

Effect of ozone exposure on allergic sensitization and airway inflammation induced by dendritic cells

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Summary

Background Epidemiological studies suggest that ozone exposure is related to increased asthma symptoms. Dendritic cells (DCs) are the principal antigen-presenting cells in the airways.

Objective We have examined whether ambient doses of ozone (100 ppb for 2 h) enhance allergic sensitization and/or airway inflammation in a mouse model.

Methods C57BL/6 mice were sensitized to inhaled ovalbumin (OVA) by intratracheal instillation of OVA-pulsed DCs on day 0. Daily exposure to OVA aerosol on days 14–20 resulted in an eosinophilic airway inflammation, as reflected in bronchoalveolar lavage fluid and lung histology. In a first experiment, mice were exposed to ozone or room air immediately prior to and following sensitization. Subsequently, we tested the effect of ozone exposure during antigen challenge in DC-sensitized mice.

Results Exposure to ozone during sensitization did not influence airway inflammation after subsequent allergen challenge. In contrast, in sensitized mice, challenge with OVA together with ozone (days 14–20) resulted in enhanced airway eosinophilia and lymphocytosis, as compared with mice exposed to OVA and room air ($1.91 \times 10^6 \pm 0.46 \times 10^6$ vs. $0.16 \times 10^6 \pm 0.06 \times 10^6$ eosinophils/mL lavage fluid; $P = 0.015$; $0.49 \times 10^6 \pm 0.11 \times 10^6$ vs. $0.08 \times 10^6 \pm 0.03 \times 10^6$ lymphocytes/mL lavage fluid; $P = 0.004$). Ozone exposure without subsequent OVA exposure did not cause airway inflammation.

Conclusion Ozone exposure does not increase allergic sensitization but enhances antigen-induced airway inflammation in mice that are sensitized via the airways.

Keywords dendritic cell, ozone, sensitization, C57BL/6 mice, airway inflammation

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Introduction

Ozone is a major air pollutant to which millions of people throughout the urbanized world are regularly exposed. The main source of ozone in the lower atmosphere is nitrogen oxides and hydrocarbons from motor vehicle exhausts, reacting in the presence of ultraviolet light [1]. The increase in asthma prevalence in westernized societies during the last few decades has been paralleled by a rise in atmospheric levels of road traffic air pollution [2–4]. Epidemiological studies have consistently shown associations between short-term asthma morbidity, as revealed by gradual deterioration or acute exacerbations leading to hospitalization, and the concomitant ambient ozone levels [5–7]. A prospective cohort study (the Adventist Health Smog (AHSMOG) study) has found an association between the development of adult-onset asthma and long-term ambient ozone concentrations [8]. Controlled human experiments have demonstrated the development of neutrophilic and eosinophilic inflammation in upper and lower airways after exposure to even

low concentrations of ozone, both in healthy as well as asthmatic volunteers [9–11]. A few studies suggest that ozone may enhance the sensitivity to inhaled allergen in previously sensitized individuals. In human asthmatic volunteers, exposure to a low level of ozone has been shown to lower the threshold of allergen-induced bronchoconstriction, and enhances eosinophilic airway inflammation [12–14]. An intriguing question is whether exposure to ambient levels of ozone also facilitates sensitization to inhaled allergen. For evident ethical reasons this can only be experimentally studied in animal models. Previous studies in which the effect of ozone on allergic sensitization in guinea-pigs was studied used very high toxic ozone concentrations and considered anaphylaxis as end-point [15,16]. We have developed a model of sensitization to ovalbumin (OVA) in C57BL/6 mice by intratracheal instillation of OVA-pulsed dendritic cells (DCs), avoiding the use of systemic adjuvant. DCs are the principal antigen-presenting cells of the airways, that have the capacity to recognize antigens and their surrounding inflammatory context [17,18]. After encounter with allergen in the airway mucosa, these cells take up the allergen and transport it to the draining lymph nodes of the lung for presentation to naïve T cells [19]. Primary sensitization by DCs in the lung leads to the formation of Th2 lymphocyte-dependent eosinophilic airway inflammation [20].

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As DCs are crucial for inducing sensitization to inhaled allergen and as these cells have the capacity to recognize antigens in an inflammatory context [21], we have examined whether simultaneous exposure to ambient levels of ozone enhanced allergic sensitization induced by DCs, and whether exposure to the same levels of ozone aggravated allergen-induced airway inflammation in previously sensitized mice.

Materials and methods

Animals

Male inbred C57BL/6 mice were obtained from Harlan CBP (Zeist, the Netherlands). Mice were about six to eight weeks old and weighed 20–25 g at the time of testing. All animals were housed in the animal research facility of the Faculty of Medicine, University Hospital (Ghent, Belgium) and received food and water *ad libitum*. Mice were allowed to acclimatize for one week before starting the exposures.

Study design (Figure 1)

Influence of ozone exposure on the sensitization to OVA by intratracheal instillation of OVA-pulsed DCs The protocol to induce sensitization to OVA using OVA-pulsed DCs has been published in detail previously [20]. Three groups of 16 C57BL/6 mice were instilled with 10^4 (group 1), 10^5 (group 2) and 10^6 OVA-pulsed DCs (group 3), respectively (day 0). Half of the mice of each group were exposed daily to ozone (100 ppb for 4 h) from two days prior (day -2) to two days following instillation (day 2); the other half of the mice received room air. From day 14 to day 20, all mice were exposed daily to 1% OVA aerosol for 30 min. On day 21, bronchoalveolar lavage was performed and lung histology was examined.

Influence of ozone on airway inflammation induced by OVA aerosol in previously sensitized mice Three groups of eight mice were studied. The first two groups (groups 4 and 5) received intratracheal instillation with 10^5 OVA-pulsed DCs, the third group (group 6) received unpulsed DCs. From day 14 to day 20, mice from group 4 and group 5 were exposed daily to 1% OVA aerosol for 30 min, immediately preceded by ozone (100 ppb for 4 h) or room air, respectively. Mice from group 6 were exposed daily to ozone (100 ppb for 4 h) followed by phosphate buffer solution (PBS) aerosol for 30 min. On day 21, bronchoalveolar lavage was performed and lung histology was examined.

Methods

Culture and stimulation of DCs DCs were isolated as previously described in detail [20]. In short, mice bone marrow cells were collected in tissue culture medium and labelled with rat monoclonal antibodies against CD4 (GK1.4), CD8 (H35-172), MHC class II (B21.2), B220 (RA3-6B2) (all from American Type Culture Collection, Manassas, VA, USA) and GR-1 (RB6-8C5, PharMingen, San Diego, CA, USA), followed by incubation with paramagnetic anti-rat Ig beads (5 beads/cell, Dynabeads M450, Dynal, Oslo, Norway). Lineage depleted cells were grown in medium supplemented with 10 ng/mL of rhFlt3-ligand, 200 ng/mL rmGM-CSF and 1×10^3 U/mL rmIL-4. After 4 days of culture,

cells were grown under serum-free conditions (Optimem-1 with Glutamax, Gibco, Invitrogen Corporation, Carlsbad, CA, USA). At day 9, non-adherent cells were harvested, and enriched by density centrifugation over metrizamide (14.5% in tissue culture medium, Sigma Chemical Co., St Louis, MO, USA).

The OVA-pulsing of the DC was performed by incubating DC with 100 µg/mL of OVA (grade V, Sigma) overnight. After antigen-pulsing, cells were extensively washed to remove free OVA, and resuspended in PBS.

Intratracheal instillation of DCs

Mice were anaesthetized (avertin 2.5% v/v in PBS) and 80 µL of cell suspension was injected intratracheally using the technique of Ho and Furst [22]. After the instillation, animals were allowed to recover on a temperature control blanket.

Ozone exposure

Mice were exposed to ozone (0.100 ppm) for 4 h in an exposure chamber made of Plexiglas, measuring 20 cm × 30 cm × 40 cm. Control animals were exposed to room air for 4 h in the same exposure chamber. Ozone was generated by means of high voltage electrostatic pulses in an Ozomat COM ozone generator (Anseros, Tübingen, Germany) which was connected with Teflon tubing to the inlet of the exposure chamber. Ozone concentrations in the exposure chamber were analysed continuously by an Ozomat MP Ozone Analyser (Anseros, Tübingen, Germany) which was calibrated against an Envitec CSI 3100 ozone analyser (SI Columbia Scientific Industries, Austin, TX, USA), which in turn was calibrated against a standard ozone source at the 'IRCEL Ykbank' in Brussels. Ozone concentrations measured in the exposure chamber varied around 100 ppb (80–120).

Aerosol exposure

Groups of 10 mice were placed in an exposure chamber connected to the outlet of an ultrasonic nebulizer that delivers an aerosol of particles with a mean diameter of 3.5 µm (Microvernebler R80, Microvernebler, Zürich, Switzerland). Dissolving 1 g OVA in 100 cc PBS made a 1% OVA-solution.

Bronchoalveolar lavage

After the animals were anaesthetized with intraperitoneal pentobarbital, a probe was inserted via tracheotomy. Bronchoalveolar lavage was performed via intratracheal instillation of two washings of 0.5 mL saline. The fluid was recovered manually by gentle aspiration with a disposable, pyrogen-free syringe while exercising gentle pressure on the thorax. The lavage fluid was immediately centrifuged (270 g for 7 min at 4 °C) and the cell pellet was suspended in 1 mL PBS.

The cells were counted manually in a Bürker chamber. Cytospin preparations (Cytospin II; Shandon, Runcorn, UK; centrifugation 11 g for 5 min) were stained with May-Grünwald-Giemsa. Differential cell count based on standard morphologic criteria was made on 300 cells.

Lung histology

After bronchoalveolar lavage was performed, fixative (4% paraformaldehyde in PBS) was gently infused through the lavage catheter by a continuous release pump under pressure- and volume-controlled conditions. The lungs were then removed

and fixed for an additional 4 h. After routine paraffin embedding, 4- μ m sections were stained with haematoxylin-eosin and examined by light microscopy for histology.

Drugs and chemicals

Pentobarbital (Abbott Laboratories, Louvain-la-Neuve, Belgium), sodium EDTA and ovalbumin (Sigma), Hanks' solution (Pasteur, Brussels, Belgium) and saline (Baxter, Lessines, Belgium) were obtained from regular commercial sources.

Statistical analysis

Reported values are expressed as mean $\pm$ standard error of the mean (SEM).

All outcome variables were compared using a *t*-test, if the values were normally distributed, or a Mann-Whitney *U*-test for not normally distributed values. *P*-values of less than 0.05 were regarded as significant. For the statistical analysis, the SPSS program was used (SPSS Inc. Chicago, IL, USA).

Results

Influence of ozone exposure on the sensitization to OVA by intratracheal instillation of OVA-pulsed DCs in naïve mice

We have previously reported that OVA-aerosol exposure induces the influx of eosinophils and lymphocytes in the BAL of mice immunized with 1×10^6 OVA-pulsed DCs, but not in those immunized with 1×10^6 PBS-pulsed DCs [20]. In the current study, we addressed the effects of concurrent ozone exposure during the period of sensitization by OVA-pulsed DCs. Figure 2 shows the leucocyte cell recovery from mice from group 1–3. All mice that were instilled with 1×10^6 OVA-pulsed DCs (group 1) developed a significant eosinophilic airway inflammation, accompanied by increased amounts of lymphocytes recovered from lavage. This was independent of whether the instillation was accompanied by exposure to ozone or room air. A few mice instilled with 10^5 OVA-pulsed DC (group 2) developed a moderate eosinophilic infiltration, accompanied by very low amounts of lymphocytes in BAL fluid. Again, no significant differences were observed between mice exposed to ozone or room air. In mice instilled with 10^4 OVA-pulsed DC (group 3), no significant bronchoalveolar lavage eosinophilia could be demonstrated. Histological examination of lung tissue was concordant with the findings in bronchoalveolar lavage fluid: all animals with eosinophilia in bronchoalveolar lavage fluid had tissue infiltration of eosinophils, to the same extent in the perivascular and peribronchiolar interstitial tissue; no eosinophilic tissue infiltration could be found in mice without lavage fluid eosinophilia.

Influence of ozone on airway inflammation induced by OVA aerosol in previously sensitized mice

Figure 3 shows the leucocyte cell recovery from mice from group 4–6. In sensitized mice, OVA exposure (days 14–20) preceded by exposure to ozone resulted in enhanced airway eosinophilia and increased levels of BAL fluid lymphocytosis, as compared with mice exposed to OVA and room air ($1.91 \times 10^6 \pm 0.46 \times 10^6$ vs. $0.16 \times 10^6 \pm 0.06 \times 10^6$ eosinophils/mL lavage fluid; $P = 0.015$; $0.49 \times 10^6 \pm 0.11 \times 10^6$ vs. $0.08 \times 10^6 \pm 0.03 \times 10^6$ lymphocytes/mL lavage fluid;

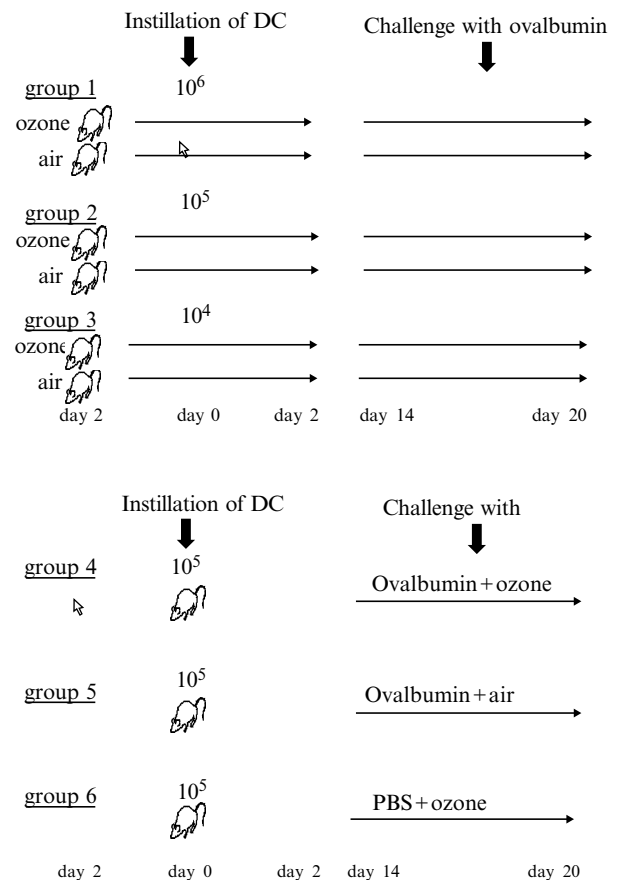


Fig. 1. Study protocol. See Materials and methods section for details. All groups of animals were examined on day 22. DC = dendritic cells.

$P = 0.004$). Ozone exposure without subsequent OVA exposure did not result in BAL fluid eosinophilia, neutrophilia or lymphocytosis (group 6).

Histological examination of lung tissue was concordant with the findings in bronchoalveolar lavage fluid. All animals with eosinophilia in bronchoalveolar lavage fluid also showed a marked tissue infiltration of eosinophils, to the same extent in the perivascular and peribronchiolar interstitial tissue; no eosinophilic tissue infiltration could be found in mice without lavage fluid eosinophilia. Since inflammation in histological specimens was scored semiquantitatively, no quantitative correlation between tissue eosinophilia and lavage fluid eosinophilia could be performed.

Discussion

In this study we have observed that exposure to ambient air levels of ozone does not modify the allergic sensitization process induced by DC but significantly increases the allergen-induced airway inflammation in previously sensitized mice.

In recent years, various animal models have been developed to study the contribution of various cell types and substances in the development of allergic airway inflammation. In many previous mice models, sensitization to antigen has been induced systemically by intraperitoneal injection of OVA, most often

emulsified in the Th2-skewing adjuvant aluminium hydroxide [23–26]. Intraperitoneal injection of antigen to sensitize naïve animals might not reflect naturally occurring sensitization to inhaled allergens. Also, the use of systemic adjuvant obscures many of the regulatory processes that occur in the airway microenvironment and serve to dampen the immune response

to inhaled allergen. In this series of experiments however, we have used intratracheal instillation of OVA-pulsed bone marrow-derived DCs in order to induce sensitization to OVA. The DC is the most important antigen-presenting cell in the airways for inducing primary immune responses *in vivo*. OVA-loaded myeloid DCs, instilled intratracheally in mice can prime naïve T lymphocytes for a subsequent recall response, as revealed by the development of an (Th2-dependent) eosinophilic airway inflammation upon repeated inhalation of OVA [18,20]. The development of this eosinophilic airway inflammation is strongly dependent on a Th2 driven response, as revealed by increased levels of Th2 but not Th1 cytokines in BAL fluid, and an expansion of CD4⁺ Th2 cells. Despite high levels of CD4 measured, no significant levels of OVA-specific IgE production can be detected in this model [20].

Our model of sensitization via the airways has enabled us to study directly the modulating effects of concurrent inhalation of air pollutants, in this case ozone, on the pulmonary immune response induced by DCs. The magnitude of the airway inflammation, as revealed by the recovery of inflammatory cells in bronchoalveolar lavage fluid, was dependent on the number of OVA-pulsed DCs instilled. A marked BAL fluid eosinophilia was observed in animals receiving 10⁶ OVA-pulsed DCs. Daily exposure to 100 ppb of ozone from two days before until two days after intratracheal instillation of DC did not influence the subsequent development of airway inflammation after allergen challenge. In mice sensitized with 10⁵ DCs, a moderate and variable inflammation was observed and there was a trend towards increased inflammation in animals concurrently exposed to ozone. This however, did not reach statistical significance, as the eosinophilic response between individual mice was quite variable.

Only some limited data are available about the effect of ozone exposure on the primary sensitization to inhaled allergen. In macaca monkeys, exposure to 1 ppm ozone (10-fold higher than in our study) concurrent with platinum salt increased the incidence of positive platinum skin tests and bronchial hyper-responsiveness to inhaled methacholine as well as inhaled platinum, vs. exposure to platinum alone [27]. In guinea-pigs, exposure to very high levels of ozone (5 ppm) prior to nebulization with OVA has been shown to enhance the development of anaphylaxis [15,16]. Although human exposure to such high concentrations of ozone may occur in industrial accidents, these levels are seldom encountered in ambient circumstances. The effects observed with exposure to high ozone concentrations

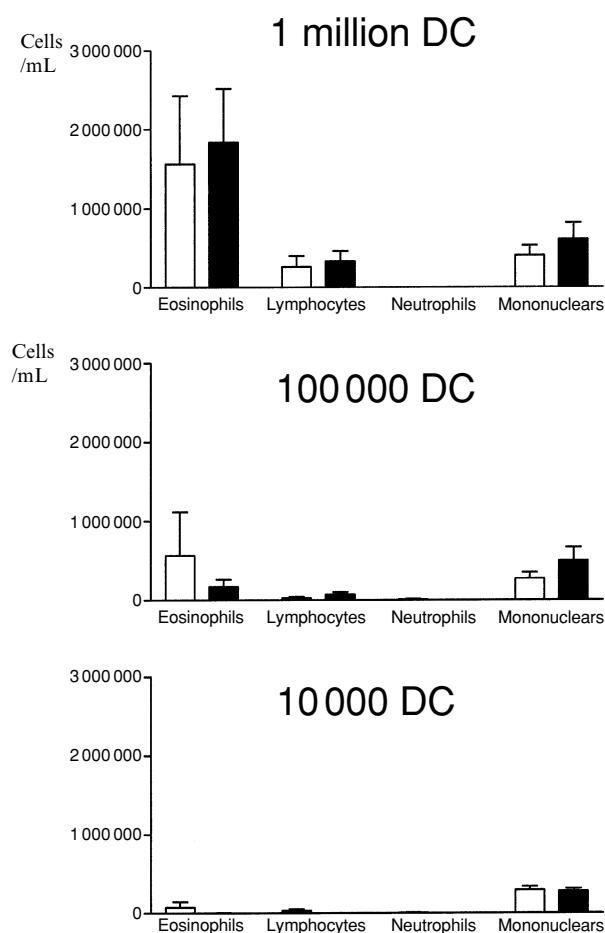


Fig. 2. Absolute cellular counts per mL bronchoalveolar lavage fluid in mice instilled with 1 000 000, 100 000 and 10 000 OVA-pulsed DC, exposed to ozone (white bars) or room air (black bars).

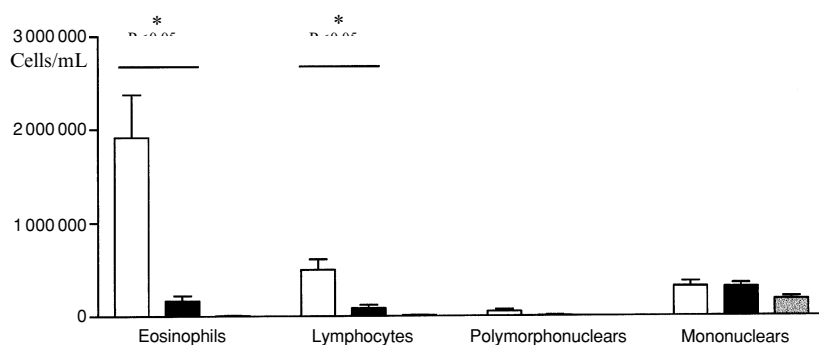


Fig. 3. Absolute cellular counts per mL bronchoalveolar lavage fluid in sensitized mice exposed to OVA and ozone (white bars) or room air (black bars), and in non-sensitized mice exposed to ozone (grey bars).

cannot be extrapolated to those of the much lower and more prolonged ozone levels that occur in the atmosphere. In apparent contrast to our findings, it has been recently reported that a 28-day concurrent exposure to inhaled OVA and 250 ppb ozone in naïve Th2-prone Balb/c mice increased the response to inhaled OVA, as it resulted in the development of a very mild airway eosinophilia (9×10^3 eosinophils per ml lavage compared with almost 2×10^6 eosinophils per ml lavage fluid in our studies) and a Th2 cytokine expression [28]. The difference in the strength of the eosinophilic response notwithstanding, the most likely explanation for the differences between our model and the model reported by Neuhaus-Steinmetz et al. lies in the difference in which sensitization via the airways was induced. In the model of Neuhaus-Steinmetz, it is very likely that sensitization to inhaled OVA is induced by endogenous DCs. However, in the absence of any stimulus, airway DCs do not present OVA in a sufficient immunogenic way to induce eosinophilic airway inflammation. Similar models that have shown the development of strong eosinophilic responses to inhaled OVA were accompanied by the expansion and activation of endogenous DCs either by adenoviral expression of the DC growth factor GM-CSF or by concurrent influenza virus infection [29,30]. Although it remains to be proven, it is possible that concurrent exposure with ozone in the studies of Neuhaus-Steinmetz led to sensitization to OVA by increasing the numbers and/or activation status of DCs in the airways. Our model of direct injection of DCs might already provide a maximal stimulus, which is no longer sensitive to the effects of ozone. One important additional difference between both models is that the sensitization phase (primary immune response) is not clearly separated from the challenge phase (recall immune response) in the model of Neuhaus-Steinmetz. Therefore it cannot be excluded that the enhancing effects were on challenge rather than on sensitization.

In our studies, the primary immune response induced by OVA-pulsed DC was clearly separated from the challenge phase 14 days later. To investigate the effect of ozone on allergen challenge, we sensitized mice with the intermediate dose of 10^5 OVA-pulsed DCs, since we expected that the resulting moderate inflammation would enable us to observe a possible modulating effect by ozone more clearly. The mean number of eosinophils recovered in bronchoalveolar lavage fluid in mice challenged with allergen and room air (group 4) was comparable with that in the first series of experiments (group 2). In sensitized mice exposed to ozone during allergen challenge (group 5), eosinophilic and lymphocyte recovery in bronchoalveolar lavage fluid was very significantly increased. Exposure to the same level of ozone without allergen (group 6) did not lead to airway inflammation, suggesting that the enhanced inflammation in mice from group 5 was the result of the priming effect of ozone on the secondary immune response, rather than caused by a direct toxic effect. By analogy, Neuhaus-Steinmetz et al. also reported that ozone exposure in C57Bl/6 mice did not induce airway eosinophilia in their model. However, chronic exposure to ozone without allergen in Balb/c mice also stimulated total IgE production and induced very mild eosinophilic airway inflammation (5×10^3 eosinophils per lavage), although significantly less than combined with allergen, which underlines the fact that this particular strain responds with a Th2-oriented inflammation to a pure toxic stimulus as well [28].

Limited data are available on the effect of ozone on allergen-induced inflammation in sensitized individuals. In dogs

sensitized to *Ascaris suum*, modulation of allergen-induced bronchial constriction has been reported, but with conflicting results, [31–33]. In two studies in allergic patients, ozone has been shown to enhance the bronchial response to allergen. In asthmatics, inhalation of 0.12 ppm ozone for 1 h increased the bronchial response to inhaled allergen [12]. In subjects with mild allergic asthma, as well as subjects with allergic rhinitis only, exposure to 0.250 ppm ozone for 3 h induced a slight increase in the responsiveness of the lower airways to an inhaled allergen [13]. Neither study provided data on the degree of airway inflammation. A few studies have addressed the effects of ozone on allergic inflammation in volunteers, without conclusive results. In asthmatics with allergen-induced nasal inflammation, exposure to 0.4 ppm ozone enhanced the late-phase eosinophilic response to allergen as indicated by increased levels of eosinophilic cationic protein in nasal lavage fluid, without however, influencing the number of eosinophils [34]. In a study by Bascom and coworkers however, exposure of patients with allergic rhinitis to 0.5 ppm ozone for 4 h prior to antigen challenge did not influence nasal inflammatory response as measured by cell counts and concentrations of mediators such as histamine [35]. Finally, when mild asthmatics were exposed to 0.120 ppm ozone prior to allergen challenge by bronchial instillation, no differences in the number of inflammatory cells could be demonstrated in bronchial biopsies of individuals exposed to ozone vs. room air [36].

The mechanisms by which ozone modulates allergic airway response are unclear and need further study. Since ozone exerts its modulating effect only in the challenge phase of our model, other mechanisms than skewing towards a Th2 response must be involved. Some preliminary data favour the airway epithelium as the crucial factor in pollution–allergen interaction. In this epithelium-centred hypothesis, pollutants may damage epithelial cells, which leads to decreased clearance of, and increased permeability for, inhaled allergen. Exposure to pollutants may also cause epithelial release of pro-inflammatory cytokines or enhance epithelial expression of adhesion molecules, resulting in infiltration with, and activation of, inflammatory cells [37]. Finally, as DCs are important both during the induction and the maintenance phase of eosinophilic airway inflammation, and as these cells have the potential to react to danger signals derived from toxic radicals and epithelial cells, the effects of ozone on DCs clearly deserve more attention.

In conclusion, our study demonstrates that low levels of ozone enhance allergic airway inflammation in mice that are sensitized via the respiratory route. These findings add to the concern that ambient levels of ozone are able to influence allergic airway inflammation in sensitized individuals. The present data do not support a modulating effect of ambient ozone levels on sensitization, but more research is needed to address this issue. Our mice model might provide a useful tool in further elucidating the interaction between allergens and air pollutants.

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