

Letter

***In vitro* study of the effect of nanoparticle-rich diesel exhaust particles on IL-18 production in splenocytes**

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ABSTRACT — It has been shown that pulmonary exposure to diesel exhaust particles (DEP) disrupts the immune system, presenting as exacerbating effects on allergic manifestations (e.g., allergic asthma). However, since a model inhalation system has not been developed, the impact of nano-level DEP on health has not been satisfactorily investigated. Our institute (the National Institute for Environmental Studies) established an “environmental nanoparticle exposure system applied in animals” in 2005 and since then, we have explored the health effects of exposure to these types of agent. The present study was conducted to investigate the *in vitro* effects of nanoparticle-rich DEP (NRDEP) on primary splenocytes from atopy-prone hosts. NC/Nga mouse-derived splenic mononuclear cells were co-cultured with NRDEP (0-50 µg/ml); thereafter, the production/release of interleukin (IL)-18 in the culture supernatants was evaluated by means of ELISA. NRDEP increased IL-18 production/release by splenocytes in a dose-dependent manner with an overall trend (with significance vs. 10 µg/ml of NRDEP). In contrast, 50 µg/ml of NRDEP inhibited production/release. These results suggest that NRDEP can activate naïve splenic mononuclear cells from atopy-prone animals in terms of IL-18 induction.

Key words: Nanoparticles, Diesel exhaust particles, Splenocytes, Interleukin-18, Nc/Nga mouse

INTRODUCTION

It has generally been recognized that diesel exhaust particles (DEP), derived from diesel engine-powered automobiles and major constituents of atmospheric particulate matter, in industrialized countries, have adverse health effects in the context of their immunotoxic potential (Diaz-Sanchez *et al.*, 1994). In addition, the adverse effects of DEP can be clearly seen in the atopy-prone milieu (Peden and Reed, 2010). Consistent with this, we and others have previously demonstrated that DEP exposure induces and/or aggravates airway inflammation in atopic/allergic animals, including NC/Nga mice (which naturally develop an atopic dermatitis-like pathophysiology; Takano *et al.*, 1997; Ichinose *et al.*, 1998; Inoue *et al.*, 2005a). Furthermore, DEP reportedly affect/disrupt several cell populations, which contributes to their

immunotoxicity, such as epithelial cells (Terada *et al.*, 1997; Takizawa *et al.*, 2003), endothelial cells (Terada *et al.*, 1999), macrophages (Beck-Speier *et al.*, 2005), eosinophils (Hirota *et al.*, 2008), dendritic cells (DC: Inoue *et al.*, 2009), and mast cells/basophils (Saneyoshi *et al.*, 1997; Devouassoux *et al.*, 2002), mainly *in vitro*. Generally, peripheral lymphoid organs such as the spleen and their resident mononuclear cells including lymphocytes, are key players in the immunopathogenesis of allergy; thus, the effects of DEP on these compartments may provide much information on their adjuvant effects. However, it remains unclarified whether various characteristic types of DEP have a similar immunological potential.

In the National Institute for Environmental studies, an inhalation chamber for environmental nanoparticles generated from a diesel engine (called “nanoparticle-rich DEP (NRDEP)”) was established in 2005 to examine

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the health-related effects of another type of DEP. Since then, the effects of inhaled environmental nanoparticles have been examined mainly *in vivo*. We have recently shown that NRDEP collected from the system significantly increases the surface expression of molecules related to dendritic cell (DC) maturation/activation such as CD11c, CD80, and CD86, and T-cell activation such as CD69 and CD40L on atopy-prone splenocytes, suggesting an immunomodulating effect of these NRDEP *in vitro* (Inoue *et al.*, 2011). However, the effects of NRDEP on immune cells and their mechanisms have not been fully explored.

Interleukin (IL)-18 is a newly-discovered proinflammatory cytokine that is structurally and functionally related to the IL-1 family (Leslie and Meldrum, 2008). IL-18 was originally identified as an interferon (IFN)- γ -inducing factor, which induces IFN- γ production in T cells and natural killer cells (Leslie and Meldrum, 2008). IL-18's proinflammatory properties include the induction of other proinflammatory mediator production such as tumor necrosis factor, IL-1, chemokines (CXC and CC), and the Fas ligand (Arend *et al.*, 2008). Furthermore, enhanced IL-18 expression has been associated with a variety of immunological abnormalities such as non-obese diabetes, experimental autoimmune encephalomyelitis, lupus syndrome, rheumatoid arthritis, and atopy (Arend *et al.*, 2008; Konishi *et al.*, 2002; Nakanishi *et al.*, 2001). Nonetheless, the impacts of PM on IL-18 by immune cells have been less studied. Furthermore, the effects of NRDEP we used have never been examined.

The aim of the present cutting-edge study was to examine the *in vitro* effects of NRDEP on splenocytes from atopy-prone NC/Nga mice (i.e., using this model to focus on responses to particulate matter in a highly sensitive host), particularly in terms of IL-18 production/release.

MATERIALS AND METHODS

Animals

Male Nc/Nga mice at 11–15 weeks of age and weighing 29–33 g (Japan Clea Co., Tokyo, Japan) were used in all experiments. Mice were fed a commercial diet (Japan Clea Co.) and provided with filtered water *ad libitum*. Mice were housed in an animal facility maintained at 24–26°C with a 55–75% relative humidity and 12-hr light/dark cycle. The studies performed here 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) (Fujitani *et al.*, 2009). An 8-L diesel engine (J08C, Hino Motors, Tokyo, Japan) that was not fitted with after-treatment devices, and that met 1997 emission regulations, was powered under steady-state conditions (speed: 2,000 rpm; engine torque: 0 Nm; diesel fuel: JIS No. 2) for 5 hr. These conditions generated nanoparticle-rich exhaust (particle concentration: 3×10^7 counts/cm³; modal diameter $\pm$ S.D.: 21.45 ± 1.46 nm) (Fujitani *et al.*, 2009). Particles were electrostatically (-27 kvolts) collected at approximately 10 m from the engine (with a dilution ratio of 13; 100 m³/min) onto dichloromethane-washed gold discs (25 mm diameter, 0.025 mm thickness) at a flow rate of 20 l/min using an SSPM-100 sampler (Shimadzu, Kyoto, Japan). Sampling was repeatedly conducted seven times using the same sampler, and the total time duration was 35 hr. Seven gold discs with NRDEP (total: 7.3476 mg) were obtained and stored in a -80°C freezer. NRDEP were dispersed (under ice conditions) from the discs into the vehicle (0.02% Tween80-R10 medium (RPMI 1640 medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (MP Biomedicals Inc., Eschwege, Germany), 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ 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 (Inoue *et al.*, 2005b).

Splenocyte preparation and NRDEP exposure

Spleens were removed from naïve Nc/Nga mice and placed in a petri dish with phosphate-buffered saline (PBS, pH 7.4). The spleens were pushed through a 200-mesh stainless steel sheet mesh. The resulting cells were suspended in PBS and centrifuged at $400 \times g$ for 10 min at 20°C . Any red blood cells were removed by incubation with hypotonic (0.87% (w/v) NH_4Cl in PBS) lysis buffer for 3 min. Cells were then washed twice with PBS and resuspended in R10. The number of viable cells was determined employing the trypan blue dye exclusion method. Splenocytes (at 1×10^6) were cultured in 1 ml of R10 containing NRDEP (1, 5, 10, or 50 $\mu\text{g}/\text{ml}$) or control solution (0.01% Tween80-R10) in 12-well plates at 37°C in a 5% CO_2 /95% air atmosphere. After 24-hr incubation, the culture medium was used for the enzyme-linked immunosorbent assay (ELISA).

ELISA for IL-18

ELISA for IL-18 (Amersham, Buckinghamshire, UK) was conducted according to the manufacturer's instructions. The secondary antibodies were conjugated to horseradish peroxidase. Values generated by subtracting readings obtained at 450 nm from those at 550 nm were converted to pg/ml using values obtained from standard curves generated in parallel. The limit of detection for this kit was 5 pg IL-18/ml.

Statistical analysis

Data are expressed as the mean $\pm$ S.E.M. of three or four animals from one experiment, each representative of three experiments. Differences among groups were analyzed by ANOVA (Statview version 4.0; Abacus Concepts, Inc., Berkeley, CA, USA). When significant differences were detected, post-hoc comparisons within each group were evaluated with Bonferroni correction. Significance was assigned to p-values smaller than 0.05.

RESULTS AND DISCUSSION

To determine the effects of NRDEP on cytokine production/release by naïve splenocytes after a 24-hr exposure, IL-18 was measured in the culture supernatants collected at that time point (Fig. 1). Overall, NRDEP increased the surface expression of these molecules in a

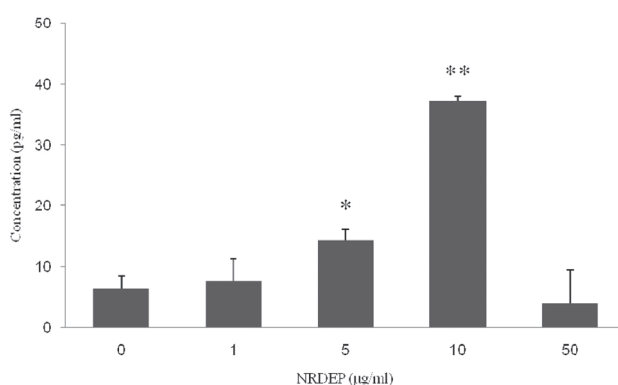


Fig. 1. Effects of nanoparticle-rich diesel exhaust particles (NRDEP) on IL-18 production by splenocytes from atopic animals. Splenocytes (1×10^6 /ml) derived from NC/Nga mice were exposed to various concentrations of NRDEP (1-50 µg/ml) for 24 hr. Thereafter, the production of IL-18 by the cells was analyzed employing ELISA. Data are the mean $\pm$ S.E.M. of four individual cultures from four animals, representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$ vs. control.

dose-dependent manner. Specifically, NRDEP at a concentration of 10 µg/ml significantly increased the expression compared to the vehicle control, whereas 50 µg/ml of NRDEP did not increase cytokine production/release compared to the vehicle control.

In the present study, we first demonstrated that nanoparticles close to those in the real world can induce IL-18 from splenic mononuclear cells derived from atopy-prone mice, suggesting their proinflammatory potential, at least in part, *in vitro*. We have recently shown that this type of DEP significantly increases 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 these cells (Inoue *et al.*, 2011), and induce IL-17A production/release at both translational and transcriptional (ROR γ t) levels (Nakamura *et al.*, in press). Taking the current and previous results into consideration, it is possible that this type of DEP has a different immunomodulating impact.

Ligands for Toll-like receptor (TLR) 4 such as lipopolysaccharides (LPS) and LPS-containing microbacteria induce IL-18 production (Nakanishi *et al.*, 2001). Moreover, TLR4 mediates the inflammatory response to DEP (Inoue *et al.*, 2006). Thus, NRDEP might induce IL-18 production via TLR4, at least *in vitro*. On the other hand, activated IL-18 can stimulate several immune cells including macrophages, NK cells, and lymphocytes (Yoshimoto *et al.*, 2003). Therefore, it can be proposed that IL-18 activated by NRDEP, in turn, stimulates these cells in the spleen to express a variety of surface molecules such as CD11c, CD80, CD86, CD69, and CD40L found in our recent study (Inoue *et al.*, 2011).

IL-18 has the capacity to stimulate innate immunity and both Th1- and Th2-mediated responses and, consequently, influence various immune disorders. In brief, in collaboration with IL-12, IL-18 stimulates Th1-mediated immune responses through the induction of interferon- γ , whereas IL-18 without help from IL-12 has the potential to induce the production of Th2 cytokines such as IL-4 and IL-13 in T cells, NK cells, mast cells, and basophils (Nakanishi *et al.*, 2001). On the other hand, IL-18 directly stimulates T and mast cells to release atopic dermatitis-associated molecules, Th2 cytokines, and histamine. This cutting-edge study suggests that NRDEP promote an atopic dermatitis-like pathophysiology in NC/Nga mice via enhanced IL-18 synthesis dependent on/independent of IgE.

It is interesting that up to 10 µg/ml of NRDEP increased the IL-18 level in a dose-dependent manner with an overall trend, whereas 50 µg/ml of NRDEP did not increase cytokine production/release by these cells. On the other

hand, NRDEP increased the surface expression of molecules related to immunity such as CD11c, CD80, CD86, CD69, and CD40L on the splenocytes in a dose-dependent manner with an overall trend (with significance vs. 50 µg/ml of NRDEP) on the same cell compartments (Inoue *et al.*, 2011). It is possible that NRDEP directly/indirectly influence cells related to the production/release/expression of these molecules, including macrophages/monocytes, epithelial cells, endothelial cells, dendritic cells, and both T and B lymphocytes at a preferential dose in each cell population. Taken together our recent study and the present study, NRDEP stimulates production of IL-18 and might promote cell activation at least partially in.

There should be a discussion about the 50 µg/ml exposure. Our recently study revealed a dose-response cytotoxicity of NRDEP (Nakamura *et al.*, in press). Thus, it can be partly explained that the marked decrease in the production/release of IL-18 induced by 50 µg/ml NRDEP compared to that by 1 or 10 µg/ml is a result of cell death. It was previously shown that relatively high dose DEP exposure lead to apoptosis in alveolar macrophage, macrophage cell line (Hiura *et al.*, 1999), and airway cell line (Ackland *et al.*, 2007), resulting to cell death. In our study, accordingly, decreased IL-18 production/release by 50 µg/ml NRDEP was likely due to significant cells' dysfunction possibly through the induction of apoptosis. In future, to validate the hypothesis, we will conduct the examination about the effect of the NRDEP exposure on the induction of apoptosis and resolve its underlying machinery.

In summary, NRDEP significantly increased IL-18 production/release by splenocytes of atopy-prone NC/Nga mice. These data highlight the possibility of another immunomodulating effect of NRDEP *in vitro*, and may provide insight into the immunotoxicity of nano-level diesel engine-generated PM.

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