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Water Soluble Multi-Walled Carbon Nanotubes Enhance Peritoneal Macrophage Activity *In Vivo*

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Due to their unique physical properties, carbon nanotubes are becoming promising novel materials in diverse areas like information technology, ultrastiff materials, biomedicine etc. The toxicological study of these materials is very imperative for the safety assessment in respect to their wide applications. The objective of the toxicity study was to find out whether water soluble multi-walled carbon nanotubes (S-MWNTs) would impair the phagocytic activity of macrophages, which play an important role in defenses against invading microbioles. The results showed that S-MWNTs did not impair the phagocytosis of macrophages; to our surprise, S-MWNT significantly enhanced this function. Increased activity of enzymes in lysosome also showed that S-MWNTs enhanced the lysosome function of macrophages. However, S-MWNTs can not influence nitric oxide secretion in macrophages and induce inflammation.

Keywords: Carbon Nanotubes, Peritoneal Macrophage, Phagocytosis Activity, Arginase, Acid Phosphatase, Nitric Oxide.

RESEARCH ARTICLE

1. INTRODUCTION

Possessing very desirable physicochemical properties, including ordered structure with high aspect ratio, ultralight weight, high mechanical strength, high electrical conductivity, high thermal conductivity, metallic or semimetallic behaviour and high surface area, carbon nanotubes (CNTs), a novel and unique material, have potential for diverse applications, from field emission, molecular electronics and energy storage, to sensors, atomic force microscope and many others.[1] In the past few years, the biomedical applications of CNTs have been proposed and great progress has been achieved by *in vitro* and *in vivo* studies, including substrate for the growth of cells for tissue regeneration and tissue engineering, delivery of drugs, proteins, peptides and nucleic acids for gene transfer or gene silencing, tumour targeting and imaging, and antineoplastic treatment.[2] Intensive research in these areas may lead to applications in medical diagnosis and therapeutics. However, as the use of CNTs in biomedical products is relatively new and most of these applications will involve the administration or implantation of CNTs or their matrices into patients, the toxicological and pharmacological profile of CNTs will have to be determined prior to any clinical studies undertaken.[3] In the past years, a great number of nano-toxicologists have made efforts on toxicity study of CNTs. Cell culture studies have shown evidence of cytotoxicity and *in vivo* studies have shown CNTs caused a dose-dependent inflammation and formation of small nodules in the lungs of animals via intratracheal instillation.[4–6] On the contrary, no skin irritation or allergy was observed as working with soot containing CNTs,[7] and no acute and chronic toxicity of functionalized CNTs were found when injected into the bloodstream of mice over several months.[8]

However, at present, only a few studies have been reported about the toxicity of CNTs on immune system or immune-related cells. Jia et al. indicated that single-walled CNTs (SWNTs) were more toxic than multi-walled CNTs (MWNTs), quartz and fullerene based on the results of alveolar macrophage cell culture study.[9] Bottini et al. reported that high concentrations of pristine and oxidized MWNTs induce massive loss of human T cells and human peripheral blood lymphocytes viability through programmed cell death *in vitro*.[10] Kiura et al. found that production of TNF-α by total mouse spleen cells and the human monocytic cell line THP-1 was induced upon incubation with non-functionalized SWNTs.[11] In contrast, some reports have shown that stable aqueous solutions of functionalized CNTs do not exert toxic effects on immortalized cell lines from different origins.[12] Last year,

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Mitchell et al. found that inhalation of MWNT **s** did not result in significant lung inflammation or tissue damage, but caused nonmonotonic systemic immunosuppression after 14 days.[13] However, Shvedova et al. showed that an inhalation exposure of mice to SWCNTs produced inflammation, fibrosis, and oxidative stress.[14]

Macrophages are essential cellular components of the immune system, and can be found in almost all tissues of the body depending. Macrophages perform a multitude of functions essential for tissue remodelling, inflammation and immunity, including phagocytosis and secretion of a wide array of cytokines, growth factors, lysozymes, proteases, complement components, coagulation factors and prostaglandins.[15] When injected or implanted into bodies, CNTs will firstly and inevitably confront the macrophages and may affect the function of these macrophages.[16] Our previous work has shown the fact that macrophages in reticuloendothelial system could readily engulf but hardly metabolize CNTs when they were injected into the bloodstream of mice.[17] In this work, in order to investigate the effects of MWNTs on macrophages *in vivo*, we carried out the peritoneal macrophages as a model and various biochemical parameters were tested to evaluate MWNT's influence on macrophages, including macrophage phagocytosis activity, nitric oxide (NO) secretion, and enzyme activity in lysosome.

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2. MATERIALS AND METHODS

2.1. Synthesis and Characterization of Water Soluble MWNTs

The raw MWNTs produced by chemical vapor deposition method with outer diameters of 10–20 nm, lengths of several μm to tens of μm, and a purity of >95% were purchased from Shenzhen Nanoharbor, China. Transmission electron microscope (TEM), thermogravimetric analysis (TGA) and ICP-MS were used to further characterize the MWNT samples. Water soluble MWNTs (S-MWNTs) used in this work were prepared and characterized as described in Ref. [17]. Briefly, 12 mg oxidated MWNTs by H_2SO_4/HNO_3 (3:1) mixture were first reaction with thionyl chloride and then the resulting dried product was dispersed in 6 ml anhydrous dimethyl formamide upon ultrasonication with 125 mg taurine added, followed by adding 280 μl $(CH_3CH_2)_3N$. The reaction mixture was stirred at 130 °C for 96 h, then passed through a 0.22 μm polycarbonate membrane to collect S-MWNTs solid which was washed with deionized water, followed by drying under vacuum at 60 °C.

2.2. Animals and Treatment

Eighty male Kunming mice (~25 g) were obtained from Second Military Medical University (Shanghai, China) and housed in polycarbonate cages under a 12-h light/dark cycle and fed with a commercial diet and tap water *ad libitum*. All animal experiments were performed in compliance with the institutional ethics committee regulations and guidelines on animal welfare.

After an acclimatization period, 40 mice were assigned randomly into four groups, including two experimental groups (0.2 and 2 mg/kg), one control group and one positive group (lipopolysaccharide, LPS), with each containing 10 mice. The experimental groups were administered with *ca* 200 μl S-MWNTs solution in sterile saline at a dose of 0.2 or 2 mg per body weight via intraperitoneal injection (*i.p.*) repeatedly for 7 days, while control group were exposed to 200 μl sterile saline, and positive group were exposed to 200 μl LPS solution in sterile saline. These mice were used for chicken red blood cell phagocytosis experiment and the peritoneal macrophages were isolated for measuring phagocytosis activity of peritoneal macrophages.

The other 40 mice were treated the same as the first 40 mice, but used for the other experiments. After mice were sacrificed, the mouse body, liver and spleen were weighed and the organ index was calculated as the organ weight (in grams)/body weight (in grams). The peritoneal macrophages were isolated for measuring Argininase (Arg), Acid Phosphatase (Acp), and NO secretion.

2.3. Chicken Red Blood Cell (CRBC) Phagocytosis Experiment

2.3.1. CRBC Suspension Preparation

CRBC was used to examine the phagocytosis of macrophages.[18] Two milliliters of blood were firstly collected from the chicken wing artery with disposable 10-ml syringe soakaged by heparin (1%) and then mixed with 5 ml saline solution in a tube. After being mildly shaken up several times, the sample tube was centrifuged at 1000 rpm for 5 min at room temperature. The sediment was collected and washed 3 times with saline solution for removing the supernate and leukocytes completely. Finally, the chicken blood cells were diluted with sterile saline to produce a final concentration of 5% CRBC suspension, and stored at 4 °C until required.

2.3.2. CRBC Phagocytosis Experiment

Twenty four hours after the last administration, each mouse from the first 40 mice was injected intraperitoneally with 0.5 ml CRBC suspension (5%). Six hours later, mouse was killed and the abdomen was cleansed with 70% ethanol. Peritoneal macrophages were collected as described by Lee et al.[19] Briefly, the abdominal skin was carefully dissected without opening the peritoneum and 5 ml of ice-cold Ca^{2+} and Mg^{2+} free Hank's balanced saline solution was injected intraperitoneally. Following the abdomen was massaged for 3 min, 3.5 ml of the

injected volume was recovered. A sample of 150 μl of the exudative cells was dropped on the glass slide to make a smear and two parallel smears were made for each mouse. Finally, the smears were incubated in a wet box at 37 °C for 30 min, rinsed with saline to remove suspension cell, and stained with wright's stain according standard procedure (Nanjing Jiancheng Biotechnology Institute, China). The fraction of macrophages that ingested CRBC was assessed by examining 100 cells or more microscopically. The phagocytic rate (PR) was calculated using the following formula:

$$PR(\%) = \frac{\text{Number of macrophage ingesting CRBC}}{\text{Number of total macrophage}} \times 100\%$$

2.4. Peritoneal Macrophages Isolation and Culture

As described above, the peritoneal macrophages from the other 40 mice were isolated from mice abdomen and kept on ice until all the mice were killed. Then each macrophage suspension was centrifuged at 1500 rpm for 15 min at room temperature and the sediment cells were collected and washed 3 times with Hank's balanced saline solution. After adding 4 ml RPMI 1640 (at room temperature) containing 10% fetal bovine serum, each sample was divided equally into two parts, seeded into 24-well plates and cultured in a 5% CO_2 atmosphere at 37 °C. One part was for the Arg and Acp assay, while the other part for NO assay.

2.5. Arg and Acp Activity Assay

After 2-h culturing, the peritoneal macrophages adhering to the plate well were gently washed with Hank's balanced saline solution, added 0.5 ml double distilled water and repeatedly frozen and thawed from −30 °C to +30 °C with each time for 30 min for getting the macrophage lysis.

Arg activity was measured on the basis of urea release.[20] Briefly, 0.2 ml of the macrophage lysis was mixed with 1.2 ml Tris-HCl buffer (pH = 9.0) and incubated at 55 °C for 1 h. The resultant mixture was added to 0.1 ml arginine solution (0.5 M) and kept for 15 min at 37 °C and then for 7 min at 100 °C to terminate the reaction. Following the addition of 1.0 ml diacetyl oxime solution (1 g in 50 ml) and 2.0 ml acidic solution (44 ml concentrated sulfuric acid, 66 ml phosphoric acid, 50 mg thiosemicarbazide and 2 g cadmium sulfate in 1000 ml), the final solution was incubated in boiling water for 10 min and cooled to room temperature for the spectrophotometry. The absorbance at 520 nm was measured by a microplate reader and the Arg activity of sample was calculated from a standard curve. One unit enzyme activity is defined as 1 nmol urea produced in one minute at the condition of 37 °C, pH = 9.0.

Acp activity was measured based on cleavage of the substrate p-nitrophenyl phosphate in citrate buffer (pH 5.0). In brief, 0.1 ml of peritoneal macrophage lysate was mixed with 1.0 ml citric acid buffer (pH = 5.0) containing p-nitrophenol phosphate with concentration of 1 mg/ml, and incubated at 37 °C for 10 min. Then 0.5 ml NaOH solution (1M) was added to terminate the reaction. The absorbance of sample at 405 nm and 546 nm was measured on a microplate reader, and Acp activity in experimental samples was calculated from a standard curve. One unit enzyme activity is defined as hydrolysis of 1 μmol p-nitrophenol phosphate in one minute at the condition of 37 °C, pH = 5.0.

2.6. Assay for NO Determination

After 24-h culture, the supernatant of macrophages in 24-well plates was collected. NO was measured indirectly by determination of NO_2^- concentration through the Griess reagent that is exclusive for measuring mouse NO_2^- concentration.[20] Briefly, the supernatant was incubated with an equal volume of Griess reagent at room temperature for 10 min. Absorption at 550 nm was measured with the spectrophotometer, and the absolute concentration was calculated by the standard curve generated through a series of standard NO_2^- stock solution.

2.7. Statistical Analysis

All values were expressed as the mean ± the standard deviation (S. D.). Statistical analysis was performed using the two-way analysis of variance (ANOVA) and an unpaired Student's *t*-test. Values of $P < 0.05$ were regarded as statistically significant, compared with the control group.

3. RESULTS

S-MWNTs were synthesized by attaching taurine molecules onto oxidated MWNTs via the amidation of carboxylic acid moieties on nanotubes. Less than 0.5% carbon atoms of MWNTs were bond to taurine, but the linked taurines make the insoluble MWNTs satisfactorily soluble. The S-MWNTs suspension in distilled water keeps stable over 6 months, and suspension in saline, 0.01 M phosphate-buffered saline remained stable after 6 h post preparation.[17] The TEM image of S-MWNTs (Fig. 1) shows that S-MWNTs varied from 100 nm to 1 μm long with the average length of 248 ± 160 nm, and the diameter is *ca* 15 nm.

In order to evaluate the effects of S-MWNTs on mouse body, liver and spleen, the weight change, liver index and spleen index were measured (Fig. 2). No statistically significant changes of body weight occur in the treated groups compared with the control groups. The liver and spleen indices indicate S-MWNTs will not cause shrinkage or

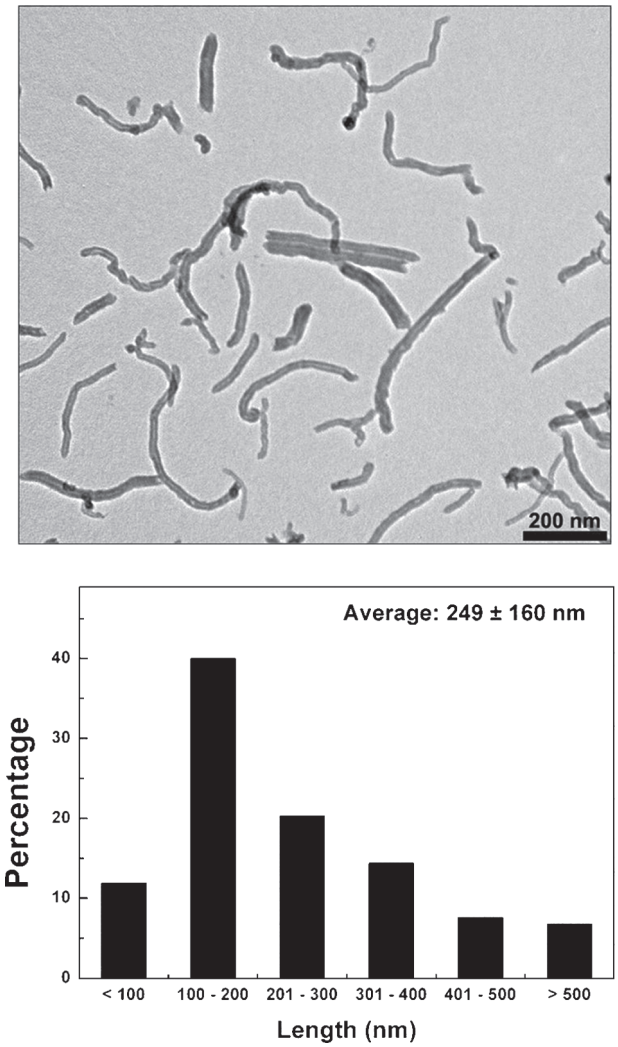

Fig. 1. TEM photo and length distribution of S-MWNTs.

swelling of the liver and spleen of mice. There are no significance of mortality and gross abnormalities noted for any animal throughout the duration of study.

CRBC are commonly used in phagocytosis assay because they are easy to observe with exceptionally nuclei stained with Giemsa and light microscope. We can quantitatively count the macrophage that phagocytized CRBC by a light microscope and calculate macrophage phagocytosis rate. Figure 3 shows that phagocytic rate of mice treated with S-MWNTs was slight increased compared with control group, and this means that S-MWNTs can enhance the phagocytosis activity of peritoneal macrophages.

Two types of enzyme in lysosome, argininase and acid phosphatase, were tested to evaluate the lysosome activity of macrophage.[22] Arg is a manganese-containing enzyme and the final enzyme of the urea cycle. Figure 4 shows the Arg activity of control, positive (LPS), high dose (2 mg/kg) and low dose (0.2 mg/kg) group. It is apparent that the Arg activity of the treated groups is significantly higher than that of the control group, but slightly lower than that of positive control. The reslut shows S-MWNTs can enhance the lysosome activity of macrophages. Acp is another important component of the macrophage lysosome enzyme, and it is also an indicator for assessing macrophage function.[22] The Acp activity of the peritoneal macrophages was measured and shown in Figure 2. Similar to the findings on Arg activity, Acp activity of the peritoneal macrophage of S-MWNT groups is higher that that of control group. Again, this result

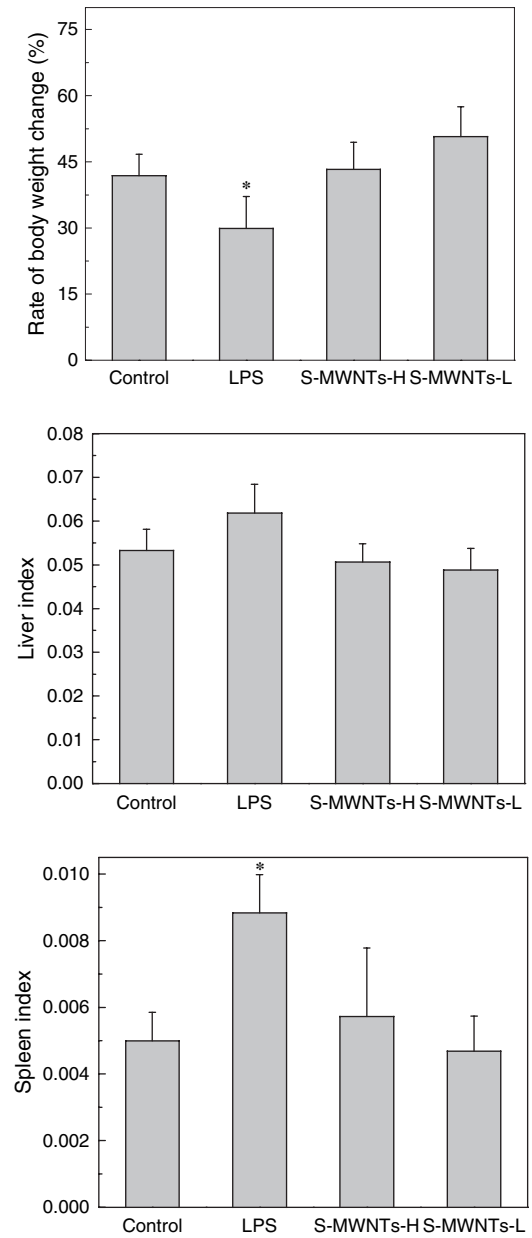

Fig. 2. Effect of S-MWNTs on the body weight change, liver index and spleen index of mice. Data are the mean ±S. D. of 10 mice. (∗represents $p < 0.05$ comparing to the control group, S-MWNTs-H and S-MWNTs-L means the mouse was exposed to S-MWNTs at dose of 2 and 0.2 mg/kg body weight, respectively. liver (or spleen) index = weight of wet liver (or spleen) /Body weight).

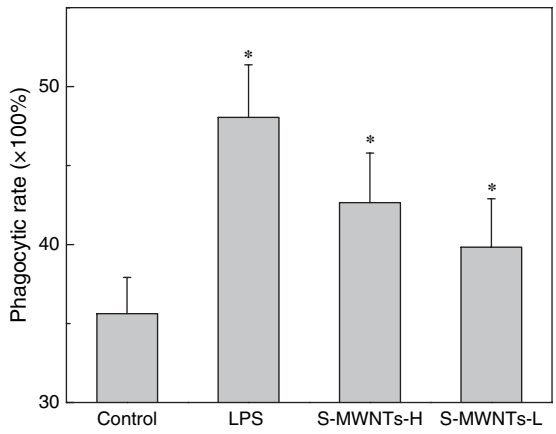

Fig. 3. Effect of S-MWNTs on phagocytic rate of peritoneal macrophages. Data are the mean ±S. D. of 10 mice. (∗represents $p < 0.05$ comparing to the control group, S-MWNTs-H and S-MWNTs-L means the mouse was exposed to S-MWNTs at dose of 2 and 0.2 mg/kg body weight, respectively).

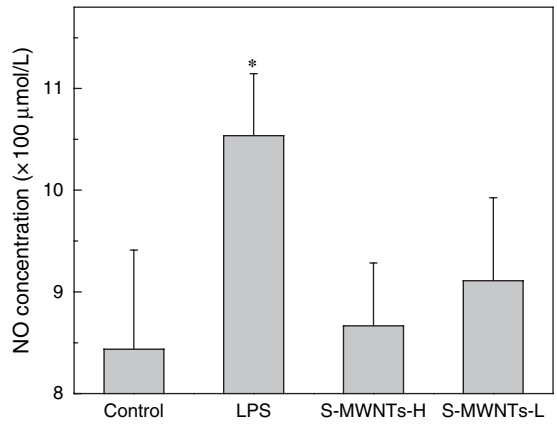

Fig. 5. Effect of S-MWNTs on NO secretion of peritoneal macrophages. Data are the mean ±S. D. of 10 mice. (∗represents $p < 0.05$ comparing to the control group, S-MWNTs-H and S-MWNTs-L means the mouse was exposed to S-MWNTs at dose of 2 and 0.2 mg/kg body weight, respectively).

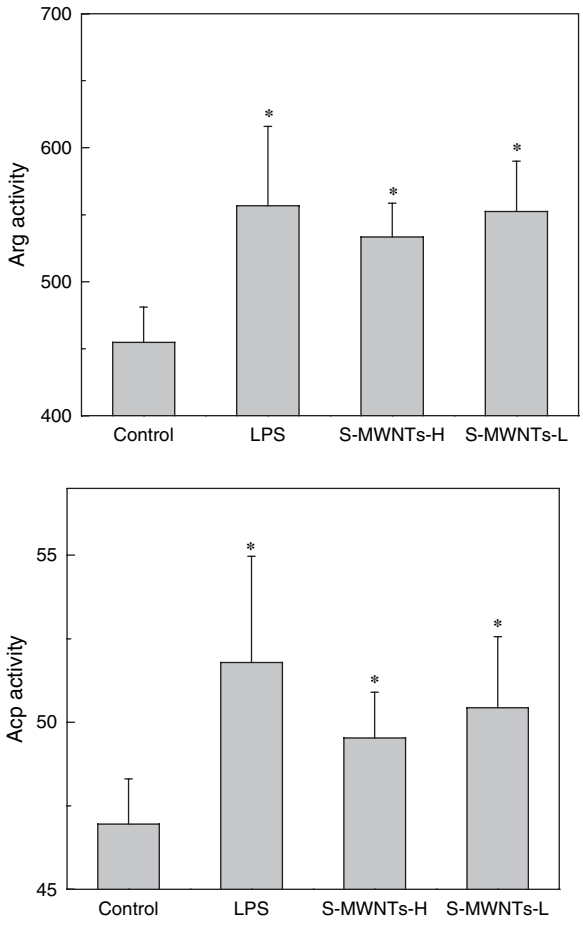

Fig. 4. Effect of S-MWNTs on the activity of Arg and Acp of peritoneal macrophages. Data are the mean ±S. D. of 10 mice. (∗represents $p < 0.05$ comparing to the control group, S-MWNTs-H and S-MWNTs-L means the mouse was exposed to S-MWNTs at dose of 2 and 0.2 mg/kg body weight, respectively).

indicates that S-MWNTs can active the digestion activity of macrophage.

Macrophages nitric oxide (NO) produced by the inducible NO synthase (iNOS) is a key inflammatory mediator and a defense mechanism against bacteria, viruses, and transformed cells. NO is a kind of radicals, with half-life of only a few seconds, and easily forms nitrate or nitrite in biological system.[23] Data of NO release by peritoneal macrophages of mice are depicted in Figure 5. As can be seen, the two doses (0.2 mg/kg and 2 mg/kg) of S-MWNTs do not influence macrophage NO production in relation to data obtained in animals of the control group.

4. DISCUSSION

Nano-sized material is greatly different from traditional material with the same composition, and may have many excellent performances and entirely new functions. Some nanomaterials have shown wide applications in many fields. Nanotechnology brings us many advantages, but a few studies also demonstrate that some nanomaterials also bring disadvantages due to their small size and large surface.[24] *In vitro* studies have demonstrated the functionalized soluble CNTs could effectively transport across the cell membrane into various types of cells.[25] *In vivo* studies show that CNTs are easily scavenged by macrophages in different tissues.[26] Macrophages, as the inductors of pathogen, play very important role in eliminating pathogen and regulating proper immune reaction. The activation of macrophages is a prerequisite to participate effectively in the immune surveillance.[16]

Phagocytic ability is a major function of macrophages that largely affects the immunological potential of lungs and other related organs.[16] Here, the method of CRBC

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experiment was used to evaluate xenogeneic phagocytosis. We clearly showed that S-MWNTs did not impair phagocytosis of macrophage, but partly enhanced the phagocytosis activity. This is not consistent with the *in vitro* result from Jia et al., who found MWNTs significantly impaired alveolar macrophage phagocytosis at a higher dose of 3.06 μg/cm.[2, 9] We infer that the conflict may come from the different surface and water-solubility of MWNTs. In our paper, taurine functionalized and water soluble MWNTs were used, while Jia et al. only used the non-functionalized MWNTs. The functionalization may improve biocompatibility of CNTs. Interestingly, Cherukur et al. reported that macrophages can ingest detergent (Pluronic)-solubilized SWNTs without showing significant toxicity through the fluorescence NIR imaging microscopy.[27]

As discovered by many reported work, the phagocytized CNTs usually relocate in the lysosomes of cells.[17] In order to assess the effect of S-MWNTs on macrophages, two types of enzyme in lysosome were measured to evaluate the lysosome function after macrophages were exposed to S-MWNTs. Acp is a hydrolase inside the lysosome and is considered to provide cytochemical criteria for evaluation of macrophage activation, while Arg is involved in anti-tumor cytotoxicity by macrophages, and both enzyme participate in a variety of lysosomal digestive function. As shown in Figure 4, the Arg and Acp activity of the S-MWNT treated groups were higher than those of control group. Again, these two lysosomal enzymatic markers give the indication that S-MWNTs can activate macrophages.

Induction of intracellular oxidative stress seems to be a key event of the biological effects of some nanomaterials.[28] Because of small in size, nanoparticles may be translocated from site of exposure to distant organs; the oxidative activity of some nanomaterials that enter the nucleus can alter pro-inflammatory genes, such as iNOS. In fact, NO acts to kill invading microbes and tumor cells in phagocytes and plays an important role in the inflammatory response to infections. In macrophages, NO is synthesized by iNOS from L-arginine.[29] In our experiment, NO was measured indirectly by determination of NO_2^- concentration and the results showed that both dose of S-MWNTs did not influence NO secretion in macrophages. This is consistent with the results from Pulskamp et al., who indicated that the CNTs did not induce the inflammatory mediators NO, TNF-α and IL-8.[30]

5. CONCLUSION

In summary, after investigating the macrophage phagocytosis rate, enzyme activity in lysosome and NO secretion, we demonstrate that S-MWNTs can activate peritoneal macrophages but not induce the inflammation. The results are very useful in evaluating the health impacts of CNTs and we believe these data will benefit the broad application of CNTs in drug delivery.

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References and Notes

1. C. Y. Wang, Y. Y. Zhang, C. M. Wang, and V. B. C. Tan, *J. Nanosci. Nanotechnol.* 7, 4221 (**2007**).
2. M. Prato, K. Kostarelos, and A. Bianco, *Acc. Chem. Res.* 41, 60 (**2008**).
3. L. Lacerda, H. Ali-Boucetta, M. Herrero, G. Pastorin, M. Prato, and K. Kostarelos, *Nanomedicine (UK)* 3, 149 (**2008**).
4. C. W. Lam, J. T. James, R. McCluskey, S. Arepalli, and R. L. Hunter, *Cri. Rev. Toxicol.* 36, 189 (**2006**).
5. J. Muller, F. Huaux, and D. Lison, *Carbon* 44, 1048 (**2006**).
6. C. C. Chou, H. Y. Hsiao, Q. S. Hong, C. H. Chen, Y. W. Peng, H. W. Chen, and P. C. Yang, *Nano Lett.* 8, 437 (**2008**).
7. A. Huczko and H. Lange, *Fullerene. Sci. Techol.* 9, 247 (**2001**).
8. M. L. Schipper, N. Nakayama-Ratchford, C. R. Davis, N. M. S. Kam, P. Chu, Z. Liu, X. M. Sun, H. J. Dai, and S. S. Gambhir, *Nature Nanotechol.* 3, 216 (**2008**).
9. G. Jia, H. F. Wang, L. Yan, X. Wang, R. J. Pei, T. Yan, Y. L. Zhao, and X. B. Guo, *Environ. Sci. Technol.* 39, 1378 (**2005**).
10. M. Bottini, S. Bruckner, K. Nika, N. Bottini, S. Bellucci, A. Magrini, A. Bergamaschi, and T. Mustelin, *Toxicol. Lett.* 160, 121 (**2006**).
11. K. Kiura, Y. Sato, M. Yasuda, B. Fugetsu, F. Watari, K. Tohji, and K. I. Shibata, *J. Biomed. Nanotechnol.* 1, 359 (**2005**).
12. H. Dumortier, S. Lacotte, G. Pastorin, R. Marega, W. Wu, D. Bonifazi, J. Briand, M. Prato, S. Muller, and A. Bianco, *Nano Lett.* 6, 1522 (**2006**).
13. L. A. Mitchell, J. Gao, R. Vander Wal, A. Gigliotti, S. W. Burchiel, and J. D. McDonald, *Toxicol. Sci.* 100, 203 (**2007**).
14. A. A. Shvedova, E. Kisin, A. R. Murray, V. J. Johnson, O. Gorelik, S. Arepalli, A. F. Hubbs, R. R. Mercer, P. Keohavong, N. Sussman, J. Jin, J. Yin, S. Stone, B. T. Chen, G. Deye, A. Maynard, V. Castranova, P. A. Baron, and V. E. Kagan, *Am. J. Physiol. Lung Cell Mol. Physiol.* 295, L552 (**2008**).
15. D. H. Bowden, *Environ. Health Perspect.* 55, 327 (**1984**).
16. S. T. Yang, W. Guo, Y. Lin, X. Y. Deng, H. F. Wang, H. F. Sun, Y. F. Liu, X. Wang, W. Wang, M. Chen, Y. Huang, and Y. Sun, *J. Phys. Chem. C* 111, 17761 (**2007**).
17. X. Y. Deng, G. Jia, H. F. Sun, H. F. Wang, X. Wang, S. T. Yang, T. C. Wang, and Y. F. Liu, *Carbon* 45, 1419 (**2007**).
18. W. Y. Zhang, Y. Wang, and Y. Y. Hou, *Int. Immunopharmacol.* 4, 461 (**2004**).
19. J. K. Lee, J. A. Byun, S. H. Park, H. J. Choi, H. S. Kim, and H. Y. Oh, *Toxicology* 211, 187 (**2005**).
20. I. M. Corraliza, M. L. Campo, G. Soler, and M. Modolell, *J. Immunol. Methods* 174, 231 (**1994**).
21. E. Y. Moon, D. K. Rhee, and S. Pyo, *Cancer Lett.* 136, 167 (**1999**).
22. J. Zhu, Z. Ji, J. Wang, R. Sun, X. Zhang, Y. Gao, H. Sun, Y. Liu, Z. Wang, A. Li, J. Ma, T. Wang, G. Jia, and Y. Gu, *Small* 4, 1168 (**2008**).
23. L. J. Ignarro, *FASEB J.* 3, 31 (**1989**).

24. K. A. D. Guzman, M. R. Taylor, and J. F. Banfield, *Environ. Sci. Technol.* 40, 1401 **(2006)**.
25. N. W. S. Kam, T. C. Jessop, P. A. Wender, and H. J. Dai, *J. Am. Chem. Soc.* 126, 6850 **(2004)**.
26. Z. Liu, C. Davis, W. B. Cai, L. He, X. Y. Chen, and H. J. Dai, *Proc. Natl. Acad. Sci. USA* 105, 1410 **(2008)**.
27. P. Cherukur, S. M. Bachilo, S. H. Litovsky, and R. B. Weisman, *J. Am. Chem. Soc.* 126, 15638 **(2004)**.
28. V. Castranova, *Free Radic. Biol. Med.* 37, 916 **(2004)**.
29. Y. Xia and J. L. Zweier, *Proc. Natl. Acad. Sci. USA* 94, 6954 **(1997)**.
30. K. Pulskamp, S. Diabate, and H. F. Krug, *Toxicol. Lett.* 168, 58 **(2007)**.

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