

Effects of Diesel Exhaust Particles on Antigen-Presenting Cells and Antigen-Specific Th Immunity in Mice

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Diesel exhaust particles (DEP) exacerbate antigen-related airway inflammation and hyperresponsiveness in mice; however, the mechanisms remain undefined. The present study characterized more precisely which pathways and cellular events of the allergic response are amplified by DEP in view of the maturation/activation/function of antigen-presenting cells (APC) and the antigen-specific Th response. We evaluated the effects of DEP on the phenotype and function of bone marrow-derived dendritic cells (BMDC) *in vitro* and on the expression pattern of APC-related molecules in the murine lung in the presence or absence of antigen *in vivo*. Also, we tested the effects of *in vivo* DEP co-exposure with antigen on the splenic antigen-specific Th response in the context of cytokine production. DEP significantly increased both allogeneic and antigen (ovalbumin: OVA)-specific syngeneic T-cell proliferation *in vitro*. In addition, an *in vivo* experiment showed that repetitive pulmonary exposure to DEP plus antigen (OVA) increased the numbers of MHC class II⁺ cells and those expressing CD11c, DEC205 (DC markers), CD80, CD86 (co-stimulatory molecules), F4/80 (a macrophage marker), and CD19 (a B-cell differentiation antigen) in the lung as compared to that of others (vehicle, DEP, or OVA). Furthermore, an *ex vivo* assay system demonstrated that splenic mononuclear cells primed by DEP plus OVA produced a greater amount of interleukin (IL)-4, IL-5, and IL-13 after *in vitro* antigen stimulation compared to those primed by the other

treatments. In conclusion, enhancement of allergic responses by DEP can be explained via two novel mechanisms, i.e., enhancement effects on APC including DC and on antigen-specific Th response, which culminate in the promotion of local and systemic dysregulated Th immunity. *Exp Biol Med* 234:200–209, 2009

Key words: diesel exhaust particles; antigen-related airway inflammation; antigen-presenting cells; dendritic cell; Th response

Introduction

Previous epidemiological studies have indicated that exposure to ambient particulate matter (PM) is linked to increases in mortality and morbidity related to cardiopulmonary diseases (1, 2). A study has shown that PM with a diameter less than 2.5 μm (PM_{2.5}) is more closely associated with both acute and chronic respiratory effects including bronchial asthma and subsequent mortality than PM₁₀ (3). Also, some data imply that PM is a risk factor for the development/exacerbation of allergic diseases including bronchial asthma, allergic rhinitis, and atopic dermatitis (4–6). In particular, during PM pollution episodes, asthmatic subjects reportedly demonstrate increased respiratory symptoms, bronchoconstriction, medication use, bronchial hyper-reactivity, and emergency care visits (7, 8). Compatible with the epidemiological investigation, we and others have experimentally demonstrated that exposure to diesel exhaust or diesel exhaust particles (DEP), representative constituents in PM_{2.5}, exacerbate experimental asthma (9–13). It has also been shown that airway exposure to DEP enhanced lung expression of Th cytokines (interleukin [IL]-4, IL-5, and/or interferon [IFN]- γ) in the presence of antigen (10, 12), indicating that DEP facilitates antigen-related airway inflammation, at least partly, through the enhanced Th response in the lung. However, it remains unclear how DEP exert their enhancing effects on antigen-related airway

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inflammation with Th response and which pathways and cellular events in this pathological condition are amplified.

The role of antigen-presenting cells (APC), including dendritic cells, macrophages, and B cells, in the pathogenesis of allergic diseases has been well defined. When antigens enter the airways, APC in the airways capture antigens and migrate to secondary lymphoid tissues such as draining lymph nodes (14). There, antigen-specific T cells differentiate into Th effector cells or regulatory T cells via an interaction with APC (15). Indeed, pathological activation of APC reportedly enhances antigen-related airway inflammation *in vivo* (16, 17). Among APC, dendritic cells (DC) are recognized to be professional cell populations having potent antigen-presenting properties (14, 15, 18, 19). Several studies have identified a critical role for DC in antigen-related airway inflammation (20, 21). Nonetheless, the effects of DEP on these cell populations have been less studied. Furthermore, there has been no report investigating their effects using both *in vitro* and *in vivo* experiments.

On the other hand, proallergic effects of DEP on T lymphocytes primed with *in vivo* antigen challenge remain obscure, although previous *in vivo* studies have demonstrated that DEP amplify the lung expression of Th cytokines (10, 12, 22). Indeed, it has been previously reported that a component of DEP (pyrene) induces a Th milieu in both human and murine T cells *in vitro* (23); however, there has been no *ex vivo* study to confirm it.

In this study, we sought to characterize more precisely which pathways and cellular events of the antigen-induced response are amplified by DEP in the context of the maturation/ activation/function of APC and the antigen-specific Th response using *ex vivo*-generated murine dendritic cells (DC) *in vitro* and a murine antigen-related airway inflammation model in both *in vivo* and *ex vivo* assay systems.

Materials and Methods

Animals. Male ICR and BALB/c mice, 11 to 15 wks of age and weighing 38 to 42 g (ICR) and 27 to 32 g (BALB/c) (Japan Clea Co., Tokyo, Japan), were used for *in vitro* experiments (two to three animals in each experiment and experiments were repeated two to four times). On the other hand, male ICR mice, 6 to 7 wks of age and weighing 29 to 33 g (Japan Clea Co.), were used for *in vivo* experiments ($n = 17$ – 18 in each group). They were fed a commercial diet (Japan Clea Co.) and given water *ad libitum*. Mice were housed in an animal facility that was maintained at 24–26°C with 55–75% humidity and a 12-hr light/dark cycle. The studies adhered to the National Institutes of Health guidelines for the experimental use of animals. All animal studies were approved by the Institutional Review Board of the National Institute for Environmental Studies.

Preparation and Generation of Bone Marrow-Derived Dendritic Cells (BMDC). Preparation of bone

marrow cells was conducted as previously described (24, 25). BMDC were differentiated using a modified protocol of Lutz *et al.* (26) as previously conducted (24, 25).

Collection of DEP and *In Vitro* Exposure. DEP were collected as previously described (12, 13, 22, 27). The DEP suspension was sonicated for 3 mins using an Ultrasonic Disrupter (UD-201; Tomy Seiko, Tokyo, Japan), just before the exposure. At day 8 of generation, immature BMDC, as stimulators for lymphocyte reaction, were treated with 50 µg/ml mitomycin C (Kyowa Hakko Kirin Co. Ltd., Tokyo, Japan) for 20 mins in a water bath at 37°C before exposure to DEP as previously described (28). Then, the cells were washed three times with R10, which was RPMI 1640 medium (GIBCO BRL, Eggenstein, Germany) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Dainippon Sumitomo Pharma Co., Ltd.), 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma Chemical, St. Louis, MO), and 50 µM 2-mercaptoethanol (GIBCO BRL). 2.4×10^6 cells were placed in 6-well plates and exposed to different concentrations of DEP (1–10 µg/ml) or to 0.05% Tween 80 (Nakalai Tesque, Kyoto, Japan: control) in 2.4 ml of R10 medium, containing 10 ng/ml recombinant mouse granulocyte macrophage-colony stimulating factor (GM-CSF; Sigma) for 24 hrs at 37°C in an atmosphere of 5% CO₂/95% air.

FACS Analysis. For FACS analysis, the following monoclonal antibodies were used: MHC class II molecules: I-A/I-E (2G9, FITC-conjugated, BD Biosciences Pharmingen, CA); co-stimulatory molecules: CD80 (16–10A1, PE-conjugated, BD Biosciences Pharmingen), CD86 (GL1, PE-conjugated, BD Biosciences Pharmingen); and DC markers: CD11c (HL3, PE-conjugated, BD Biosciences Pharmingen), DEC-205 (NLDC-145, PE-conjugated, Cedarlane Labs, Ontario, Canada). Immunostaining was performed as previously described (24, 25), and flow cytometry was performed using a FACSCalibur (Becton, Dickinson and Company, NJ). Fluorescence data were expressed as the percentage of positive cells. This experiment was repeated four times using two to three animals in each group.

Allogeneic Mixed Lymphocyte Reaction (MLR). After exposure to DEP, BMDC derived from ICR or BALB/c mice were washed twice with R10 medium and resuspended in R10 medium. Allogeneic T cells were isolated from pooled C57BL/6 spleen cells as previously described (25). At day 9 of generation for BMDC, DEP-exposed BMDC (from 2.5×10^3 to 1×10^4) and allogeneic T cells (2×10^5) were co-cultured for 4 days in 96-well flat-bottom plates (200 µl/well), and thereafter, T-cell proliferation was measured by using a Cell-Proliferation-ELISA Kit (Roche Molecular Biochemicals, Mannheim, Germany) as previously described (25). This experiment was repeated three times using three animals in each group.

Preparation of Antigen-Sensitized T Cells and Antigen-Specific Lymphocyte Reaction. Ovalbumin (OVA: Grade IV; Sigma Chemical)-sensitized T cells were derived from a pool of spleens from OVA-sensitized

syngeneic (ICR and BALB/c) mice as previously described (24, 25). At day 9 of generation for BMDC, DEP-exposed BMDC (from 2.5×10^3 to 1×10^4) and OVA-specific T cells (2×10^5) were co-cultured in the presence of OVA (40 μ g) for 4 days, and T-cell proliferation was measured by the same manner as the MLR experiment. This experiment was repeated three times using three animals in each group.

In Vivo and Ex Vivo Protocol. Male ICR mice were divided into four experimental groups. The vehicle group received PBS containing 0.05% Tween 80 every 2 wks for 4 wks (total 3 times). The OVA group received 2 μ g of OVA dissolved in the same vehicle every 2 wks for 4 wks (total 3 times). The DEP group received 100 μ g of DEP suspended in the same vehicle every 2 wks for 4 wks (total 3 times). This DEP dosage (100 μ g/body) was confirmed to significantly facilitate this type of allergic asthma when intratracheally administered several times in our previous studies; in contrast, 50 μ g of DEP was not sufficient ([10] and unpublished observation). The DEP + OVA group received the combined treatment using the same protocol as the OVA and DEP groups, respectively. In each group, vehicle, OVA, DEP, or DEP + OVA was dissolved in 0.1-ml aliquots and inoculated by the intratracheal route through a polyethylene tube under anesthesia with 4% halothane (Hoechst, Japan, Tokyo, Japan). Five hrs after the last intratracheal administration, mice were sacrificed and sera, the lungs, and spleen were collected for lung histology, FACS analysis, antigen-specific antibody assay, and *ex vivo* experiments, respectively.

Preparation of the Lung Cells and FACS Analysis. The lung cells were prepared as previously described (28, 29). The numbers of viable cells were determined by the trypan blue (Gibco Laboratories, Grand Island, NY) exclusion method.

For FACS analysis, the following monoclonal antibodies were used: I-A/I-E, CD80, CD86, CD11c, DEC205; a macrophage marker: F4/80 (CI:A3-1, PE-conjugated, Serotec, Oxford, UK); and a B-cell differentiation antigen: CD19 (6D5, PE-conjugated, Southern Biotechnology, Birmingham, AL). FACS analysis was performed using the same protocol as in the *in vitro* study ($n = 14$ – 15 in each group).

Antigen-Specific Igs Determination. Antigen-specific IgG₁, IgG_{2a}, or IgE antibodies were measured by ELISA using sera obtained from blood. OVA-specific IgG₁ and IgG_{2a} were measured according to the methods applied in our previous reports (12, 22) ($n = 3$ – 4 in each group). OVA-specific IgE were measured by ELISA kit (Dainippon Pharmaceutical Co.) according to the manufacturer's instructions ($n = 3$ – 4 in each group).

Splenocytes Culture. After removal of the lungs, spleens were harvested, and splenic mononuclear cells were isolated and resuspended in R10, at a concentration of 4×10^6 cells/ml, and cultured in medium, or two concentrations of OVA (40 or 400 μ g/ml). After 5 days of culture,

supernatants were collected for cytokine measurement as previously conducted (30).

Cytokine Measurements. Levels of IL-4 (Amersham, Buckinghamshire, UK), IL-5 (Endogen, Cambridge, MA), IL-13 (R&D Systems, Minneapolis, MN), and IFN- γ (R&D Systems) were measured by ELISA according to the manufacturer's instructions as previously conducted (22, 25) ($n = 6$ for IL-4, $n = 14$ – 15 in each group, respectively).

Statistical Analysis. Data were reported as mean $\pm$ SEM. Differences were determined using analysis of variance (Stat view version 4.0; Abacus Concepts, Inc., Berkeley, CA). If differences between groups were significant ($P < 0.05$), Bonferroni's protected least significant difference test was used to distinguish between pairs of groups.

Results

Effects of DEP on DC Maturation and Function

In Vitro. We first elucidated the effects of DEP exposure on DC maturation/activation using BMDC derived from ICR or BALB/c mice *in vitro*. DEP increased the percentage of MHC class II⁺ DEC205⁺ and that of MHC class II^{high} DEC205^{high}, on BALB/c-derived BMDC as compared with the control on day 9 (Fig. 1). To further examine the effects of DEP on DC activation, we examined the expression of co-stimulatory molecules such as CD80 and CD86, and CD11c. However, the expression of these molecules was not significantly different between control-treated and DEP treated BMDC (Fig. 2). In addition, IL-18 level in culture supernatants tended to be higher in DEP-treated BMDC than in control-treated BMDC (NS; data not shown).

We next tested the effects of DEP on the immunostimulatory capacity of BMDC using allogeneic MLR (Fig. 3) and OVA-specific syngeneic lymphocyte reaction (Fig. 4). Allogeneic T-cell proliferation was increased by the addition of BMDC in a concentration-dependent manner (data not shown). DEP exposure significantly enhanced the ability of BMDC from both strains of mice to proliferate T cells when 2.5% of ICR-BMDC and 1.25% of BALB/c-BMDC were co-cultured. Also, OVA-specific T-cell proliferation was increased by the addition of syngeneic BMDC in a concentration-dependent manner (data not shown). DEP exposure enhanced the antigen-presenting activity of BMDC from both strains of mice to proliferate OVA-specific T cells when 2.5% of both BMDC were co-cultured (Fig. 4: $P < 0.05$ for ICR), although no dose-dependent response was observed. Furthermore, Th cytokine levels in culture supernatants were found to show similar tendency as these T-cell proliferation tests (data not shown).

Effects of DEP on Lung Inflammation Related to Antigen In Vivo. We next examined whether DEP enhanced antigen-related airway inflammation in a histological examination on this model. DEP alone slightly induced the infiltration of inflammatory leukocytes. Intratracheal instillation with OVA led to the recruitment of

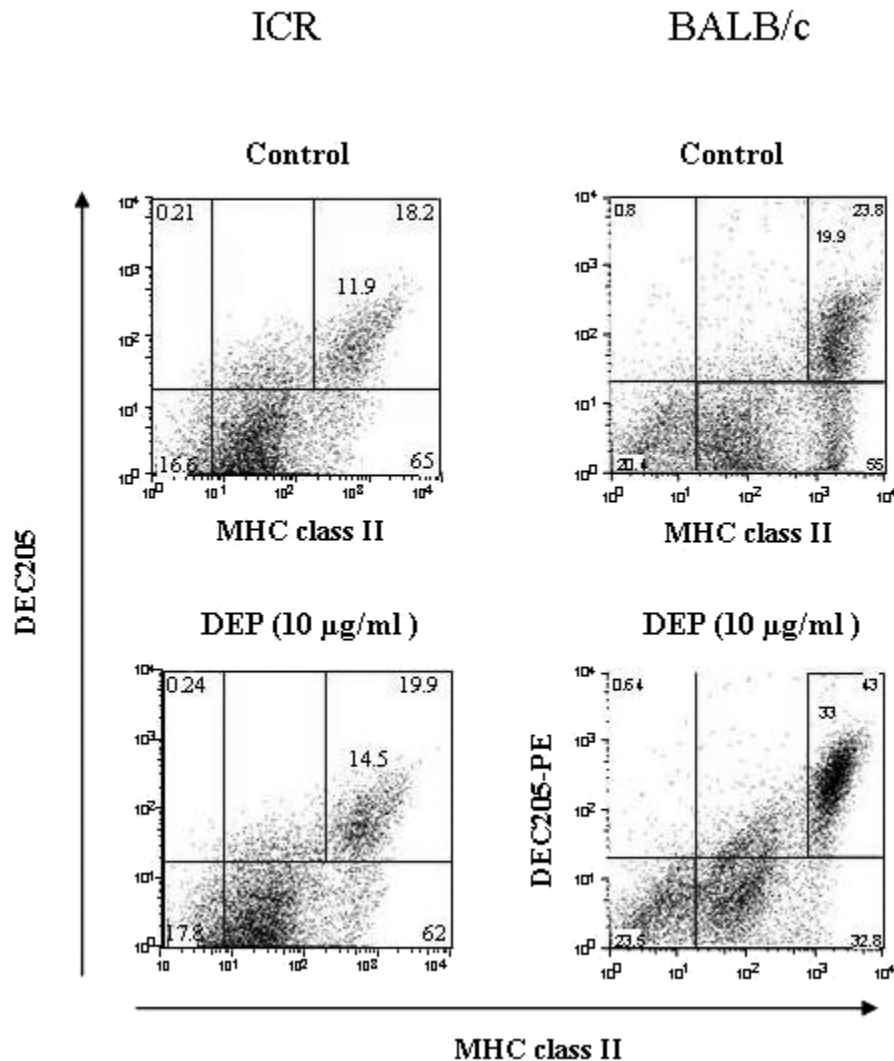


Figure 1. Effects of diesel exhaust particles (DEP) on bone marrow-derived dendritic cell (BMDC) maturation in terms of surface expressions of MHC class II and DEC205. Bone marrow (BM) cells were derived from each (ICR, BALB/c) of the mice and cultured as described in Materials and Methods. At day 8 of generation, 1×10^6 cells/ml BMDC was further cultured for the next 24 hrs with 0.05% Tween 80 (control) or 10 µg/ml DEP in R10 medium containing 10 ng/ml GM-CSF. Thereafter, MHC class II and DEC205 expressions on BMDC were analyzed by a flow cytometry. Percentage of MHC class II⁺ DEC205⁺ cells is shown in the upper right quadrant. The boxes denote the percentage of MHC class II^{high} DEC205^{high} cells. This experiment was repeated four times using two to three animals in each group. One representative data set is shown.

eosinophils around the airways, whereas vehicle did not. The magnitude of peribronchial and perivascular inflammation in the DEP + OVA group was greater than that in the OVA group as found in our previous studies on similar protocol (12, 22).

The number of total cells in the lung was greater in the DEP + OVA group than in the vehicle ($P < 0.01$), DEP (NS), or OVA group (NS) (Table 1). On the other hand, each challenge with DEP, OVA, and DEP plus OVA similarly did not affect the cell viability (more than 90%) of lung cells (data not shown).

Effects of *In Vivo* Exposure to DEP on the Expression of MHC Class II and Costimulatory Molecules in Lung Cells. The number of MHC class II⁺ cells was greater in the DEP + OVA group than in the

vehicle group (Fig. 5; $P < 0.05$). Also, the number of the cells tended to be greater in the DEP + OVA group than in the DEP or OVA group (NS). The numbers of CD80⁺ and CD86⁺ cells were greater in the DEP + OVA group than in the vehicle group ($P < 0.05$ for CD80, $P < 0.01$ for CD86). In addition, the numbers tended to be greater in the DEP + OVA group than in the DEP or OVA group (NS). Furthermore, challenge with DEP + OVA also increased numbers of MHC class II⁺ CD80⁺ and MHC class II⁺ CD86⁺ cells as compared to vehicle challenge ($P < 0.05$ for CD80, $P < 0.01$ for CD86). Also, the numbers tend to be greater in the DEP + OVA group than in the DEP or OVA group (NS). The number of CD11c⁺ cells was greater in the DEP + OVA group than in the vehicle group ($P < 0.01$). The number was also greater in the DEP + OVA group than

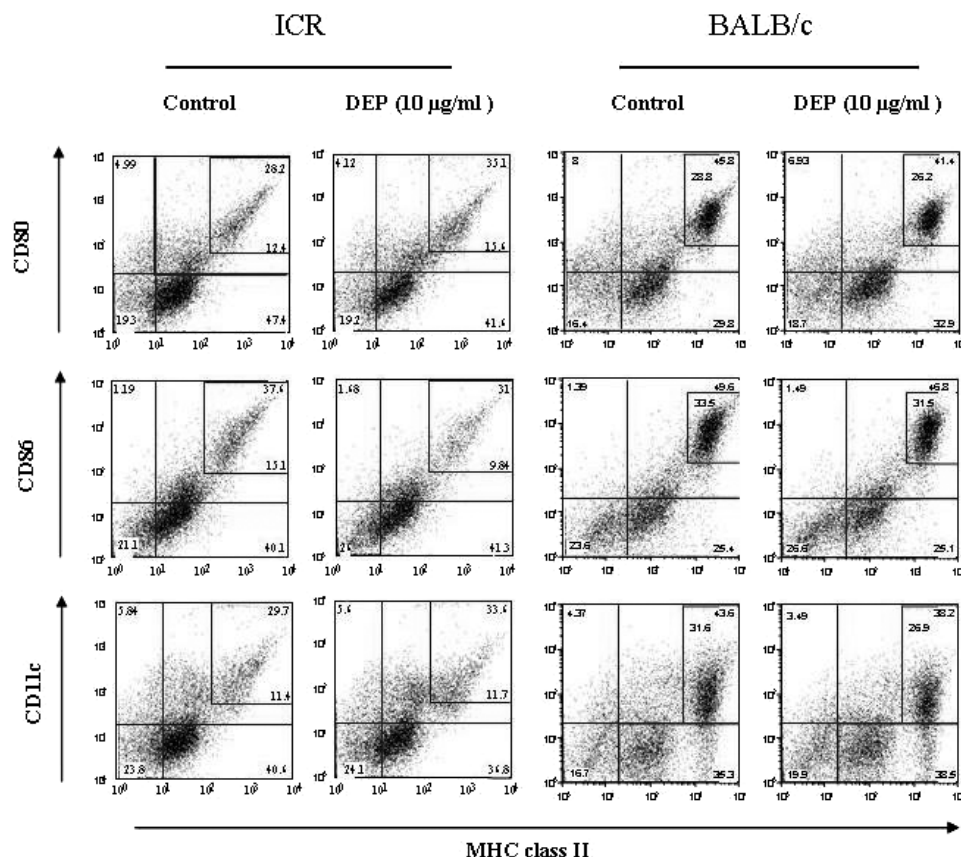


Figure 2. Effects of DEP on BMDC maturation/activation in terms of surface expressions of MHC class II and CD80, CD86, or CD11c. BM cells were derived from each (ICR, BALB/c) of the mice and cultured with medium (control) or DEP (10 µg/ml) as described in Figure 1. Thereafter, MHC class II expression and CD80, CD86, or CD11c expression on BMDC were analyzed by a flow cytometry. Percentage of MHC class II⁺ cells expressing CD80, CD86, or CD11c is shown in the upper right quadrant. The boxes denote the percentage of MHC class II^{high} cells expressing CD80^{high}, CD86^{high}, or CD11c^{high}. This experiment was repeated four times using two to three animals in each group. One representative data set is shown.

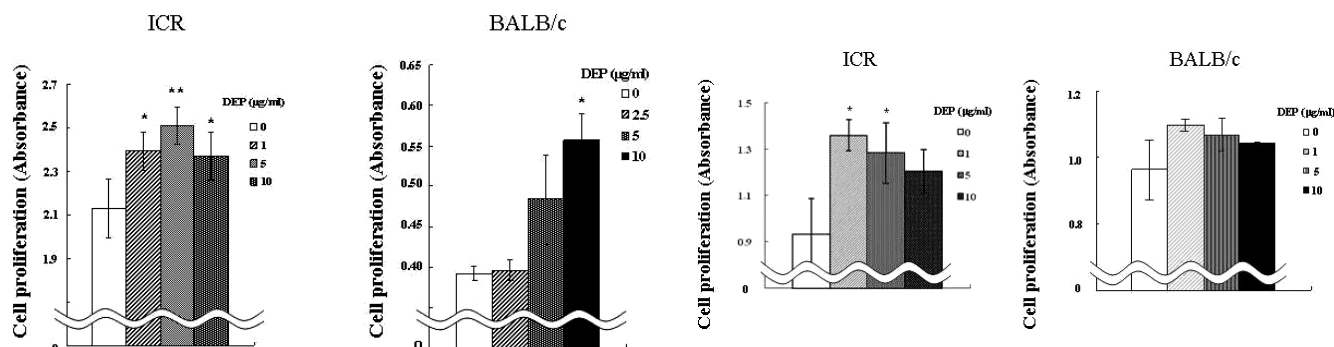


Figure 3. Allogeneic T-cell proliferation in response to DEP-exposed BMDC. At day 9 of generation for BMDC, DEP-exposed BMDC were washed with fresh medium two times. Then, allogeneic T cells (2×10^5) isolated from C57BL/6 splenocytes were stimulated with DEP-exposed BMDC (5×10^3 for ICR, 2.5×10^3 for BALB/c) as described in Methods. Thereafter, T-cell proliferation was measured as described in Methods. This experiment was repeated three times using three animals in each group. Data represent the mean $\pm$ SE ($n = 3$ different BMDC animals). * $P < 0.05$ vs. corresponding control, ** $P < 0.01$ vs. corresponding control.

Figure 4. Ovalbumin (OVA)-specific syngeneic T-cell proliferation in response to DEP-exposed BMDC. At day 9 of generation for BMDC, DEP-exposed BMDC were washed with fresh medium two times. Then, T cells (2×10^5) isolated from splenocytes of OVA-sensitized syngeneic mice were stimulated with DEP-exposed BMDC (5×10^3) in the presence of OVA (40 µg) as described in Methods. Thereafter, T-cell proliferation was measured as described in Methods. This experiment was repeated three times using three animals in each group. Data represent the mean $\pm$ SE ($n = 3$ different BMDC animals). * $P < 0.05$ vs. corresponding control, ** $P < 0.01$ vs. corresponding control.

Table 1. Effects of Diesel Exhaust Particles (DEP) on the Body Weight and the Number of Total Cells in the Lung.^a

Group	Body weight (g)	Number of total cells (1×10 ⁷ /lung tissue)
Vehicle	42.46 ± 0.53	2.028 ± 0.263
DEP	41.16 ± 0.58	2.457 ± 0.344
OVA	41.81 ± 0.82	2.591 ± 0.201
DEP + OVA	40.35 ± 1.06	3.679 ± 0.492*

^a Results are presented as mean ± SE. * $P < 0.01$ vs. vehicle.

in the DEP ($P < 0.05$) or OVA group (NS). The number of MHC class II⁺ CD11c⁺ cells was greater in the DEP + OVA group than in the vehicle group ($P < 0.05$). The number tended to be greater in the DEP + OVA group than in the DEP or OVA group (NS).

Effects of *In Vivo* Exposure to DEP on the Number of APC in the Lung. The number of cells expressing DEC205 was significantly greater in the DEP + OVA group than in the vehicle group (Fig. 6; $P < 0.01$). The number was further greater in the DEP + OVA group than in the DEP (NS) or OVA ($P < 0.05$) group. Challenge with DEP + OVA increased the number of MHC class II⁺ DEC205⁺ cells as compared to vehicle challenge ($P < 0.05$). The number of cells expressing F4/80, a maker of macrophages, was also greater in the OVA ($P < 0.05$) and DEP + OVA ($P < 0.05$) groups than in the vehicle group. The number tended to be greater in the DEP + OVA group than in the DEP group (NS). Challenge with OVA or DEP + OVA also tended to increase the number of MHC class II⁺ F4/80⁺ cells as compared to vehicle challenge in the same manner as those of F4/80, although it did not reach significance. The number of cells expressing CD19, a maker of B-cells, was significantly greater in the DEP + OVA group than in the vehicle group ($P < 0.01$). The number tended to be greater in the DEP + OVA group than in the DEP or the OVA group, without significance. Challenge with DEP + OVA increased the number of MHC class II⁺ CD19⁺ cells as compared to vehicle challenge in the same manner as those of CD19⁺ cells ($P < 0.01$).

Effects of DEP on Adjuvant Activity for Antigen-Specific Igs Production. To examine whether DEP have adjuvant activity for antigen-specific Igs production in this assay system, we measured OVA-specific IgG₁, IgG_{2a}, and IgE titers 4 hrs after the last intratracheal instillation (Fig. 7). There was a tendency for the titer of these Igs to be greater in the OVA group than in the vehicle group (NS). The combination of DEP plus OVA increased the production as compared with OVA alone ($P < 0.05$ for IgG₁ and IgG_{2a}, $P = 0.055$ for IgE).

Effects of *In Vivo* Exposure to DEP on the Splenic Th2 Response. Next, to elucidate the impacts of pulmonary exposure to DEP on the extrathoracic antigen-specific Th2 response, we compared the levels of Th2 (IL-4,

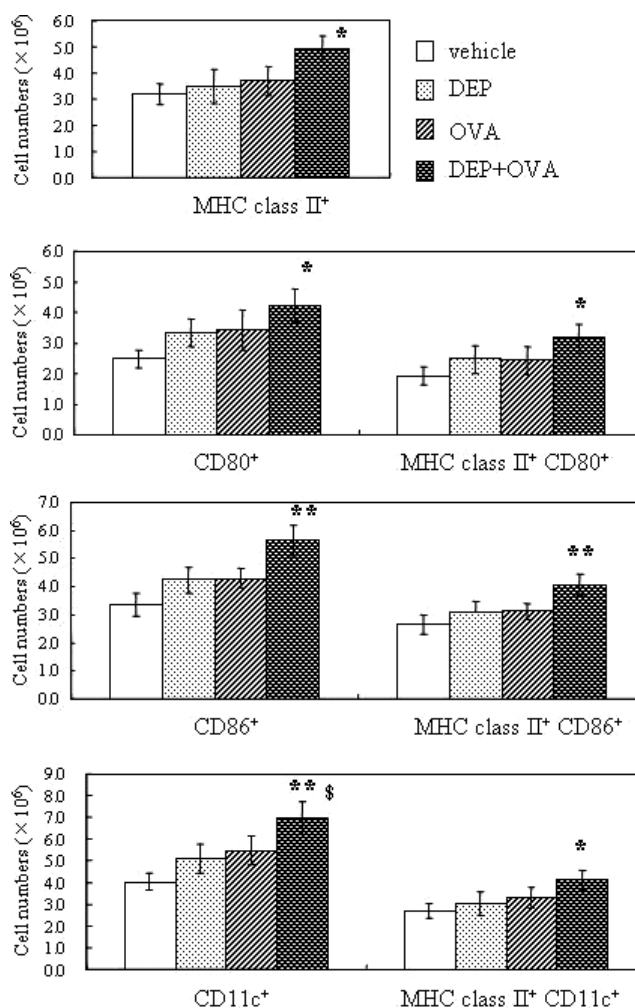


Figure 5. Effects of DEP on the number of cells expressing MHC class II and co-stimulatory molecules in the lung. Four groups of ICR mice were intratracheally administered with vehicle, DEP, ovalbumin (OVA), or DEP + OVA for 4 wks. Lung cells were prepared 5 hrs after the last intratracheal instillation. The expression of cell surface molecules was analyzed by a flow cytometry. The numbers of each cell type in the lung are shown. Values are presented as mean ± SE of 14–15 animals. * $P < 0.05$ vs. vehicle, ** $P < 0.01$ vs. vehicle, \$ $P < 0.05$ vs. DEP.

IL-5, and IL-13) and a Th1 (IFN- γ) cytokine production by splenocytes derived from each group (vehicle, DEP, OVA, or DEP + OVA) after *in vitro* OVA stimulation (Fig. 8). All levels of Th2-associated cytokines produced by splenocytes from the DEP + OVA group were higher than those from the vehicle ($P < 0.01$), DEP ($P < 0.05$ for IL-5, $P < 0.01$ for IL-13 and IL-4), or OVA ($P < 0.05$ for IL-4) group after *in vitro* stimulation with OVA. OVA-stimulated IFN- γ production was not significantly different among cells from the experimental groups.

Discussion

In previous studies from us and others, it was reported that pulmonary exposure to DEP aggravates airway inflammation (10, 12, 22) and hyperresponsiveness (9, 11,

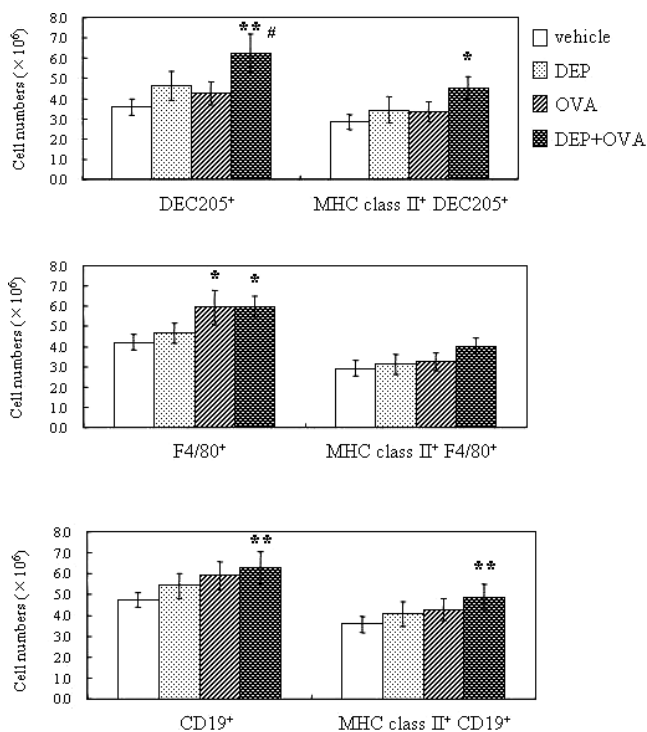


Figure 6. Effects of DEP on the number of antigen-presenting cells in the lung. Four groups of ICR mice were intratracheally administered with vehicle, DEP, OVA, or DEP + OVA for 4 wks. Lung cells were prepared 4 hrs after the last intratracheal instillation. The expression of cell surface molecules was analyzed by a flow cytometry. The numbers of each cell type in the lung were shown. Values are presented as mean $\pm$ SE of 14–15 animals. * $P < 0.05$ vs. vehicle, ** $P < 0.01$ vs. vehicle, # $P < 0.05$ vs. OVA.

13) in rodent asthma models. The exacerbated antigen-related airway inflammation is concomitant with amplified lung expression of Th cytokines (10, 12, 22). The allergic responses involve a series of distinct events mediated by a number of different cell types and cellular interactions, yet the influences of DEP on those are not fully understood. Our current study extends the previous findings to those that DEP exposure facilitates: 1) the maturation/activation/function of APC including DC, and 2) an extrathoracic antigen-specific Th response.

APC including DC, macrophages, and B cells play essential roles in the pathogenesis of asthma (16, 17). In particular, DC are recognized to be professional APC, exhibiting a potent antigen-presenting ability (31). Therefore, we evaluated the effects of DEP exposure on the phenotype and function of DC *in vitro*. Regarding the effects of DEP on cultured DC, Bleck and colleagues showed that DEP did not affect the human monocyte-derived DC phenotype and subsequent T-cell activation (32). Likewise, Verstraelen *et al.* demonstrated that DEP did not alter the pattern of the surface expression of HLA-DR on human monocyte-derived DC in the presence or absence of a DC stimulator (lipopolysaccharide) (33). Our present *in vitro* study, in which DEP did not significantly enhance the expression of surface markers (DEC205, CD80, and CD86)

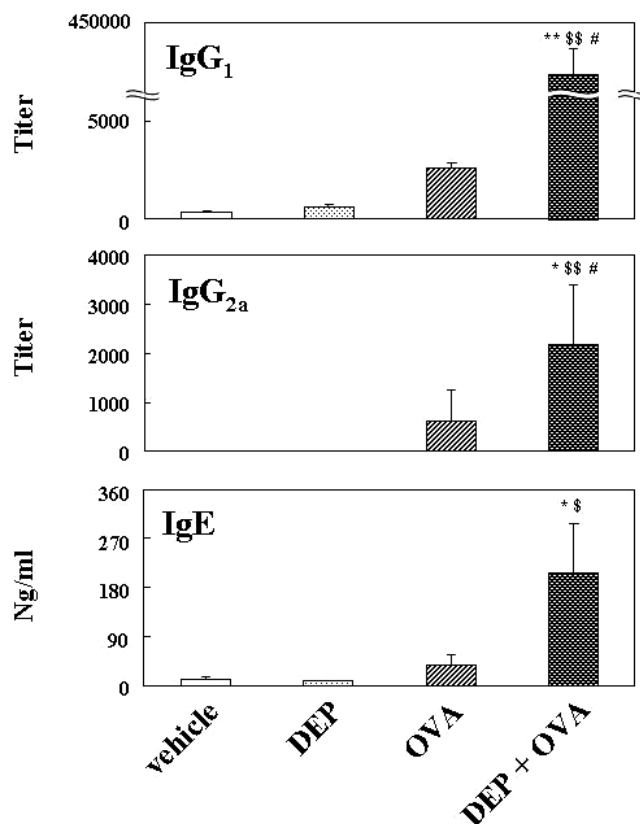


Figure 7. Effects of DEP on antigen-specific Ig productions. Four groups of ICR mice were intratracheally administered with vehicle, DEP, OVA, or a combination of DEP and OVA for 4 wks. Serum samples were retrieved 4 hrs after the last intratracheal instillation. Antigen-specific both IgG₁, IgG_{2a} and IgE values were analyzed using ELISA. Results are expressed as mean $\pm$ SE ($n = 3-4$ in each group). * $P < 0.05$ vs. vehicle, ** $P < 0.01$ vs. vehicle, \$ $P < 0.05$ vs. DEP, \$\$ $P < 0.01$ vs. DEP, # $P < 0.05$ vs. OVA.

as an activation maker and accessory molecules on BMDC, was similar to these previous reports. On the other hand, however, DEP exposure amplified both allogeneic and antigen-specific syngeneic T-cell reactivity against BMDC from both strains of mice. The variety of effects of DEP on BMDC phenotype and function might be due to the protocol of maturation and/or timing of co-culture. Moreover, the differences between the previous reports (32, 33) and our present results, and ununified effects of DEP might be explained by variations in the cell source (species and strain, peripheral monocyte-derived or bone marrow mononuclear cell-derived), protocol for DC culture (stimulators, duration, end point, perhaps co-culture with other cell types, e.g., epithelial cells, B cells), character of DEP (engine type, methodology for collection, nature, components, activity including oxidative stress), and/or DEP exposure method (solution, homogeneity, beginning point and duration). In any case, the present *in vitro* results suggest that DEP can considerably modify the functional activation of BMDC.

The above-mentioned *in vitro* results, however, were not directly linked to the aggravating effects of DEP on allergic asthma demonstrated by previous studies (12, 13,

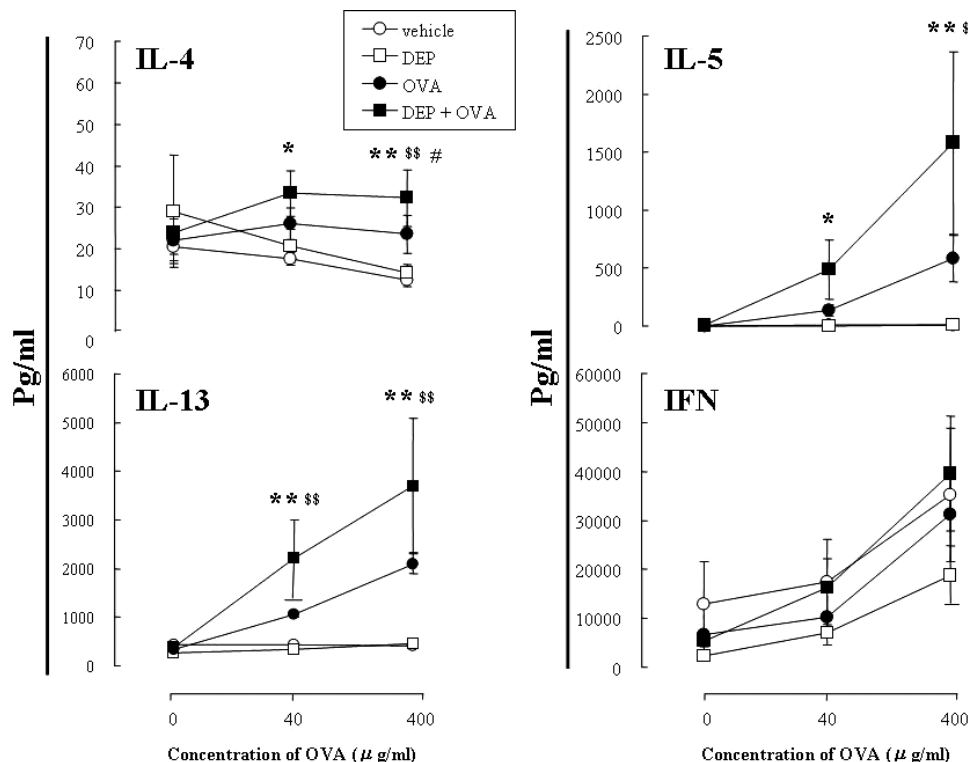


Figure 8. Effects of *in vivo* exposure to DEP in the presence or absence of OVA on production of interleukin (IL)-4, IL-5, IL-13, and interferon (IFN)- γ by splenic mononuclear cells after *in vitro* stimulation with OVA or medium alone. Four groups of ICR mice were intratracheally administered vehicle, DEP, OVA, or a combination of DEP + OVA for 4 wks. Spleen was removed 24 hrs after the last intratracheal instillation. Cytokine production by splenocytes was determined by ELISA ($n = 6$ for IL-4, $n = 14-15$ for IL-5, IL-13, and IFN- γ in each group). Results are presented as mean $\pm$ SE. * $P < 0.05$ vs. vehicle, ** $P < 0.01$ vs. vehicle, \$ $P < 0.05$ vs. DEP, \$\$ $P < 0.01$ vs. DEP, # $P < 0.05$ vs. OVA.

22), since the effects were not always on APC (DC) present in the lung microenvironment but on BM-generated DC. Additionally, there have been no published studies elucidating the effects of pulmonary exposure to ambient PM on pulmonary APC in the presence or absence of antigen *in vivo*. We, therefore, next sought to examine the effects of pulmonary exposure to DEP on the expression of APC-related molecules in the lung *in vivo*. In the present study, the numbers of cells expressing MHC class II and/or co-stimulatory molecules (CD80 and CD86) in the lung were greater in the DEP + OVA group than in the other groups. Furthermore, the numbers of DC, macrophages, and B cells bearing MHC class II in the lung were also increased by the administration of DEP to antigen-sensitized mice. It can be proposed that the enhanced expression of these APC-related molecules and increased pulmonary numbers of APC in the presence of antigen induced by DEP can, at least in part, play a role in the aggravating effects of DEP on the allergic response in the airway as reported previously (12, 13). Otherwise, the current data may lead to the scenario that DEP can synergistically increase the number of APC recruited into the lung in asthmatic mice, and in turn, can amplify functional activation of DC, which confer synergism for the enhancement of asthma pathophysiology. Furthermore, we suggest that exposure to PM, including DEP, is able to enhance APC migration/activation in the

airway, especially in the presence of antigen *in vivo*. Similarly, taking the current *in vitro* and *in vivo* results into consideration, it is likely that B cells and macrophages as well as DC play important roles as APC in proallergic responses in the airways.

We previously reported the enhanced lung expression of GM-CSF, IL-2, and macrophage inflammatory protein (MIP)-1 α by pulmonary exposure to DEP plus OVA as compared to OVA alone using a similar protocol as in the present study (12, 22). IL-2 and MIP-1 α play roles as chemotactic factors in the recruitment of DC into the lung, and GM-CSF can promote DC activation. Therefore, the enhanced expression of MHC class II and co-stimulatory molecules elicited by DEP + OVA may be explained, at least in part, by the enhanced expressions of these chemotactic factors and consequent strengthened migration of these APC into the lung. On the other hand, CXCR3 is reportedly up-regulated on activated B cells and its ligands (CXCL9, CXCL10, CXCL11) are implied as chemotactic factors for these cells (34). Thus, analyses of these interactions warrant providing evidence for the enhanced lung expression of MHC class II and CD19 in the DEP + OVA group.

The increase in activated B cells reportedly promotes Ig class switch to the IgE type (35). Furthermore, the CD21/CD19 coreceptor on B cells and its signaling can regulate B-

cell survival via activation of B-cell receptor signals (36). On the other hand, CD86 is implicated in the development of airway inflammation and airway hyperresponsiveness related to antigen (37). A recent study has demonstrated that inhaled CD86 antisense oligonucleotide suppresses allergic airway inflammation and hyperresponsiveness (38). In the current study, a comparatively striking increase in the numbers of (MHC class II⁺) CD86⁺ and (MHC class II⁺) CD19⁺ was found in the lungs of the DEP + OVA group. Therefore, the increase of these cell phenotypes could contribute to the enhancing effects of DEP on allergic manifestation including dysregulated humoral immunity. As well, adjuvant effects of DEP on antigen-specific IgS production found in previous studies (9, 12, 39, 40) and the current result (Fig. 7) could support this concept, although it cannot be denied that DEP directly influence B cell activation and/or differentiation toward plasma cells.

Increased activity of APC triggers an enhanced T-cell response via the immunological synapse. Compatible with this, our *in vitro* study showed that DEP enhanced the response/pathway as demonstrated by the allogeneic MLR and in particular, antigen-specific syngeneic lymphocyte reaction. Also, we previously demonstrated that pulmonary exposure to DEP and their components amplified lung expressions of Th cytokines *in vivo* (12, 13). However, these studies failed to address whether *in vivo* DEP exposure alters T-cell functions. Furthermore, there has been no investigation examining T-cell responses on *in vivo* antigen-primed lymphocytes with or without DEP. In the present study, when splenic mononuclear cells from the DEP + OVA group were stimulated with OVA, we found that they produced a greater amount of Th cytokines than those from the other groups. Accordingly, the *ex vivo* results suggest that the enhancing effects of DEP on allergic asthma can be explained, at least in part, by skewing T cells toward a Th phenotype, especially during antigen priming. Furthermore, our *in vitro* and *in vivo* studies suggest that this Th-skewed susceptibility found in DEP + OVA-primed lymphocytes could be mediated, at least in part, through amplified APC (DC) activity. Alternately, it is also possible that DEP directly influence (T) lymphocyte differentiation independent of immunological synapse including APC activation, since pyrene, a DEP component, reportedly induces IL-4 production by naïve T cells through activation of its promoter (23). This issue should be further clarified and has being examined using murine naïve T cells and a human T cell line in our laboratory. In addition, as a hint for possible contributors to the effects, the enhanced extra-thoracic Th response brought about by DEP might be predominantly induced by chemical components in DEP dissolved into the circulation rather than residual carbonaceous nuclei, since we found that *in vivo* carbon nanoparticles + OVA primed splenic mononuclear cells did not increase the response as compared with OVA-only primed ones using a similar protocol as employed in the present model (unpublished observations). As for Th1/Th2 polar-

ized activation by DEP, on the other hand, we and others showed that DEP (12, 41) and their components (22, 42) possess complicated Th1/Th2 regulating properties (for example, carbonaceous particles and a quinone in DEP can promote Th1, in turn, chemical components extracted with benzene-ethanol do Th2). In accordance with these previous findings, in the present study, the combined *in vivo* exposure to DEP + OVA profoundly increased the production of both Th1 (IgG_{2a}) and Th2 (IgG1) associated IgS as compared with that to OVA alone. Thus, it may be proposed that DEP induce both Th1 and Th2-mediated immunopathology with or without APC activation.

In conclusion, the present study using *in vitro*, *in vivo*, and *ex vivo* assay systems has shown evidence that DEP exposure facilitates APC activity including DC and extrathoracic antigen-specific Th responses. These results shed light on the mechanisms regarding the adverse effects of DEP on allergic diseases, in particular allergic asthma.

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