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Direct Effects of Carbon Nanotubes on Dendritic Cells Induce Immune Suppression Upon Pulmonary Exposure

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Abstract

Pharyngeal aspiration of single walled carbon nanotubes (SWCNT) caused inflammation, pulmonary damage and an altered cytokine network in the lung. Local inflammatory response *in vivo* was accompanied by modified systemic immunity as documented by decreased proliferation of splenic T cells. Pre-incubation of naïve T cells *in vitro* with SWCNT-treated dendritic cells reduced proliferation of T cells. Our data suggest that *in vivo* exposure to SWCNT modify systemic immunity by modulating dendritic cell function.

Keywords

dendritic cells; nanoparticles; immune system; immunosuppression; nanotubes

Carbonaceous nanoparticles and their derivatives have been widely used for a number of applications, including energy storage devices, super-conducting products, magnets, biologic materials and catalysts. Numerous studies have focused on toxicity issues associated with nanoparticle (NP) exposure. However, very limited information is currently available regarding their immunomodulating potential. The early phase of lung innate immune response to many pathogens is characterized by onset of inflammation mediated by phagocytic cells, *i.e*., polymorphonuclear leukocytes (PMNs), alveolar macrophages (AM), and antigen presenting dendritic cells (DC) populating the lungs. The latter are mobilized

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Supporting Information Available: Pulmonary damage and cytokine release following SWCNT exposure. This material is available free of charge via the Internet at http://pubs.acs.org.

and recruited into inflammation sites where they serve their primary duty – antigen recognition, capture and subsequent presentation.[1] Stimulation of T cells by DC is crucial for adaptive immune responses. The nature of encountered antigen(s) as well as environmental signals during antigen uptake by DC shape the subsequent T cell response. The inflammatory milieu in the lung following NP exposure provides a range of signals required for activation and potential polarization of local DC subsets. These signals are presented in the context of cytokines and chemokines, damaged cells/cell fragments, and, perhaps, specific features of the NP itself. However, as of now, it is not yet clear if the exposure of DC to NP affects DC maturation/function. NP given to the lung along with antigens displayed adjuvant properties and enhanced respiratory and systemic allergic responses.[2–5] Nevertheless, detailed mechanisms of these adjuvant effects have not been disclosed. Noteworthy, pristine SWCNT, in contrast to carboxylated or coated SWCNT, do not carry charges on their surface and are poorly recognized by DC.[6] It has been reported that some carbonaceous particles, *e.g.*, carbon black or diesel exhaust particles, may stimulate functional activity of DC *in vitro*.[7–9] Recently, it has been shown that exposure of mice to respirable multi-walled carbon nanotubes (MWCNT) did not elicit pulmonary inflammation yet caused suppression of T cell proliferation.[10,11]

In the current study, we evaluated site-specific pulmonary inflammation and systemic immune response in mice after the pharyngeal aspiration of SWCNT. Here we present evidence that the mechanisms of altered systemic immunity found in SWCNT-exposed mice may be, to some extent, due to direct effects of SWCNT on DC.

RESULTS

Characterization of SWCNT employed in the study

SWCNT (CNI, Houston, TX) produced by the high-pressure CO disproportionation (HiPco) process, employing CO in a continuous-flow gas phase as the carbon feedstock and $Fe(CO)_5$ as the iron-containing catalyst precursor and purified by acid treatment to remove metal contaminates were used in the study. Analysis performed by NMAM 5040 and ICP-AES revealed that SWCNT comprises 99.7% (wt) elemental carbon and 0.23% (wt) iron. For purity assessment of HiPco SWCNT, we used several standard analytical techniques, including thermo-gravimetric analysis with differential scanning colorimetry (TGA-DSC), thermo-programming oxidation (TPO), and Raman and near-infrared (NIR) spectroscopy. Comparative analytical data obtained by TGA-DSC, TPO, NIR, and Raman spectroscopy revealed that >99% of carbon content in the SWCNT HiPco product was accountable in a carbon nanotube morphology. Morphology of SWCNT is presented at Figure 1(A,B). The length of the individual SWCNT is approximately 1–3 μm as confirmed by transmission electron microscopy (TEM). Surface area was determined by Brunauer, Emmett, and Teller analysis. Zeta potential and particle size were determined on the Malvern Zetasizer Nano (Malvern Instruments, Westborough, MA). Zeta potential was measured at −42.3 ± 0.9 mV. The mean diameter and surface area of SWCNT were 1–4 nm and 1040 m^2/g, respectively. Stock suspensions (1 mg/ml) were prepared before each experiment in PBS or culture medium, pH was adjusted to 7.0. To obtain a more homogenous and dispersed suspension, SWCNT were ultrasonicated (30 sec × 3 cycles). The dispersity of sonicated SWCNT assessed as previously described[12] was 83%.

Cell counts in the lungs of mice exposed to SWCNT

The degree of inflammatory response induced by the aspirated particles was estimated by the number of total cells, macrophages, PMNs, and lymphocytes recruited into the mouse lungs and recovered in the broncho-alveolar lavage (BAL) fluid. Experiments were conducted under a protocol approved by the Animal Care and Use Committee of NIOSH.

SWCNT were administered by pharyngeal aspiration (40 or 120 μg/mouse). At days 1 and 7 post exposure, mice were weighed, sacrificed and BAL fluid was collected in sterile centrifuge tubes. Cell counts were performed with an electronic cell counter. AM, PMNs and lymphocytes were identified by their typical cell shape and morphology in the cytospin preparations stained with a Hema-3 kit (Fisher Scientific, Pittsburgh, PA). Pulmonary DC were analyzed in non-lavaged lungs by flow cytometry. DC counts were normalized by the total numbers of lung cells upon digestion. Pulmonary exposure to SWCNT significantly increased the number of PMNs in BAL fluid on day 1 post exposure, with an almost 500-fold increase at the high dose of SWCNT, and up to a 37-fold increase in response to the lower dose as compared to controls (Figure 2B). On day 7 post exposure, PMNs, although markedly decreased as compared to day 1 post exposure, still remained at the elevated levels up to 22-fold and 6-fold over controls at the two respective doses ($p<0.05$). AM displayed a different homing pattern with significant increases observed only after day 7 post exposure (Figure 2A). Lymphocyte recruitment into the lung was not detected on day 1 post exposure. A relatively small number of lymphocytes were found later on day 7 post exposure in BAL fluid ($p<0.05$) (Figure 2C). Notably, elevated numbers of DC were found in the lung at 1d and 7d post exposure ($p<0.05$) (Figure 2D).

Pulmonary exposure to SWCNT suppresses T cell responsiveness in the spleen of mice

To investigate if pulmonary exposure to SWCNT (in the absence of specific antigen) altered systemic immunity, we tested the proliferative response of splenic T cells stimulated with Concavalin A. In SWCNT-exposed animals we observed the dose-dependent decrease of splenocyte proliferation, as evidenced by decrease of both proliferation and expansion indices (Figure 3). The proliferation index was decreased at all doses of SWCNT, reaching its maximum (~15% decrease) at 120 μg/mouse dose while the expansion index showed ~23% decrease at that dose, as compared to control.

Suppressed systemic immunity in SWCNT-exposed mice is associated with the direct effects of SWCNT on DC—To assess if DC could be responsible for modulation of systemic immunity in SWCNT-treated mice, we evaluated the ability of SWCNT-exposed DC to alter T cell responses *in vitro*. Internalization of SWCNT by DC was observed at 48h of exposure, as evidenced by transmission electron microscopy (Figure 4).

We found that SWCNT-exposed DC are able to modulate T cell response *in vitro*. Notably, co-culturing of T cells with SWCNT- exposed DC was shown to suppress the T cell proliferation response upon re-stimulation with freshly generated, unexposed DC (Figure 5). T cells that have been pre-incubated with SWCNT-exposed DC, had a proliferation indices of 1.6±0.07 or 1.4±0.01 (for DC exposed to 6.25 or 25 μg/ml SWCNT, respectively) as compared to 1.8±0.01 in controls ($p<0.05$). Pre-incubation of T cells with LPS-exposed DC (1.0 μg/ml) used as a positive control, resulted in increase of T cell proliferation (2.3±0.03, $p<0.05$). Simultaneous treatment of DC with LPS (1.0 μg/ml) and SWCNT (25N μg/ml) was found to eliminate LPS-induced DC activation and subsequent T cell proliferation (1.8±0.2%) (Figure 5A). Expansion index, reflecting the expansion of the whole culture showed the same pattern as the proliferation index (Figure 5B). We found that SWCNT-exposed DC suppress T cell responses to antigenic stimuli, thus suggesting that the direct effects of SWCNT on DC may cause immune suppression observed *in vivo*.

Exposure to SWCNT does not alter DC phenotype—To investigate if DC-suppressed T cell responsiveness was due to altered DC phenotype, we evaluated expression of maturation markers/co-stimulatory molecules and MHC class II on DC exposed to SWCNT. We did not observe any significant changes in the expression of CD80, CD86,

CD40 or MHC class II molecules by DC after 48h of exposure (6.25 or 25 μg/ml) of SWCNT (Table 1). Treatment of DC with *E. coli* LPS, a positive control, induced phenotypical maturation of DC (increased expression of CD80, CD86, CD40, MHCII molecules). Noteworthy, DC exposed to both LPS and SWCNT (25 μg/ml) had the phenotype indistinctive from LPS-only exposed DC, suggesting that SWCNT do not affect LPS-induced DC maturation. Therefore, other mechanism(s), beyond DC phenotypical alterations, might be involved in suppression of T cells by SWCNT-exposed DC.

DISCUSSION

Results from several laboratories have demonstrated SWCNT-driven robust inflammation, granulomatous lesions and interstitial fibrosis in the lungs of exposed animals.[13–17] Complex interactions between the pathophysiologic mechanisms—inflammatory response and oxidative stress, that can synergistically amplify each other and cause enhanced pulmonary toxicity - have been revealed.[18] In several recent field studies airborne concentration of nanotubes was reported as high as 53 $\mu g/m^3$/SWCNT or 430 $\mu g/m^3$/MWCNT.[19,20] Thus, under manufacturing settings, lung burdens comparable to used in our study (adjusted by lung surface area) can be achieved by workers in 2.3–19.3 years (53–430 $\mu g/m^3$, lung ventilation of 9.6 m^3/day,[21] deposited pulmonary fraction of 10%[22]). Lifetime human lung burdens at these nanotube concentrations in the air (53 or N430$\mu g/m^3$) will be 0.57 g or 4.54 g, respectively. This will bring us to assessed exposure levels in rodents that are equivalents to 0.28 or 2.23 mg/mouse. In the current study, a bolus SWCNT delivery protocol, pharyngeal aspiration, was used to expose mice. This technique provides widespread delivery of particles throughout the lung at a single time point.[23,24] It has been demonstrated that at comparable particle burdens pulmonary responses to bolus instillation reflected the pulmonary response to inhalation.[25,26] There is compelling evidence that poorly soluble carbonaceous nanoparticles *e.g.* nanotubes are not appreciably cleared from the lungs following intratracheal instillation or inhalation.[27,28] Therefore, the doses of SWCNT utilized in the current study (40–120 μg/mouse) are relevant to the actual workplace and certainly less than those that could be achieved during life-time work exposures (8hr/d, 5 d/wk, 45 yrs).[29]

Our data are in line with these previous reports confirming that SWCNT are capable of inducing pulmonary inflammation. In fact, we observed a dose dependent increase in cell counts including macrophages and PMNs in BAL fluid after the animals exposure to SWCNT. Notably, SWCNT-induced inflammation facilitated the recruitment of DC to the lung tissues (Figure 2D), increasing chances of direct DC/SWCNT interactions. The acute phase of lung inflammation (on day 1 post exposure) was characterized by an increase of both pro-inflammatory cytokines (TNF-α, IL-6) as well as chemotactic factors (MCP1). Our assessment of pulmonary damage, measured by LDH and protein release in the BAL fluid, correlates well with the observed pulmonary inflammation (see suppl., Figures S1, S2). Altogether, SWCNT-induced inflammation favors the recruitment of immune cells to the lung and provides additional signals for APC activation and maturation in the context of “danger signals” and a specific cytokine/chemokine environment.[30]

In vivo, SWCNT induced a pro-inflammatory milieu in the lungs that could lead to T cell activation and systemic immunostimulation. However, our data suggest that in SWCNT-exposed animals splenic T cell responses were suppressed (Figure 3).

Several lines of evidence indicate that local inflammation following SWCNT exposure is able to modify immune responses. Recent *in vivo* studies revealed the adjuvant effects of various NP administered along with a specific antigen/allergen. Increased serum levels of ovalbumin (OVA)-specific IgE, as well as elevated eosinophil counts in BAL following

MWCNT or SWCNT + OVA exposure were reported.[2,5,31] As for the response to infectious agents, it has been demonstrated that SWCNT and diesel exhaust particles (DEP) exposure increased the severity of *Listeria monocytogenes* infection *in vivo*.[32,33] Pulmonary exposure to MWCNT caused suppressed responses of spleen cells to mitogen stimulation, as reported by Mitchell *et al.*[10,11] In these studies, no significant pulmonary inflammation was documented. However, TGF-β release from the lung was proposed to be the mechanism of T cell dysfunction and impaired systemic immunity. Here we provide evidence that DC might be involved in translation of immune suppressive signal(s) from the inflamed lung to peripheral lymphoid tissues modulating systemic immunity.

DC are the major bridge between the innate and adaptive immune responses. DC are APC, that are highly efficient in antigen presentation and stimulation of T lymphocytes.[34] DC take-up and process antigens, migrate from peripheral tissues to lymphoid organs, present antigens, produce cytokines and express co-stimulatory molecules critical for efficient activation of T cells required for the development of adaptive immune responses. DC exist in two functionally and phenotypically distinct states, immature and mature. Immature DC are widely distributed throughout the body and occupy sentinel positions in many non-lymphoid tissues including the lung. They constantly test their environment for antigens by phagocytosis, macropinocytosis, and pinocytosis.[35] Immature DC express relatively low levels of MHC class I, class II, and co-stimulatory molecules.[36] After engulfing antigens and activation by proinflammatory cytokines and other signals (like bacterial LPS), immature DC differentiate into mature cells. Mature DC have a reduced potential for antigen uptake but a high capacity for antigen presentation and T cell stimulation.[37,38] Notably, it was previously reported that a fraction of DC may translocate from the lung to the spleen.[39]

While it is unlikely that SWCNT are recognized as full antigens by APC, it is plausible that SWCNT share some similarities with bacterial/viral components and are capable of triggering pattern recognition receptors on DC thus promoting DC maturation and migration. It was previously reported[9] that exposure of DC to carbon black stimulated DC maturation and T cell proliferation *in vitro.* A [Gd@C(82)(OH)(22)](n) fullerene derivative was also reported to induce maturation of DC and stimulate cytokine production by DC, including IL-12p70.[40] In the report by Inoue *et al*.,[5] SWCNT have been shown to increase the number of CD86+ cells in bone marrow-derived DC culture at 5 and 10 μg/ml doses. However, in that study in DC-T cell co-cultures, OVA-specific T cell proliferation was decreased at these SWCNT doses. In our study, the expression of CD80, CD86, CD40 or MHC class II molecules was not changed on DC following 48h SWCNT exposure *in vitro.* It appears that SWCNT do not provide sufficient signals to activate DC *in vitro.* Current findings are supported by data of Palomaki *et al*., who also did not observe significant effects of MWCNT or SWCNT on maturation of cultured DC *in vitro*.[41]

In our study, exposure of DC to *E. coli* LPS induced phenotypical maturation of DC (Table 1). When LPS-exposed DC were mixed with T cells we observed facilitated T cell proliferation (Figure 5). Administration of LPS + SWCNT to DC did not change LPS-induced DC phenotypical maturation (Table 1). Indeed, when T cells were mixed with LPS +SWCNT treated DC we observed decreased proliferation (Figure 5). Combined, these findings suggest that SWCNT do not interfere with recognition of LPS by DC. We can speculate that SWCNT exposure may intervene with antigen capture/processing and/or presentation, thereby leading to compromised DC/T cell interactions. Recently, it has been shown that SWCNTs may interfere with the cytoskeleton actin, in the absence of acute cytotoxicity.[42] In this context, our previous studies have shown that SWCNT are not acutely cytotoxic to primary human macrophages but exposure to SWCNT resulted in marked suppression of the ability of macrophages to ingest apoptotic cells, a process that relies heavily on reorganization of the actin cytoskeleton.[43,44] Such effects could have a negative

impact on the ability of DCs to stimulate T cell proliferation. Moreover, as has been published earlier, DC could suppress T cells *via* expression of B7-H1/PD-1 and CTLA-4 molecules.[45] Previous reports have also implicated the induction of indoleamine 2,3-dioxygenase (IDO), IL-10, arginase, NO, SOCS, Foxo3 in mechanisms by which antigen-presenting cells may regulate T-cell responses.[46–51] In our preliminary experiments, we have not seen changes in IL-10 release in DC/T cells co-cultures (data not shown). Identification of potential mechanisms of T cell suppression by SWCNT-exposed DC is a subject of our further studies.

The immune suppression observed in the current study may have significant biological relevance as a defense mechanism against unnecessary and potentially harmful cytotoxic or antibody-mediated immune responses to a “harmless”, non-proliferating pathogen – SWCNT. The T cell suppression and early granuloma formation[13] concomitantly lessen the acute phase of inflammation. Involvement of DC in SWCNT-induced granulomatous lesions and fibrosis warrants further investigations. Interestingly, according to our preliminary data, the T cell suppression was sustained 2 weeks after exposure to SWCNT (not shown). Obviously, the safe immunological outcome of an exposure to a “harmless pathogen” would be a tolerogenic adaptive response. However, one cannot exclude a possible role of surfactant lipid and protein components that can strongly adhere to the SWCNT surface, and hence completely change their recognition profile by DC resulting in a different processing and subsequent antigen presentation to T cells. For example, we recently demonstrated that coating of SWCNT with anionic phospholipids - phosphatidylserine and diacylphophatidylglycerol - may markedly stimulate recognition and uptake of SWCNT by professional phagocytes, including DC.[6] This may in turn affect functional responses *i.e.* antigen processing and T cell presentation. Moreover, our recent studies have indicated that coating of SWCNT with components of lung surfactant impacts the ability of phagocytes to internalize SWCNT (Kagan *et al*., unpublished data). Thus, the nanoparticle-modified self- or foreign proteins can be adversely recognized by APC leading to the breakage of normal self-tolerance and the development of autoimmune or allergic responses.

In conclusion, suppressed immune responsiveness following pulmonary exposure to SWCNT is likely to augment host susceptibility to infections and may also facilitate tumor progression. The detailed mechanisms by which SWCNT-exposed DC suppress T cell proliferation require further investigations.

METHODS

Animals

Specific pathogen–free adult female BALB/c and C57BL/6 mice (7–8 wk old) were supplied by Jackson Labs (Bar Harbor, ME). Animals were individually housed in the National Institute for Occupational Safety and Health (NIOSH) facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Mice were acclimated for 1 wk before use. Sterile Beta Chip bedding (Northeastern Products, Warrensburg, NY) was changed weekly. Animals were supplied with tap water and food (Harlan Teklad, 7913, Harlan Teklad, Madison, WI) *ad libitum* and housed under controlled light, temperature, and humidity conditions. Experiments were conducted under a protocol approved by the Animal Care and Use Committee of NIOSH.

Animal exposure – pharyngeal aspiration

Pharyngeal aspiration was used to introduce SWCNT to the mouse (BALB/c) lung. In brief, after anesthesia with a mixture of ketamine and xylazine (Phoenix, St. Joseph, MO) (62.5 and 2.5 mg/kg subcutaneous in the abdominal area), the mouse was placed on a board in a

near-vertical position, and the tongue was gently extended with lined forceps. A suspension of SWCNT (40, 80 or 120 μg/mouse; 60 μl in PBS) was placed posterior in the throat, and the tongue was held until the suspension was aspirated into the lungs. Five or six animals per study group were used. Particles were sterilized before administration by autoclaving the particle suspension.

Bronchoalveolar lavage

At days 1 and 7 post exposure, mice were weighed and anesthetized with intraperitoneal injection of sodium phenobarbital and exsanguinated. The trachea was cannulated with a blunted 22-gauge needle, and bronchoalveolar lavage (BAL) was performed with cold sterile Ca^{2+} and Mg^{2+}-free PBS at a volume of 0.7 ml for first lavage (kept separate) and 0.8 ml for subsequent lavages. Approximately 5 ml of BAL fluid per mouse were collected and pooled in sterile centrifuge tubes. Pooled BAL cells were washed in Ca^{2+} and Mg^{2+}-free PBS by alternate centrifugation (800 × g for 10 min at 4°C) and resuspension. Cell-free first-fraction BAL aliquots were frozen and kept until processed.

BAL cell counting and differentials

The degree of inflammatory response induced by the aspirated particles was estimated by the total cells, macrophages, PMNs, and lymphocytes recruited into the mouse lungs and recovered in the BAL fluid. Cell counts were performed with an electronic cell counter equipped with a cell sizing attachment (Coulter model Multisizer II with a 256C channelizer; Coulter Electronics, Hialeah, FL). Alveolar macrophages, neutrophils, and lymphocytes were identified by their characteristic cell shape in cytospin preparations stained with a Hema-3 kit (Fisher Scientific, Pittsburgh, PA), and differential counts of BAL cells were performed. Three hundred cells per slide were counted.

Pulmonary DC counts

For pulmonary DC analysis, non-lavaged lungs were dispersed using 2% collagenase A and 0.75% DNAse I in RPMI 1640 with 10% FBS at 37°C for 1h and labeled with anti-CD11b and anti-CD11c antibodies (BD Biosciences, CA) directly conjugated to FITC or PE. Percent of $CD11c^{+}/CD11b^{+}$ cells was obtained by flow cytometry. DC counts were normalized by total cell numbers in the digested lung.

Spleen harvest and cell isolation

Spleens from exposed or non-exposed BALB/c mice (day 7 post exposure) were aseptically harvested into 5 ml of sterile supplemented medium in a sterile culture dish. Harvested spleens were ground and suspension filtered through a cell strainer. Isolated splenocytes were centrifuged at 300 g for 10 min. Red blood cells were lysed with lysing buffer (155 mM NH4Cl in 10 mM Tris-HCl buffer (pH 7.5), 25°C) for 3 min. After lysis, splenocytes were washed with complete RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 [g/ml streptomycin, 10 mM HEPES, 10% heat-inactivated FBS, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate (Invitrogen Life Technologies) (further referred as complete medium) and pellets were resuspended in 4 ml of complete medium. T cells (utilized in MLR assay) were obtained from spleen cell suspensions by passage through the nylon wool columns, counted using hemacytometer, diluted in the complete medium and used immediately for subsequent assays.

DC generation

Murine DC were generated from hematopoietic progenitors isolated from bone marrow. Bone marrow cells were collected from tibias and femurs of C57BL/6 mice, passed through a nylon cell strainer to remove debris. Bone marrow cells were then depleted of red blood

cells with lysing buffer for 3 min. The single-cell suspensions were then incubated with anti-mouse B220, CD4 and CD8 antibodies for 1 h at 4 °C followed by incubation with guinea pig complement for 30 min at 37 °C to deplete B and T lymphocytes. Cells were then cultured overnight (37°C, 5% CO_2) in six-well plates (Falcon) at the concentration of 1×10^6 cells/ml in complete medium. The nonadherent cells were collected and seeded at a concentration of 2×10^5 cells/ml in six-well plates in complete medium in the presence of recombinant mouse GM-CSF (1000 U/ml) and IL-4 (1000 U/ml) (PeproTech, Rocky Hill, NJ).

Splenocyte proliferation, ex-vivo

Splenocytes were obtained from exposed (40, 80 or 120 μg/mouse) or non-exposed BALB/c mice (day 7 post exposure). Cells were labeled with 5-(and-6)-carboxyfluorescein diacetate (CFSE) at 5μM concentration for 5 min (Invitrogen, Carlsbad, CA), counted using a hemacytometer, diluted in complete medium (1×10^6 cells/ml) and stimulated with 5 μg/ml Concavalin A (Sigma, St. Louis, MO) for 4 days in 24 well plates in triplicates. The proliferation response was measured using flow cytometry (BD FACSCalibur instrument, BD, NJ). Dead cells were excluded from the assay with propidium iodide staining preceding the flow cytometry. The background fluorescence readings were subtracted during the analysis. Proliferation index is the average number of cell divisions that the responding T cells underwent. Only responding T cells are reflected in the proliferation index. Expansion index determines the fold-expansion of the overall culture. This statistic is identical to that obtained by manual cell counting, and is predicted to be closely related to a 3H-T assay of the culture (http://www.flowjo.com/v9/html/proliferation.html). The proliferation and expansion indices were calculated from flow cytometry data using Flowjo software package (Tree Star Inc, Ashland, OR).

Mixed leukocyte reaction (MLR)

Cultured bone marrow-derived (C57BL/6 mice) DC were exposed to SWCNT (6.25 or 25 μg/ml), *E. coli* LPS (1 μg/ml, Sigma, St. Louis, MO), both (1 μg/ml LPS + 25 μg/ml SWCNT), or vehicle only for 48h on day 5 of DC culture. Non-cytotoxic doses of SWCNT were selected in the preliminary experiments (>95% viability after 48h exposure as determined by trypan blue exclusion). Sterile diluted suspensions of SWCNT in complete medium were sonicated and added to the cells (8×10^5) to reach final concentrations of 6.25 or 25 [g/ml. The content of the wells was gently mixed and cultured for additional 48h. At day 7, exposed DC were collected, washed twice in complete medium, counted and aliquoted. Unexposed mouse (BALB/c) T lymphocytes were used as responder cells in MLR. MLR was set at a 1:30 DC:T cell ratio (after preliminary optimization). Following 72h of co-culture, exposed DC 16 (CD11c positive cells) were depleted utilizing magnetic separation. Co-cultured cells were collected, labeled with CD11c MicroBeads (MiltenyiBiotec GmbH, Germany) according to the manufacturer's protocol and passed through LD columns mounted on a MidiMACS separator (MiltenyiBiotec GmbH, Germany). The effluent T cells were collected and labeled with 5-(and-6)-carboxyfluorescein diacetate (CFSE) at 5μM concentration for 5 min (Invitrogen, Carlsbad, CA). The labeled DC-depleted T cells were mixed with newly generated unexposed allogeneic DC (day 7 of DC culture) at a 1:30 DC:T cell ratio. The T cell proliferation response was measured on day 5 of MLR using flow cytometry (BD FACSCalibur instrument, BD, NJ). The proliferation and expansion indices were calculated from flow cytometry data using Flowjo software package (Tree Star Inc, Ashland, OR).

DC phenotype

To determine DC phenotype, cells were exposed for 48h to SWCNT, washed in FACS medium (Hanks Balanced Salt Solution containing 0.1% BSA and 0.1% NaN_3) and stained

with appropriately diluted antibodies (BD Biosciences, CA) directly conjugated with FITC or PE, followed by fixation in 2% paraformaldehyde. Control samples were labeled with isotype-matched antibodies conjugated with the same fluorochrome. Expression of CD11c, CD80, CD86, CD40 and MHCII (IAB) was measured using the BD FACS Calibur instrument.

Statistical analysis

Results were compared by one-way ANOVA and Student's unpaired t test with Welch's correction for unequal variances. All results are presented as means ± SEM. P values of < 0.05 were considered to be statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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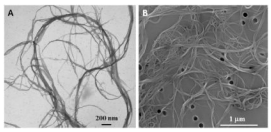

Figure 1.
Transmission (A) and scanning (B) electron microscopic images of SWCNT.

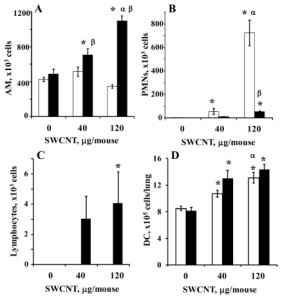

Figure 2.

Inflammatory response in the lung of mice after pharyngeal aspiration of SWCNT. Open bars – 1 day post exposure; closed bars – 7 days post exposure. Data are shown as means ± SEM (3 experiments, 6 animals/group); *p<0.05 *vs*. $^{\alpha}$ p<0.05 *vs*. 40 μg/mouse SWCNT exposure, $^{\beta}$p<0.05 *vs*. 1d post exposure.

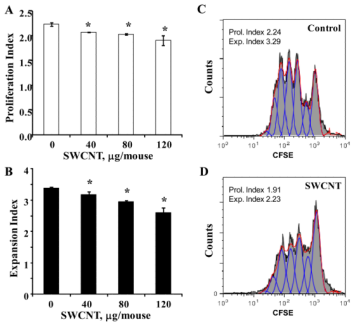

Figure 3.

Suppressed splenic T cell proliferation following pharyngeal aspiration of SWCNT. A – Proliferation index. B – Expansion index. C – Representative cell division profile from control animal. D - Representative cell division profile from SWCNT-exposed animal (120 μg/mouse). Data are shown as means ± SEM (2 experiments, 5 animals/group); *p<0.05 vs. control.

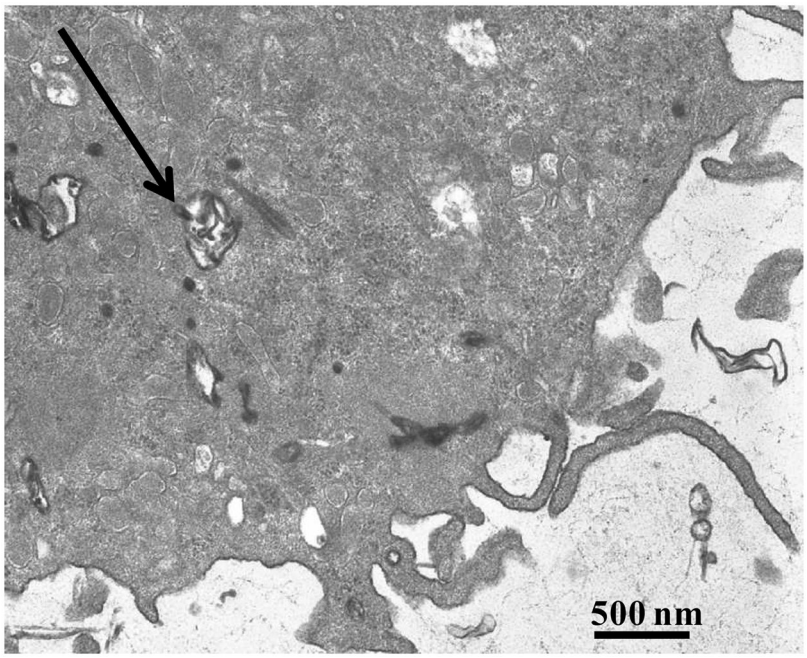

Figure 4.
A typical TE micrograph illustrating internalization of SWCNT (arrow) by DC (*in vitro*) at 48h of exposure.

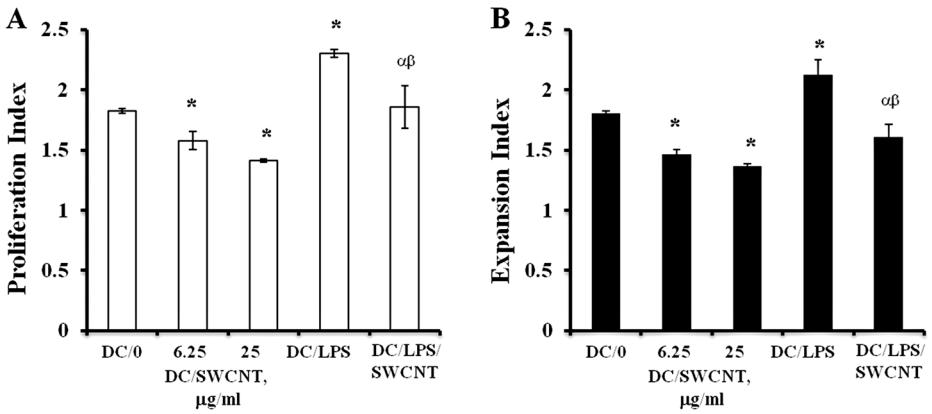

Figure 5.

Co-culturing of T cells with SWCNT exposed DC suppresses the T cell proliferation response. A – Proliferation Index. B – Expansion Index. T cells were incubated for 48h with SWCNT-exposed DC, LPS-exposed DC, or LPS+SWCNT exposed DC. Following co-incubation, exposed DC were depleted and freshly generated DC were added as stimulatory cells. Responder T cell proliferation was measured in 48h. T cells co-incubated with SWCNT-exposed DC showed impaired proliferation. T cells co-incubated with LPS-exposed DC showed increased proliferation. Proliferation of T cells co-incubated with LPS +SWCNT-treated DC was decreased to the control levels. In other words, exposure to SWCNT abrogated the stimulatory effect of LPS on DC. Data are shown as means ± SEM (3 experiments); *$p<0.05$ vs. control, $^{\alpha}p<0.05$ vs. LPS exposure, $^{\beta}p<0.05$ vs. 25 μg/ml SWCNT exposure.

Table 1

Expression of maturation markers/co-stimulatory molecules on DC *(in vitro)* is not altered by SWCNT exposure. Note, that SWCNT-only exposure failed to promote DC maturation. LPS-induced DC maturation was not affected by SWCNT. Data (geometric mean fluorescence intensity) are shown as mean ± SEM (3 experiments);

	CONTROL	SWCNT (6.25 μg/ml)	SWCNT (25 μg/ml)	LPS (1 μg/ml)	LPS/SWCNT (1 μg/ml/25 μg/ml)
CD80	120±2.1	117.6±3.4	128.3±1.8	223.5±4.5$^{*\alpha}$	229.5±9.5$^{*\alpha}$
CD86	159.1±5.5	140.5±13.2	171.1±6.3	278.0±20.1$^{*\alpha}$	295.5±7.5$^{*\alpha}$
CD40	12.8±0.5	13.5±0.3	14.5±0.6	23.5±1.5$^{*\alpha}$	25.5±0.5$^{*\alpha}$
MHC class II	46.13±1.63	43.90±1.32	44.27±2.32	84.16±2.20$^{*\alpha}$	85.93±2.60$^{*\alpha}$

*p<0.05 vs. control,

$^{\alpha}$p<0.05 vs. SWCNT exposure.

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