

RESEARCH ARTICLE

# Nitrogen dioxide: no influence on allergic sensitization in an intranasal mouse model with ovalbumin and diesel exhaust particles

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## Abstract

The role of traffic-related air pollution in the development of allergic diseases is still unclear. We therefore investigated if NO<sub>2</sub>, an important constituent of traffic-related air pollution, promotes allergic sensitization to the allergen ovalbumin (OVA). We also examined if NO<sub>2</sub> influenced the allergy adjuvant activity of diesel exhaust particles (DEP). For this purpose, mice were exposed intranasally to OVA with or without DEP present, immediately followed by exposure to NO<sub>2</sub> (5 or 25 parts per million [ppm]) or room air for 4 h in whole body exposure chambers. Eighteen hours after the last of three exposures, the lungs of half of the animals were lavaged with saline and markers of lung damage and lung inflammation in the bronchoalveolar lavage fluid (BALF) were measured. Three weeks later, after intranasal booster immunizations with OVA, the levels of OVA-specific IgE and IgG2a antibodies in serum were determined. Both NO<sub>2</sub> (25 ppm) and DEP gave lung damage, measured as increased total protein concentration in BALF, whereas only NO<sub>2</sub> seemed to stimulate release of the proinflammatory cytokine tumor necrosis factor alpha (TNF-α). In contrast, only DEP significantly increased the number of neutrophils. Furthermore, DEP in combination with OVA stimulated the production of serum allergen-specific IgE antibodies. NO<sub>2</sub>, however, neither increased the production of allergen-specific IgE antibodies, nor influenced the IgE adjuvant activity of DEP. Thus, based on our findings, NO<sub>2</sub> seems to be of less importance than combustion particles in the development of allergic diseases after exposure to traffic-related air pollution.

**Keywords:** Traffic-related air pollution, nitrogen dioxide, diesel exhaust particles, lung inflammation, allergic sensitization, intranasal mouse model

## Introduction

Diesel engines generate large amounts of combustion particles and oxides of nitrogen, which mainly include NO and NO<sub>2</sub> (Ahlvik et al., 2008). Traffic-related air pollution has been reported to aggravate existing asthma and allergic diseases (Lin et al., 2002; Janssen et al., 2003; Halonen et al., 2008). The role of traffic-related air pollution in the development of allergic diseases, however, is still unclear (Janssen et al., 2003; Brauer et al., 2007; Oftedal et al., 2007; Morgenstern et al., 2008; Nordling et al., 2008; Rosenlund et al., 2009; Gehring et al., 2010). Allergic sensitization is a crucial risk factor for the development of allergic diseases. Several epidemiological studies in European children failed to show a relation

between air pollutants and allergic sensitization (Oftedal et al., 2007; Rosenlund et al., 2009; Gehring et al., 2010), whereas other studies have indicated an association between exposure to particulate matter early in life and allergic sensitization later in life (Brauer et al., 2007; Morgenstern et al., 2008; Nordling et al., 2008). An experimental study has confirmed that diesel exhaust particles (DEP) can promote allergic sensitization to a neoantigen in the human nasal mucosa (Diaz-Sanchez et al., 1999). Furthermore, DEP stimulate the production of allergen-specific IgE antibodies in mice (Muranaka et al., 1986; Takafuji et al., 1987; Løvik et al., 1997; Nilsen et al., 1997; Takano et al., 1997) and rats (Steerenberg et al., 1999; Dong et al., 2005). Two of the epidemiological studies

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mentioned above also indicated that traffic-related NO<sub>x</sub> was associated with allergic sensitization (Brauer et al., 2007; Nordling et al., 2008). There was, however, a strong positive correlation between NO<sub>2</sub> and PM<sub>2.5</sub> ( $r=0.93$ ), which makes it difficult to separate the relative contribution from each of these air pollutants (Brauer et al., 2007). Experimental studies have shown that short-time exposure to NO<sub>2</sub> can promote sensitization to allergens in mice and rats (Gilmour et al., 1996; Siegel et al., 1997; Bevelander et al., 2007). However, the ability of NO<sub>2</sub> to promote allergic sensitization seems to be dependent on the experimental protocol. The aim of this study was to further elucidate the role of NO<sub>2</sub> in promoting allergic sensitization in an intranasal mouse model. Moreover, we have taken into consideration the fact that when present as a mixture, the component toxicity could differ from that found in investigations of the individual pollutants. We therefore also examined the combined effects of NO<sub>2</sub> and DEP.

## Materials and methods

### Experimental design

An overview of the experimental design is given in Figure 1. Mice were exposed intranasally for three consecutive days (0, 1, and 2) to Hank's balanced salt solution (HBSS), ovalbumin (OVA, 10 µg per animal per day), DEP (67.7 µg per animal per day), or OVA (10 µg per animal per day) and DEP (67.7 µg per animal per day). Mice were anesthetized by intraperitoneal injections of a mixture of Ketalar (80 mg/kg; Pfizer AB, Sollentuna, Sweden) and Narcoxyl vet (10 mg/kg; Intervet International BV, Boxmeer, The Netherlands), and were held horizontally while a total volume of 35 µL per animal per day was deposited on both nostrils with a pipette. Each instillation was immediately followed by exposure to NO<sub>2</sub> (5 or 25 ppm in two separate experiments) or room air for 4 h in whole body exposure chambers. On Day 3, 18 h after the last of three exposures, half of the animals were euthanized and the lungs lavaged with saline. In bronchoalveolar lavage



Figure 1. An overview of the experimental design. Six-week-old female Balb/cA mice were anesthetized and exposed intranasally for three consecutive days (0, 1, and 2) to Hank's balanced salt solution (HBSS), ovalbumin (OVA), diesel exhaust particles (DEP), or OVA and DEP. Each instillation was immediately followed by exposure to NO<sub>2</sub> (5 or 25 ppm in two separate experiments) or room air for 4 h in whole body exposure chambers. On Day 3, half of the animals in each group were euthanized and the lungs lavaged with saline. After 3 weeks (Days 22, 23, and 24), the remaining animals were booster-immunized intranasally with OVA or HBSS. On Day 27, the animals were bled by cardiac puncture under CO<sub>2</sub> anesthesia.

fluid (BALF), markers of lung damage and lung inflammation were determined, and the BAL cells were enumerated by differential counting. After 3 weeks (Days 22, 23, and 24), the remaining animals were booster-immunized intranasally with OVA (10 µg per animal per day, OVA-, and OVA- and DEP-immunized mice) or HBSS (HBSS- and DEP-immunized mice). Three days after the last exposure (Day 27), animals were bled by cardiac puncture under CO<sub>2</sub> anesthesia, and sera were prepared and stored at -20°C until analysis for OVA-specific antibodies.

### Animals

Inbred 6-week-old female Balb/cANNCrI mice were obtained from Charles River (Sulzfeld, Germany). Mice were distributed into groups of 20 animals per cage containing Beekay GLP bedding (B&K Universal AS, Nittedal, Norway), and rested for 1–2 weeks before entering the experiments. The cages were placed in 0.65-m<sup>3</sup> inhalation chambers and the mice were exposed to a 12-h/12-h light/dark cycle, an overall temperature of 22.9 ± 0.3°C (mean ± standard deviation, SD), and a relative humidity of 73.0 ± 1.9%. Tap water and food pellets (RM1; SDS, Essex, UK) were provided *ad libitum*. Mean animal weights (±SD) increased from 18.1 ± 0.3 g at start of exposure to 20.0 ± 0.4 g after 27 days, and no significant weight differences were observed between treatment groups. The experiments were performed in conformity with the laws and regulations controlling experiments with live animals in Norway, and were approved by the local officer of the Experimental Animal Board under the Ministry of Agriculture in Norway.

### Allergens and particulate matter

OVA (Gal d1; chicken egg albumin, grade VII; Sigma-Aldrich, St. Louis, MO) was used as allergen after removal of endotoxin by Detoxi-Gel™ Endotoxin Removing Gel (Pierce, Rockford, IL). The final endotoxin level was 3.34 ng/mg OVA determined by Limulus Amebocyte Lysate Kinetic QCL Kit (Cambrex, Walkersville, MD). Standard reference material 2975, forklift diesel particulate matter, was obtained from National Institute of Standards and Technology (Gaithersburg, MD). OVA and particles were suspended in HBSS (GIBCO® Invitrogen, Invitrogen Corp. Carlsbad, CA) to a final concentration of 0.286 and 1.905 mg/mL, respectively. When appropriate, allergen was added to the suspension simultaneously with particles. The particle suspensions were stirred at 18°C overnight using a magnetic stirrer.

### Inhalation exposure to NO<sub>2</sub>

Exposure of animals was performed in 0.65-m<sup>3</sup> inhalation chambers. Fresh air was drawn through the chambers by the building ventilation system creating a negative pressure of 2–5 mmHg inside the chambers. The total flow of air through the chambers was regulated individually for each chamber to an overall

mean of  $20.7 \pm 1.8$  (SD) air changes per hour. The animals were exposed to  $4.9 \pm 0.2$  or  $25.3 \pm 0.4$  ppm  $\text{NO}_2$  in air (Aga Gas AB, Stockholm, Sweden) for 4 h/day for 3 days via the main airstream through the chambers. The  $\text{NO}_2$  concentration was monitored with Q RAE+ Multi Gas Monitor PGM-2000 (RAE Systems, Sunnyvale, CA) calibrated with 0 and 5 ppm  $\text{NO}_2$  (RAE Systems Europe, Copenhagen, Denmark) 7 weeks before start of the exposure period.

### BALF collection, preparation, and analyses

Mice were euthanized by an overdose of pentobarbital given intraperitoneally, and the lungs flushed *in situ* three times by injection into the trachea of a single volume of 1 mL ice-cold phosphate-buffered saline (10 mM). The BALF was centrifuged at 280 g (Eppendorf centrifuge 5810R; Eppendorf AG, Hamburg, Germany) for 10 min at 4°C, and the supernatant from the first lavage was stored at -80°C for later biochemical analysis. The cells from all three lavages were pooled and counted in a Coulter Counter Z1 (Beckman Coulter Incorporated, Fullerton, CA). Cell smears were prepared by spinning 200  $\mu\text{L}$  cell suspension (125,000 cells/mL) onto cytoslides at 70 g for 6 min at 4°C using a Cytospin 3 Cytocentrifuge (Shandon Scientific Ltd., Astmoor, Runcorn, England). The cells were stained using the Hemacolor rapid staining blood smears kit according to the manufacturer's protocol (Merck KGaA, Darmstadt, Germany). Differential cell counts were performed on 200–300 cells by standard morphological characteristics. The total protein and tumor necrosis factor alpha ( $\text{TNF-}\alpha$ ) concentrations in BALF were determined according to the manufacturers' recommendations using the Pierce Protein Assay Kit (Pierce, Rockford, IL) and mouse  $\text{TNF-}\alpha$ /TNFSF1A DuoSet ELISA Development kit (R&D Systems, Minneapolis, MN), respectively. The concentrations of protein and  $\text{TNF-}\alpha$  were determined from the optical density values using a quadratic equation.

### ELISA for detection of IgE and IgG2a anti-OVA antibodies

OVA-specific IgE antibodies were detected in a capture ELISA as previously described by Løvik et al. (1997) and modified by Ormstad et al. (1998), whereas OVA-specific IgG2a antibodies were determined using indirect ELISA as described by Nygaard et al. (2004). The sera to be tested were for both purposes diluted 1:10. In short, microtiter plates coated with rat anti-mouse IgE (LO-ME-3; Experimental Immunology Unit, University of Louvain, Belgium) or OVA were allowed to capture IgE or OVA-specific antibodies, respectively, in serum. Before capture of the anti-OVA antibodies, the microtiter plates were blocked with skimmed milk. Capture of IgE and anti-OVA antibodies was followed by incubation with biotinylated OVA or biotinylated rat anti-mouse IgG2a antibodies (BD Biosciences Pharmingen, San Diego, CA), respectively, and thereafter with preformed complexes of streptavidin and biotinylated alkaline phosphatase, and

finally with *p*-nitrophenyl phosphate. Optical density was measured at 405 nm on a MRX Microplate Reader (Dynatech Laboratories, Chantilly, VA) connected to a PC using Revelation software (Thermo Labsystems, Chantilly, VA). Antibody concentrations were calculated by means of standard curves made from duplicates of diluted IgE standard (mouse anti-OVA IgE; AbD Serotec, Oxford, UK) or serum pools from mice immunized with OVA and CpG oligonucleotides for IgG2a.

### Statistical analysis

Statistical analysis was performed using Minitab 15 (Minitab Inc., State College, PA). All datasets were transformed using the natural log to become normally distributed (evaluated by means of histograms and normal probability plots), but normal distribution was not obtained for the antibody datasets, which therefore were analyzed using ranked values. To be able to rank values in the groups where nearly all values were below the detection limit of the ELISA, and to fulfill the assumption of normal distribution and equal variance, variance was introduced by creating random data with a normal distribution. Random IgE and IgG2a data were created based on the lower quantitative detection limits of the ELISA, with mean values of  $61.0 \pm 0.02$  ng/mL and  $0.48 \pm 0.01$  AU/mL, respectively. The multifactorial design was analyzed using the General Linear Model with OVA, DEP, and  $\text{NO}_2$  as independent variables. Statistically significant interactions obtained using this model were analyzed using the Tukey's post hoc test. Only the relevant comparisons of the post hoc analyses are presented in the "Results" section.

To determine if there was an association between the allergen-specific IgE levels and early BAL responses, Pearson's correlation analysis was performed. Correlation coefficients below 0.6 were not considered. In general, the limit for statistical significance was set at  $P \leq 0.05$ .

## Results

### Lung inflammation

The early inflammatory response in the lungs was measured as the total protein and  $\text{TNF-}\alpha$  concentration, as well as the number of neutrophils and macrophages in BALF 18 h after the last of three exposures. In the experiment using 5 ppm  $\text{NO}_2$  one of 10 animals in the  $\text{NO}_2$  group died during acclimatization, and in the experiment using 25 ppm  $\text{NO}_2$  one of 10 animals in the  $\text{NO}_2$  group died for unknown reasons after the third immunization. DEP (Figure 2A and 2B) and the highest concentration of  $\text{NO}_2$  (25 ppm, Figure 2B) caused lung injury, measured as significantly increased total protein concentrations in BALF. However, the Tukey's post hoc analysis of the interaction between DEP and  $\text{NO}_2$  showed that  $\text{NO}_2$  in the presence of HBSS or OVA increased the protein concentration significantly more than in the presence of DEP alone (Figure 2B,  $P \leq 0.001$ ). The highest concentration of  $\text{NO}_2$  also stimulated release of the proinflammatory

cytokine TNF- $\alpha$  (Figure 2D) small, but statistically significant increase in the TNF- $\alpha$  concentration was also observed after exposure to DEP in the experiment using 25 ppm NO<sub>2</sub> (Figure 2D), but not in the one using 5 ppm NO<sub>2</sub> (Figure 2C). The background level of TNF- $\alpha$  in the experiment using 5 ppm NO<sub>2</sub> was higher than in the experiment using 25 ppm NO<sub>2</sub>. NO<sub>2</sub> had no significant effect on the number of neutrophils and macrophages regardless of concentration (Figure 3). In contrast, DEP significantly increased the number of neutrophils (Figure 3A and 3B), and decreased the number of macrophages in BALF (Figure 3C and 3D). Pearson's correlation analysis showed that the number of neutrophils

and macrophages in both experiments were negatively correlated ( $r = -0.7$ ,  $P \leq 0.001$ , Table 1). Moreover, there was a significant positive and negative correlation between the number of neutrophils and macrophages, respectively, and the concentration of protein in BALF in the experiment using 5 ppm NO<sub>2</sub> ( $r = 0.9$  and  $-0.6$ , respectively,  $P \leq 0.001$ , Table 1). The Tukey's post hoc tests of the interaction between DEP and NO<sub>2</sub> showed a small, but statistically significant decrease in the total number of neutrophils after exposure to DEP in the presence of NO<sub>2</sub> (5 ppm) compared with DEP alone ( $P \leq 0.01$ , Figure 3A). DEP significantly decreased the total number of macrophages, but in one of two experiments the

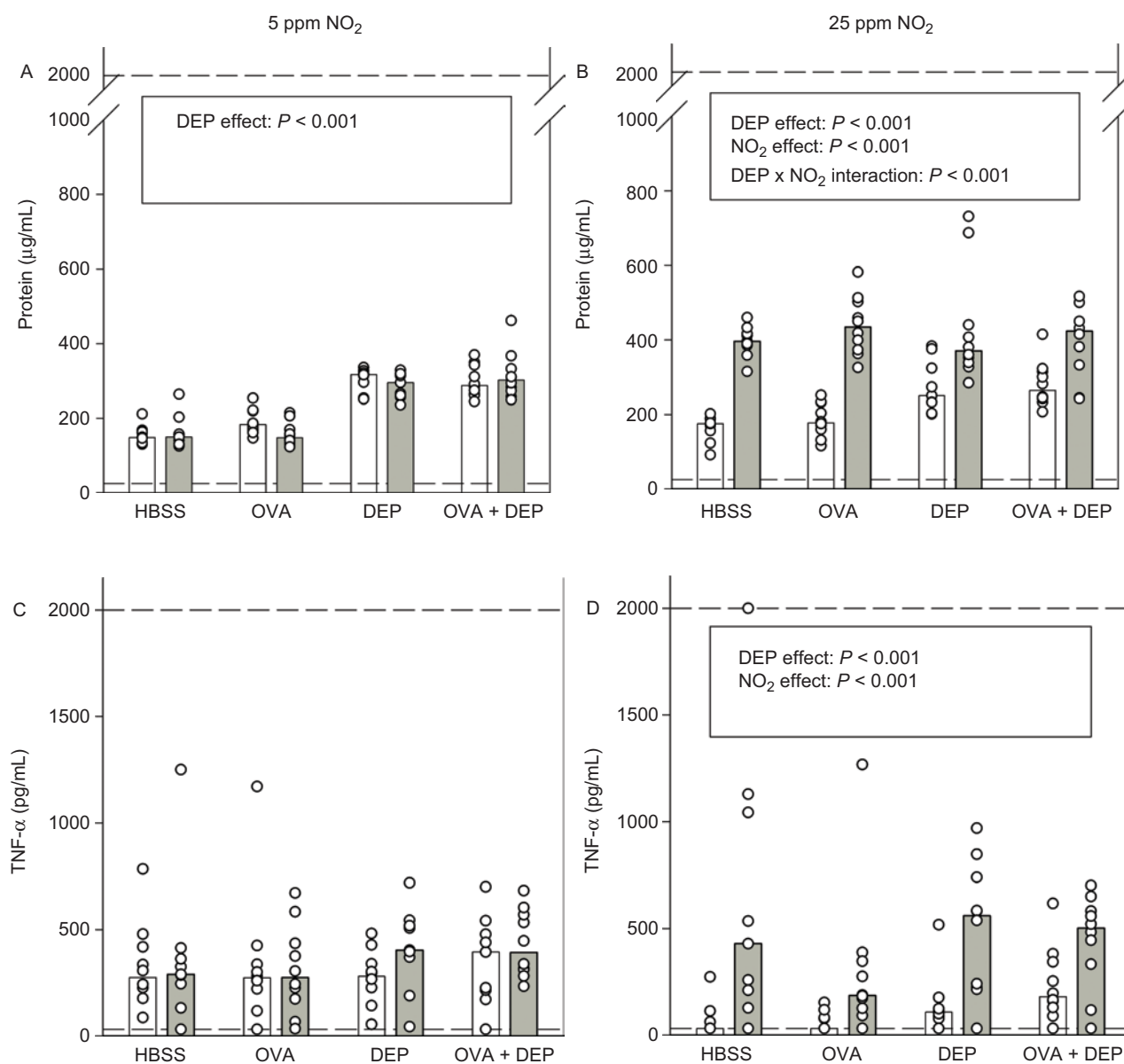


Figure 2. The total protein (A and B) and TNF- $\alpha$  (C and D) concentrations in bronchoalveolar lavage fluid (BALF) 18h after intranasal exposure of mice for three consecutive days (0, 1, and 2) with a solution containing Hank's balanced salt solution (HBSS), ovalbumin (OVA), diesel exhaust particles (DEP), or OVA and DEP. Immediately after the intranasal instillation, mice were exposed to NO<sub>2</sub> (5 or 25 ppm, gray bars in the left and right panels, respectively) or room air (white bars) for 4h. Individual results (circles) and medians (bars) for groups of 10 mice are shown. The dotted lines indicate the upper and lower quantitative detection limits of the assays. The statistically significant effect of OVA, DEP, and NO<sub>2</sub>, and the interactions between them, are presented in each panel.

Table 1. Pearson's correlation coefficients of early inflammatory responses in bronchoalveolar lavage fluid (BALF) (Day 3) and allergen-specific antibody levels after booster immunizations with ovalbumin (OVA, or Hank's balanced salt solution [HBSS], Day 27).

| NO <sub>2</sub> exposure (ppm) | Response      | Protein           | TNF- $\alpha$ | Neutrophils       | Macrophages | OVA-IgE |
|--------------------------------|---------------|-------------------|---------------|-------------------|-------------|---------|
| 5                              | TNF- $\alpha$ | 0.0               |               |                   |             |         |
| 25                             |               | 0.5               |               |                   |             |         |
| 5                              | Neutrophils   | 0.9 <sup>a</sup>  | 0.1           |                   |             |         |
| 25                             |               | 0.3               | 0.3           |                   |             |         |
| 5                              | Macrophages   | -0.6 <sup>a</sup> | 0.0           | -0.7 <sup>a</sup> |             |         |
| 25                             |               | -0.2              | -0.3          | -0.7 <sup>a</sup> |             |         |
| 5                              | OVA-IgE       | 0.4               | 0.0           | 0.5               | -0.2        |         |
| 25                             |               | 0.2               | 0.2           | 0.5               | -0.2        |         |
| 5                              | OVA-IgG2a     | 0.4               | 0.1           | 0.3               | -0.3        | 0.3     |
| 25                             |               | 0.0               | 0.1           | 0.3               | -0.1        | 0.4     |

<sup>a</sup>Statistically significant,  $P \leq 0.001$ .

decrease was less pronounced in the presence of OVA ( $P \leq 0.01$ , Figure 3D). Thus, the early inflammatory effect of DEP may be influenced by the concurrent exposure to NO<sub>2</sub> and OVA.

### Allergic sensitization

The ability of DEP and NO<sub>2</sub> to promote allergic sensitization to OVA was determined as the production of OVA-specific IgE antibodies in serum after booster immunizations with OVA (or HBSS). In the experiment using 5 ppm NO<sub>2</sub>, two of 10 animals in the HBSS group died because of technical problems. DEP in combination with OVA significantly increased the production of OVA-specific IgE antibodies compared with all other groups ( $P \leq 0.001$ , Figure 4A and 4B) underscoring that DEP acted as an adjuvant on allergic sensitization to OVA. In contrast, NO<sub>2</sub> did not increase the production of OVA-specific IgE antibodies regardless of concentration, and did not influence the IgE adjuvant activity of DEP. Independent of NO<sub>2</sub> exposure, small, but statistically significant amounts of OVA-specific IgG2a antibodies were produced after exposure to OVA and DEP in combination compared with OVA alone ( $P \leq 0.05$ , Figure 4C and 4D).

To determine if there was an association between the early inflammatory BAL responses and allergen-specific IgE levels, Pearson's correlation analysis was performed. No significant correlation between the early inflammatory BAL responses and the allergen-specific IgE levels was detected (Table 1).

### Discussion

The role of traffic-related air pollution for the development of allergic diseases and sensitization is still unclear (Janssen et al., 2003; Brauer et al., 2007; Oftedal et al., 2007; Morgenstern et al., 2008; Nordling et al., 2008; Rosenlund et al., 2009; Gehring et al., 2010). The aim of this study was to investigate if NO<sub>2</sub>, an important constituent of traffic-related air pollution, promotes allergic sensitization to OVA, and/or influences the allergic adjuvant activity of DEP in an intranasal mouse model. In accordance with previous findings (Muranaka et al., 1986; Takafuji et al., 1987; Løvik et al., 1997; Nilsen et al., 1997; Takano et al.,

1997; Diaz-Sanchez et al., 1999; Steerenberg et al., 1999; Dong et al., 2005), DEP stimulated the production of OVA-specific IgE antibodies in the present study. Very low, but statistically significant concentrations of anti-OVA IgG2a antibodies in serum were also detected after exposure to DEP in combination with OVA. We found, however, no indication that short-term exposures to NO<sub>2</sub> promoted allergic sensitization in mice, or that NO<sub>2</sub> influenced the IgE adjuvant activity of DEP.

In agreement with our findings, Bevelander et al. (2007) reported that similar concentrations of NO<sub>2</sub> did not promote allergic sensitization if its exposure came after exposure to OVA. However, according to Bevelander, NO<sub>2</sub> promoted allergic sensitization if its exposure preceded exposure to OVA. In the experiments of Bevelander et al., NO<sub>2</sub> before allergen stimulated the production of OVA-specific IgE antibodies in C57BL/6 mice, and also eosinophilic inflammation, mucus cell metaplasia, Th2 cytokine response, and airway hyperresponsiveness. Thus, the ability of NO<sub>2</sub> to promote allergic sensitization may be dependent on the timing of exposure relative to allergen. It should be noticed that Bevelander et al. used a different mouse strain and a different mode of allergen exposure, so their experiments are not directly comparable with ours.

In the real-life situation, humans are exposed to ambient particles and NO<sub>2</sub> simultaneously. Allergen exposure may take place simultaneously with, or separated in time from the exposure to traffic-related air pollutants. In our experimental setup, the exposure to OVA and DEP was slightly separated in time from the exposure to NO<sub>2</sub> due to the use of different modes of exposure. However, OVA is probably present in the lung at the time of NO<sub>2</sub> exposure since the half-life of OVA in the mouse lung is estimated to 6 h (Hansen et al., 2007). Moreover, polystyrene particles have been shown to stimulate the production of OVA-specific IgE antibodies, albeit more weakly, even when given 1 day after the OVA exposure (Granum et al., 2001). The instillation volume (35  $\mu$ L) for intranasal exposures was selected based on optimal lung deposition (Southam et al., 2002). Thus, it seems likely that all three components, NO<sub>2</sub>, OVA, and DEP, are present in the alveolar regions of the lung (Heyder et al., 1986; Siegel

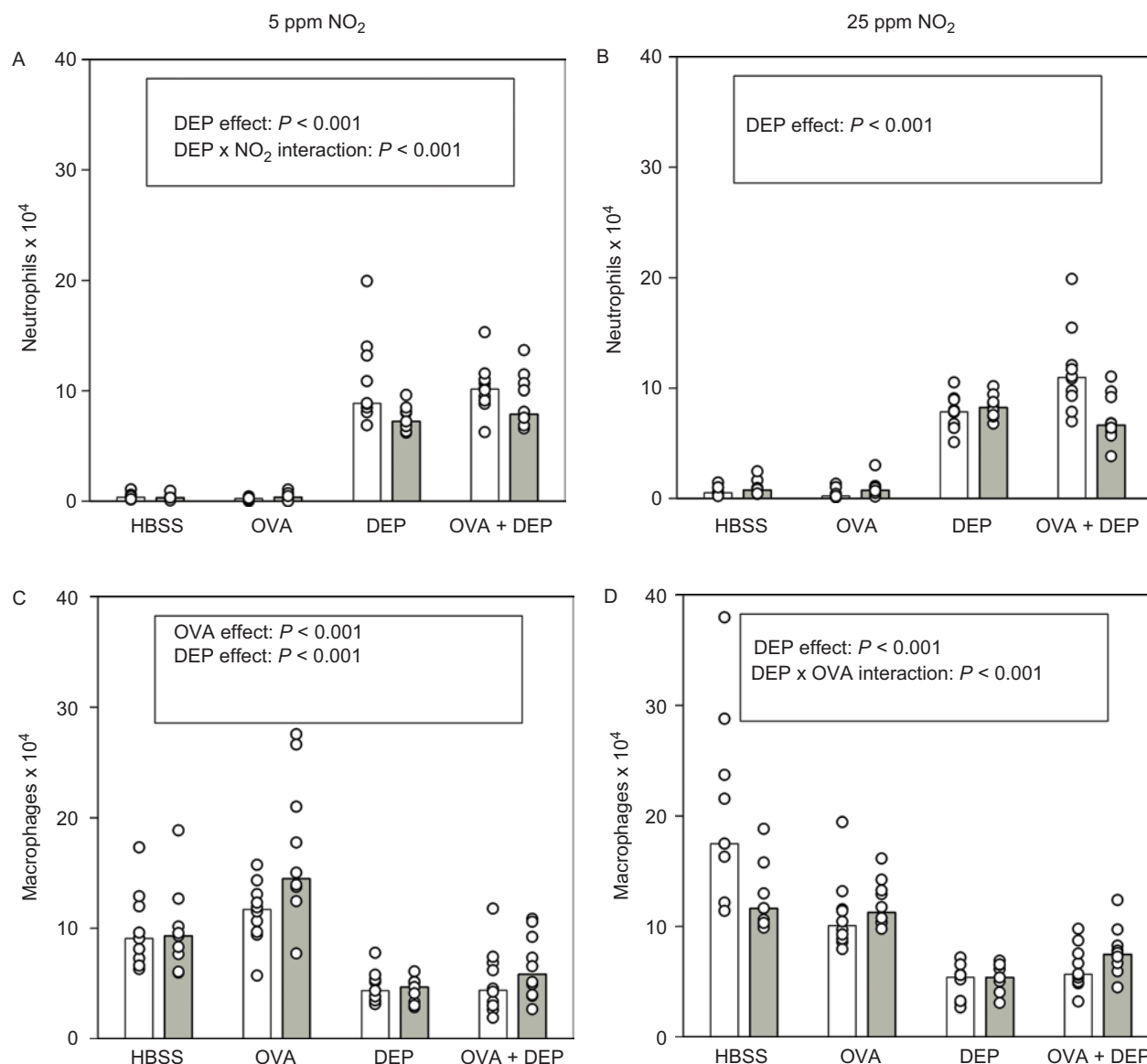


Figure 3. The number of neutrophils (A and B) and macrophages (C and D) in the bronchoalveolar lavage fluid (BALF) 18 h after intranasal instillation of mice for three consecutive days (0, 1, and 2) with a solution containing Hank's balanced salt solution (HBSS), ovalbumin (OVA), diesel exhaust particles (DEP), or OVA and DEP. Mice were subsequently exposed to NO<sub>2</sub> (5 or 25 ppm, gray bars in the left and right panels, respectively) or room air (white bars) for 4 h. Individual results (circles) and medians (bars) for groups of 10 mice are shown. The statistically significant effect of OVA, DEP, and NO<sub>2</sub>, and the interactions between them, are presented in each panel.

et al., 1997) simultaneously in the present study, giving NO<sub>2</sub> the opportunity to influence the immune response to OVA, as well as the adjuvant activity of DEP.

The allergic adjuvant activity of ultrafine particles has been linked to their potential to elicit an acute inflammatory response in the lung (de Haar et al., 2005). We therefore examined the early lung effects of DEP and NO<sub>2</sub> and found that DEP, and the highest concentration of NO<sub>2</sub>, caused increased lung permeability measured as increased protein concentrations in BALF. Others have also reported that NO<sub>2</sub> cause increased lung permeability in mice (Kleeberger et al., 1997; Hussain et al., 2004; Poynter et al., 2006) and in rats (Siegel et al., 1997; Garn et al., 2003). The epithelium is of great importance in controlling the function of dendritic cells in the lung through

release of Th2 cell-promoting cytokines (Lambrecht and Hammad, 2009). Thus, damage to epithelial cells may modulate the Th2 immune response. In the present study, both DEP and NO<sub>2</sub> caused increased lung permeability, but only DEP promoted allergic sensitization, indicating that destruction of the epithelium is not the only mechanism of importance for allergic sensitization.

The highest concentration of NO<sub>2</sub> significantly increased the release of the proinflammatory cytokine TNF- $\alpha$  in the present study. Pathmanathan et al. (2003) have reported that relatively low concentrations of NO<sub>2</sub> (2 ppm) had little effect on TNF- $\alpha$  production in healthy humans, and exposure of rats to NO<sub>2</sub> (10 ppm) even decreased the TNF- $\alpha$  levels 3 days after exposure (Garn et al., 2003). Thus, only relatively high concentrations

of  $\text{NO}_2$  seem to stimulate  $\text{TNF-}\alpha$  release. The ability of DEP to stimulate the production of  $\text{TNF-}\alpha$  in the present study was not consistent. Others have reported that DEP does not stimulate the production of  $\text{TNF-}\alpha$  in mice (Bachoual et al., 2007; Samuelsen et al., 2009). In contrast, Saber et al. (2005) found increased levels of  $\text{TNF-}\alpha$  in mice after exposure to DEP. In the present study,  $\text{NO}_2$  (25 ppm) stimulated the release of  $\text{TNF-}\alpha$  18 h after the last of three exposures, whereas DEP apparently did not. Conversely, DEP, but not  $\text{NO}_2$ , promoted allergic sensitization. Thus,  $\text{TNF-}\alpha$  does not seem to be of importance for allergic sensitization. In contrast to our finding, Lambert et al. (2001) have reported that  $\text{TNF-}\alpha$  enhanced allergic sensitization to house dust mite in

rats. Still, other factors seem to substitute this function when  $\text{TNF-}\alpha$  activity is blocked (Lambert et al., 2001).

Neutrophil influx is an important marker of lung inflammation. In agreement with others, we found an increase in the number of neutrophils in BALF after DEP exposure (Saber et al., 2005, 2006; Bachoual et al., 2007). Furthermore, DEP led to a decrease in the number of macrophages, which may be due to a stronger macrophage adherence to the epithelium after uptake of DEP. In contrast to others, we observed no increase in the number of neutrophils after exposure to  $\text{NO}_2$  (Kleeberger et al., 1997; Siegel et al., 1997; Garn et al., 2003; Hussain et al., 2004; Poynter et al., 2006; Hesterberg et al., 2009).  $\text{NO}_2$  is presumably present in the lungs for a much shorter time

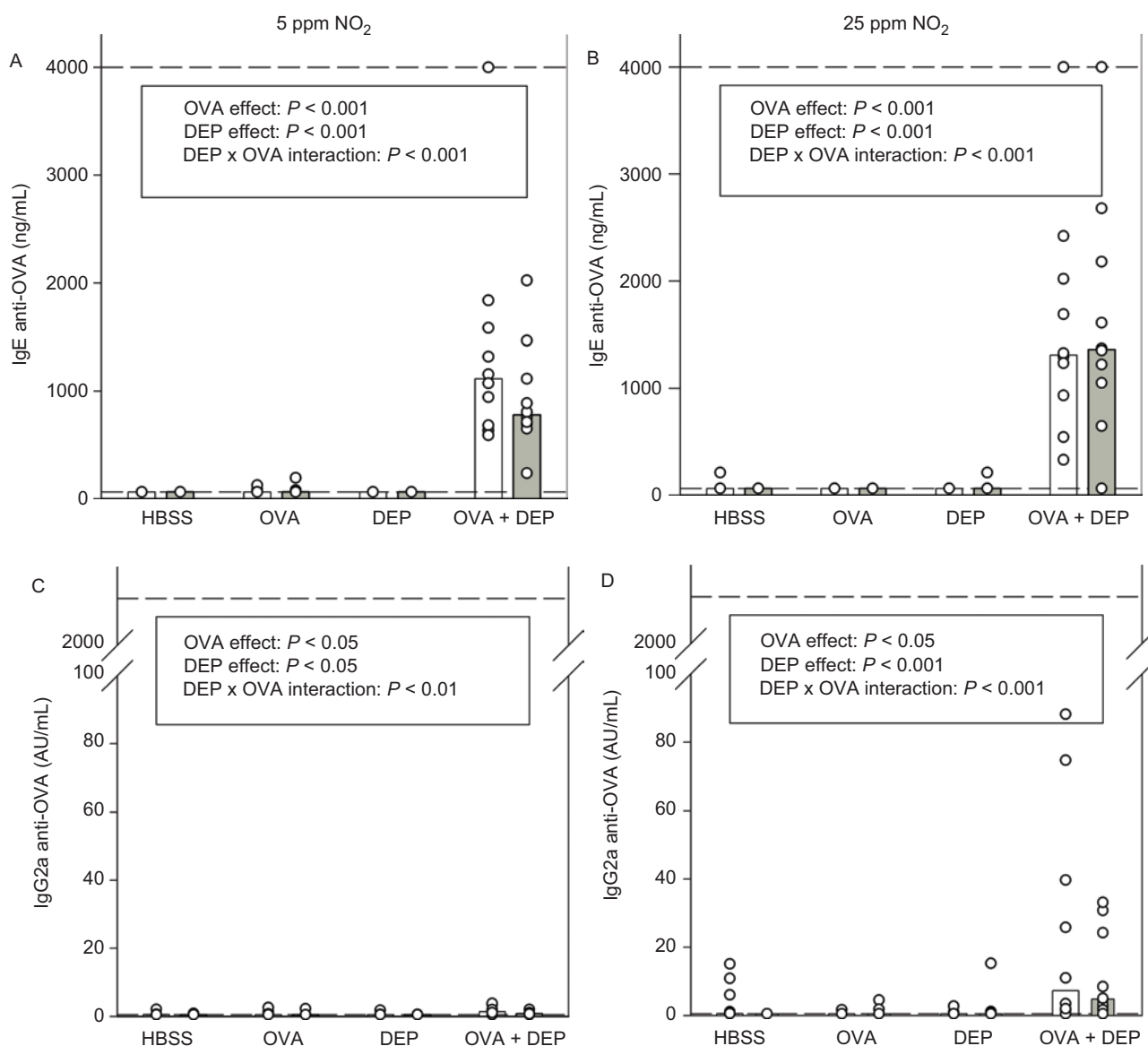


Figure 4. The serum levels of ovalbumin (OVA)-specific IgE (A and B) and IgG2a antibodies (C and D) on Day 27. Mice were instilled intranasally for three consecutive days (0, 1, and 2) with a solution containing Hank's balanced salt solution (HBSS), OVA, diesel exhaust particles (DEP), or OVA and DEP. Each instillation was followed by exposure to  $\text{NO}_2$  (5 or 25 ppm, gray bars in the left and right panels, respectively) or room air (white bars) for 4 h. On Days 22, 23, and 24 mice were again instilled with OVA (10  $\mu\text{g}$  per animal per day) or HBSS. Individual results (circles) and medians (bars) for groups of 10 mice are shown. The dotted lines indicate the upper and lower quantitative detection limits of the assays. The statistically significant effect of OVA, DEP, and  $\text{NO}_2$ , and the interactions between them, are presented in each panel.

than DEP (which is observed in the lungs at Day 27). Thus, a neutrophil influx caused by NO<sub>2</sub> may disappear earlier than one caused by DEP. According to Kleeberger et al. (1997), the number of neutrophils in mice were increased 6 h after NO<sub>2</sub> exposure, but had returned to control levels after 24 h. Thus, we can not exclude that NO<sub>2</sub> induced an early neutrophil influx in the present study, but that the number of neutrophils had returned to normal when measured, 18 h after the last of three exposures. It should also be noticed that interstrain variations in the response to NO<sub>2</sub> have been observed in mice, the weakest responses being detected in Balb/c mice and the strongest responses in C57BL/6 mice (Kleeberger et al., 1997). These strain variations may explain the observed discrepancy regarding neutrophil influx in mice after acute NO<sub>2</sub> exposure. In the present study, DEP led to neutrophil influx and allergic sensitization, whereas NO<sub>2</sub> neither caused neutrophil influx nor allergic sensitization. In agreement with others (Siegel et al., 1997; Nygaard et al., 2009), no correlation between the number of early neutrophils and the levels of allergen-specific IgE antibodies was observed.

To summarize, we found no indications that short-term exposures to NO<sub>2</sub> immediately after exposure to allergen, and in concentrations well above those encountered in ambient outdoor atmosphere, promoted allergic sensitization. One should, however, be aware that short-time exposure to air pollutants might underestimate the effect of long-term exposure to polluted air. Short-term exposure to DEP stimulated allergic sensitization in our intranasal mouse model, but under the experimental conditions applied, NO<sub>2</sub> did not influence the allergic adjuvant activity of DEP. Thus, based on our findings NO<sub>2</sub> seems to be of less importance than combustion particles in the development of allergic diseases after exposure to traffic-related air pollution.

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## Declaration of interest

The authors report no declarations of interest.

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