

Potential adjuvant effect of intranasal urban aerosols in mice through induction of dendritic cell maturation

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ABSTRACT

Urban air pollution is a crucial environmental problem in industrialized and developing countries. Although epidemiologic studies have associated exposure to urban aerosols with exacerbations of allergic airway diseases, the underlying mechanism of toxicity is largely unknown. Here, we evaluated the effect of urban aerosols from China, on the induction of allergic diseases *in vivo* and on the function of dendritic cells (DCs) *in vitro*. Mice were intranasally given urban aerosol plus ovalbumin (OVA), and the levels of OVA-specific antibodies in the plasma were determined. Urban aerosol induced higher OVA-specific immunoglobulin (Ig) G and IgE responses than OVA alone. Furthermore, urban aerosol plus OVA induced high levels of histamine production, indicating that exposure to the aerosol could cause serious allergic symptoms. Next, we examined the effect of urban aerosol on DCs. The aerosol enhanced cell-surface expression of co-stimulatory molecules such as CD80 and CD86 and the production of interleukin (IL)-1 β and IL-6 on DCs. In addition, allogeneic T-cell-stimulation assay showed that the urban aerosol could activate T cells through maturation of DCs. These results indicate that urban aerosols can induce allergic airway diseases through activation of DCs.

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1. Introduction

A major concern in environmental public health is the global increase in air pollution. Urban air pollution is a crucial environmental problem in industrialized and developing countries. Increased levels of air pollution have been associated with a wide range of health effects. Epidemiological studies have suggested that increased levels of particulate matter (PM) in ambient air are asso-

ciated with adverse effects in the respiratory and cardiovascular systems (Mauderly and Chow, 2008). In fact, respiratory-associated morbidity and mortality, such as increased exacerbation of asthma, have been observed the day after exposure to elevated PM levels (Dominici et al., 2005). Therefore, it has been hypothesized that airborne PM are contributing to the increased incidence of and morbidity from allergic airway diseases such as asthma in adults and children in urban areas (Dominici et al., 2005).

Allergic airway diseases, including asthma, are immune diseases. Activation of the Th2 response, including production of interleukin (IL)-4, IL-5, and IL-13 from CD4⁺ T cells and immunoglobulin (Ig) G1 or IgE from B cells, is responsible for many of the pathologic features of allergic inflammation (Finkelman et al., 2010). Dendritic cells (DCs), which are the most potent antigen-presenting cells, are believed to be indispensable to the initiation of T-cell immunity (Lambrecht and Hammad, 2009). In the mucosal environment, airway DCs or pulmonary DCs, while retaining their ability to stimulate CD4⁺ T cells, also have the capacity to capture airborne antigens via cellular processes that extend across the airway mucosal barrier (Lambrecht and Hammad, 2009).

Abbreviations: Ab, antibody; ANOVA, analysis of variance; BM, bone marrow; BMDCs, bone marrow-derived dendritic cells; DCs, dendritic cells; DEPs, diesel exhaust particles; ELISA, enzyme-linked immunosorbent assay; GM-CSF, granulocyte-macrophage colony-stimulating factor; Ig, immunoglobulin; IL, interleukin; LPS, lipopolysaccharide; MLR, mixed lymphocyte reaction; OVA, ovalbumin; PM, particulate matter.

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The immune-modulating effects of PM have been demonstrated in both animal and human studies (Sandstrom, 1995; Gilliland et al., 2004; Matsumoto et al., 2006; Stevens et al., 2008). In humans it has been shown that intranasal instillation of PM can enhance environmental antigen-induced IgE and IL-4 production (Sandstrom, 1995; Gilliland et al., 2004). Animal studies have demonstrated that inhalation or intratracheal instillation of PM can enhance immune responses to experimental allergens (Matsumoto et al., 2006; Stevens et al., 2008). In contrast, it is becoming increasingly apparent that different types of air pollution can have qualitatively different effects on human health (Ryan et al., 2005), because the chemical and physical properties of PM vary greatly with time, region, meteorology, and source (Becker et al., 2005; Schlesinger, 2007). However, although a number of animal models have been established to study the immune-activating effects of diesel exhaust particles (DEPs) on allergic sensitization (Riedl and Diaz-Sanchez, 2005), there are few data for urban aerosols from several places. Furthermore, the immunological mechanism of PM-induced exacerbations of allergic airway diseases are not fully understood (Saxon and Diaz-Sanchez, 2005). A mechanistic understanding of the immune-activating effect of each urban aerosol could help to clarify whether, and how, PM increases the prevalence of asthma.

To investigate the immunological mechanisms involved in asthma-like airway allergic symptoms induced by exposure to urban aerosols, we examined the allergic response to exposure to urban aerosol from Beijing, China, *in vivo* and the effects of the aerosol on DCs *in vitro*.

2. Materials and methods

2.1. Characteristics of the urban aerosol

Urban aerosol from Beijing, China, was obtained from the National Institutes for Environmental Studies, Japan. The chemical analysis was already reported (Mori et al., 2008). The urban aerosol was suspended in distilled water. We confirmed that the mean secondary particle diameters of the urban aerosol measured by dynamic laser scatter analysis was about 1 μm . The endotoxin level was quantified by a Limulus Amebocyte Lysate Pyrogen Plus (Lonza, Walkersville, MD, USA). The endotoxin content of the urban aerosol was ≤ 1250 pg/mg aerosol.

2.2. Mice

Female BALB/c mice were purchased from Nippon SLC (Hamamatsu, Japan) and used at 6–8 weeks of age. All of the animal experimental procedures in this study were performed in accordance with the National Institute of Biomedical Innovation guidelines for the welfare of animals.

2.3. Immunization protocols

Female BALB/c mice were intranasally immunized with a 20 μl aliquot (10 μl per nostril) containing 10 μg of ovalbumin (OVA; Sigma Chemical Co., St. Louis, MO, USA) as antigen plus 16, 80 or 400 μg /mouse of the urban aerosol on days 0, 1 and 2. On days 25, 26 and 27, the nasal challenge was performed by intranasal droplet application of 20 μl aliquot (10 μl per nostril) containing 10 μg of OVA.

2.4. Detection of antigen-specific antibody (Ab) responses by enzyme-linked immunosorbent assay (ELISA)

On day 21, plasma was collected to assess antigen-specific Ab responses. Antigen-specific IgG, IgG1 and IgG2a Ab levels in plasma on day 21 were determined by ELISA. ELISA plates (Maxisorp, type 96F; Nalge Nunc International, Naperville, IL, USA) were coated with 10 $\mu\text{g}/\text{ml}$ OVA and incubated overnight at 4 °C. The plates were incubated with 4% blocking solution (Block Ace; Dainippon Sumitomo Pharmaceuticals, Osaka, Japan) at 37 °C for 2 h, and plasma dilutions were added to the antigen-coated plates. After incubation at 37 °C for 2 h, the coated plates were washed with PBS-0.05% Tween 20 and incubated with a horseradish peroxidase-conjugated goat anti-mouse IgG solution (Southern Biotechnology Associates, Birmingham, AL, USA) solution at 37 °C for 2 h. After incubation, the color reaction was developed with tetramethylbenzidine (MOSS, Inc. Pasadena, MD, USA), stopped with 2 N H_2SO_4 , and measured by OD_{450–655} on a micro plate reader. OVA-specific IgE Abs levels in plasma on day 21 were determined by commercial ELISA kits (Dainippon Sumitomo Pharma, Osaka, Japan).

2.5. Measurement of histamine release

Histamine levels in plasma on day 27 were measured by commercial ELISA kits according to the manufacturer's instructions (Immunotech, Marseille, France).

2.6. Generation of mouse bone marrow (BM)-derived DCs (BMDCs)

DCs were generated from bone marrow cells as described elsewhere (Inaba et al., 1993). Briefly, bone marrow cells were isolated from BALB/c mice and were cultured in RPMI 1640 (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum, 50 μM 2-mercaptoethanol, 1% antibiotic cocktail (Nacalai Tesque, Kyoto, Japan) and 40 ng/ml mouse granulocyte-macrophage colony-stimulating factor (GM-CSF). After 8–16 days of culture, non-adherent cells were collected and used as DCs.

2.7. Flow cytometry analysis of the urban aerosol-treated BMDC phenotype

BMDCs (5×10^5 cells/well) were treated with either 1 $\mu\text{g}/\text{ml}$ lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO, USA) or 10 $\mu\text{g}/\text{ml}$ the urban aerosol. At 24 h after treatment, cell phenotype was confirmed by flow cytometry analysis. All staining procedures were performed in PBS containing 0.5% BSA and 0.1% azide. To minimize nonspecific bindings, single cell suspensions were pre-incubated with anti-CD16/CD32 Abs (2.4G2). Cells were labeled with combinations of FITC-conjugated CD40 (3/23), PE-conjugated CD80 (16–10A1), biotin-conjugated H-2D^d (34–2–12), biotin-conjugated I-A^d (AMS-32.1) (all BD Pharmingen, San Diego, CA, USA), and APC-conjugated CD86 (GL-1) (eBioscience, San Diego, CA, USA). The cells were resuspended in staining buffer containing PE-Cy7-conjugated streptavidin and then the stained cells were analyzed for surface phenotype, using FACS Canto flow cytometer (BD Pharmingen) and FlowJo (TreeStar, Eugene, OR, USA) software.

2.8. Measurement of cytokine productions in the urban aerosol-treated BMDC

BMDCs (5×10^5 cells/well) were treated with either 1 $\mu\text{g}/\text{ml}$ LPS or 10–250 $\mu\text{g}/\text{ml}$ urban aerosol. 250 $\mu\text{g}/\text{ml}$ is equal to 125 $\mu\text{g}/\text{cm}^2$; 50 $\mu\text{g}/\text{ml}$ is equal to 25 $\mu\text{g}/\text{cm}^2$; and 10 $\mu\text{g}/\text{ml}$ is equal to 5 $\mu\text{g}/\text{cm}^2$. At 24 h after treatment, pro-inflammatory cytokines, IL-1 β and IL-6 productions in the culture supernatants were measured by commercial ELISA kits according to the manufacturer's instructions (BD Pharmingen).

2.9. Preparation of T cells

Spleens were aseptically removed from C57BL/6 mice. Briefly, a single cell suspension of splenocytes was prepared as described previously (Kayamuro et al., 2009). T cells were enriched by passage through a nylon-wool column (Wako Chemicals, Osaka, Japan).

2.10. Allogeneic mixed lymphocyte reaction (MLR)

The T cell stimulatory ability of BMDCs was assessed in an allogeneic MLR. BMDC (1×10^4 cells/well) from BALB/c mice were exposed to 2–250 $\mu\text{g}/\text{ml}$ urban aerosol or 1 $\mu\text{g}/\text{ml}$ LPS. After 24 h, BMDCs (1×10^4 cells) were co-cultured with responder T-cells (1×10^5 cells) from C57BL/6 mice for 5 days and subsequently pulsed with [³H] thymidine (Perkin-Elmer, Wellesley, MA, USA) to quantify T-cell proliferation during the last 24 h of culture. Radioactive incorporation was measured using a TopCount NXT microplate scintillation luminescence counter (Perkin-Elmer).

2.11. Statistical analysis

All results are expressed as means $\pm$ SEM or SD. Differences were compared using Bonferroni's method after analysis of variance (ANOVA).

3. Results

3.1. Antigen-specific immune responses to urban aerosol

To investigate the potential of the urban aerosol to enhance allergic diseases, we examined its effects on the production of allergen-specific Abs *in vivo*. On days 0, 1, and 2, mice were intranasally treated with OVA (10 $\mu\text{g}/\text{mouse}$) plus 16 μg , 80 μg , or 400 $\mu\text{g}/\text{mouse}$ urban aerosol. On day 21, we examined the anti-OVA IgE and IgG Ab responses in the plasma by ELISA (Fig. 1). The OVA-specific IgE and IgG Ab responses in mice treated with OVA plus urban aerosol were higher than in mice given OVA alone and increased in a dose-dependent manner (Fig. 1).

Plasma IgG subclass responses have been used to assess the types of immune responses elicited by urban aerosols. IgG1 production is indicative of a Th2-type response, whereas IgG2a production is indicative of a Th1-type response. To assess the type of immune

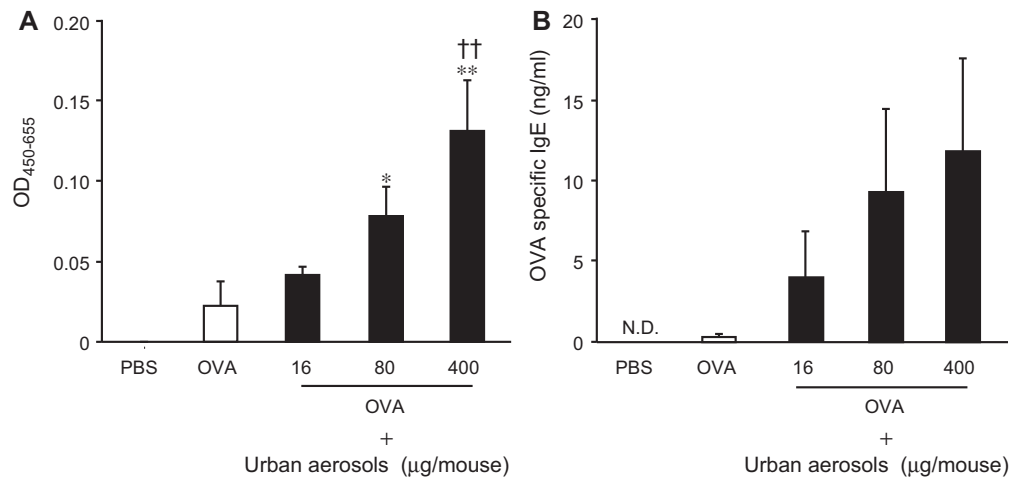


Fig. 1. Plasma OVA-specific IgG and IgE Abs responses after nasal exposure to OVA plus urban aerosol. BALB/c mice were intranasally exposed to OVA alone or OVA plus urban aerosol on days 0, 1, and 2. Plasma was collected on day 21 and analyzed by ELISA for: (A) OVA-specific IgG responses and (B) OVA-specific IgE responses. Data represent mean absorbances at a wavelength of 450 nm (reference wavelength, 655 nm). N.D., not detected. Data are presented as means $\pm$ SEM ($n = 6-8$; * $P < 0.05$, ** $P < 0.01$, †† $P < 0.01$ versus value by ANOVA versus group treated with OVA alone).

response induced by the urban aerosol, we examined plasma OVA-specific IgG subclass responses (Fig. 2). OVA-specific IgG1 Ab levels in mice given OVA plus urban aerosol were higher than those in mice given OVA alone (Fig. 2A). In contrast, levels of OVA-specific IgG2a were low in all groups (Fig. 2B). These results suggested that the urban aerosol induced the production of allergen-specific Abs and immune responses with an antigen-specific Th2 component.

3.2. Histamine release after nasal challenge

Mast cell degranulation and histamine release are major mechanisms underlying anaphylactic reactions in allergic airway diseases. Plasma histamine levels are correlated with the severity of anaphylactic reactions (Li et al., 2000). To investigate the effect of the urban aerosol on the production of histamine, mice treated with OVA plus 400 μ g/mouse urban aerosol intranasally on days 0, 1 and 2 were exposed to OVA (10 μ g/mouse) intranasally on days 26, 27, and 28. On day 28, we examined the histamine levels in the plasma by ELISA (Fig. 3). Mice treated with the urban aerosol plus OVA had significantly higher histamine levels in their plasma than mice given OVA alone ($P < 0.05$; Fig. 3). These results indicate that exposure to the urban aerosol plus an allergen has the potential to cause serious allergic symptoms.

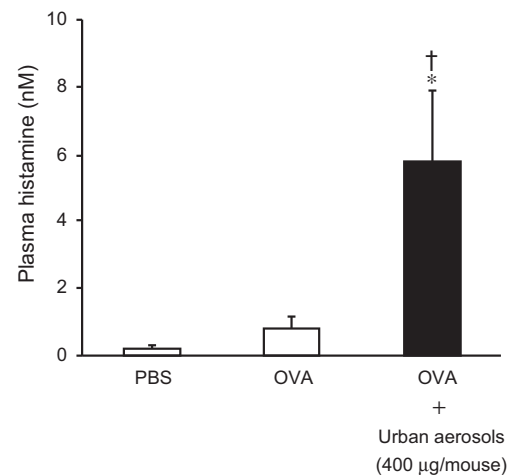


Fig. 3. Systemic histamine response when nasally challenged with OVA after intranasal exposure to OVA plus urban aerosol. BALB/c mice were intranasally exposed to OVA alone or OVA plus urban aerosol on days 0, 1 and 2. Nasal challenge with OVA was given on days 25, 26, and 27. Plasma was collected 10 min after the last OVA challenge and analyzed by ELISA for plasma histamine levels. Data are presented as means $\pm$ SEM ($n = 6-8$; * $P < 0.05$ by ANOVA versus group treated with PBS alone, † $P < 0.05$ versus value by ANOVA versus group treated with OVA alone).

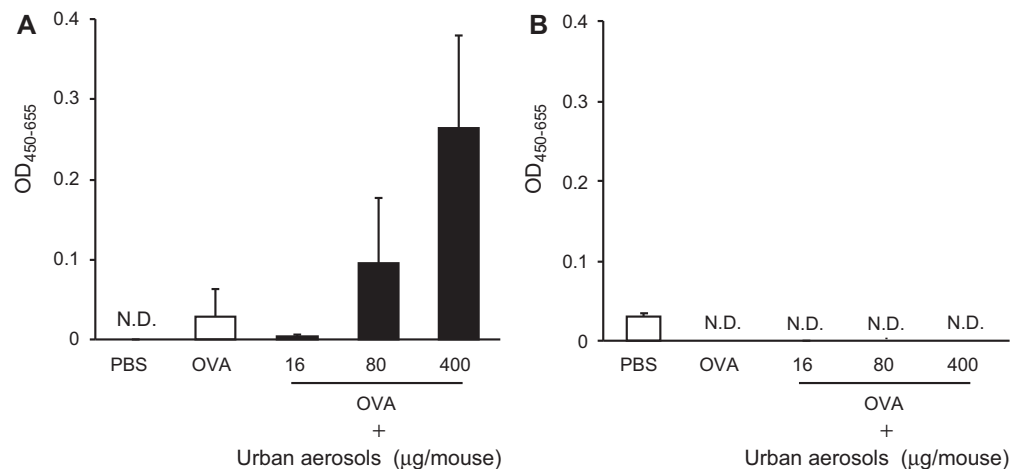


Fig. 2. Plasma OVA-specific IgG subclass Ab responses after nasal exposure to OVA plus urban aerosol. BALB/c mice were intranasally exposed to OVA alone or OVA plus urban aerosol on days 0, 1 and 2. Plasma was collected on day 21 and analyzed by ELISA for: (A) OVA-specific IgG1 and (B) OVA-specific IgG2a subclass responses. Data represent mean absorbances at a wavelength of 450 nm (reference wavelength, 655 nm). N.D., not detected. Data are presented as means $\pm$ SEM ($n = 6-8$).

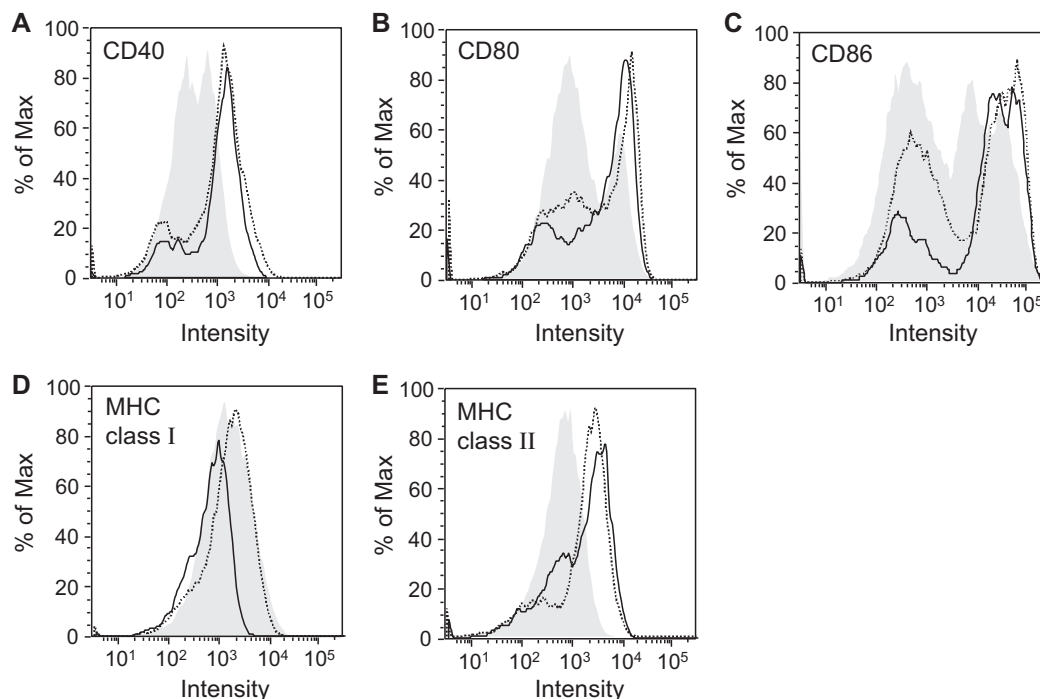


Fig. 4. Maturation and activation of BMDCs treated with urban aerosols. BMDCs were exposed to urban aerosols at 10 µg/ml (solid lines) for 24 h. BMDCs treated with 1 µg/ml LPS (dotted lines) for 24 h were used as positive controls for phenotypic DC maturation. Twenty-four hours later, (A) CD40, (B) CD80, (C) CD86, (D) MHC class I, and (E) MHC class II expression on BMDCs was determined by flow cytometry. Gray shaded histograms represent controls.

3.3. Effect of urban aerosol on expression of co-stimulatory molecules and production of cytokines on BMDCs

DCs are crucial in regulating immune responses and play an important role in both the induction and maintenance of asthma (Lambrecht and Hammad, 2009). However, little is known about whether and how urban aerosols affect the function of DCs. We hypothesized that the immune-activating effects of the urban aerosol were mediated through DCs. Therefore, we next assessed whether the urban aerosol could directly induce maturation of DCs. We examined the expression of co-stimulatory molecules and the production of cytokines on BMDCs in response to the aerosol (Fig. 4), because DC maturation is defined by the enhanced cell-surface expression of co-stimulatory molecules, enhanced antigen

presentation, and increased cytokine production (Lanzavecchia and Sallusto, 2001). GM-CSF-cultured BMDCs were exposed to medium (vehicle control), 10 µg/ml urban aerosol, or 1 µg/ml LPS (positive control). Exposure of these BMDCs to the aerosol tended to induce upregulation of MHC class II molecules and the co-stimulatory molecules CD40, CD80, and CD86 when compared with exposure to medium alone (Fig. 4). Aerosol exposure did not affect the expression of MHC class I. Upregulation of the costimulatory molecules by the urban aerosol was similar to that of BMDCs exposed to LPS.

Next, we examined cytokine production after stimulation of BMDCs by urban aerosol at 10–250 µg/ml. The aerosol induced greater production of IL-6 and IL-1β on BMDCs than did LPS (Fig. 5). These results indicated that the urban aerosol could induce activation of T-cells by maturation of DCs.

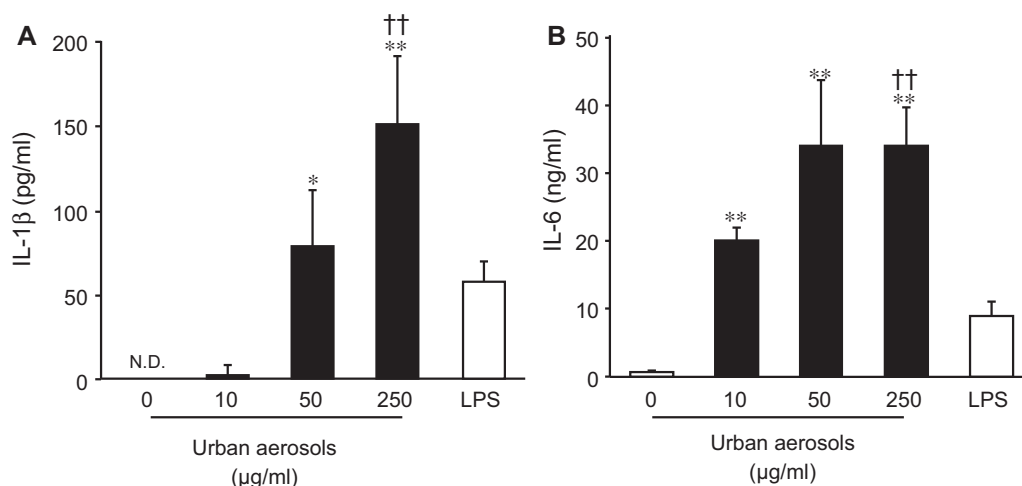


Fig. 5. Cytokine production in BMDCs exposed to urban aerosol. BMDCs were exposed to 10–250 µg/ml urban aerosol or 100 ng/ml LPS for 24 h. (A) IL-1β and (B) IL-6 production in the culture supernatants were determined by ELISA. N.D., not detected. Data are presented as means ± SD ($n = 3$; ** $P < 0.01$, * $P < 0.05$ by ANOVA versus untreated group, †† $P < 0.01$ versus value by ANOVA versus group treated with LPS).

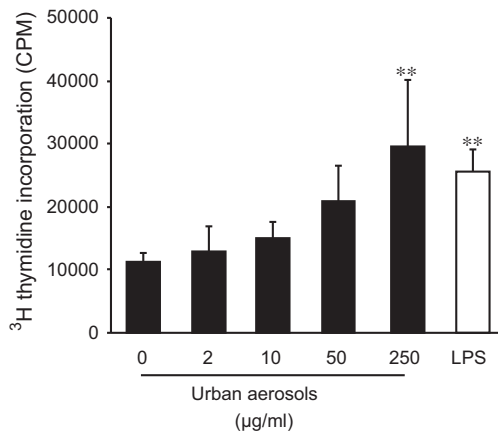


Fig. 6. Allogeneic T-cell-stimulation capacity of BMDCs exposed to urban aerosol. BMDCs were prepared from BALB/c mice and exposed to 2–250 µg/ml urban aerosol or 1 µg/ml LPS. After 24 h, γ-irradiated BMDCs (1×10^4 cells) were cocultured with allogeneic T cells (1×10^5 cells) from C57BL/6 mice. After 5 days, cells were pulsed with [^{3} H] thymidine overnight and the incorporated radioactivity was determined. Data are means $\pm$ SD ($n = 4$; ** $P < 0.01$ by ANOVA versus untreated group).

3.4. Effect of urban aerosol on allogeneic T-cell-stimulation capacity of BMDCs

The hallmark of an activated DC is its ability to stimulate alloreactive T cells. Therefore, finally, we used co-cultures of BMDCs with allogeneic T-cells to study how exposure to the urban aerosol altered the ability of DCs to induce T-cell proliferation (Fig. 6). T-cell proliferation was significantly increased in a dose-dependent manner by exposure to the urban aerosol at 50 or 250 µg/ml (Fig. 6). In a separate series of experiments, we directly stimulated CD4⁺ T cells with urban aerosol and confirmed that it did not alter the proliferation of T cells (data not shown). We therefore considered that the urban aerosol could activate T cells through the maturation of DCs.

4. Discussion

The potential links between exposure to PM and immune dysregulation require further study, although it has been suggested that urban air pollution is a likely risk factor for airway allergic diseases. Unfortunately, although most of the published *in vivo* studies that have addressed the immune-modulating effects of PM have used DEPs, few have looked at urban aerosols. Here, we examined the immune-activating effects of urban aerosol collected by particle concentrators in Beijing, China.

In Beijing, the concentration of urban aerosol is estimated at about 120 µg/m³ (Guo et al., 2009). The people of Beijing are therefore likely to be exposed to urban aerosols at 1800 µg/day, assuming a daily respiratory volume of 15 m³ for an adult. About 1 µg/mouse is a dose equivalent to this estimated level of human adult exposure; the doses of urban aerosol used in our *in vivo* study were about 10–400 times higher than this. However, we believe that they were within the physiological range, because the aerosol might accumulate and be hardly metabolized in the body.

We observed increase in plasma OVA-specific IgE and IgG1 levels (Figs. 1 and 2) and enhancement of the production of histamine (Fig. 3) in mice exposed to urban aerosol plus OVA. These results raise the possibility that long-term urban aerosol exposure increases the prevalence of asthma and allergic diseases. However, the mechanism of the adjuvant effect of the urban aerosol is not clear. Although a variety of mechanisms have been shown to explain the adverse respiratory effects of PM, there is accumulating evidence that oxidative stress plays a role in the proinflammatory

and immune-activating effects of PM (Li et al., 2003). In fact, some reports have shown that administration of antioxidants suppresses the immune-activating effects of, and inflammatory responses to, PM in mice (Whitekus et al., 2002). Furthermore, it has been suggested that the organic chemical fraction of PM could play an important role in its immune-activating effects through the ability to generate reactive oxygen species in the respiratory tract (Li et al., 2003; Nel et al., 2006). Therefore, we need to investigate the effects of oxidative stress on organic and inorganic compounds in urban aerosols. On the other hand, we found that the endotoxin content of the urban aerosol was ≤ 1250 pg/mg aerosol. It was reported that exposure to endotoxins during sensitization and allergen challenge influenced allergen-induced IgE production and allergic responses in a time and dose-dependent manner (Delayre-Orthez et al., 2004). Therefore, it is needed to investigate the effect of endotoxin more precisely.

In recent years, it has become clear that pulmonary DCs are crucial mediators of the immune response in the lung and that these DCs bridge the innate and adaptive immune responses. In asthma, for example, DCs are important in both induction and maintenance of the disease (Hammad and Lambrecht, 2008). In addition, PM has been observed within biopsies of bronchial lymph nodes in humans (Pinkerton et al., 2000). Therefore, we hypothesized that pulmonary DCs phagocytosed the urban aerosol, became activated, and migrated toward the lymph nodes. We demonstrated here that urban aerosol induced DC activation and maturation, including increased expression of co-stimulatory molecules and inflammatory cytokine production (Figs. 4 and 5). In addition we showed that mature DCs exposed to urban aerosol were essential for the activation of allogeneic T cells *in vitro* (Fig. 6). However, some controversy exists about the ability of PM to activate DCs directly or indirectly. Previously, some reports have shown that PM such as DEPs activates DCs directly, consistent with our observation (Porter et al., 2007). In contrast, other reports have shown that DEPs do not directly trigger the maturation of DCs but instead induce DC maturation through the release of cytokines by epithelial cells (Provoost et al., 2010; Bleck et al., 2006, 2008). Although the precise reason for these contradictory results remains unclear, it may be associated with differences in the characteristics of PM – such as region, meteorology, and source – or in DC subsets. Pulmonary tissue contains DC subsets that differ by anatomical location and function – i.e., conventional DCs and plasmacytoid DCs (Wikstrom and Stumbles, 2007; GeurtsvanKessel and Lambrecht, 2008). Conventional DCs are important in inducing sensitization, and plasmacytoid DCs are involved in the induction of tolerance (de Heer et al., 2004). Recently, Provoost et al. reported that exposure to DEPs increased the abundance of conventional DCs in the lungs, suggesting that exposure to DEPs could shift the balance between conventional DCs and plasmacytoid DCs in favor of sensitization (Provoost et al., 2010). Currently, we are trying to identify and characterize the urban aerosol-induced DCs subsets within the lung.

We also showed that mice exposed to urban aerosol produced significantly higher OVA-specific IgG1 and IgE Abs responses than mice exposed to OVA alone, indicating that the urban aerosol induced allergen-specific Th2-type immune responses *in vivo* (Figs. 1 and 2). It is not clear why the urban aerosol would induce Th2-polarized immunity. When DCs were stimulated with the urban aerosol *in vitro*, they upregulated their expression of MHC class II molecules and the costimulatory molecules CD40, CD80, and CD86 (Fig. 4). Several studies have suggested that CD86 is involved in the priming of naive T cells into Th2 cells and in the subsequent development of allergic airway inflammation (van Rijt et al., 2004). Therefore, upregulation of CD86 might be one mechanism of the Th2-type immune response to urban aerosol. In addition, recent studies have shown that crystalline silica, also known as an environmental pollutant, disturbs host immune systems by stimulating

the production of IL-1 β *via* activation of the cytoplasmic receptor NLRP3 (Dostert et al., 2008). Other groups have suggested that NLRP3-dependent IL-1 β production by DCs skews the adaptive cellular immune response toward a Th2-type response (Kool et al., 2008). Because the urban aerosol induced strong IL-1 β production on DCs it might therefore induce Th2 immune responses *via* NLRP3.

In conclusion, we showed that urban aerosol had the potential to induce allergic airway diseases. In addition, we showed that it could promote DC maturation and activation, which could be a mechanism in urban aerosol-enhanced allergic responses. Our findings might provide useful information for clarifying whether and how urban aerosols are promoting an increase in asthma prevalence.

Conflict of interest

The authors declare that there are no conflicts of interest.

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