

RESEARCH ARTICLE

In vitro effects of nanoparticle-rich diesel exhaust particles on splenic mononuclear cells

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Abstract

It has been shown that pulmonary exposure to diesel exhaust particles (DEP) disrupt immune systems, presenting as exacerbating effects on allergic manifestations (i.e., allergic asthma). However, since inhalation system could not be developed, impact of nano-level DEP on health has not been satisfactorily elucidated. Our institute (National Institute for Environmental Studies) established the “environmental nanoparticle exposure system applied in animals” in 2005, and, since then, we have explored the health effects of the exposure. As part of our ongoing research, the present study was aimed to investigate the effects of nanoparticle-rich DEP (NRDEP) on the characterization of primary atopy-prone splenocytes *in vitro*. NC/Nga mouse-derived splenic mononuclear cells were co-cultured with NRDEP (0–50 µg/ml); thereafter, the surface expression of CD11c, CD80, CD86, CD69, and CD40L was evaluated by means of flow cytometry. NRDEP increased the surface expression of these molecules on the splenocytes in a dose-dependent manner with an overall trend (with significance vs. 50 µg/ml of NRDEP). These results suggest that NRDEP can activate naïve splenic mononuclear cells from atopy-prone animals.

Keywords: Nanoparticles, diesel exhaust particles, splenocyte, dendritic cell, T cell

Introduction

It has been generally recognized that diesel exhaust particles (DEP), derived from diesel engine-powered automobiles and major constituents of atmospheric particulate matter, have adverse health effects in the context of immunotoxic potential.⁽¹⁾ In particular, the adverse effects of DEP can be clearly seen in the atopy-prone milieu.⁽²⁾ Biologically, consistent with this, we and others have previously demonstrated that DEP exposure induces/aggravates airway inflammation in atopic/allergic animals.^(3–5) Likewise, DEP reportedly affect/disrupt several cell populations, which contributes to their immunotoxicity, such as epithelial cells^(6,7), endothelial cells⁽⁸⁾, macrophages⁽⁹⁾, eosinophils⁽¹⁰⁾, and mast cells/basophils^(11,12), mainly *in vitro*. Especially, peripheral lymphoid organs and their resident mononuclear cells, including lymphocytes, are key players in the immunopathogenesis of allergy; accordingly, the effects of DEP on these compartments might provide much information

on their adjuvant effects. However, it remains unclarified whether various characteristic types of DEP have a similar immunological potential.

Recently, DEP sizes are getting smaller and smaller due to advancement of the automobile industry, at least in part, resulting in the production of particles less than 100 nm in mass median aerodynamic diameter (called “nanoparticles”). The trend may increase ambient levels of nanoparticles and another health concerns.^(13–15) Nanoparticles have been suggested to adversely affect the cardiopulmonary system.^(16,17) Nanoparticles are able to penetrate deeply into the respiratory tract, and they have a larger surface area than particles with a larger size; thus, they induce a greater inflammatory response.^(18,19) In consistence, there are many reports regarding the toxic effects of “model nanoparticles” such as titanium, gold, carbon black (CB), and latex *in vitro* and *in vivo*.^(20,21) Previously, we and others showed that CB nanoparticles have facilitating effects on allergic

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features *in vivo* and *in vitro*.^(22–24) However, few studies have examined the immunological impact of existing environmental nanoparticles. In the National Institute for Environmental studies, an inhalation chamber for environmental nanoparticles generated from a diesel engine (called as “nanoparticle-rich DEP [NRDEP]”) was established in 2005. Since then, the health effects of inhaled environmental particles have been examined mainly *in vivo*. However, to comprehensively estimate/examine the effects of the nanoparticles, *in vitro* research is also required.

The aim of the present cutting edge study was to examine the *in vitro* effects of NRDEP on atopy-prone splenocytes from NC/Nga mice (naturally developing atopic dermatitis-like pathophysiology) in terms of immunotoxicity.

Materials and methods

Animals

Male Nc/Nga mice at 11–15 weeks of age and weighing 29 to 33 g (Japan Clea Co., Tokyo, Japan) were used in all experiments. They were fed a commercial diet (Japan Clea Co.) and given water *ad libitum*. Mice were housed in an animal facility 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.

NRDEP collection and preparation for *in vitro* studies

NRDEP were collected at the National Institute for Environmental Studies Japan (Tsukuba, Ibaraki, Japan: Ref 25). An 8L-diesel engine (J08C, Hino Motors, Tokyo, Japan) that was not fitted with after-treatment devices and that met 1997 emission regulations was driven under steady-state conditions (speed: 2000 rpm; engine torque: 0 Nm; diesel fuel: JIS No. 2) for 5 h. These conditions generate nanoparticle-rich exhaust (particle concentration: 3×10^7 counts/cm³; modal diameter $\pm$ S.D.: 21.45 ± 1.46 nm).⁽²⁵⁾ Particles were collected at ~10 m from the engine with a dilution ratio of 13 (100 m³/min) on dichloromethane-washed, gold discs (25 mm in diameter, 0.025 mm in thickness) electrostatically (–27 kV) at a flow rate of 20 L/min using a sampler (SSPM-100, Shimadzu, Kyoto, Japan). Sampling was repeatedly conducted seven times on the same sample and total duration of time was 35 h. Seven gold discs with NRDEP (total: 7.3476 mg) were provided and stored in a refrigerator (–80°C). NRDEP were dispersed from the discs into the vehicle (0.02% Tween80-R10: GIBCO RPMI 1640 medium [Invitrogen, Grand Island, NY, USA] supplemented with 10% heat-inactivated fetal bovine serum [FBS; MP Biomedicals Inc., Eschwege, Germany], 100 U/ml penicillin, 100 µg/ml streptomycin [Sigma Chemical, St. Louis, MO, USA], and 50 µM 2-mercaptoethanol [Invitrogen]) using an ultrasonic disrupter (UD-201, Tomy

Seiko, Tokyo, Japan) under ice-cold conditions for 3 min, as previously described.^(22,23)

Splenocyte preparation and NRDEP exposure

Spleens were removed from the naïve ICR mice and placed in a petri dish with phosphate buffered saline. The spleens were pushed through a 200 mesh stainless steel sheet, and the resulting cells were suspended in PBS. Cells were collected by centrifugation at 400g for 10 min at 20°C. Thereafter, red blood cells were removed by incubating with hypotonic lysis buffer for 3 min. Cells were washed twice with PBS and resuspended in R10. The number of viable cells was determined by the trypan blue dye exclusion method. Splenocytes (1×10^6) were cultured in 1 ml of R10 containing NRDEP (1, 5, 10, or 50 µg/ml) or control (0.01% Tween80-R10) in 12-well plates at 37°C in a 5% CO₂ / 95% air atmosphere. After 24-h incubation, cells were analyzed for fluorescence-activated cell-sorting (FACS).

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, USA), CD11c (HL3, PE-conjugated, BD Biosciences Pharmingen), CD80 (16-10A1, PE-conjugated, BD Biosciences Pharmingen), CD86 (GL1, PE-conjugated, BD Biosciences Pharmingen), CD4 (GK1. 5, PE-conjugated, BD Biosciences Pharmingen), CD69 (H1. 2F3, BD Biosciences Pharmingen), and CD40 Ligand (L) (CD154: gp39: MR1, PE-conjugated, BD Biosciences Pharmingen). Cells ($1\text{--}3 \times 10^5$) were resuspended in 100 µl of PBS with 0.3% BSA and 0.05% sodium azide (Wako Pure Chemical Industries), and stained with antibodies at 1 µg for 30 min on ice. After incubation, the cells were washed and fluorescence was measured using a FACSCalibur (Becton, Dickinson and Company, NJ, USA). For each sample, fluorescence data from 10,000 cells were collected and positive cells were expressed as % total events.

Statistical analysis

Data are expressed as the mean $\pm$ SEM of three or four animals from one experiment, representative of three experiments. Differences among groups were analyzed by analysis of variance (ANOVA; Statview version 4.0; Abacus Concepts, Inc., Berkeley, CA). When significant differences were detected, post hoc comparisons within each group were evaluated with Fisher's LSD test. Significance was assigned to *P* values smaller than 0.05.

Results

Effects of NRDEP on surface expression of immunity-related molecules on naïve splenocytes

To determine the effects of exposure to NRDEP on the surface expression of molecules related to immunity (dendritic cell maturation and lymphocyte activation), 24 h after the culture of the naïve splenocytes, we

analyzed expression patterns of CD11c, CD80, CD86, CD69, and CD40L by FACS (Figure 1). Overall, NRDEP increased the surface expression of these molecules in a dose-dependent manner. Specifically, NRDEP at a concentration of 50 µg/ml significantly increased the expression compared to the vehicle control. On the other hand, the expression of MHC class II and CD4 was not significantly different among the experimental groups (data not shown).

Discussion

This cutting edge study demonstrates that NRDEP exposure induces several surface markers related to immunity such as CD11c, CD80, CD86 (DC maturation), CD69, and CD40L (lymphocyte activation) on mouse naïve mononuclear cells (splenocytes) from atopy-prone mice *in vitro*.

DEP have immune-disrupting effects, which likely depend on their characteristics, such as particle size, surface charge/components, aggregation state, and/or solubility. Therefore, the health impacts of various types of DEP should be examined to properly estimate their toxicity. On the other hand, increases in ambient levels and the adverse health effects of environmental nano-level particles have become a concern worldwide. For example, previous measurements indicate that nanoparticle numbers in ambient air range from 2×10^4 to $2 \times 10^5/\text{cm}^3$, with mass concentrations of more than

50 µg/m³ near major highways.^(14,15) Epidemiologically, nanoparticles have been implicated in cardiopulmonary system effects.^(16,17) Compared to larger particles, nanoparticles have a higher deposition rate in the peripheral lung, they can cross the pulmonary epithelium and reach the interstitium⁽²⁶⁾, and may thus be distributed/affected systemically⁽²⁷⁾. Indeed, we and others experimentally showed that CB nanoparticles induce/augment lung inflammation as “model” nanoparticles, which is more prominent with smaller particles based on the overall trend.^(22,23,28,29) However, since there was no existing inhalation system, the health effects of exposure to environmentally relevant types of nanoparticles have not been sufficiently examined.

On the other hand, the immunotoxic effects of DEP varied from immunosuppression to immunoactivation.^(3,4,30) Among them, we focused on their potential to enhance allergic reactions/pathophysiology. Indeed, DEP and their components can directly activate B cells to enhance IgE production^(31,32), chemical constituents of DEP induce IL-4 production/release by human-derived basophils⁽¹²⁾, and diesel exhaust (DE) exposure decreases IL-12 (Th1 cytokine) production from alveolar macrophages or a macrophage cell line (RAW264.7 cell) *in vitro*⁽³³⁾ and that in lung homogenates *in vivo*⁽³⁾. Furthermore, it has been reported that DEP suppress T-bet expression and interferon-γ production by isolated T cells from healthy humans.⁽³⁴⁾ As well, we have recently demonstrated that

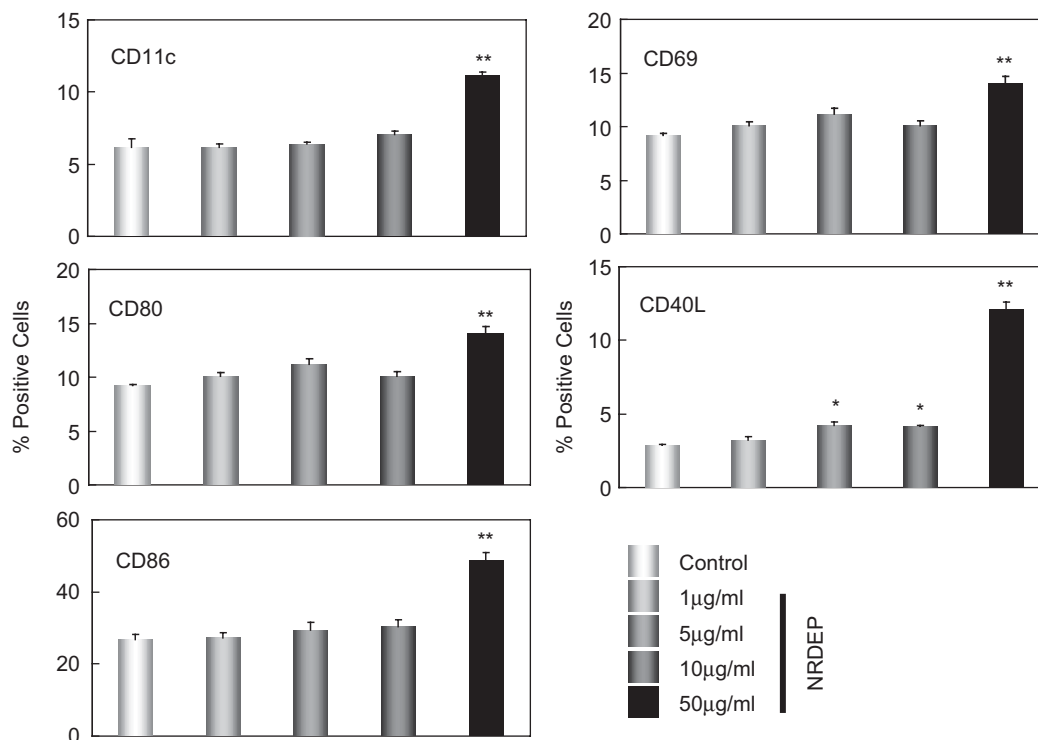


Figure 1. Effects of nanoparticle-rich diesel exhaust particles (NRDEP) on the surface expression of CD11c, CD80, CD86, CD69, or CD40L on splenocytes from atopic animals. Splenocytes ($1 \times 10^6/\text{ml}$) derived from NC/Nga mice were exposed to various concentrations of NRDEP (1–50 µg/ml) for 24 h. Thereafter, the expression of CD11c, CD80, CD86, CD69, or CD40L on the cells was analyzed by flow cytometry. Data are the mean $\pm$ SEM of four individual cultures from four animals, representative of three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. control.

DEP generated from another diesel engine (average size of particles: 400 nm) and their components differentially increase surface expression of IL-4R, CD69, and CD40L on mouse splenic mononuclear cells with amplified IL-4 production/release.⁽³⁵⁾ Taken together, DEP generally promote Th2-skewed responses both *in vitro* and *in vivo*.^(30,34) Nevertheless, whether nano-level DEP have a similar immunological potential remains unclarified. In the present study, we first demonstrated that NRDEP generated by an inhalation exposure system can potentiate immune cell activation in splenic mononuclear cells from atopy-prone mice (with a trend to suppress cell viability [determined by WST-1]: data not shown), suggesting their proallergic potential, in particular, in sensitive subjects.

Antigen-presenting cells (APC) are recognized to be important for both innate and adaptive types of immunity. The APC-mediated uptake of foreign antigens and subsequent presentation for T cells is the first step in immune responses. The maturation of DC, professional APC, is an important step for polarized antigen presentation and the consequent Th response.⁽³⁶⁾ On the other hand, the generation of oxidative stress at the APC level reportedly yields Th2-skewing.⁽³⁷⁾ We previously showed that DEP and CB nanoparticles activate bone marrow-generated DC *in vitro*⁽³⁸⁾ and pulmonary APC in asthmatic mice *in vivo*⁽³⁹⁾. Furthermore, DEP chemicals induce oxidative stress in DC with the suppression of Th1 cytokine release *in vitro*,⁽⁴⁰⁾ suggesting that particulate matters (PM) modify APC characterization/function favoring a Th2 milieu. CD69 is an early activation marker of hematopoietic cells including lymphocytes. Further, CD40–CD40L (CD154) interaction is crucial in the regulation of cross talk between both DC-T and DC-B cells.⁽⁴¹⁾ In the present study, exposure to NRDEP at a concentration of 50 µg/ml markedly up-regulated CD69 and CD40L on splenocytes. Therefore, it is attractive to speculate that DC, T cells, and/or their interaction, including immunological synapse, might be target cells/networks for NRDEP.

In turn, it should be important to elucidate the effects of inhaled exposure to nanoparticles on the immunopathophysiology, in particular, in the respiratory system. We previously examined the effects of inhaled DE (derived from another diesel engine: average particle size: 400 nm) at a soot concentration of 0.3, 1.0, or 3.0 mg/m³ on an allergic asthma model *in vivo* and demonstrated that this type of DE exacerbated lung inflammation related to allergen with an overall trend.⁽⁴²⁾ On the other hand, the inhaled exposure did not exacerbate but rather ameliorated acute lung inflammation induced by endotoxin exposure⁽⁴³⁾; thus, the facilitating effects of PM may depend on the inflammation type. Indeed, using this *in vivo* exposure system, we demonstrated that NRDE exacerbates acute lung inflammation elicited by endotoxin.⁽⁴⁴⁾ Currently, we are analyzing the effects of inhaled NRDE on an allergic asthma model in mice.

The Nc/Nga mouse has been established as an animal model for human atopic dermatitis.^(45,46) This strain

of mouse spontaneously develops dermatitis associated with IgE overproduction under conventional conditions; thus, their compartments as for immunity may be applicable for testing immunodisrupting potentials. In fact, we previously demonstrated that immune cells derived from Nc/Nga mice are sensitive to pollutants which can facilitate allergic traits in terms of DC phenotypic and functional maturation.^(47,48) Combining the current and previous results, it is possible that this type of culture system using atopy-prone splenocytes becomes an alternative and useful testing item for immunotoxicity.

In summary, NRDEP significantly increased the surface expression of molecules related to DC maturation/activation such as CD11c, CD80, and CD86, and T-cell activation such as CD69 and CD40L on splenocytes. These data demonstrate the immunomodulating effects of NRDEP on immune cells in the context of APC and lymphocytes *in vitro*, and may provide an insight into the immunotoxicity of nano-level diesel engine-generated PM.

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Declaration of interest

The authors have no financial conflicts of interest.

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