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Original Contribution

Repeated pulmonary exposure to single-walled carbon nanotubes exacerbates allergic inflammation of the airway: Possible role of oxidative stress

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ABSTRACT

The development of nanotechnology has increased the risk of environmental exposure to types of particles other than those derived from combustion, namely, industrial nanomaterials. Patients with bronchial asthma are sensitive to inhaled substances, including particulate matter. This study examined the effects of pulmonary exposure to a type of nano-sized carbon nanotube (single-walled nanotubes (SWCNT)) on allergic airway inflammation and sought their cellular mechanisms. In the in vivo experiments, ICR mice were divided into four experimental groups that were repeatedly administered vehicle, SWCNT (50 μg/animal), ovalbumin (OVA; an allergen), or OVA + SWCNT through an intratracheal route and thereafter assayed. SWCNT aggravated allergen-induced pulmonary inflammation with mucus hyperplasia. SWCNT with allergen amplified lung protein levels of T helper (Th) cytokines and chemokines related to allergy and exhibited adjuvant activity for allergen-specific IgG_1 (and IgE) compared with allergen alone. SWCNT accentuated the level/activity of oxidative stress-related biomarkers in the airways in the presence of allergen. In vitro, SWCNT can partially promote/strengthen the maturation/activation/function of bone marrow-derived dendritic cells (DC). Together, these results suggest that SWCNT can exacerbate murine allergic airway inflammation via enhanced activation of Th immunity and increased oxidative stress. In addition, this exacerbation may be partly through the inappropriate activation of antigen-presenting cells, including DC.

Allergic asthma is a chronic inflammatory disorder of the airway in which several cell types play a role [1]. Recruited inflammatory cells in the airway have the capacity to produce reactive oxygen species (ROS), which play a crucial role in the initiation/development/deterioration of airway inflammation [2]. Accordingly, disruptors of the redox balance as well as inflammatory status might be risky for asthma pathology. Atmospheric inhalable particulate matter (PM) is one of the important environmental factors leading to the aggravation of allergic airway diseases, including bronchial asthma, and an increase in their patient numbers [3]. It has also been epidemiologically recognized that, during PM-polluting events, asthmatic subjects exhibit increases in respiratory symptoms, bronchoconstriction, medication use, bronchial hyperreactivity, and emergency room visits [4,5]. We and others have experimentally shown that exposure to diesel exhaust or diesel exhaust particles (DEP), representative constituents in PM with a diameter less than 2.5 μm, exacerbates allergic asthma models, with significant adjuvant activity for T helper (Th)-related Ig synthesis in vivo [6–10]. Furthermore, we have also demonstrated that airway inflammation and hyperresponsiveness by DEP involve a marked generation of ROS such as superoxide and nitric oxide (NO) in the airways [11].

Nanotechnology is now advancing at an incredible pace, such that it has created an alternative industrial revolution over the past few years [12]. Consistent with this, the use of engineered nanomaterials has been rapidly increasing in commercial applications. As these materials have become more widespread, many questions have arisen regarding the effects they may have on the environment as alternative inhalable toxicants. Carbon nanotubes (CNT) are one of the most attractive nanomaterials because of their unique physical and chemical characteristics, including their size (1–20 nm width and many micrometers in length), strength, and surface chemistry [13,14]. 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 diverse areas ranging from the semiconductor industry to composite

Abbreviations: ROS, reactive oxygen species; PM, particulate matter; DEP, diesel exhaust particles; Th, T helper; CNT, carbon nanotubes; SWCNT, single-walled CNT; MWCNT, multiwalled CNT; APC, antigen-presenting cells; DC, dendritic cells; CNI, Carbon Nanotechnologies, Inc.; PBS, phosphate-buffered saline; OVA, ovalbumin; CB, carbon black; BAL, bronchoalveolar lavage; BALF, BAL fluid; PAS, periodic acid–Schiff; IL, interleukin; IFN, interferon; TARC, thymus and activation-regulated chemokine; MDC, macrophage-derived chemokine; MCP, macrophage chemoattractant protein; KC, keratinocyte-derived chemoattractant; MPO, myeloperoxidase; LPO, lipid hydroperoxide; HO, heme-oxygenase; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; NT, nitrotyrosine; BMDC, bone marrow-derived DC; ANOVA, analysis of variance; PMN, polymorphonuclear leukocytes.

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materials, electrochemical devices, and biomedical applications [15], and, simultaneously, the levels are reportedly increasing, particularly in indoor environments [16]. CNT show marked cytotoxicity, such as the induction of oxidative stress, cellular proliferation, and apoptosis/necrosis in several types of cell, including keratinocytes, fibroblasts, and macrophages, in vitro [16–24]. In addition, there is in vivo evidence implicating CNT in the induction of inflammatory lung disorders including oxidative tissue injury [25,26]. Currently, however, less research is being conducted to examine the effects of CNT on predisposing respiratory disorders in view of them as an exacerbating factor.

In this study, we examined the effects of single-walled CNT (SWCNT), a type of CNT, on allergic inflammation, a "PM-sensitive respiratory state," in the airway in vivo and sought the underlying cellular mechanisms in vivo and in vitro in the context of Th immunity, the maturation/activation/function of antigen-presenting cells (APC) (using ex vivo-generated murine dendritic cells (DC)), and, in particular, the oxidative potential.

Materials and methods

Animals

Male ICR mice 6–7 weeks of age (weighing 29 to 33 g; Japan Clea Co., Tokyo, Japan) were used for the in vivo studies; mice 11–15 weeks of age (38–42 g) were used for the in vitro studies. The 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.

SWCNT

Two types of SWCNT, purchased from Carbon Nanotechnologies, Inc. (CNI; Houston, TX, USA) and SES Research (Houston, TX, USA), respectively, were used in this study. SWCNT from CNI (Lot R0554) were produced using the high-pressure CO disproportionation process technique, employing CO in a continuous-flow gas phase as the carbon feedstock and $Fe(CO)_5$ as the iron-containing catalyst precursor. The SWCNT ranged from 0.8 to 1.2 nm in diameter and 100 to 1000 nm in length and contained <35% (by weight) iron. According to the information supplied by the manufacturer (http://www.sesres.com/index.asp), SWCNT from SES Research were formed in the arc process and ranged from 1.2 to 2 nm in diameter and 1 to 15 μm in length. The SWCNT contain up to 75% nanotubes, with the remaining material consisting of amorphous carbon and other carbon nanoparticles. The catalytic iron is entrapped within the CNT structure and does not leach out under neutral pH conditions. The levels of metallic impurities in both types of SWCNT were analyzed using inductively coupled plasma-atomic emission spectrometry after acid digestion of the samples, according to the method described previously [27], and are shown in Table 1. Both types of SWCNT 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. Lipopolysaccharide activity in the autoclaved SWCNT, determined by the *Limulus* amebocyte lysate assay (Seikagaku Biobusiness Corp., 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 [28]; thus, the degree of agglomeration is critical when evaluating the adverse effects of CNT. Serum [29] or Tween 80 [30] has been used to disperse CNT for in vivo studies, and polyoxyethylene sorbitan monooleate (PS80 or Tween 80) [28] or pluronic surfactant [31] may be an appropriate dispersing medium for in vitro studies. Therefore, we used Tween 80 for SWCNT dispersal. Nonetheless, CNT occur, at least in part, in agglomerated forms because of van der Waals forces, regardless of the concentration of the detergent used.

Table 1
Single-walled nanotube characterization

Element	ng/mg CNT	
	CNI	SES Research
Al	660	1,550
As	ND	ND
Cd	ND	ND
Co	4.07	8.00
Cr	ND	(10)[a]
Cu	92.3	658
Fe	233,000	480
K	ND	ND
Mg	2.2	16.4
Mn	15.6	9.30
Mo	ND	3.90
Ni	144	65,800
Pb	ND	ND
Ti	14.5	30.8
V	46.6	21.3
Y	ND	8,570
Zn	46.7	409

ND, nondetectable value.
[a] Semiquantitive value.

In vivo studies

Study protocol

Mice were divided into four experimental groups (Fig. 1). The vehicle group received phosphate-buffered saline (PBS) at pH 7.4 (Invitrogen, Carlsbad, CA, USA) containing 0.05% Tween 80 (Nacalai Tesque, Kyoto, Japan) weekly for 6 weeks. The OVA group received 1 μg of ovalbumin (Grade IV; Sigma Chemical, St. Louis, MO, USA) dissolved in the same vehicle every 2 weeks and only PBS containing 0.05% Tween 80 every other 2 weeks for a total of 6 weeks (Fig. 1). The SWCNT group received 25 or 50 μg of SWCNT suspended in the same vehicle weekly for 6 weeks. The OVA + SWCNT group received combined treatment using the same protocols as for the OVA and SWCNT groups ($n = 12$ or 13 in each group).

Vehicle, SWCNT, OVA, or OVA + SWCNT 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 DEP or carbon black (CB) nanoparticles on allergic airway inflammation at a dose of 50 or 100 μg/animal in vivo [32–34]. Based on the previous studies from our laboratory, we chose the doses of 25 and 50 μg/animal for SWCNT.

The animals were sacrificed and studied 24 h after the final intratracheal administration for bronchoalveolar lavage (BAL) cellularity, lung histology, gene expression analysis in the lung, protein profiles of cytokines and chemokines in the lung tissue supernatants, and systemic (serum) Ig production. 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

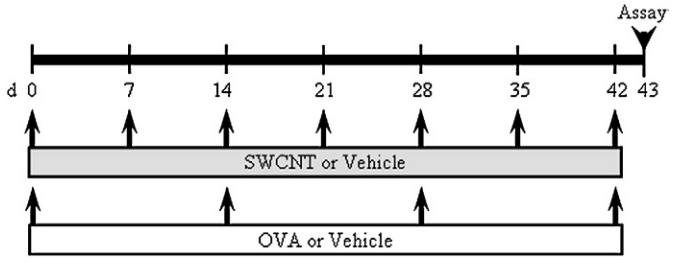

Fig. 1. Experimental design for in vivo study. SWCNT, single-walled carbon nanotubes; OVA, ovalbumin.

(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) [32–35]. After the BAL procedure, the lungs were removed, snap-frozen in liquid nitrogen, and stored at −80 °C until assayed for cytokines and chemokines. All BAL fluid (BALF) supernatants were then analyzed for the (cytokine) protein assay ($n=8$ in each group).

Histologic evaluation

After exsanguination, the lungs were fixed and all sections (at 4 μm) were prepared. For histologic studies, all sections were then stained with hematoxylin and eosin or periodic acid–Schiff (PAS), as previously described [32,34].

Morphometric analysis of the numbers of neutrophils, eosinophils, mononuclear cells, and goblet cells around airways

The length of the basement membrane of airways was measured using a videomicrometer (Olympus, Tokyo, Japan) in each sample slide. The numbers of neutrophils, eosinophils, and mononuclear cells around the airways were counted with a micrometer under oil immersion on all lung sections stained with hematoxylin and eosin. The results are expressed as the number of inflammatory leukocytes per millimeter of basement membrane, as described previously [32].

To quantify mucus hyperplasia, the number of goblet cells in the bronchial epithelium was counted on lung sections stained with PAS. The results are expressed as the number of goblet cells per millimeter of basement membrane, as described previously [32].

Quantitation of cytokine and chemokine protein levels, levels of NO, and lipid peroxide levels in the lung tissue and/or BALF supernatants

The frozen lungs (after BAL) were homogenized as described previously [32,34,35]. ELISAs for interleukin (IL)-1β (R&D Systems, Minneapolis, MN, USA), IL-4 (Amersham, Buckinghamshire, UK), IL-5 (Endogen, Cambridge, MA, USA), IL-6 (Invitrogen), IL-13 (R&D Systems), interferon (IFN)-γ (Endogen), IL-17A (BioLegend, San Diego, CA, USA), IL-23 (R&D Systems), 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), keratinocyte-derived chemoattractant (KC; R&D Systems), and myeloperoxidase (MPO; Hycult Biotechnology, Uden, Netherlands) in the lung tissue homogenates and/or BALF were conducted according to the manufacturer's instructions ($n=8$ in each group for each protocol). Total NO synthesis in the BALF and lipid peroxidation in the lung was measured using the NO assay (Assay Designs, Ann Arbor, MI, USA) and lipid hydroperoxide (LPO) assay (Cayman Chemical, Ann Arbor, MI, USA) kits, respectively, according to the manufacturer's instructions ($n=8$ in each group).

Allergen-specific Ig determination

Allergen (OVA)-specific IgG_1, IgG_{2a}, or IgE antibodies were measured by ELISA using sera obtained from each mouse. OVA-specific IgG_1 and IgG_{2a} were measured according to the methods employed in our previous reports [32,34] ($n=13$ in each group). OVA-specific IgE were measured using an ELISA kit (Dainippon Sumitomo Pharma, Osaka, Japan) according to the manufacturer's instructions ($n=13$ in each group).

Extraction of mRNA and real-time RT-PCR analysis

In another experiment, total RNAs in the lung were extracted with ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. cDNA synthesis was conducted according to the manufacturer's protocol. The quantitation of mRNA expression was carried out employing real-time RT-PCR using the ABI Prism 7000 sequence detection system (TaqMan; Perkin–Elmer, Foster City, CA, USA) according to the manufacturer's instructions. cDNAs were amplified using the thermal profile of 50 °C for 2 min, then 95 °C for 10 min, followed by up to 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Specific primers and probes were obtained from Applied Biosystems. The sequences of Muc5ac, heme-oxygenase (HO)-1, and 18S rRNA, which were purchased from Perkin–Elmer, were not disclosed by the manufacturer. The quantitation of gene expression was derived from the cycle number at which the fluorescent signal crossed a threshold in the exponential phase of the PCR using the standard curve method according to the manufacturer's protocol. The relative quantitation of mRNA was normalized to an endogenous control gene (18S rRNA; $n=6$–8 in each group).

Immunohistochemistry

The expression and localization of 8-hydroxy-2′-deoxyguanosine (8-OHdG), nitrotyrosine (NT), and pentosidine in the lung were detected by immunohistochemistry using anti-8-OHdG polyclonal antibody (concentration as primary antibody: 0.5 μg/ml; Japan Institute for the Control of Aging, Shizuoka, Japan), anti-NT polyclonal antibody (concentration as primary antibody: 5 μg/ml; Upstate Biotechnology, Lake Placid, NY, USA), and anti-pentosidine antibody (concentration as primary antibody: 7.5 μg/ml; Trans Genic, Kumamoto, Japan) with a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's protocol ($n=5$ in each group). For each of the lung specimens, the extent and intensity of staining with both anti-8-OHdG and anti-NT antibodies were graded on a scale of 0–4+ (immunohistochemical score) by two blinded observers on two separate occasions using coded slides, as previously described [32] ($n=5$ in each group).

In vitro studies

Preparation and generation of bone marrow-derived DC (BMDC)

The preparation of bone marrow cells from naïve ICR mice was conducted as previously described [36,37]. BMDC were differentiated using a modified protocol of Lutz et al. [38], as previously conducted [37].

Exposure to SWCNT

On day 8 of culturing, the immature BMDC (as stimulators of the lymphocyte reaction) were treated with 50 μg/ml mitomycin C (Kyowa Hakko Kirin, Tokyo, Japan) for 20 min in a water bath at 37 °C before exposure to SWCNT, as previously described [36,37]. The cells were then washed three times with R10 (RPMI 1640 medium (GIBCO BRL, Eggenstein, Germany) supplemented with 10% heat-inactivated fetal bovine serum (Dainippon Sumitomo Pharma), 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma Chemical), and 50 μM 2-mercaptoethanol (GIBCO BRL)). Cells (5×10^5) were then placed in 24-well plates and exposed to various concentrations of SWCNT (0.1–10 μg/ml) in vehicle (0.5 ml of R10 medium, containing 10 ng/ml recombinant mouse granulocyte macrophage colony-stimulating factor (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.

Fluorescence-activated cell sorting (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, San Jose, CA, USA); costimulatory molecules CD80 (16-10A1, PE-conjugated; BD Biosciences Pharmingen), CD86 (GL1, PE-conjugated; BD Biosciences Pharmingen); and DC markers CD11c (HL3, PE-conjugated; BD Biosciences Pharmingen), DEC205 (NLDC-145, PE-conjugated; Cedarlane Laboratories, ON, Canada). Immunostaining was carried out as previously described [35,36], and flow cytometry was performed using a FACSCalibur (Becton–Dickinson, Rutherford, NJ, USA). Fluorescence data are expressed as the

percentage of positive cells. This experiment was repeated three times using three or four animals in each experiment.

Preparation of allergen-sensitized T cells and the antigen-specific lymphocyte reaction

OVA-sensitized T cells were derived from a pool of spleens from OVA-sensitized syngeneic (ICR) mice, as previously described [36,37]. Thereafter, OVA-specific T cells (2×10^5) were cocultured with BMDC (5×10^3) in the presence of OVA (20 μg) in 200 μl of R10 medium 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 [37]. This experiment was repeated twice using three animals in each experiment.

Statistical analysis

Data are reported as means ± SE. Differences between groups were determined using analysis of variance (ANOVA; StatView version 4.0; Abacus Concepts, Berkeley, CA, USA). If differences between groups were significant ($P<0.05$) on one-way ANOVA, Bonferroni correction was used for multiple comparison.

Results

Effects of SWCNT (from CNI) on lung immune cellular profiles

We investigated the cellular profile of BALF and show representative data (50 μg/animal of SWCNT; Fig. 2A). The numbers of total cells and neutrophils were significantly greater in the SWCNT, OVA, and OVA + SWCNT groups than in the vehicle group ($P<0.05$ for SWCNT and OVA, $P<0.01$ for OVA + SWCNT). The number was greater in the OVA + SWCNT group than in the SWCNT or OVA group ($P<0.05$). Exposure to OVA or OVA + SWCNT significantly increased the number of eosinophils compared with vehicle exposure ($P<0.01$). The number was significantly greater in the OVA + SWCNT group compared to the SWCNT ($P<0.01$) or OVA ($P<0.05$) group. The number of lymphocytes was

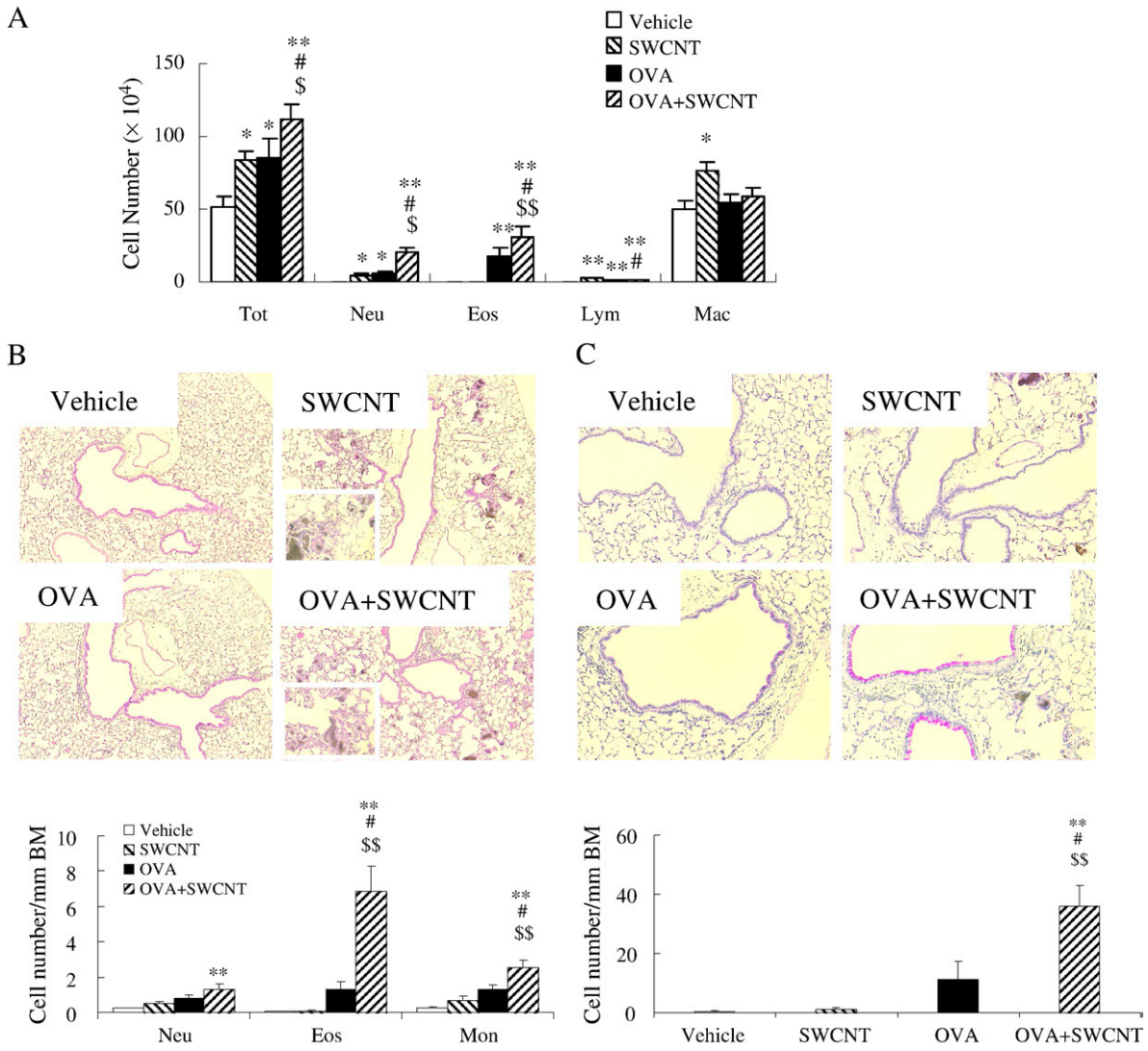

Fig. 2. Effects of SWCNT (from CNI) on inflammatory cell recruitment and mucus hypersecretion in the presence or absence of ovalbumin (OVA). (A) Total (Tot) and differential counts of bronchoalveolar lavage (BAL) cells from each mouse. BAL was performed 24 h after the final intratracheal administration of the vehicle, SWCNT (50 μg/treatment), OVA (1 μg/treatment), or OVA + SWCNT over a period of 6 weeks. Differential cell counts were performed on 500 cells to identify neutrophils (Neu), eosinophils (Eos), lymphocytes (Lym), and macrophages (Mac). Results are means ± SE (n = 8 in each group). (B) Histological examination of lung inflammation conducted using hematoxylin and eosin staining (original magnification × 100, inset × 400) and (C) mucus hypersecretion (original magnification × 200) assessed by periodic acid–Schiff staining of lungs obtained 24 h after the final intratracheal administration of the vehicle, SWCNT, OVA, or OVA + SWCNT. (B and C, bottom) Quantitative analyses of inflammatory leukocyte infiltration and mucus production in lung sections were performed as described under Materials and methods. BM, base membrane; Mon, mononuclear cells. Results are means ± SE ($n = 5$ in each group). *$P<0.05$, **$P<0.01$ vs vehicle, #$P<0.05$ vs OVA, $$P<0.05$, $$$P<0.01$ vs SWCNT.

greater in the SWCNT, OVA, and OVA + SWCNT groups than in the vehicle group ($P<0.01$). The number was greater in the OVA + SWCNT group than in the OVA group ($P<0.05$). The number of macrophages was greater in the SWCNT group than in the vehicle group ($P<0.05$). In experiments using 25 μg/animal of SWCNT, the data were similar to those using 50 μg/animal, although the level of significance was not markedly different except for eosinophil and neutrophil numbers ($P<0.05$, OVA + SWCNT vs OVA or SWCNT; data not shown).

Effects of SWCNT 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. 2B). No pathological change was seen in the lungs obtained from the vehicle group. The infiltration of neutrophils was moderately increased in the lungs of hosts in the SWCNT and OVA groups. On the other hand, the infiltration of eosinophils was moderate in lungs from the OVA group. Combined

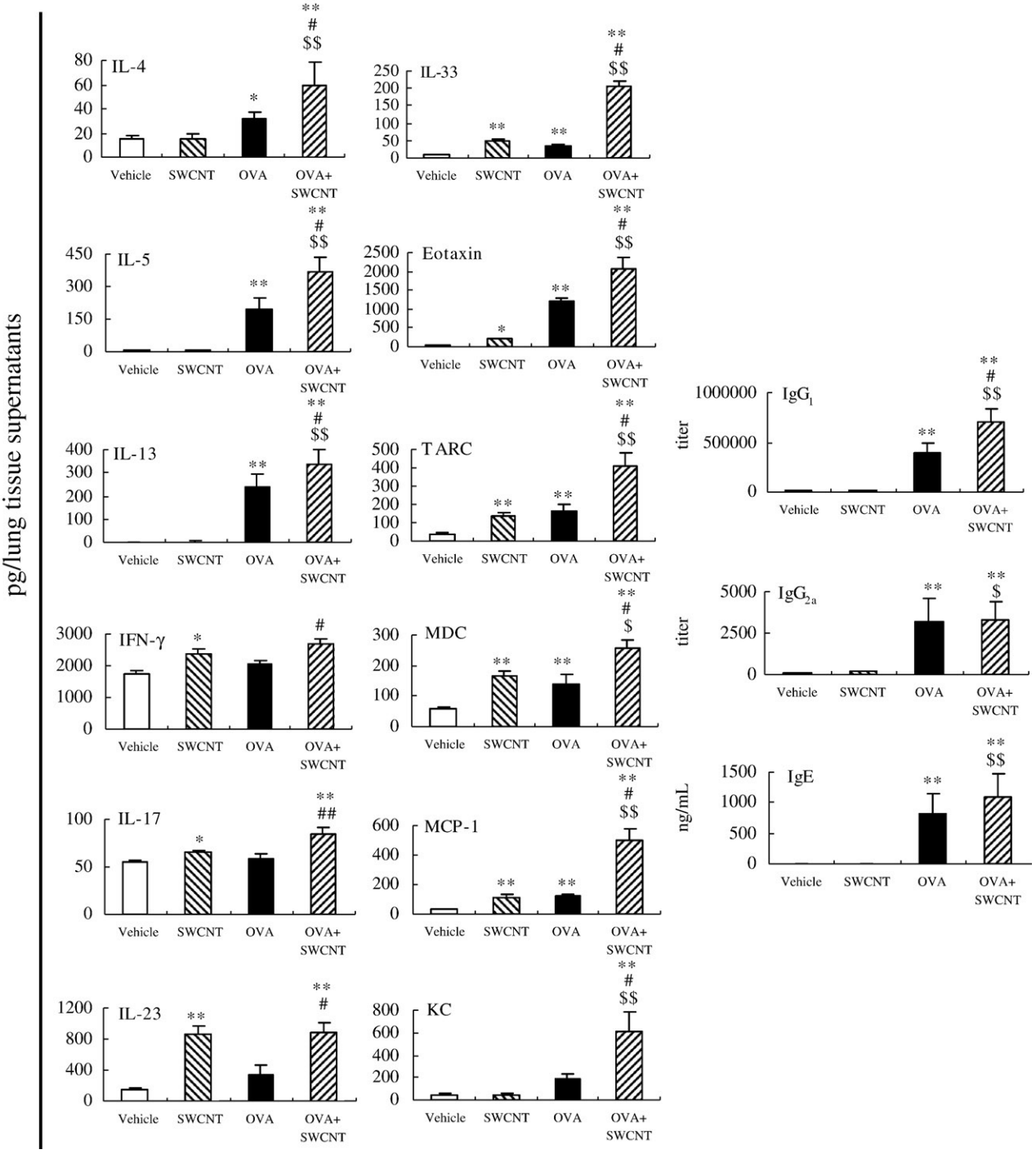

Fig. 3. Effects of SWCNT on lung cytokine and chemokine levels and serum Ig values in the presence or absence of OVA. Four groups of ICR mice were intratracheally administered vehicle, SWCNT, OVA, or a combination of OVA + SWCNT over a period of 6 weeks. Lungs were removed 24 h after the last intratracheal instillation and homogenized; thereafter, cytokine and chemokine protein levels were determined by ELISA. Results are means ± SE ($n = 8$ in each group). Serum samples were retrieved 24 h after the last intratracheal instillation. OVA-specific IgG_1, IgG_{2a}, and IgE were analyzed using ELISA. Results are expressed as the means ± SE ($n = 12$ or 13 in each group). $^{*}P<0.05$, $^{**}P<0.01$ vs vehicle, $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs OVA, $^{\$}P<0.05$, $^{\$\$}P<0.01$ vs SWCNT.

treatment with OVA + SWCNT seemed to worsen polymorphonuclear leukocyte (PMN; mainly neutrophils and eosinophils) and mononuclear cell (mainly lymphocytes) sequestration into the lung parenchyma, compared with OVA treatment alone. Of note, marked granuloma formation was observed in both SWCNT and OVA + SWCNT groups (Fig. 2B, inset), as previously reported by other laboratories [26,29,39].

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 numbers of neutrophils, eosinophils, and mononuclear cells were significantly greater in the OVA + SWCNT group compared to the vehicle group ($P<0.01$). The numbers were also greater in the OVA + SWCNT group than in the SWCNT ($P<0.01$ for eosinophil and mononuclear cells) or OVA ($P<0.01$ for eosinophils, $P<0.05$ for mononuclear cells) group.

SWCNT potentiates mucus hyperplasia

To evaluate mucus hyperplasia, lung sections were stained with PAS (Fig. 2C). OVA moderately and OVA plus SWCNT significantly induced goblet cell hyperplasia in the airway compared with the vehicle. Semiquantitative analyses also showed that OVA + SWCNT significantly increased the number of goblet cells compared with vehicle ($P<0.01$), SWCNT ($P<0.01$), or OVA ($P<0.05$). Further, the transcript level of Muc5ac, an important mucin-producing gene, was significantly higher in the OVA + SWCNT group than in the vehicle ($P<0.01$) and SWCNT ($P<0.05$) groups (see Supplementary Fig. S1).

Impacts of SWCNT on local expression of cytokines and chemokines in the presence of allergen

We quantified protein levels of allergic response-related cytokines, such as IL-4, IL-5, IL-13, IFN-γ, IL-17A, IL-23, and IL-33, and chemokines, such as eotaxin, TARC, MDC, MCP-1, and KC, in the lung-tissue homogenates (Fig. 3) and of inflammatory cytokines, such as IL-1β, IL-6, and IL-23, in the BALF (see Supplementary Table S1). As for cytokine and chemokine profiles in the lung tissue homogenates, SWCNT (IFN-γ, IL-17A, and IL-23) or OVA (IL-4, IL-5, and IL-13) exposure significantly increased the cytokine level compared with vehicle challenge. On the other hand, the levels of IL-33, eotaxin, TARC, MDC, and MCP-1 were significantly greater in both SWCNT and OVA groups. With the exception

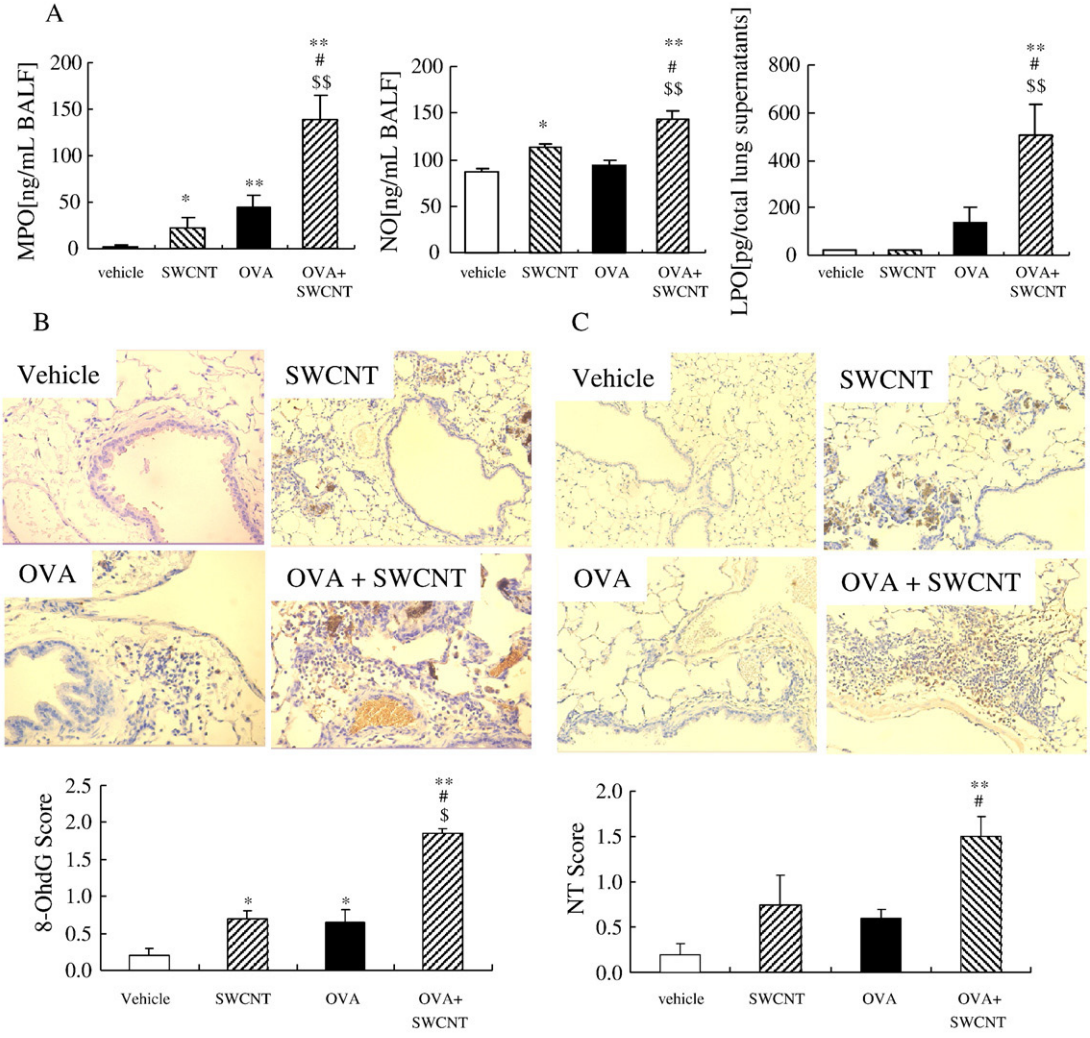

Fig. 4. Effects of SWCNT on oxidative stress-related molecules/adducts in the lung in the presence or absence of OVA. (A) Levels of myeloperoxidase (MPO) and total nitric oxide (NO) in the BAL fluids and lipid peroxide (LPO) in the lung homogenates obtained from the vehicle, SWCNT, OVA, and OVA + SWCNT groups. BAL was performed 24 h after the final intratracheal administration. After BAL, the lungs were removed. MPO, NO, and LPO values were determined using measuring kits. Results are means ± SE ($n=8$ in each group). Immunohistological staining of (B) 8-hydroxy-2′-deoxyguanosine (8-OHdG) and (C) nitrotyrosine (NT) in the lung from each group. Lungs were removed 24 h after the last intratracheal instillation of vehicle, SWCNT, OVA, or combination of OVA + SWCNT over a period of 6 weeks. Original magnification × 300. (B and C, bottom) Scoring analyses of immunoreactivity in lung sections were performed as described under Materials and methods ($n=5$ in each group). Results are expressed as the means ± SE. $^{*}P<0.05$, $^{**}P<0.01$ vs vehicle, $^{\#}P<0.05$ vs OVA, $^{\$}P<0.05$, $^{\$\$}P<0.01$ vs SWCNT.

of IFN-γ and IL-23, the levels of almost all of the cytokines tested were significantly higher in the OVA + SWCNT group relative to the other three test groups. The levels of IL-1β and IL-6 in the BALF (Table S1) were increased in the OVA + SWCNT group compared to the vehicle ($P<0.05$ for IL-1β, $P<0.01$ for IL-6), OVA ($P<0.01$), or SWCNT ($P<0.05$) group. On the other hand, the level of IL-23 in the BALF was significantly higher in the SWCNT ($P<0.01$), OVA ($P<0.05$), and OVA + SWCNT ($P<0.01$) groups compared to the vehicle group and was significantly higher in the OVA + SWCNT group compared to the OVA group ($P<0.01$), but the level was comparable between the SWCNT and the OVA + SWCNT groups.

Effects of SWCNT on the allergen-specific production of Ig's

We measured allergen-specific IgG_1, IgG_{2a}, and IgE levels (Fig. 3). The levels of allergen-specific IgG_1, IgG_{2a}, and IgE were significantly greater in the OVA and OVA + SWCNT groups compared to the vehicle group ($P<0.01$). Both IgG_1 and IgE were greater in the OVA + SWCNT group than in the OVA ($P<0.05$ for IgG_1) or SWCNT ($P<0.01$) group; however, the IgG_{2a} value was comparable between the two groups.

Effects of SWCNT on allergen-induced oxidative stress in the lung

To elucidate the effects of SWCNT on oxidative stress in the lung, we first measured MPO, NO, and LPO levels in the BALF/lung homogenates (Fig. 4A). MPO activity was higher in the SWCNT ($P<0.05$), OVA ($P<0.01$), and OVA + SWCNT ($P<0.01$) groups than in the vehicle group. The activity was higher in the OVA + SWCNT group than in the SWCNT ($P<0.01$) or OVA ($P<0.05$) group. The total NO level was greater in the SWCNT ($P<0.05$) and OVA + SWCNT ($P<0.01$) groups than in the vehicle group. The level was even higher in the OVA + SWCNT group than in the SWCNT ($P<0.01$) or OVA group ($P<0.05$). The LPO level was significantly greater in the OVA + SWCNT group than in the vehicle ($P<0.01$), SWCNT ($P<0.01$), and OVA ($P<0.05$) groups.

Next, we measured the transcript levels of the HO-1 gene in the lung using real-time RT-PCR (see Supplementary Fig. S2). The HO-1 transcript level was greater only in the OVA + SWCNT group compared to the vehicle, SWCNT, and OVA groups ($P<0.05$).

We next studied immunoreactivity for 8-OHdG (Fig. 4B), NT (Fig. 4C), and pentosidine (see Supplementary Fig. S3) in the lung. In the vehicle group, positive staining with 8-OHdG was barely detectable. SWCNT or OVA treatment induced moderate staining for 8-OHdG. On the other hand, OVA plus SWCNT resulted in intense immunoreactive 8-OHdG staining compared to SWCNT or OVA alone. As typically shown in the OVA + SWCNT group, we identified the expression of 8-OHdG in macrophages phagocytosing SWCNT as well as in PMN. Regarding NT and pentosidine immunoreactivity, the tendency resembled that of 8-OHdG.

We performed morphometric analysis to quantitate the extent and intensity of immunoreactive 8-OHdG among the experimental groups. Compared to vehicle treatment, SWCNT or OVA treatment significantly increased the immunohistochemical score ($P<0.05$). The score was greater in the OVA + SWCNT group than in the vehicle ($P<0.01$), SWCNT ($P<0.05$), and OVA ($P<0.05$) groups. NT immunoreactivity was also greater in the SWCNT and OVA group than in the vehicle group. The reactivity was significantly greater in the OVA + SWCNT group compared to the vehicle ($P<0.01$) or OVA group ($P<0.05$).

Action of SWCNT on functional activation of BMDC in vitro

Immature BMDC were exposed to SWCNT for 24 h. The expression of MHC class II, CD80, CD86, CD11c, and DEC205 on the cells was analyzed in the maturation/activation of BMDC. The percentage of $CD86^+$ cells was significantly increased by the addition of 5 and 10 μg/ml SWCNT (Fig. 5A). The expression (or coexpression) of other molecules tended to be increased by SWCNT exposure, although the difference did not reach

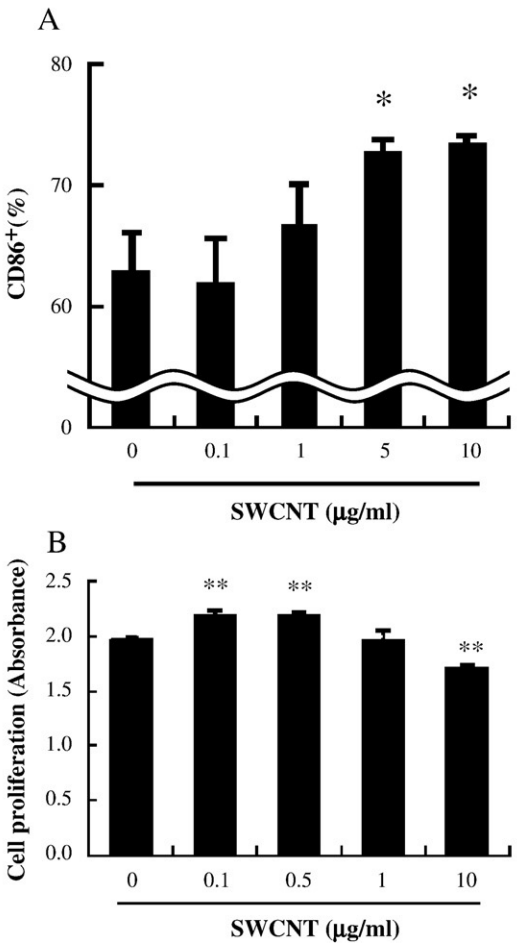

Fig. 5. Effects of SWCNT on the expression of CD86 on bone marrow-generated dendritic cells (BMDC) and their antigen-specific syngeneic T-cell-stimulating capacity. Immature BMDC were exposed to SWCNT (0.1–10 μg/ml) for 24 h as described under Materials and methods. (A) After exposure, the expression of CD86 on the BMDC was analyzed by flow cytometry. Percentages of $CD86^+$ cells in the BMDC population are shown. (B) In another experiment, the BMDC were cocultured with splenic T cells (2×10^5) from OVA-sensitized ICR mice in the presence of OVA (20 μg) in 200 μl of R10 medium for 4 days. Thereafter, T cell proliferation was evaluated. Data represent the means ± SE of three animals from one experiment, representative of two or three experiments (*$P<0.05$, **$P<0.01$ vs corresponding control).

significance (data not shown). Next, BMDC function was assessed via their capacity to stimulate antigen-specific syngeneic T cell proliferation (Fig. 5B). 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 and 0.5 μg/ml SWCNT ($P<0.01$), whereas it was decreased by exposure to 10 μg/ml SWCNT ($P<0.01$). In addition, Th cytokine levels in the culture supernatants showed a tendency similar to that in these T cell proliferation tests (data not shown).

Effects of another type of SWCNT (from SES Research) on allergic airway inflammation in vivo

To assess the specificity of SWCNT, we investigated the impacts of another type of SWCNT (purchased from SES Research; 50 μg/animal) on the allergic airway inflammation model in the context of BAL profile (Fig. 6A), histological findings (Fig. 6B and Supplementary Fig. S4), Th2 cytokine and LPO levels in the lung (Fig. 6C and Supplementary Fig. S5), and specific Ig production (Fig. 6D). As a result, the findings with the SES variant of SWCNT were almost identical to those observed with the SWCNT from CNI except for LPO. Accordingly, these results indicate that SWCNT have considerable

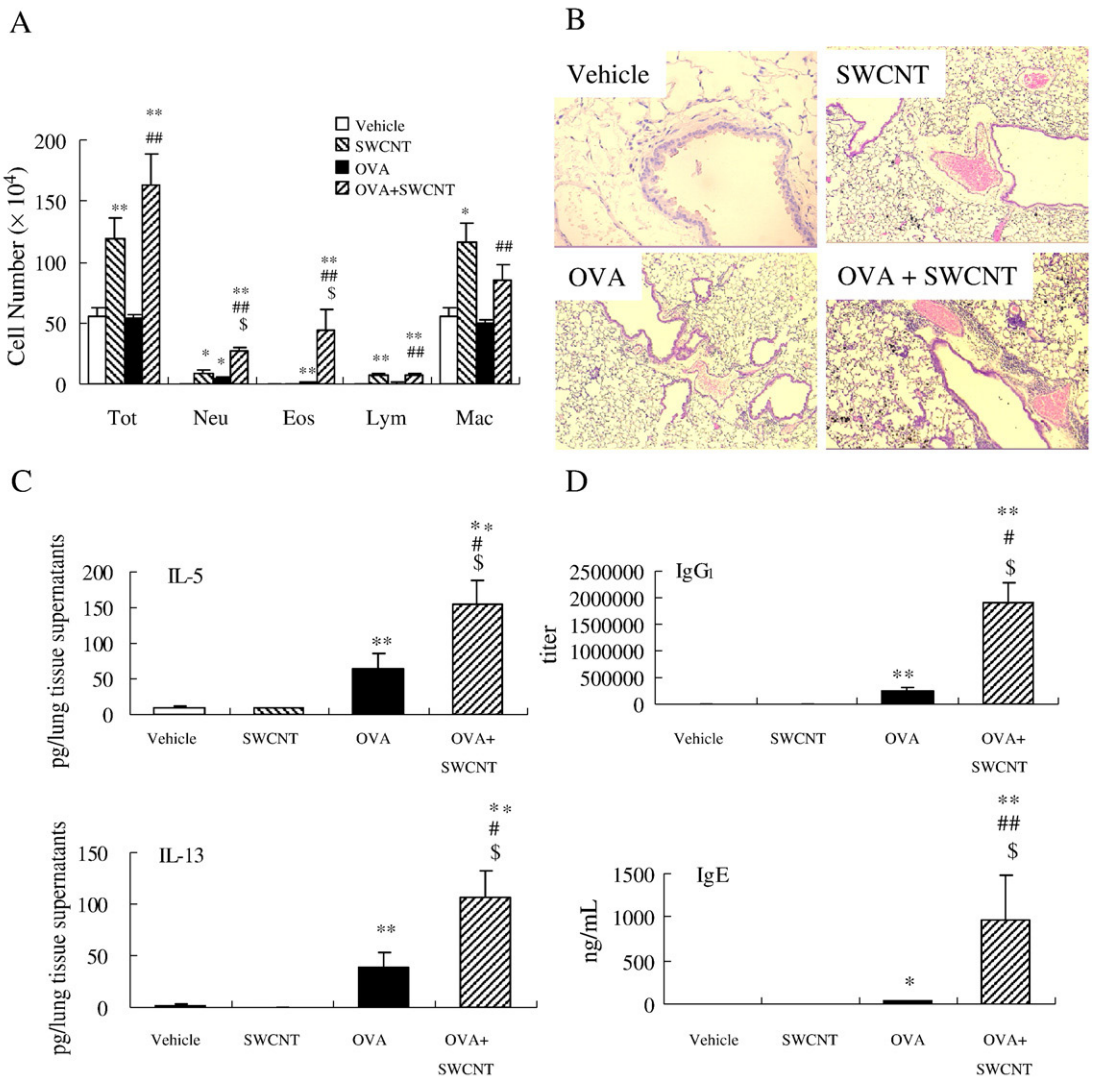

Fig. 6. Effects of SWCNT (from SES Research) on OVA-related lung inflammation and T helper (Th) response. (A) Differential counts of BAL cells from each mouse. BAL was performed 24 h after the final intratracheal administration of the vehicle, SWCNT (50 μg/treatment), OVA, or OVA + SWCNT over a period of 6 weeks. Differential cell counts were performed on 500 cells to identify eosinophils (Eos), macrophages (Mac), neutrophils (Neu), and lymphocytes (Lym). Results are means ± SE ($n = 8$ in each group). (B) Histological examination of lung inflammation (original magnification × 200) conducted through the hematoxylin and eosin staining of lungs obtained 24 h after the final intratracheal administration of vehicle, SWCNT, OVA, or OVA + SWCNT. Representative photomicrographs are shown of lung sections from five animals in each group. (C) Lungs were removed 24 h after the last intratracheal instillation and homogenized; thereafter, Th2 cytokine (IL-5 and IL-13) protein levels were determined by ELISA. Results are means ± SE ($n = 8$ in each group). (D) Serum samples were retrieved 24 h after the last intratracheal instillation. OVA-specific IgG_1 and IgE were analyzed using ELISA. Results are expressed as the means ± SE ($n = 12$ or 13 in each group). $^{*}P<0.05$, $^{**}P<0.01$ vs vehicle, $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs OVA, $^{\$}P<0.01$ vs SWCNT.

facilitating potentials on OVA-related airway inflammation with humoral immunity probably regardless of their characteristics.

Discussion

This study has shown that two different SWCNT administered intratracheally equally deteriorate allergen-related airway inflammation with mucus hyperplasia in mice. The deterioration is concomitant 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; Th17 cytokines such as IL-17A and IL-23 (a pivotal cytokine for the maintenance of Th17 cells); Th2 chemokines such as TARC and MDC; and chemokines related to the migration/maturation of eosinophils, T lymphocytes, and neutrophils such as eotaxin, MCP-1, and KC, respectively. Also, SWCNT exhibit adjuvant activity for the allergen-specific production of IgG_1 (and IgE). Furthermore, SWCNT potentiated the formation/activity of oxidative stress-related biomarkers such as HO-1, MPO, NO, LPO, 8-OHdG, NT, and pentosidine in the lung in the presence of allergen. Finally, SWCNT can partially promote the maturation/activation of BMDC in terms of the surface expression of CD86 and allergen-specific T cell reactivity in vitro.

With respect to the deleterious effects of nano-sized materials on health, focusing on the facilitation of allergy, we and others have examined their impacts on allergic asthma pathobiology in vivo, showing that CB nanoparticles and CNT have the potential to worsen it [32,40–43]. As well, titanium dioxide nanoparticles (14–29 nm in size) showed more prominent adjuvant effects on allergic traits than fine (250–260 nm in size) particles [44]. Recently, on the other hand, we have found that latex nanoparticles (15–100 nm in size) do not facilitate the same allergic asthma model as those in the present study [45]. These previous observations suggest that each nano-level particle/material has different effects on airway pathology, which may depend on their characteristics and/or state of suspension (in fact, the same weight of multiwalled CNT (MWCNT), another type of CNT, in the same experimental setting as used here, more aggressively induced airway inflammation, with a Th2 response even in the absence of allergen [41], compared to SWCNT in this study). Likewise, the exposure pattern should be important when one considers their

effects, i.e., instillation vs inhalation [46]. For instance, regarding MWCNT, we found that instilled MWCNT significantly exacerbated allergic airway inflammation, with an aberrant DC-Th response [41], whereas Ryman-Rasmussen et al. demonstrated that inhaled MWCNT did not affect the pathophysiology with Th responses, but augmented airway fibrosis [47]. To address this issue, our institute is developing an inhalation system for CNT. Based on the present data, in any case, we propose that SWCNT exposure can be a risk factor for the aggravation of allergic pathophysiology of the airway. Further, 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-1β, IL-6, IL-23, MDC, and KC), epithelial cells (IL-1β, IL-6, KC, MCP-1, and eotaxin), neutrophils (IL-1β and MCP-1), T cells (IL-4, IL-5, IL-6, IL-13, IL-17A, IFN-γ, TARC, and MDC), fibroblasts (IL-1β, IL-6, eotaxin, and KC), and endothelial cells (IL-1β, IL-6, TARC, and KC), the aggravation should be associated with the widespread influence of SWCNT in the presence or absence of allergen.

The predominance of Th cell direction accompanied by the enhancing effects of inhalable PM on allergy remains enigmatic. Probably because of the complicated mixture of carbonaceous particles, metals, and organic chemicals, for example, DEP may act as both Th1 and Th2 adjuvants on OVA-related airway inflammation [6,34]. Furthermore, we have recently demonstrated that MWCNT have both Th1 and Th2 adjuvanticity [41]. In this study, OVA-specific IgG_1 was significantly higher in the OVA + SWCNT group compared to the OVA group; in turn, OVA-specific IgG_{2a} was not, although the lung IFN-γ level was significantly higher (alternatively indicating that the adjuvant potential for Th1 may be somewhat weaker in the SWCNT than in the MWCNT in our previous experiment [41]). Recently, it was reported that the IL-23–IL-17 axis is an alternative key modulator in allergic pathophysiology, including asthma [48,49]. We first showed that the lung IL-17A level was significantly greater in the OVA + SWCNT group compared to the other groups, and the IL-23 level was significantly higher in the OVA + SWCNT group compared to the OVA group, implicating the IL-23–IL-17 axis in the SWCNT-mediated exacerbation of allergic airway inflammation. Taking these results together, SWCNT might optimally drive the Th2 and probably the Th17, and partially the Th1, response in this allergic inflammation model.

Oxidative stress is thought to play an important role in the pathogenesis of various types of lung inflammation, including allergic asthma [2]. In particular, ROS are, in turn, capable of modifying the activity of proteins at the posttranslational level and regulating gene expression via important transcriptional factors such as activator protein-1 and nuclear factor-κB [50–52], which confers an additional inflammatory cascade. On the other hand, SWCNT reportedly generate oxidative stress, possibly due to constituent catalytic metals such as iron, cobalt, and nickel, present in SWCNT [53,54], as shown in Table 1, as well as their proinflammatory properties [39]. SWCNT treatment impairs the activity of radical-scavenging enzymes such as superoxide dismutase, catalase, and glutathione peroxidase in macrophages as a result of the increase in local oxidative stress associated with H_2O_2 accumulation [39]. Actually, antioxidants such as vitamin E can reduce the SWCNT-induced lung inflammatory response in vivo [55]. However, it remained unknown whether oxidative stress plays a role in facilitating the effects of CNT on allergic airway inflammation. We first conducted a comprehensive analyses of the redox state in this assay and showed that gene expressions of HO-1, a surrogate biomarker of oxidative stress [56]; immunoreactive 8-OHdG, a marker of DNA modification; and lipid peroxide in the lung were significantly greater in the OVA + SWCNT group compared to the OVA group. Furthermore, immunoreactivity for pentosidine and *N*ε-(carboxymethyl)lysine, advanced glycation end products (and final products of oxidative stress [57]), in the lung tended to be greater in the OVA + SWCNT group than in the OVA group (Supplementary Fig. S4 and unpublished observation). Taken together, one can envision that the OVA + SWCNT group was exposed to greater levels of oxidative stress in the lungs than the OVA group. On the other hand, protein tyrosine nitration is a posttranslational modification that occurs under conditions of oxidative stress and may play a role in the pathogenesis of inflammation [58]. NO released from BM-derived mononuclear cells reportedly promote vasodilation and vessel permeability, thereby increasing the inflammatory leukocyte influx [59]. In this study, OVA + SWCNT-treated mice showed a significantly higher NO level in the BALF and immunoreactive NT expression in the lung compared to other mice; thus, reactive nitrogen species and consequent nitration events could also be important in the aggravating effects of SWCNT on allergic inflammation. This concept is partly supported by the phenomenon that the MPO level in the BALF was significantly greater in the OVA + SWCNT group compared to the other groups, because MPO can generate NT in cooperation with ROS in vitro [60]. On the other hand, a comparative evaluation of the oxidative stress caused by these different CNT is important to determine which physicochemical characteristics of CNT are implied. We conducted linear regression analysis of the metal concentration in SWCNT and MWCNT (used in the previous study; Ref. [41]) vs the degree of oxidative status evaluating the lipid peroxide content and immunoreactivity for NT. As a result, the iron concentration showed a correlation with these parameters, although this did not reach significance (K.-i. Inoue, unpublished observation). It seems likely that complicated factors contributed to the redox imbalance as well as other pathological traits, which remains to be clarified in the future.

APC-mediated uptake of foreign antigens and subsequent presentation for T cells is the first step in the immune response. The maturation of DC, professional APC, is an important step for polarized antigen presentation and the consequent Th response [61]. On the other hand, generation of oxidative stress at the APC level reportedly yields Th2 skewing [62]. We have previously shown that DEP and CB nanoparticles activate BMDC in vitro [36] and pulmonary APC in asthmatic mice in vivo [63]. Furthermore, asbestos reportedly activates APC [64], and DEP chemicals induce oxidative stress in DC with suppression of Th1 cytokine release in vitro [65], suggesting that PM or fibrous materials modify APC characterization/function, favoring a Th2 milieu. In this study, exposure to SWCNT significantly increased the expression of CD86 on BMDC and amplified their OVA-specific T cell reactivity. Further, the NO level in culture supernatants from SWCNT-exposed BMDC was higher than that from control-exposed BMDC (data not shown). Therefore, it is possible that the adverse effects of SWCNT on allergic pathophysiology might account, at least partly, for the inappropriate maturation/activation of APC including DC.

In summary, SWCNT can exacerbate allergic airway inflammation with accentuated Th immunity including Th2 and Th17, possibly through the inappropriate activation of APC. Further, the exacerbation is associated with increased oxidative stress in the airway. These results suggest that inhalable SWCNT may become one of the important environmental risk factors for allergic airway inflammatory disorders such as bronchial asthma in the future.

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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.freeradbiomed.2010.01.013.

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