Toxicology Letters 217 (2013) 91–101

Contents lists available at SciVerse ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Immunomodulatory properties of multi-walled carbon nanotubes in peripheral blood mononuclear cells from healthy subjects and allergic patients

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HIGHLIGHTS

- Carbon nanotubes do not modulate cytokine secretion by resting PBMC.
- Carbon nanotubes increase TLR agonist-induced cytokine secretion by PBMC.
- Carbon nanotubes increase PHA-induced T cell cytokine release from PBMC.
- Carbon nanotubes inhibit allergen-induced IL-5 release by PBMC from allergic patients.
- Carbon nanotubes have the capacity to blunt dendritic cell differentiation.

ARTICLE INFO

Article history:
Received 8 March 2012
Received in revised form
11 December 2012
Accepted 12 December 2012
Available online 20 December 2012

Keywords:
Carbon nanotube
Peripheral blood mononuclear cell
Immunotoxicity
Allergy
T cell
Dendritic cell

ABSTRACT

In the present study, we investigated the immunomodulatory activity of multi-walled carbon nanotubes (MWCNTs) in peripheral blood mononuclear cells (PBMCs) from healthy donors and mite-allergic subjects. Freshly prepared PBMCs, stimulated or not with Toll-like receptor (TLR)1–9 agonists, a T cell mitogen (phytohemagglutinin A) or mite allergen extract were cultured in the presence or absence of MWCNTs. Secretion of TNF-α, IL-2, IL-5, IL-6, IL-12/23p40 or IFN-γ was quantified in the culture supernatants by ELISA. Basal secretion of all the cytokines was not altered by MWCNTs in PBMCs from both healthy donors and allergic subjects. In PBMCs from healthy donors, TNF-α, IL-6 and IL-12/23p40 secretion in response to the TLR4 agonist, lipopolysaccharide was however increased in a dose-dependent manner by MWCNTs. Significant increases in the release of these cytokines were also observed in PBMCs stimulated with a TLR2 or TLR3 agonist. MWCNTs also increased the release of IL-2 and IFN-γ by PBMCs stimulated with a T cell mitogen. In contrast, MWCNTs inhibited allergen-induced IL-5 secretion by PBMCs from mite-allergic subjects. As well, MWCNTs altered the capacity of PBMC-derived monocytes to differentiate into functional dendritic cells. All together, our data suggest that according to its immune cell target, MWCNTs may either promote or suppress immune responses in humans. Further investigations are necessary to fully understand the complexity behind interactions of engineered nanoparticles with the immune system.

1. Introduction

Carbon nanotubes (CNTs) are nanosized particles that consist of one (single-walled CNT; SWCNT) or more (multi-walled CNT; MWCNT) graphite carbon sheets rolled into cylinders. CNTs, and in particular MWCNTs, have unique mechanical, electrical, optical and thermal properties and a huge potential for industrial and biomedical applications (Kostarelos et al., 2009; Paradise and Goswami, 2007). The development of nanotechnologies raises however concerns, as on the one hand the growing production and use of nanomaterials are likely to increase the human exposure to nanoparticles and on the other hand, nanosized particles have been shown to exhibit greater adverse health effects than larger particles (Maynard et al., 2004; Oberdorster et al., 2005). Among others, nanomaterials have been reported to stimulate or suppress immune responses, which can result

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http://dx.doi.org/10.1016/j.toxlet.2012.12.008

in pathological conditions, such as life-threatening infections, autoimmune diseases, hypersensitivity or cancer (Dobrovolskaia and McNeil, 2007). Considering the huge potential of MWCNTs for industrial or biomedical applications, a better understanding of their immunomodulatory properties in humans is of importance.

Epidemiological and experimental studies on air pollution have provided substantial evidence that exposure to particulate matters is associated with a variety of adverse health effects, including airway inflammation, allergic sensitization and exacerbation of asthma. Among particulate matters, ultrafine particles, and in particular diesel exhaust particles (DEPs) have a higher potency to induce inflammation and display adjuvant activity (de Haar et al., 2006; Riedl and Diaz-Sanchez, 2005). As MWCNTs share physicochemical characteristics with airborne ultrafine particles, their potential adverse effects on the respiratory tract retained attention of toxicologists over the last years. Thus, upon administration in the lung of laboratory animals, MWCNTs have been shown to trigger the recruitment of macrophages and neutrophils and the secretion of cytokines such as IL-1β, TNF-α and IL-6 (Muller et al., 2005; Park et al., 2009; Porter et al., 2010; Ronzani et al., 2012). As well, they have been reported to promote the inflammatory response evoked by bacterial infection, the bacterial component lipopolysaccharide (LPS), or allergens, and to behave as adjuvant of the allergic response (Inoue et al., 2008, 2009; Nygaard et al., 2009). These observations suggest that inhalation of MWCNTs may induce airway inflammation and increase respiratory disorders such as life-threatening infections or allergic asthma in exposed individuals. However, MWCNTs were also proposed to suppress systemic immune function, as splenic T cell functions were inhibited in mice exposed to inhaled MWCNTs for 2 weeks (Mitchell et al., 2009). Similarly, *in vitro* studies investigating the ability of MWCNTs to trigger an inflammatory response in human or mouse monocytes, macrophages, T cells or DCs, or to modulate the function of these immune cells gave contradictory results (Bottini et al., 2006; Fiorito et al., 2009; Inoue et al., 2009; Palomaki et al., 2010; Thurnherr et al., 2009; Wang et al., 2009). Therefore, current data remain insufficient to fully characterize the immunomodulatory properties of MWCNTs in humans, and particularly in subjects with pre-existing respiratory disorders such as asthma.

Peripheral blood mononuclear cells (PBMCs) in culture are largely used to assess the immunomodulatory properties of chemical or particulate entities in healthy or allergic human subjects (Fahy et al., 2000; Kooijman et al., 2010; Laverny et al., 2009). Indeed, this cell system composed of lymphocytes and monocytes acting as accessory cells can be stimulated by innate or adaptive immune stimulus to study the secretion of cytokines involved in immunity. Therefore, in the present study, we used PBMCs from healthy donors and mite-allergic subjects to investigate the immunomodulatory activity of MWCNTs.

2. Materials and methods

2.1. Study design

PBMCs from healthy donors and mite-allergic subjects were stimulated with Toll-like receptor (TLR) agonists, a T cell mitogen and/or a specific allergen to characterize the potency of MWCNTs to modulate innate and adaptive immune response. Mixed lymphocyte reactions (MLRs) were performed with allogeneic PBMCs from healthy donors to further study the impact of MWCNTs on T cell activation. As well, differentiation, maturation and function of monocyte-derived dendritic cells (MDDCs) were assessed in the presence or absence of MWCNTs, to provide information on whether MWCNTs preferentially target antigen-presenting cells (APCs).

2.2. Donors and subjects

PBMCs from blood donors (Etablissement Français du Sang, Strasbourg, France) were used in the TLR agonist and polyclonal T cell stimulation experiments and the MLR assays. Blood from healthy donors was used also for the generation of MDDCs. Allergen stimulation was performed on PBMCs from mite-allergic and non-atopic subjects. Venous blood was collected from 9 mite-allergic subjects. These subjects (Table 1) had a clinical history of asthma, a positive skin-prick test towards house dust mite (HDM) allergen extract from *Dermatophagoides pteronyssinus* and specific IgE higher than 0.7 kU/L (ImmunoCAP, Phadia, Sweden). Mite-allergic subjects could exhibit positive skin-prick tests towards other usual aeroallergens. They were allowed to use only short-acting β2 mimetics as treatment during the study period. None had received antihistaminic and/or oral or inhaled corticosteroids within one month before inclusion. As controls, venous blood was also obtained from 9 non-atopic subjects with negative skin-prick tests to common aeroallergens and no symptom of allergy or asthma (Table 1). Active and passive smokers were excluded from the study. The local Ethic's committee approved the research protocol (2009-A00575-52, cpp 09/26) and written informed consent was obtained from all subjects.

Table 1
Subject characteristics according to clinical status.

	Mite-allergic asthmatic	Non-atopic
Number of subjects	9	9
Age (years)	25.1 [19–31]	29.8 [23–34]
Male/female	3/6	3/6
Der p skin prick test	+	–
Serum Der p specific IgE (kUA/L)	26.9 [3.0–100]	<0.1

Der p, *Dermatophagoides pteronyssinus*; mean value [range].

2.3. Multi-walled carbon nanotubes

MWCNTs used in this study (Graphistrengh C100) were provided by Arkema (Colombes, France). They were synthesized by chemical vapor deposition. MWCNT specifications and endotoxin and trace metal content were as previously described (Ronzani et al., 2012).

2.4. Preparation and characterization of the MWCNT dispersions

MWCNT dispersions were prepared just before their use on cultures by adding MWCNTs to complete PBMC culture medium at a final concentration of 1 mg/mL. The resulting suspension was briefly vortexed, dispersed by sonication in a bath (Bioblock Scientific, France) at 56 W and 40 kHz for 30 min, and further diluted in complete medium before addition to cultures. The dispersions were characterized just after their preparation, as previously described (Ronzani et al., 2012). At first, potential formation of MWCNT coarse agglomerates was observed by light microscopy (Axiovert 25 microscope, Carl Zeiss, Le Pecq, France). The size distribution of the dispersed particles was analysed by DLS using a Zetasizer Nano ZEN3600 (Malvern Instruments, Paris, France) and results were expressed as a representative particle size distribution deduced from the intensity distribution graph. At last, organization of dispersed MWCNTs was investigated by transmission electronic microscopy (TEM). Drops of the MWCNT suspensions were deposited onto carbon-coated grids and allowed to air dry before observation using a CM 12 Philips microscope operated at 80 kV. Image acquisition was realized using an Orius 1000 CCD camera (Gatan).

2.5. PBMC isolation

PBMCs were isolated from buffy coats from blood donors (Etablissement Français du Sang, Strasbourg, France) or from venous blood from mite-allergic and non-atopic subjects by ficoll gradient centrifugation (Ficoll-Paque™ Plus, GE Healthcare, France) and suspended in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% (v/v) heat-inactivated foetal bovine serum, 1% non-essential amino acids, 0.5 mg/mL gentamicin and 1 mM sodium pyruvate (complete culture medium with all reagents from Invitrogen, France).

2.6. Transmission electron microscopy on PBMCs

PBMCs were seeded on coverslips made from 7.8 mil Aclar® sheets (EMS), and fixed with 2.5% glutaraldehyde, 4% formaldehyde in 0.1 M phosphate buffer. Cells were then post-fixed with 1% osmium tetroxide, dehydrated in a graded series of acetone and resin-embedded in epoxy resin. Ultrathin tissue sections (60 nm) were collected on grids and observed by TEM using a CM12 Philips microscope operated at 80 kV. Image acquisition was done with an Orius 1000 CCD camera (Gatan).

2.7. Effect of MWCNTs on PBMC viability

PBMCs (2×10^5/200 μL/well) were cultured in 96-well flat-bottom plates in complete medium alone or in medium containing increasing concentrations (0–100 μg/mL) of MWCNTs. After 48 h of culture at 37 °C in a humidified atmosphere containing 5% CO_2, PBMC viability was assessed by trypan blue exclusion assay. Data were expressed as percentage of controls.