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

Gilles Laverny[a], Anne Casset[a], Ashok Purohit[b], Evelyne Schaeffer[c], Coralie Spiegelhalter[d], Frédéric de Blay[b], Françoise Pons[a,*]

[a] Laboratoire de Conception et Application de Molécules Bioactives, CNRS-Université de Strasbourg, Faculté de Pharmacie, Illkirch, France
[b] Unité de Pneumologie, d'Allergologie et de Pathologie respiratoire de l'environnement, Hôpitaux Universitaires de Strasbourg, Strasbourg, France
[c] UPR 9021 CNRS, Institut de Biologie Moléculaire et Cellulaire, Strasbourg, France
[d] Centre d'Imagerie, Institut de Génétique et de Biologie Moléculaire et Cellulaire, 1 rue Laurent Fries, Illkirch, France

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.

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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

* Corresponding author at: UMR 7199, Université de Strasbourg, Faculté de Pharmacie, 74 route du Rhin, CS 60024, 67401 Illkirch Cedex, France.
Tel.: +33 3 68 85 42 03; fax: +33 3 68 85 43 06.
E-mail address: pons@unistra.fr (F. Pons).

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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.

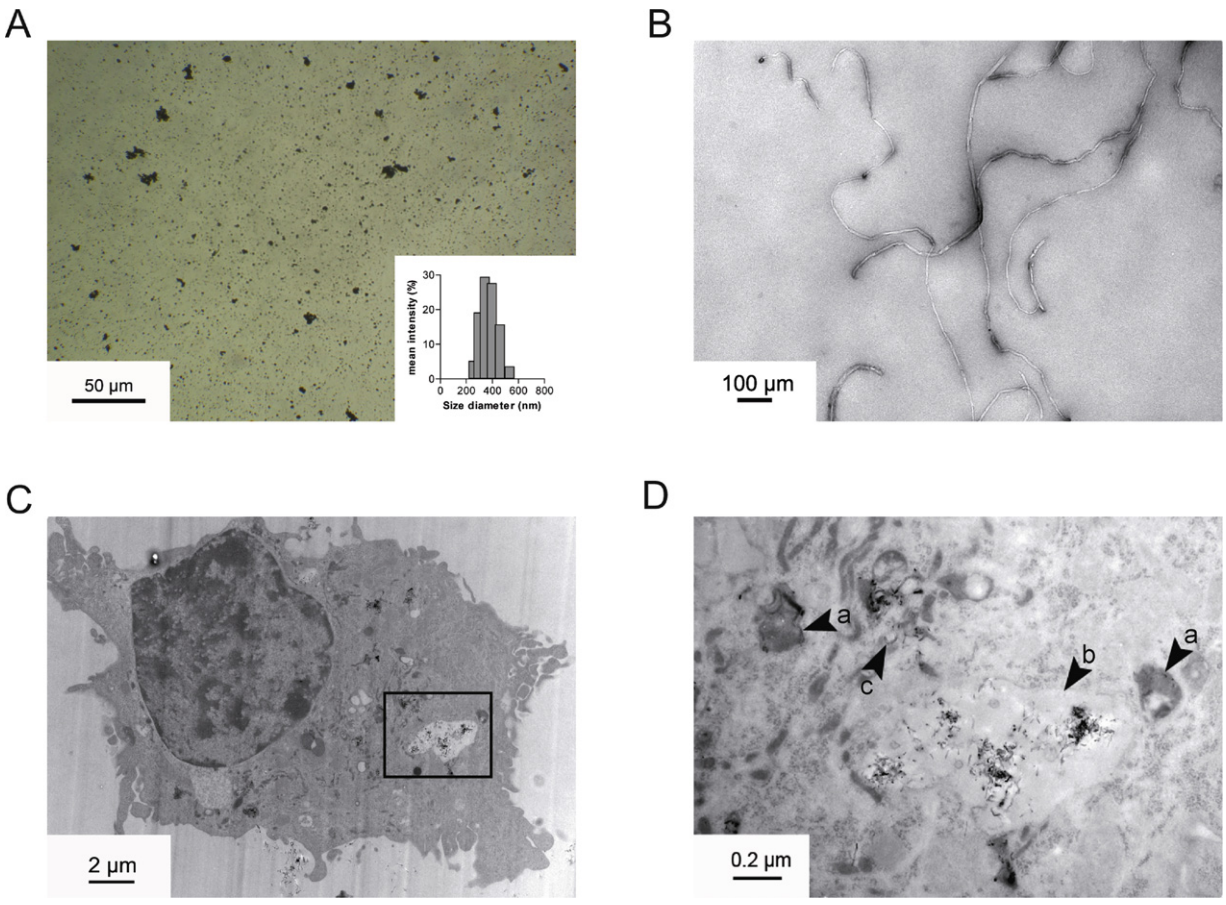

Fig. 1. MWCNT dispersions and uptake by PBMCs. (A and B) light microscopy image (panel A), size distribution as analysed by DLS (inset in panel A) and transmission electronic micrographs of MWCNT dispersion (120 μg/mL) in culture medium. (C and D) Uptake of MWCNTs by monocytes, as illustrated by transmission electronic microscopy images. The image on panel D is a magnification of the panel C marked area. Arrows indicate MWCNTs in (a) lysosome, (b) phagosome and (c) cytoplasm.

2.8. Stimulation of PBMCs with TLR agonists in the presence or absence of MWCNTs

PBMCs (2×10^5/200 μL/well) were cultured for 24 or 48 h in 96-well flat-bottom plates in the presence or absence of MWCNTs in complete culture medium alone or in medium containing *Escherichia coli* (*E. coli*) 055:B5 LPS (0.01, 0.1 or 1 μg/mL, Sigma–Aldrich, France) or other TLR agonists (Human TLR1-9 Agonist kit, InvivoGen, France) including Pam3CSK4 (TLR1/2 agonist, 1 μg/mL), heat-killed preparation *Listeria monocytogenes* (HKLM, TLR2 agonist, 10^8 cells/mL), poly(I:C) (TLR3 agonist, 10 μg/mL), *E. coli* K12 LPS (TLR4 agonist, 1 μg/mL), *Salmonella typhimurium* flagellin (TLR5 agonist, 1 μg/mL), FSL1 (TLR6/2 agonist, 1 μg/mL), imiquimod (TLR7 agonist, 2.5 μg/mL), ssRNA40 (TLR8 agonist, 2.5 μg/mL) and oligodeoxynucleotide 2006 (ODN2006, TLR9 agonist, 5 μM). MWCNTs were added on cells at the beginning of the culture. After 24 or 48 h of culture at 37 °C in a humidified atmosphere containing 5% CO_2, cell supernatants were harvested and stored at −80 °C until cytokine analysis.

2.9. Stimulation of PBMCs with a T cell mitogen in the presence or absence of MWCNTs

PBMCs (2×10^5/200 μL/well) were cultured in 96-well flat-bottom plates in the presence of absence of MWCNTs in complete culture medium alone or in medium containing 1 μg/mL phytohemagglutinin A (PHA, Sigma–Aldrich, France). MWCNTs were added to cultures at day 0. After 3 days of culture at 37 °C in a humidified atmosphere containing 5% CO_2, culture supernatants were harvested and stored at −80 °C until cytokine analysis.

2.10. Stimulation of PBMCs from mite-allergic and non-atopic subjects with allergen in the presence or absence of MWCNTs

PBMCs (5×10^5/500 μL/well) from mite-allergic and non-atopic subjects were cultured in 24-well flat-bottom plates in the presence or absence of MWCNTs in complete medium alone or medium containing HDM allergen extract from *D. pteronyssinus* (GREER® Laboratories Inc., Lenoir, NC). This extract contained a Der p 1 (a major allergen of *D. pteronyssinus*) concentration of 210 μg/mL (Der p 1 ELISA kit, Indoor Biotechnologies, Charlottesville, VA) and an endotoxin level of 12.5 ng/mL (Chromo LAL, Associates of Cape Cod Inc., East Falmouth, CA). PBMCs were stimulated with an extract concentration corresponding to 0.1 μg/mL Der p 1. All throughout the article, HDM concentrations refer to Der p 1 concentrations. MWCNTs were added to cultures at day 0 or 3. After 5 days of culture at 37 °C in a humidified atmosphere containing 5% CO_2, cell supernatants were harvested and stored at −80 °C until IL-5 analysis.

2.11. Mixed lymphocyte reaction in the presence or absence of MWCNTs

The same number (3×10^5/200 μL/well) of allogeneic PBMCs from two different healthy donors was co-cultured in 96-well flat-bottom plates in complete medium alone or in medium containing MWCNTs. MWCNTs were added to cultures at day 0 or 3. As negative controls, PBMCs from each donor were cultured independently. After 3 or 5 days of culture at 37 °C in a humidified atmosphere containing 5% CO_2, cell supernatants were harvested and stored at −80 °C until cytokine analysis.

2.12. Differentiation, maturation and function of monocyte-derived dendritic cells in the presence or absence of MWCNTs

iDCs were differentiated from adherent monocytes obtained from PBMCs from healthy donors in complete medium supplemented with 10 ng/mL rhGM-CSF and 10 ng/mL rhIL-4 (R&D Systems, France). Fresh medium containing GM-CSF and IL-4 was added every other day. After 7 days of culture, iDCs were counted and cell viability was assessed by trypan blue exclusion. To obtain mDCs, iDCs were stimulated with 1 μg/mL LPS for 48 h. iDCs cultured for an additional 48 h in complete medium without LPS were used as controls. DC maturation in response to LPS was assessed by measuring IL-12/23p40 secretion in culture supernatant. MWCNTs were added to culture medium during MDDC differentiation or maturation. Function of mDCs was assessed by unidirectional MLR. CD3+ T cells were purified from PBMCs from healthy subjects by negative selection using T cell enrichment columns (R&D Systems, France) according to the manufacturer's instructions. mDCs or iDCs were then co-cultured with allogeneic CD3+ T cells (ratio 1:10) in complete medium in 24-well flat-bottom plates. After 5 days of culture, culture supernatants were harvested and stored at −80 °C until cytokine analysis.

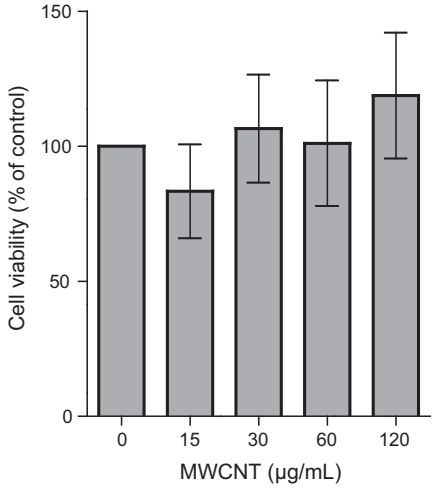

Fig. 2. Effect of MWCNTs on PBMC viability. PBMC viability was assessed by trypan blue exclusion assay after 48 h of culture in the presence or absence of the indicated concentrations of MWCNTs. Data were expressed in percent of controls. Each point represents the mean ± SEM of $n = 3$ healthy donors.

2.13. FACS analysis

Cells (3×10^4) were washed twice with PBS supplemented with 2% foetal bovine serum and stained for 30 min at 4 °C using the following mouse mAb (all from BD Pharmingen, France): anti-CD86 IgG1 (APC), IgG2a anti-MHC-II (FITC), IgG1 anti-CD83 (FITC), IgG1 CD14 (PE), IgG2b anti-CD209 (PerCp) and IgG1 anti-CD1a (APC). The corresponding isotypes were used as controls. After the staining, cells were washed twice and resuspended in 350 µL PBS supplemented with 2% foetal bovine serum. Cells were then analysed by flow cytometry (FACSCalibur®). Data were acquired using the CellQuest 3.3 software (Becton Dickinson, Pont de Claix, France) and processed with the FlowJo software.

2.14. Cytokine quantification

Cytokines (IFN-γ, TNF-α, IL-6, IL-12/23p40, IL-10, IL-5 and IL-2) were quantified by enzyme-linked immunosorbent assays (BD OptEIA™ set, BD Biosciences, France) according to manufacturer's instructions.

2.15. Statistical analysis

Data were analysed with Graph Prism (Graph Pad software, USA) and curves were generated with the appropriated nonlinear fit regression. For one to one comparisons, data were analysed by *t*-test. For multiple comparisons, analysis of variance (ANOVA) followed by a *post hoc* test was used. Data had to follow a normal distribution before being tested for significance. Differences were considered as statistically significant when $p < 0.05$.

3. Results

3.1. Characterization of MWCNT dispersions

Raw MWCNTs tend to aggregate upon suspension in aqueous physiological media (Bihari et al., 2008; Buford et al., 2007; Ronzani et al., 2012). Consequently, characterization of MWCNT dispersions is an important issue when evaluating the toxicological impact of the nanomaterial in biological systems. Observation of MWCNT preparations by light microscopy revealed the presence of sparse micrometric MWCNT agglomerates, as shown in Fig. 1. DLS analysis showed one particle population of mean diameter of 370 nm, indicating monodispersity of the preparation (inset in Fig. 1A). At last, TEM investigations revealed the presence of individualized MWCNTs (Fig. 1B). Thus, although sparse micrometric agglomerates were present in our preparations, most MWCNTs appeared as dispersed.

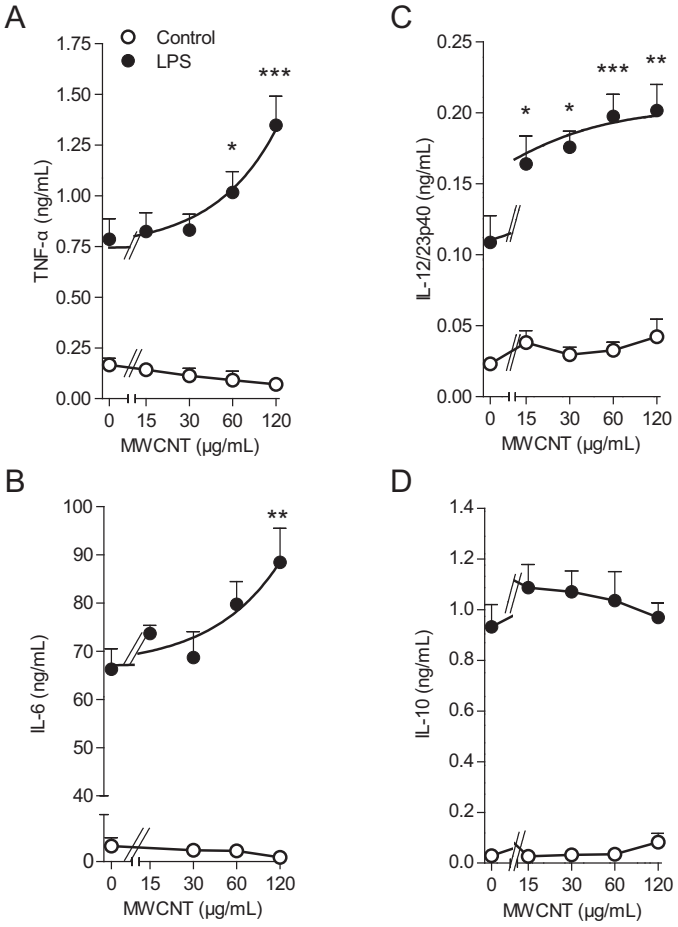

Fig. 3. Effect of MWCNTs on cytokine secretions by LPS-stimulated PBMCs. Secretion of the pro-inflammatory cytokines TNF-α (A), IL-6 (B) and IL-12/23p40 (C), and the anti-inflammatory cytokine IL-10 (D) by PBMCs from healthy donors after a 48 h stimulation with (closed circles) or without (open circles) lipopolysaccharide (LPS, 1 µg/mL) in the presence or absence of the indicated concentrations of MWCNTs. Each point represents the mean ± SEM of $n = 6$ healthy donors. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ *vs.* cytokine secretion in absence of MWCNTs.

3.2. Uptake of MWCNTs by PBMCs

Inflammatory cytokine production by professional phagocytes in response to nanoparticles can result from particle engulfment (Dobrovolskaia and McNeil, 2007). To get insight into the uptake of MWCNTs by PBMCs, cell cultures were observed by TEM. This investigation provided evidence of an uptake of MWCNTs by monocytes (Fig. 1C). Bundles and individualized MWCNTs were observed in phagosomes, lysosomes and cytoplasm of the cells (Fig. 1D).

3.3. Effect of MWCNTs on PBMC viability

Studies carried out so far on human enriched primary immune cells reported some loss of viability at MWCNT concentrations as high as 250–400 µg/mL (Bottini et al., 2006; De Nicola et al., 2009). In this context, we explored whether PBMC viability was affected in the presence of MWCNT concentrations in the range of those usually used in *in vitro* studies, namely 0–120 µg/mL. As shown in Fig. 2, no significant decrease in viability was observed when PBMCs were incubated for 48 h with 0–120 µg/mL MWCNTs.

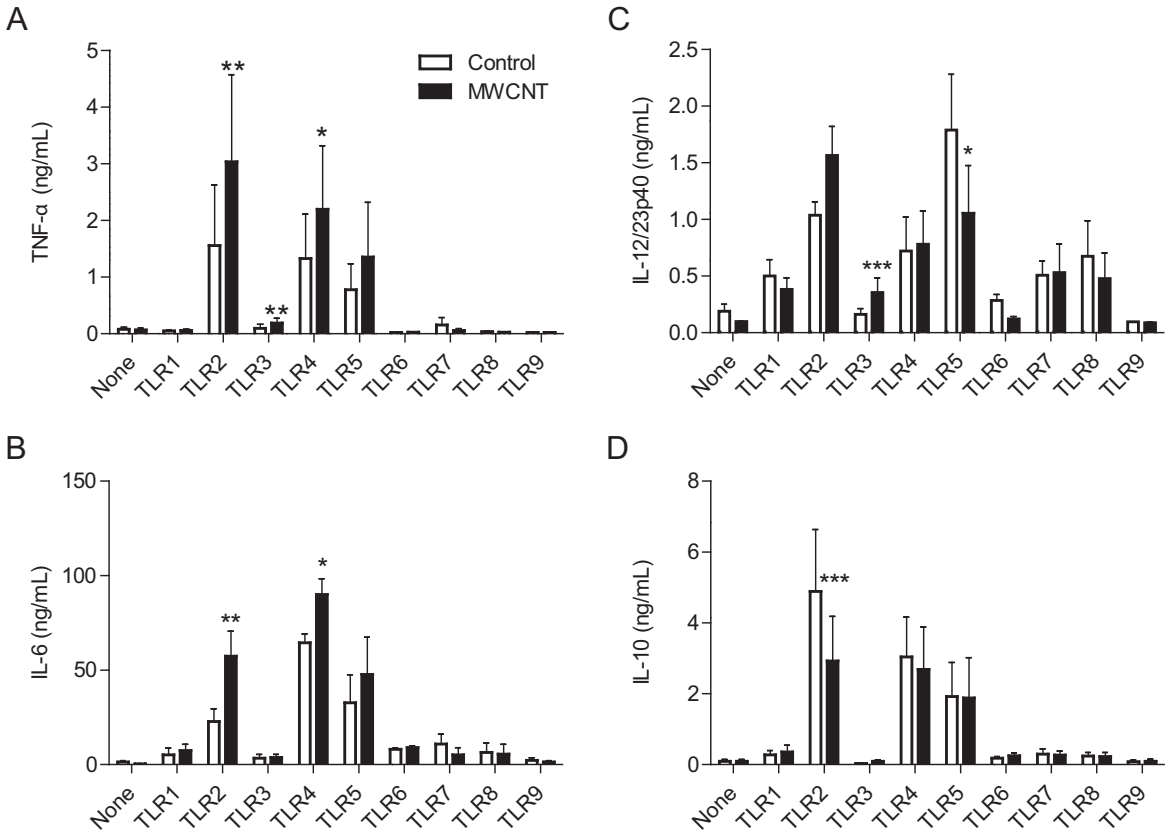

Fig. 4. Effect of MWCNTs on cytokine secretions by Toll-like receptor agonist-stimulated PBMCs. Secretion of the pro-inflammatory cytokines TNF-α (A), IL-6 (B) and IL-12/23p40 (C), and the anti-inflammatory cytokine IL-10 (D) by PBMCs from healthy donors after a 48 h stimulation with TLR agonists in the presence (black bars) or absence (white bars) of 120 μg/mL MWCNTs. Each point represents the mean ± SEM of $n = 4$ healthy donors. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ *vs.* cytokine secretion in absence of MWCNTs.

3.4. MWCNTs increase the release of cytokines from TLR-stimulated PBMCs

We first analysed the potency of MWCNTs to modulate immunity by assessing the release of the cytokines TNF-α, IL-12/23p40, IL-6 and IL-10 by resting- and TLR stimulated-PBMCs from healthy donors. As shown in Fig. 3, MWCNTs did not influence the basal secretion of TNF-α, IL-6, IL-12/23p40 or IL-10 from resting PBMCs after 48 h of culture. So far nine TLR members (TLR1–9) and their ligands have been identified in humans (Kawai and Akira, 2010). At first, we focused our investigations on TLR4, a founding member of the TLR family, the ligand of which, the bacterial endotoxin LPS is widely used as an inducer of the innate immune response in *in vitro* and *in vivo* studies. MWCNTs increased significantly and dose-dependently the secretion of the pro-inflammatory cytokines TNF-α, IL-6 and IL-12/23p40 induced by 1 μg/mL LPS from *E. coli* 055:B5 (Fig. 3A–C). Similar results were observed with lower concentrations of LPS (0.01 and 0.1 μg/mL) or shorter (24 h) stimulation (data not shown). In contrast, the secretion of the anti-inflammatory cytokine IL-10 evoked by LPS remained unchanged in the presence of MWCNTs (Fig. 3D). To extend our results on TLR4 and determine whether MWCNTs could affect the response of other TLRs, we carried out the same experiments with selective agonists from all TLR family members. Fig. 4 represents the data obtained with the highest MWCNT concentration tested (120 μg/mL). MWCNTs enhanced the secretion of the pro-inflammatory cytokines TNF-α and IL-6 in PBMCs stimulated with the *E. coli* K12 LPS (TLR4 agonist), consolidating our previous data on LPS from *E. coli* 055:B5. The secretion of TNF-α, IL-6 and IL-12/23p40 was also increased by MWCNTs in PBMCs stimulated with a TLR2 agonist. Similarly, increases in the release of TNF-α and IL-12/23p40 were observed in PBMCs treated with a TLR3 agonist, although IL-6 secretion was not altered. After TLR5 stimulation, TNF-α secretion by PBMCs tended to increase in the presence of MWCNTs, while IL-12/23p40 release was inhibited and IL-6 remained unchanged. MWCNTs did not interfere however with the cytokine secretions measured in PBMCs stimulated with TLR1 and TLR6–9 agonists. By the way, these TLR agonists evoked no significant increase in the levels of any cytokine. As previously observed, IL-10 secretion remained unchanged in the presence of MWCNTs in PBMCs stimulated with most of the TLR agonists and even was significantly decreased after stimulation with a TLR2 agonist.

3.5. MWCNTs increase the release of cytokines from PBMCs stimulated with a T cell mitogen

In order to extend our evaluation of the capacity of MWCNTs to modulate immunity, we next focused on T cell, one of the main effector cells of adaptive immune responses. We thus analysed the potency of MWCNTs to modulate cytokine release evoked by PHA, a specific T cell mitogen in PBMCs from healthy donors. As read out of a 72 h stimulation with PHA, we measured the secretion by PBMCs of IL-2, the key cytokine required for T cell proliferation and of IFN-γ, a T cell effector cytokine. As shown in Fig. 5, IL-2 secretion from PHA-treated PBMCs was significantly and dose-dependently increased in the presence of MWCNTs. Similarly, MWCNTs enhanced the secretion of IFN-γ evoked by PHA (Fig. 5B).

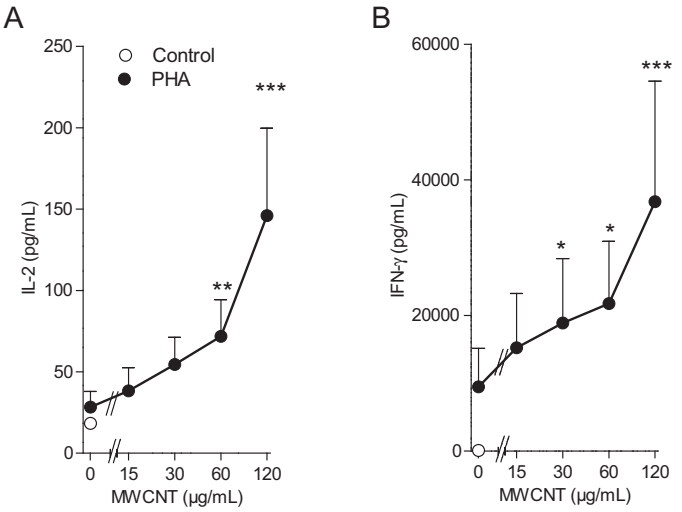

Fig. 5. Effect of MWCNTs on cytokine secretions by PBMCs after a T cell mitogen stimulation. Secretion of the proliferative cytokine IL-2 (A) and the T cell signature cytokine IFN-γ (B) by PBMCs from healthy donors after a 72 h stimulation with (closed circles) or without (open circles) the T cell mitogen phytohemagglutinin A (PHA) in the presence or absence of the indicated concentrations of MWCNTs. Each point represents the mean ± SEM of $n = 6$ healthy donors. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ *vs.* cytokine secretion in absence of MWCNTs.

3.6. MWCNTs inhibit IL-5 secretion in PBMCs from mite-allergic subjects after allergen stimulation

To further investigate the modulatory activity of MWCNTs on the adaptive immune response, we focused on the response to an allergen of PBMCs from allergic subjects. Since HDMs are the most prevalent cause of sensitization leading to asthma symptoms (Thomas et al., 2010), we carried out these investigations on PBMCs purified from mite-allergic subjects and non-atopic subjects, as controls (Table 1). PBMCs from all subjects were stimulated with 0.1 μg/mL HDM extract for 5 days in the presence or absence of MWCNTs. IL-5 was used as the cytokine read-out, since in allergic asthma, it is produced by activated T cells and involved in driving the airway inflammatory response. As expected, HDM stimulation significantly increased IL-5 secretion after 5 days of culture in PBMCs from mite-allergic subjects, but not in those from non-atopic subjects (Fig. 6A). However, IL-5 secretion induced by HDMs in PBMCs from mite-allergic subjects was significantly inhibited in the presence of MWCNTs (Fig. 6A).

Since this observation was unexpected in regards to the data obtained so far in PBMCs from healthy donors, we assessed the effect of MWCNTs on the response evoked by LPS in PBMCs from mite-allergic and non-atopic subjects. In agreement with the observations made in cells from healthy donors, LPS-induced TNF-α secretion was increased by MWCNTs in PBMCs from both non-atopic and mite-allergic subjects (Fig. 6B). Similarly, MWCNTs increased PHA-induced IFN-γ secretion in PBMCs from both groups of subjects (data not shown). Thus, we report here that in the PBMC model, MWCNTs are able to inhibit an allergen-specific T cell-mediated immune response, despite their ability to increase a polyclonal T cell response. One hypothesis to explain inhibition of allergen-specific T cell-mediated immune response would be that MWCNTs target APCs.

3.7. MWCNTs inhibit the initiation of the mixed lymphocyte reaction

In order to consolidate our above hypothesis, we used an *in vitro* model of T cell stimulation targeting an extent T cell population, the MLR. At first, allogeneic PBMCs from two different healthy donors were co-cultured for 5 days with or without MWCNTs added at the initiation of the MLR (day 0). The main read-out of the MLR was the T cell signature cytokine, IFN-γ. As shown in Fig. 7A, MWCNTs did not affect IFN-γ secretion from resting PBMCs from each donor (negative control). However, in agreement with our previous observation in PBMCs from allergic subjects, MWCNTs inhibited significantly and dose-dependently, the IFN-γ secretion evoked after a 5-day MLR, suggesting here again that MWCNTs target preferentially APCs rather than T cells to inhibit adaptive immune responses.

Initiation of a MLR involves recognition of the major histocompatibility complex molecules of one donor's cells, usually the APCs, by T cells of the second donor, resulting in the proliferation and secretion of cytokines by the activated T cells (O'Keefe and Ashman, 1982). As shown in Fig. 7 (data in absence of MWCNTs), whereas an important IFN-γ secretion was observed at day 5 of the MLR, the T cell signature cytokine was not secreted at day 3. Therefore, in order to assess our hypothesis that MWCNTs target preferentially the APCs rather than T cells to inhibit adaptive immune responses in the PBMC model, MLR were conducted in the presence of MWCNTs added at day 0 or 3. Whereas, as expected, MWCNT addition at day 0 resulted in the inhibition of the IFN-γ secretion (as measured at day 5), MWCNT addition at day 3 provoked a significant increase in IFN-γ (Fig. 7B). To validate these findings, the same experiment was carried out on PBMCs from mite-allergic subjects. Likewise, we found that IL-5 secretion evoked by allergen stimulation after 5 days of culture was not inhibited when MWCNTs were added at day 3 of culture (data not shown). All together, these results reinforce the idea that MWCNTs act on APCs to inhibit T cell mediated-immune responses in the PBMC model.

3.8. Effect of MWCNTs on antigen-presenting cell maturation

To investigate our hypothesis that MWCNTs could target the APCs, we assessed the effect of MWCNTs on DCs, the most potent APCs. DC maturation, characterized by enhanced cell surface expression of co-stimulatory molecules, reduced endocytosis, enhanced antigen presentation, stimulation of T cell proliferation and increased cytokine production, is a crucial step of the induction of most adaptive immune responses. Therefore, we first investigated the impact of MWCNTs on DC maturation. MDDCs were matured with or without 1 μg/mL LPS for 48 h in the presence or absence of MWCNTs (15 or 120 μg/mL). The efficiency of DC maturation was then assessed by analyzing expression of the co-stimulatory molecules CD83, CD86 and MHC-II at the cell surface and measuring IL-12/23p40 in the culture supernatants. MWCNTs alone did not induce MDDC maturation (data not shown). Similarly, MWCNTs did not interfere with LPS-induced MDDC maturation, since iDCs matured with LPS in the presence of MWCNTs exhibited similar levels of co-stimulatory molecules (Fig. 8A) and secreted comparable levels of IL-12/23p40 (Fig. 8B) when compared to control mDCs. To evaluate the function of DCs matured in the presence or absence of MWCNTs, we co-cultured these mDCs with allogeneic CD3+ T cells purified from healthy donor PBMCs, and measured IFN-γ secretion. iDCs maturated in the presence of MWCNTs exhibited no changes in function, since they were as potent as control mDCs to induce IFN-γ secretion by allogeneic CD3+ T cells (Fig. 8C).

3.9. Effect of MWCNTs on antigen-presenting cell differentiation

To investigate in more details the impact of MWCNTs on APCs, we next focused on MDDC differentiation. iDCs were differentiated from adherent monocytes by culture with GM-CSF and IL-4 for 7 days in the presence or absence of 15, 30, 60 or 120 μg/mL MWCNTs. In order to evaluate the impact of MWCNTs on MDDC differentiation, we first assessed the number and viability of

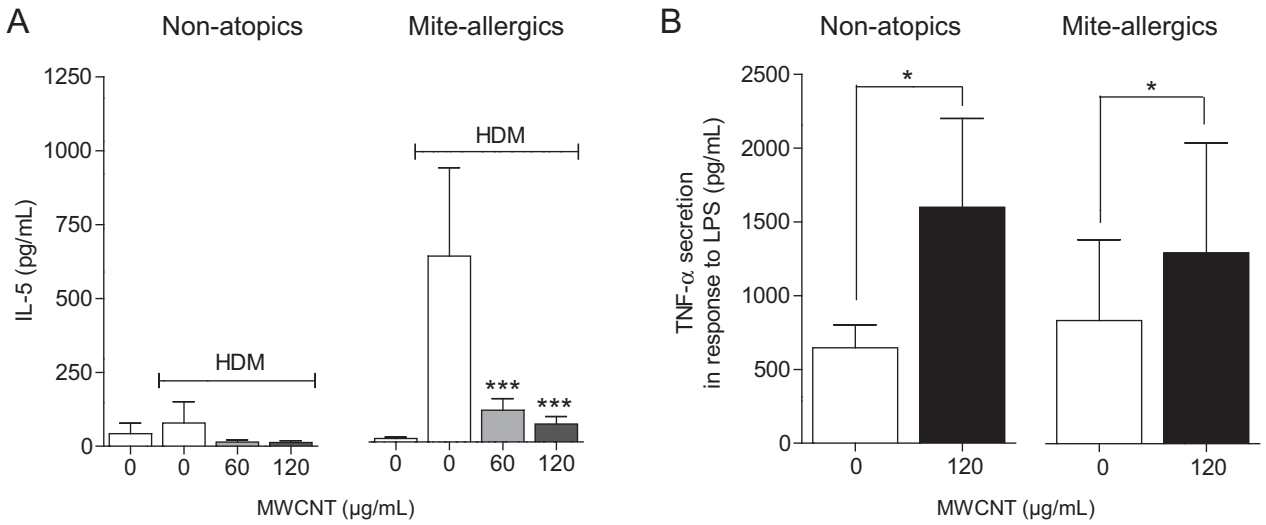

Fig. 6. Effect of MWCNTs on cytokine secretions by PBMCs from mite-allergic subjects after allergen stimulation. Secretion by PBMCs from mite-allergic or non-atopic subjects of (A) the T cell signature cytokine IL-5 after a 5 day stimulation with 0.1 μg/mL house dust mite extract (HDM) or (B) TNF-α after a 24 h stimulation with 1 μg/mL LPS in the presence or absence of the indicated concentrations of MWCNTs. Each point represents the mean ± SEM of $n = 9$ (A) and $n = 4$ (B) subjects. $^{*}p < 0.05$; $^{***}p < 0.001$ *vs.* cytokine secretion in absence of MWCNTs.

non-adherent cells by trypan blue assay. Less iDCs were differentiated in the presence of 30–120 μg/mL MWCNTs although they were viable (data not shown). In contrast, MWCNTs did not affect the number of differentiated MDDCs at the concentration of 15 μg/mL. Therefore, this concentration was used to evaluate the impact of MWCNTs on DC differentiation. As shown in Fig. 9, expression of the MDDC-specific surface marker molecules CD1a, CD209 and MHC-II was reduced in MDDCs differentiated in the presence of 15 μg/mL MWCNTs when compared to control MDDCs, suggesting that the presence of MWCNTs during differentiation influenced MDDC phenotype. We next investigated whether these modifications in phenotype could result in altered DC maturation. MDDCs differentiated in the presence of MWCNTs were matured with LPS for 48 h (MWCNT mDC). MDDCs differentiated in the absence of MWCNTs and treated with and without LPS were used as positive (control mDC) and negative (iDC) controls, respectively. The mDCs obtained from MDDCs differentiated in the presence of MWCNTs showed lower expression of CD83, CD86 and MHC-II when compared to control mDCs (data not shown). Likewise, as shown in Fig. 9, MDDCs differentiated in the presence of MWCNTs secreted significantly lower levels of IL-12/23p40 after LPS-induced maturation when compared to control mDCs (56 ng/mL *vs.* 359 ng/mL, respectively), although they were still able to secrete significantly more IL-12/23p40 when compared to the iDCs (56 ng/mL *vs.* 2.3 ng/mL, respectively). These data suggest that the changes in phenotype of iDCs induced by the presence

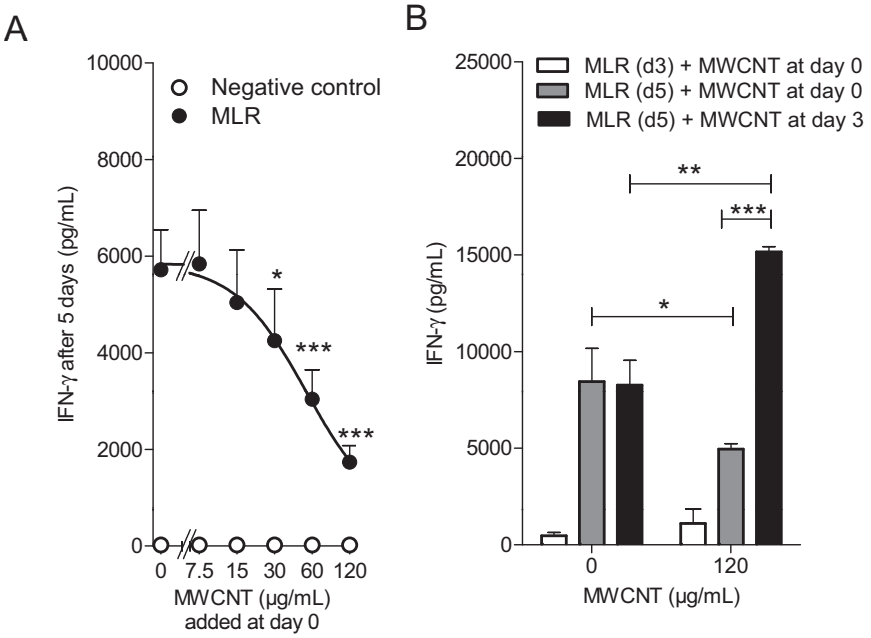

Fig. 7. Effect of MWCNTs on IFN-γ secretion in the mixed lymphocyte reaction. (A) Secretion of IFN-γ after a 5-day MLR in the presence or absence of the indicated concentrations of MWCNTs added at day 0. As negative control, PBMCs from each donor were cultured independently. (B) Secretion of IFN-γ after 3 days (d3) or 5 days (d5) of MLR in the presence or absence of MWCNTs added at day 0 or day 3 of the MLR. Each point represents the mean ± SEM of $n = 3$–9 experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ *vs.* cytokine secretion in absence of MWCNTs (A) or the indicated group (B).

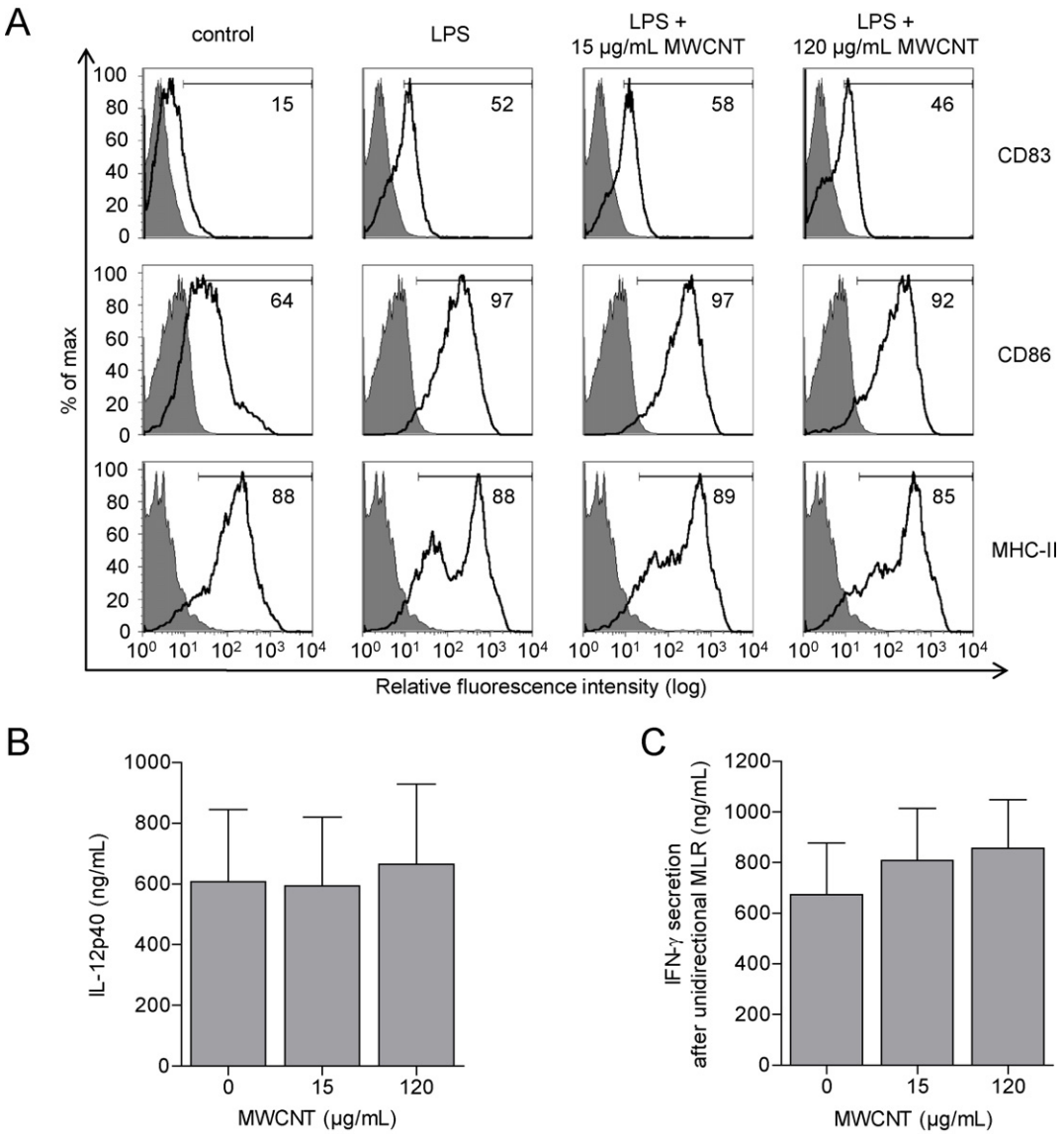

Fig. 8. Effect of MWCNTs on LPS-induced maturation of monocyte-derived dendritic cells. (A) Expression of the co-stimulatory molecules CD83, CD86 and MHC-II by monocyte-derived dendritic cells (MDDCs) maturated for 48 h with LPS in the presence or absence of the indicated concentrations of MWCNTs. The isotype control is represented in light grey. The number in the top right quadrant represents the percentage of positive cells. (B) IL-12/23p40 secretion by MDDCs maturated for 48 h with LPS in the presence or absence of the indicated concentrations of MWCNTs. (C) Capacity of MDDCs maturated for 48 h with LPS in the presence or absence of the indicated concentrations of MWCNTs to induce allogeneic CD3+ T cell stimulation, as assessed by IFN-γ secretion after 5 days of co-culture. (A) Data shown are representative of $n = 4$ healthy donors. (B and C) Each point represents the mean ± SEM of $n = 6$ healthy donors (B) or $n = 10$ MLR (C).

of MWCNTs during their differentiation influence mDC function. Therefore, we then investigated the potency of mDCs to induce a T cell-mediated immune response. mDCs differentiated in the presence of MWCNTs still had the capacity to activate T cells as shown by a significant increase in IFN-γ secretion when compared to iDCs. However, this IFN-γ secretion was significantly lower compared to mDCs differentiated in the absence of MWCNTs (Fig. 9C). All together, these experiments suggest that MWCNTs can alter DC differentiation into iDCs and subsequent function of mDCs.

4. Discussion

In the present study, we investigated the potency of MWCNTs to modulate innate, polyclonal T cell and allergen-induced cytokine responses in human PBMCs from healthy donors and mite-allergic subjects. Our results showed that MWCNTs increase the cytokine response evoked by different TLR agonists in PBMCs from healthy donors. However, we found also that MWCNTs inhibit the cytokine response evoked by an allergen in PBMCs from allergic subjects, despite their ability to increase a polyclonal T cell response. As well, we demonstrated that MWCNTs alter human DC differentiation into iDCs and subsequent function of mDCs.

Raw MWCNTs tend to aggregate upon suspension in aqueous physiological media (Bihari et al., 2008; Buford et al., 2007; Ronzani et al., 2012). Therefore, before any investigations on PBMC cultures, MWCNT dispersions were characterized. This characterization showed that sparse micrometric agglomerates were present in the preparations. As demonstrated by Budford et al. in their comparative study, total agglomerate avoidance is a very difficult task to achieve when dispersing highly hydrophobic CNTs into aqueous physiological media (Buford et al., 2007). Although sparse micrometric agglomerates were present in our preparations, most MWCNTs appeared to be dispersed. This dispersion may be explained by the presence of 10% foetal bovine serum in the PBMC

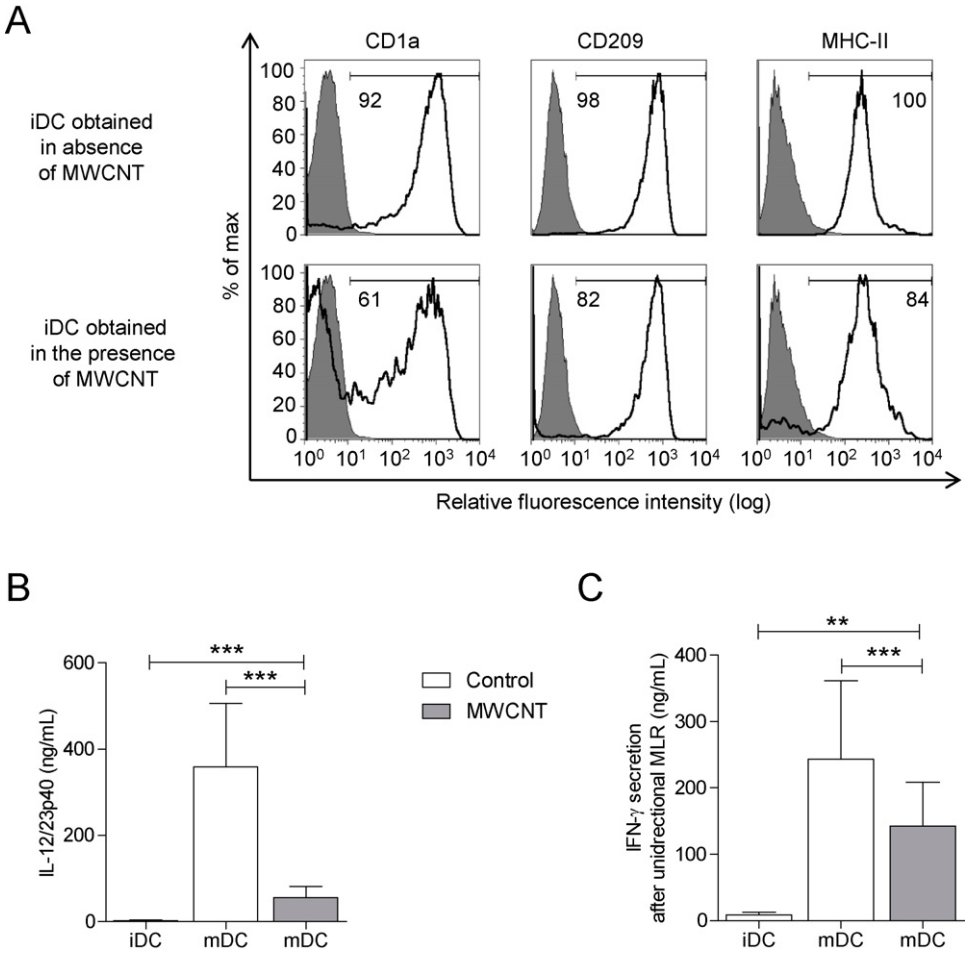

Fig. 9. Effect of MWCNTs on differentiation of monocyte-derived dendritic cells. (A) Expression of CD1a, CD209 and MHC-II on the surface of MDDC differentiated in the presence or absence of 15 μg/mL MWCNTs. The isotype control is represented in light grey. The number in the top right quadrant represents the percent of positive cells. (B) IL-12/23p40 secretion by MDDCs differentiated in the presence or absence of 15 μg/mL MWCNTs and further cultured for 48 h with or without LPS leading to mDCs or iDCs, respectively. (C) Capacity of iDCs or mDCs differentiated in absence of 15 μg/mL MWCNTs (white bars) and of mDCs differentiated in the presence of MWCNTs (grey bars) to induce allogeneic CD3+ T cell stimulation, as assessed by IFN-γ after 5 days of co-culture. (A) Data shown are representative of $n = 3$ healthy donors. (B and C) Each point represents the mean ± SEM of $n = 6$ healthy donors (B) or $n = 10$ MLR (C). $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs.* the indicated group.

culture medium, all the more this serum was heat-inactivated. Indeed, nanoparticles including CNTs bind serum proteins as albumin (Heister et al., 2010; Nel et al., 2009; Ronzani et al., 2012) and albumin unfolding by heat-inactivation may promote this binding by increasing exposure of inner hydrophobic protein domains (Matsuura et al., 2006).

When investigating the immunomodulatory activity of these MWCNT dispersions in PBMCs from healthy donors, we found that basal secretion of all the cytokines we assessed was not altered by the presence of MWCNTs. In contrast, MWCNTs increased significantly and dose-dependently the release of TNF-α, IL-6 and/or IL-12/23p40 evoked by different TLR agonists, the production of IL-10 remaining unchanged. The cytokines TNF-α, IL-12/23p40 and IL-6 are required for the recruitment and activation of immune cells such as phagocytes during infections, whereas IL-10 is able to inhibit the secretion of pro-inflammatory cytokines and mediators by macrophages and DCs (Mosser and Zhang, 2008). In addition, all the cytokines cited above have the potency to modulate adaptive immune responses. Therefore, MWCNTs could increase immune responses involving TLR signalling in humans. This hypothesis is in agreement with the observations made in human or mouse macrophages or peripheral blood monocytes *in vitro* or in mouse lung *in vivo* by other groups (Fiorito et al., 2009; Inoue et al., 2008; Palomaki et al., 2010).

Nanoparticle engulfment by professional phagocytes can result in inflammatory cytokine production (Dobrovolskaia and McNeil, 2007). In the present study, we showed MWCNT uptake by monocytes. Indeed, in agreement with previous data (Gao et al., 2011; Mu et al., 2009), MWCNTs were observed in phagosomes, lysosomes and cytoplasm of these cells. However, uptake of MWCNTs cannot explain alone their immunostimulatory activity, as the nanomaterials failed to trigger cytokine release in resting PBMCs. Several mechanisms have been proposed for cell internalization of CNTs (Gao et al., 2011; Mu et al., 2009). Taking into account the presence of serum in our culture medium, albumin possibly adsorbed onto MWCNTs could trigger uptake of the nanomaterials through a scavenger receptor-mediated pathway, as previously suggested for SWCNTs (Dutta et al., 2007). Absence of inflammatory cytokine release in resting PBMCs treated with MWCNTs is in favour of this internalization pathway (Dobrovolskaia and McNeil, 2007; Gao et al., 2011). In the present study, MWCNTs increased the inflammatory response evoked by cell membrane TLRs (TLR2, 4 and 5), but also by a TLR located in the endosomal compartment, TLR3. This suggests that MWCNTs could act intracellularly to modulate TLR signalling cascade. One mechanism by which particles, including pollution-derived particulate matter and engineered nanoparticles, may induce an inflammatory response is the generation of reactive oxygen species (ROS), which are key messengers in intracellular

signalling cascades leading to activation of the transcription factor NF-kB (Li et al., 2008). We did not address the role of these mediators in the responses evoked by MWCNTs in our model. However, there is some evidence in the literature for their possible involvement. Indeed, oxidative stress was reported in human airway epithelial cells in response to MWCNTs (Thurnherr et al., 2011). An increase in ROS levels was observed in human monocytes exposed to silver nanoparticles (Greulich et al., 2011). Furthermore, ROS were proposed to play a role in the aggravation of allergic inflammation evoked by MWCNTs in a mouse asthma model (Inoue et al., 2010).

When investigating the release of cytokines by PBMCs in response to PHA, we found that IL-2 and IFN-γ release was significantly and dose-dependently increased in the presence of MWCNTs. These data suggest that MWCNTs could enhance T cell function and thus increase adaptive immune responses in humans. In agreement with this hypothesis, MWCNTs were shown to slightly increase IL-2 secretion by activated Jurkat T cells (Thurnherr et al., 2009). As well, MWCNTs administered intravenously in mice were reported to stimulate the function of macrophages, which in turn activated the adaptive immune response by producing IL-12 (Grecco et al., 2011). Our observations are however in opposition to previous *in vitro* data showing inhibition of T cell-dependent antibody response by MWCNTs in mouse splenic T cells (Mitchell et al., 2009). A difference in the type of T cell activation could explain this divergence of results. In the present study, no MWCNT was found in T cells upon TEM analysis of PBMC cultures. In lines with our results, uptake of silver nanoparticles in human PBMCs was found to be cell-type specific, occurring in monocytes but not in T cells (Greulich et al., 2011). On another hand, it has been reported that MWCNTs are taken up by Jurkat T cells without affecting their proliferation (Thurnherr et al., 2009). Therefore, uptake of MWCNTs by T cells may not account for their stimulatory effect in PHA-treated PBMCs. T cell activation involves triggering of the T cell antigen receptor and intracellular signalling through the recruitment of tyrosine kinases and the phosphorylation of intracellular proteins (Smith-Garvin et al., 2009). In the Jurkat T cell line, MWCNTs were shown to have some stimulatory activity on intracellular tyrosine phosphorylation (Bottini et al., 2006). This mechanism could account for the stimulatory effect of MWCNTs in PHA-treated PBMCs. Besides, adsorption of PHA on the MWCNTs could also be involved. Indeed, Fadel et al. (2008) demonstrated that anti-CD3 antibodies induce effective T cell stimulation when adsorbed onto SWCNTs, the secretion of IL-2 evoked by the SWCNT-antibody complex being more pronounced than the one triggered by the free antibodies (Fadel et al., 2008). All together, our results suggested that MWCNTs could enhance T cell function and thus adaptive immune responses in humans.

To our knowledge, no study investigated so far the impact of CNTs or other engineered nanoparticles on PBMCs from allergic patients. When investigating the immunomodulatory activity of MWCNTs in PBMCs from mite-allergic subjects, we found that MWCNTs inhibited HDM-induced secretion of IL-5, a major player in the immunological and pathological features of allergic asthma. This observation is surprising, since both SWCNTs and MWCNTs have been reported to exacerbate allergic inflammation in mice (Inoue et al., 2009, 2010; Nygaard et al., 2009). One hypothesis to explain the divergence of our data with this literature is the difference in models (*i.e. in vitro vs. in vivo*) or systems (blood-derived cells *vs*. lung). Asthmatics are known to be highly sensitive to air pollutants, and in particular to particulate matters such as DEPs (Riedl and Diaz-Sanchez, 2005). PBMCs were used to analyse the effect of diesel exhausts on the response to an allergen in humans (Fahy et al., 2000). In agreement with epidemiological and experimental data describing an exacerbating effect of DEPs in asthma, this study found a synergistic effect between diesel exhausts and mite allergen on the release of cytokines and chemokines by PBMCs from allergic patients. However, this work was conducted on DEP organic extracts and not on the whole particle, which makes comparison with our own data difficult. One hypothesis to explain our observation would be that MWCNTs inhibit HDM-induced IL-5 secretion in PBMCs by inhibiting APC activation. In lines with this hypothesis, we observed a decrease in IFN-γ secretion in an allogeneic MLR when MWCNTs were present at the initiation of the reaction, whereas this secretion was increased when MWCNTs were added at day 3 of the reaction. Initiation of an allergen-specific memory T cell response requires presentation of the allergenic peptide by the APC to T cells, signalling by the co-stimulatory molecules expressed on the membrane of the two cell subtypes and release of stimulatory cytokines by APCs. Among others, inhibition of an allergen-specific T cell-mediated immune response could thus result from an impairment of the allergen internalization and presentation by APCs. Indeed, ultrafine carbon particles and DEPs have been demonstrated to impair the phagocytosis mediated by scavenger, mannose, Fc and complement receptors by macrophages *in vitro* (Lundborg et al., 2006). However, one can also hypothesize an inhibitory effect of MWCNTs on the signalling by co-stimulatory molecules or the release of stimulatory cytokines by APCs.

We studied at last the effect of MWCNTs on the differentiation and maturation of DC, a key cell in the initiation of adaptive immune responses. We first observed that MWCNTs do not influence phenotypic maturation and subsequent function of human MDDCs. In agreement with us, most studies found that MWCNTs have no modulatory activity on DC maturation and function (Inoue et al., 2009; Palomaki et al., 2010; Wang et al., 2009). Our data showed however that MWCNTs alter monocyte differentiation into iDCs. A recent study on mouse bone marrow-derived DCs proposed that SWCNTs may suppress T cell activation as well by acting on DCs (Tkach et al., 2011). Indeed, this work demonstrated that co-culturing T cells with SWCNT-exposed DCs suppressed T cell proliferation response upon restimulation with fresh and unexposed DCs, suggesting that SWCNTs may trigger immunosuppression by inducing T cell anergy. In the present work, we suggest that MWCNTs may inhibit adaptive immune responses by altering DC differentiation leading to iDCs with impaired phenotype and therefore impaired capacity to activate T cells.

In conclusion, our study suggests that MWCNTs could aggravate innate and adaptive immune responses in humans by increasing TLR-mediated responses or T cell activation. This study provides also evidence that MWCNTs could as well suppress allergen-induced response by targeting APCs or blunting DC differentiation. All together, this work underlines the complexity that stands behind the interactions of engineered nanoparticles with the immune system. Further investigations are necessary to fully understand these interactions.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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