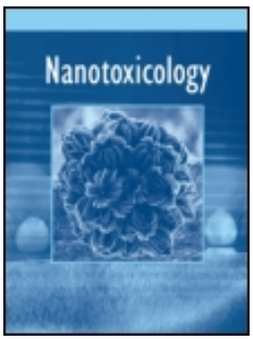

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Immune responses of BALB/c mice to subcutaneously injected multi-walled carbon nanotubes

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Abstract

Carbon nanotubes have been shown to have the ability to transport therapeutic and detective reagents into cells. However, the rapid advances in new carbon nanotube-based materials and technologies have raised concerns about their safety. Such concerns require a fundamental understanding of the toxicological properties of carbon nanotubes. In particular, the use of carbon nanotubes as drug or probe delivery platforms may depend on the prevention of stimulatory side-effects to the immune system. In this study, we investigated the immunological properties of oxidized water dispersible multi-walled carbon nanotubes (MWCNTs) in healthy BALB/c mice. We injected the MWCNTs subcutaneously, and the immune responses of the mice were monitored over time. We show that the MWCNTs induce complement activation and the production of pro-inflammatory cytokines early after injection of the mice, and that the levels of complement and cytokines return to normal levels over time. With the exception of the lymph nodes, there was no obvious accumulation of MWCNTs observed in the liver, spleen, kidney, or heart. In addition, we did not observe injury in the organs or lymph nodes. Our results indicate that local, subcutaneous administration of MWCNTs induces obvious short-term immunological reactions, which can be eliminated over time.

Keywords: *Nanotubes, nanotoxicity, nanomedicine, biocompatibility*

Introduction

In recent years, carbon nanotubes have attracted increased attention from the biomedical field. These nanotubes have been shown to exhibit the ability to penetrate cells and to deliver therapeutic and detective reagents into cells (Kam et al. 2004; Bianco et al. 2005; Kam and Dai 2005; Kam et al. 2006; Chen et al. 2008; Foldvari and Bagonluri 2008; Klingeler et al. 2008; Liu et al. 2008a; Pastorin 2009; Mehra et al. 2008) and to protect nucleic acids from nucleic enzymes (Jia et al. 2007; Cheng et al. 2008; Krajcik et al. 2008; Wu et al. 2008; Yandar et al. 2008; Liu et al. 2009). In addition, they have also been shown to have the potential to be applied as novel photoacoustic agents in molecular imaging (Zerda et al. 2008).

However, the rapid advances in new carbon nanotube-based materials and technologies have raised concerns about their safety. Such concerns require a fundamental understanding of their toxicological properties. In particular, the use of carbon nanotubes as drug or probe delivery platforms may depend on the prevention of stimulatory side-effects to the immune system. A number of groups have reported that carbon nanotubes can impact the immune system by activating the complement system (Salvador-Morales et al. 2006, 2008; Hamad et al. 2008) and promoting inflammation (Chou et al. 2008; Murray et al. 2009). The induction of immune responses and toxicity by carbon nanotubes largely depends on their route of administration. Administration of carbon nanotubes by intratracheal instillation leads to overt inflammation and fibrosis in the lungs (Warheit et al. 2004; Mitchell et al. 2007, 2009; Muller et al. 2008) and may promote allergic responses in mice (Journeay et al. 2008; Nygaard et al. 2009). Poland et al. (2008) showed that the introduction of long multi-walled carbon nanotubes into the abdominal cavity of mice leads to inflammation and the formation of granulomas. In contrast, intravenous administration of single-walled carbon nanotubes was reported to exhibit low toxicity.

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For example, Yang et al. (2008) investigated the effects and toxicity of long-term accumulation of single-walled carbon nanotubes administrated intravenously into mice. They reported that the nanotubes accumulated in the lungs, liver and spleen and provoked low-level inflammatory cell infiltration into the lungs. In addition, in a pilot study using a small number of mice, Schipper et al. (2008) reported that single-walled carbon nanotubes do not induce obvious toxicity when administered intravenously.

In this study, we investigated the immunological properties of oxidized water dispersible multi-walled carbon nanotubes (MWCNTs) injected subcutaneously into healthy BALB/c mice. In particular, we examined the immune responses of the mice to the nanotubes over time.

Materials and experiment

Preparation and characterization of MWCNTs

MWCNTs were purchased from Chengdu Organic Chemicals Co. Ltd (Chengdu, China). The MWCNTs were prepared using an oxidation/sonication procedure; these were characterized by scanning electron microscopy (SEM) and X-ray photon spectroscopy (XPS), as described previously (Meng et al. 2009), to examine their morphology and surface chemistry. The purity of the MWCNTs, which was determined to be 98.6%, was examined by inductively coupled plasma mass spectrometry (ICP-MS). The MWCNTs and all other materials used for biological experiments were sterilized by autoclaving. The sterilized MWCNTs were dispersed in water using a sonication probe.

Animals and injection schedule

Eight-week-old female BALB/c mice were maintained at the Experimental Animal Center at the Institute of Basic Medical Sciences of the Chinese Academy of Medical Sciences (Beijing, China) under specific pathogen-free conditions. The mice were fed tap water and autoclaved food pellets. All the animal experiments reported herein were carried out in compliance with the national regulations governing animal experiments. Following acclimatization, naïve female BALB/c mice were randomly divided into two groups according to weight. One group received injections of MWCNTs (CNT, n = 36), and the control group (Ctrl, n = 12) received injections of pure water in same volumes as the CNT group. The CNT group was further divided into three subgroups as subgroup 1, subgroup 2, and subgroup 3 (n = 12 for each subgroup). The mice in the three subgroups received two s.c injections (neck subcutis) with MWCNT, once a week. In single injection, 0.05 mg, 0.3 mg and 0.5 mg of MWCNTs was given to each mouse in subgroup 1, subgroup 2, and subgroup 3, respectively. The total MWCNTs for each mouse in subgroup 1, subgroup 2, and subgroup 3 was 0.1 mg (0.05 mg × 2), 0.6 mg (0.3 mg × 2) and 1.0 mg (0.5 mg × 2), respectively. Dosage and time points in the all experiments are summarized in Supplementary Table S3 and Table S4 respectively (available online).

Histology and immunohistochemistry

The mice which received the highest dose of MWCNTs (total dose of 1.0 mg) were sacrificed by designed time point post-injection within 90 days. The livers, spleens, kidneys, hearts and lymph nodes of the mice in the CNT and Ctrl groups were excised and fixed in 4% paraformaldehyde overnight. The tissue surrounding the injection sites were also collected and fixed. All the tissues were then embedded in paraffin. Multiple 5 μm thick tissue sections were cut using a microtome and stained with hematoxylin and eosin (H&E) using standard methodology. For immunohistochemical staining, the sections were dewaxed, rehydrated, treated with 0.1 mg/ml trypsin and incubated at 37°C to retrieve the antigens. The macrophage glycoprotein F4/80 was detected in the sections by treating them with a rat monoclonal anti-F4/80 IgG antibody (BMA Biomedicals).

Mouse inflammation cytokine array

Blood samples from four mice in the CNT group, which received the highest dose of MWCNTs, and the Ctrl group were diluted for the preparation of serum. The levels of cytokines in the serum were then examined using a mouse inflammation cytokine antibody array kit (RayBiotech, Inc., Norcross, GA, USA) according to the manufacturer's instructions. In this cytokine array, biotin-conjugated IgG is used to produce positive control spots, which are used to identify the orientation and to compare the relative expression levels of the cytokines in the different membranes. The cytokines on the membranes were visualized using a chemiluminescent imaging system (Alpha FluorChem). The positive signals on the same membrane, visualized as spots using chemiluminescent imaging software, were averaged and used as a positive control for normalization. Each spot was normalized to the positive control on the same membrane; the normalized relative expression was calculated as following:

$$I_{normalized\ sample} = I_{sample\ spot}/I_{positive\ control\ spot}$$

where I represents the spot intensity, *sample* means any one of the cytokines from CNT group or from Ctrl group.

The relative expression of Ctrl group was set to 1, and then the variation of relative expression level of each cytokine for CNT group was calculated as following formula and presented as the n-fold change (the threshold was set as 2-fold):

$$\text{Variation in n - fold change} = I_{normalized\ CNT}/I_{normalized\ Ctrl}$$

Detection of complement 3 (C3) and C5a proteins

During the experiment, blood samples were collected from the tails of the mice on days 2, 7, 30, and 45 after injection of MWCNTs. The blood samples were clotted at room temperature and centrifuged at 3000 rpm for 30 min to separate the serum. The serum samples were stored at -80°C for later use.

The serum samples were coated in triplicate onto 96-well plates (Costar) and incubated at 4°C overnight. Rat anti-mouse C3 antibody of 0.1 mg/ml (HyCult Biotechnology) was diluted to 0.025 μg/ml and added to the wells, and the plates were incubated at 37°C for one hour. Horseradish peroxidase (HRP)-conjugated anti-Rat IgG antibody (Rockland Immunochemicals, Inc.) was added to the wells, and HRP substrate was used for detection. When the reaction was stopped, the absorbance of the samples was read at 450 and 630 nm wavelengths (Synogen 4). The background absorbance of the samples at 630 nm was subtracted from their absorbance at 450 nm to obtain the final values. To measure the levels of C5a in the serum of the mice, we performed a sandwich enzyme-linked immunosorbent assay (ELISA) using a similar protocol.

Statistical analysis

Single-factor analysis of variance (ANOVA) was employed to assess the statistical significance of the results. All data are presented as the mean ± SD. P-values of < 0.05 were considered statistically significant.

Results

Accumulation and histological observation of MWCNTs

Anatomically, the skin is composed of three primary layers: The epidermis, dermis and the subcutis (also known as the hypodermis or subcutaneous layer). The subcutis is the innermost layer of the skin. Here, we injected the MWCNTs into the subcutis. To document any pathological changes in the area of the subcutis where the MWCNTs of 1 mg was injected, the mice in the CNT group were sacrificed on days 2, 7, 30, 60 and 90 post-injection, and the tissue surrounding the injection site was excised and processed for H&E stained. An anatomic optical graph of the tissues 90 days after injection (Figure 1a) revealed that the area surrounding the injection site exhibited a black color. For comparing the amount of MWCNTs in the injection site over time, an anatomic optical graph and H&E examination with the mice received subcutaneous injection for 1 hour is provided by Supplementary Figure S1, available online. As Supplementary Figure S1 shows, there are large amounts of MWCNTs in the injection site, many of which have been taken up by the cells. There was not obvious acute inflammation or appreciable necrosis in the subcutis around the injection site. Comparing the black color of the subcutis between 1 h and 90 days post injection, the color had little variation during the experimental period, implying that major part of the injected MWCNTs stayed around the injection site.

Macrophages are sentinel cells of the innate immune system, and they have a significant influence on the overall development of the body's immune response. They are one of the most phagocytic cells of the immune system and are responsible for the clearance of invading foreign particles or pathogens. In addition, they secrete a wide variety of cytokines that are vital to the generation of an immune response (Klimp et al. 2002). Figure 1b-1 shows an H&E-stained image of the tissues at the injection site that corresponds to Figure 1a. Analysis of H&E-stained sections revealed that a large number of black cells, which engulfed the injected MWCNTs, gathered around the injection sites. Because macrophages are larger than lymphocytes and have irregular shapes, unlike round lymphocytes, it is likely that majority of the black cells are macrophage cells. We confirmed the identity of these black cells by staining the tissue sections with an antibody against the macrophage membrane glycoprotein F4/80. We found that the cells that engulfed the MWCNTs were mostly macrophages, as shown by the brown-colored staining of many areas of the tissue with F4/80 antibody (Figure 1b-2). In the H&E stained sections (Figure 1b-1) slight fibrosis due to collagen deposition could be observed in the subcutis around the injection site (fiber-like substance in pink color, pointed by yellow arrow heads) as well as a small number of lymphocytes, showing that there was low-level inflammation at the site of MWCNTs injection.

The MWCNTs are highly stable in water. In contrast, they are likely to aggregate in PBS buffer or

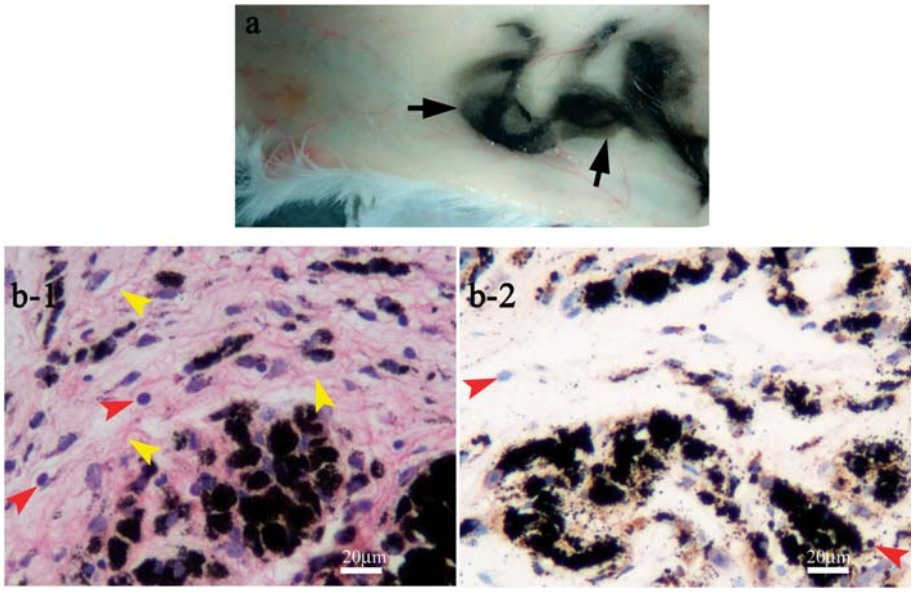

Figure 1. Anatomic and histological analyses of subcutis around injection sites of MWCNTs 90 days post injection. (a) A representative anatomic photograph. Black arrows point to MWCNTs aggregation in the subcutis tissue. (b-1) Histological observation of the subcutis. (b-2) Immunohistochemistry analysis of the subcutis. Red arrows in b-1 and b-2 point to lymphocytes. MWCNTs aggregation remains mostly in macrophages ($F4/80^{+}$cells in b-2) cells in subcutis.

saline because salt ions in these solutions can decrease the interactions between the water molecules and the MWCNTs. Therefore, the physiological environment of the subcutis can affect the stability of the MWCNTs. When injected into a biological environment, the MWCNTs will aggregate due to the influence of various salt ions; large amounts of the remaining MWCNTs are engulfed by macrophages attracted to the injection site.

Interestingly, the axillary lymph nodes exhibited a light black color, indicating that the subcutaeously injected MWCNTs migrated from the subcutis to the nearest skin-draining lymph nodes. Figure 2a-1 shows an example of some of these partially black axillary lymph nodes, which were excised 90 days after the injection of MWCNTs, implying some of the MWCNTs stayed in the lymph nods for at least three months. Figure 2a-2 presents H&E image of the axillary lymph nodes that were not exposed to the MWCNTs. Figure 2b-1 shows that, in axillary lymph nodes excised 30 days after injection, we could observe scattered aggregation of MWCNTs mainly in the subcapsular sinuses, which are macrophge-rich regions of the lymph nodes. The MWCNTs were also detected, to a lesser extent, in the cortical sinuses. Sixty days after injection, more obvious aggregation of MWCNTs could be observed in the cortex region and in the medullary region (Figure 2b-2). Ninety days after injection, the MWCNTs in the cortex were observably less than at 60 days post-injection, but the aggregates were larger in size (Figure 2b-3). Figure 2b-4 (left) shows a whole view of the distribution of MWCNTs in subcapsular sinuses, cortical sinuses, and medullary region of axillary lymph nodes. Figure 2b-4 (right) provides the distribution of MWCNTs in medullary region in a magnified view. As it has been demonstrated that there are large amounts of macrophage cells engulfing the MWCNTs in the injection site, we would consider that a part of the engulfed MWCNTs was carried away by the macrophage cells and entered into the lymph nods via the lymph ducts.

Figure 3 shows representative histological images of sections of the liver, kidney, spleen and heart of mice in the CNT group 2, 7, 30, 60, and 90 days after injection of 1 mg of MWCNTs. For the mice scarified on day 2 post injection MWCNTs, the hepatocytes in the liver were little pale stained swollen with few inflammatory cells and sinusoids were congestion. The vessels in the glomeruli of kidney presented congestion and perivascular edema. On day 7 post MWCNTs injection, the hepatocytes almost reversed to normal morphology and the edema of the kidney was also reduced. On day 30, the liver and kidney of CNT group exhibited normal histology. The above observations implied that the subcutaneously injected MWCNTs induced short-term immune responses. The tissues collected on day 30 and 60 post injection exhibited normal histology, showing no obvious signs of immune reactions as well as tissue degeneration,

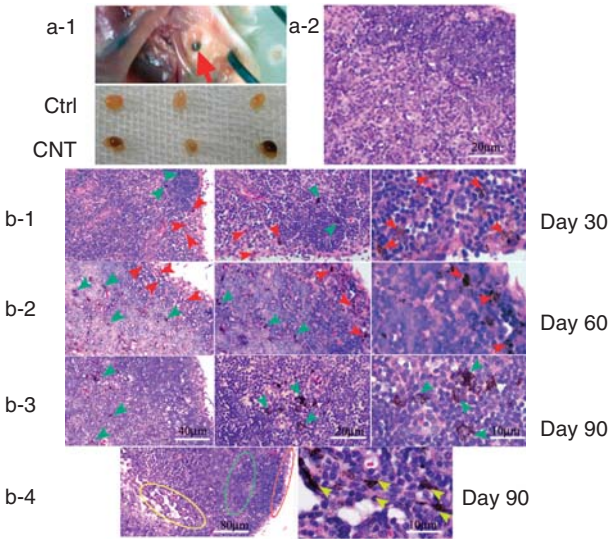

Figure 2. Histological observations of axillary lymph nodes of the mice up to 90 days post subcutaneous injection of MWCNTs. (a-1 [up]) Anatomic graph of mice 90 days post injection of MWCNTs; the red arrow points to an axillary lymph node that becomes black. (a-1 [bottom]) Photograph of axillary lymph nodes isolated from on day 90 from mice of Ctrl and CNT group. (a-2) H&E analysis of axillary lymph nodes of Ctrl group. (b-1 to b-4) The MWCNT distribution in the axillary lymph nodes. Red arrows point to the MWSNTs distributed in the subcapsular sinuses, green arrows point to the MWCNT in the cortical sinuses, and yellow arrows ponits to the MWCNTs in the medullary region. The red, green and yellow circle indicats the regions of subcapsular, cortical and medullary sinuses respectively.

necrosis or fibrosis. However, on day 90 after injection, we noted larger gaps between the cells and lighter staining of the cells H&E in the liver, suggesting that subcutaenous injection of MWCNTs has a minor influence on the histology of the liver. Another noteworthy observation is that, unlike occured in the axillary lymph nodes, there was no obvious accumulation of MWCNTs in the liver, spleen, kidney, and heart. We also compared the weight of these organs excised from mice in the CNT and Ctrl groups on days 2, 7, 30, 60, and 90 post-injection and found no significant differences (see Supplementary Table S1, available online).

Inflammatory cytokines in the serum of mice treated with MWCNTs

The major function of macrophages is to phagocytose particulate matter. Upon their activation, macrophages induce the production of inflammatory cytokines, which influence many processes of the innate and adaptive immune response, including inflammation, defense against infection, proliferation of antigen-specific T and B cells, and regulation of differentiated function of T and B cells.

To analyze the cytokine profiles in the serum of the mice that were injected with MWCNTs, we employed a cytokine array to detect the relative expression levels of 40 different inflammatory cytokines 2 and 7 days post-injection. Representative arrays are shown in Figure 4a, and the analysis of the arrays and the cytokine expression profiles are shown in Figure 4b, and Supplementary Table S2 (available online). Among the 40 cytokines, 19 were clearly up-regulated to more than 2-fold change 2 days after injection with MWCNTs. Among these, interleukin (IL)-17 was the most significantly up-regulated, as indicated by its 5.41-fold increase in expression in the CNT group; I-TAC, IL-1β, and IFN-γ exhibited a more than 3-fold increase in expression, and IL-1α, IL-2, IL-3, IL-6, IL-10, IL-13, CD30L, G-CSF, GM-CSF, KC, leptin, MIG, TIMP-2, and TNF-α were modestly up regulated, as shown by a 2-fold increase in their expression. Seven days after injection of MWCNTs, most of the cytokines returned to almost normal levels, with only slight variations when compared to the levels of the Ctrl group. Analysis of the cytokine array indicated that the MWCNTs induce acute inflammation within 2 days after injection. This phenomenon is consistent with a previous report (Park et al. 2009) in which cytokines in the blood, including IL-1, IL-6 and IL-10, reached their highest levels before the third day after a single intratracheal instillation of nanotubes. It is important to note that, here, the acute inflammatory response that ensued in the mice apparently disappeared and the cytokine levels returned to normal seven days after injection of MWCNT.

The complement system is a major component of the innate immune system and is comprised of more than 30 serum proteins and cell surface receptors that interact to recognize, opsonize and clear or kill invading microorganisms, altered host cells and other foreign materials. The activation of complement is organized into three separate pathways, the classical, lectin and alternative pathways, all of which converge at the third complement component (C3). C3 is enzymatically cleaved into C3a and C3b, which are deposited on the surface of cells or microbes. This process, in turn, leads to the cleavage of C5 and the release of C5a, which act to recruit immune cells to the site of inflammation (Dunkelberger and Song 2010). Therefore, consumption of C3 and production of C5a are indicative of complement activation. We investigated whether subcutaneously injected MWCNTs induced the activation of the complement system by assaying the levels of C3 and C5a in the serum of the mice. As shown in Figure 5a, the levels of C3 decreased in the serum two days after injection of MWCNTs, further

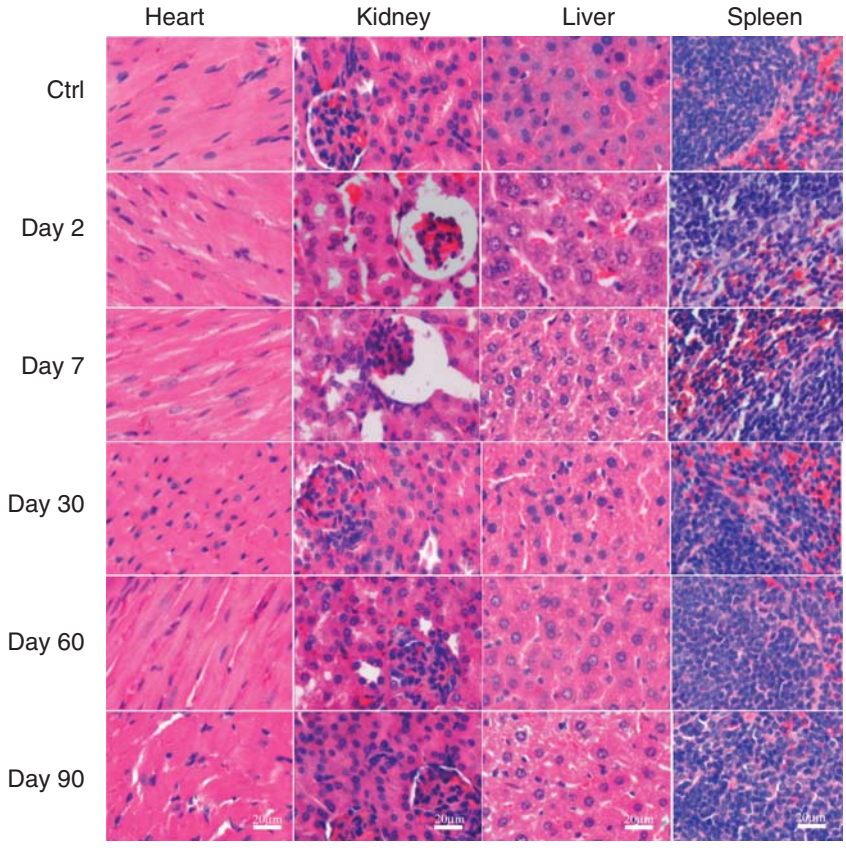

Figure 3. Histological observations of spleen, liver, kidney and heart of the Ctrl and CNT group on day 2, 7, 30, 60 and 90 after received subcutaneous injection of MWCNTs of 1.0 mg per mouse.

decreased 7 days post-injection and then returned to normal levels 30 days after injection. The levels of C5a displayed an increased trend (Figure 5b). These results indicate that MWCNTs activate the complement system. Although the levels of C3 and C5a varied over time, we found no statistically significant differences between mice that received different doses of MWCNTs. Based on our results, we speculate that the variations observed in the levels of C3 and C5a caused by MWCNTs are likely to be time but not dose dependent.

Discussion

The immune system is the major defense against invading pathogens and foreign particulate matter. Nanoparticles and many of nanoparticle-based biological therapeutics are often immunostimulatory. As such, the immune system efficiently recognizes them as foreign substances and mounts a multilevel immune response against them, which raises concerns about their safety. For example, complement activation by systemically administered drugs is responsible for some tissue injury and hypersensitivity (allergic) reactions and anaphylaxis, which is a life-threatening condition (Dobrovolskaia and McNeil 2007).

We administered MWCNTs into mice via subcutaneous injection. The dynamic variation in the levels of C3 and C5a in the serum indicates that this route of administration induces complement activation shortly after injection of MWCNTs. However, the levels of these complement proteins return to normal levels one week after injection. The profile of inflammatory cytokine expression is in agreement with the activation of complement. Thus, our results suggest that subcutaneous administration of MWCNTs is relatively safer than systemic administration. To use carbon nanotubes for biomedical applications, it is necessary that the acute inflammation that they induce resolves over time. Our histological observations indicate that there were no appreciable pathological changes in the liver, spleen, kidney and heart of the mice 90 days after injection of MWCNTs.

High levels of complement activation are known to generate inflammatory responses and promote the formation of granulomas. Our histological analyses showed that only a minor inflammatory reaction was

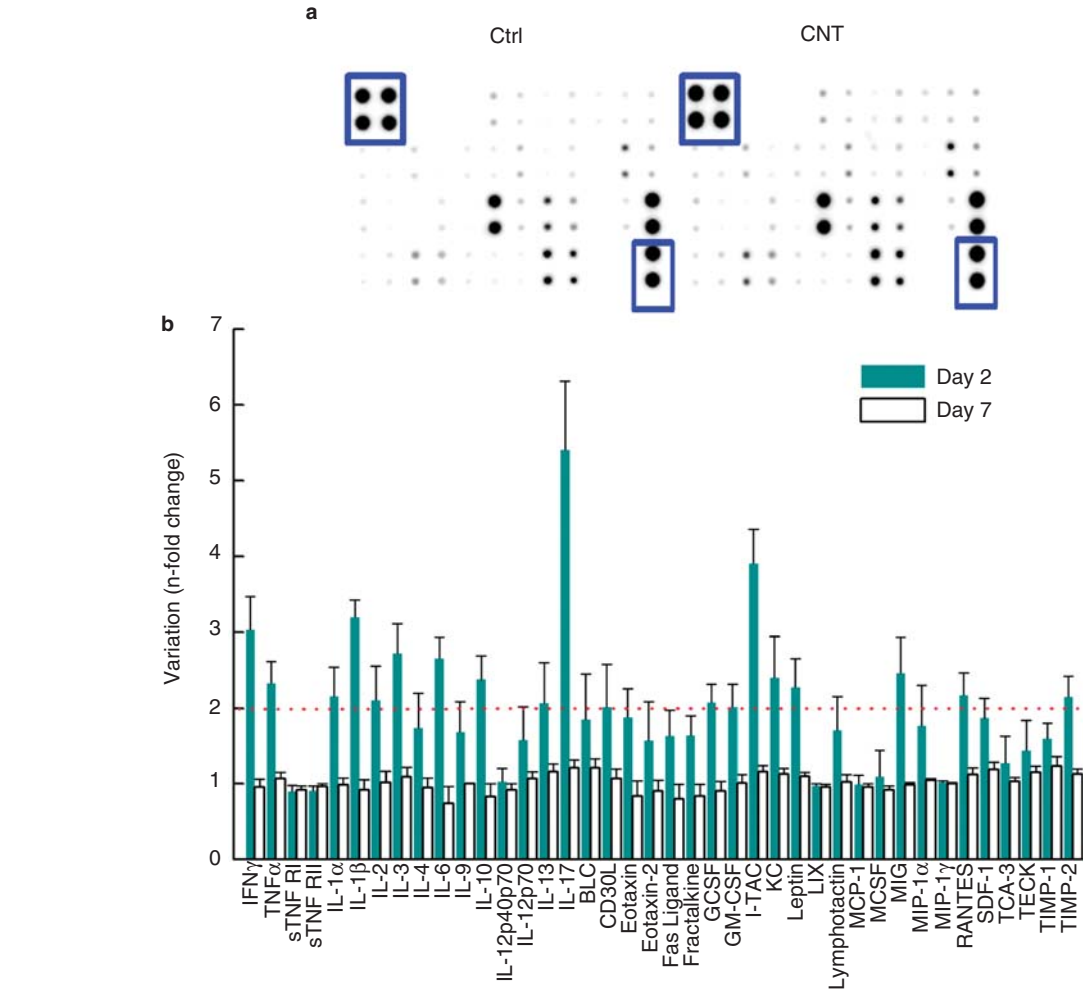

Figure 4. Variation of the inflammation cytokines profile for mice received subcutaneous injection of MWCNTs of 1.0 mg per mouse. (a) The representative arrays, positive spots indicate by blue rectangles; (b) The variations for the 40 cytokines level.

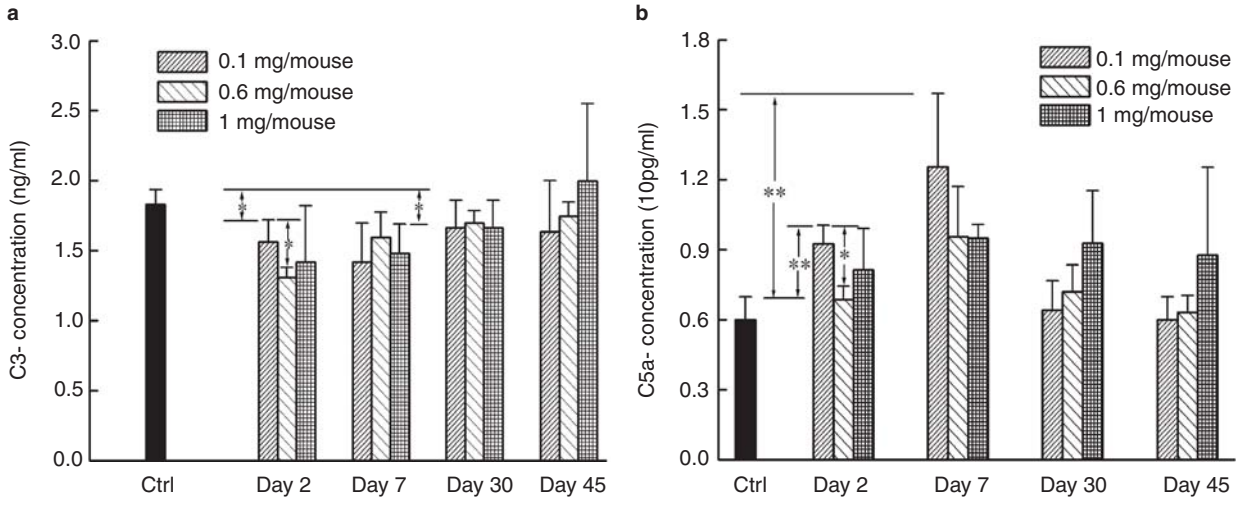

Figure 5. Complement activation induced by subcutaneous injection of MWCNTs at different time points, applying three different CNT dosages. (a) C3, (b) C5a. Difference was considered statistically significant at $p < 0.05$ ($^{*}P < 0.05$, $^{**}P < 0.01$).

produced at the injection site, and no granulomas were observed over time. These results are opposite to what has been shown to occur when carbon nanotubes are intratracheally or intraperitoneally instilled, which induces the formation of granulomas within 90 days post-administration (Poland et al. 2008). This low-level inflammatory reaction correlates with the relatively low levels of complement activation observed shortly after injection, which resolves at a later time point.

It was hard to determine whether all CNT injected subcutaneously were ingested by macrophage cells because there were no obvious free MWCNTs in the subcutis found by intensive histological observations. We would suggest that the majority of injected MWCNTs were likely captured by the cells. The accumulation of MWCNTs in the organs is an additional concern for their safety. Histological analyses revealed that there was no obvious aggregation of MWCNTs in the liver, spleen, kidney and heart. This result indicates that subcutaneous administration of MWCNTs does not lead to their significant accumulation in the organs. This result is very different from what has been reported for intravenous administration of single-walled carbon nanotubes, which accumulate in the lung, liver and spleen, and induce a low-level inflammatory response in the lungs (Yang et al. 2008).

The accumulation of MWCNTs in the axillary lymph nodes has two implications. The lymph nodes are one of the major sites of cancer metastases. The ability of MWCNTs to migrate to and accumulate in the lymph nodes makes them potential candidates for use as adjuvants or delivery vehicles to target these immune organs. However, the long-term effects of the accumulation of MWCNTs in the lymph nodes were previously unknown. According to our histological observations, over time, MWCNTs migrated from the subcapsular sinus to the cortical sinus and then to the medullary region. We would suggest that the MWCNTs in the lymph nods might migrate over time in a very slow rate because they could be observed in the lymph nodes three months post the injection. As the amount of MWCNTs in the lymph nodes is quite small, and their migration in the lymph nodes seemed very slow, it is hard to find appreciable MWCNTs accumulated in the liver by histological observation. As such, MWCNTs have the potential to be used for *in vivo* applications. However, this possibility warrants further investigation.

Elimination of metal residues from carbon nanotubes-based products has been a big technical challenge as well as an important issue. Strong oxidation procedure is helpful to remove the majority of metals loaded on the outside and internal surfaces of nanontubes, but does not work for metals packed inside the surrounding carbon fragments. Recent studies suggest that the encapsulated metals are non-bioavailable for at least two months (Liu et al. 2007, 2008b). Therefore we would consider that the remaining impurity contributed little to the toxicity in the current study.

Conclusions

We have shown that, shortly after administration, subcutaneously injected MWCNTs induces the activation of complement and the production of inflammatory cytokines in healthy BALB/c mice. However, the levels of active complement components and cytokines return to normal levels within three months after injection of MWCNTs. With the exception of the lymph nodes, there was no obvious accumulation of MWCNTs in the liver, spleen, kidney and heart. The MWCNTs that accumulated in the lymph nodes reached their highest levels two months after injection and then decreased three months post-injection. In addition, no obvious tissue injury was observed in the organs and lymph nodes. Altogether, our results suggest that subcutaneous injection of MWCNTs induces a short-term immunological reaction and that this route of administration is relatively safer than systemic administration of carbon nanotubes.

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***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Supplementary material available online

Tables S1–4
Figure S1.