀ by intranasal instillation to BALB/c mice that had been sensitised to ovalbumin and repeatedly challenged with a low ($\approx 3 \text{ mg/m}^3$) mass concentration of aerosolised ovalbumin for 4 weeks. Responses were compared to animals administered carbon black as a negative control, or a moderate ($\approx 30 \text{ mg/m}^3$) concentration of ovalbumin to simulate an allergen-induced acute exacerbation of airway inflammation.

Delivery of PM₁₀ to mice, in which experimental mild chronic asthma had previously been established, elicited characteristic features of enhanced allergic inflammation of the airways, including eosinophil and neutrophil recruitment, similar to that in the allergen-induced exacerbation. In parallel, there was increased expression of mRNA for interleukin (IL)-33 in airway tissues and an increased concentration of IL-33 in bronchoalveolar lavage fluid.

Administration of a monoclonal neutralising anti-mouse IL-33 antibody prior to delivery of particulates significantly suppressed the inflammatory response induced by Sydney PM₁₀, as well as the levels of associated pro-inflammatory cytokines in lavage fluid. We conclude that IL-33 plays a key role in driving airway inflammation in this novel experimental model of an acute exacerbation of chronic allergic asthma induced by exposure to PM₁₀.

Introduction

Asthma is characterised by chronic allergic inflammation of the airways, with superimposed episodes of acute inflammation (1). These acute exacerbations are the most clinically significant feature of asthma and are the main contributor to direct healthcare costs associated with the disease (2). Exacerbations are typified by increased distal airway inflammation, with exaggeration of symptoms such as cough, chest tightness and dyspnoea (3, 4). As many as 80% of acute exacerbations of asthma are triggered by respiratory viral infections, most commonly human rhinovirus (5, 6). Allergens and other triggers may also be important. Notably, there is accumulating epidemiological evidence that high levels of atmospheric pollutants, including particulates and oxides of nitrogen, can trigger acute exacerbations of asthma (7-10). However, little is understood about the mechanisms involved.

Airborne particulate matter (PM) may be solid or liquid material, which can be generated as a result of natural phenomena such as dust storms, bush and forest fires, or volcanic eruptions, as well as by human activity. Environmental PM with the potential to cause respiratory effects is categorised on the basis of size as PM₁₀, PM_{2.5}, PM₁ or PM_{0.1} i.e. all particles less than a median aerodynamic diameter of 10, 2.5, 1 or 0.1 μm respectively (11). A major source of 'man-made' particulate matter is the combustion of fossil fuels, typically associated with vehicular emissions and industrial activity. Thus, a number of experimental studies have examined the effects of diesel exhaust particulates (DEP) on cells of the respiratory tract and on acute allergic inflammation of the airways (12-17).

However, although DEP represent an important component of urban air pollution, exposure to DEP does not accurately represent the widely varying nature of particulate pollutants in urban environments. Ambient urban PM has a bimodal size distribution, with peaks at approximately 1 μm and 5 μm median aerodynamic diameter (18, 19). Whereas DEP primarily contribute to PM_{2.5} (20), at least half of the airborne particulate mass is in the PM_{2.5-10} range. These so-called coarse particles have a higher metal content (19) and may have a greater potential to cause injury to cells of the respiratory system (21). Thus, experimental assessment of the relationship between particulate air pollution and

exacerbations of asthma needs to focus on ambient particulate matter in an animal model of pre-existing mild chronic asthma. However, to date no such studies have been performed.

We have previously described a model of mild chronic allergic asthma in mice (22).

In this well-characterised animal model, we have demonstrated chronic asthmatic inflammation of the airways and changes of structural remodelling in mice that were systemically sensitised to ovalbumin (OVA) and received long-term inhalational challenge with a controlled low mass concentration of aerosolised OVA. Importantly, lesions are confined to the conducting airways and there is minimal parenchymal inflammation, unlike conventional short-term models of allergic airway inflammation. We have shown that this model of chronic asthma can be used to assess the effects of potential triggers of an acute exacerbation. Thus, following exposure to a single moderate-level challenge with aerosolised OVA, acute inflammatory changes simulating an allergen-induced asthmatic exacerbation are elicited, with rapid and enhanced accumulation of eosinophils and neutrophils around intrapulmonary airways (23). In the present study, we show that ambient particulate matter (PM₁₀) collected from the geographical centre of the Sydney region provokes enhanced allergic airway inflammation in mice in which experimental chronic asthma had previously been established. Furthermore, we provide evidence that interleukin-33 (IL-33) plays a key role in the development of a PM₁₀-induced acute exacerbation of airway inflammation.

Materials and Methods

Animals

Specific pathogen-free female BALB/c mice aged 7-8 weeks were obtained from the Biological Resources Centre, University of New South Wales. Animals were held in individually ventilated cages, exposed to a 12-hour light/dark cycle and were provided with autoclaved water and food *ad libitum*. All experimental procedures complied with the requirements of the Animal Care and Ethics Committee of the University of New South Wales (reference number: 12/108B).

Particulate matter

Ambient particulate matter (PM₁₀) was collected on Teflon sampling filters by the Air Quality Monitoring Unit, Office of Environment and Heritage, New South Wales Government, Sydney, Australia. Particulates were released from filters and dispersed in solution by ultrasonication in endotoxin-free 0.9% saline for 30 min. To quantify the mass of released particles, filters were dried for a minimum of 48 hours and weighed on a precision balance before and after ultrasonication. PM₁₀ were resuspended in saline at a concentration of 1 mg/ml.

These particulates have previously been shown to be associated with a variety of trace metals, notably including Mg, Al, Fe, Zn and Cu; as well as with alkanes (C₁₀-C₃₀, with major peaks in the range C₁₂-C₁₄) and various aromatic hydrocarbons. Suspensions of PM₁₀ contained <3 ng/ml bacterial endotoxin as assessed by a *Limulus* lysate assay (24).

Sensitisation and challenge of mice

The protocol for sensitisation and inhalational challenge has previously been described in detail (23). Briefly, animals were systemically sensitised by intraperitoneal injection of 50 µg of alum-precipitated chicken egg ovalbumin (OVA) (Grade V, ≥98% pure, Sigma, Sydney, Australia) 21 and 7 days prior to the commencement of inhalational challenge. Mice were placed in a whole-body inhalation chamber (Unifab Corporation, Kalamazoo, MI) and challenged with a low mass concentration of aerosolised ovalbumin (3 mg/m³) for 30 min/day, 3 days/week on alternate weekdays for 4 weeks, to establish a background of chronic asthmatic inflammation (Fig. 1). Aerosol concentration was assessed throughout all inhalational challenges, using a DustTrak 8520 instrument (TSI, St Paul, MN).

At the end of the 4 week period, animals received 50 µg of Sydney PM₁₀ intranasally in 50 µl of saline, or an equivalent amount of carbon black (Langridge Colours, Yarraville, Victoria, Australia) as negative control particulates. Additional groups of mice received a single 30 min challenge with a moderate level of aerosolised OVA (30 mg/m³) to simulate an allergen-induced acute exacerbation, as a positive control. Further controls included animals that received only chronic challenge with a low mass concentration of OVA aerosol for 4

weeks, as well as naïve mice that received either Sydney PM10 or carbon black. Each experimental group comprised 6 animals.

Assessment of inflammatory response

At 4 hours after the final airway challenge, mice were sacrificed by exsanguination following an overdose of sodium pentobarbital. This time point was selected on the basis of our earlier studies using this model, which established that in animals which had previously been chronically challenged, cytokine expression and cellular recruitment occurred more rapidly than in conventional short-term models (23, 25). Bronchoalveolar lavage (BAL) was performed with 2×1 ml of PBS, for total and differential cell counts and measurement of concentrations of pro-inflammatory cytokines. Intrapulmonary accumulation of eosinophils was assessed using a colorimetric assay of eosinophil peroxidase activity in lung tissue, adapted from previously described methods (26), which we have demonstrated to yield results equivalent to direct counts of eosinophils. Intrapulmonary neutrophil accumulation was assessed by immunostaining in frozen lung sections, using rat anti-Gr-1 (RB6-8C5, BD Bioscience, Sydney, Australia). Cells were enumerated in a minimum of 10 microscopic fields of lung tissue in 1-2 sections per animal. All counting/grading was performed by a single observer blinded to the identity of the samples and slides were examined in a random order.

Cytokine expression in airway tissue

Proximal airway tissue was isolated by blunt dissection as previously described (27). RNA was extracted using TriReagent. Samples were treated with DNase (Turbo DNase, Ambion, Scoresby, Australia) and reverse transcribed into cDNA using Superscript III (Invitrogen). Quantitative real time PCR was used to assess expression of IL-4 and IL-33, with detection of amplified products using SYBR green (BioLine, Tauton, MA). Reactions were performed using a LightCycler 480 (Roche Diagnostics, Sydney, Australia) and expression was normalised to HPRT.

Protein immunoassays

The concentrations of IL-33, TNF- α and CXCL1 in BAL fluid were assessed using enzyme-linked immunoassays (R&D systems, Australia) according to the manufacturer's instructions.

Neutralisation of IL-33

To assess the role of IL-33 in the development of a particulate-induced acute exacerbation, mice received an intraperitoneal injection of 100 µg of either a blocking monoclonal antibody to IL-33 (clone 1F11, MBL, Nagano, Japan) (28) or a control monoclonal antibody to β-galactosidase (βGL-113) (29), 30 minutes prior to the final moderate-level challenge.

Statistical analysis

Data are presented as arithmetic means ± SEM. Results were analysed by a one-way ANOVA followed by a Holm-Sidak multiple comparison test. The software package GraphPad Prism 6.02 (GraphPad Software, San Diego, CA) was used for all data analysis and preparation of graphs.

Results

Ambient particulates promote asthmatic airway inflammation

In preliminary experiments, naïve animals that received intranasal Sydney PM10 or carbon black did not exhibit evidence of airway inflammation at 4 hours, in terms of either the total number of cells in BAL fluid or the percentage of neutrophils in BAL (total cell counts for naïve animals administered saline = $15.6 \pm 1.9 \times 10^4$, carbon black = $18.8 \pm 3.5 \times 10^4$, PM10 = $17.1 \pm 2.1 \times 10^4$; percentage of neutrophils for naïve animals administered saline = 0.0 ± 0.0 , carbon black = 0.0 ± 0.0 , PM10 = 0.1 ± 0.1). Animals that received intranasal Sydney PM10 at the end of the 4-week period of chronic challenge had a significant increase in the total number of cells in BAL fluid as compared to the carbon black negative control group, in which cell numbers were comparable to the naïve and chronic challenge control groups (Fig. 2A) (analysis by one-way ANOVA including all groups and post-hoc testing for differences compared to the relevant control group). The increase was similar to that observed in the group of mice in which moderate-level challenge with aerosolised OVA was used to simulate an allergen-induced acute exacerbation; this group served as a positive control. The percentage of neutrophils in BAL was also significantly increased in the PM group compared to the carbon black negative control group (Fig. 2B) and again this was comparable to the increase observed in the allergen-induced acute exacerbation group, relative to the chronic

challenge group. The percentage of lymphocytes in BAL was similarly increased and that of macrophages was correspondingly decreased in the PM group, analogous to the allergen-induced acute exacerbation group (Fig. 2C, D).

In lung tissue, there was a parallel increase in numbers of intrapulmonary neutrophils as revealed by immunohistochemical staining (Fig. 2E). Again, the increase was significant compared to the carbon black negative control group, which remained as low as the naïve and chronic challenge control groups, and was equivalent to that observed in the allergen-induced acute exacerbation group. Importantly, there was also an increase in intrapulmonary eosinophils in the animals that received intranasal Sydney PM10, as demonstrated by the assay for eosinophil peroxidase activity (Fig. 2F). Once more, these changes were equivalent to those seen in the allergen-induced acute exacerbation group.

Ambient particulates increase expression of IL-4 and IL-33 in airways

Following the 4-week period of chronic challenge, intranasal administration of particulate matter significantly increased the expression of mRNA for both IL-4 and IL-33 in airway wall tissue, to an extent comparable to that observed in animals in which an acute exacerbation of airway inflammation was induced by moderate-level exposure to aerosolised ovalbumin (Fig. 3A,B). This was paralleled by a significant increase in the concentration of IL-33 in BAL fluid (Fig. 3C) which again was similar to that seen in the allergen-induced acute exacerbation group. No such increases were seen in relevant control groups.

Inhibition of IL-33 suppresses particulate matter-induced airway inflammation

Compared to treatment with the control antibody, treatment of animals with anti-IL-33 prior to induction of an acute exacerbation with PM10 inhibited recruitment of inflammatory cells to the airways (Fig. 4A). Although the decrease in cell numbers in BAL fluid was not statistically significant, there was a significant reduction in the percentage of neutrophils in BAL fluid in animals pre-treated with anti-IL-33, compared to the control antibody-treated group that received PM10 (Fig. 4B). This was paralleled by a significant reduction in the numbers of intrapulmonary neutrophils (Fig. 4C). There was also significant inhibition of the recruitment of eosinophils into the lung tissue associated with exposure to particulates, demonstrated using the assay for eosinophil peroxidase activity (Fig. 4D).

Treatment with the anti-IL-33 neutralising antibody also significantly diminished levels of TNF- α and CXCL1 proteins in BAL fluid (Figs. 4E, F) compared to animals which received the control antibody prior to challenge with particulate matter.

Discussion

In this report, we provide the first experimental evidence that ambient particulate matter can trigger an acute exacerbation of airway inflammation in mice with experimental mild chronic asthma. Exposure to ambient particulates induced changes in the airways similar to those of an allergen-induced exacerbation of airway inflammation, including up-regulation of expression of IL-4 as well as recruitment of both eosinophils and neutrophils, consistent with an augmented Th2-biased immunological response. This was paralleled by increased levels of pro-inflammatory cytokines in BAL fluid. Importantly, these particulates did not induce airway inflammation when administered to naïve animals. The data thus highlight the ability of ambient particulates, a non-allergic trigger, to elicit an antigen-independent allergic response in the setting of pre-existing mild asthmatic inflammation.

While epidemiological data published to date have been somewhat controversial, our findings are consistent with those studies that have suggested an association between exposure to high levels of ambient particulates and aggravation of asthma, potentially requiring increased medication use and/or hospital admission (7, 9, 10, 30). In this context, it is noteworthy that our studies used ambient particulate matter collected by air sampling in the geographical centre of the Sydney region, including all particulates with median aerodynamic diameter less than 10 μm (PM10). Most recently published experimental studies of the effects of airborne particulate pollutants on allergic inflammation and asthma have focused on PM2.5, which include the majority of particulates derived from vehicular emissions (16, 17, 31, 32). While these clearly pose a significant risk to human health, the larger so-called coarse particulates of median aerodynamic diameter 2.5-10 μm have to some extent been overlooked. However, PM10 particulates are potentially more hazardous (21), possibly because they have a higher content of metal oxides (19), which may be related to the much higher fraction of particulates of geological origin.

The relevance of our findings is strengthened by the fidelity of the animal model of asthma employed in this study. Most animal models of asthma typically involve systemic sensitisation to a protein, followed by short-term, high-level inhalational exposure to the aerosolised protein. Although these models trigger the development of allergic airway inflammation, they fail to replicate the background features of chronic inflammation classically seen in human disease (33). The model which has been developed in our laboratory is one of long-term, low-level inhalational challenge which mimics many of the features of mild chronic human asthma, including airway-specific acute-on-chronic inflammation and airway wall remodelling (22). Importantly, in our model the animals do not develop inflammation of the lung parenchyma, a feature present in many of the short-term models but absent in human disease. Thus our findings are more likely to reflect the cellular and molecular events associated with particulate-induced exacerbations in asthmatic patients.

Our results may help to explain the mechanisms by which particulate matter is able to promote an acute exacerbation of airway inflammation in chronic asthma. Typical airborne particulates are comprised of a relatively inert carbonaceous core, on to which a variety of polycyclic aromatic hydrocarbons, quinones, metals, and volatile organic compounds may be adsorbed (34, 35). Many of these compounds are capable of inducing oxidative stress and in the context of developing a model of the induction phase of childhood asthma, we have previously shown that the ambient particulates used in this study elicit an oxidative stress response in airway epithelial cells (24). In turn, this may lead to the production of pro-inflammatory cytokines, potentially important among which is IL-33, a member of the IL-1 cytokine superfamily (36, 37). IL-33 is expressed by airway epithelial cells and by resident macrophages, and can drive production of Th2 associated cytokines such as IL-4, IL-5 and IL-13, as well as of chemokines relevant to allergic inflammation (38-42), which are likely to be important in the inflammation associated with an exacerbation of chronic allergic asthma. Because it can be generated in response to both allergic and non-allergic stimuli (43), IL-33 may be an important mechanism of cross-talk between innate host defences and the adaptive immune response. Such cross-talk is increasingly being recognised as important in the

pathogenesis of respiratory inflammation, including in acute exacerbations of asthma (44, 45).

Using our model of moderate-level allergen challenge on a background of low-level chronic challenge with OVA, we have recently demonstrated that IL-33 plays a key role in an experimental acute exacerbation of asthma, with suppression of airway inflammation following treatment with a neutralising antibody (46). Therefore, we hypothesised that this cytokine may similarly play an important role in the novel experimental model described in this study. This concept was supported by evidence of increased expression of IL-33 in the airways and BAL fluid following challenge with Sydney PM10. We found that prior administration of a neutralising antibody against IL-33 largely suppressed the particulate matter-induced exacerbation of airway inflammation. Specifically, there was a significant reduction in neutrophil recruitment and in BAL fluid levels of pro-inflammatory cytokines such as TNF- α and CXCL1. Of importance in this context is that whereas previous reports have suggested that the endotoxin content of ambient particulates may account for their capacity to induce an inflammatory response, at least on the basis of in vitro studies using macrophages (34, 47), the preparations of particulates that we used had a low endotoxin content (24). Thus each animal would have received a dose of <0.2 ng intranasally, which would have been insufficient to explain the resultant airway inflammation. Moreover, administration of particulates to naïve animals did not directly induce inflammation.

Our study has limitations. For technical reasons, we were unable to assess whether the exacerbation of airway inflammation induced by PM10 was associated with airway hyper-responsiveness, as previously demonstrated in an allergen-induced model of an asthmatic exacerbation (23). We were also unable to generate a complete profile of the changes in concentrations of pro-inflammatory and Th2 cytokines in BAL fluid, as we had obtained in previous work (23, 25).

Nevertheless, our data emphasise the importance of IL-33 in the development of a PM10-induced exacerbation of airway inflammation, analogous to our previous findings in an experimental allergen-induced exacerbation, suggesting that it may therefore be a relevant therapeutic target. However, as yet the relative contributions of airway epithelial cells and

macrophages to the increased levels of this cytokine remain undefined. Furthermore, additional studies will also be required to define the cellular mechanisms by which IL-33 exerts its effects. An intriguing possibility is that IL-33 may promote a Th2 cytokine response by IL-5/IL-13-producing innate lymphoid cells (ILCs), which appear to have a crucial role in Th2 allergic responses, including in the setting of established allergic inflammatory disease of the airways (48, 49). While both IL-25 and IL-33 can stimulate Th2 cytokine production by ILCs, IL-33 is particularly effective in inducing IL-13-producing cells (50) .

In conclusion, we have demonstrated that in a setting of experimental chronic asthma in mice, exposure to ambient particulate matter induces airway inflammation similar to an allergen-induced exacerbation of disease. This is mediated at least in part by IL-33, which may thus illustrate the role of cross-talk between innate host defences and the adaptive immune response in particulate-induced acute exacerbations of chronic asthma.

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Author contributions

R.K.K. and C.H. conceived and designed the experiments. A.M.S and C.H. performed the experiments. R.K.K., A.M.S. and C.H. analysed the data and wrote the paper.

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Conflict of interest

All authors declare they have no actual or potential conflict of interest.

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Legends to Figures

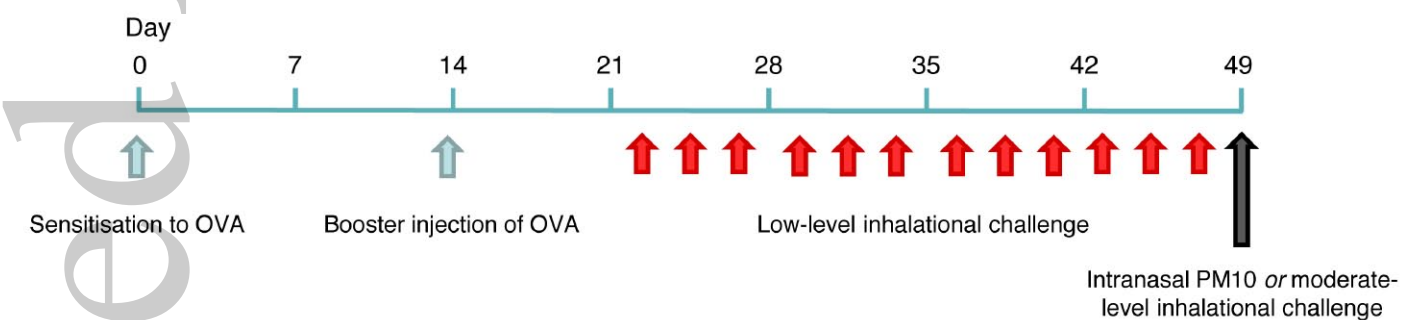
Fig. 1 Model of particulate-induced exacerbation of asthma: timeline for sensitisation, inhalational challenges with ovalbumin and intranasal challenge with particulates.

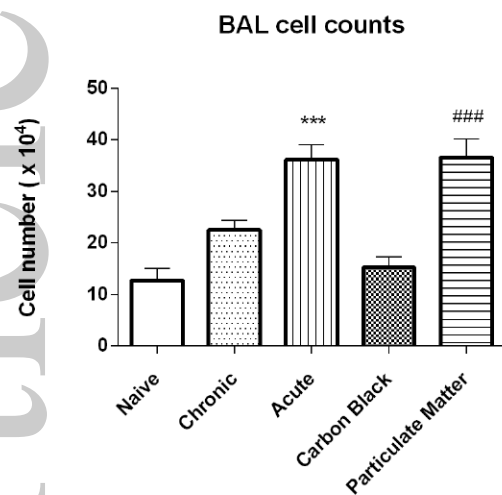
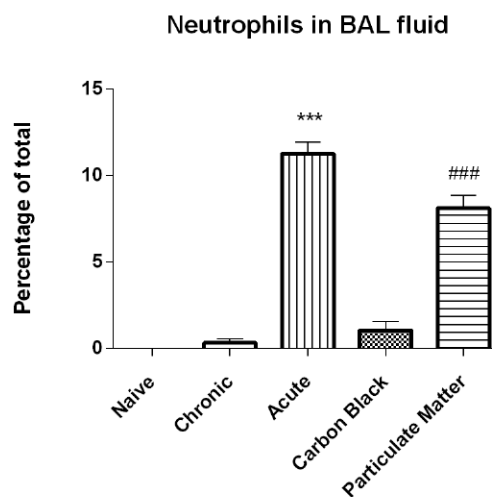
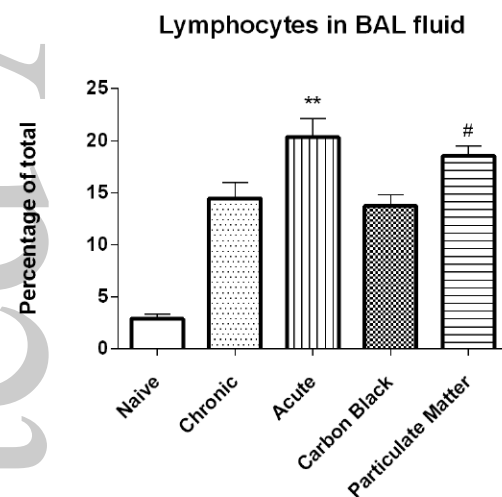
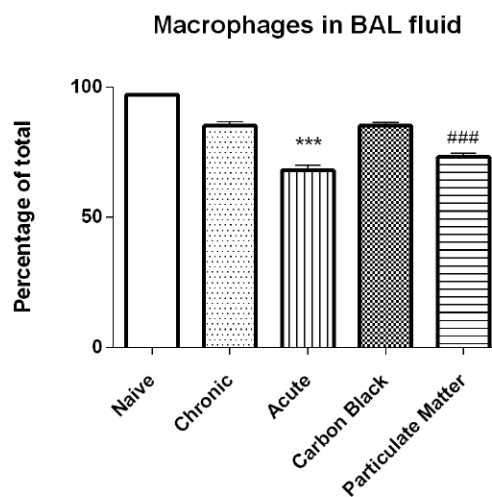
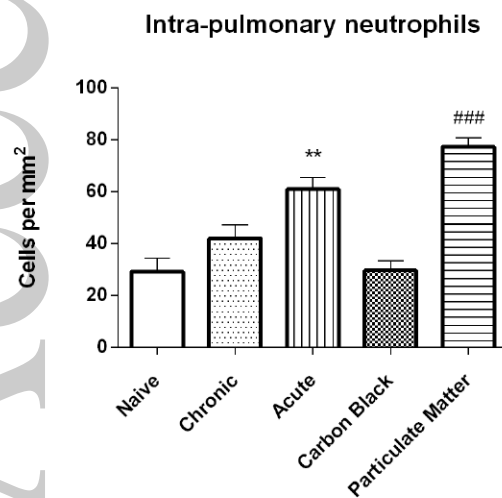
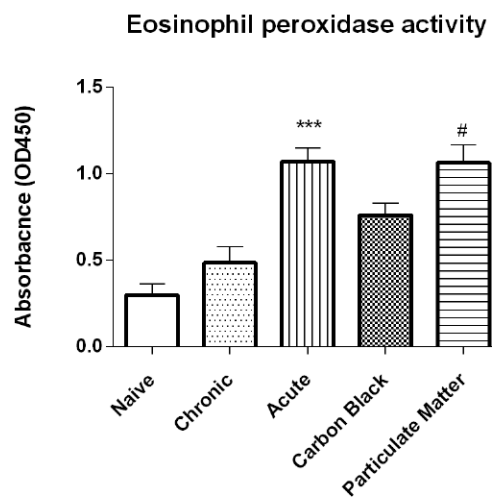
Fig. 2 Airway inflammation in the model of a particulate-induced exacerbation of asthma. (A) Numbers of cells in BAL fluid (B) Percentage of neutrophils in BAL fluid (C) Percentage of lymphocytes in BAL fluid (D) Percentage of macrophages in BAL fluid (E) Numbers of neutrophils in lung tissue, assessed by immunostaining (F) Relative numbers of eosinophils in lung tissue, assessed by colorimetric assay for EPO in lung tissue. Data are mean $\pm$ SEM ($n = 6$ samples per group). In this and subsequent figures, the acute group refers to the allergen-induced acute exacerbation group, which served as a positive control. Significant differences between the chronic and acute groups shown as ** ($p < 0.01$) and *** ($p < 0.001$); between the carbon black and PM10-treated groups shown as # ($p < 0.05$) and ### ($p < 0.001$).

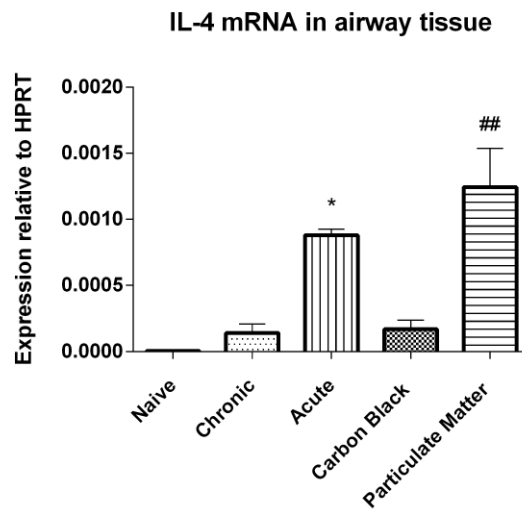
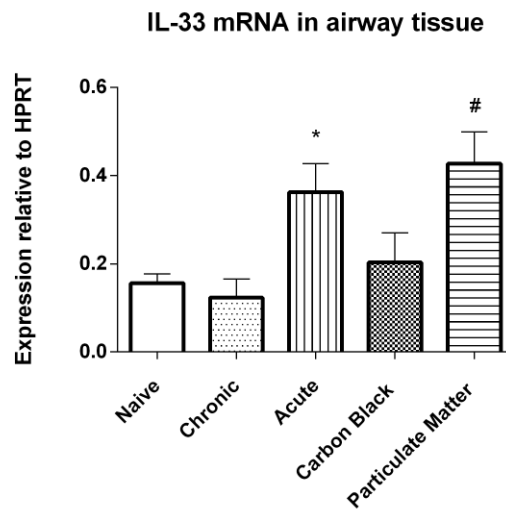
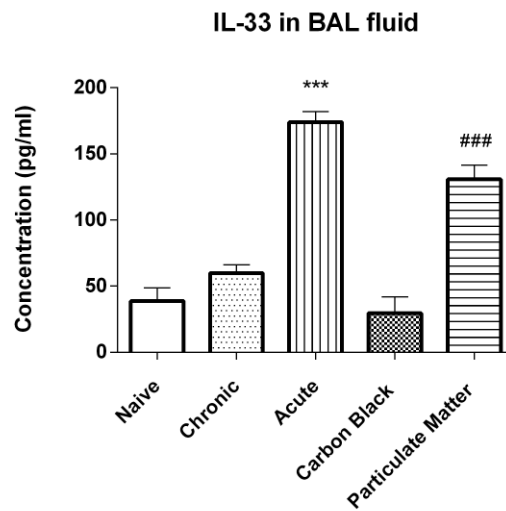
Fig. 3 Cytokine expression in the model of a particulate-induced exacerbation of asthma. (A) Relative expression of mRNA for IL-4 in airway wall tissue (B) Relative expression of mRNA for IL-33 in airway wall tissue (C) Concentration of IL-33 in BAL fluid. Data are mean $\pm$ SEM ($n = 6$ samples per group). Significant differences between the chronic and acute groups shown as * ($p < 0.05$) and *** ($p < 0.001$); between the carbon black and PM10-treated groups shown as # ($p < 0.05$) and ### ($p < 0.001$).

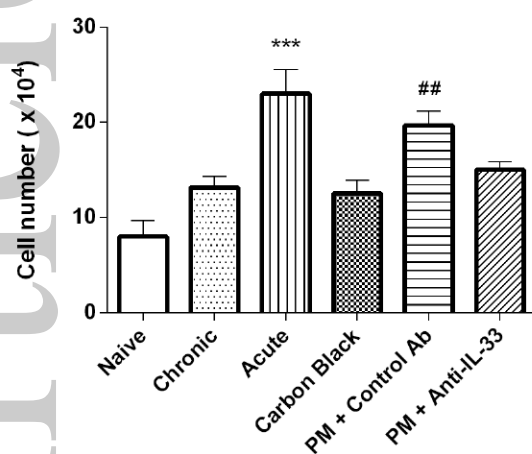
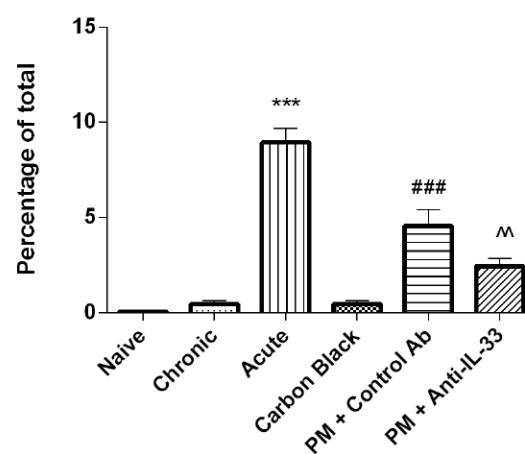
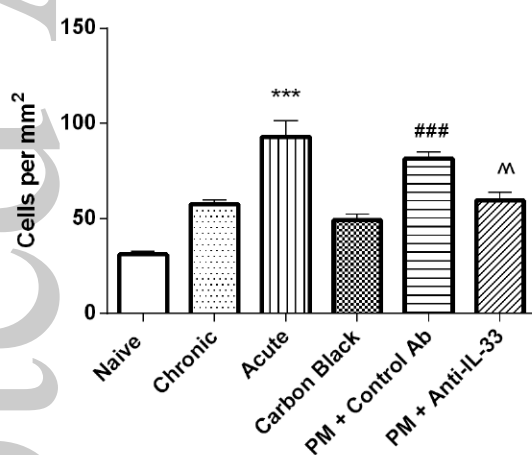
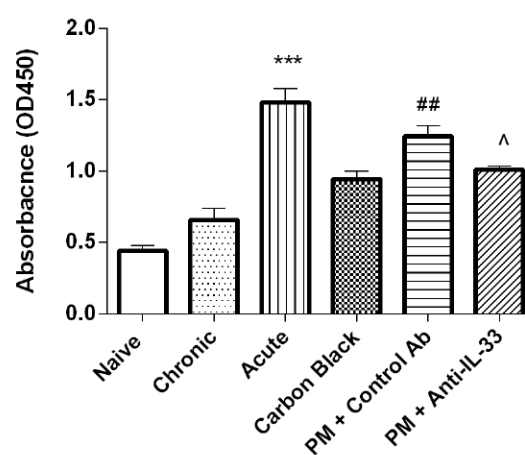
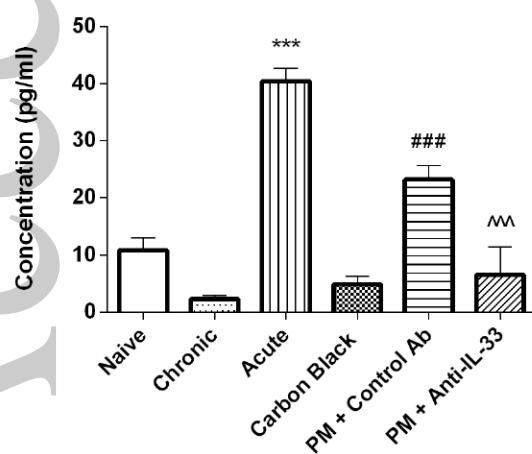
Fig. 4 Effect of anti-IL-33 on airway inflammation in the model of a particulate-induced exacerbation of asthma. (A) Numbers of cells in BAL fluid (B) Percentage of neutrophils in BAL fluid (C) Numbers of neutrophils in lung tissue, assessed by immunostaining (D) Relative numbers of eosinophils in lung tissue, assessed by colorimetric assay for EPO in lung tissue (E) TNF- α concentration in BAL fluid (B) CXCL1 concentration in BAL fluid. Data are mean $\pm$ SEM ($n = 6$ samples per group). Significant differences between the chronic and acute groups shown as *** ($p < 0.001$); between the carbon black and PM10 groups treated with control

antibody shown as ## ($p<0.01$) and ### ($p<0.001$); and between the PM10 group treated with control antibody and the PM10 group treated with anti-IL-33 as ^ ($p<0.05$), ^^ ($p<0.01$) and ^^^ ($p<0.001$).



A**B****C****D****E****F**

A**B****C**

A**BAL cell counts****B****Neutrophils in BAL fluid****C****Intra-pulmonary neutrophils****D****Eosinophil peroxidase activity****E****TNF- α in BAL Fluid****F****CXCL1 in BAL Fluid**