Journal of
Advanced Microscopy Research
Vol. 8, 93–99, 2013

RESEARCH ARTICLE

Acid-Treated Multi-Walled Carbon Nanotubes Coated with Lung Surfactant Protein SP-A Do Not Induce a Lung Inflammatory Response

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The potential toxicity of carbon nanotubes (CNTs) in the lungs has not been widely investigated at the protein level. Previous studies have focused on addressing pulmonary toxicity primarily at the tissue and cellular levels. However, studying the interaction between carbon nanotubes and the lung surfactant proteins A (SP-A) and D (SP-D), two versatile and potent innate immune molecules in the lungs, can yield important information about the mechanisms involved in lung toxicity. Here, we investigated whether SP-A-coated acid treated multi-walled carbon nanotubes ($MWCNT_{ox}$) induced an inflammatory response. We found that although SP-A coated $MWCNT_{ox}$ were avidly taken up by alveolar macrophages, they did not induce an inflammatory response as measured by the enhancement of levels of nitric oxide, a key marker for inflammation, when carbon nanotubes were incubated with IFN-γ primed alveolar macrophages. Thus, the binding of SP-A to carbon nanotubes does not trigger inflammation.

Keywords: Carbon Nanotubes, Lung Surfactant, SP-A, SP-D, Alveolar Macrophages, Nitric Oxide.

1. INTRODUCTION

The inhalation of airborne particles may lead to lung dysfunction including airway inflammation and alterations in breathing.[1] Small particles, particularly in the nano-scale size range, are perhaps the most dangerous as they penetrate to the lung alveoli. The industrial-scale production of nanomaterials such as carbon nanotubes (CNTs) is increasing rapidly. Therefore, there is a need to assess possible hazardous effects of these nanomaterials.

Many studies report adverse biological responses in the presence of CNTs.[2–6] The most frequently reported risks associated with these nanomaterials in the context of entry into the lungs are formation of granulomas[2–4] and pulmonary fibrosis.[5, 6] For example, it has been shown that pulmonary exposure to single-walled carbon nanotubes (SWCNTs) caused persistent changes in pulmonary function and decreased bacterial clearance.[6]

To date, the lung toxicity aspect of carbon nanotubes has been investigated mainly at the tissue and cellular levels. Very little research has been conducted on the interaction, which occurs between carbon nanotubes and the lung surfactant proteins SP-A and SP-D.[7–9] Such study could help understand if administration of nanotubes can interfere with the functions of key defense molecules in the lungs such as SP-A.

Lung surfactant proteins SP-A and SP-D are the most abundant hydrophilic proteins in pulmonary surfactant. They are produced primarily by type II alveolar epithelial cells and belong to a family of proteins called collectins (Fig. 1),[10, 11] the major function of which is to bind to an array of carbohydrate structures on the surfaces of various pathogens and allergens via their carbohydrate recognition domains or *C*-type lectin domains.[12] SP-A acts as an

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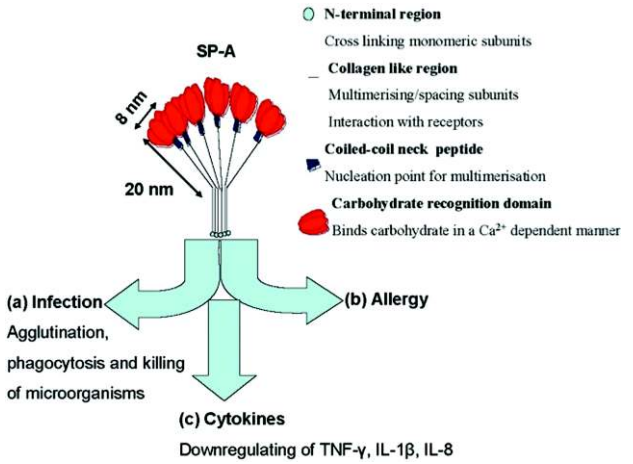

Fig. 1. Schematic representation of SP-A and its role in lung immune defense. SP-A assembles as octadecamers consisting of six trimeric subunits of 20-nm arm length. SP-A has several functions in the lung including phagocytosis, regulation of reactive oxygen species production, and handling allergenic particles.

opsonin and enhances attachment of a variety of pathogens to alveolar macrophages, stimulating phagocytosis. *In vitro* studies show that in the lung, SP-A interacts with alveolar macrophages and modulates the lung defense system through production of cytokines, chemokines, and nitric oxide (NO).[13, 14]

In an effort to identify some potential causes of the lung toxicity associated with carbon nanotubes from a protein perspective, we previously reported, for the first time, the binding of SP-A and SP-D to acid-treated carbon nanotubes. The study showed that SP-A and SP-D bound to carbon nanotubes via oxygen-containing functional groups on their surface generated by the acid treatment.[7] Since the binding of SP-A and SP-D to the acid-treated carbon nanotubes was prominent we speculated that such interaction could prevent SP-A and SP-D from performing their normal functions, which are mainly involved in offering resistance to microbial infection and allergenic challenge.

This work aims to investigate whether acid-treated multi-walled carbon nanotubes coated with SP-A ($MWCNT_{ox}$ + SP-A) induce an inflammatory response. To achieve this objective we used confocal microscopy and a nitric oxide assay as the main research techniques.

2. MATERIALS AND METHODS

2.1. Synthesis of Carbon Nanotubes

MWCNTs were purchased from Nanostructured and Amorphous Materials Inc. (Houston, Texas, USA). Characteristics of these MWCNTs were as follows: diameter greater than 10 nm, 5–15 μm in length, 95% purity. Ten milligrams of MWCNTs were placed in a test tube containing a mixture of concentrated sulphuric and nitric acid (3:1). The test tube was sonicated for 8 h at 50 °C in an ultrasonic bath. After this shortening process, carbon nanotubes (now referred to as $MWCNT_{ox}$) were placed in Opti-MEM culture medium (without added serum) (Life technologies, US) to test their dispersibility properties.

2.2. Purification of Native SP-A Using Affinity and Superose 6 Gel Filtration Chromatographic Steps

Native SP-A was purified from the SP-A-rich surfactant pellet obtained from centrifugation of human alveolar proteinosis broncho-alveolar lavage fluid (BALF).[15] Briefly, a 10 g surfactant pellet was resuspended in 20 ml of wash buffer containing 5 mM Tris-HCl, 2 mM EGTA, 1 mM $MgCl_2$, pH 7.4 to release SP-A. After mixing for 1 hour at 4 °C, the solution was centrifuged at 10000 g for 1 hour at 4 °C, and the SP-A-containing supernatant was decanted. Two ml of this supernatant was then loaded onto a Superose 6 (HR 10/30) gel filtration column (GE Healthcare, UK) that had been equilibrated with 5 mM Tris-HCl, 2 mM EDTA, pH 7.4. The elution was run at a flow rate of 1 ml/min, and 1 ml fractions were collected.

2.3. Labelling of Carbon Nanotubes with Streptavidin-Fluorescein Isothiocyanate and Coating with SP-A or BSA

$MWCNT_{ox}$ (10 mg) were suspended in 2 ml of deionized water. The mixture was stirred for a short time at room temperature. Subsequently 1.0 ml of 500 mM MES buffer (pH 6.1) and 2.3 ml of a 50 mg/ml *N*-Hydroxysuccinimide (NHS) (Aldrich, US) aqueous solution was added to the above suspension and mixed. Under fast stirring, 1.2 ml fresh *N*-(3-Dimethylaminopropyl)-*N*′-ethyl-carbodiimide hydrochloride (EDC-HCl) (Fluka, US) aqueous solution (10 mg/ml) was added quickly and the mixture was continually stirred at room temperature for 30 min. The suspension was centrifuged (10000 g, 5 min) and rinsed thoroughly with a 50 mM MES buffer, pH 6.1 to remove excess EDC, NHS and byproduct urea. The treated carbon nanotubes were resuspended in 0.5 ml of 50 mM MES buffer. An equal volume (0.5 ml) of pentylamine-Biotin (6 mg/ml final concentration) in 50 mM MES buffer (Pierce, US) was added to the carbon nanotubes. This mixture was stirred for 2 h at room temperature. The suspension was centrifuged and rinsed scrupulously with 50 mM MES buffer (pH 6.1). To remove non-reacted Pentylamine-Biotin and EDC-NHS byproducts from the biotinylated $MWCNT_{ox}$, dialysis against 50 mM MES buffer for 24 h was performed. Subsequently 3 μl of Streptavidin-fluorescein isothiocyanate with a concentration of 0.8 mg/ml (Invitrogen, US) was incubated with biotinylated $MWCNT_{ox}$ for 1 h at room temperature followed by dialysis overnight in deionized water.

Then, the labeled $MWCNT_{ox}$ (50 μg in 250 μl of 10 mM HEPES, 140 mM NaCl, 0.15 mM $CaCl_2$, pH 7.0) were incubated with purified SP-A (30 μg) for 2 h at room

temperature (RT). SP-A was previously dialysed in 10 mM HEPES, 140 mM NaCl, 0.15 mM $CaCl_2$, pH 7.0. Fifty μg of labeled $MWCNT_{ox}$ suspended in 250 ul of PBS were also incubated with BSA (30 μg) and incubated for 2 h at RT. In addition, fifty μg of uncoated labeled $MWCNT_{ox}$ were incubated for 2 h at RT. These samples were washed four times with the appropriate buffer in order to remove unbound material.

2.4. Microscopy

2.4.1. Transmission Electron Microscopy

The $MWCNT_{ox}$ + SP-A sample was prepared with approximately 200 μg of $MWCNT_{ox}$ suspended in 10 mM HEPES, 140 mM NaCl, 0.15 $CaCl_2$, pH 7.0 and incubated with SP-A (50 μg/ml final concentration in the sample buffer). The mixture was incubated overnight at 4 °C. After incubation, the sample was washed several times with 10 mM HEPES, 140 mM NaCl, 0.15 $CaCl_2$ mM, pH 7.0 to remove unbound materials. After the final wash this sample was resuspended in 500 μl of the above buffer. Ten μl of this preparation was placed on a copper TEM grid. After a few seconds of absorption, the grid was negatively stained with 2% uranyl acetate. The copper grid was blotted and examined with a FEI Tecnai 12 operating at 120 kV. The $MWCNT_{ox}$ sample was prepared with 200 μg of $MWCNT_{ox}$ suspended in 500 μl of ddH_2O and sonicated for 3 min in a bath sonicator. Ten μl of this sample was placed on a copper grid and stained with 2% uranyl acetate solution. This sample was also examined with FEI Tecnai 12 operating at 120 kV. The SP-A sample was prepared with (5 μg/ml final concentration in the sample buffer). Ten μl of this sample was placed on a copper grid and stained also with uranyl acetate followed by its examination with FEI Tecnai 12 operating at 120 kV.

2.4.2. Confocal Microscopy

Mouse alveolar macrophage cells MH-S were purchased from ATCC (cat # CRL-2019) and cultured following the ATCC protocol (ATCC formulated RPMI 1640 Medium, cat #302001, made 0.05 mM 2-mercaptoethanol and 10% v/v fetal bovine serum). This culture medium preparation was called complete medium. Next, cells were plated at 5×10^5 cells/well on glass coverslips in complete medium of 24-well plates, and incubated overnight. After the incubation period, cells were washed three times with sterile PBS. Then, 300 μl of labeled uncoated and coated multi-walled samples in Opti-MEM (without added serum) were added to each well and incubated for 3 h at 37 °C. After the incubation step, cells were washed three times with PBS, and 300 μl of Opti-MEM alone or Opti-MEM + Cell tracker (fluorescence probe CMTPX (1 μM)) (cat # C34 552) (life sciences, US) were added to the wells and incubated at 37 °C for 30 min. The cell tracker stains mainly the cytoplasm, but also part of the cell membrane. Subsequently, cells were washed once with PBS and fixed with 4% paraformaldehyde for 30 min at RT. Cells were then washed three times with PBS. Immediately, cells were stained with DAPI (cat # R37606) (Life Sciences, US) and incubated at 37 °C for 10 min followed by two washes with PBS. The glass coverslips were mounted on glass and examined with a confocal microscope (Nikon C1 plus), 60 × oil objective, lasers at 405, 488 and 561 nm.

2.5. Nitric Oxide Assay

To investigate whether $MWCNT_{ox}$ coated with SP-A induced an inflammatory response, production of nitric oxide was measured. Alveolar macrophages (Cat # CRL-2019) were cultured in complete medium as described above. Cells were lifted with PBS-0.5 mM EDTA and plated at 2×10^5 cells per well in 96-well plates in complete medium. After 24 h in culture, cells were washed three times with 100 μl of PBS per well. Subsequently, alveolar macrophages were stimulated with 75 μl of IFN-γ (40 ng/ml) (R&D systems, Minneapolis, MN, USA) before adding the ligands. Seventy-five microliters of each ligand was added to cells. These ligands were $MWCNT_{ox}$ (250 μg/ml), $MWCNT_{ox}$ + BSA (250 μg/ml), $MWCNT_{ox}$ + SP-A (250 μg/ml), LPS (200 μg/ml) and Zymosan (250 μg/ml). The last two were used as positive controls. Zymosan was purchased from Sigma-Aldrich, while LPS was from *Salmonella minnesota*, purchased from InvivoGen (San Diego, California, USA). IFN-γ was purchased from R&D Systems, Minneapolis, MN.

The carbon nanotubes ligands were prepared as follows: $MWCNT_{ox}$ and $MWCNT_{ox}$ coated with SP-A or BSA suspended in endotoxin-free water, 10 mM HEPES, 140 mM NaCl, 0.15 mM $CaCl_2$, pH 7.0 and PBS respectively were incubated in an orbital shaker at 37 °C for 1 h. After the incubation period, each sample was washed several times with the appropriate buffer or water to remove unbound material. Subsequently, these carbon nanotubes samples were dispersed in Opti-MEM (without added serum) (250 μg/ml) and sonicated for 3 min in an ultrasonic bath. Next, 75 μl of ligand suspension in Opti-MEM (without added serum) was added to IFN-γ alveolar macrophages in Opti-MEM without added serum and incubated at 37 °C, 5% CO_2 for 24 h. After 24 h incubation period, 100 μl of each supernatant was transferred to 96-well V-bottom plates and spun down for 5 min at 1500 rpm to remove cells and debris. Subsequently, supernatants were transferred to 96-well flat-bottom plates for nitric oxide assay according to the manufacturer's instructions (Griess Reagent System, Promega Corporation, US). The Griess reagent system measures nitrite (NO_2^-), which

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is a primary stable and nonvolatile breakdown product of nitric oxide (NO).

3. RESULTS AND DISCUSSION

Acid treatment cuts carbon nanotubes and generates both carboxylates and ester functional groups on the walls and tips of MWCNTs and SWCNTs.[16–18] The purpose of cutting the MWCNTs was (1) to create carboxylic acid groups that mediate their binding to SP-A, and (2) to facilitate the dispersion of carbon nanotubes in different aqueous environments. The latter is a very important experimental step since an accurate evaluation of the toxicological aspect of carbon nanotubes depends on many factors including their ability to disperse in aqueous phase. $MWCNT_{ox}$ dispersed in Opti-MEM (with no added serum) remained in suspension less than 1 h (Fig. 2, left side), whereas $MWCNT_{ox}$ coated with SP-A ($MWCNT_{ox}$ + SP-A) remained in suspension longer than 1 h (Fig. 2, right side). Purity of native SP-A was verified by SDS-PAGE under reducing conditions (Fig. 3). In this gel figure, SP-A is visible as two bands of ~35 kDa, and ~66 kDa, representing the monomer and non-reducible dimer, respectively. Transmission electron microscopy studies were conducted to visualize the shortened carbon nanotubes and their interaction with SP-A (Fig. 4). SP-A molecules and $MWCNT_{ox}$ were used as control samples. Figure 4(A) shows many individual SP-A molecules stained with 2% uranyl acetate, while Figure 4(B) shows several shortened $MWCNT_{ox}$. The shortening procedure produced $MWCNT_{ox}$ with a wide variety of lengths. On average, $MWCNT_{ox}$ between 200 nm and 1 μm were produced after the acid treatment (Figs. 4(B)–(C)).

Fig. 2. Dispersibility test of acid-treated MWCNTs in Opti-MEM. Acid-treated carbon nanotubes ($MWCNT_{ox}$, left side) and $MWCNT_{ox}$ coated with SP-A ($MWCNT_{ox}$ + SP-A, right side) suspended in Opti-MEM (without added serum) for 1 h after five min of sonication.

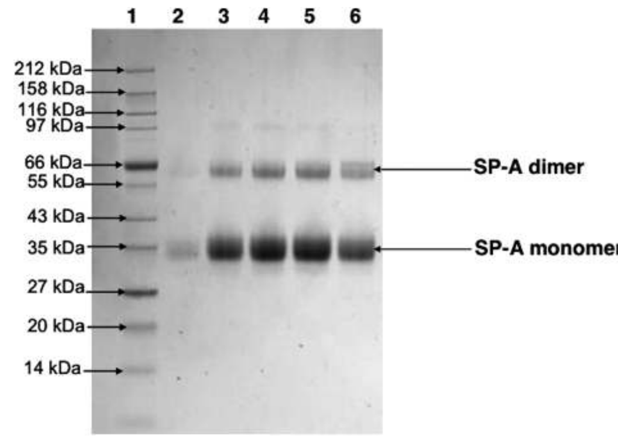

Fig. 3. Native human SP-A purification on Superose 6. SDS-PAGE analysis (reducing conditions) of the fractions of SP-A taken from gel filtration and stained with coomassie blue. Lane 1: Molecular weight marker, lane 3–6: purified SP-A, individual fractions from chromatography.

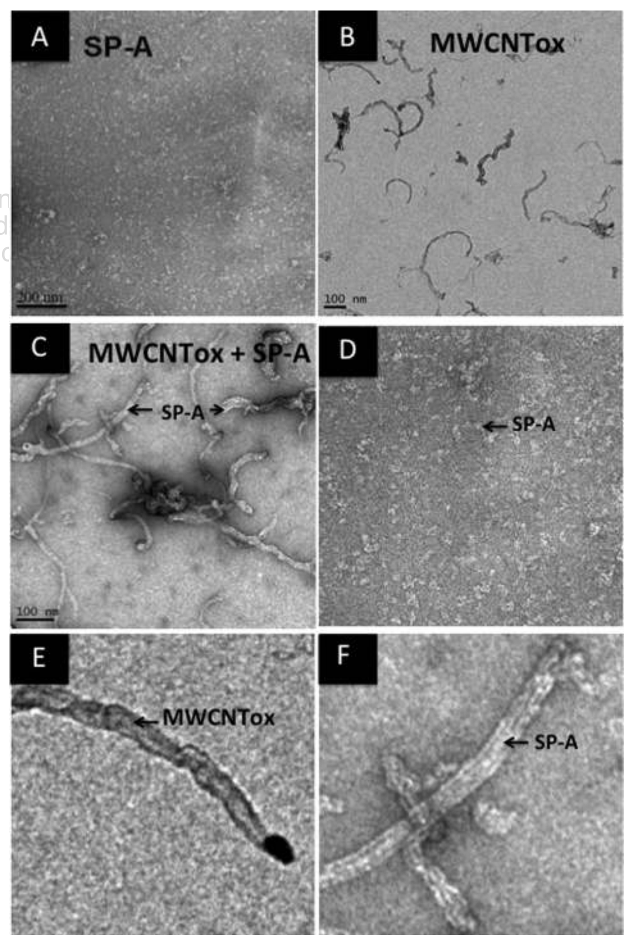

Fig. 4. Transmission electron micrographs show the binding of SP-A to $MWCNT_{ox}$. (A) SP-A alone, (B) $MWCNT_{ox}$, (C) $MWCNT_{ox}$ + SP-A. The acid treatment greatly reduces the size of MWCNTs. The average size of shortened $MWCNT_{ox}$ is between 300 nm and 1 μm. The walls of $MWCNT_{ox}$ are completely coated with SP-A as indicated by black arrows. (E)–(F) shows a magnified view of (A)–(C).

This interaction is of high affinity since the walls of $MWCNT_{ox}$ are completely coated with SP-A (Fig. 4(C)). $MWCNT_{ox}$ appear white because of the presence of SP-A. The black regions in this image indicate an excess of uranyl acetate, which was used to stain the sample. Magnified views clearly show the presence of single SP-A molecules (Fig. 4(D)), shortened uncoated $MWCNT_{ox}$ (Fig. 4(E)) and shortened $MWCNT_{ox}$ coated with SP-A (Fig. 4(F)). The high level of binding of SP-A to acid-treated MWCNT is consistent with the suggestion that binding takes place via oxygen-containing functional groups.[7]

The uptake of $MWCNT_{ox}$ + SP-A by alveolar macrophages is the first step to assess the pulmonary toxicity of carbon nanotubes at the protein and cell level. The nuclei of the macrophages were stained with DAPI whereas the cytoplasm was stained with the fluorescent probe CMTPX (Fig. 5). Uncoated $MWCNT_{ox}$ and $MWCNT_{ox}$ coated with Bovine serum albumin (BSA) and SP-A proteins penetrated both cytoplasm and nucleus of the alveolar macrophages. The majority of carbon nanotubes of these three samples was found in the cytoplasm as indicated by the green color. Of the three samples, $MWCNT_{ox}$ + SP-A were the ones that accumulated the most in the cytoplasm of the cell. This phenomenon might be due to the opsonization role of SP-A in lung immunity.

After the cellular uptake of $MWCNT_{ox}$, $MWCNT_{ox}$ + BSA and $MWCNT_{ox}$ + SP-A by alveolar macrophages, we investigated if such uptake induced an inflammatory response. We tested these samples in IFN-γ stimulated alveolar macrophages. The inflammatory response was assessed by measuring the enhancement levels of nitric oxide generated during the incubation time of carbon

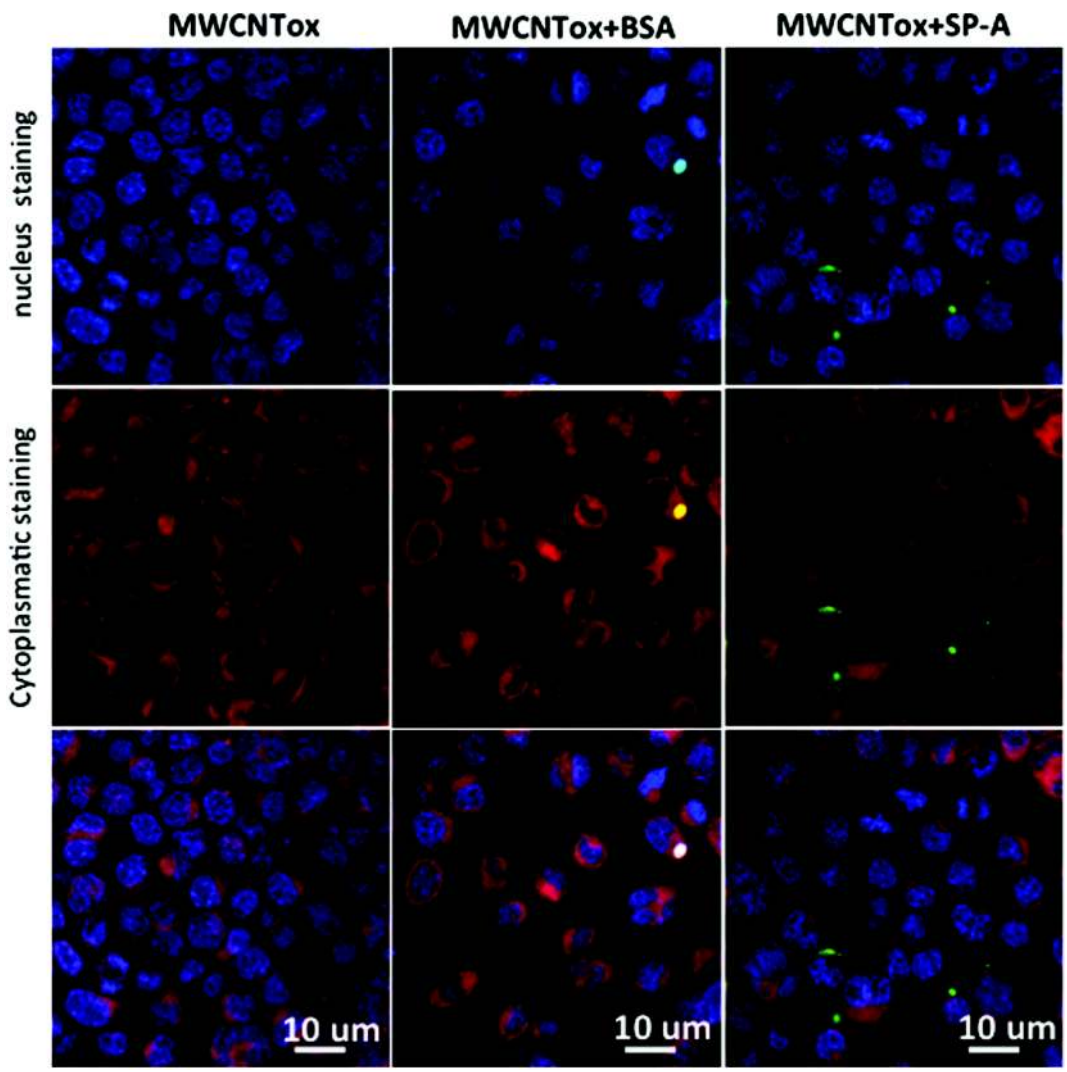

Fig. 5. Confocal microscopy images show the uptake of uncoated and coated $MWCNT_{ox}$ by mouse alveolar macrophages. The nucleus of the cell was stained with DAPI while the cytoplasm of alveolar macrophages was stained with CMTPX. $MWCNT_{ox}$ labeled with streptavidin-FITC appear as green fluorescence in the cytoplasm. From left to right, $MWCNT_{ox}$ (control), $MWCNT_{ox}$ + BSA (control), $MWCNT_{ox}$ + SP-A penetrate both the nucleus and cytoplasm of alveolar macrophages. $MWCNT_{ox}$ + SP-A sample displays the greatest cellular uptake of carbon nanotubes by alveolar macrophages as observed by the presence of green-labeled carbon nanotubes in the cytoplasm. The scale bar is the same for all images.

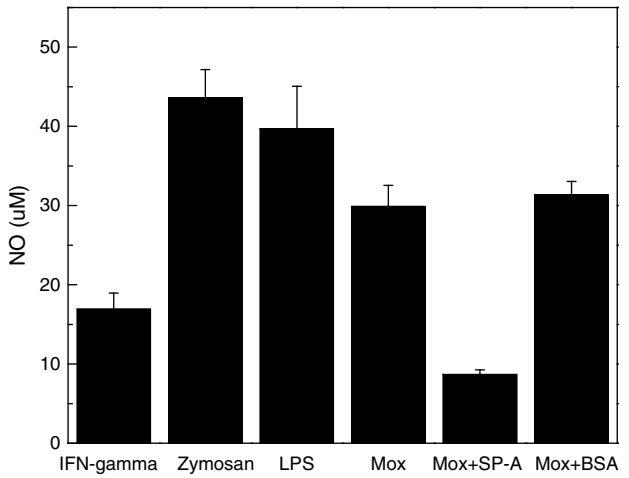

Fig. 6. Nitric oxide (NO) levels induced in IFN-γ-stimulated alveolar macrophages by the presence of uncoated and coated MWCNTs. LPS and Zymosan are the positive controls of this experiment. $MWCNT_{ox}$ were pre-coated (or not) with SP-A or BSA. $MWCNT_{ox}$ + SP-A induced the lowest level of nitric oxide, lower than the IFN-γ control. Each value represents the mean + standard deviation of three independent experiments.

nanotubes and stimulated alveolar macrophages. Nitric oxide, quantified by the accumulation of nitrite in the cell culture medium was measured using the Griess reaction. $MWCNT_{ox}$ and $MWCNT_{ox}$ + BSA induced levels of nitric oxide comparable with Zymosan and LPS, which are the positive control samples of this experiment (Fig. 6). In contrast, the levels of nitric oxide induced by $MWCNT_{ox}$ + SP-A were the lowest among the tested samples, despite the avid uptake of $MWCNT_{ox}$ + SP-A by alveolar macrophages. It is important to mention that we tested our samples for endotoxin contamination before conducting the nitric oxide assay as we know that endotoxin is a very potent immunostimulant and can induce nitric oxide secretion when it is present in picogram quantities.[19] Therefore, the levels of nitric oxide observed in our experiment are induced by the samples and not by a source of endotoxin contamination.

The lowest level of nitric oxide induced in alveolar macrophages by $MWCNT_{ox}$ + SP-A led us to suggest that this interaction does not induce a lung inflammatory response. Other recent studies of acid treated multi-walled carbon nanotubes, which were introduced into mouse lungs by oropharyngeal aspiration[8] reported that the levels of SP-A in the brochoalveolar lavage fluid (BALF) did not decrease in the presence of carbon nanotubes, but the levels of SP-D were greatly reduced. The authors suggested that an increased production of SP-D in mice treated with MWCNTs may represent a defensive reaction of the respiratory tract.[8] In addition, recently Kapralov et al. reported the *in vivo* adsorption of the SP-D on SWCNTs in an animal model.[9] The authors of this paper reported that the presence of SP-D on SWCNTs markedly enhanced the *in vitro* uptake of SWCNTs by macrophages.[9]

SP-A has been shown to have protective functions in the lungs. For example, SP-A is known to regulate the inflammatory response of the alveolar macrophages when challenged with particles, pathogens and other pulmonary insults. This suppression of inflammatory process is mediated by a negative regulation of TLR-4 mediated generation of proinflammatory transcription factor, NFκB.[20] Also, SP-A has recently been shown to reduce the cytotoxicity of human beta defensin 3 in the lungs.[21] With emerging role of SP-A as a downregulator of inflammatory response in the lungs, it is clear that interaction between SP-A and carbon nanotubes can benefit the therapeutic outcomes intended as a drug delivery vehicle. Preclinical trials in murine models using SP-A opsonized carbon nanotubes will help address and assess the beneficial effect of SP-A.

4. CONCLUSIONS

In conclusion, we report that although $MWCNT_{ox}$ coated with SP-A are avidly taken up by alveolar macrophages, they do not trigger inflammation. This finding might be due to the role of SP-A as a downregulator in inflammation. Further studies are required to be conducted to investigate in depth the precise role that the binding of SP-A to $MWCNT_{ox}$ plays in the lung inflammatory response.

Acknowledgments: We thank Dr. Maria A. Aronova (National Institute of Health) for the assistance with the TEM imaging. Also, we thank Mr. Barry Neun (Nanotechnology Characterization Lab, SAIC-Frederick Inc.) for conducting the endotoxin test on the multi-walled carbon nananotubes that were used for the confocal experiments and nitric oxide assay. This work was supported by CSM start-up fund #162904.

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Received: 14 June 2013. Accepted: 20 July 2013.