

RESEARCH ARTICLE

Effects of nanoparticle-rich diesel exhaust particles on IL-17 production *in vitro*

Ryosuke Nakamura¹, Ken-ichiro Inoue¹, Yuji Fujitani², Masako Kiyono¹, Seishiro Hirano², and Hirohisa Takano³

¹Department of Public Health and Molecular Toxicology, School of Pharmacy, Kitasato University, Tokyo, ²Research Center for Environmental Risk, and ³Environmental Health Sciences Division, National Institute for Environmental Studies, Ibaraki, Japan

Abstract

It has been shown that pulmonary exposure to diesel exhaust particles (DEP) disrupt immune systems, presenting as exacerbating effects on allergic manifestations (e.g., allergic asthma). To date, the impact of nano-level DEP on health has not been fully elucidated. Our institute (the National Institute for Environmental Studies) established an 'environmental nanoparticle exposure system applied in animals' in 2005 and, since then, the health effects of exposures to these types of agents have been explored. The present study was designed 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, cell viability/proliferation was evaluated via a WST-1 assay, production/release of interleukin (IL)-17A in the culture supernatants by ELISA, and expression of ROR γ t (retinoic acid-related orphan receptor- γ t) in cell lysates by Western blot analyses. The results indicated that NRDEP reduced cell viability/proliferation in a dose-related manner—significantly so at a level of 50 µg/ml NRDEP. In contrast, up to 10 µg NRDEP/ml increased ROR γ t expression in the splenocytes and subsequent IL-17A production/release by the cells in a dose-dependent manner with an overall trend (with significance vs 1 µg NRDEP/ml and 10 µg NRDEP/ml for IL-17A); 50 µg NRDEP/ml tended to inhibit the transcription factor expression and cytokine production/release. These results suggest that NRDEP can activate naïve splenic mononuclear cells from atopy-prone animals in terms of ROR γ t and IL-17A induction (T_H17 response).

Keywords: Nanoparticle, diesel exhaust particles, IL-17, ROR γ t

Introduction

It has been generally 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, our group and other investigators have previously demonstrated that DEP exposure induces and/or aggravates airway inflammation in atopic animals, including NC/Nga mice (that 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), 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.

Address for Correspondence: Ken-ichiro Inoue, MD, PhD, Department of Public Health and Molecular Toxicology, School of Pharmacy, Kitasato University, 9-1, Shirokane 5-Chome, Minato-ku, Tokyo 108-8641, Japan. Tel: 81357916264; Fax: 81334424146; E-mail: inouek@pharm.kitasato-u.ac.jp

(Received 30 June 2011; revised 08 September 2011; accepted 03 October 2011)

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 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 splenocytes, suggesting an immunomodulatory 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.

T_H17 cells are defined by their secretion of the pro-inflammatory cytokine interleukin (IL)-17. The observation that T_H17 cells are a distinct lineage of T-cells with a unique cytokine and chemokine/chemokine receptor profile led to the discovery of ROR γ t, which encodes retinoid orphan nuclear receptor, as a transcriptional factor for the differentiation of T_H17 cells (Ivanov et al., 2006; Wilson et al., 2007). T_H17 cells have reportedly been linked to the development of immune disorders such as autoimmune, inflammatory bowel, and atopic diseases (Ivanov et al., 2006; Wilson et al., 2007). Nonetheless, the impacts of particulate matters on T_H17 cells and/or production of IL-17 have been less extensively studied. Furthermore, the effects of NRDEP we used have never been examined.

Thus, the aim of the present cutting-edge study was to examine *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-17A production/release and its underlying transcriptional control.

Materials and methods

Animals

Male NC/Nga mice, 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 filtered water *ad libitum*. Mice were housed in an animal facility maintained at 24–26°C with 55–75% relative humidity and a 12-h light/dark cycle. All 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). An 8 L-diesel engine (J08C, Hino Motors, Tokyo) that was not fitted with after-treatment devices, and that met 1997 emission regulations, was powered under steady-state

conditions (speed=2000 rpm; engine torque=0 Nm; diesel fuel=JIS No. 2) for 5 h. These conditions generated nanoparticle-rich exhaust (particle concentration = 3×10^7 counts/cm³; modal diameter [$\pm$ SD] = 21.45 ± 1.46 nm) (Fujitani et al., 2009). Particles were electrostatically (~ 27 kVolts) collected at ≈ 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 a SSPM-100 sampler (Shimadzu, Kyoto, Japan). Sampling was repeatedly conducted seven times using the same sample, and the total time duration was 35 h.

Seven gold discs with NRDEP (total = 7.3476 mg) were obtained and stored in a -80°C freezer. NRDEP were dispersed (under ice-cold conditions) from the discs into the vehicle (0.02% Tween80-R10 medium [RPMI 1640 medium (Invitrogen, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (MP Biomedicals, Eschwege, Germany), 100 U penicillin/ml, 100 μg streptomycin/ml (Sigma, St. Louis, MO), and 50 μM 2-mercaptoethanol (Invitrogen)]) using an ultrasonic disrupter (UD-201, Tomy Seiko, Tokyo) for 3 min as previously described (Inoue et al., 2005b, 2006).

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 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] NH₄Cl 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 using a trypan blue dye exclusion method. Splenocytes (at 1×10^6) were cultured in 1 ml of R10 containing NRDEP (1, 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% CO₂/95% air atmosphere. After a 24-h incubation, the culture medium and cells were harvested; medium was used for enzyme-linked immunosorbent assays (ELISA) while the cells were processed to yield lysate for Western blot analyses.

WST-1 assay

Splenocytes prepared as above were plated into 96-multiwell plates (Costar, Corning, NY) and incubated at 37°C for 24 h before being exposed to various levels (1–50 $\mu\text{g}/\text{ml}$) of NRDEP test suspensions for 24 h. A negative control was provided using cells that had received culture medium only. At the end of the latter incubation, the media in each well was removed and replaced with 100 μl of new complete media. Thereafter, WST-1 solution (10 μl , final concentration/well = 0.5 $\mu\text{g}/\text{ml}$) was added to each well, and the plated incubated at 37°C for a further 3 h. The absorbance at 490 nm (reference at 630 nm) in each well was then measured using a microplate reader (BioTek ELX800, BioTek Instruments, Winooski, VT).

The percentage of cell viability was calculated in terms of absorbance of the cells treated with NRDEP relative to that in cells exposed to culture media only.

ELISA for IL-17

An ELISA for IL-17A (BioLegend Inc., San Diego, CA) 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 2.7 pg IL-17A/ml.

Preparation of whole protein and Western blot analysis

Cells were washed twice in PBS and lysed in RIPA buffer (Nacalai Tesque Inc., Kyoto) on ice for 30 min. The lysate was centrifuged at $10,000\times g$ for 15 min, and the supernatant was used as a total protein sample. The protein content was determined using a Quant-iT protein assay kit (Molecular Probes Inc., Eugene, OR). A total of 25 μ g protein/sample was loaded onto and electrophoresed over a 10% SDS-PAGE gel; the resolved proteins were then electrotransferred to a polyvinylidene-difluoride (PVDF) membrane. The membrane was then blocked with Blocking One solution (Nacalai Tesque) for 1 h at room temperature, rinsed, and then incubated with anti-ROR γ t (retinoic acid-related orphan receptor- γ t) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-GAPDH antibody (Cell Signaling Technology, Beverly, MA) overnight at 4°C. Blots were then washed with Tris-buffered saline-0.1% Tween-20 (TBST, pH 7.6) to remove non-adherent primary antibody and then incubated with horseradish peroxidase-labeled anti-mouse IgG (GE Healthcare, Buckinghamshire, UK) for 1 h at room temperature. After washes with TBST, the membrane was developed using the ECL-plus enhanced chemiluminescence light detecting kit (GE Healthcare), according to manufacturer's instructions. For quantification, the bands present in photographs of each fluorescent blot were scanned using a Las-4000miniEPUV luminescent image analyzer (Fujifilm, Tokyo).

Statistical analysis

Data are expressed as the mean ($\pm$ SEM) of three 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). When significant differences were detected, *post-hoc* comparisons within each group were evaluated with Bonferroni correction. Significance was assigned to *p*-values < 0.05.

Results and discussion

At first, to examine the effects of NRDEP exposure on the viability/proliferation of splenocytes isolated from

NC/Nga mice, a WST-1 assay was performed. It was seen that NRDEP suppressed cell viability/proliferation in a dose-related manner. Of the three doses tested in that assay, only a concentration of 50 μ g NRDEP /ml significantly decreased these values compared to those seen with control cells (Figure 1).

To then determine the effects of NRDEP on one type of cytokine production/release by naïve splenocytes after a 24-h exposure, IL-17A was measured in the culture supernatants collected at that timepoint (Figure 2A). A concentration of 1 or 10 μ g NRDEP/ml significantly increased IL-17A levels compared to those seen with (vehicle) control cells. Further, consistent with the results for IL-17A, Western blot analysis (for transcriptional studies) revealed that 1 or 10 μ g NRDEP/ml induced ROR γ t expression in these cells (Figure 2B). In contrast, 50 μ g NRDEP/ml tended to inhibit the transcription factor expression and cytokine production/release.

The immunotoxic effects of DEP (diesel exhaust particles) vary from immunosuppression to immunoactivation (Takano et al., 1997; Ichinose et al., 1998; Ohtani et al., 2005). On the other hand, the impacts of DEP on T-helper cell (T_H)-related immunity remain obscure. Reportedly, DEP and their components can directly activate B-cells to enhance IgE production (Takenaka et al., 1995; Tsien et al., 1997), whereas the chemical constituents of DEP promote interleukin (IL)-4 production/release by human-derived basophils (Devouassoux et al., 2002). Moreover, DE (diesel exhaust) exposure decreases IL-12 (T_H1 cytokine) production from alveolar macrophages or a macrophage cell line (RAW264.7 cells) *in vitro* (Saito et al., 2002) and in lung homogenates *ex vivo* (Takano et al., 1997). Furthermore, it has been shown that DEP suppress T-bet activation and interferon- γ production by isolated T-cells from healthy humans (Sasaki et al., 2009). Similarly, we recently demonstrated that DEP generated from another diesel engine (average size of particle:

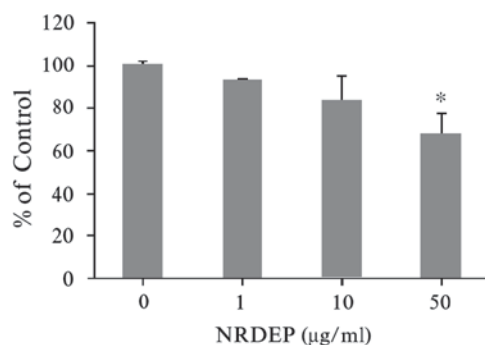


Figure 1. Effects of nanoparticle-rich diesel exhaust particles (NRDEP) on viability/proliferation of splenocytes from atopic animals. Splenocytes (1×10^6 /ml) derived from NC/Nga mice were exposed to various concentrations of DEP (1–50 μ g/ml) for 24 h. Thereafter, cell viability/proliferation was assessed using a WST-1 assay. The percentage of viable cells was calculated from the ratio of absorbance values at 490 nm of the treated vs control cells. Data shown are the mean ($\pm$ SEM) of four individual cultures from three animals, representative of three independent experiments. **p* < 0.05 vs control.

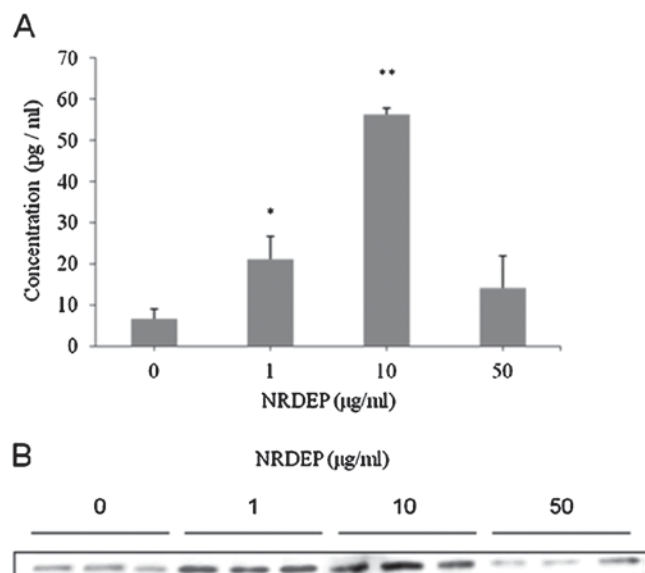


Figure 2. Effects of nanoparticle-rich diesel exhaust particles (NRDEP) on IL-17A production by, and ROR γ t expression in, splenocytes from atopic animals. Splenocytes (1×10^6 /ml) derived from NC/Nga mice were exposed to various concentrations of NRDEP (1–50 μ g/ml) for 24 h. Thereafter, the levels of (A) IL-17A in culture supernatants and (B) ROR γ t expression in lysates of the cells was analyzed by ELISA and immunoblotting, respectively. Data shown are the mean ($\pm$ SEM) of four individual cultures from three animals, representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$ vs control.

400 nm) and their components differentially increase the surface expression of IL-4R, CD69, and CD40L on mouse splenic mononuclear cells with amplified IL-4 production/secretion (Inoue et al., 2010). Taken together, DEP, particularly at non-toxic doses, generally promote T_H2-skewed responses both *in vitro* and *in vivo* (Takenaka et al., 1995; Tsien et al., 1997; Sasaki et al., 2009).

Nevertheless, whether nano-level DEP exhibit a similar immunological potential remains unclarified. Furthermore, there is no study regarding the effects of DEP on T_H17 differentiation. In the present study, we demonstrated for the first time that NRDEP generated by an inhalation exposure system can induce IL-17A on splenic mononuclear cells from atopy-prone mice, suggesting their potential to guide lymphocyte differentiation toward T_H17 cells, at least in part, *in vitro*. It is also interesting that this type of DEP induces IL-17A production/release from the components of a T_H2-biased animal (Nc/Nga mouse). We previously demonstrated that repeated pulmonary exposure to DEP (average particle size: 400 nm) induces lung inflammation with elevated IL-4 expression in Nc/Nga (Inoue et al., 2006). Taking the current and previous results into consideration, it is possible that this type of DEP has a different immunomodulatory impact compared to previously employed DEP, although further investigation is needed in the future.

ROR γ t is an important transcriptional factor required for the generation/differentiation of T_H17 cells (Ivanov et al., 2006; Wilson et al., 2007). ROR γ t-depleted mice

exhibit a reduced number of IL-17-secreting cells, and, furthermore, their IL-17-secreting cells are unresponsive to stimulation by IL-23 (Ivanov et al., 2006), reportedly an important cytokine for the survival and population expansion of T_H17 cells (Wilson et al., 2007). In addition, the forced expression of ROR γ t can induce IL-17 in naïve CD4⁺ T cells *in vitro* (Ivanov et al., 2006). In the present study, consistent with the results for IL-17A, NRDEP at preferential concentrations (1 and 10 μ g/ml) induced ROR γ t expression in these cells. Nonetheless, searching for signals/molecules up- and downstream of ROR γ t and/or other machinery for the 'forced' T_H17 milieu promoted by NRDEP in this assay system requires further investigation.

We believe it is important to discuss the 50 μ g NRDEP/ml exposure-related effects seen here. An examination of cytotoxicity in the cells using WST-1 revealed a dose-related cytotoxic effect with the particles. Thus, the marked decrease in production/release of IL-17 induced by 50 μ g/ml NRDEP compared to that by 1 or 10 μ g/ml can be partly explained to be a result of overt cell death. It was previously shown that a relatively high-dose DEP exposure led to apoptosis (and cell death) in alveolar macrophages, macrophage cell lines (Hiura et al., 1999), and airway cell lines (Ackland et al., 2007). As such, in the study here, the decreased IL-17 production/release caused by 50 μ g NRDEP/ml was also likely due to cell dysfunction resulting, in part, from an induction of apoptosis. Future studies to validate this hypothesis will examine the effect of NRDEP exposure on induction of apoptosis and examine underlying mechanisms for this outcome.

Finally, we would be remiss if we did not note that, while there might be no argument with the cell types that were used here as examples of immune system-related cells, some might question our reasoning for the use of the mouse splenocytes at all to assess effects from host inhalation exposure to NRDEP. Clearly, an argument can be made that NRDEP might translocate to the spleen; however, since DEP are very sticky, the number of these particles would be very small. It is also possible that soluble mediators/chemicals from any inhaled NRDEP could move into the systemic circulation and, consequently—albeit at low concentrations—influence the spleen and/or splenocytes. Therefore, to assess the reliability of these and other *in vitro* studies that use/will use splenocytes to assess potential NRDEP immunotoxicities, future studies are needed to assess the impact of inhaled/instilled NRDEP on the spleen overall and on splenocytes *in vivo* and/or *ex vivo*.

In summary, NRDEP significantly increased IL-17A production/release by splenocytes from atopic mice, which was concomitant with the enhanced ROR γ t expression in the cells. These data highlight the possibility of another immunomodulatory effect of NRDEP in the context of T_H17 domination *in vitro*, and may provide insight into the immunotoxicity of nano-level diesel engine-generated PM.

Acknowledgements

This study was supported by Grants-in-Aid for Scientific Research (B) 18390188 (to K. Inoue) from the Japan Society for the Promotion of Science. We thank Dr Eiko Koike, for her assistance.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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