*Immunopharmacology and Immunotoxicology*, 2009; 31(1): 71–74

informa
healthcare

**RESEARCH ARTICLE**

# Effects of pulmonary exposure to diesel exhaust particles on extrathoracic CD4 polarization in asthmatic mice

Ken-ichiro Inoue, Eiko Koike, Rie Yanagisawa, and Hirohisa Takano

*Environmental Health Sciences Division, National Institute for Environmental Studies, Tsukuba, Ibaraki, Japan*

**Abstract**

Although diesel exhaust particles (DEP) exacerbate murine allergic asthma, the mechanisms remain unclarified. This study elucidated the effects of pulmonary exposure to DEP on extrathoracic allergen-induced CD4 polarization using an *ex vivo* system in asthmatic mice. Mice were divided into 4 groups, and vehicle, DEP, ovalbumin (OVA), or DEP + OVA was intratracheally instilled biweekly 5 times. Twenty-four h after the last intratracheal instillation, the spleen was removed. Mononuclear cells were isolated from the spleen and stimulated *in vitro* with OVA; thereafter, the number of separated $CD4^+$ cells was counted. The number of separated $CD4^+$ cells was significantly greater in the OVA or the DEP + OVA group than in the vehicle group. Furthermore, the number was significantly greater in the DEP + OVA than in the DEP or the OVA group. Taken together, *in vivo* DEP exposure amplified extrathoracic CD4 polarization in asthmatic subjects.

**Keywords:** *Diesel exhaust particles; allergic asthma; mechanism; CD4; ex vivo*

Immunopharmacology and Immunotoxicology Downloaded from informahealthcare.com by Instituto Superiore di Sanita on 05/31/10
For personal use only.

## Introduction

Some epidemiological data have implied that particulate matter (PM) is a risk factor for the initiation/development of allergic diseases, including bronchial asthma, allergic rhinitis, and atopic dermatitis.[1-3] To support this, we have experimentally demonstrated that pulmonary exposure to diesel exhaust particles (DEP), representative constituents of PM with mass median aerodynamic diameter ≤ 2.5μm ($PM_{2.5}$), exacerbates murine asthma[4,5]. In these studies,[4] we found that DEP amplified the lung expression of Th2 cytokines (interleukin [IL]-4, IL-5) in the presence of allergen, implicating the pathological activation of intrathoracic Th2 response as the key events of the exacerbation. Furthermore, this was recently supported by our latest studies in which *in vivo* DEP exposure augmented allergen-specific intrathoracic[6] and extrathoracic (Inoue et al., in submission) Th2 response using *ex vivo* systems. However, the mechanisms of how *in vivo* DEP exposure facilitates asthma pathobiology with an enhanced Th2-skewed response have not been fully resolved.

It is reported that an increased number of T lymphocytes is found in the airways of patients with asthma[7] and that the majority of T lymphocytes bear CD4-receptors.[8,9] CD4-expressing T lymphocytes differentiate into Th2 cells, essential effector cells for allergic inflammation, dependent on allergen stimulation. Reportedly, $CD4^+$ cells are also functionally important for the deterioration of systemic allergic reactions *in vivo*[10]; therefore, the pathogenesis of allergic diseases, including asthma, is thought to involve both a local and systemic increase in the number and dysregulated activation of $CD4^+$ T lymphocytes.

In this study, we sought to elucidate the impact of *in vivo* DEP exposure on the extrathoracic T lymphocyte milieu in view of $CD4^+$ polarization using an *ex vivo* system in a murine allergic asthma model.

*Address for Correspondence*: Ken-ichiro Inoue, M.D., Ph.D., Environmental Health Sciences Division, National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba 305–8506, Japan; E-mail: inoue.kenichirou@nies.go.jp

*(Accepted 21 July 2008)*

ISSN 0892-3973 print/ISSN 1532-2513 online © 2009 Informa UK Ltd
DOI: 10.1080/08923970802355835



RIGHTSLINK

Immunopharmacology and Immunotoxicology Downloaded from informahealthcare.com by Instituto Superiore di Sanita on 05/31/10
For personal use only.

## Materials and methods

### *Animals*

Male ICR, 6 wk of age and weighing 28 to 33 g, were purchased (Japan Clea Co., Tokyo, Japan), fed a commercial diet (Japan Clea Co.), and given water ad libitum. Mice were housed in an animal facility that was maintained at 24 to 26 °C with 55 to 75% humidity and a 12-h 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.

### *Collection of DEP*

A 4JB1-type, light-duty, four-cylinder, 2.74-liter, Isuzu diesel engine (Isuzu Automobile Company, Tokyo, Japan) under computer control was connected to a dynamometer (Meiden-sha, Tokyo, Japan). The engine was operated on standard diesel fuel at 1500rpm under a load of 10 torques (kg/m). DEP were collected as previously described.[11] The mass median aerodynamic diameter of DEP was 0.4μm by analysis with an Anderson Air Sampler (Shibata Science Technology, Tokyo, Japan).

### *Protocol*

Male ICR mice were divided into four experimental groups (n = 4–5 in each group). The vehicle group received phosphate-buffered saline (PBS) at pH 7.4 (Invitrogen Co., Carlsbad, CA, USA) containing containing 0.025% Tween 80 (Nacalai Tesque, Kyoto, Japan) and 0.25% dimethyl sulufoxide (DMSO: Nacalai Tesque; total: 5 times). The OVA group received 2μg of OVA dissolved in the same vehicle biweekly for 8 wk (total: 5 times). The DEP group received 100μg of DEP suspended in the same vehicle biweekly for 8 wk (total: 5 times). This DEP dosage (100μg/body) was confirmed to significantly facilitate this type of allergic asthma when intratracheally administered several times in our previous studies; in contrast, 50μg was not sufficient[ref 4,5, and unpublished observation]. The DEP + OVA group received the combined treatment using the same protocol as the OVA and DEP groups, respectively. The DEP suspension was sonicated for 3 min using an Ultrasonic Disrupter (UD-201; Tomy Seiko, Tokyo, Japan). 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) as previously conducted.[4,5,12] Twenty-four h after the last intratracheal administration, mice were sacrificed and the spleen was removed for an *ex vivo* experiment.

### *Splenocyte culture and isolation of CD4⁺ cells*

The collected spleen was pushed through a sterile stainless wire mesh (200 mesh) and red blood cells were lysed with ammonium chloride. Thereafter, isolated mononuclear cells were resuspended in R10, which was RPMI 1640 medium (GIBCO BRL, Eggenstein, Germany) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Dainippon Sumitomo Pharma Co., Ltd, Osaka, Japan), 100 U/ml penicillin, 100μg/ml streptomycin (Sigma, St. Louis, MO, USA), and 50μM 2-mercaptoethanol (GIBCO BRL), at a concentration of $1 \times 10^8$ cells/ml, and cultured in medium or allergen (OVA: 200μg/ml). After 72 h incubation, the cells were washed three times with R10 medium. $CD4^+$ cells were separated from the incubated mononuclear cells ($5 \times 10^7$ cells) by magnetic separation using a MACS Separator (Miltenyi Biotec Inc., Auburn, CA, USA) and $CD4^+$ T cell Isolation Kit (Miltenyi Biotec Inc.). The number of $CD4^+$ cells separated by mononuclear cells from each experimental group was counted.

### *Statistical analysis*

Data are reported as the mean ± SE. Differences were determined using analysis of variance (Statview version 4.0; Abacus Concepts, Inc., Berkeley, CA, USA). 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

The numbers of mononuclear cells isolated from the spleen and those after stimulation with or without OVA for 72 h were comparable among the experimental groups (data not shown). On the other hand, the number of cells expressing CD4, which were separated from mononuclear cells following *in vitro* stimulation with OVA, was significantly greater in the OVA or the DEP + OVA group than in the vehicle group (Fig. 1: $P < 0.01$). The number was even greater in the DEP + OVA group than in the DEP or the OVA group ($P < 0.01$).

## Discussion

In our previous studies, we have reported that pulmonary exposure to DEP aggravates airway inflammation[4,12] and hyperresponsiveness[5] induced by repetitive intratracheal instillation with allergen, likely through amplified lung expression of Th2 cytokines.[4,12] The allergic responses involve a series of distinct events mediated by a number of different cell types and cellular interactions,

RIGHTSLINK

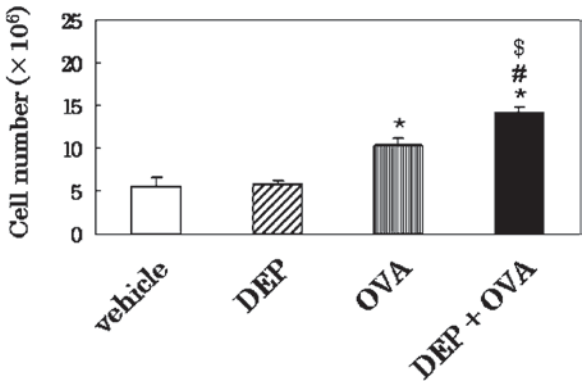


**Figure 1.** Effects of *in vivo* exposure to diesel exhaust particles (DEP) in the presence or absence of ovalbumin (OVA) on the number of $CD4^+$ cells separated from splenic mononuclear cells after *in vitro* stimulation with OVA. Four groups of mice were intratracheally administered vehicle, DEP, OVA, or a combination of DEP plus OVA biweekly, 5 times for 8 wks. The spleen was removed 24 h after the last intratracheal instillation. Mononuclear cells were isolated from the spleen and stimulated *in vitro* with OVA; thereafter, separated $CD4^+$ cells were counted. Results are presented as the mean ± SE (n = 4–5 in eac/LE> group). *$P < 0.01$ vs. vehicle, #$P < 0.05$ vs. OVA, $$P < 0.01$ vs. DEP.

yet the influences of DEP on those events are not fully understood *in vivo*. Recently, we have demonstrated that *in vivo* repetitive pulmonary exposure to DEP, if combined with allergen, enhances *ex vivo* allergen-specific Th2 response in the lung compared to that to allergen alone.[6,in press] Our current study extends the previous findings to that *in vivo* pulmonary exposure to DEP facilitates extrathoracic allergen-specific CD4 polarization in asthmatic subjects. In relation to the present finding, furthermore, we have recently shown that *in vivo* pulmonary exposure to DEP plus OVA augmented activation of intrathoracic antigen-presenting cells and extrathoracic Th2 response as compared to that to OVA or DEP in a similar protocol (Inoue et al., manuscript in submission).

CD4 positive T lymphocytes play an important role in the initiation/development/deterioration of allergic diseases, including asthma. Fujimaki et al. have previously shown that pharmacological blockade of $CD4^+$ cells abrogates the adjuvant effects of DEP on allergen-specific Ig production,[10] implying a pivotal role of these cell populations in facilitating effects of DEP on Th2 immunity; however, strictly speaking, their model is not an asthma model, since the animals were sensitized and boosted with allergen intraperitoneally in conjugation with or without DEP.

Our model is more relevant to human asthma since humans are primed and sensitized with allergen, not through the intraperitoneal route, but intratracheally, and in the absence of an adjuvant (also in the present study, we observed apparent allergic airway inflammation in the OVA or the DEP + OVA group [data not shown]). Accordingly, this is the first demonstration that pulmonary exposure to DEP plus allergen induces a significant extrathoracic (systemic) CD4-prone milieu as compared to that to allergen alone in an asthma model. In other words, the enhancing effects of DEP on asthma pathophysiology found previously[4,5,12] can be explained, at least in part, by the extrathoracic CD4 polarization of T lymphocytes; however, the mechanisms by which DEP promote CD4 polarization remain unobscure. In addition, whether the effect was resulted from a direct action of DEP on T lymphocytes or an antigen-presenting cell-mediated reaction should be addressed in the future.

Another important issue to be clarified is whether the (OVA-specific) $CD4^+$ T lymphocytes *ex vivo* generated from the DEP + OVA group serve to augment/exacerbate allergic reactions/pathobiology more prominent than those from the OVA group. Thus, separated $CD4^+$ cells from each group were adoptively transferred into naïve mice followed by OVA administration and the data, including bronchoalveolar lavage (BAL) cellularity and lung histology are being analyzed [for the present, we have observed that polymorphonuclear cell numbers recovered into the BAL fluid was greater in the mice transferred with $CD4^+$ cells from DEP + OVA-exposed mice and challenged with OVA than in the mice transferred with the cells from OVA-exposed mice and challenged with OVA: unpublished data]. We will report this functional facilitation by DEP on allergen-specific $CD4^+$ T lymphocytes if significant results are obtained.

In summary, *in vivo* pulmonary exposure to DEP amplified extrathoracic allergen-specific CD4 polarization in asthmatic subjects. The result suggests the mechanistic pathways aggravating impact of DEP on allergic diseases, and anticipates future molecular research into PM-induced immunotoxicity.

## Acknowledgment

***Declaration of interest*:** The authors report no conflicts of interest.

## References

1. Ring, J.; Eberlein-Koenig, B.; Behrendt, H. Environmental pollution and allergy. Ann Allergy Asthma Immunol. 2001; 87:2–6.
2. Lee, Y.L.; Shaw, C.K.; Su, H.J.; et al. Climate, traffic-related air pollutants and allergic rhinitis prevalence in middle-school children in Taiwan. Eur. Respir. J. 2003; 21:964–70.
3. Heinrich, J.; Wichmann, H.E. Traffic related pollutants in Europe and their effect on allergic disease. Curr. Opin. Allergy Clin. Immunol. 2004; 4 (3):41–8.
4. Takano, H.; Yoshikawa, T.; Ichinose, T.; Miyabara, Y.; Imaoka, K.; Sagai, M. Diesel exhaust particles enhance antigen-induced airway inflammation and local cytokine expression in mice. Am. J. Respir. Crit. Care Med. 1997; 156:36–42.
5. Takano, H.; Ichinose, T.; Miyabara, Y.; Yoshikawa, T.; Sagai, M. Diesel exhaust particles enhance airway responsiveness

Immunopharmacology and Immunotoxicology Downloaded from informahealthcare.com by Instituto Superiore di Sanita on 05/31/10
For personal use only.

RIGHTSLINK

following allergen exposure in mice. Immunopharmacol. Immunotoxicol. 1998; 20:329–36.
6. Inoue, K.; Koike, E.; Yanagisawa, R.; Takano, H. Impact of diesel exhaust particles on Th2 response in the lung in asthmatic mice. J. Clin. Biochem. Nutr.
7. Azzawi, M.; Johnston, P.W.; Majumdar, S.; Kay, A.B.; Jeffery, P.K. T lymphocytes and activated eosinophils in airway mucosa in fatal asthma and cystic fibrosis. Am. Rev. Respir. Dis. 1992; 145:1477–82.
8. Corrigan, C.J.; Hamid, Q.; North, J.; et al. Peripheral blood CD4 but not CD8 t-lymphocytes in patients with exacerbation of asthma transcribe and translate messenger RNA encoding cytokines which prolong eosinophil survival in the context of a Th2-type pattern: effect of glucocorticoid therapy. Am. J. Respir. Cell Mol. Biol. 1995; 12:567–78.
9. Bousquet, J.; Jeffery, P.K.; Busse, W.W.; Johnson, M.; Vignola, A.M. Asthma. From bronchoconstriction to airways inflammation and remodeling. Am. J. Respir. Crit. Care Med. 2000; 161:1720–45.
10. Fujimaki, H.; Ui, N.; Ushio, H.; Nohara, K.; Endo, T. Roles of CD4+ and CD8+ T cells in adjuvant activity of diesel exhaust particles in mice. Int. Arch .Allergy Immunol. 2001; 124:485–96.
11. Sagai, M.; Furuyama, A.; Ichinose, T. Biological effects of diesel exhaust particles (DEP). III. Pathogenesis of asthma like symptoms in mice. Free Radic. Biol. Med. 1996; 21:199–209.
12. Yanagisawa, R.; Takano, H.; Inoue, K.I.; et al. Components of diesel exhaust particles differentially affect Th1/Th2 response in a murine model of allergic airway inflammation. Clin. Exp. Allergy 2006; 36:386–95.

Immunopharmacology and Immunotoxicology Downloaded from informahealthcare.com by Instituto Superiore di Sanita on 05/31/10
For personal use only.

RIGHTSLINK