



Pro-inflammatory and potential allergic responses resulting from B cell activation in mice treated with multi-walled carbon nanotubes by intratracheal instillation

Eun-Jung Park^a, Wan-Seob Cho^b, Jayoung Jeong^b, Jongheop Yi^c, Kyunghye Choi^d, Kwangsik Park^{a,*}

^a College of Pharmacy, Dongduk Women's University, 23-1, Wolgok-dong, Seongbuk-gu, Seoul 136-714, Republic of Korea

^b Division of Toxicological Research, National Institute of Toxicological Research, Korea Food and Drug Administration, Seoul, Republic of Korea

^c School of Chemical and Biological Engineering, Seoul National University, 599 Gwanangno, Gwanak-gu, Seoul 151-742, Republic of Korea

^d Environmental Research Complex, Kyungseo-dong, Seo-gu, Incheon 404-708, Republic of Korea

ARTICLE INFO

Article history:

Received 5 January 2009

Received in revised form 19 February 2009

Accepted 19 February 2009

Available online 4 March 2009

Keywords:

MWCNTs

Pro-inflammation

Cytokines

B cell activation

IgE

ABSTRACT

The increased application of engineered carbon nanotubes (CNTs) has also raised the level of public concern regarding possible toxicities caused by exposure to these nanostructures. In this study, pulmonary and systemic immune responses induced by intratracheal instillation of multi-walled carbon nanotubes (MWCNTs) were investigated in mice. Total numbers of immune cells in bronchoalveolar lavage (BAL) fluid were significantly increased in treated groups (5, 20, and 50 mg/kg doses of MWCNTs) and the distribution of neutrophils was elevated at day 1 after instillation. Pro-inflammatory cytokines (IL-1, TNF- α , IL-6, IL-4, IL-5, IL-10, IL-12, and IFN- γ) were also increased in a dose-dependent manner, both in BAL fluid and in blood. Most of the cytokines showed the highest levels at day 1 after instillation and then decreased. Th2-type cytokines (IL-4, IL-5, and IL-10) were elevated in the treated group to levels higher than those of the Th1-type cytokines (IL-12 and IFN- γ). Furthermore, distributions of B cells in spleen and blood were significantly increased at day 1 after instillation, indicating that Th2-type cytokines had activated B cells, causing them to proliferate. Along with the additional numbers of B cells, granuloma formation in the lung tissue and IgE production were also observed, with an intensity dependent on the dose of MWCNTs instilled. Based on these observations, it is suggested that MWCNTs may induce allergic responses in mice through B cell activation and production of IgE.

© 2009 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Carbon nanotubes (CNTs), which structurally resemble rolled-up graphite sheets with one capped end, exist in various forms including single-walled CNTs (SWCNTs), double-walled CNTs, and multi-walled CNTs (MWCNTs). They are allotropes of carbon with a nanostructure that can have a length-to-diameter ratio as large as 28,000,000:1. They are conveniently obtained by carrying out the pyrolysis of hydrocarbons such as ethylene and acetylene over nanoparticles of iron, cobalt, and other metals dispersed over a solid substrate. This is the reason why metals exist in carbon nanotubes (Rao and Govindaraj, 2002). The properties of CNTs suggest a possible accumulation along the food chain and high persistence. The kinetics and toxicity of CNTs depend on the inherent physical and chemical characteristics (Helland et al., 2007). CNTs are now widely used and are the major building blocks in nanotechnology because of their unique physical, chemical, mechanical, thermal, and electrical properties. However, the widespread application of

CNTs may result in escalation of environmental, occupational, and human exposures, resulting in increased public concern regarding the potential toxicity of CNTs (Helland et al., 2007; Vairavapandian et al., 2008). According to the published toxicity data for CNTs, they may exert adverse effects through oxidative stress and inflammation following human exposure.

Intratracheal instillation of SWCNTs in rats resulted in the production of chemicals typical of inflammatory responses in bronchoalveolar lavage (BAL) fluid, and the appearance of fibrosis/multifocal granulomas in lung tissue (Lam et al., 2004; Warheit et al., 2004). Pharyngeal aspiration of SWCNTs also induced fibrotic lesions within the alveolar region of the lung, concomitant with an increase in platelet-derived growth factors (Mangum et al., 2006). In mice, dose-dependent increases in protein level and activities of lactate dehydrogenase and L-glutamyl transferase in bronchoalveolar lavage fluids have been observed, along with the accumulation of 4-hydroxy nonenal (a biological marker for oxidative stress) and depletion of glutathione. Neutrophil accumulation, with the influx of lymphocytes and macrophages, and accompanied by early elevation of pro-inflammatory cytokines such as TNF- α , IL-1 β , TGF- β 1, NF-kB, and AP-1, have also been reported (Shvedova et al., 2005, 2007; Chou et al., 2008). A single intrapharyngeal instillation of

* Corresponding author. Tel.: +82 2 940 4522; fax: +82 2 940 4159/4295.

E-mail address: kspark@dongduk.ac.kr (K. Park).

SWCNTs induced the activation of heme oxygenase-1 (HO-1) in lung, aorta, and heart tissues in transgenic HO-1 reporter transgenic mice. Plaque formation in brachiocephalic arteries was found, together with increased mitochondrial damage, in SWCNT-treated mice (Li et al., 2007a).

Pro-inflammatory responses induced by MWCNTs as well as by SWCNTs have been reported. When MWCNTs were administered intratracheally to rats, both inflammatory and fibrotic reactions were induced. Pulmonary lesions induced by MWCNTs were characterized by the formation of collagen-rich granulomas that protruded into the bronchial lumen, in association with alveolitis in the surrounding tissues (Muller et al., 2005). Comparative study of pathological lesions induced by MWCNTs by intratracheal instillation and inhalation has been previously conducted by Li et al. (2007b), who showed that MWCNTs aggregated into clumps in bronchi and alveoli following instillation. These clumps led to inflammation of the lining of the wall of the bronchi, resulting in lesions that were more similar to those induced by mechanical injury. However, when MWCNTs were inhaled, most of the aggregations in the alveoli were smaller and the general alveolar structure was not affected. Furthermore, MWCNT inhalation at concentrations from 0.3 to 5 mg/m³ did not result in significant lung inflammation or tissue damage, although it did cause systemic immune function alterations (Mitchell et al., 2007). Therefore, the actual effects of inhaled MWCNTs seem to be debatable (Lison and Muller, 2008; McDonal and Mitchell, 2008), but the observed differences can most likely be explained by differences in biological and toxicological responses due to variations in the experimental procedures such as dosages, routes of dosing, and types of morphology, among others.

In the current study, assessment of pulmonary pro-inflammatory responses by intratracheal instillation of MWCNTs was conducted to determine the induction level of Th1-type/Th2-type cytokines and to estimate the possible allergic responses through Th2-type cytokine-induced B cell activation.

2. Materials and methods

2.1. Animals and materials

ICR male mice were purchased from Orient-Bio Animal Company (Seongnam, Gyunggi-do, Korea) and were allowed to adapt to the animal room conditions prior to initiation of the study. The environmental conditions were a temperature of 23 ± 1 °C; relative humidity of 55 ± 5%; and a 12-h light/dark cycle. All the animals used in this study were cared for in accordance with the principles outlined in the "Guide for the Care and Use of Laboratory Animals" issued by the Animal Care and Use Committee of NVRQS (National Veterinary Research and Quarantine Service). MWCNTs (Cat No. 659258) were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA) and confirmed by TEM prior to use. According to the information provided by the company, inner diameter is less than 5 nm and outer diameter is 110–170 nm. Length of MWCNT is 5–9 μm, surface area is 12.83 m²/g and carbon content is more than 90%. The particles were suspended in phosphate buffered saline and were sonicated prior to use to ensure a uniform suspension.

2.2. Intratracheal instillation and sample preparation

MWCNTs were delivered with a 24-gauge catheter, at doses of 5, 20, or 50 mg/kg, by intratracheal instillation under light tiletamine anesthesia (day 0). The animals were sacrificed at 1, 3, 7, or 14 days after treatment (*n* = 10–12 per group) (Cho et al., 2007).

At the selected time intervals after instillation, blood was collected from the retro-orbital venous plexus into heparinized capillary tubes. Serum was obtained by centrifugation at 3000 rpm for 20 min. Serum obtained from each mouse of control or treated group was pooled for cytokine assay. BAL fluid collection was performed by cannulating the trachea and lavaging the lungs with 1 mL of cold sterile (Ca²⁺ plus Mg²⁺)-free PBS. Approximately 500–600 μL BAL fluid was harvested per mouse and this was centrifuged at 3000 rpm for 10 min prior to measurement of cytokines. BAL fluid obtained from each mouse of control or treated group was pooled for cell count, cytokine measurement, NO measurement, and IgE.

For the isolation of splenocytes, the spleen was aseptically removed from an ICR mouse, and suspended by passage through a sterile plastic strainer in Dulbecco's modified Eagle's medium (DMEM) with 2% fetal bovine serum (FBS). After centrifugation at 1500 rpm for 3 min, the supernatant was discarded, and the pellet was

briefly vortexed in 500 μL distilled water, then resuspended in DMEM with 2% FBS. The cells were filtered through a nylon mesh to yield the final splenocyte preparation (Byun et al., 2006; Vendrame et al., 2006; Manosroi et al., 2005; Cho et al., 2007).

2.3. BAL fluid analysis

Cytospinning was performed with 500 μL BAL fluid harvested at the selected time intervals. After cytospinning, slides were fixed in methanol and stained with Diff-Quick (Thermo Shandon, PA). Cell totals were recorded and different cell types in the BAL fluid were quantified by their characteristic morphologies (Cho et al., 2007).

2.4. Measurement of cytokines

The concentrations of each cytokine in the supernatant of BAL fluid and in serum were determined using commercially available ELISA kits (eBioscience, San Diego, CA, USA). Briefly, each well of a microplate was coated with 100 μL of capture antibody, and incubated overnight at 4 °C. After washing and blocking with assay diluent, BAL fluid, serum or standard cytokines were added to individual wells and the plates were maintained for 2 h at room temperature. The plates were washed, then biotin-conjugated detecting mouse antibody was added to each well and incubated at room temperature for 1 h. The plates were washed again and further incubated with avidin-HRP for 30 min before detection with TMB solution. Finally, reactions were stopped by adding 1 M H₃PO₄, and the absorbance at 450 nm was measured with an ELISA reader (Molecular Devices, Sunnyvale, CA, USA). The amount of cytokine was calculated from the linear portion of the generated standard curve (Byun et al., 2006).

2.5. Nitric oxide (NO) production

Nitric oxide production in BAL fluid was quantified spectrophotometrically using Griess reagent (1% sulfanilamide, 2.5% H₃PO₄, 0.1% N-1-naphthylethylenediamine dihydrochloride). The absorbance was measured at 540 nm and the nitrite concentration was determined using a calibration curve prepared with sodium nitrite as the standard (Chen et al., 1995).

2.6. Immunophenotyping

All monoclonal antibodies were purchased from eBioscience (San Diego, CA, USA). T cells (CD3, 1:50), B cells (CD19, 1:50), NK cells (DX5, 1:100), CD4⁺ T cells (CD4⁺, 1:160), and CD8⁺ T cells (CD8⁺, 1:50) were identified using directly conjugated anti-mouse antibodies. Briefly, splenocytes at (3–5) × 10³ cells/mL and serum were blocked with Fc-block (eBioscience, San Diego, CA, USA) to reduce non-specific antibody binding. Cells were then incubated in the dark with 10 μL of the appropriate fluorochrome-conjugated antibody for 20 min at 4 °C. Cells were then washed with 500 μL FACS (Fluorescence Activated Cell Sorter) buffer. The blood was lysed for 5 min with FACS lysis buffer (BD Bioscience, Franklin Lakes, NJ, USA) at room temperature, then re-washed with FACS buffer. Finally, each sample was fixed with 1% paraformaldehyde until further analysis.

Flow cytometry analysis was performed on the FACSCalibur system (BD Biosciences, Franklin Lakes, NJ, USA). Control samples were matched for each fluorochrome. Data were analyzed using CellQuest software (Becton Dickinson, Franklin Lakes, NJ, USA) (Silva et al., 2005; Vendrame et al., 2006).

2.7. Measurement of IgE

The concentration of IgE in serum and in the supernatant of BAL fluid was determined using commercially available IgE ELISA kits (Komabiotec, Seoul, Korea). Briefly, each well of a microplate was coated with coating antibody and incubated overnight at 4 °C. After washing and blocking with blocking solution for 1 h at room temperature, BAL fluid, serum and IgE standards were added to individual wells and left for 1 h at room temperature. The plates were then washed, detection antibody was added to each well and the plates were incubated at room temperature for 1 h. After washing, the plates were further incubated with color development solution. Finally, the reaction was terminated by addition of 2 M H₂SO₄, and the absorbance was measured at 450 nm with an ELISA reader (Molecular Devices, Sunnyvale, CA, USA). The amounts of secreted IgE were calculated from the linear portion of the prepared standard curve (Fletcher et al., 2000).

2.8. Histopathology

Lungs from mice of control group and treated group were fixed with 10% neutral buffered formalin and processed using routine histological techniques. After paraffin embedding, 3-μm sections were cut and stained with hematoxylin and eosin (H&E) for histopathologic evaluation. For the assessment of lung injury, the airways, terminal bronchioles and lung parenchyma were examined microscopically for evidence of cellular changes and inflammation. Pathologic scores of 0 (no histopathologic change) to 4+ (severe abnormalities) were assigned to each lung tissue based on the degree of alveolitis, bronchiolitis, bronchitis, fibrosis and the extent of involvement of each, as previously described (Pryhuber et al., 2003).

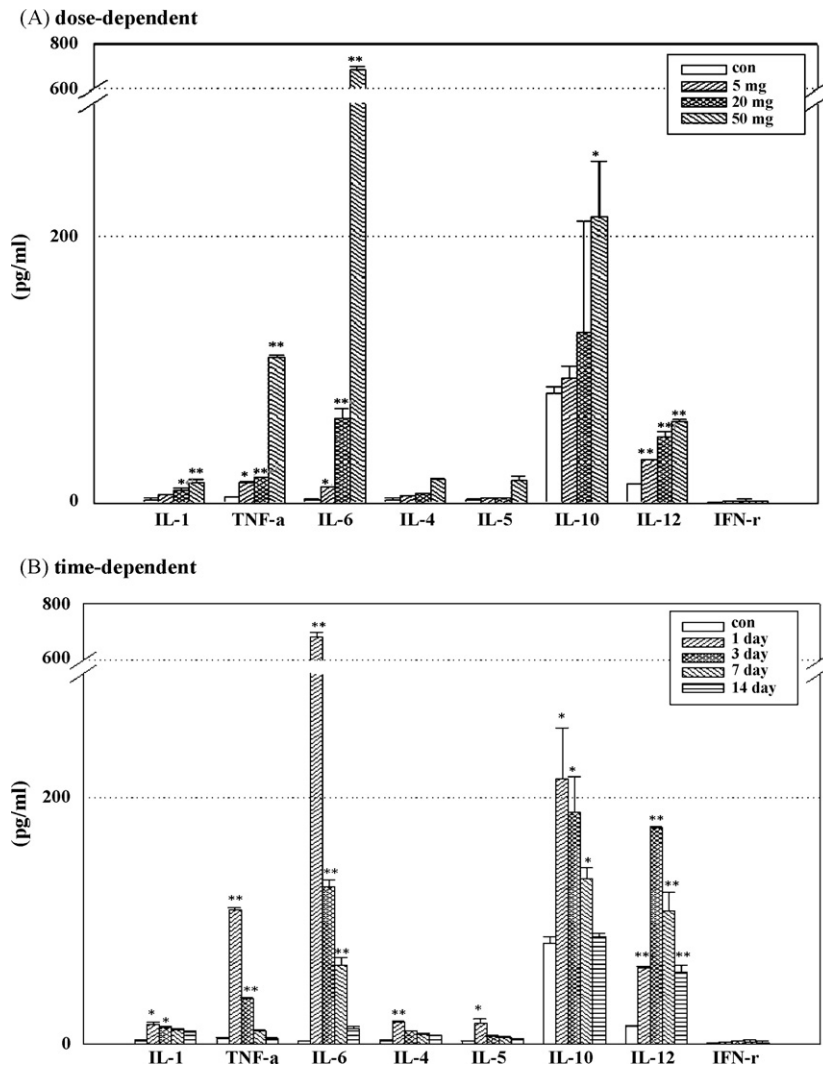


Fig. 1. Changes in cytokine levels in BAL fluid after a single intratracheal instillation of MWCNTs. The concentrations of cytokines in BAL fluid harvested on the respective sacrifice days after instillation were determined using ELISA kits. (A) Dose-dependent changes at day 1 and (B) time-dependent changes. Significantly different from control group: * $P < 0.05$ and ** $P < 0.01$.

2.9. Statistical analysis

The results obtained from the chemically treated groups were compared to those of the control group. The values were compared using the Student's *t*-test, and levels of significance were represented for each result.

3. Results

3.1. Cell composition in BAL fluid

The instillation of MWCNTs significantly increased the total number of cells recovered in BAL fluid and this showed a dose-dependency at day 1 after instillation. With the increase in total cell numbers, the percentage composition of neutrophils was also significantly increased by MWCNT doses of 20 and 50 mg/kg. When mice were treated with a 50 mg/kg dose, the increase in total number of cells in the BAL fluid was maintained over the duration of the experiment for 14 days after a single instillation, while the composition percentage of neutrophils was restored to the level of the control group by day 14 (Table 1).

Body weight was not changed by MWCNT instillation except at the highest dose of 50 mg/kg. When mice were treated at this dosage, bodyweight at day 1 was decreased to 88% of the level measured at day 0 before instillation. This loss in weight was sus-

tained until day 3 after instillation (data not shown). In this group, mice also showed sedation and decreased movement until day 3 but these abnormal behaviors disappeared after day 3.

3.2. Cytokines in BAL fluid

To identify inflammatory responses induced by MWCNT instillation, the concentrations of pro-inflammatory cytokines, such as IL-1, IL-6, TNF- α , Th1-type cytokines (IL-12 and IFN- γ), and Th2-type cytokines (IL-4, IL-5, and IL-10), were measured in the BAL fluid. A significant increase in pro-inflammatory cytokines was observed in BAL fluid following intratracheal MWCNT instillation. Secretion of IL-1 increased in a dose-dependent manner at day 1, and was detected at levels of 6.1, 9.8, and 15.9 pg/mL at MWCNT dosages of 5, 20, and 50 mg/kg, respectively. TNF- α also increased in a dose-dependent manner at day 1 and showed levels of 15.6, 19.6, and 109.2 pg/mL following treatment with 5, 20, and 50 mg/kg dosages of MWCNTs, respectively. Levels of IL-6, which plays an important role in induction of acute inflammation, were also increased by MWCNT treatment. In this study, the average increase in the level of IL-6 in the group with a 50 mg/kg MWCNT dose was 682 pg/mL, or 272.9-fold higher than the level in control group at day 1 (Fig. 1A). The time-courses for the observed changes

Table 1

Analysis of cell composition in BAL fluid after a single intratracheal instillation of MWCNTs.

Groups	Total cells ($\times 10^5$)	Percentage of cells in BALF		
		Macrophages	Lymphocytes	Neutrophils
Control	5.03 $\pm$ 0.41	96.02 $\pm$ 8.29	2.88 $\pm$ 0.19	1.09 $\pm$ 0.01
Carbon nanotubes				
1 day (5 mg/kg)	14.56 $\pm$ 2.97*	89.28 $\pm$ 5.34	4.39 $\pm$ 3.60	6.32 $\pm$ 3.95
1 day (20 mg/kg)	10.52 $\pm$ 0.88*	80.61 $\pm$ 11.29	1.78 $\pm$ 0.01	19.39 $\pm$ 2.12*
1 day (50 mg/kg)	12.04 $\pm$ 1.74*	89.70 $\pm$ 18.34	2.01 $\pm$ 0.02	10.30 $\pm$ 1.15*
3 days (50 mg/kg)	12.88 $\pm$ 3.12*	87.73 $\pm$ 11.78	6.21 $\pm$ 5.61	6.06 $\pm$ 5.50
7 days (50 mg/kg)	12.08 $\pm$ 2.57*	93.54 $\pm$ 7.26	2.81 $\pm$ 3.20	3.64 $\pm$ 5.09
14 days (50 mg/kg)	15.78 $\pm$ 4.79*	89.35 $\pm$ 8.99	10.65 $\pm$ 8.21	1.52 $\pm$ 0.01

Total numbers of cells in the BAL fluid were quantified by hemacytometric counting. Alveolar macrophages, neutrophils, and lymphocytes were identified by their characteristic cell shapes.

* Significantly different from age-matched control group ($P < 0.05$).

in IL-1, TNF- α , and IL-6 are shown in Fig. 1B. At day 1 after a single instillation, the level was maximally induced and then decreased over the remainder of the experimental period.

In general, activated macrophages secrete IL-12 or IL-10 to allow differentiation of naïve CD4 T cells into Th1 or Th2 cells, respectively. In the current study, the IL-12 level was 14.5 pg/mL in the

control group and this level was significantly elevated in a dose-dependent manner in the treated groups (Fig. 1A). A time-course of IL-12 elevation in the 50 mg/kg dose group indicated that the level of IL-12 reached its highest value at day 3 and then decreased (Fig. 1B). However, after only a single instillation, the level was still elevated above that of the control at day 14. In the case of

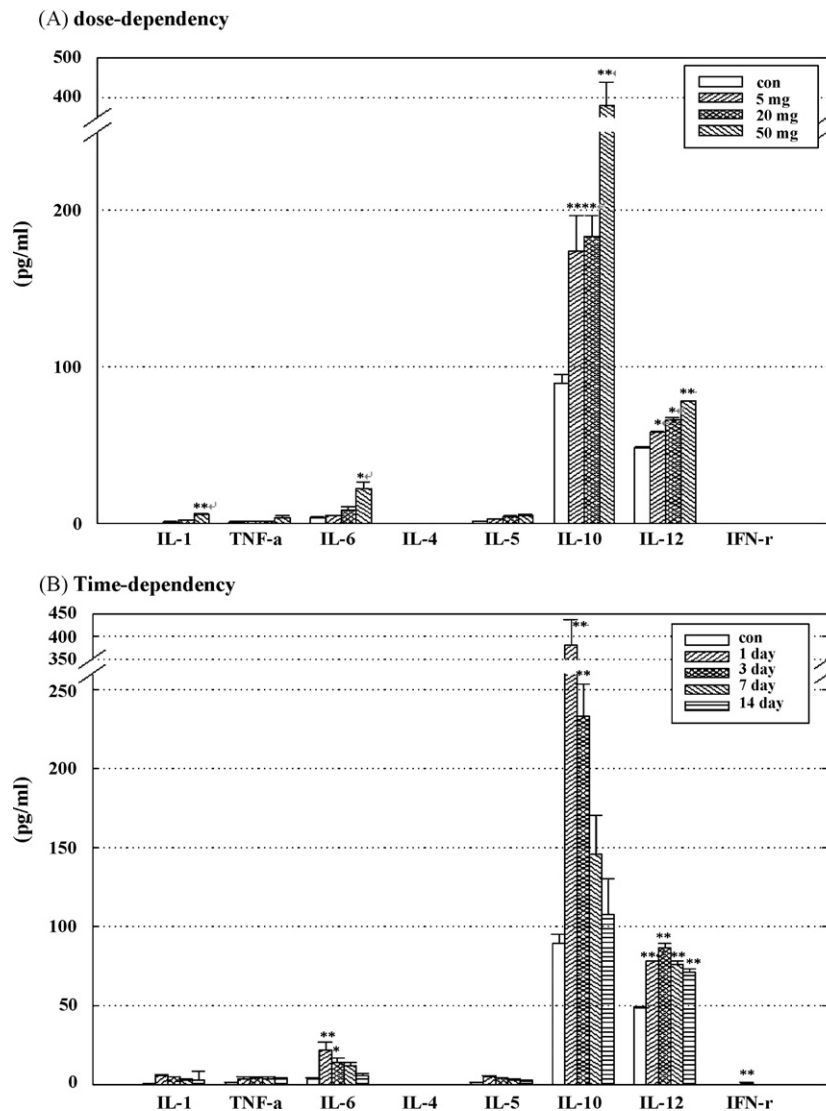


Fig. 2. Changes in cytokine levels in blood after a single intratracheal instillation of MWCNTs. The concentrations of cytokines in blood harvested at the respective sacrifice days after instillation were determined using ELISA kits. (A) Dose-dependent changes at day 1 and (B) time-dependent changes. Significantly different from control group: * $P < 0.05$ and ** $P < 0.01$.

IL-10, which a Th2-type cytokine, the level in the control group was 82.2 pg/mL, while the maximally induced level of IL-10, which appeared at day 1 in the 50 mg/kg dose group, was 214.7 pg/mL (Fig. 1A). IL-2 was also elevated maximally at day 1 and decreased after that time (Fig. 1B).

Th1 cells also secrete IFN- γ , which in turn stimulates the Th1 cells themselves and inhibits activation of the Th2-type cytokines. In this study, IFN- γ was induced by MWCNT treatment, but the level detected was very low, ranging from 0.46 pg/mL in the control group to 2.51 pg/mL in the treated group. The induction level reached a maximum at day 3 after a single instillation of MWCNTs at the 50 mg/kg dose (Fig. 1B). IL-4 and IL-5 secretion by Th2 cells were also induced by MWCNT treatment. Levels of IL-4 ranged from 2.6 pg/mL in the control group to 18.3 pg/mL in the treated group, while IL-5 levels ranged from 2.7 pg/mL in the control group to 17.2 pg/mL in the treated group. Both of these cytokines reached their highest levels at day 1 after a single instillation of MWCNTs at the 50 mg/kg dose.

3.3. Cytokines in the blood

As shown Fig. 2, higher blood levels of IL-1, TNF- α , IFN- γ , IL-4, and IL-5 were induced by a single intratracheal instillation of MWCNTs. Compared to the levels in BAL fluid in treated groups, the blood levels of these cytokines were generally lower. As shown in Fig. 2A, cytokines IL-6, IL-10, and IL-12 in the blood samples of the treated groups were present at much higher levels than in the control group. For IL-6, levels ranged from 3.5 pg/mL in the control group to 22.1 pg/mL in the treated group. Detected levels of IL-10 ranged from 89.7 pg/mL in the control group to 379.8 pg/mL in the treated group, while for IL-12 these values ranged from 48.3 pg/mL in the control group to 86.9 pg/mL in the treated group. IL-6 and IL-10 reached their highest levels at 1 day, while IL-12 reached its highest level at day 3, after a single intratracheal instillation.

3.4. NO secretion in BAL fluid

The effect of MWCNT instillation on nitric oxide production in BAL fluids was also examined. As shown in Fig. 3A, increased NO generation was only observed in the group treated with the 20 mg/kg MWCNT dose. In case of the 50 mg/kg dose group, the average value was higher than that of control group but was not statistically significant. The time-course for induction after a single instillation of a 20 mg/kg dose showed that maximal induction occurred at day 1 and the level was gradually decreased throughout the rest of the experimental period (Fig. 3B).

3.5. Histopathology of lung tissue

MWCNTs were found in the bronchiole and alveoli of the lung at day 1 after instillation (Fig. 4). The numbers of distributed nanoparticles in the lung were well correlated with the dosage at day 1 (Fig. 4A–D). Granulomatous lesions were evident in the alveoli and most of the nanoparticles were entrapped by the fibrous tissues by the third day after treatment (Fig. 4E). The granulomatous lesions persisted until day 14 of the experiment (Fig. 4F), while the number of nanoparticles slightly decreased as time progressed. Some nanoparticles could be found in the cytoplasm of alveolar macrophages, in the alveolar epithelial cells and also simply distributed in the interstitium of the alveoli (Fig. 4G). Scoring of lung injury induced by nanoparticles showed that the degree of lung injury was increased at day 1 after instillation in a dose-dependent manner and peaked at day 3 for the highest treatment

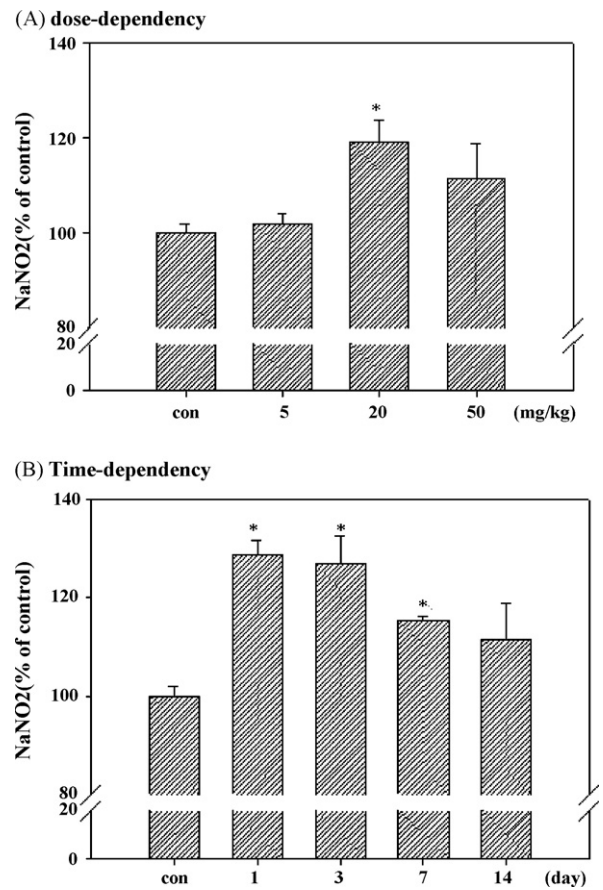


Fig. 3. Changes in nitric oxide in BAL fluid after a single intratracheal instillation of MWCNTs. The concentrations of nitric oxide in BAL fluid harvested at the respective sacrifice days after instillation were determined using Greiss reagent. (A) Dose-dependent changes at day 1 and (B) time-dependent changes. Significantly different from control group: * $P < 0.05$.

level, persisting throughout the duration of the 14 day experiment (Fig. 4H).

3.6. Lymphocyte phenotype

A single instillation of a MWCNT dose of 50 mg/kg significantly changed lymphocyte phenotypes and caused an increase in the proportion of B cells in both blood and spleen. The proportion of B cells in blood lymphocytes was 45.97% in the control group and 80.84% in the MWCNT-treated group. In the spleen, B cells comprised 56.97% of the lymphocytes in the control and 68.93% in the spleens of treated animals (Fig. 5). This increased proportion of B cells was maintained for the duration of the experiment (data not shown). Concomitant with the increase in B cells, a decrease in the proportions of NK and T cells also occurred in the treated groups, as shown in Fig. 6. The ratio of CD4⁺/CD8⁺ T cells was decreased in the treated animals, from 4.67 to 3.86 in blood and from 4.66 to 4.20 in spleen.

3.7. IgE secretion

IgE concentrations in BAL fluid and in blood were measured after the examination of B cell proliferation. As shown in Fig. 7, IgE levels were significantly increased by a single intratracheal instillation of MWCNTs at 50 mg/kg. The IgE production continued to increase with time following the instillation. At the end of the experiment at day 14, the blood IgE levels were 3.79 pg/mL in the control group and 30.54 pg/mL in the treated group (Fig. 7).

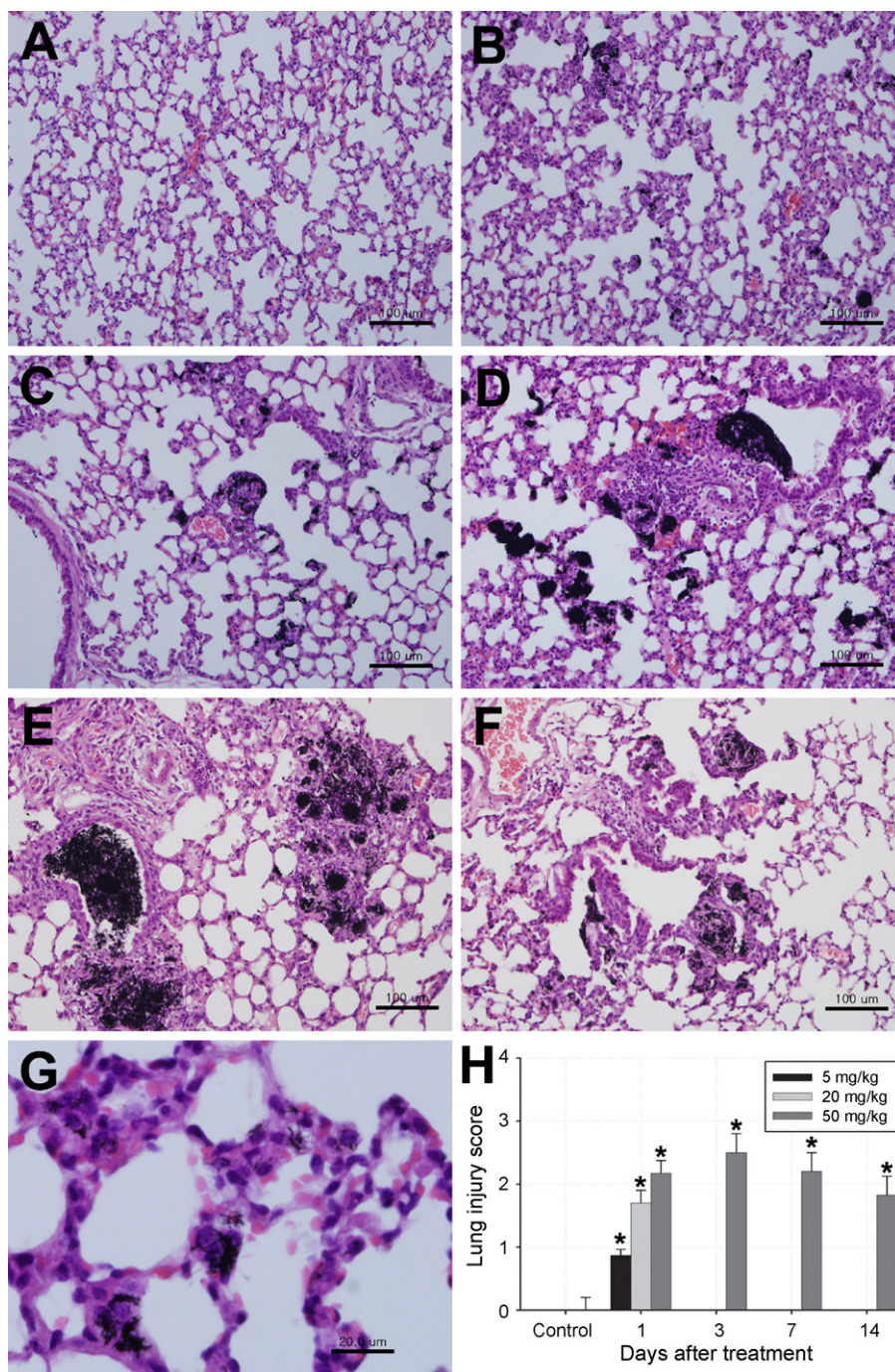


Fig. 4. Histopathology of lung tissues after a single intratracheal instillation of MWCNTs. Lung sections were stained with hematoxylin and eosin stains (200 $\times$). MWCNTs were instilled at doses of 5, 20 or 50 mg/kg at day 0. (A) Vehicle control mice, (B) 5 mg/kg at day 1, (C) 20 mg/kg at day 1, (D) 50 mg/kg at day 1, (E) 50 mg/kg at day 3, and (F) 50 mg/kg at day 14. (G) Higher magnification (1000 $\times$) of (F). (H) Lung injury score was derived based on a light microscopic examination of the degree of abnormality in the alveoli, bronchioles and bronchi, as described in Section 2.

4. Discussion

With the swift adoption of engineered MWCNTs in numerous commercial applications because of its physico-chemical properties such as strength, telescoping property, and thermal conductivity, the potential for inhalation or other exposures of the general population to these nanotubes is also rapidly increasing. However, the toxicities and mechanisms by which MWCNTs become health risks are not yet fully elucidated. In this study, we investigated the pro-inflammatory responses and potential aller-

gic responses induced in mice by exposure to MWCNTs through a single intratracheal instillation.

As shown in Table 1, significant increases in total cell numbers in BAL fluid were observed in all treated mice. Thus, it appeared that alveolar macrophages were activated by MWCNTs and that they attracted immune cells such as neutrophils, T cells and monocytes from the blood stream. This increase of total numbers of cells has also been previously observed in mice in response to SWCNT and to silica treatments (Barbarin et al., 2005; Shvedova et al., 2007). Furthermore, the activated macrophages secreted cytokines that

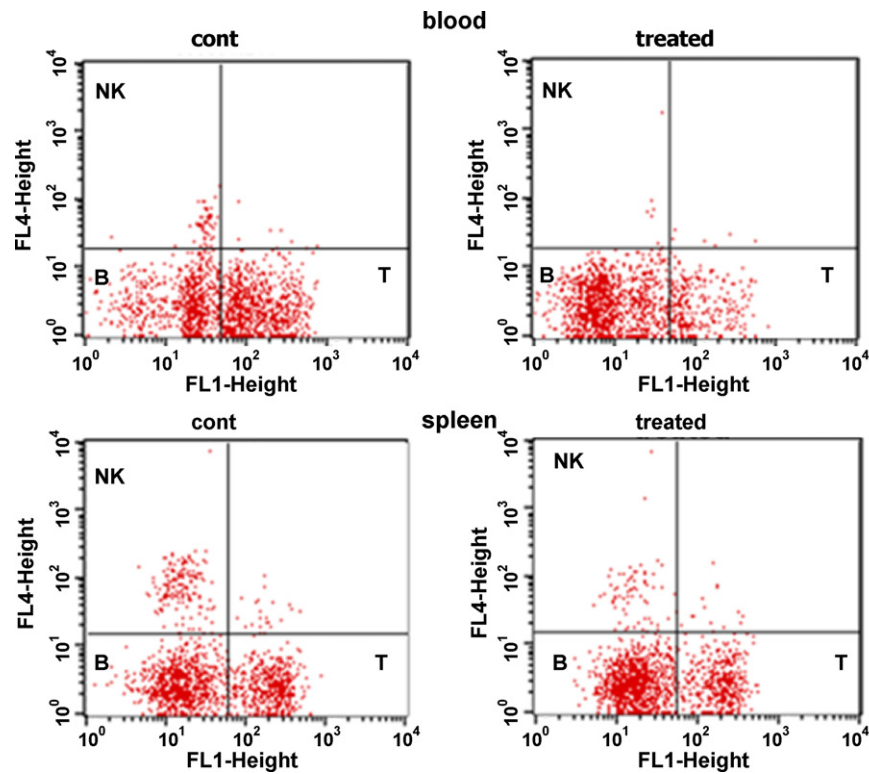


Fig. 5. Analysis of lymphocyte phenotypes in blood and spleen at day 1 after a single intratracheal instillation of MWCNTs. All monoclonal antibodies were identified using directly conjugated anti-mouse antibodies, and flow cytometry analysis was performed on the FACSCalibur system. Control samples were matched for each fluorochrome.

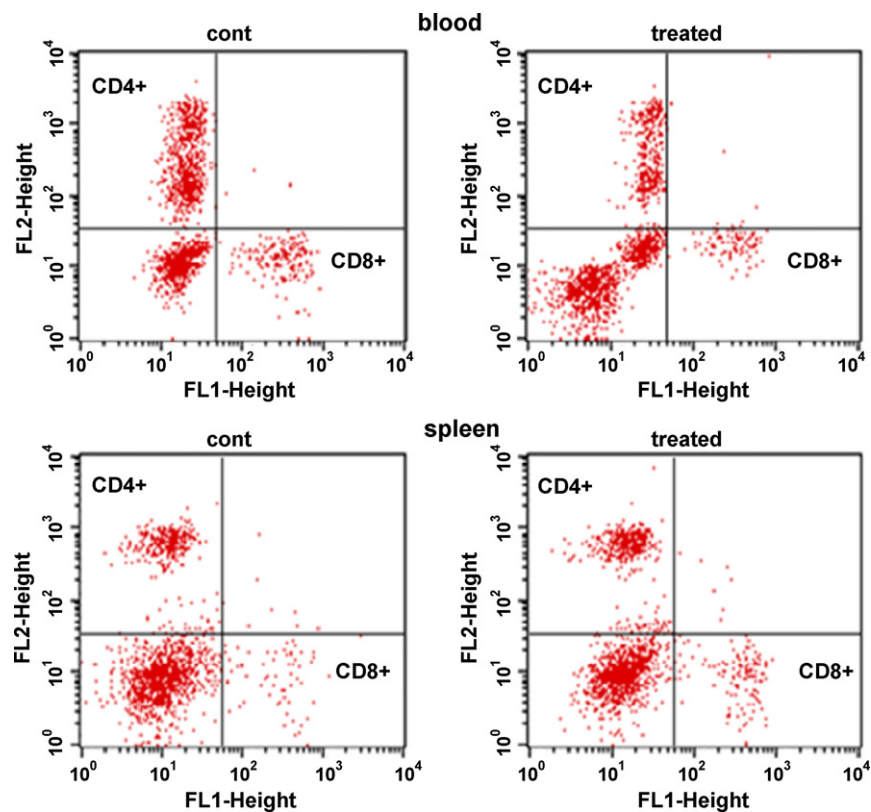


Fig. 6. Analysis of T cell subtypes in blood and spleen at day 1 after a single intratracheal instillation of MWCNTs. All monoclonal antibodies were identified using directly conjugated anti-mouse antibodies, and flow cytometry analysis was performed on the FACSCalibur system. Control samples matched for each fluorochrome.

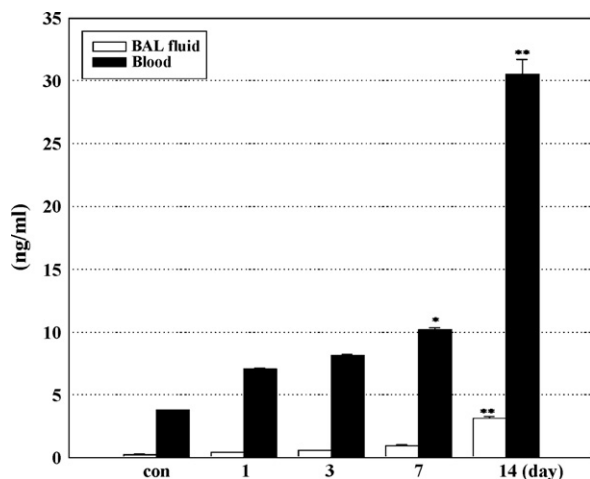


Fig. 7. Levels of IgE in BAL fluid and in blood after a single intratracheal instillation of MWCNTs. The concentrations of IgE in BAL fluid and in blood harvested at the respective sacrifice days after instillation were determined using ELISA kits. Significantly different from control group: * $P < 0.05$ and ** $P < 0.01$.

affected the behavior of other cells. For example, IL-1 can serve as an initiator of the further induction of inflammatory responses by the creation of positive feedback loops following binding with its receptor IL-1R. Similarly, TNF- α plays a critical role in local inflammatory responses, while IL-6 acts as a major pro-inflammatory cytokine released during acute inflammation (Zhu and Paul, 2008; LeBien and Tedder, 2008; Dienz and Rincon, 2009). In the current study, the IL-1 level of the 50 mg/kg dose group was increased 6.0-fold compared to the control group, rising from 2.6 to 15.9 pg/mL in the BAL fluid collected at day 1 after instillation. TNF- α also increased 22.5-fold, from 4.9 to 109.2 pg/mL, and IL-6 increased 272.9-fold, from 2.5 to 682.1 pg/mL.

It has been previously shown that IL-6 increased in a THP-1 cell line treated with SWCNTs (Chou et al., 2008). Gold nanoparticles have not been shown to induce pro-inflammatory cytokines but when they were conjugated with peptides they induced macrophage activation and gene expression of pro-inflammatory cytokines such as IL-1, IL-6 and TNF- α (Bastus et al., 2008). In addition to the increase in cytokine levels in the BAL fluid, the cytokines IL-1, TNF- α , and IL-6 were also increased in blood stream. It may be possible that MWCNTs in the alveolar region were translocated into the bloodstream, which then activated blood macrophages to secrete cytokines (Shimada et al., 2006).

Activated macrophages, both in the BAL fluid and in the blood, secrete cytokines that stimulate CD4 $^{+}$ T cells, which provide a protective and survival mechanism against xenobiotics or foreign biomaterials. CD4 $^{+}$ T cells have two subtypes, Th1 and Th2, and the fate of a naïve CD4 $^{+}$ T cells is determined by the cytokines that are secreted by the antigen presenting cells (Zhu and Paul, 2008). In particular, IL-4 is essential for the development of Th2 responses such as activation of B cells, production of IgE, and allergic responses. IFN- γ and IL-12 are important in the physiology of the development of Th1 cells, which are responsible for many cell-mediated functions including delayed-type hypersensitivity, promotion of excessive inflammation, and tissue injury. However, the overall inflammatory responses are a result of a balance of mixed responses of both Th1 and Th2 cells. This balance is further regulated by other cytokines. For example, IFN- γ , a product of Th1 cells, can prevent the activation of Th2 cells and can also stimulate the macrophages by positive feedback regulation for further inflammatory cascades. In contrast, IL-10, a product of Th2 cells, can in turn inhibit the development of Th1 cells (Strid et al., 2006).

In this study, it appeared that alveolar macrophages activated by MWCNT induced pro-inflammatory responses to attract immune cells such as neutrophils and that these cells secreted various cytokines such as IL-6, TNF- α , and IL-1 for pro-inflammation and differentiation of naïve T cells. The naïve T cells were then differentiated into CD4 $^{+}$ or CD8 $^{+}$ T cells. The levels of IL-12 and IL-4 in both BAL fluid and blood were significantly increased (Figs. 1 and 2) and these cytokines may have activated the CD4 $^{+}$ T cells to Th1 and Th2 cells, respectively. By the activation of Th1 cells and Th2 cells, Th1-type (IL-12 and TNF- α) or Th2-type (IL-4, IL-5, and IL-10) cytokines were also secreted in a significant amounts. Levels of IL-10, a Th2-type cytokine, were markedly elevated in BAL fluid and blood (Figs. 1 and 2). IL-10 may be one of the cytokines that is responsible for the activation of B cells and it is one cytokine that is apparently over-expressed in silica-treated mice and quartz-treated rats (Barbarin et al., 2005; Driscoll et al., 1998).

With the increase of Th2-type cytokines, an increased B cell distribution was observed, as shown in Fig. 5. Furthermore, B cell activation enhanced the production of IgE, as shown in Fig. 7, which is very important in allergic responses to xenobiotics. Based on these data, it can be proposed that MWCNTs may exert allergic responses by causing an amplification of IgE production. The rise in pro-inflammatory responses, such as elevation of cytokine levels, NO generation, the increase in the numbers of immune cells, and granuloma formation, all commonly observed in chronic inflammatory disease, were seen in the all of the groups exposed to MWCNTs (Fig. 4). The granuloma formation was dose-dependent at day 1 after instillation and was maximal at day 3 for the group treated with 50 mg/kg MWCNTs. Granuloma formation has also been observed in previous studies in response to treatment with SWCNTs or carbon black nanoparticles (Chou et al., 2008; Koike et al., 2008; Li et al., 2007b). Granuloma formation was also significantly increased in C57BL/6 mice exposed to asbestos (~ 13.3 mg/m ^{3}) for 3 h/day for 3 days and in rats treated with SWCNTs at a dose of 5 mg/kg for 24 h (Rosenthal et al., 1998; Warheit et al., 2004).

The change in lymphocyte phenotypes, observed at day 1 as an increase in B cells (Fig. 5), was maintained until day 14 after instillation. The distribution ratios of B cells in blood were 45.97% in control samples, 89.12% at day 7, and 81.45% at day 14 after instillation. Similarly, these ratios in spleen were 56.97% in controls, 61.40% at day 7 and 72.19% at day 14 after instillation. The distribution of T cells was apparently decreased concomitant with the increase in B cell distribution. In blood, these changes were associated with the time-dependent increase in IgE (Fig. 7). IgE plays an essential role in foreign molecule-induced allergy. The increased level of IgE by MWCNTs may reflect the activation of mast cells. IgE generated in the allergic individuals bind to the corresponding receptor on the surface of the mast cells, which provoke the mast cells to degranulate and release active mediator such as histamine, 5-hydroxytryptamine and some cytokines, leading to a typical hypersensitivity (Sun et al., 2008).

In general, the expression of CD4 and CD8 defines two major functional subpopulations of T lymphocytes, the helper T cells and the cytotoxic T cells. CD4 $^{+}$ T cells generally function as T helper cells (Th) and are class-II restricted, while CD8 $^{+}$ T cells function primarily as T cytotoxic cells (Tc) and are class-I restricted. The ratio of CD4 $^{+}$ and CD8 $^{+}$ T cells is approximately 2:1 in normal human peripheral blood, but it may be significantly altered by inflammation (Pahwa et al., 2008). The ratio of CD4 $^{+}$ and CD8 $^{+}$ in normal mice was about 4.7 both in blood and spleen, but the ratio was decreased by MWCNT treatment, which signifies that pro-inflammatory responses were induced by the nanoparticles (Fig. 6). The meaning of this change caused by MWCNT instillation needs further investigation.

In summary, MWCNT instillation activated alveolar macrophages, resulting in attraction of immune cells to the BAL fluid. Pro-inflammatory cytokines (IL-1, TNF- α , and IL-6),

Th1-type cytokines (IL-12 and IFN- γ), and Th2-type cytokines (IL-4, IL-5, and IL-10) were increased by the immune cells both in BAL fluids and in blood. Differentiation of CD4+ T cell to Th1 cells and Th2 cells were triggered by IL-12 and IL-4, respectively. The elevated numbers of B cells activated by IL-10 were then involved in the production of IgE. Taken together, this combination of responses may have caused the allergic reactions observed in the mice treated with MWCNTs.

Conflict of interest

There are none.

Acknowledgement

This subject was supported by Ministry of Environment as the Eco-technopia 21 project.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tox.2009.02.009.

References

- Barbarin, V., Xing, Z., Delos, M., Lison, D., Huaux, F., 2005. Pulmonary overexpression of IL-10 augments lung fibrosis and Th2 responses induced by silica particles. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 288, 841–848.
- Bastus, N.G., Sanchez-Tillo, E., Pujals, S., Farrera, C., Kogan, M.J., Giralt, E., Celada, A., Lloberas, J., Puentes, V., 2008. Peptides conjugated to gold nanoparticles induce macrophage activation. *Mol. Immunol.*, doi:10.1016/j.molimm.2008.08.277.
- Byun, J.A., Ryu, M.H., Lee, J.K., 2006. The immunomodulatory effects of 3-monochloro-1,2-propanediol on murine splenocyte and peritoneal macrophage function in vitro. *Toxicol. In Vitro* 20, 272–278.
- Chen, F., Sun, S.C., Kuh, D.C., Gaydos, L.J., Demers, L.M., 1995. Essential role of NF- κ B activation in silica-induced inflammatory mediator production in macrophages. *Biochem. Biophys. Res. Commun.* 214, 985–992.
- Cho, W.S., Choi, M., Han, B.S., Cho, M., Oh, J., Park, K., Kim, S.J., Kim, S.H., Jeong, J., 2007. Inflammatory mediators induced by intratracheal instillation of ultrafine amorphous silica particles. *Toxicol. Lett.* 175, 24–33.
- Chou, C.C., Hsiao, H.Y., Hong, Q.S., Chen, C.H., Peng, Y.W., Chen, H.W., Yang, P.C., 2008. Single-walled carbon nanotubes can induce pulmonary injury in mouse model. *Nano Lett.* 8 (2), 437–445.
- Dienz, O., Rincon, M., 2009. The effects of IL-6 on CD4 T cell responses. *Clin. Immunol.* 130, 27–33.
- Driscoll, K.E., Carter, J.M., Howard, B.W., Hassenbein, D., Burdick, M., Kunkel, S.L., Strieter, R.M., 1998. Interleukin-10 regulates quartz-induced pulmonary inflammation in rats. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 275, 887–894.
- Fletcher, M., Miguez-Burbano, M.J., Shor-Posner, G., Lopez, V., Lai, H., Baum, M.K., 2000. Diagnosis of human immunodeficiency virus infection using an immunoglobulin E-based assay. *Clin. Diagn. Lab. Immunol.* 7 (1), 55–57.
- Helland, A., Wick, P., Koehler, A., Schmid, K., Som, C., 2007. Reviewing the environmental and human health knowledge base of carbon nanotubes. *Environ. Health Perspect.* 115 (8), 1125–1131.
- Koike, E., Takano, H., Inoue, K.I., Yanagisawa, R., Kobayashi, T., 2008. Carbon black nanoparticles promote the maturation and function of mouse bone marrow-derived dendritic cells. *Chemosphere* 73, 371–376.
- Lam, C., James, J.T., McCluskey, R., Hunter, R.L., 2004. Pulmonary toxicity of single-wall carbon nanotubes in mice 7 and 90 days after intratracheal instillation. *Toxicol. Sci.* 77, 126–134.
- LeBien, T.W., Tedder, T.F., 2008. B lymphocytes: how they develop and function. *Blood* 112, 1570–1580.
- Li, Z., Huldeman, T., Salmen, R., Chapman, R., Leonard, S.S., Young, S., Shvedova, A., Luster, M.I., Simeonova, P.P., 2007a. Cardiovascular effects of pulmonary exposure to single-wall carbon nanotubes. *Environ. Health Perspect.* 115 (3), 377–382.
- Li, J.G., Li, W.X., Xu, J.Y., Cai, X.Q., Liu, R.L., Li, Y.J., Zhao, Q.F., Li, Q.N., 2007b. Comparative study of pathological lesions induced by multiwalled carbon nanotubes in lungs of mice by intratracheal instillation and inhalation. *Environ. Toxicol.* 22, 415–421.
- Lison, D., Muller, J., 2008. To the editor. *Toxicol. Sci.* 101 (1), 179–180.
- McDonal, J., Mitchell, L., 2008. To the editor. *Toxicol. Sci.* 101 (1), 181–182.
- Mangum, J.B., Turpin, E.A., Matao-Menezes, A., Cesta, M.F., Bermudes, E., Bonner, J.C., 2006. Single-walled carbon nanotube (SWCNT)-induced interstitial fibrosis in the lungs of rats is associated with increased levels of PDGF mRNA and the formation of unique intercellular carbon structures that bridge alveolar macrophages in situ. *Particle Fiber Toxicol.* 3, 15.
- Manosroi, A., Saraphanchotiwitthaya, A., Manosroi, J., 2005. In vitro immunomodulatory effect of *Pouteria cambodiana* (Pierre ex Dubard) Baehni extract. *J. Ethnopharmacol.* 101, 90–94.
- Mitchell, L.A., Gao, J., Wal, R.V., Gigliotti, A., Burchiel, S.W., McDonal, J.D., 2007. Pulmonary and systemic immune response to inhaled multiwalled carbon nanotubes. *Toxicol. Sci.* 100 (1), 203–214.
- Muller, J., Huaux, F., Moreau, N., Misson, P., Heilier, J.F., Delos, M., Arras, M., Fonseca, A., Nagy, J.B., Lison, D., 2005. Respiratory toxicity of multi-wall carbon nanotubes. *Toxicol. Appl. Pharmacol.* 207, 221–231.
- Pahwa, S., Read, J.S., Yin, W., Matthews, Y., Shearer, W., Diaz, C., Rich, K., Mendez, H., Thompson, B., 2008. CD4+/CD8+ T cell ratio for diagnosis of HIV-1 infection in infants: women and infants transmission study. *Pediatrics* 122 (2), 331–339.
- Pryhuber, G.S., Huyck, H.L., Baggs, R., Oberdorster, G., Finkelstein, J.N., 2003. Induction of chemokines by low-dose intratracheal silica is reduced in TNFR1 (p55) null mice. *Toxicol. Sci.* 72, 150–157.
- Rao, C.N.R., Govindaraj, A., 2002. Carbon nanotubes from organometallic precursors. *Acc. Chem. Res.* 35, 998–1007.
- Rosenthal, G.J., Corsini, E., Simeonova, P., 1998. Selected new developments in asbestos immunotoxicity. *Environ. Health Perspect.* 106, 159–169.
- Shimada, A., Kawamura, N., Okajima, M., Kaewamatawong, T., Inoue, H., Morita, T., 2006. Translocation pathway of the intratracheally instilled ultrafine particles from the lung into the blood circulation in mice. *Toxicol. Pathol.* 34 (7), 949–957.
- Shvedova, A.A., Kisin, E.R., Mercer, R., Murray, A.R., Johnson, V.J., Pptapovich, A.I., Tyurina, Y.Y., Gorelik, O., Arepalli, S., Schwegler-Berry, D., Hubbs, A.F., Antonini, J., Evans, D.E., Ku, B., Ramsey, D., Maynard, A., Kagan, V.E., Castranova, V., Baron, P., 2005. Unusual inflammatory and fibrogenic pulmonary responses to single-walled carbon nanotubes in mice. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 289, L698–L708.
- Shvedova, A.A., Kisin, E.R., Murray, A.R., Gorelik, O., Arepalli, S., Castranova, V., Young, S.H., Gao, F., Tyurina, Y.Y., Oury, T.D., Kagan, V.E., 2007. Vitamin E deficiency enhances pulmonary inflammatory response and oxidative stress induced by single-walled carbon nanotubes in C57BL/6 mice. *Toxicol. Appl. Pharmacol.* 221, 339–348.
- Silva, I.A., Graber, J., Nyland, J.F., Silbergeld, E.K., 2005. In vitro HgCl₂ exposure of immune cells at different stages of maturation: effects on phenotype and function. *Environ. Res.* 98, 341–348.
- Strid, J., Callard, R., Strobel, S., 2006. Epicutaneous immunization converts subsequent and established antigen-specific T helper type 1 (Th1) to Th2-type responses. *Immunology* 119, 27–35.
- Sun, P., Li, D., Li, Z., Dong, B., Wang, F., 2008. Effects of glycinin on IgE-mediated increase of mast cell numbers and histamine release in the small intestine. *J. Nutr. Biochem.* 19, 627–633.
- Vairavapandian, D., Vichchulada, P., Lay, M.D., 2008. Preparation and modification of carbon nanotubes: review of recent advances and applications in catalysis and sensing. *Anal. Chim. Acta* 626, 119–129.
- Vendrame, M., Gemma, C., Pennypacker, K.R., Bickford, P.C., Davis, Sanberg, C., Sanberg, P.R., Willing, A.E., 2006. Cord blood rescues stroke-induced changes in splenocyte phenotype and function. *Exp. Neurol.* 199, 191–200.
- Warheit, D.B., Laurence, B.R., Reed, K.L., Roach, D.H., Reynolds, G.A., Webb, T.R., 2004. Comparative pulmonary toxicity assessment of single-wall carbon nanotubes in rats. *Toxicol. Sci.* 77, 117–125.
- Zhu, J., Paul, W.E., 2008. CD4 T cells: fates, functions, and faults. *Blood* 112, 1557–1569.

<Supplementary data>

Table 1. Dimension of MWCNT

ITEMS	M65
Outer diameter	110-170 nm
Inner diameter	<5 nm
Length	5-9 μm
Surface area*	12.83 m ² /g
Carbon content	>90 %

Fig.1

Morphology of MWCNT by TEM

