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 (SWCNTs) 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. Preincubation 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 modifies 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, superconducting 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 (AMs), and antigen presenting dendritic cells (DCs) populating the lungs. The latter are mobilized and recruited into inflammation sites, where they serve their primary duty: antigen recognition, capture, and subsequent presentation.[1] Stimulation of T cells by DCs is crucial for adaptive immune responses. The nature of encountered antigen(s) as well as environmental signals during antigen uptake by DCs 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 DCs to NP affects DC maturation/function. NPs 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 SWCNTs, in contrast to carboxylated or coated SWCNTs, do not carry charges on their surface and are poorly recognized by DCs.[6] It has been reported that some carbonaceous particles, *e.g.*, carbon black or diesel exhaust particles, may stimulate functional activity of DCs *in vitro*.[7−9] Recently, it has been shown that exposure of mice to respirable multiwalled carbon nanotubes (MWCNTs) 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 SWCNTs. 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 SWCNTs on DCs.

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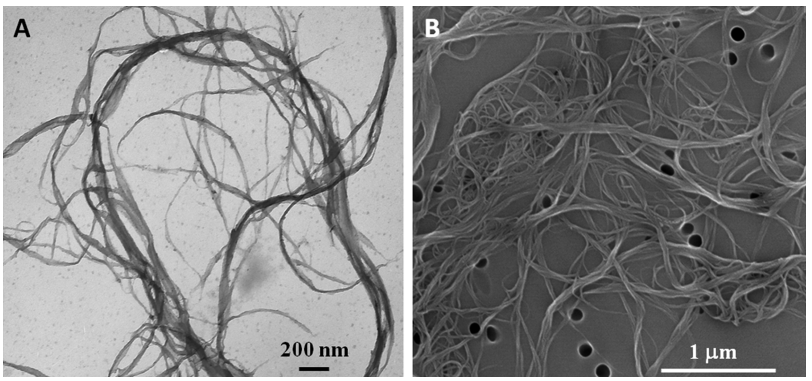

Figure 1. Transmission (A) and scanning (B) electron microscopic images of SWCNT.

RESULTS

Characterization of SWCNT Employed in the Study. SWCNTs (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 SWCNTs comprises 99.7% (wt) elemental carbon and 0.23% (wt) iron. For purity assessment of HiPco SWCNTs, 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 the carbon nanotube morphology. The morphology of SWCNTs is presented in Figure 1A,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 SWCNTs 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, and the pH was adjusted to 7.0. To obtain a more homogeneous and dispersed suspension, SWCNTs were ultrasonicated (30 s × 3 cycles). The dispersity of sonicated SWCNTs assessed as previously described[12] was 83%.

Cell Counts in the Lungs of Mice Exposed to SWCNTs. 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 bronchoalveolar lavage (BAL) fluid. Experiments were conducted under a protocol approved by the Animal Care and Use Committee of NIOSH. SWCNTs were administered by pharyngeal aspiration (40 or 120 μg/mouse). At days 1 and 7 postexposure, mice were weighed and sacrificed, and BAL fluid was collected in sterile centrifuge tubes. Cell counts were performed with an electronic cell counter. AMs, 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 DCs were analyzed in nonlavaged lungs by flow cytometry. DC counts were normalized by the total numbers of lung cells upon digestion. Pulmonary exposure to SWCNTs significantly increased the number of PMNs in BAL fluid on day 1 postexposure, with an almost 500-fold increase at the high dose of SWCNTs and up to a 37-fold increase in response to the lower dose as compared to controls (Figure 2B). On day 7 postexposure, PMNs, although markedly decreased as

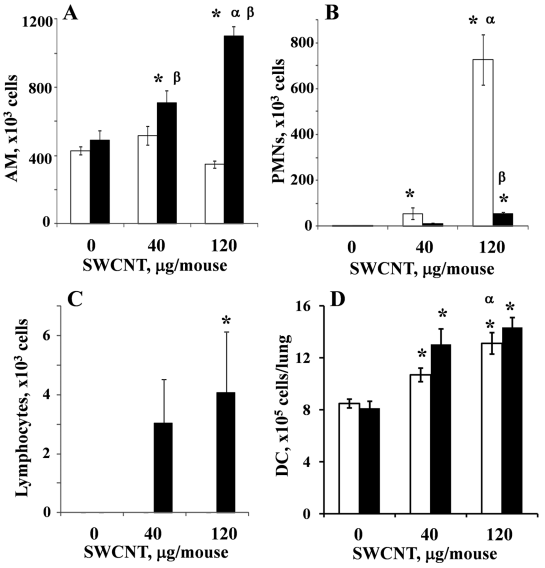

Figure 2. Inflammatory response in the lung of mice after pharyngeal aspiration of SWCNTs. Open bars: 1 day postexposure; solid bars: 7 days postexposure. Data are shown as means ± SEM (3 experiments, 6 animals/group); *$p < 0.05$ *vs* control. $^{\alpha}p < 0.05$ *vs* 40 μg/mouse SWCNT exposure, $^{\beta}p < 0.05$ *vs* 1 day postexposure.

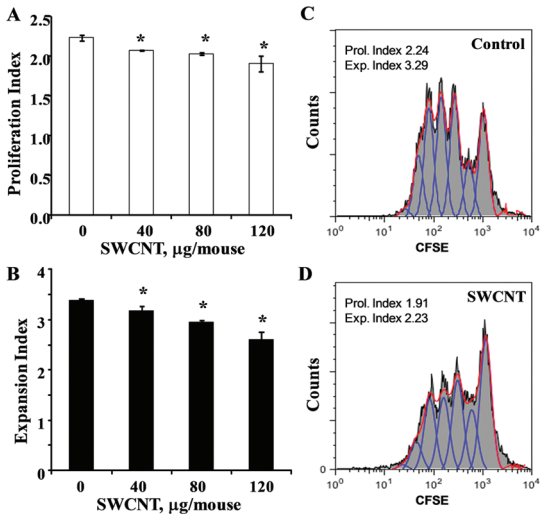

Figure 3. Suppressed splenic T cell proliferation following pharyngeal aspiration of SWCNTs. (A) Proliferation index. (B) Expansion index. (C) Representative cell division profile from control animal. (D) Representative cell division profile from SWCNT-exposed animal (120 μg/mouse). Data are shown as means $\pm$ SEM (2 experiments, 5 animals/group); *$p < 0.05$ *vs* control.

compared to day 1 postexposure, still remained at elevated levels up to 22-fold and 6-fold over controls at the two respective doses ($p < 0.05$). AMs displayed a different homing pattern, with significant increases observed only after day 7 postexposure (Figure 2A). Lymphocyte recruitment into the lung was not detected on day 1 postexposure. A relatively small number of lymphocytes were found later on day 7 postexposure in BAL fluid ($p < 0.05$) (Figure 2C). Notably, elevated numbers of DCs were found in the lung at 1 day and 7 day postexposure ($p < 0.05$) (Figure 2D).

Pulmonary Exposure to SWCNTs Suppresses T Cell Responsiveness in the Spleen of Mice. To investigate if pulmonary exposure to SWCNTs (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 SWCNTs, reaching its maximum (~15% decrease) at 120 μg/mouse dose, while the expansion index showed a ~23% decrease at that dose, as compared to the control.

Suppressed Systemic Immunity in SWCNT-Exposed Mice Is Associated with the Direct Effects of SWCNTs on DCs. To assess if DCs could be responsible for modulation of systemic immunity in SWCNT-treated mice, we evaluated the ability of SWCNT-exposed DCs to alter T cell responses *in vitro*. Internalization of SWCNTs by DCs was observed at 48 h of exposure, as evidenced by transmission electron microscopy (Figure 4).

We found that SWCNT-exposed DCs are able to modulate T cell response *in vitro*. Notably, co-culturing

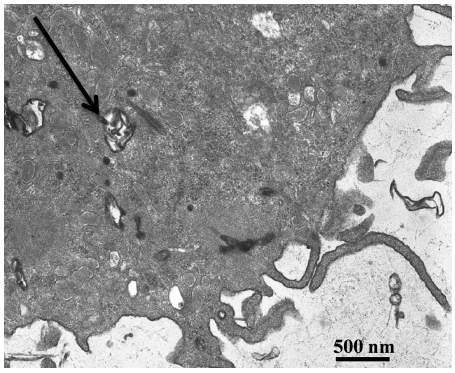

Figure 4. Typical TE micrograph illustrating internalization of SWCNTs (arrow) by DCs (*in vitro* at 48 h of exposure).

of T cells with SWCNT-exposed DCs was shown to suppress the T cell proliferation response upon restimulation with freshly generated, unexposed DCs (Figure 5). T cells that have been preincubated with SWCNT-exposed DCs had a proliferation index of 1.6 ± 0.07 or 1.4 ± 0.01 (for DCs exposed to 6.25 or 25 μg/mL SWCNTs, respectively) as compared to 1.8 ± 0.01 in controls ($p < 0.05$). Preincubation of T cells with LPS-exposed DCs (1.0 μg/mL) used as a positive control resulted in an increase of T cell proliferation (2.3 ± 0.03, $p < 0.05$). Simultaneous treatment of DCs with LPS (1.0 μg/mL) and SWCNTs (25 μg/mL) was found to eliminate LPS-induced DC activation and subsequent T cell proliferation ($1.8 \pm 0.2\%$) (Figure 5A). The expansion index, reflecting the expansion of the whole culture, showed the same pattern as the proliferation index (Figure 5B). We found that SWCNT-exposed DCs suppress T cell responses to antigenic stimuli, thus suggesting that the direct effects of SWCNTs on DCs 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 SWCNTs. We did not observe any significant changes in the expression of CD80, CD86, CD40, or MHC class II molecules by DCs after 48 h of exposure (6.25 or 25 μg/mL) of SWCNTs (Table 1). Treatment of DCs with *E. coli* LPS, a positive control, induced phenotypical maturation of DCs (increased expression of CD80, CD86, CD40, MHCII molecules). Noteworthy, DCs exposed to both LPS and SWCNT (25 μg/mL) had a phenotype indistinctive from LPS-only exposed DCs, suggesting that SWCNTs 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 DCs.

DISCUSSION

Results from several laboratories have demonstrated SWCNT-driven robust inflammation, granulomatous

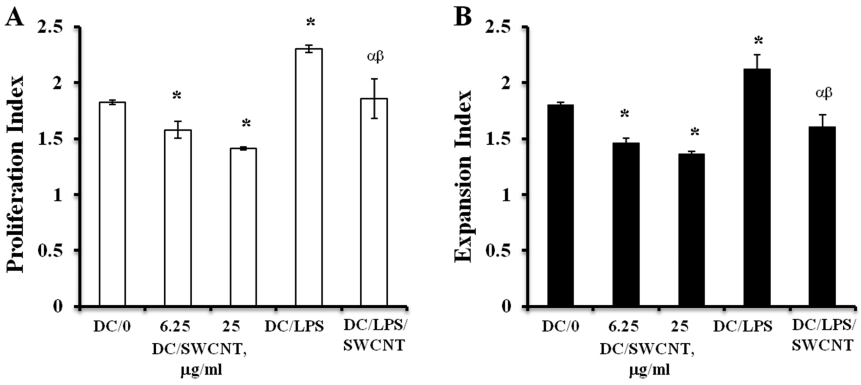

Figure 5. Co-culturing of T cells with SWCNTs exposed DCs suppresses the T cell proliferation response. (A) Proliferation index. (B) Expansion index. T cells were incubated for 48 h with SWCNT-exposed DCs, LPS-exposed DCs, or LPS+SWCNT-exposed DCs. Following co-incubation, exposed DCs were depleted, and freshly generated DCs were added as stimulatory cells. Responder T cell proliferation was measured in 48 h. T cells co-incubated with SWCNT-exposed DCs showed impaired proliferation. T cells co-incubated with LPS-exposed DCs showed increased proliferation. Proliferation of T cells co-incubated with LPS+SWCNT-treated DCs was decreased to the control levels. In other words, exposure to SWCNTs abrogated the stimulatory effect of LPS on DCs. 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 DCs (*in Vitro*) Is Not Altered by SWCNT Exposure[a]

	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^{b,c}	229.5 ± 9.5^{b,c}
CD86	159.1 ± 5.5	140.5 ± 13.2	171.1 ± 6.3	278.0 ± 20.1^{b,c}	295.5 ± 7.5^{b,c}
CD40	12.8 ± 0.5	13.5 ± 0.3	14.5 ± 0.6	23.5 ± 1.5^{b,c}	25.5 ± 0.5^{b,c}
MHC class II	46.13 ± 1.63	43.90 ± 1.32	44.27 ± 2.32	84.16 ± 2.20^{b,c}	85.93 ± 2.60^{b,c}

[a] Note that SWCNT-only exposure failed to promote DC maturation. LPS-induced DC maturation was not affected by SWCNTs. Data (geometric mean fluorescence intensity) are shown as mean ± SEM (3 experiments). $^{b}p < 0.05$ *vs* control. $^{c}p < 0.05$ *vs* SWCNT exposure.

lesions, and interstitial fibrosis in the lungs of exposed animals.[13−17] Complex interactions between the pathophysiologic mechanisms—inflammatory response and oxidative stress, which can synergistically amplify each other and cause enhanced pulmonary toxicity—have been revealed.[18] In several recent field studies the airborne concentration of nanotubes was reported as high as 53 μg/m^3/SWCNT or 430 μg/m^3/MWCNT.[19,20] Thus, under manufacturing settings, lung burdens comparable to those used in our study (adjusted by lung surface area) can be achieved by workers in 2.3−19.3 years (53−430 μg/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 430 μg/m^3) will be 0.57 or 4.54 g, respectively. This will bring us to assessed exposure levels in rodents that are equivalent 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 SWCNTs 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 lifetime work exposures (8 h/d, 5 d/wk, 45 yr).[29]

Our data are in line with the previous reports confirming that SWCNTs 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 SWCNTs. Notably, SWCNT-induced inflammation facilitated the recruitment of DCs to the lung tissues (Figure 2D), increasing chances of direct DC/SWCNT interactions. The acute phase of lung inflammation (on day 1 postexposure) was characterized by an increase of both pro-inflammatory cytokines (TNF-α, IL-6) and 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 Supporting Information, 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, SWCNTs 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 NPs 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 particle exposure increased the severity of *Listeria monocytogenes* infection *in vivo*.[32,33] Pulmonary exposure to MWCNTs 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 DCs might be involved in translation of immune-suppressive signal(s) from the inflamed lung to peripheral lymphoid tissues modulating systemic immunity.

DCs are the major bridge between the innate and adaptive immune responses. DCs are antigen presenting cells (APC), which 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. DCs exist in two functionally and phenotypically distinct states: immature and mature. Immature DCs are widely distributed throughout the body and occupy sentinel positions in many nonlymphoid tissues including the lung. They constantly test their environment for antigens by phagocytosis, macropinocytosis, and pinocytosis.[35] Immature DCs 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 DCs differentiate into mature cells. Mature DCs 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 DCs may translocate from the lung to the spleen.[39]

While it is unlikely that SWCNTs are recognized as full antigens by APCs, it is plausible that SWCNTs share some similarities with bacterial/viral components and are capable of triggering pattern recognition receptors on DCs, thus promoting DC maturation and migration. It was previously reported[9] that exposure of DCs 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 DCs and stimulate cytokine production by DCs, including IL-12p70.[40] In the report by Inoue *et al*.,[5] SWCNTs 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 DCs following 48 h SWCNT exposure *in vitro*. It appears that SWCNTs do not provide sufficient signals to activate DCs *in vitro*. Current findings are supported by data of Palomaki *et al*., who also did not observe significant effects of MWCNTs or SWCNTs on maturation of cultured DCs *in vitro*.[41]

In our study, exposure of DCs to *E. coli* LPS induced phenotypical maturation of DCs (Table 1). When LPS-exposed DCs were mixed with T cells, we observed facilitated T cell proliferation (Figure 5). Administration of LPS + SWCNT to DCs did not change LPS-induced DC phenotypical maturation (Table 1). Indeed, when T cells were mixed with LPS+SWCNT-treated DCs, we observed decreased proliferation (Figure 5). Combined, these findings suggest that SWCNTs do not interfere with recognition of LPS by DCs. 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 SWCNTs are not acutely cytotoxic to primary human macrophages, but exposure to SWCNTs 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, DCs 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, and 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 cell co-cultures (data not shown). Identification of potential mechanisms of T cell suppression by SWCNT-exposed DCs 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", nonproliferating pathogen: SWCNT. The T cell suppression and early granuloma formation[13] concomitantly lessen the acute phase of inflammation. Involvement of DCs 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 SWCNTs (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 DCs, resulting in a different processing and subsequent antigen presentation to T cells. For example, we recently demonstrated that coating of SWCNTs with anionic phospholipids—phosphatidylserine and diacylphophatidylglycerol—may markedly stimulate recognition and uptake of SWCNTs by professional phagocytes, including DCs.[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 SWCNTs with components of lung surfactant impacts the ability of phagocytes to internalize SWCNTs (Kagan *et al.*, unpublished data). Thus, the nanoparticle-modified self- or foreign proteins can be adversely recognized by APCs, 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 SWCNTs is likely to augment host susceptibility to infections and may also facilitate tumor progression. The detailed mechanisms by which SWCNT-exposed DCs 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 Laboratories (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 SWCNTs 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 postexposure, 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 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 was 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, nonlavaged lungs were dispersed using 2% collagenase A and 0.75% DNase I in RPMI 1640 with 10% FBS at 37 °C for 1 h 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 nonexposed BALB/c mice (day 7 postexposure) 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 NH_4Cl 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 μg/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 to 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 nylon wool columns, counted using a hemacytometer, diluted in the complete medium, and used immediately for subsequent assays.

DC Generation. Murine DCs were generated from hematopoietic progenitors isolated from bone marrow. Bone marrow cells were collected from tibias and femurs of C57BL/6 mice and 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 a 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 nonexposed BALB/c mice (day 7 postexposure). Cells were labeled with 5-(and-6)-carboxyfluorescein diacetate at 5 μM 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 the Flowjo software package (Tree Star Inc., Ashland, OR).

Mixed Leukocyte Reaction (MLR). Cultured bone marrow-derived (C57BL/6 mice) DCs were exposed to SWCNTs (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 48 h on day 5 of DC culture. Noncytotoxic doses of SWCNTs were selected in the preliminary experiments (>95% viability after 48 h exposure as determined by Trypan blue exclusion). Sterile diluted suspensions of SWCNTs in complete medium were sonicated and added to the cells (8×10^5) to reach final concentrations of 6.25 or 25 μg/mL. The content of the wells was gently mixed and cultured for an additional 48 h. At day 7, exposed DCs 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 72 h of co-culture, exposed DCs (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 at 5 μM concentration for 5 min (Invitrogen, Carlsbad, CA). The labeled DC-depleted T cells were mixed with newly generated unexposed allogeneic DCs (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 the Flowjo software package (Tree Star Inc., Ashland, OR).

DC Phenotype. To determine DC phenotype, cells were exposed for 48 h to SWCNTs, 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. Data expressed as geometric mean fluorescence intensity.

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.

Disclosure: The findings and conclusions in this report are those of the authors and do not necessarily represent the view of the National Institute for Occupational Safety and Health.

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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.

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