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Effects of multi-walled carbon nanotubes on a murine allergic airway inflammation model

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ABSTRACT

The development of nanotechnology has increased the risk of exposure to types of particles other than combustion-derived particles in the environment, namely, industrial nanomaterials. On the other hand, patients with bronchial asthma are sensitive to inhaled substances including particulate matters. This study examined the effects of pulmonary exposure to a type of nano-sized carbon nanotube (multi-walled nanotubes: MWCNT) on allergic airway inflammation *in vivo* and their cellular mechanisms *in vitro*. *In vivo*, ICR mice were divided into 4 experimental groups. Vehicle, MWCNT (50 μg/animal), ovalbumin (OVA), and OVA + MWCNT were repeatedly administered intratracheally. Bronchoalveolar lavage (BAL) cellularity, lung histology, levels of cytokines related to allergic inflammation in lung homogenates/BAL fluids (BALFs), and serum immunoglobulin levels were studied. Also, we evaluated the impact of MWCNT (0.1–1 μg/ml) on the phenotype and function of bone marrow-derived dendritic cells (DC) *in vitro*. MWCNT aggravated allergen-induced airway inflammation characterized by the infiltration of eosinophils, neutrophils, and mononuclear cells in the lung, and an increase in the number of goblet cells in the bronchial epithelium. MWCNT with allergen amplified lung protein levels of Th cytokines and chemokines compared with allergen alone. MWCNT exhibited adjuvant activity for allergen-specific IgG_1 and IgE. MWCNT significantly increased allergen (OVA)-specific syngeneic T-cell proliferation, particularly at a lower concentration *in vitro*. Taken together, MWCNT can exacerbate murine allergic airway inflammation, at least partly, via the promotion of a Th-dominant milieu. In addition, the exacerbation may be partly through the inappropriate activation of antigen-presenting cells including DC.

Introduction

Bronchial asthma is recognized as a chronic type of airway inflammation with an associated hyperresponsiveness. Intensive research has accumulated evidence regarding the molecular mechanisms for therapeutic targets against the pathology; in contrast, relatively fewer investigations on the (environmental) exacerbating factors have been conducted. However, the latter type of research should be focused on, since morbidity due to bronchial asthma has markedly increased over the past few decades and the large environmental changes occurring throughout the world may be a causal factor (Umetsu et al., 2002; Matsui et al., 2008).

Atmospheric inhalable particulate matter (PM) is one of the important environmental factors leading to the aggravation of asthma and an increase in patient numbers (Leikauf, 2002). In particular, during PM-polluting events, asthmatic subjects reportedly exhibit increases in respiratory symptoms, bronchoconstriction, medication use, bronchial hyperreactivity, and emergency care visits (Schwartz et al., 1993; Boezen et al., 1998). Compatible with epidemiological studies, we and others have experimentally demonstrated that exposure to diesel exhaust or diesel exhaust particles (DEP; representative constituents in $PM_{2.5}$) exacerbate allergic asthma *in vivo* (Takano et al., 1997, 1998; Hao et al., 2003; Dong et al., 2005; Matsumoto et al., 2006). Extensive efforts are being made by several governments to reduce atmospheric PM in the environment throughout the world.

To date, in contrast, the development of nanotechnology has increased the exposure risk to other types of particles in addition to combustion-derived ones, namely, engineered nanomaterials (Oberdorster et al., 2005). As these materials have become more widespread, many questions have arisen regarding the effects they may have on the environment. In fact, previous reports have shown that the full, or even partial, impact of manufactured nanomaterials on health and the environment has yet to be explored in depth (Borm, 2002; Colvin, 2003; Guzman et al., 2006; Nel et al., 2006). It has been reported that nanomaterial exposure itself induces lung inflammation

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(Warheit et al., 2004, 2006; Shvedova et al., 2005; Chen et al., 2006). We previously demonstrated that industrial carboneous particles (carbon black [CB] nanoparticles) enhance allergic asthma hallmarks (Inoue et al., 2005, 2006c, 2007b), suggesting that these inhalable nano-level materials/particles may also promote the pathophysiology. However, the facilitating effects of nanomaterials on subjects with predisposing inflammatory disorders including asthma have not been sufficiently examined.

Among these nanomaterials, carbon nanotubes (CNT) are unique. CNT possess significant characteristics in size (1–20 nm width, and many microns in length), strength, and surface chemistry (Mitchell et al., 2007). Notably, their length/width (aspect) ratios of >1000, reactive surface chemistry, and/or poor solubility raise concerns linked to previous experiences involving hazardous fibers, including asbestos. CNT are being applied in wide-ranging areas from the semiconductor industry to drug delivery and super-light and strong composite materials (de Jonge and Bonard, 2004), and, simultaneously, levels are implicated to be increasing, in particular in the indoor environment (Murr et al., 2005). Furthermore, there is *in vivo* data implicating CNT in the induction of inflammatory lung disorders (Muller et al., 2005; Shvedova et al., 2005).

In this study, we examined the effects of multi-walled CNT (MWCNT), a type of CNT, on allergic airway inflammation *in vivo* and the underlying cellular mechanisms in the context of the maturation/activation/function of antigen-presenting cells (APC) and the allergen-specific T-cell response (using *ex vivo*-generated murine dendritic cells [DC] *in vitro*).

Materials and methods

Animals. Male ICR mice 6–7-wk-of-age (weighing 29 to 33 g: Japan Clea Co., Tokyo, Japan) were used for the *in vivo* studies; mice 11–15-wk-of-age (38–42 g) were used for *in vitro* studies. 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, and fed a commercial diet (Japan Clea Co.) and given water ad libitum.

MWCNT. Two types of MWCNT, purchased from Bussan Nanotech Research (Ibaraki, Japan) and SES Research (TX), respectively, were used in this study. We previously reported the characterization of MWCNT (XNRIWMVT-7, Lot# 05072001K28) prepared by a catalytic chemical vapor deposition method (Kim et al., 2005) from Bussan Nanotech Research (Hirano et al., 2008). In brief, the nominal characteristics of the MWCNT were as follows: average diameter, 67 nm; surface area, 26 m^2/g; carbon purity, 99.79 wt.%; fiber length, not specified. The precise length distribution of the MWCNT varies from 3 to approximately 30 μm when the fiber suspension was filtered through a Nuclepore® filter with a pore size of 3 μm. According to the manufacturer's disclosing information (http://www.sesres.com/index.asp), MWCNT from SES Research were formed in the arc process and ranged from 2–20 nm in diameter, and 100 nm to several microns long, with 5–20 graphitic layers. The MWCNT contain 40–50% nanotubes with the remaining material consisting of Amorphous carbon and other carbon nano-particles. The catalytic iron is entrapped within the CNT structure and does not leach out under neutral pH conditions. The amount of metallic impurities of both types of MWCNT was analyzed using nitric acid dissolution and inductively coupled plasma-atomic emission spectrometry (ICP-AES), performed according to the method described previously (Okamoto, 1987) and shown in Table 1. Both MWCNT were autoclaved at 250 °C for 2 h before use, and the suspension was sonicated for 3 min using an Ultrasonic Disrupter (UD-201; Tomy Seiko, Tokyo, Japan) immediately before exposure. LPS activity in the autoclaved MWCNT, determined by the Limulus Amebocyte Lysate assay (Seikagaku-kogyo, Tokyo, Japan), was lower than the assay's detection limit (0.001 EU/mg CNT). It has been demonstrated that well-dispersed CNT are less toxic than agglomerated or pelleted CNT in a mesothelioma cell line (Wick et al., 2007); thus, the degree of agglomeration is critical in evaluation of the adverse effects of CNT. Serum (Lam et al., 2004) or Tween 80 (Warheit et al., 2004) has been used to disperse CNT for *in vivo* studies, and polyoxyethlene sorbitan monooleate (PS80 or Tween 80) (Wick et al., 2007) or Pluronic surfactant (Cherukuri et al., 2004) may be proper dispersing media for *in vitro* studies. Therefore, we used Tween 80 for dispersing these MWCNT. Nonetheless, CNT occur, at least in part, in agglomerated forms due to van der Waals forces, no matter what concentration of detergent is used.

Table 1
Multi-walled nanotubes (MWCNT) characterization.

Element	ng/mg CNT	
	Bussan Nanotech Research	SES Research
A1	<300	<400
As	(20)	ND
Cd	(10)	ND
Co	ND	(10)
Cr	19.5	ND
Cu	(10)	19.2
Fe	3633	146
K	ND	ND
Mg	20.1	26.2
Mn	(10)	ND
Mo	ND	ND
Ni	(20)	(20)
Pb	65.7	(100)
Ti	12.5	27.0
V	(10)	ND
Y	ND	ND
Zn	32.3	284

ND: Undetectable value.
( ): Semi quantitative value.

In vivo studies

Study protocol. Mice were divided into 4 experimental groups (Fig. 1). The vehicle group received phosphate-buffered saline (PBS) at pH 7.4 (Invitrogen Co., Carlsbad, CA) containing 0.05% Tween 80 (Nacalai Tesque, Kyoto, Japan) once a week for 6 wk. The OVA group received 1 μg of OVA (Grade IV: Sigma Chemical, St. Louis, MO) dissolved in the same vehicle every 2 wk and only PBS containing 0.05% Tween 80 every another 2 wk for totally 6 wk (Fig. 1). The MWCNT group received (25 or) 50 μg of MWCNT suspended in the same vehicle every week for 6 wk. The OVA + MWCNT group received combined treatment using the same protocol as for the OVA and MWCNT groups ($n = 12$–13 in each group).

Vehicle, MWCNT, OVA, or OVA + MWCNT were suspended in 0.1-ml aliquots, and inoculations were delivered by the intratracheal route through a polyethylene tube, under anesthesia with 4% halothane (Takeda Chemical Industries, Osaka, Japan). We previously examined the effects of diesel exhaust-derived particles or carbon nanoparticles on allergic airway inflammation at a dose of 50 or 100 μg/animal *in vivo* (Inoue et al., 2005, 2006c; Yanagisawa et al., 2006). Based on (and referred to) the previous studies from our laboratory, we chose the dosage of 25 and 50 μg/body of MWCNT.

The animals were euthanized and studied 24 h after the final intratracheal administration, with bronchoalveolar lavage (BAL) cellularity, lung histology, protein levels of cytokines and chemokines in the lung tissue supernatants, and systemic Ig production then being analyzed. The studies adhered to the National Institutes of Health guidelines for the experimental use of animals according to the Institutional Animal Care and Use Committee (IACUC: www.iacuc.org). All animal studies were approved by the Institutional Review Board.

BAL process. BAL and cell counts were conducted as previously described ($n = 8$ for each group for each experiment) (Takano et al.,

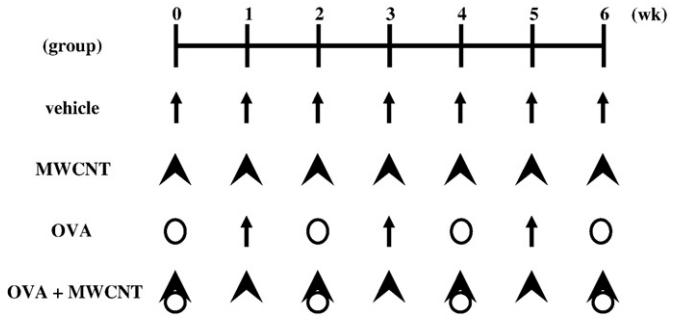

Fig. 1. Protocol for *in vivo* study. MWCNT: multi-walled carbon nanotubes, OVA: ovalbumin.

2002; Inoue et al., 2006a, 2006b, 2006c; Yanagisawa et al., 2006). After the BAL procedure, the lungs were removed, snap-frozen in liquid nitrogen, and stored at −80 °C until assays of cytokines and chemokines. On the other hand, all the BAL fluid (BALF) supernatants were then analyzed for the (cytokine) protein assay ($n=8$ in each group).

Histologic evaluation. After exsanguinations, the lungs were fixed and all the sections (at 4 μm) were prepared. For histologic studies, all the sections were then stained with hematoxylin and eosin or periodic acid-Schiff (PAS), as previously described (Takano et al., 1997, 2002; Inoue et al., 2009a).

Morphometric analysis of the numbers of polymorphonuclear leukocytes (PMNs), mononuclear cells (MONs), and goblet cells around the airways. All the sections of the lungs were stained with Diff-Quik to quantify the numbers of infiltrated PMNs. The length of the basement membrane of airways was measured using a videomicrometer (Olympus, Tokyo, Japan) in each sample slide. The number of PMNs and MONs around the airways was counted with a micrometer under oil immersion. The results are expressed as the number of inflammatory cells per millimeter of basement membrane, as described previously (Takano et al., 1997; Inoue et al., 2005).

To quantify goblet cells, all the sections of the lungs were stained with PAS. The number of goblet cells in the bronchial epithelium was counted by employing a micrometer. The results are expressed as the number of goblet cells per millimeter of basement membrane, as described previously (Inoue et al., 2005).

Quantitation of cytokine and chemokine protein levels in the lung tissue and BALF supernatants. The frozen lungs (after lavaging) were homogenized as described previously (Inoue et al., 2006a and 2006b). ELISAs for tumor necrosis factor (TNF)-α (Endogen, Cambridge, MA), interleukin (IL)-1β (R&D Systems, Minneapolis, MN), IL-4 (Amersham, Buckinghamshire, UK), IL-5 (Endogen), IL-6 (Invitrogen Co.), IL-13 (R&D Systems), IFN-γ (Endogen), IL-18 (MBL, Nagoya, Japan), IL-33 (R&D Systems), eotaxin (R&D Systems), thymus and activation-regulated chemokine (TARC: R&D Systems), macrophage-derived chemokine (MDC: R&D Systems), macrophage chemoattractant protein (MCP)-1 (R&D Systems), and keratinocyte-derived chemoattractant (KC: R&D Systems) in the lung tissue homogenates/BALFs were conducted according to the manufacturer's instructions. All values are expressed in pg/total lung supernatants ($n=8$ in each group for each protocol).

Allergen-specific Ig determination. Allergen (OVA)-specific IgG_1 or IgE antibodies were measured by ELISA using sera obtained from each mouse. OVA-specific IgG_1 were measured according to the methods employed in our previous reports (Inoue et al., 2005; Yanagisawa et al., 2006) ($n=12$–13 in each group). OVA-specific IgE were measured using an ELISA kit (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan) according to the manufacturer's instructions ($n=12$–13 in each group).

In vitro studies

Preparation and generation of bone marrow-derived dendritic cells (BMDC). The preparation of bone marrow cells from naïve ICR mice was conducted as previously described (Koike et al., 2008a and 2008c). BMDC were differentiated using a modified protocol of Lutz et al. (1999), as previously conducted (Koike et al., 2008c).

Exposure to MWCNT. On Day 8 of their culturing, the immature BMDC (as stimulators of the lymphocyte reaction) were treated with 50 μg/ml mitomycin C (Kyowa Hakko Kirin Co., Ltd., Tokyo, Japan) for 20 min in a water bath at 37 °C before exposure to MWCNT, as previously described (Koike et al., 2008a and 2008c). The cells were then washed three times with R10 (RPMI 1640 medium [GIBCO BRL, Eggenstein, Germany] supplemented with 10% heat-inactivated fetal bovine serum [FBS; Dainippon Sumitomo Pharma Co., Ltd.], 100 U/ml penicillin, 100 μg/ml streptomycin [Sigma Chemical], and 50 μM 2-mercaptoethanol [GIBCO BRL]). Cells (2.4×10^6) were then placed in 6-well plates, and exposed to different concentrations of MWCNT (0.1–1 μg) in vehicle (2.4 ml of R10 medium, containing 10 ng/ml recombinant mouse granulocyte macrophage-colony stimulating factor [GM-CSF; Sigma Chemical] plus 0.05% Tween 80) or vehicle alone (control) for 24 h at 37 °C in an atmosphere of 5% CO_2/95% air.

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); co-stimulatory molecules: CD80 (16-10A1, PE-conjugated, BD Biosciences Pharmingen), CD86 (GL1, PE-conjugated, BD Biosciences Pharmingen), DC markers: CD11c (HL3, PE-conjugated, BD Biosciences Pharmingen), DEC-205 (NLDC-145, PE-conjugated, Cedarlane Labs, Ontario, Canada). Immunostaining was performed as previously described (Koike et al., 2008a and 2008b), and flow cytometry was performed using a FACSCalibur (Becton, Dickinson and Company, NJ). Fluorescence data are expressed as the percentage of positive cells. This experiment was repeated three times using three animals in each experiment.

Preparation of allergen-sensitized T-cells and antigen-specific lymphocyte reaction. OVA-sensitized T-cells were derived from a pool of spleens from OVA-sensitized syngeneic (ICR) mice, as previously described (Koike et al., 2008a and 2008c). Thereafter, OVA-specific T-cells (2×10^5) were co-cultured with BMDC (5×10^3) in the presence of OVA (40 μg) in 96-well flat-bottom plates for 4 days, and T-cell proliferation was then measured using a Cell-Proliferation-ELISA Kit (Roche Molecular Biochemicals, Mannheim, Germany), as previously described (Koike et al., 2008c). This experiment was repeated twice using three animals in each experiment.

Statistical analysis. Data are reported as the mean ± SE. Differences between experimental groups were determined using analysis of variance (Stat view version 4.0; Abacus Concepts, Inc., Berkeley, CA) followed by the post-hoc test (Fisher's protected least significant difference test). Significance was assigned to *P*-values smaller than 0.05.

Results

Effects of MWCNT (from Bussan Nanotech Research) on lung immune cell profiles

We investigated the cellular profile of BALF and showed representative data (50 μg/animal of MWCNT; Fig. 2). The number of total cells was significantly greater in the MWCNT group, OVA group, or OVA + MWCNT group than in the vehicle group ($P<0.05$

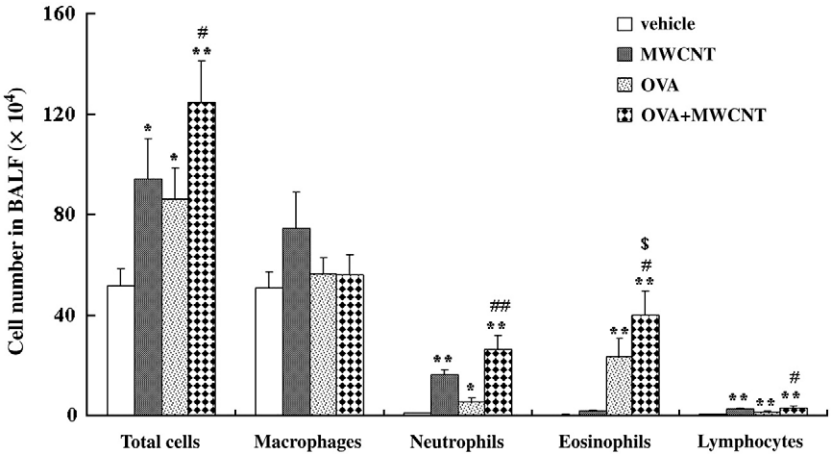

Fig. 2. Cellularity in bronchoalveolar lavage fluid (BALF) after intratracheal challenge. Twenty-four hours after the final intratracheal administration of the vehicle, multi-walled carbon nanotubes (MWCNT [50 μg/treatment]), ovalbumin (OVA), or OVA + MWCNT over a period of 6 wk, lungs were lavaged for the analysis of BALF. Total cell counts were determined in fresh fluid specimens using a hemocytometer. Differential cell counts were assessed in cytologic preparations stained with Diff-Quik. Results are means ± SE ($n = 8$ in each group). $^{*}P<0.05$ vs. vehicle group, $^{**}P<0.01$ vs. vehicle group, $^{\#}P<0.05$ vs. OVA group, $^{\#\#}P<0.01$ vs. OVA group, $^{\$}P<0.01$ vs. MWCNT group.

for MWCNT and OVA, $P<0.01$ for OVA + MWCNT). The number was greater in the OVA + MWCNT group than in the MWCNT group (N. S.) or OVA group ($P<0.05$).

Exposure to OVA or OVA + MWCNT significantly increased the number of eosinophils as compared with vehicle exposure ($P<0.01$). The number was significantly greater in the OVA + MWCNT group than in the MWCNT group ($P<0.01$) or OVA group ($P<0.05$). The number of lymphocytes was greater in the MWCNT group, OVA group or OVA + MWCNT group than in the vehicle group ($P<0.01$). The number was greater in the OVA + MWCNT group than in the OVA group ($P<0.05$) or the MWCNT group (N. S.). The number of neutrophils was greater in the MWCNT group ($P<0.01$), OVA group ($P<0.05$), or OVA + MWCNT group ($P<0.01$) than in the vehicle group. The number was higher in the OVA + MWCNT group than in the OVA group ($P<0.01$) or MWCNT group (N. S.). The number of macrophages did not significantly differ among the experimental groups. In experiments using 25 μg/animal of MWCNT, the data are similar to those using 50 μg/animal, although the level of significance was not marked except for eosinophil number ($P<0.01$, OVA + MWCNT vs. OVA or MWCNT) (data not shown).

Effects of MWCNT on allergen-related histological changes in the lung

We evaluated lung specimens stained with hematoxylin and eosin 24 h after the final intratracheal instillation (Fig. 3). No pathological change was seen in lungs obtained from the vehicle group. The infiltration of neutrophils was moderately increased in the lungs of hosts in the MWCNT group or OVA group. On the other hand, the infiltration of eosinophils was moderate in lungs from the OVA group. Combined treatment with OVA + MWCNT appeared to worsen PMN (mainly neutrophils and eosinophils) and MON (mainly lymphocytes) sequestration into the lung parenchyma, as compared with OVA treatment alone.

To quantify the infiltration of inflammatory leukocytes around the airways, we expressed the number of these cells per length of basement membrane of the airways. The number of PMNs was greater in the MWCNT group (N. S.), OVA group ($P<0.05$), or OVA + MWCNT group ($P<0.01$) than in the vehicle group. The number was greater also in the OVA + MWCNT group than in the OVA group or MWCNT group ($P<0.01$). The number of MONs was larger in the MWCNT group, OVA group ($P<0.05$), or OVA + MWCNT group ($P<0.01$) than in the vehicle group. The number was also higher in the OVA + MWCNT group than in the MWCNT group ($P<0.05$) or OVA group (N. S.).

MWCNT potentiates goblet cell hyperplasia

To evaluate airway epithelial injury and the hypersecretion of mucus, lung sections were stained with PAS (Fig. 4). MWCNT or OVA alone significantly induced goblet cell hyperplasia in the airway as compared with the vehicle. However, the phenomenon had markedly progressed in the OVA + MWCNT group compared to the other groups. Semi-quantitative analyses also showed that MWCNT or OVA alone significantly increased the number of goblet cells as compared with the vehicle ($P<0.01$). The number was significantly greater in the OVA + MWCNT group than in the vehicle group ($P<0.01$), OVA group ($P<0.05$), or MWCNT group (N. S.).

Impact of MWCNT on local expressions of cytokines and chemokines in the presence of allergen

We quantified protein levels of cytokines such as IL-4, IL-5, IL-13, IFN-γ, IL-18, and IL-33 (Table 2), and chemokines such as eotaxin, TARC, MDC, MCP-1, and KC (Table 3) related to allergic inflammation in the lung tissue homogenates and proinflammatory cytokines such as IL-1β, TNF-α, and IL-6 in the BALFs (Table 4). As for cytokine and chemokine profiles in the lung tissue homogenates, OVA ($P<0.05$) or OVA + MWCNT ($P<0.01$) exposure increased the level of IL-4 as compared with vehicle challenge. The level was greater in the OVA + MWCNT group than in the OVA group or MWCNT group ($P<0.01$). The levels of IL-5 and IL-13 were significantly greater in the MWCNT group, OVA group, or OVA + MWCNT group than in the vehicle group ($P<0.01$). The levels were further higher in the OVA + MWCNT group than in the OVA group (N. S.) or MWCNT group ($P<0.01$). The level of IFN-γ was significantly greater in the MWCNT group or OVA + MWCNT group than in the vehicle group ($P<0.01$). Although the level was significantly larger in the OVA + MWCNT group than in the OVA group ($P<0.01$), it tended to be lower in the OVA + MWCNT group than in the MWCNT group (N. S.). The level of IL-18 was significantly higher in the MWCNT group or OVA + MWCNT group than in the vehicle group ($P<0.01$). The level was greater in the OVA + MWCNT group than in the OVA group ($P<0.01$). The level of IL-33 was significantly greater in the MWCNT group, OVA group, or OVA + MWCNT group than in the vehicle group ($P<0.01$). The level was greater in the OVA + MWCNT group than in the OVA group ($P<0.01$). Eotaxin level was greater in the MWCNT group, OVA group, or OVA + MWCNT group than in the vehicle group ($P<0.01$).

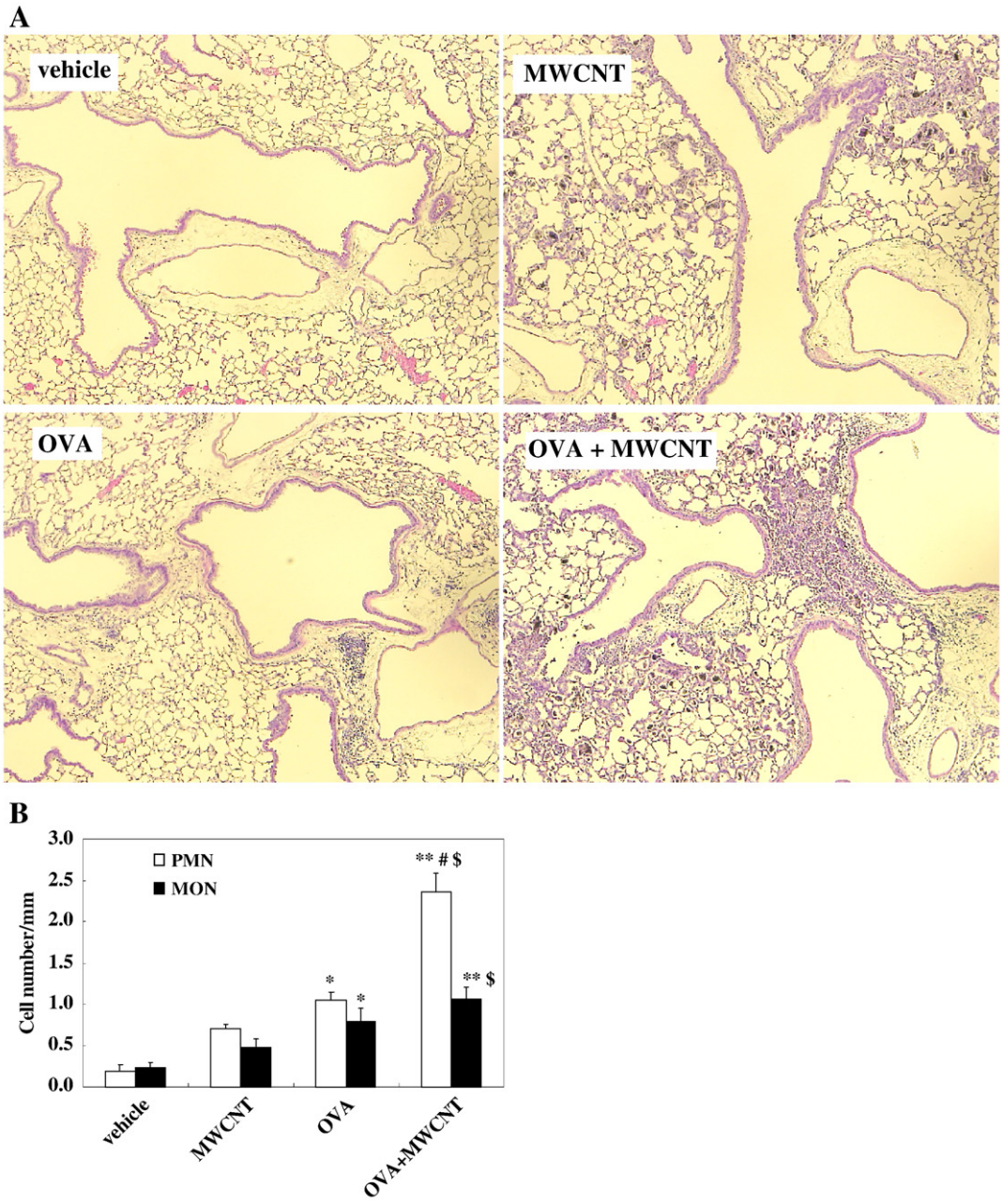

Fig. 3. Representative histological findings of hematoxylin and eosin-stained lungs obtained from the vehicle, MWCNT (50 μg/treatment), OVA, or OVA + MWCNT groups 24 h after the final intratracheal administration (A), and semi-quantitative analysis of polymorphonuclear leukocyte (PMN) and mononuclear cell (MON) sequestration into the lung (B; $n = 4$–5 in each group). Animals received intratracheal instillation of vehicle, MWCNT, OVA, or OVA + MWCNT over a period of 6 wk. Lungs were removed and fixed 24 h after the last intratracheal administration. Original magnification × 100. PMN and MON infiltration was assessed by averaging the number of PMNs and MONs per millimeter of basement membrane. Values are the mean ± SE in each group. $^{*}P<0.05$ vs. vehicle group, $^{**}P<0.01$ vs. vehicle group, $^{\#}P<0.05$ vs. OVA group, $^{\$}P<0.05$ vs. MWCNT group.

The level was further greater in the OVA + MWCNT group than in the OVA group (N. S.) or MWCNT group ($P<0.01$). The levels of TARC, MDC, MCP-1, and KC were higher in the MWCNT group, OVA group, or OVA + MWCNT group than in the vehicle group ($P<0.01$ except for KC vs. OVA [$P<0.05$]). The level was greater in the OVA + MWCNT group than in the OVA group or MWCNT group ($P<0.01$ except for MDC vs. MWCNT [$P<0.05$]).

IL-1β and IL-6 levels (Table 4) in the BALFs were greater in the MWCNT group ($P<0.05$) or OVA + MWCNT group ($P<0.01$) than in the vehicle group. The levels were further significantly greater in the OVA + MWCNT group than in the MWCNT group ($P<0.05$) or OVA group ($P<0.01$). TNF-α level was not different among the experimental groups (data not shown).

Effects of MWCNT on allergen-specific production of Igs

We measured allergen-specific IgG_1 and IgE levels (Fig. 5). These levels were significantly greater in the OVA group or OVA + MWCNT group than in the vehicle group ($P<0.01$). The titers were further greater in the OVA + MWCNT group than in the OVA group ($P<0.01$ for IgG_1 and $P = 0.07$ for IgE) or MWCNT group ($P<0.01$).

Action of MWCNT on the expression of cell surface molecules in BMDC

Immature BMDC were exposed to MWCNT for 24 h. The expression of MHC Class II, CD86, CD80, CD11c, and DEC205 (Fig.

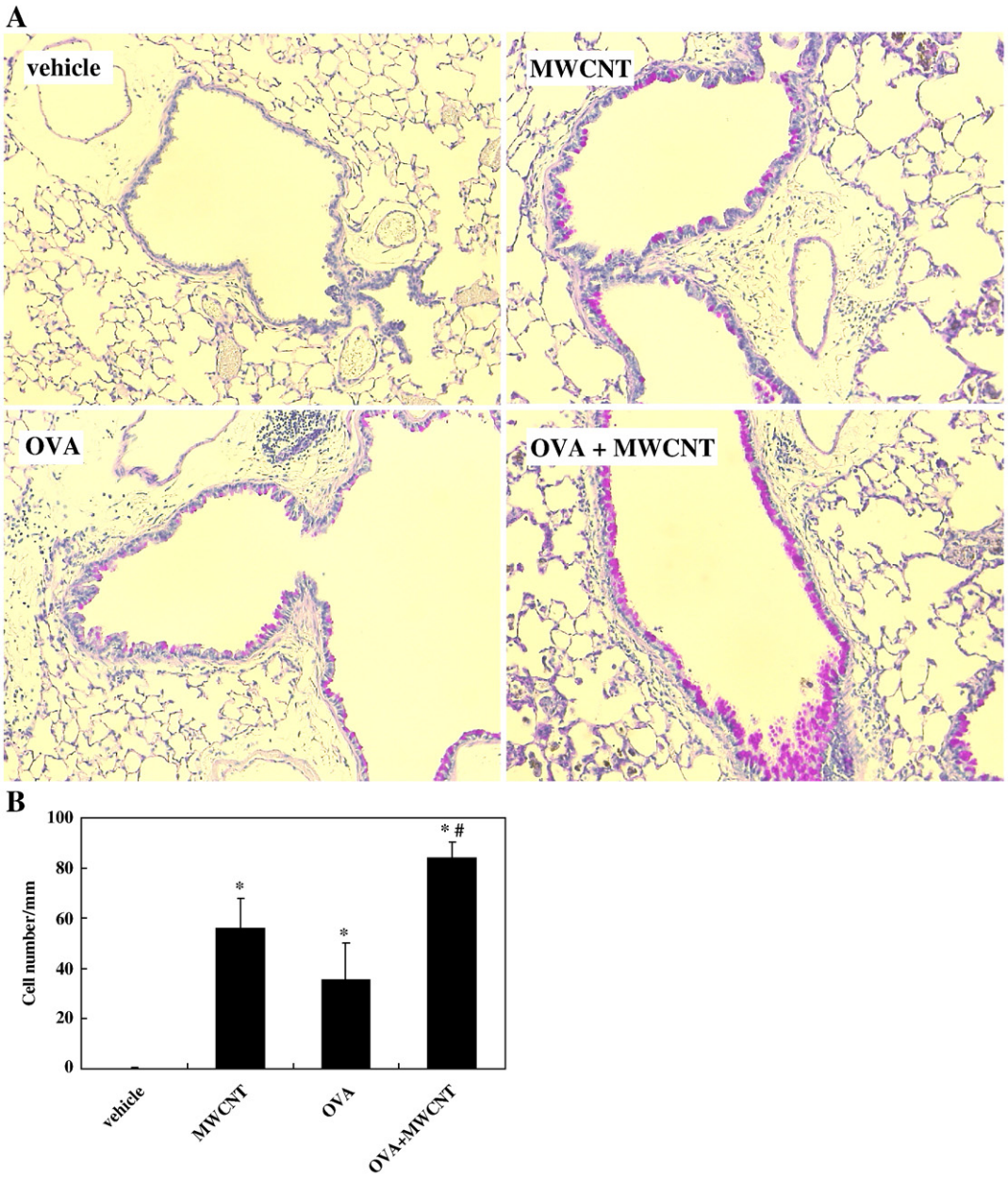

Fig. 4. Representative histological findings of the periodic acid-Schiff (PAS)-stained lungs obtained from the vehicle, MWCNT (50 μg/treatment), OVA, or OVA + MWCNT group (A), and semi-quantitative analysis of the number of goblet cells in the lung (B; $n = 4$–5 in each group). Animals received intratracheal instillation of vehicle, MWCNT, OVA, or OVA + MWCN over a period of 6 wk. Lungs were removed and fixed 24 h after the last intratracheal administration. Original magnification × 200. The number of goblet cells in the bronchial epithelium was expressed as the number of PAS-positive cells per millimeter of basement membrane. Values are the mean ± SE in each group. $^{*}P<0.01$ vs. vehicle group, $^{\#}P<0.05$ vs. OVA group.

6) on the cells was analyzed on the maturation/activation of BMDC. The percentage of MHC Class II^{+} cells (Fig. 6A) and $CD86^{+}$ cells (Fig. 6B) was significantly increased by the addition of 0.1 and 1 μg/ml of MWCNT. Also, the percentage of MHC Class $II^{+}CD86^{+}$ cells (Fig. 6C) was significantly increased by the addition of 0.1 μg/ml of MWCNT. The expression (or co-expression) of other

Table 2
Protein levels of cytokines in lung tissue supernatants after the final intratracheal challenge.

Treatment	IL-4	IL-5	IL-13	IFN-γ	IL-18	IL-33
	(pg/total lung tissue supernatants)					(ng/Total lung tissue supernatants)
Vehicle	14.9 ± 2.59	84.3 ± 0.64	2.09 ± 0.85	1724.0 ± 125.0	1167.8 ± 101.5	8.0 ± 1.4
MWCNT	17.9 ± 3.91	27.1 ± 1.8**	74.1 ± 9.58**	4295.6 ± 264.9**	3716.4 ± 335.1**	264.8 ± 18.7**
OVA	32.2 ± 4.95*	196.0 ± 54.6**	235.8 ± 56.7**	2028.4 ± 128.5	922.8 ± 70.2	35.1 ± 6.2**
OVA + MWCNT	96.8 ± 12.3**#$	385.9 ± 58.9**$	343.8 ± 61.2**$	3628.6 ± 124.9**#	2972.1 ± 399.8**#	275.6 ± 26.6**#

Results are expressed as the mean ± SE ($n = 8$ in each group). $^{*}P<0.05$ vs. vehicle group, $^{**}P<0.01$ vs. vehicle group, $^{\#}P<0.01$ vs. ovalbumin (OVA) group, $^{\$}P<0.01$ vs. multi-walled nanotubes (MWCNT) group.

Table 3
Protein levels of chemokines in lung tissue supernatants after the final intratracheal challenge.

Treatment	Eotaxin	TARC	MDC	MCP-1	KC
	(pg/total lung tissue supernatants)				
Vehicle	51.8 ± 6.60	37.8 ± 6.39	59.9 ± 6.33	33.4 ± 4.22	51.8 ± 18.0
MWCNT	950.8 ± 99.9**	248.8 ± 11.1**	248.4 ± 16.1**	620.2 ± 46.2**	565.3 ± 50.6**
OVA	1204.6 ± 272.3**	162.8 ± 36.2**	138.6 ± 31.8**	117.5 ± 17.2*	200.3 ± 47.2*
OVA + MWCNT	2061.6 ± 287.6**$	526.7 ± 43.7**#$	410.4 ± 24.3**#$	1617.7 ± 231.8**#$	1151.3 ± 105.2**#$

Results are expressed as the mean ± SE ($n = 8$ in each group). *$P<0.05$ vs. vehicle group, **$P<0.01$ vs. vehicle group, #$P<0.01$ vs. OVA group, $$P<0.05$ vs. MWCNT group, $$$P<0.01$ vs. MWCNT group.

molecules tended to increase after the addition of MWCNT, although the difference did not reach significance.

Effect of MWCNT on antigen-specific syngeneic T-cell-stimulating capacity of BMDC

BMDC function was assessed via their capacity to stimulate antigen-specific syngeneic T-cell proliferation (Fig. 7). The proliferation of T-cells (responder cells) was increased only by the addition of BMDC (stimulator cells). The reaction was significantly increased by exposure to 0.1 μg/ml of MWCNT ($P<0.01$). Treatments with higher concentrations of the nanotubes failed to modulate the activity from levels seen with the untreated cells.

Effects of another type of MWCNT (from SES Research) on allergic airway inflammation and reactivity in vivo

To assess the specificity of MWCNT, we investigated the impacts of other types of MWCNT (50 μg/animal) on the asthma model in the context of BAL cellularity (S-Fig. 1) and specific Ig production (S-Fig. 2). The number of total cells was significantly greater in the OVA + MWCNT group than in the vehicle group ($P<0.01$). The number was greater in the OVA + MWCNT group than in the MWCNT group (N. S.) or OVA group ($P<0.05$). Exposure to OVA or OVA + MWCNT significantly increased the number of eosinophils as compared with vehicle exposure ($P<0.01$). The numbers were significantly greater in the OVA + MWCNT group than in the MWCNT group ($P<0.01$) or the OVA group ($P<0.05$). The number of lymphocytes was greater in the MWCNT group, OVA group, or OVA + MWCNT group than in the vehicle group ($P<0.01$). The number was greater in the OVA + MWCNT group than in the OVA group ($P<0.01$) or the MWCNT group (N. S.). The number of neutrophils was greater in the MWCNT group, OVA group, or OVA + MWCNT group than in the vehicle group ($P<0.01$). The number was higher in the OVA + MWCNT group than in the OVA group ($P<0.01$) or the MWCNT group (N. S.). The number of macrophages was significantly greater in the OVA + MWCNT group than in the vehicle group, OVA group or MWCNT group ($P<0.01$).

For Ig production, allergen-specific IgG_1 and IgE were significantly greater in the OVA or OVA + MWCNT group than in the vehicle group ($P<0.01$). The titers were further greater in the OVA + MWCNT group than in the OVA group ($P<0.05$) or MWCNT group ($P<0.01$). Accordingly, these results indicate that MWCNT have considerable facilitating properties on OVA-related airway inflammation with specific Ig productions likely regardless of their characteristics (external and internal components).

Table 4
Protein levels of cytokines in bronchoalveolar lavage fluid (BALF) after the final intratracheal challenge.

Treatment	IL-1β	IL-6
	(pg/mg BALF)	
Vehicle	0 ± 0	51.1 ± 7.7
MWCNT	8.5 ± 2.7*	177.8 ± 34.2*
OVA	0 ± 0	37.6 ± 7.8
OVA + MWCNT	32.3 ± 8.1**#$	443.2 ± 79.5**#$

Results are expressed as the mean ± SE ($n = 8$ in each group).
*$P<0.05$ vs. vehicle group, **$P<0.01$ vs. vehicle group,
#$P<0.01$ vs. OVA group, $$P<0.05$ vs. MWCNT group.

Discussion

The present study has shown that MWCNT administered by an intratracheal route deteriorate allergic airway inflammation in mice, which is characterized by the infiltration of inflammatory PMNs in both the bronchoalveolar spaces and lung parenchyma. MWCNT also significantly induce and moderately exacerbate goblet cell metaplasia in the presence or absence of allergen. The enhancing effects are associated with the increased lung levels of proinflammatory cytokines such as IL-1β and IL-6, Th2 cytokines such as IL-4, IL-5, and IL-13, Th2 chemokines such as TARC and MDC, and chemokines related to the migration/maturation of T lymphocytes and neutrophils such as MCP-1 and KC, respectively. Also, MWCNT exhibit adjuvant activity for the allergen-specific production of IgG_1 and IgE. Finally, MWCNT can partially promote the maturation/activation and function of BMDC *in vitro*.

Despite being less studied, we and others have examined the effects of exposure to several types of nanoparticle/material on allergic asthma *in vivo*, showing that carbon black nanoparticles have the potential to advance allergic asthma *in vivo* (de Haar et al., 2005; Inoue et al., 2005). In addition, nano-sized TiO_2 (14–29 nm in size) have reportedly more prominent adjuvant effects on allergen-specific responses with Ig production than fine (250–260 nm in size) ones *in vivo* (de Haar et al., 2006). On the other hand, we have recently found that latex nanoparticles (15–100 nm in size) do not facilitate allergic asthma employing the same protocol as in the present study (Inoue et al., 2009a). These previous observations suggest that each nano-level

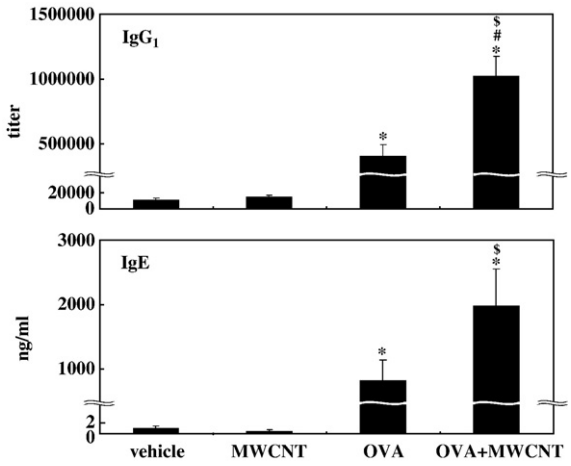

Fig. 5. Allergen-specific IgG_1 and IgE values. Four groups of mice were intratracheally administered vehicle, MWCNT (50 μg/treatment), OVA, or a combination of OVA + MWCNT over a period of 6 wk. Serum samples were retrieved 24 h after the last intratracheal instillation. Antigen-specific IgG_1 and IgE were analyzed using ELISA. Results are expressed as the mean ± SE ($n = 12$–13 in each group). *$P<0.01$ vs. vehicle group, #$P<0.01$ vs. OVA group, $$P<0.01$ vs. MWCNT group.

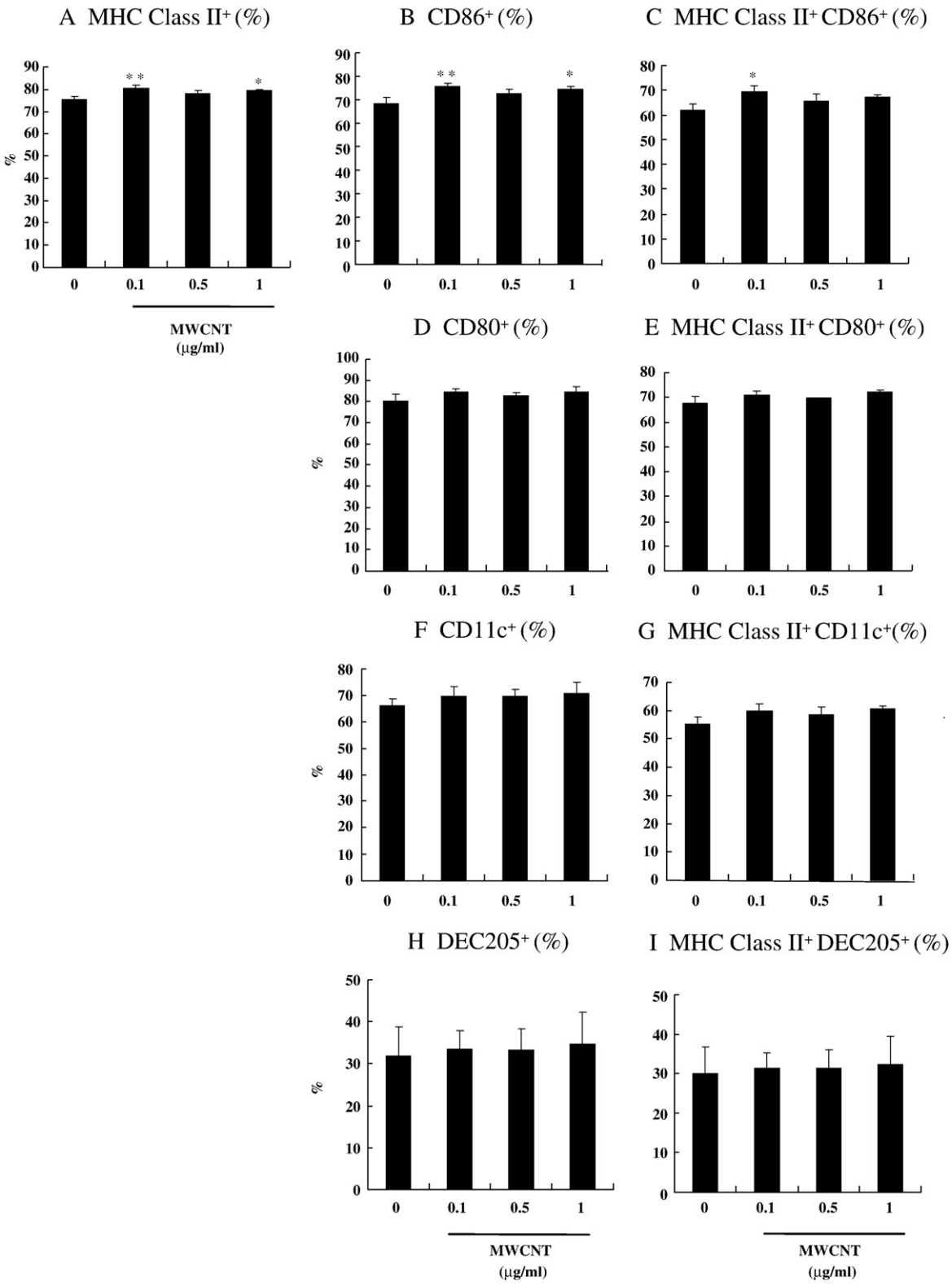

Fig. 6. Effects of MWCNT on the expression of molecules related to antigen presentation in bone marrow-derived dendritic cells (BMDC). Immature BMDC from ICR mice were exposed to MWCNT (0.1–1 μg/ml) as described in Materials and methods. After exposure, the expression of molecules related to antigen presentation was analyzed by flow cytometry. Percentages of each cell type in the BMDC population are shown. (A) MHC Class II$^+$, (B) CD86$^+$, (C) MHC Class II$^+$CD86$^+$, (D) CD80$^+$, (E) MHC Class II$^+$CD80$^+$, (F) CD11c$^+$, (G) MHC Class II$^+$CD11c$^+$, (H) DEC205$^+$, (I) MHC Class II$^+$DEC205$^+$. Data represent the mean ± SE of three animals from one experiment, representative of three experiments ($^*P<0.05$, $^{**}P<0.01$ vs. corresponding control).

particle/material has different effects on this pathology, which may depend on their characteristics. Based on the current data, we propose that MWCNT exposure is a risk factor for the development/aggravation of allergic asthma. Further, enhanced lung expression of allergy-related molecules including IL-4, IL-5, IL-13 (Th2 cytokines), TARC, and MDC (Th2 chemokines) could be important contributors to the

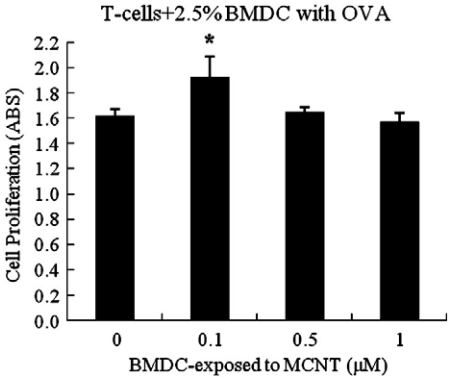

Fig. 7. Effects of MWCNT on antigen-specific syngeneic T-cell-stimulating capacity of BMDC. Immature BMDC from ICR mice were exposed to MWCNT (0.1–1 μg/ml) in the same manner as shown in Fig. 5. Splenic T-cells (2×10^5) from OVA-sensitized ICR mice were co-cultured with BMDC (5×10^3, to be present at 2.5% of the splenic T-cell level) from ICR mice for 4 days. Thereafter, T-cell proliferation was evaluated. Data represent the mean ± SE of three animals from one experiment, representative of two experiments ($^*P<0.05$ vs. corresponding control).

etiology of the aggravation. Regarding the outcome, the unique characteristics of the fibrous shape may be responsible for the allergic asthma-promoting effects, since two kinds of MWCNT showing different characteristics of their components similarly enhance allergic airway inflammation with significance. However, these are not described in reports on the adverse effects of pathogenic fibers such as asbestos on allergic diseases in humans and *in vivo*, although fibrous materials (asbestos and its component silica) have been implicated in the promotion of asthma pathobiology in terms of inducing/enhancing mucin production dependent on the *mclca3* gene (Sabo-Attwood et al., 2005) and local Th2 cytokine expression (Choi et al., 2009) *in vivo*, and IL-4 production with a consequent Th2 response including IgE synthesis in humans (Lange et al., 1995). These implications can be partly linked to our present findings, in which MWCNT alone induced significant goblet cell hyperplasia and amplified IL-5 and IL-13 expressions in the lung. Thus, the (nano-level) material size in addition to its unique (fibrous) shape might be important to cause induction/facilitation in allergic phenotypes. Of course, it is also possible that attached metals and chemical components in these MWCNT potentiated the induction/facilitation in association with their nano-size and unique shape.

The cellular points and underlying molecular mechanistic pathways in which MWCNT potentiate allergic airway inflammation remain obscure. Because the measured cytokine and chemokine levels in lung homogenates (and partially in BALF supernatants) representing products of multiple cell types exist in the distal airways, e.g., macrophages (IL-18, MDC, KC, IL-1β, IL-6), epithelial cells (KC, MCP-1, eotaxin, IL-1β, IL-6), neutrophils (IL-1β, MCP-1), T cells (IL-4, IL-5, IL-13, IL-33, IFN-γ, TARC, MDC, IL-6), fibroblasts (eotaxin, KC, IL-1β, IL-6), and endothelial cells (TARC, KC, IL-1β, IL-6), the data suggest the widespread influence of MWCNT in the presence or absence of allergen. From a few *in vitro* studies, furthermore, CNT has been shown to activate the signaling of mitogen-activated protein kinases, nuclear factor-κB, activator protein-1, and protein serine–threonine kinase, common mechanisms for cytokine expansion *in vitro* (Manna et al., 2005; Pacurari et al., 2008) and *in vivo* (Chou et al., 2008), as is often the case with asbestos (Mossman et al., 1997; Cacciotti et al., 2005). Thus, it appears plausible that the inhaled MWCNT cooperating with allergen synergistically exacerbate allergic inflammation/damage through direct/indirect effects on the local pulmonary microenvironment interacting with these effector cells, possibly through several signaling pathways related to inflammation.

APC including DC, macrophages, and B cells play fundamental roles in the pathogenesis of asthma (Upham, 2003; Kato et al., 2006). APC-mediated phagocyting and subsequent antigen presentation for T-cells is the first step in both primary and secondary immune responses. In particular, DC are recognized to be professional APC, exhibiting a potent antigen-presenting ability (Rossi and Young, 2005). The maturation of DC is an important step for polarized antigen presentation and the consequent Th response (de Jong et al., 2005). We have previously shown that CB nanoparticles activate BMDC employing the same protocol as in the present study *in vitro* (Koike et al., 2008a), and enhance intrapulmonary APC activation (Koike et al., 2008b) commensurate with asthma pathophysiology *in vivo* (Inoue et al., 2005), fully indicating that the effects of nanoparticles on allergic asthma can be mediated, at least in part, through the activation of APC. In addition, we have recently shown that DEP significantly enhance BMDC-mediated antigen-presenting activity at certain concentrations *in vitro* (Inoue et al., 2009b). Furthermore, fibrous particles such as asbestos reportedly activate APC *in vitro* (Hamilton et al., 2004). Taken together, it is likely that inhalable PM or fibrous materials modify APC characterization/function. In the present study, exposure to MWCNT significantly increased the percentage of MHC Class II^+, $CD86^+$, and MHC Class II^+CD86^+ cells, although the response did not clearly exhibit dose-dependency. Furthermore, MWCNT exposure significantly amplified OVA-specific syngeneic T-cell reactivity mediated by BMDC. Therefore, the present *in vitro* results suggest that MWCNT can markedly regulate the phenotypic and functional activation of BMDC at certain doses. In addition, of note, the levels of mediators produced/released by phagocytes (macrophage and dendritic cell: IL-1β) in the BALF supernatants were greater in the OVA + MWCNT group than in the OVA group *in vivo*. Therefore, it is possible that the exacerbating effects of MWCNT on allergic pathophysiology can account for, at least partly, the amplified maturation/activation of APC including DC.

Allergic asthma represents a Th2-dominant response. However, environmental toxicants sometimes deteriorate allergic asthma models not through augmentation of the Th2 response alone, but also through the activation of both Th1 and Th2 responses (Takano et al., 1997; Larsen et al., 2002 and 2004; Yanagisawa et al., 2006; Inoue et al., 2007a). This phenomenon might be explained by their make-up, comprising a complicated mixture of large amounts of organic and inorganic components which stimulate/activate various immune responses. Furthermore, asbestos reportedly can up-regulate both Th1 and Th2 immunity, likely through APC activation *in vitro* (Hamilton et al., 2004). In accordance with these previous findings, in the present study, the combined *in vivo* exposure to OVA + MWCNT profoundly increased the production of both Th1 (IgG_{2a}: data not shown)- and Th2 (IgG_1 and IgE: Fig. 4)-associated Igs as compared with that to OVA alone. Alternate, MWCNT alone might trigger the mixed Th milieu, which subsequently assists in the hyperproduction of allergen-specific Igs, since lung expression levels of IFN-γ as well as IL-5 and IL-13 were elevated by MWCNT exposure in the presence or absence of allergen. On the other hand, a recent report showed that IL-18 and allergen (OVA) activate Th1 cells to produce Th2 cytokines and induce "Th1"-type allergic asthma characterized by the predominance of neutrophilic airway inflammation *in vivo* (Hayashi et al., 2007). Of interest, our recent study has shown that latex nanoparticles amplified lung expressions of IFN-γ (unpublished observation) and IL-18, and subsequent exacerbation neutrophilic airway inflammation related to allergen (OVA) (Inoue et al., 2009a). Similarly in the present study, MWCNT significantly elevated IL-18 expression in the lung in the presence or absence of allergen, aggravated neutrophilic airway inflammation *in vivo*, and tended to increase cytokine production in culture supernatants from MWCNT-exposed BMDC cocultured with T cells *in vitro* (data not shown). Thus, one can lead to the scenario that MWCNT in the presence of allergen independently triggers IL-18 production/release in the airway and subsequent Th1-type airway inflammation, which culminate in the promotion of allergic pathology via a mixed pattern of "Th1/Th2 immune responses", or alternatively, a

"mixed Th response". Nonetheless, to clarify the issue, future systematic investigations should be carried out using *in vitro* and *ex vivo* assays focusing on Th cells.

During the preparation of our manuscript, a study on the effects of MWCNT on another murine asthma model was reported (Ryman-Rasmussen et al., 2009). In their study, MWCNT did not exacerbate allergic airway inflammation with specific Th responses, but augmented airway fibrosis accompanied by inflammation. The results differing from ours, are interesting and may be explained by significant differences from our study regarding many points such as the materials and methods, e.g., characteristics of the MWCNT, mouse strain (C57BL/6), experimental asthma (sensitized and challenged by intraperitoneal injection and inhalation of OVA), MWCNT exposure style (nebulizer, timing), and point of termination (28 days after sensitization). On the other hand, they did not examine the impacts on airway hyperresponsiveness (we examined and found that MWCNT slightly strengthened allergen-related cholinergic airway hyperresponsiveness; data not shown), whereas, we neither compared fibrous changes in the lung structure nor examined local and systemic levels of growth factors including transforming growth factor-β in the current model; thus, one cannot view these two sets of results as opposite outcomes. In other words, taken these positive findings from us and Ryman-Rasmussen's group into consideration, it is proposed that MWCNT can affect/facilitate both "early (induction, effector)" and "late (airway remodeling)" phases of allergic asthma.

In sum, the present study showed that MWCNT can exacerbate allergic airway inflammation with augmented humoral immunity. The enhancing effects are concomitant with the increased lung expression of Th cytokines and chemokines related to inflammatory leukocyte recruitment/infiltration. These results suggest that inhalable MWCNT may become one of the important environmental risk factors of allergic asthma.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.taap.2009.04.003.

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