

Original Article

Effects of exposure to nanoparticle-rich or -depleted diesel exhaust on allergic pathophysiology in the murine lung

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ABSTRACT — Although it has been shown that exposure to diesel exhaust (DE) is linked to the induction or exacerbation of respiratory disorders, the major components responsible have not been fully identified. We examined the effects of airway exposure to nanoparticle-rich DE (NR-DE) or DE without particles on allergic pulmonary inflammation in mice. We also investigated the cellular responses to intratracheal instillation of NR-DE particles (NR-DEP). ICR mice inhaled one of four different mixtures (control air, low-concentration DE, high-concentration DE, and high-concentration DE without particles) for 8 weeks in the presence or absence of repeated intratracheal administration of ovalbumin (OVA). In a separate study, NR-DEP and/or OVA were repeatedly administered intratracheally to mice. High-concentration NR-DE or DE without particles substantially exacerbated OVA-induced eosinophilic airway inflammation. This exacerbation was concomitant with increases in lung levels of Th2 cytokines such as interleukin (IL)-4, IL-5, and IL-13 and of chemokines such as monocyte chemoattractant protein-1. Furthermore, in the presence of allergen, both DE without particles and high-concentration NR-DE strongly enhanced the production and release of myeloperoxidase into the alveolar spaces. Repeated administration of NR-DEP did not substantially affect the allergic asthma. These results strongly suggest that gaseous compounds in NR-DE aggravate murine allergic airway inflammation, mainly via amplification of the Th2 response.

Key words: Nanoparticle-rich diesel exhaust, Allergic airway inflammation, Eosinophils, Th2-cytokine, Myeloperoxidase

INTRODUCTION

The incidence of allergic diseases is increasing in urban areas worldwide (Calvert and Burney, 2005; O'Connor *et al.*, 2008), and this allergic sensitization may be occurring through exposure to various environmental factors, such as particulate matter (PM), the products of fossil-fuel combustion, or gaseous compounds in ambient air (D'Amato *et al.*, 2005). Several epidemiological studies have demonstrated that traffic-related air pollution is associated with asthma (Patel *et al.*, 2011) and chronic obstructive pulmonary disease (COPD) (Andersen *et al.*,

2011). However, the factors in urban air that cause allergic responses have remained to be identified.

An intriguing aspect of the epidemiological data is that the health impacts of PM of mass median aerodynamic diameter < 2.5 μm (PM_{2.5}) are predominantly seen in subjects with predisposing factors, including pneumonia, bronchial asthma, or COPD, and in those with compromised immune systems (Dockery *et al.*, 1993). Diesel exhaust particles (DEP) that include PM_{2.5} are epidemiologically accepted to be harmful to the respiratory system (Patel *et al.*, 2010). Asthma is a chronic airway inflammatory disease, and patients with asthma are well known

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to be sensitive to PM (Kappos *et al.*, 2004). In addition, nitric oxides (e.g., NO and NO₂) are not only good indicators of traffic-related air pollution (Eckel *et al.*, 2011) but are also associated with asthma in children (Carlsten *et al.*, 2011). Already, diesel exhaust (DE) has been demonstrated to have respiratory toxicity in the presence or absence of predisposing factors *in vivo*, both experimentally and epidemiologically (Ichinose *et al.*, 1995; Takano *et al.*, 1997; Maejima *et al.*, 2001). These findings suggest that DEP—and especially PM_{2.5}—aggravate asthma in patients via modulation of the immune system. However, although PM_{2.5} are believed to contain components that enhance the allergic response, our previous experimental observations have suggested that components other than PM_{2.5} contained in diesel exhaust enhance this response (Inoue *et al.*, 2007a; Kierstein *et al.*, 2008). Here, we aimed to reveal experimentally the components in DE that are responsible for this enhancement and to determine the mechanisms by which the enhancement occurs.

DE contains particles with a mass median aerodynamic diameter < 100 nm (defined as nanoparticles) and reactive gaseous components such as nitric oxides, aldehydes and quinones (Inoue *et al.*, 2007b). Because PM_{2.5} have been recognized as harmful to health (Carlsten *et al.*, 2011), the automobile industry now intends progressively to decrease the PM_{2.5} content of diesel engine exhaust through innovations in engine technology, but nanoparticles are exhausted not only from diesel engines but also from gasoline-powered engines. The increasing use of automobiles may in fact raise the ambient levels of nanoparticles and consequently pose even greater health risks (Timonen *et al.*, 2004; Zhu *et al.*, 2007; Terzano *et al.*, 2010), since nanoparticles are able to reach the circulatory system by easily penetrating the respiratory tract (MacNee and Donaldson, 2000; Nemmar *et al.*, 2001). However, there is no conclusive experimental evidence of nanoparticle-mediated mechanistic effects on the pathophysiology of the allergic response, and it remains unclear whether gaseous components in DE, rather than nanoparticles, enhance the allergic response.

Here, we examined the effects of either nanoparticle-rich DE (NR-DE) or DE from which the particulate components had been removed on allergic airway inflammation in mice following 8 weeks of repeated inhalation. Our aim was to elucidate the effects of both the particulate compounds in NR-DE (referred to here as “NR-DEP”) and the gaseous components of NR-DE.

MATERIALS AND METHODS

Animals

Female ICR mice aged 6 weeks (weighing 29 to 33 g; Japan Clea Co., Tokyo, Japan) were used; the mice were housed in an animal facility maintained at 24 to 26°C with 55% to 75% humidity and a 12-hr light-dark cycle and fed a commercial diet (Japan Clea Co.) with water *ad libitum*. All animal studies were approved by the Institutional Review Board of the National Institute for Environmental Studies, Tsukuba Japan.

Generation of nanoparticle-rich diesel exhaust (NR-DE) or -depleted diesel exhaust inhalation systems

An 8-L diesel engine (J08C, Hino Motors Ltd., Tokyo, Japan) was used to generate the nanoparticles. The engine was operated under steady-state conditions for 5 hr per 24 hr. Engine speed was 2,000 rpm, and engine torque was 0 Nm—conditions that can generate nanoparticles easily (Fujitani *et al.*, 2009). We designed the exhaust pipe connecting the engine and dilution tunnel to be as short as possible, and exhaust was immediately diluted with clean air to prevent changes in particle size due to coagulation during transport from the engine to the inhalation chamber. The air was then passed through a dilution system (Fujitani *et al.*, 2009). Clean air or NR-DE diluted with clean air was supplied in four inhalation chambers. One was a control chamber (control air: CA), and the NR-DE chambers were set to low particle concentration (average, 36 µg/m³: D1), high particle concentration (average, 169 µg/m³: D2). We set nanoparticle-depleted DE chamber (D3), in which the particles were removed and gas concentration was the same to that in high particle concentration chamber; Exposed air for D3 chamber was supplied after passage through an ultra-low-penetration air particulate (ULPA) filter (collection efficiency 99.999% at particle sizes of 0.1–0.2 µm) which located before the chamber. The temperature in each chamber was maintained at 20°C and the relative humidity at 50%. The internal pressure of the inhalation chambers was maintained at -100 Pa to protect the operator from incidental exposure to DE. Hoppers were placed upstream and downstream of each inhalation chamber to stabilize the particle concentration; the total volume of each inhalation chamber, including the hoppers, was 3.496 m³. Further details of the inhalation systems can be seen in the paper by Fujitani *et al.* (2009). The average concentrations of gases (CO, SO₂, NO_x (NO₂ and NO) and CO₂) and nanoparticles and the average diameter of nanoparticles in the four mixtures during the exposure experiments are given in Table 1. We

Table 1. Exposure chamber components of nanoparticle-rich diesel exhaust (NR-DE)

		Control-air	NR-DE exposure		
		CA	Low NR-DE (D1)	High NR-DE (D2)	High NR-DE except for particles (D3)
Gaseous matter	CO (ppm)	0.32	1.2	3.3	3.3
	SO ₂ (ppm)	N.D.	0.0030	0.0090	0.0090
	NO _x (ppm)	N.D.	0.41	1.4	1.4
	NO ₂ (ppm)	N.D.	0.15	0.51	0.51
	NO (ppm)	N.D.	0.27	0.92	0.92
	CO ₂ (%)	0.060	0.060	0.080	0.080
NR-DEP	Average of mode diameter(nm)	99	26	27	N.D.
	Mass concentration (μg/m ³)	3.6	36	169	N.D.
	Number concentration (cm ⁻³)	3.4	510000	1400000	N.D.

N.D. : Not Detected

have reported particle size distribution in each inhalation chamber (Win-Shwe *et al.*, 2012). The temperature in the chambers was maintained at 20°C and the relative humidity at 50%.

NR-DEP collection and preparation for intratracheal instillation

Particulate components in NR-DEP were collected from diluted diesel exhaust on dichloromethane-washed gold discs and dispersed or suspended from these discs into the appropriate media [for details, see Supplemental Material, p. 1].

In vivo studies

Study protocol 1 [NR-DE exposure]

Mice inhaled one of four different gas-nanoparticle mixtures (CA, D1, D2, and D3) for 5 hr/day, 5 days a week for 8 weeks. During inhalation exposure, the animals were intratracheally administered ovalbumin (OVA: Grade IV; Sigma Chemical, St. Louis, MO, USA) at 1 μg/mouse, or vehicle (PBS), biweekly (total 5 times). The mice were thus allocated to eight treatment groups (Fig. 1) The vehicle and the OVA were suspended in 0.1-ml aliquots, and inoculations were delivered intratracheally through a polyethylene tube with the mouse under anesthesia with 4% halothane (Takeda Chemical Industries, Osaka, Japan). The animals were euthanized and studied 24 hr after the final intratracheal instillation, as previously described, (Ichinose *et al.*, 2004; Inoue *et al.*, 2007a; Yanagisawa *et al.*, 2006).

Study protocol 2 [NR-DEP exposure]

Mice were allocated to four experimental groups (Fig. 1), which intratracheally received vehicle, NR-DEP,

OVA, or NR-DEP+OVA (50 μg/mouse) for 6 weeks [for details, see Supplemental Material, p. 1]. The animals were euthanized and studied 24 hr after the final intratracheal instillation.

Bronchoalveolar lavage (BAL) and analysis

After exsanguination (both protocol 1 and 2 animals), determination of cell counts in BAL fluid (BALF), and removal of lungs were conducted as previously reported (n = 12 (protocol 1) or n = 8 (protocol 2) in each group) (Takano *et al.*, 1997) [for details, see Supplemental Material, p. 2].

Histological evaluation

In another experiment, after exsanguination (both protocol 1 and 2 animals), the lungs were fixed and stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) (n = 4 in each group) [for details, see Supplemental Material, p. 2].

Quantitation of cytokine and chemokine protein levels and levels of myeloperoxidase (MPO), lipid peroxidase (LPO), and nitric oxide (NO) in lung tissue or BALF supernatants

Lungs frozen after BAL were homogenized as described previously (Inoue *et al.*, 2005a) [for details, see Supplemental Material, p. 2]. ELISA for cytokine and chemokine protein levels in the lung tissue homogenate was conducted by using commercial kits [for details, see Supplemental Material, p. 2] in accordance with the manufacturers' instructions (n = 6 (protocol 1) or n = 8 (protocol 2) in each group). MPO, LPO, and total NO synthesis in the BALF or lung homogenates were measured by using commercial kits in accordance with the manufac-

turers’ instructions [for details, see Supplemental Material, p. 2]. The levels of cytokine and chemokine protein or LPO in the lung homogenate were indicated as pg/total lung tissue supernatants or nmol/total lung tissue supernatants as previously reported (Takano *et al.*, 1997)

Allergen-specific immunoglobulin (Ig) determination

Serum levels of allergen (OVA)-specific IgE and IgG1 antibodies were measured by ELISA using the appropriate ELISA kits (IgE-kit, Dainippon Sumitomo Pharma, Osaka Japan; IgG₁ kit AKRIE-040, Shibayagi, Gunma, Japan) in accordance with the manufacturers’ instructions (n = 10 (protocol 1) or n = 8 (protocol 2) in each group) [for details, see Supplemental Material, p. 2].

Immunohistochemistry

Expression and localization of nitrotyrosine in the lungs were analyzed by immunohistochemistry (n = 4 in each group) [for details, see Supplemental Material, p. 2].

Assay of serum histamine

Serum histamine levels were measured with a histamine EIA kit (SPI-BIO, Montigny-le-Bretonneux, France) in accordance with the manufacturer’s instructions.

Statistical analysis

Data are reported as means ± S.E. Differences between groups were determined by using analysis of ANOVA and Bonferroni-type multiple *t*-test variance. Statistical significance was assigned to *P*-values smaller than 0.05.

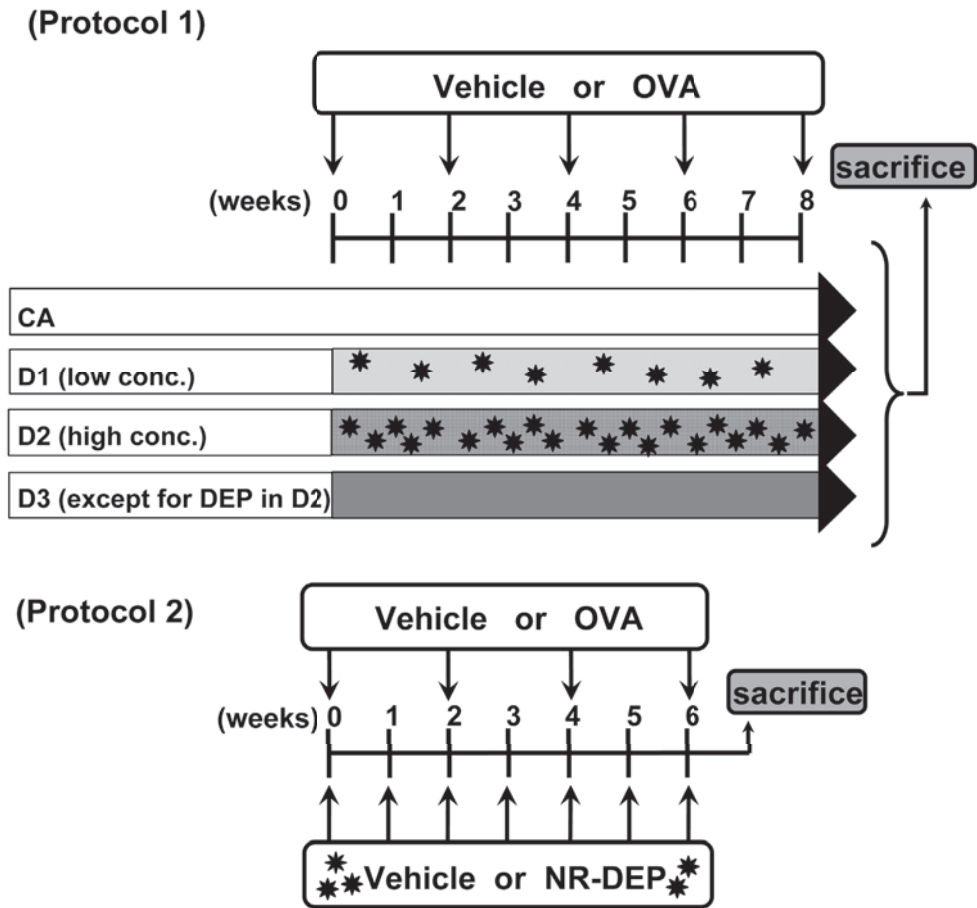


Fig. 1. Experimental design for *in vivo* studies (Protocols 1 and 2). Protocol 1: Study of NR-DE and nanoparticle-depleted DE. CA (control air), D1 (low gas + nanoparticle conc.), D2 (high gas + nanoparticle conc.), D3 (high gas conc. + no nanoparticles). Protocol 2: Study of NR-DEP. Vehicle, NR-DEP, OVA and NR-DEP+OVA.

RESULTS

Effects of NR-DE and nanoparticle-depleted DE inhalation on allergen-provoked immune cellular profiles in the lung

We quantified the numbers of infiltrating cells in OVA groups comparing with vehicle groups following NR-DE (low concentrations of both gases and nanoparticles (D1) and high concentrations of both gases and nanoparticles (D2)) inhalation and nanoparticle-depleted DE (D3) inhalation for clarifying the effect of co-existing gas pollutants. The numbers of eosinophils in BALF were significantly greater in all of the OVA groups than in the corresponding vehicle groups (Fig. 2A). Moreover, eosinophil numbers were significantly greater in the D2- and D3-OVA group than in the CA-OVA group. Exposure to D1- or D2-OVA significantly increased the number of neutrophils compared with the corresponding vehicle groups. The number of lymphocytes was significantly greater in the CA-D2 group than in the corresponding vehicle group. Despite these increases in granulocyte numbers, macrophage numbers were not increased by OVA treatment.

Allergen-related histological changes in the lung NR-DE and nanoparticle-depleted DE

H&E staining of the lungs (Fig. 2B) revealed no apparent pathological changes in the lungs from mice in the vehicle groups, whereas minimal inflammatory cell infiltration was seen in those from mice in the CA- and D1-OVA groups. Infiltration of polymorphonuclear cells around the bronchioles and vasculature was moderately potentiated in the lungs from mice in the D2- and D3-OVA groups compared with that in lungs from the CA-OVA group (arrows in Fig. 2B). PAS staining of the lung specimens (Fig. 2C) showed no substantial mucus production in the lungs from the vehicle groups. By contrast, induction of mucus hyperplasia was observed in the OVA groups. This phenomenon was very apparent in the D2- and D3-OVA groups compared with the CA-OVA group (strong red staining; arrows in Fig. 2C).

Analysis of cytokines and chemokines in lung tissues

We have already demonstrated that exposure to DEP containing PM_{2.5} exacerbates airway inflammation (Takano *et al.*, 1997). Moreover, airway exposure to DEP enhances lung expression of Th cytokines (IL-4, IL-5, and/or IFN- γ) in the presence of antigen (Takano *et al.*, 1997; Matsumoto *et al.*, 2006). We next quantified the levels of allergy-related cytokines, chemokines, and proinflammatory cytokines in the lung homogenates (Fig. 3),

revealing that production of typical Th2 cytokines (IL-4, IL-5, and IL-13) was enhanced by inhalation of D2 or D3. The level of IL-4 was significantly greater in the D3-OVA group than in the CA- and D2-OVA groups and the corresponding vehicle group. Levels of IL-5 and IL-13 were significantly greater in the D2- and D3-OVA groups than in the CA-OVA group, and those in the D1-, D2-, and D3-OVA groups were significantly higher than in the corresponding vehicle groups. The level of IL-6 was significantly greater in the D3-OVA group than in both the corresponding vehicle group and the CA-OVA group; that in the D2-OVA group was significantly greater only than the level in the D2-vehicle group.

Recently, it has been reported that the IL-23/IL-17 axis is alternative key modulator of allergic pathophysiology (Wakashin *et al.*, 2009). Production of IL-17A was significantly greater in the D3-OVA group than in the corresponding vehicle group. Inhalation of D2 or D3 along with OVA treatment increased the production of IL-23 to significantly higher levels than in the CA-OVA group; the level in the D3-OVA group was also higher than that in the D2-OVA group and the D3-vehicle group. Levels of IFN- γ , a Th1 cytokine, in the CA- and D1-OVA groups were significantly greater than those in the corresponding vehicle groups, but those in the D2- and D3-OVA groups were not elevated. The level of another Th1 cytokine, IL-1 β , was significantly greater in the D3-OVA group than in the corresponding vehicle group.

We also examined the levels of chemokines in the lungs of vehicle and OVA groups following inhalation. Production of MCP-1 was enhanced significantly in the CA-, D2-, and D3-OVA groups compared with the corresponding vehicle groups, and it was significantly greater in the D2- and D3-OVA groups than in the CA-OVA group. Inhalation of D2 or D3 in OVA-treated groups did not cause significant elevations in the levels of the monocyte-stimulating chemokines macrophage-derived chemokine (MDC) and thymus and activation-regulated chemokine (TARC) compared with the levels in the CA-OVA group, although the levels of these chemokines were significantly greater in the CA- (TARC only), D2-, and D3-OVA groups than in the vehicle groups. Levels of eotaxin, which stimulates eosinophil migration, were significantly greater in the D1-, D2-, and D3-OVA groups than in the corresponding vehicle groups. The level of the neutrophil-stimulating chemokine keratinocyte-derived chemoattractant (KC) in the lung was also significantly higher in the D3-OVA group than in the CA-OVA group and significantly greater in the D2- and D3-OVA groups than in the corresponding vehicle groups.

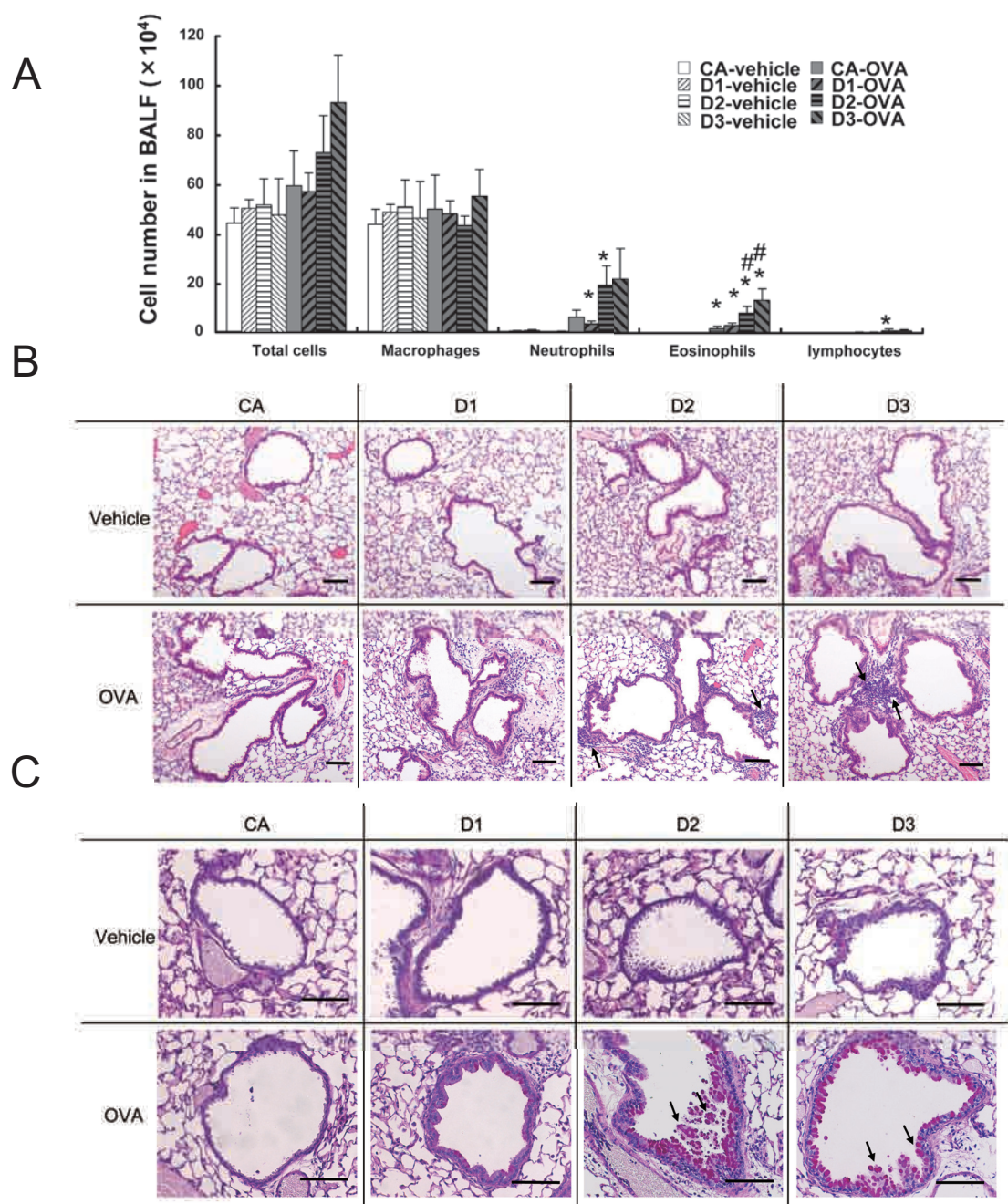


Fig. 2. Effects of inhalation exposure to NR-DE and nanoparticle-depleted DE on cellularity in bronchoalveolar lavage fluid (BALF) (**A**), and histological findings in the lung (**B**, H&E; and **C**, PAS staining). ICR mice inhaled one of four gas–nanoparticle mixtures (control air [CA], DE with low concentrations of gases and nanoparticles [D1], DE with high concentrations of gases and nanoparticles [D2], or DE with high concentrations of gases but no nanoparticles [D3]) over a period of 8 weeks and were also periodically given vehicle or ovalbumin (OVA) intratracheally. Bronchoalveolar lavage was performed 24 h after the final intratracheal administration, and the cellularity of the BALF was analyzed. Results are means $\pm$ S.E. ($n = 12$ in each group). $*P < 0.05$ vs. corresponding vehicle, $\#P < 0.05$ vs. CA-OVA. For H&E or PAS staining, the lungs ($n = 4$ in each group) were removed 24 h after the final intratracheal administration, then fixed. Representative photomicrographs of lung sections are shown. Arrows in panel (**B**) and (**C**) indicate infiltration of polymorphonuclear cells around the bronchioles and vasculature, and induction of mucus hyperplasia, respectively. Scale bars = 100 μ m.

Allergen-specific production of IgE and IgG1

We quantified OVA-specific IgE in the serum of OVA-treated mice. The level in the D3-OVA group was significantly greater than in the corresponding vehicle group or the CA- and D2-OVA groups (Fig. 3). The level of OVA-specific IgG1 was significantly greater in the CA-, D1-, D2-, and D3-OVA groups than in the corresponding vehicle groups (Fig. 3).

Enhanced induction of oxidative stress in the lungs of OVA-treated mice, and histamine production/release by NR-DE and nanoparticle-depleted DE inhalation

Oxidative stress is thought to play a dominant role in the pathogenesis of various types of lung inflammation, including allergic asthma (Andreadis *et al.*, 2003). To elucidate the role of oxidative stress in the NR-DE-provoked facilitation of allergic airway inflammation, we next measured the levels of the oxidative stress markers MPO and NO in BALF and of LPO in the lung homogenates (Fig. 4). Levels of MPO were significantly greater in the D2- and D3-OVA groups than in the CA-OVA group and significantly greater in the D1-, D2- and D3-OVA groups than in the corresponding vehicle groups. The level of LPO was significantly greater in the D3-OVA group than in the CA- and D2-OVA groups and the corresponding vehicle group. The level of NO was not significantly greater in the D1-, D2- and D3-OVA groups than in the corresponding vehicle groups or in the CA-OVA group. Immunoreactivity for nitrotyrosine (NT), a histological marker of oxidative stress, in the lung specimens (see Supplemental Material, Fig. 1) was analyzed in the vehicle and OVA groups. Slight positive staining for NT was detected in the airway epithelia of the CA-vehicle and -OVA groups; inhalation of D2 or D3 induced moderate staining for NT in the OVA groups, but D1 did not induce it. As a consequence of exacerbation of airway inflammation, serum histamine levels were expected to be elevated. The serum histamine level was significantly higher only in the D3-OVA group than in the corresponding vehicle group (see Supplemental Material, Fig. 2).

Action of NR-DEP on allergen-related airway inflammation

As shown in the inhalation study, both high-concentration NR-DE (D2) and particle-depleted high-concentration DE (D3) enhanced the allergic inflammation induced in OVA-treated mice. Therefore, in the second protocol study we aimed to further investigate whether nanoparticles in the DE were the cause of this enhancement of inflammation. We examined the effects of repeated intratracheal instillation of NR-DEP collected from NR-DE on the allergic airway inflammation model.

Examination of the cellular profiles of BALF (Fig. 5A) showed that the numbers of eosinophils and neutrophils were significantly greater in the OVA and NR-DEP+OVA groups than in the vehicle group, but the number of lymphocytes was significantly greater than with vehicle only in the OVA group. NR-DEP instillation in OVA-treated mice did not increase the numbers of macrophages, eosinophils, neutrophils, or lymphocytes, but total cell numbers and the numbers of eosinophils and lymphocytes were significantly greater in the NR-DEP+OVA group than in the NR-DEP group. Histological examination revealed no marked pathological changes in the lungs from the vehicle group. Moderate infiltration of polymorphonuclear leukocytes was seen in lungs from the OVA and NR-DEP+OVA groups, but the degree of infiltration did not differ between the two (arrows in Supplemental Material, Fig. 3 (H&E)). Histology with PAS staining showed similar degrees of mucus hyperplasia in the OVA and NR-DEP+OVA groups.

Levels of IL-5, IL-13, and eotaxin were significantly higher in the OVA and NR-DEP+OVA groups than in the vehicle group, but a significant increase in the level of IFN- γ compared with vehicle was observed only in the OVA group (Fig. 5B). There were no significant differences in the levels of IL-5 and IL-13 between the NR-DEP+OVA and OVA groups, but the level of IFN- γ was significantly less in the NR-DEP+OVA group than in the OVA group. Production of KC was significantly enhanced in the NR-DEP+OVA group compared with the NR-DEP group. We also examined the induction of OVA-specific immunoglobulin. As expected, the levels of both OVA-specific IgE and IgG1 were significantly elevated by OVA treatment in the presence or absence of NR-DEP. However, there were no significant differences in the levels of OVA-specific IgE or IgG1 between the NR-DEP+OVA and OVA groups (Fig. 5B). The level of histamine was not significantly greater in the NR-DEP+OVA group than in the vehicle or OVA group (see Supplemental Material, Fig. 4).

Effect of NR-DEP on oxidative stress in the lung of OVA-treated mice

To elucidate the role of NR-DEP in oxidative stress, we next measured the levels of the oxidative stress markers MPO and NO in BALF and LPO in lung homogenates (see Supplemental Material, Fig. 4). The level of MPO in BALF was significantly higher in the NR-DEP+OVA group than in the vehicle group or NR-DEP group. Levels of LPO and NO in the NR-DEP+OVA group did not differ significantly from those in the vehicle group. These

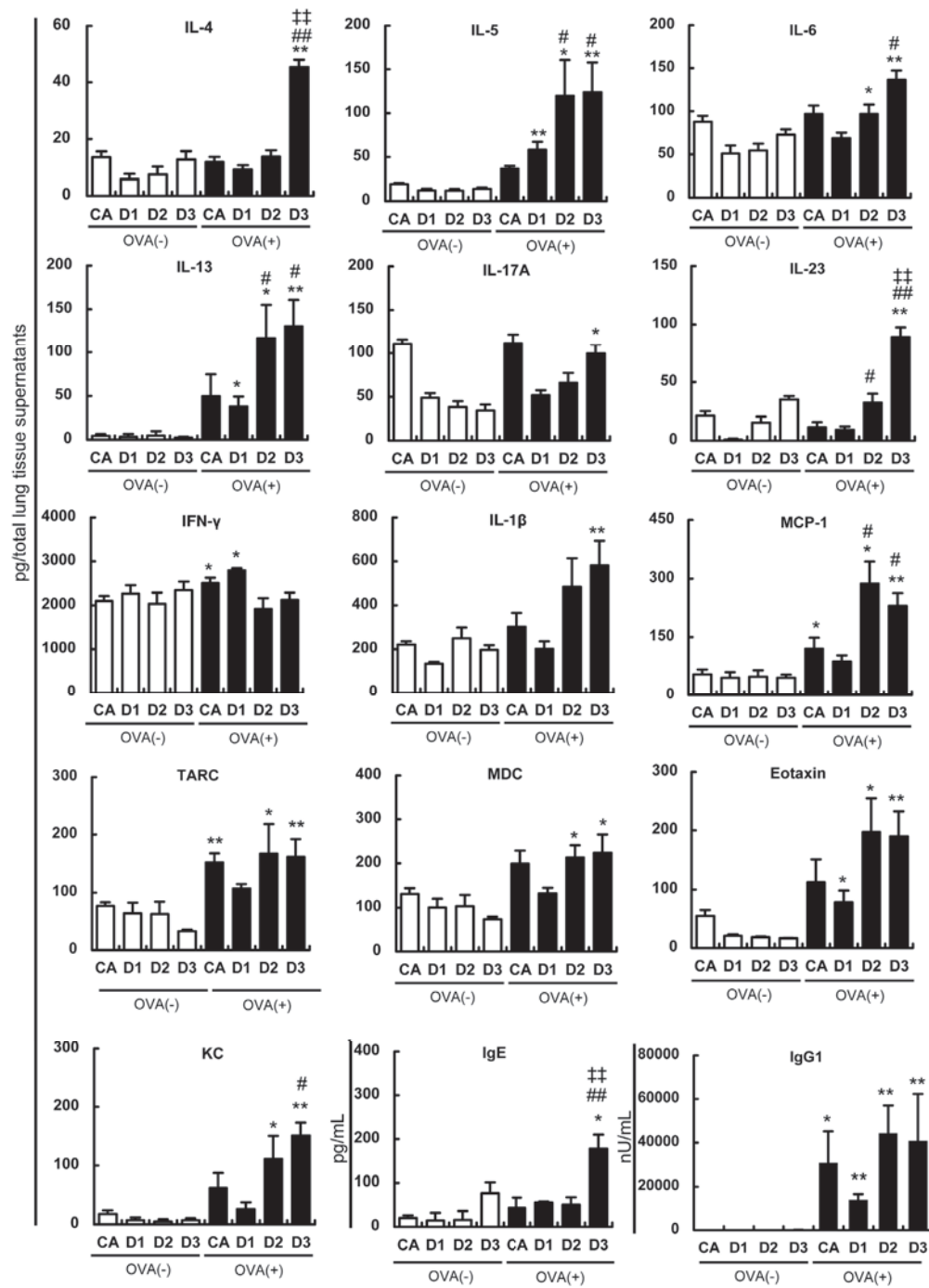


Fig. 3. Effects of inhalation exposure to NR-DE and nanoparticle-depleted DE on lung levels of cytokines and chemokines and serum levels of IgE and IgG1. ICR mice inhaled one of four gas–nanoparticle mixtures over a period of 8 weeks (see caption to Fig. 2) and were also periodically given vehicle or ovalbumin (OVA) intratracheally. Serum and lungs were retrieved 24 h after the final intratracheal administration and the lungs were homogenized. All values in the total lung tissue supernatants and serum were determined by using the appropriate assay kits. Results are means $\pm$ S.E. ($n = 6$ in each group for lung homogenates; $n = 10$ in each group for serum). * $P < 0.05$ vs. corresponding vehicle; ** $P < 0.01$ vs. corresponding vehicle; # $P < 0.05$ vs. CA-OVA; ## $P < 0.01$ vs. CA-OVA. ## $P < 0.01$ vs. D2-OVA.

Pathophysiology of nanoparticle-rich or -depleted diesel exhaust

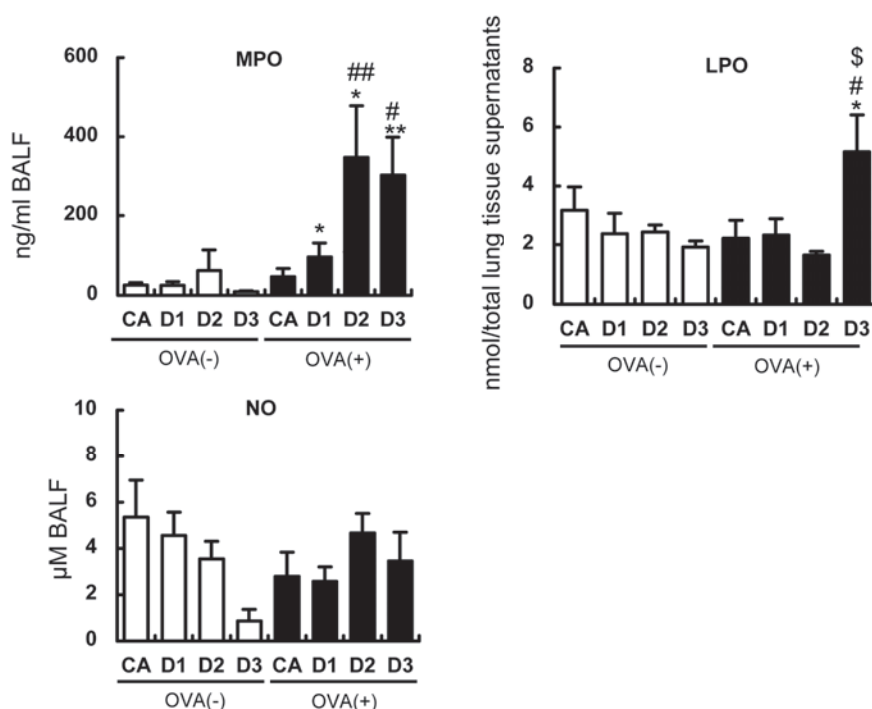


Fig. 4. Effects of inhalation exposure to NR-DE and nanoparticle-depleted DE on oxidative stress-related molecules or adducts in the airways. ICR mice inhaled one of four gas-nanoparticle mixtures over a period of 8 weeks and were also periodically given vehicle or ovalbumin (OVA) intratracheally. The bronchoalveolar lavage fluid (BALF) and lungs were retrieved 24 hr after the final intratracheal administration and the lungs were homogenized. Thereafter, all values in BALF or total lung tissue supernatants were determined by using the appropriate assay kits. Results are means $\pm$ S.E. ($n = 6$ in each group for lung homogenates, $n = 10$ in each group for BALF). * $P < 0.05$ vs. corresponding vehicle; ** $P < 0.01$ vs. corresponding vehicle; # $P < 0.05$ vs. CA-OVA; ## $P < 0.01$ vs. CA-OVA; \$ $P < 0.05$ vs. D2-OVA. MPO, LPO and NO indicates myeloperoxidase, lipid peroxidase and nitric oxide, respectively.

results showed that administration of NR-DEP via the lung did not increase in the levels of inflammation markers, suggesting that NR-DEP did not exacerbate the allergic inflammation induced in the asthma model by OVA treatment.

DISCUSSION

We and others have previously reported that DEP (mean particulate size, about 400 nm) or DE containing these DEP exacerbate allergen-associated airway inflammation and hyperresponsiveness *in vivo* (Takano *et al.*, 1997; Miyabara *et al.*, 1998; Maejima *et al.*, 2001). However, there have been no reports on the immunomodulatory effects of environmentally relevant types of DEP composed mainly of particles less than 100 nm in diameter on allergic airway inflammation models. Here, we examined the inflammatory effects of NR-DE containing nanoparticles at the concentrations of 36 or 169 $\mu\text{g}/\text{m}^3$; this

is comparable to the concentration of nanosize particles in the atmosphere previously reported (nanoparticle concentrations in ambient air range from 2×10^4 to 2×10^5 cm^{-3} and mass concentrations exceed 50 $\mu\text{g}/\text{m}^3$ in sight of major highways: Timonen *et al.*, 2004; Zhu *et al.*, 2007; Westerdahl *et al.*, 2005). Collectively, we first showed that DE containing actual concentrations of nanoparticles can exacerbate allergic airway inflammation.

As in our previous observations in mouse lungs exposed to DE containing DEP with a mean particulate size of about 400 nm (Takano *et al.*, 1997, 1998), we showed here that the count of eosinophils in the BALF of OVA-treated mice was significantly increased by inhalation of NR-DE with high concentrations of both pollutant gases and nanoparticles (i.e., D2); however, an increase in eosinophil count was also observed with inhalation of DE with high pollutant-gas concentrations but no nanoparticles (i.e., D3) (Fig. 2A). Thus migration and infiltration of eosinophils into the pulmonary alveoli and bronchi were

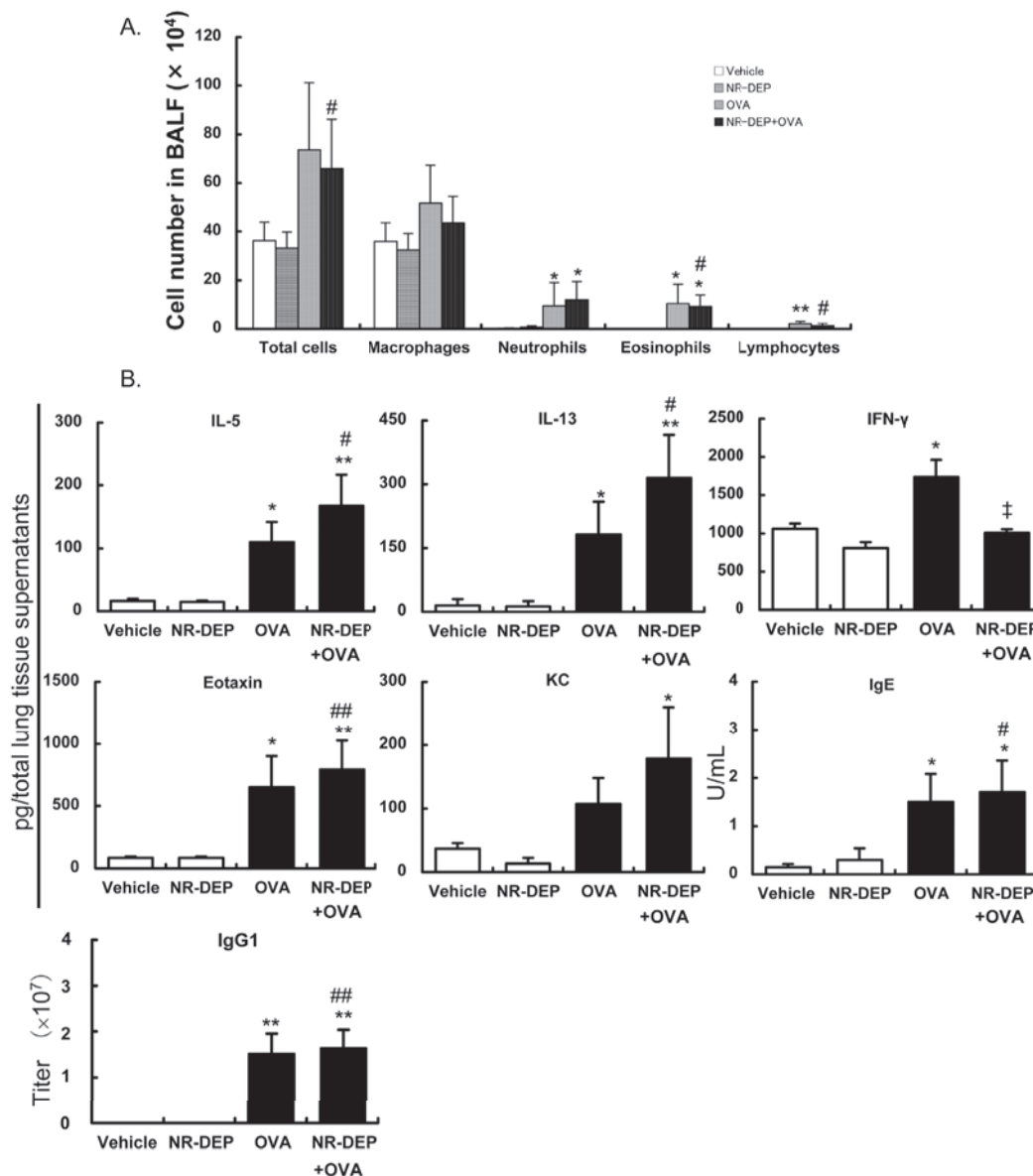


Fig. 5. Effects of intratracheal instillation of NR-DEP on (A) cellularity of bronchoalveolar lavage fluid (BALF) and (B) lung levels of cytokines and chemokines and serum levels of immunoglobulins. ICR mice were intratracheally given vehicle, NR-DEP, OVA, or NR-DEP+OVA over a period of 6 weeks. Bronchoalveolar lavage was performed 24 hr after the final intratracheal administration and the cellularity of the BALF was analyzed ($n = 8$ in each group). The lungs were then removed and homogenized. All values of cytokine and chemokine in the total lung tissue supernatants and levels of immunoglobulins in serum were determined by ELISA. Results are means $\pm$ S.E. ($n = 8$ in each group). $^*P < 0.05$ vs. vehicle; $^{**}P < 0.01$ vs. vehicle; $^{\#}P < 0.05$ vs. NR-DEP; $^{##}P < 0.01$ vs. NR-DEP; $^{\dagger}P < 0.05$ vs. OVA.

activated by the inhalation of either D3 or D2. Elevated neutrophil counts were observed in the BALF of OVA-treated mice that inhaled either D2 or D3, but these elevations were not significant compared with CA. Infiltration

of polymorphonuclear cells and induction of mucus hyperplasia were observed in the lungs of mice from both the D2- and the D3-OVA group, unlike in those from the CA-OVA group. The exacerbation of eosinophilic air-

way inflammation in mice that inhaled the high-concentration DE without particulates (D3) suggested that gaseous components in the DE stimulated allergic responses in the lung.

We showed that production of the eosinophil-activating chemokine eotaxin increased significantly in OVA-treated mice with inhalation of either D2 or D3, compared with inhalation of the respective vehicles. Production of the neutrophil-activating chemokine KC showed a trend similar to that of eotaxin except that there was a significant difference between D3-OVA and CA-OVA. MCP-1 contributes to the migration or infiltration of monocytes and macrophages (Gordon, 2000), and recently this chemokine was shown to activate eosinophils (Inoue *et al.*, 2010). Already, we and others have demonstrated that lung MCP-1 levels parallel allergic asthma severity in humans and experimental animals (Gordon, 2000; Inoue *et al.*, 2005b, 2010). Here, we found that MCP-1 levels in the lung were significantly higher in the D2- and D3-OVA groups than in the CA-OVA group; thus, exacerbation of allergic airway inflammation by high-concentration NR-DE or DE without particles (i.e., D2 and D3) exposure is likely mediated by enhanced lung expression of MCP-1.

Migration or infiltration of eosinophils and neutrophils causes inflammation via the induction of oxidative stress (Inoue *et al.*, 2010). We showed here that the level of MPO, a marker of oxidative stress induction, increased significantly in BALF with inhalation of D2 or D3 compared with inhalation of the respective vehicles or of CA-OVA (Fig. 4). Our observations suggest that eosinophils and neutrophils accumulated in the lung induce oxidative stress. MPO is a peroxidase enzyme, which is abundant in neutrophils, and produces hypochlorous acid from hydrogen peroxide and chloride anion during the neutrophils' respiratory burst. Increases in neutrophil numbers and MPO levels are sometimes associated with asthmatic pathology (Nabe *et al.*, 2011). An increase in LPO level in the lung tissue also occurred here in OVA-treated mice that inhaled D3. Therefore, the enhancing effects of NR-DE high in both gases and nanoparticles (D2) and of DE high in gases but depleted of nanoparticles (D3) on allergic airway inflammation might be due, at least in part, to enhanced synthesis or activity of these peroxidases. In contrast, there were no significant differences in the lung production of NO, although inhalation of D2 or D3 induced moderate staining for NT in the OVA groups. The role of oxidative stress needs to be addressed in further detail in future research.

One theory of the immunological basis underlying the pathogenesis of asthma—a typical allergic response—is dysregulation of T-helper 1 (Th1) and 2 (Th2) balance.

OVA-induced lung histopathological changes are associated with a Th2-predominant inflammatory response (Finkelman *et al.*, 2010). Not only DEP promoted Th2 conditions in the lungs of atopic animals (Inoue *et al.*, 2005b); they also exacerbated allergen-elicited Th2-type airway inflammation (Diaz-Sanchez *et al.*, 1997; Takano *et al.*, 1997). Here, we examined whether the production of cytokines and immunoglobulins (e.g., IgE) associated with the Th2 response was enhanced by inhalation of NR-DE or DE depleted of nanoparticles. As expected, inhalation of high-concentration NR-DE (D2) or high-concentration DE depleted of nanoparticles (D3) significantly increased the levels of Th2 cytokines such as IL-5 and IL-13 in lung homogenates in the presence of OVA as compared to without OVA, whereas inhalation of either D2 or D3 tended to decrease levels of the Th1 cytokine IFN- γ . The particle-depleted high-concentration DE (D3) was the only one to enhance lung expression of IL-4 and the production of allergen-specific IgE; the same mixture that had not been depleted of nanoparticles (D2) did not cause these enhancements. This showed that the gaseous components of DE had a potent influence in tipping the Th1/Th2 balance toward a Th2 milieu. This might play a role in aggravating the effects of NR-DE on the allergic asthma model, but the possibility cannot be ruled out that the nanoparticles in NR-DE also enhance Th2-related responses.

Recently, subtypes of helper T-cell other than Th1/Th2 have been suggested to function in the inflammatory response. For example, some T-cell compartments are activated by IL-23 to produce a proinflammatory mediator, IL-17A, and to differentiate into another T-cell subset defined as “Th17” cells (Yen *et al.*, 2006). In our study, IL-23 level in the lung was increased in OVA-treated mice by exposure to either high-concentration NR-DE (D2) or high-concentration DE depleted of nanoparticles (D3) compared with CA-OVA exposure, and the IL-23 level was significantly greater with D3 exposure than with D2 exposure. Th17 differentiation is regulated by the aryl hydrocarbon receptor, the ligands of which are polycyclic aromatic hydrocarbons (PAHs, e.g., benzo[a]pyrene) contained in DEP, thus suggesting that DEP activate the differentiation of Th17 cells. In fact, we have just reported elsewhere that IL-17A is induced in splenic cells following treatment with NR-DEP (Nakamura *et al.*, 2012). Here, our analysis of the effect of NR-DE inhalation on IL-17A levels showed that they were markedly decreased in both vehicle- and OVA-treated mice by NR-DE inhalation (D1 and D2) compared with CA inhalation (Fig. 3), although there were no differences in IL-17A levels between OVA-treated mice inhaling CA or D3. These

observations suggest that NR-DE disrupts the IL-23/IL-17 axis, a critical regulator of Th17 differentiation and maturation and related inflammatory diseases, including allergic ones (Zhao *et al.*, 2010). Accordingly, future detailed studies are needed to determine the impacts of NR-DE and NR-DEP on the Th1/Th2/Th17 immune system.

Repeated intratracheal administration of NR-DEP did not substantially aggravate the allergic response or pathophysiology: in OVA-treated mice there were no significant increases in the numbers of migrated or infiltrated eosinophils or the induction of interleukins (IL-5 and IL-13) and IgE in response to NR-DEP administration (Fig. 5). However, NR-DEP exposure of OVA-treated mice significantly decreased IFN- γ levels in the lung homogenates.

Taking these results into consideration, it seems that the presence of nanoparticles in NR-DE does not affect IgE production. It is likely that each component in NR-DE plays a different role in facilitating allergic airway inflammation. Gaseous pollutants such as NO_x and O₃ have been associated with the appearance of allergic processes (Ring *et al.*, 2001). Nitrogen dioxide gas in DE increases tissue IL-4 and IgE levels in mice (Maejima *et al.*, 2001), whereas SO₂ exposure is related to the exacerbation of asthma through the induction of epidermal growth factor and its receptor, as well as cyclooxygenase-2, at transcriptional levels *in vivo* (Ruijin and Meng, 2007). These gases might therefore be candidate causes of the marked enhancement of asthma pathology resulting from exposure to the particle-depleted mixture (D3) in the current asthma model. Besides the classical air pollutant gases (e.g., NO_x, O₃, and SO₂), physiologically active volatile compounds such as quinones (Inoue *et al.*, 2007b) and aldehydes are also present in DE. Actually, the concentration of formaldehyde, acetaldehyde, acrolein, methane and total hydrocarbon other than methane in D2 chamber was estimated to be 140 $\mu\text{g}/\text{m}^3$, 100 $\mu\text{g}/\text{m}^3$, 9.6 $\mu\text{g}/\text{m}^3$, 330 $\mu\text{g}/\text{m}^3$ and 2.5 ppm(v/v), respectively (Fujitani Y. unpublished results). These volatile compounds may play roles in acceleration of the allergic process by DE inhalation.

Interestingly, in our inhalation study of D2 and D3 (Protocol 1), the levels of IL-4 and IgE production were significantly elevated in OVA-treated mice by D3 inhalation but not by D2 inhalation, while IL-5 and IL-13 production and infiltration of eosinophils were stimulated in OVA-treated mice by inhalation of both D2 and D3. This observation suggests that gaseous components in NR-DE enhanced Th2 response, but the exposure of nanoparticles lost or markedly attenuated the production of IL-4 and IgE in the presence of OVA, because the only difference here appeared to be the absence (D3) or pres-

ence (D2) of nanoparticles. However, as both D2 and D3 inhalation also stimulated infiltration of neutrophils as well as eosinophils in OVA-treated mice, Th2 cells themselves without relation to IgE production and neutrophils might contribute to exacerbation of airway inflammation by NR-DE and gaseous components in NR-DE. Previously, Fujimaki *et al.* (1997) reported that inhalation of DE containing DEP with a mean particulate size of about 400 nm at concentration of 6 mg/m³ enhanced the production of OVA-specific IgE, reasonably concluding that repeated exposure to nano-particles, which consist of oil aerosol, contained in our model NR-DE may be weaker inducer of antigen-specific IgE and less harmful than DEP, which mainly consist of carbon black, with about 400 nm particle size. Of course, the possibility that DEP with 400 nm particle size caused adverse effects by inhalation at higher concentration of particles compared to inhalation of nano-particles cannot be ruled out. Many investigators have focused on the investigation whether Th2 cytokines is involved in nanoparticle exposure models, but our findings seem to argue against this perspective. Further investigations are needed to resolve the puzzling finding whereby, unlike exposure to D3, exposure to D2 did not increase lung IL-4 levels and serum OVA-specific IgE levels.

Our findings revealed that inhalation of high-gas-content DE in the presence (D2) or absence (D3) of high concentrations of nanoparticles exacerbated allergic airway inflammation in mice, suggesting that the gaseous compounds in NR-DE exacerbate inflammation. NR-DE was previously thought to exacerbate allergic airway inflammation via IgE, but our novel findings demonstrated that NR-DE exacerbated allergic airway inflammation via an increase in eosinophil levels.

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