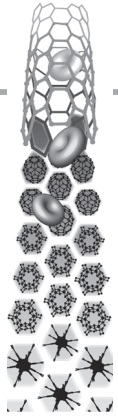

Ex vivo impact of functionalized carbon nanotubes on human immune cells

Aim: Different studies, carried out by us and others, have investigated the impact of carbon nanotubes (CNTs) *in vitro* and in animal models. To date, only a few studies have been performed on human cells *ex vivo*. There is also a lack of comparison between CNTs with varied functionalization and structural properties and their impact on different cell types. **Materials & Methods:** The present *ex vivo* human study focuses on the impact of a series of functionalized multiwalled CNTs on human T and B lymphocytes, natural killer (NK) cells and monocytes. **Results:** Smaller diameter nanotubes are internalized more efficiently. Viability assays displayed the absence of cytotoxicity of all multiwalled CNTs used. Activation assay demonstrated a strong effect on monocytes and NK cells. **Conclusion:** Our results, on human cells *ex vivo*, confirmed previous studies demonstrating appropriately functionalized CNTs are nontoxic. The effects on cell functionality were significant for the monocytes and NK cells. These findings encourage the possible use of CNTs for biomedical applications either as carriers of therapeutic molecules or as immune modulator systems.

KEYWORDS: activation ▪ cytotoxicity ▪ functionalized carbon nanotubes ▪ human cells ▪ immune system ▪ PBMCs ▪ uptake

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Nanomedicine is an emerging field of research that deals with the development and use of different classes of nanomaterials and nanoparticles for therapeutic and diagnostic purposes [1–5]. Several nanosystems, including dendrimers [6], gold nanoparticles [7,8], nanoshells [4] and porous metal-organic nanoparticles seem to show promise for the detection and the treatment of diseases [9]. Within nanomaterials, carbon nanotubes (CNTs) are gaining increased interest [5,10–14]. CNTs, with their unique and extraordinary physicochemical properties, are currently being explored for biomedical and tissue engineering [11,14], as nanoinjectors [15], biosensors [16] or drug delivery systems [5]. Interfacing CNTs with living systems has raised concerns about the toxicity of this new material. This is an important issue that should render attentive the researchers when thinking about the potential use of CNTs in nanomedicine. It has been reported that pristine, nonfunctionalized CNTs are toxic [17–19]. However, different *in vitro* and *in vivo* studies demonstrated that chemical functionalization of the surface of CNTs reduces their toxicity [20–23]. The effects of CNTs seem to depend on both the type of functionalization and the type of cells and/or organs analyzed [11]. In spite of the high number of studies performed, most of them remain inconsistent due to the difficulty of comparing different batches of nanotubes and the subsequent organic modifications. There is also a limited comparison between the effects on different cell types. To further expand the investigation of the impact of functionalized CNTs at the cellular level, we have focused our attention on human immune cells and their activation. Several studies on the effect of CNTs on the immune system have been carried out [20,24,25]. Dumortier *et al.* discovered that functionalized water soluble CNTs do not affect the immunoregulatory function of primary cells isolated from mouse lymphoid organs [20]. Nygaard *et al.* exposed mice to pristine single-walled (SWCNTs) and multiwalled CNTs (MWCNTs) during sensitization with the allergen ovalbumin [24]. They observed that CNTs promote allergic immune responses. Salvador-Morales *et al.* showed that CNTs could enhance complement activation. In the field of biomedicine, the research is generally a multistep process, which starts from an *in vitro* cell line investigation, and subsequently evolves towards preclinical *in vivo* animal tests [25]. From there the following two most important steps are *ex vivo* human studies and eventually *in vivo* clinical trials. Within this context, we have concentrated our attention on the assessment of the impact of functionalized CNTs on human immune cells *ex vivo*. We have explored the effects of a series of functionalized CNTs on several types of immune cells, including T cells, B cells, natural killer (NK) cells and monocytes. Six different types of functionalized

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MWCNTs were prepared and incubated with peripheral blood mononuclear cells (PBMCs). A recent review has summarized the results from several studies discussing the doses used in the different experiments [13]. Most authors used low concentrations of CNTs that probably do not induce significant effects. Therefore, we decided to use a relatively high amount of functionalized CNTs (100 µg/ml). We decided to use MWCNTs as they are easily available compared to SWCNTs, and their dimensions can be better controlled and characterized. Diverse parameters have been analyzed, including cellular uptake, cell viability and activation. Overall, no signs of cytotoxicity were observed, thus reinforcing the need to process CNTs by organic functionalization to render them biocompatible. Towards the assessment of cell functionality, we have found activation in the case of monocytes and NK cells treated with MWCNTs.

Material & methods

CNTs

Purified MWCNTs were purchased from NanoAmor (Nanostructured & Amorphous Materials Inc., TX, USA). MWCNTs used in this study were 95% pure (stock No. 1240XH). The outer average diameter was between 20 and 30 nm, and length between 0.5 and 2 µm before oxidative treatment. These nanotubes were used to synthesize functionalized MWCNTs (Figure 1). The second type of nanotubes, produced by the catalytic carbon vapor deposition process, was instead obtained as purified material from Nanocyl (Sambreville, Belgium; Thin MWCNT 95+% C purity, Nanocyl 3100®, batch n° 071119). The average diameter and length were 9.5 nm and 1.5 µm, respectively. These nanotubes were used to synthesize functionalized MWCNTs (Figure 2). Oxidation and functionalization of the nanotubes with the ammonium groups and a fluorescent probe (FITC) were performed as described by Samorì *et al.* [26] and Gaillard *et al.* [27]. Characterizations of the different functionalized MWCNTs were performed using transmission electron microscopy, thermogravimetric analysis and Kaiser test [26,27]. Transmission electron microscopy was performed on a Hitachi 600 microscope with an accelerating voltage of 75 kV (images acquired using a Hamamatsu CCD camera).

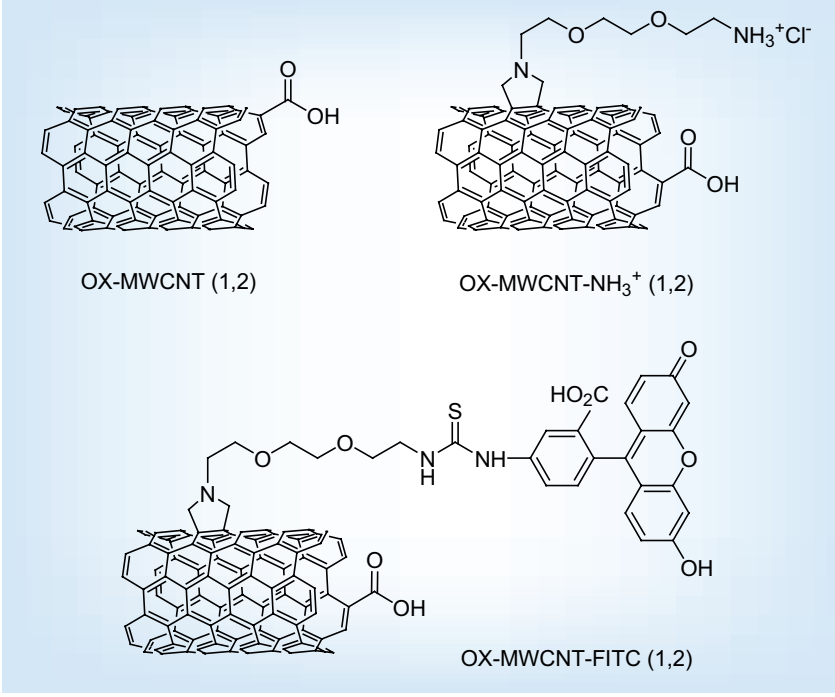

Figure 1. Molecular structures of the functionalized carbon nanotubes.
Six different functionalized multiwalled carbon nanotubes were used for the experiments of cell uptake, cytotoxicity and cell activation.
FITC: Fluorescein isothiocyanate; MWCNT: Multiwalled carbon nanotube; OX: Oxidized.

Cell culture

Human cells were obtained from informed healthy male donors (aged 25–50 years). Cell separation and experiments were performed immediately after blood drawing. PBMCs were isolated from the fresh heparinized blood by Ficoll-Paque PLUS (GE Healthcare) density gradient centrifugation. PBMCs were cultured in RPMI 1640 medium containing 1% antibiotic-antimycotic mixture and 10% heat-inactivated fetal bovine serum (Invitrogen). CNTs were homogeneously dispersed at 1 mg/ml in sterile ultrapure water. Initially, CNTs were sonicated for 45 min with a Branson 3200 water bath sonicator and vortexed for a few seconds. An additional sonication was performed for 15 min before each experiment. The experiments were performed on blood samples from at least three different donors to average the high variation of the immune system. Moreover, PBMC incubations were performed in triplicate.

Uptake experiments

In order to analyze MWCNT uptake, PBMCs were incubated in a 48-well plate (7×10^5cells/well) with increasing amounts of MWCNT-FITC (1, 10 and 100 µg/ml) for 24 h. The time course experiment was performed at 6, 12 and 24 h with 100 µg/ml CNTs. Uptake of monocytes was also performed at 10, 30 and 90 min using a concentration of 100 µg/ml CNTs. Cells were then washed in phosphate

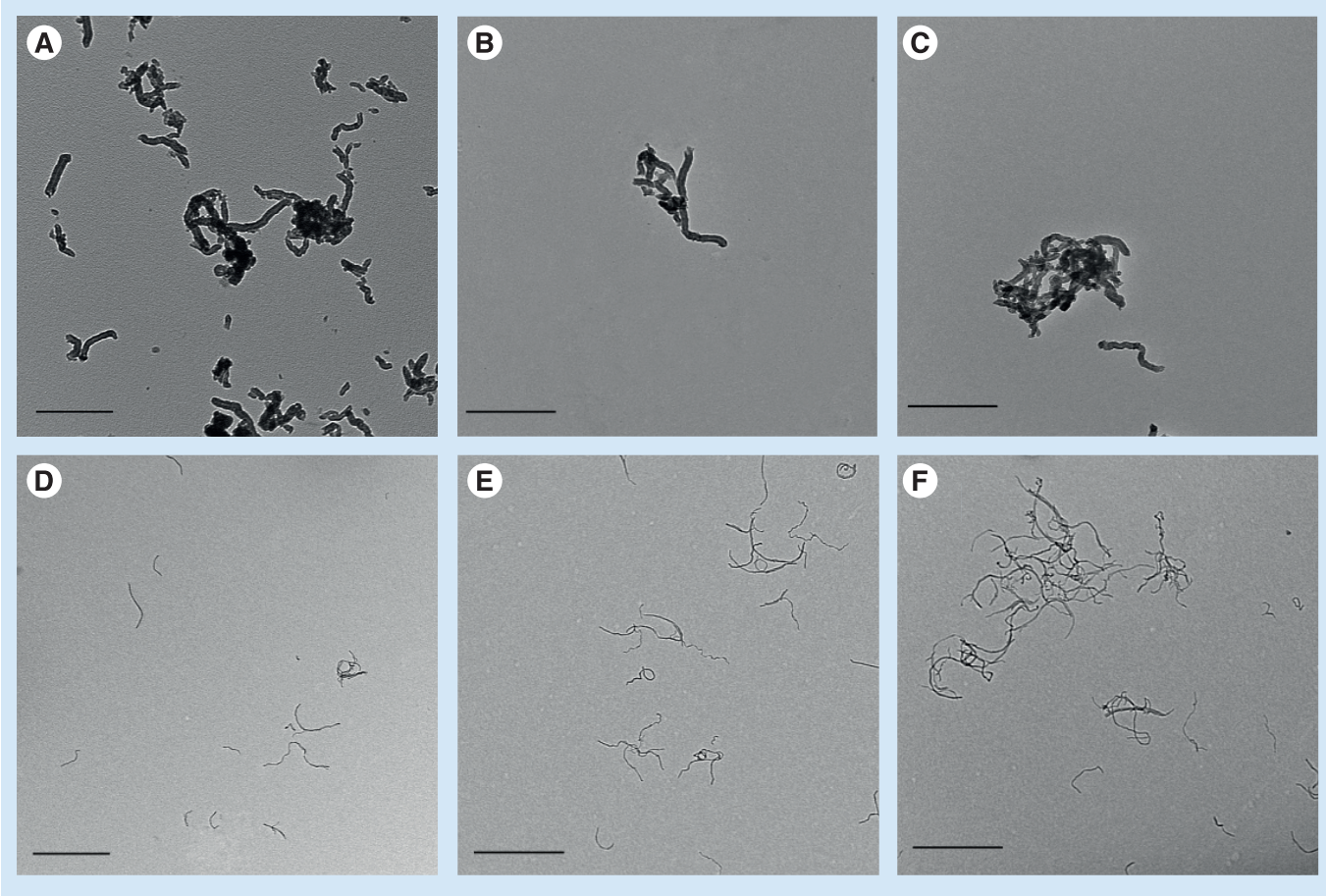

Figure 2. Transmission electron microscopy images of the functionalized carbon nanotubes. (A) OX-MWCNT 1; **(B)** OX-MWCNT-NH_3^+ 1; **(C)** OX-MWCNT-FITC 1; **(D)** OX-MWCNT 2; **(E)** OX-MWCNT-NH_3^+ 2; **(F)** OX-MWCNT-FITC 2. Scale bars correspond to 500 nm.
FITC: Fluorescein isothiocyanate; MWCNT: Multiwalled carbon nanotube; OX: Oxidized.

buffered saline (PBS) containing 1% bovine serum albumin, cytocentrifuged onto a microscope slide and visualized by a Microradiance Nikon Eclipsa from Biorad (40×). Images were analyzed with the ImageJ software. Flow cytometry measurements were performed using a FACSCalibur® and analyzed with CELLQuest software (BD Biosciences). Cell fluorescence was measured after washing with 0.4% (w/v) trypan blue to quench fluorescein signal from noninternalized CNTs [28], from 10,000 to 50,000 events were collected.

Identification of immune cell populations & analysis of their activation status

PBMCs (250,000 cells per sample) were cultured in a 96-well plate for 24 h with 100 µg/ml CNTs. After MWCNT treatment and PBS washing, major immune cell populations were identified by flow cytometry according to the expression of specific cell surface markers (clusters of differentiation [CD]) that were detected with fluorescently labeled monoclonal antibodies. Fluorescein isothiocyanate (FITC)-, phycoerythrin-, peridinin chlorophyll protein-, or allophycocyanin-conjugated anti-CD3 (SK7 clone), anti-CD4 (SK3 clone), anti-CD8 (SK1 clone), anti-CD14 (Mϕ9 clone), anti-CD16 (3G8 clone), anti-CD20 (L27 clone), anti-CD69 (L78 clone), anti-CD25 (2A3 clone), anti-HLA DR (L243 clone), anti-CD161 (DX12 clone) were purchased from BD Biosciences (Mountain View, CA, USA). Cell typing was performed using the above mentioned antibodies to recognize the major population of PBMCs: $CD3^+$/$CD4^+$ and $CD3^+$/$CD8^+$ for T cells, $CD20^+$ for B cells, $CD14^+$ for monocytes and $CD16^+$/$CD3^-$ for NK cells. To assess the expression of cell surface markers, cells were washed twice with 0.5 % bovine serum albumin in PBS pH 7.2 and were incubated for 20 min in dark conditions with fluorochrome-conjugated monoclonal antibodies. Cells were then washed and analyzed by flow cytometry. As CNTs are inside the cells, we exclude possible interference with the fluorescent antibodies, which are cell surface markers and therefore not interacting with the nanotubes.

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Viability assay

The viability assay was performed using LIVE/DEAD® green stain kit (Invitrogen, Milan, Italy). The kit employs an amine-reactive fluorescent dye to assess cell viability by fluorescence activated cell sorting (FACS) analysis. In the cells with compromised membranes (late apoptotic and necrotic), the dye reacts with free amines both inside the cell and on the cell surface, yielding intense fluorescent staining. In viable cells, the dye reactivity is restricted to the cell surface amines, resulting in less intense fluorescence. The green-fluorescent reactive dye (L23101) has excitation and emission maximas of approximately 495/520 nm. Briefly, PBMCs (1×10^6cells/well) were incubated for 24 h with MWCNTs or left untreated. After washings, the cells were stained with the green reactive dye in PBS and incubated for 30 min before flow cytometry analysis. A sample of cells was incubated for a few seconds with 70% of ethanol as a positive control.

Apoptosis assay

To detect cells undergoing apoptosis, Annexin-V FITC staining was employed. The assay is based on the binding of Annexin V to phosphatidylserines found on the external side of the membrane in early apoptotic cells. Cells were incubated for 24 h with MWCNTs or left untreated. A positive control of cell apoptosis was performed using a solution of 100 µM H_2O_2. The cells were then collected and washed with PBS pH 7.2. The staining with Annexin-V-FITC and cell subset-specific antibodies was performed for 20 min in the dark. After washing, the cells were analyzed by flow cytometry.

Activation assay

Peripheral blood mononuclear cells were cultured in the presence or in the absence of MWCNTs, concanavalin A (ConA; 4 µg/ml) or bacterial endotoxin lipopolysaccharides (LPS; 1 µg/ml). ConA and LPS were purchased from Sigma-Aldrich. After 24 h of incubation, PBMCs were stained to identify immune cell populations and analyze activation marker expression (i.e., CD25 and CD69). Staining with fluorochrome-conjugated monoclonal antibodies was performed in the dark for 20 min. After washing, cells were then analyzed by flow cytometry.

Monocytes were isolated from PBMCs to assess IL-6 secretion. Briefly, PBMCs (5×10^5 cells) were incubated in a 48-well plate in RPMI. After 24 h, monocytes attached to the bottom of the wells were washed six times with RPMI without fetal bovine serum. Isolated monocytes were incubated with 100 µg/ml of MWCNTs or left untreated; LPS was used as a positive control. After 24 h, supernatants were collected and IL-6 secretion was measured using human IL-6 ultrasensitive ELISA kit from Invitrogen (Italy).

Statistical analysis

Statistical analyses for FACS gated events were performed using Student's t-test for paired data. Data indicated with a star were considered statistically significant (p-value < 0.05). Data are presented as mean ± SD (n = 3), with the exception of the data on the uptake of OX-MWCNT-FITC by human primary immune cells where the obtained results are representative of one experiment out of at least three different incubations for each sample.

Results

Preparation of functionalized MWCNTs

For this study, we have compared the behavior of two types of functionalized MWCNTs synthesized starting from CNTs provided by NanoAmor and Nanocyl companies, respectively. These nanotubes were both initially oxidized to obtain OX-MWCNTs 1 and OX-MWCNTs 2 (Figure 1), and subsequently submitted to the 1,3-dipolar cycloaddition reaction to generate positively charged ammonium functionalized OX-MWCNTs-NH_3^+ 1 and 2 [27]. The nanotubes were isolated and fully characterized using different spectroscopic and microscopic techniques [26,27,29]. Figure 2 shows representative transmission electron microscopy images of the six different nanotubes used in this study. As can be easily observed, the series of nanotubes (NanoAmor 1 and Nanocyl 2) differ mainly by their dimensions. In particular, oxidized MWCNTs 1 are thicker, with a diameter between 20 and 30 nm, and an average length of 403 nm [27,30], while oxidized MWCNTs 2 have a smaller diameter (~9.5 nm) and an average length of 396 nm [26]. Following the 1,3-dipolar cycloaddition and FITC labeling, the morphology of the tubes was not affected. The amount of ammonium groups was calculated by Kaiser test and corresponded to 82 and 168 µmol/g for MWCNTs 1 and 2, respectively. Part of the nanotubes with the ammonium groups was labeled with FITC to prepare OX-MWCNT-FITC 1 and 2 for confocal microscopy experiments. The amount of FITC was assessed again by Kaiser test and corresponded to 50 and 128 µmol/g for MWCNTs 1 and 2, respectively.

These nanotubes were then used to assess their impact on human immune cells.

Uptake of MWCNTs by human primary immune cells

The ability of CNTs, harboring different types of functional groups, to penetrate the cell membrane has already been assessed using immortalized cell lines [5,30,31]. In this study, we analyzed the effect of six different types of functionalized MWCNTs on several immune cell subsets from human peripheral blood. We first investigated the uptake of OX-MWCNT-FITC by PBMCs (**Figure 3 & Supplementary Figure 1**, see online www.futuremedicine.com/doi/suppl/10.2217/nnm.11.101). For this purpose, human cells were incubated for 24 h with fluorescently labeled OX-MWNT-FITC 1 and 2 using increasing concentrations, 1, 10 and 100 µg/ml. This range of concentrations demonstrates effects of a low/medium dose, which can be considered the most suitable for therapeutic applications, to a high dose, which might affect cell viability and activation, as we observed in other experiments using different functionalized carbon nanomaterials [32,33]. Cells were then washed with trypan blue to quench the fluorescence, which may derive from cell surface-adsorbed CNTs [5,28]. Cell FITC-fluorescence was diminished from 0 down to 20% after trypan blue washing (data not shown). After trypan blue treatment, FACS analysis showed that the nanotubes were internalized in a dose-dependent manner with the highest fluorescence signals obtained at 100 µg/ml (**Figure 3A**). Uptake of OX-MWCNT-FITC 2 seemed to be more efficient than OX-MWCNT-FITC 1 already at 1 µg/ml. The same cells were also analyzed by confocal microscopy (**Figure 3B & 3C**). **Figure 3C** corresponds to different z-stacks of the same cell incubated with 100 µg/ml, confirming that the tubes were inside. Cell uptake was also assessed by investigating the changes on cell morphology. It has been previously reported that the sideward scatter signal (corresponding to the inner cell complexity) can be used to probe the interaction between CNTs and cells [1,13,34]. The intensity of the sideward scatter increases as the nanotubes cross the cell membrane and localize inside the cell. This is associated with a change in cell morphology, and in particular to an increase of granularity [35]. **Figure 3D** displays the dot plots of human monocytes among PBMCs incubated with MWCNTs or left untreated. The four major types of immune cells within PBMCs, namely T cells, B cells, NK cells and monocytes, were stained using specific fluorescently labeled monoclonal antibodies to identify the different populations. Monocytes were identified by CD14 expression and sideward scatter indicating granularity was observed. No significant difference in the percentage of $CD14^+$ monocytes was detected. However, sideward scatter showed a relevant increase for cells incubated with both types of functionalized MWCNTs 1, as compared to control cells or cells incubated with MWCNTs 2. This is not surprising as MWCNTs 1 are bigger in diameter, consequently increasing the granularity of monocytes once internalized. On the contrary, the dot plots for T cells, B cells and NK cells, which are nonphagocytic cells, did not exhibit any clear change in scattering upon incubation with the different samples of MWCNTs (data not shown).

As the changes in morphology were clearly indicative of uptake by at least one class of PBMCs, we wanted to analyze in more detail the uptake capacity of all cell types. PBMCs were incubated with the different fluorescent MWCNTs at 1, 10 and 100 µg/ml for 24 h, washed and stained with specific antibodies to identify the different cell populations (**Figure 3E**). Additionally, a time response experiment was performed with 100 µg/ml for 6, 12 and 24 h. We observed that nanotube internalization was both dose and time dependent. OX-MWCNT-FITC 2 showed a better uptake by all cell subsets at low concentration and already at shorter times of incubation in comparison to type 1. As for phagocytic cells, for which shorter incubation times are more relevant, we observed that OX-MWCNT-FITC 1 and 2 were also rapidly internalized by monocytes following exposure at 10, 30 and 90 min (**Supplementary Figure 1**). This is in agreement with the experiment performed on total PBMCs (**Figure 3A**). The recurring observation that the cells incubated with MWCNTs 1 are less fluorescent than those treated with MWCNTs 2 can be explained by the two following reasons: the amount of FITC in 1 is about half of 2 (50 µmol/g and 128 µmol/g, respectively); and the preferential uptake of FITC-labeled MWCNTs 2 may be due to the smaller diameter (9.5 nm) of these tubes compared to MWCNTs 1 (20–30 nm). These results suggest that the nanotube dimensions might at least partly influence their uptake. We decided to use an incubation time of 24 h and a concentration of 100 µg/ml CNTs. These conditions were chosen mainly for two reasons: because at this concentration the amount of internalized

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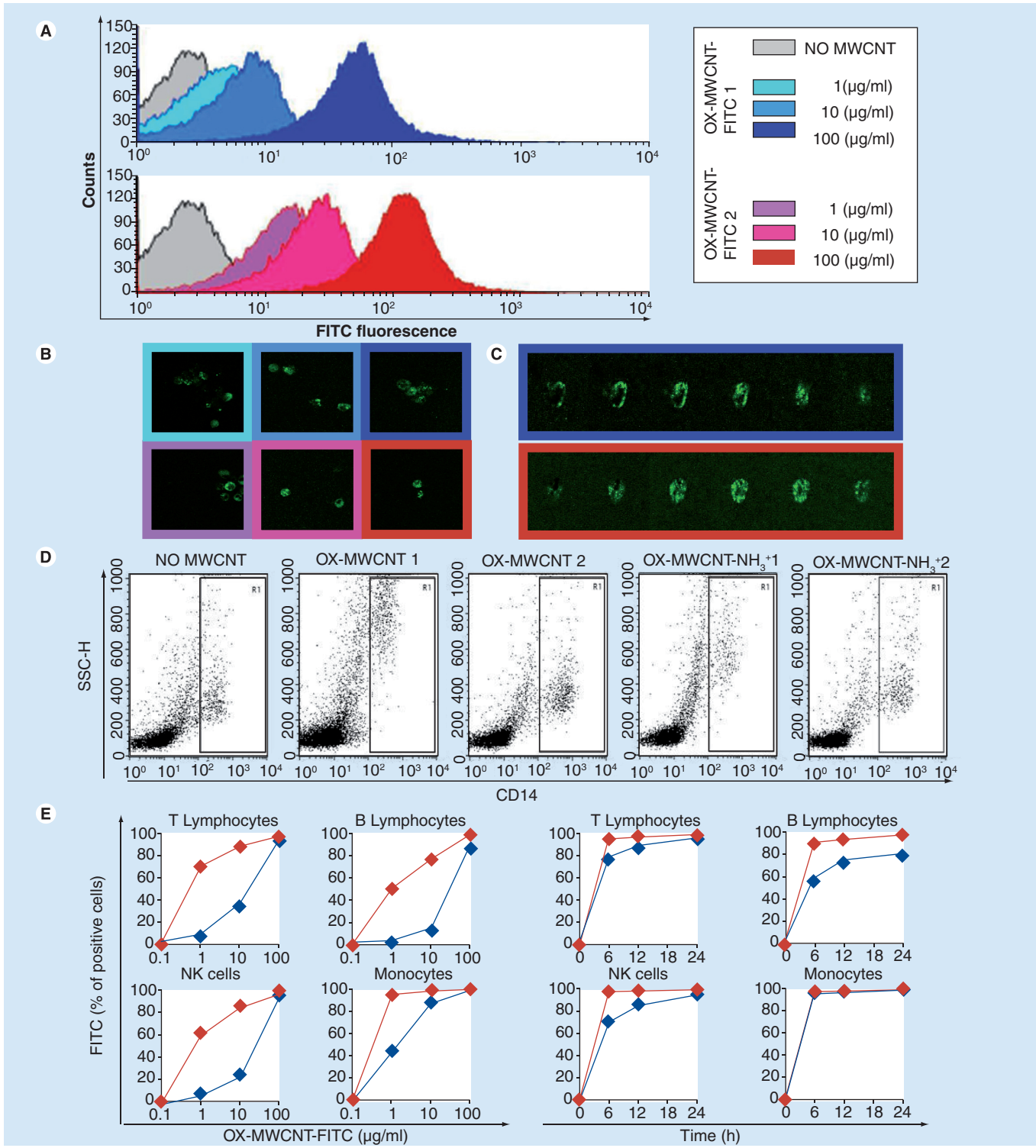

Figure 3. Uptake of OX-MWCNT-FITC by human primary immune cells. Peripheral blood mononuclear cells were either left untreated or incubated for 24 h with 1, 10 and 100 µg/ml of OX-MWCNT-FITC 1 and 2. **(A)** Samples were analyzed by flow cytometry. Cell fluorescence is reported in arbitrary units. The graphics show that CNTs are internalized in a dose-dependent manner. Data are derived from one representative experiment out of three. **(B & C)** CNT uptake was also assessed by confocal microscopy. **(B)** Representative cells incubated with 100 µg/ml. **(C)** Images correspond to consecutive plans from the top to the bottom of cells and show the presence of CNTs into the cytoplasm. Experiments were repeated three times with similar results. **(D)** The cells were left untreated or incubated with 100 µg/ml of OX-MWCNT 1 and 2 and OX-MWCNT-NH_3^+1 and 2 for 24 h. To identify monocytes and evaluate their morphology, a staining with CD14 antibody was then performed. R1 indicates the area corresponding to $CD14^+$ cells. Data are derived from one representative experiment out of three. **(E)** Penetration of increasing doses of fluorescent CNTs (OX-MWCNT-FITC 1 [blue diamond] and 2 [red diamond]) was investigated into T cells, B cells, NK cells and monocytes after 24 h incubation (left side). The highest uptake was detected at 100 µg/ml concentration. A time course (right side) was performed at 6, 12 and 24 h of incubation with 100 µg/ml of OX-MWCNT-FITC 1 (blue diamond) and 2 (red diamond). FITC: Fluorescein isothiocyanate; MWCNT: Multiwalled carbon nanotube; NK: Natural killer; OX: Oxidized.

nanotubes is very high and because we wanted to observe possible MWCNT effects (if any) at cellular level.

Effect of functionalized MWCNTs on the relative percentage of immune cell subsets & on their viability

We previously found that SWCNTs modified via 1,3-dipolar cycloaddition or via oxidation/amidation reactions were nontoxic and preserved the functionality of T cells, B cells and macrophages isolated from mice [20]. To extend this research and to try to gather further data aimed at favoring future biomedical applications of CNTs in humans, we have started to analyze the effect of these highly water dispersible MWCNTs 1 and 2 on human immune cells in a series of *ex vivo* experiments. First, we investigated the influence of CNT exposure on the percentage of cells expressing the markers CD4/CD3, CD8/CD3, CD20, CD16 and CD14 that allow identification of $CD4^+$ and $CD8^+$ T cells, B cells, NK cells and monocytes, respectively (Figure 4). The percentage of cells was not affected by incubation with the different nanotubes in the case of T cells ($CD3^+$) and monocytes ($CD14^+$). The rather low percentage of CD14 cells is probably due to the fact that after 24 h of incubation some of the monocytes attach to the plate and we did not use any mechanic (scraper) or chemical (ethylenediaminetetraacetic acid [EDTA]) method to detach them as this may affect the subsequent activation assay. Regarding $CD20^+$ B cells, we found a decrease in the number of cells in comparison to the control, while in the case of NK cells we detected an increase of the cell percentage. To our knowledge, this is the first observation of an effect of CNTs on the relative amount of $CD20^+$ B cells and $CD16^+$ NK cells. All the results showed in Figure 4 were also confirmed on absolute cell numbers (data not shown).

Toxicity is still one of the major issues related to the use of nanotubes in medicine [36,37]. Several studies are present in the literature addressing this important aspect [13]. Most works remain inconsistent because CNTs are not carefully characterized and there is a lack in comparing the effects of different CNTs using different cell types. In this context, we have decided to assess the cytotoxic effects of our four types of water dispersible MWCNTs on human immune cells *ex vivo*. PBMCs were incubated with CNTs at 100 µg/ml for 24 h and viability, particularly assessing membrane integrity, was investigated on all cells using the LIVE/DEAD stain kit and on each cell subset using Annexin V staining (Figure 5). Figure 5A shows the viable PBMCs left upon incubation with the different MWCNTs. Ethanol was used as a positive control. The graphic shows a complete overlap between histograms of the cells treated with MWCNTs and the negative control, meaning that none of the nanotubes employed in this experiment affected cell viability. Similar results were obtained analyzing the different cell populations (i.e., T cells, B cells, NK cells and monocytes; data not shown). In Figure 5B we have

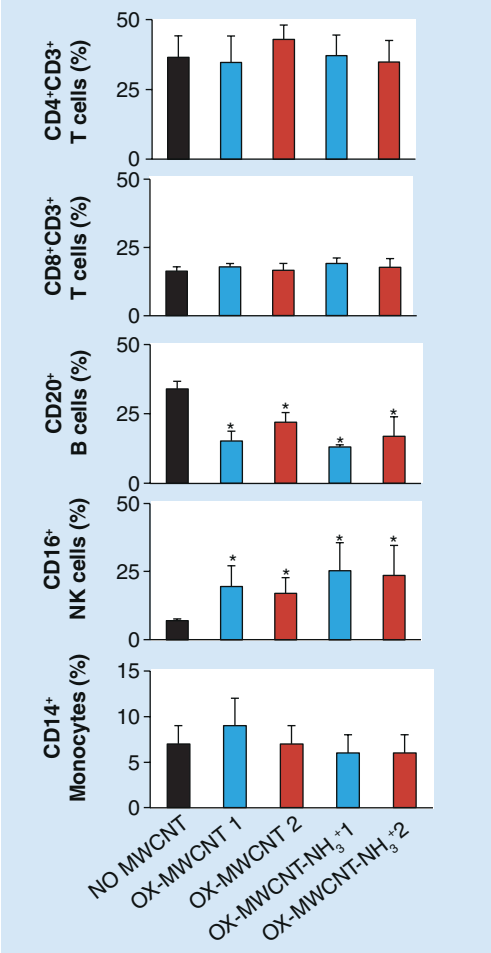

Figure 4. Relative percentage of T cells, B cells, natural killer cells and monocytes incubated with the different multiwalled carbon nanotubes. Cells were either left untreated or incubated for 24 h with 100 µg/ml of OX-MWCNT 1 and 2 and OX-MWCNT-NH_3^+ 1 and 2. A staining of the different clusters of differentiation was performed to identify cell subsets and the samples were analyzed by flow cytometry gating on viable peripheral blood mononuclear cells. Statistical significance compared to untreated cells (Student's t-test) is indicated by * = $p < 0.05$.
MWCNT: Multiwalled carbon nanotube; OX: Oxidized.

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displayed the percentage of apoptotic cells for each cell subset. Incubation with H_2O_2 100 µM for 24 h was used as a positive control. None of the MWCNTs tested caused a significant enhancement of the number of apoptotic cells. Oxidized nanotubes OX-MWCNT 1 and 2, which are rich in carboxylic functions, seem to have a protective effect on monocytes. Different studies showed an increased apoptosis for cells treated with pristine and oxidized CNTs. De Nicola *et al.* showed that nonfunctionalized MWCNTs induced a significant effect in the modulation of apoptosis on human monocytes treated with the chemotherapic agent topoisomerase II inhibitor etoposide [38]. Bottini *et al.* described an increased apoptosis for T cells incubated with oxidized CNTs [39]. On the other hand, we and others reported that functionalized CNTs did not affect cell apoptosis [20,40]. These results confirmed the lack of cytotoxicity of CNTs in isolated human cells when they are appropriately functionalized, becoming highly water dispersible and biocompatible. Our observations are in agreement with previous studies carried out *in vivo* and *in vitro* [37,41–43].

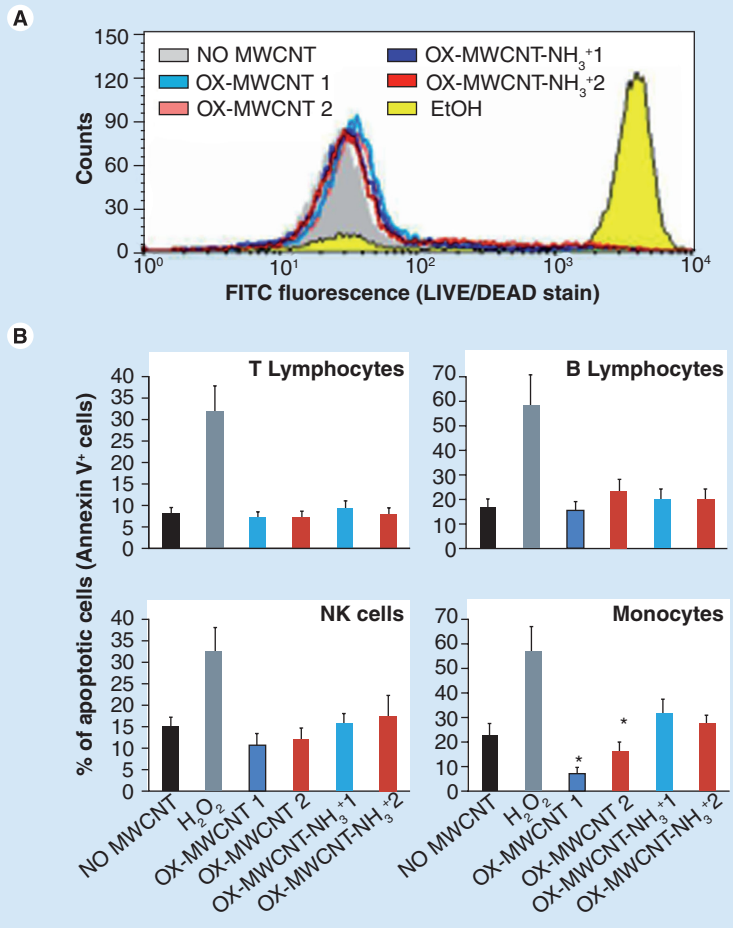

Figure 5. LIVE/DEAD staining and apoptosis assay on human primary immune cells. The viability and apoptosis of human primary immune cells was assessed after incubation with MWCNTs. LIVE/DEAD staining was performed after 24 h on peripheral blood mononuclear cells using 100 µg/ml of carbon nanotubes **(A)**. To investigate apoptosis, cells were left untreated or either incubated for 24 h with 100 µg/ml of carbon nanotubes, stained with Annexin V and with specific antibodies for population identification **(B)**. Statistical significance compared to untreated cells (Student's t-test) is indicated by * = $p < 0.05$.
FITC: Fluorescein isothiocyanate; MWCNT: Multiwalled carbon nanotube; NK: Natural killer.

Effect of MWCNTs on human immune cell activation

Preservation of normal immune functions is of fundamental importance using CNTs for the treatment of any disease. Activation is one of the best parameter to look at the functionality of immune cells. For this reason, we first investigated the expression of the activation-related surface molecule CD25, the α-chain of the IL-2 receptor (Figure 6A). Activation was assessed by CD25 staining on immune cells incubated with the different CNTs or left untreated. ConA and LPS, due to their well-known activation property, were used as positive controls for T cells and for B cells and monocytes, respectively. CNTs were again used at a concentration of 100 µg/ml for 24 h. The bar charts for $CD4^+$ and $CD8^+$ T lymphocytes and B cells show that the samples treated with CNTs were not activated. However, we found a strong statistically significant activation of the monocytes incubated with OX-MWCNT-NH_3^+ 1 and 2. To verify the obtained data we performed the experiment incubating CNTs and control samples directly in whole blood. We obtained similar results in all types of cells. On monocytes we detected an increased activation also using OX-MWCNT 1 (data not shown). To better explore the effects of MWCNTs 1 and 2 on isolated monocytes, we measured the secretion of IL-6 (Figure 6B), which is one of the most important interleukins secreted by activated monocytes during inflammation. Here again, we found a significant increasing of IL-6 secretion for samples treated with OX-MWCNT-NH_3^+ type 1 and 2, but not with OX-MWCNTs 1 and 2. We reasoned that the impact on monocyte activation using OX-MWCNT-NH_3^+ maybe due to the type of functionalization. To better investigate the possible activation process of human primary immune cells, we also examined the expression of the CD69 marker (Figure 6C). We did not observe any CNT correlated activation for $CD4^+$ and $CD8^+$ T lymphocytes and B cells (data not shown). However, in MWCNT-treated samples, the percentage of CD69 expressing NK cells was at least

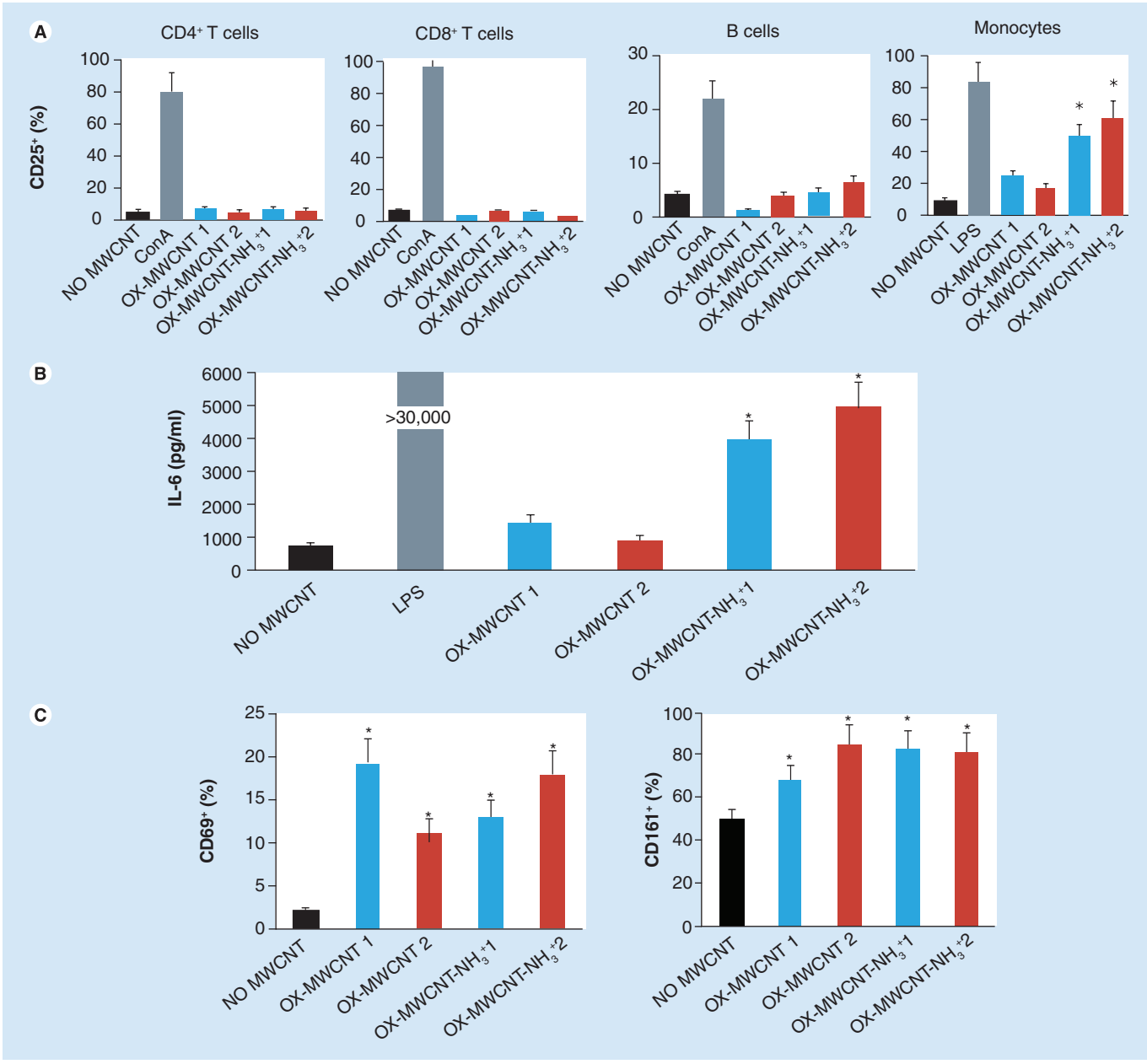

Figure 6. Impact of multiwalled carbon nanotubes on human immune cell activation. Peripheral blood mononuclear cells were incubated for 24 h with 100 µg/ml MWCNTs or left untreated. Cell activation was assessed looking at the expression of CD25. **(A)** Concanavalin A (4 µg/ml) and lipopolysaccharides (2 µg/ml) were used as positive controls for T cells, B cells and monocytes, respectively. CD25 expression on different cell subsets was assessed by flow cytometry. Cell activation on isolated monocytes was further assessed looking at the secretion of IL-6 by ELISA. **(B)** Natural killer cell activation was investigated by flow cytometry through the expression of CD69 and CD161 surface markers. **(C)** Statistical significance compared to untreated cells (Student's t-test) is indicated by * = $p < 0.05$.
LPS: Lipopolysaccharide; MWCNT: Multiwalled carbon nanotube.

three-times higher than in the control. To further explore the effects of MWCNTs 1 and 2 we measured the CD161 expression, a major phenotypic and activation marker of NK cells also present on T cells (Figure 6C). We did not observe any MWCNT-related modulation of CD161 for $CD4^+$ and $CD8^+$ T lymphocytes (data not shown). On the contrary, we found a higher expression of CD161 on CNT-treated NK cells compared to the control. In both cases (CD69 and CD161), the observed effect on NK cells was independent from the type of MWCNTs tested.

Discussion

In this study we have reported the first comprehensive analysis of the impact of functionalized CNTs on human cells *ex vivo*. In particular,

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we have focused our attention on the effects of a series of functionalized, water dispersible MWCNTs, on a wide variety of human primary immune cells, including T cells, B cells, NK cells and monocytes. Looking specifically at the immune system, we have addressed several concerns regarding CNT cytotoxicity and correlated the functionalization and the morphological properties of CNTs with their interactions with the different cell populations. We first investigated the uptake of the different MWCNTs by human primary immune cells. A few years ago we found that cellular uptake of functionalized CNTs was independent of the functional groups and cell type, but the study did not attempt to quantify the amount of internalized nanotubes and the dependence of the uptake on the incubation time [30]. Here, the observations confirm that the cellular uptake is not affected by the functional group introduced on CNTs, but it is likely linked to the different physical and structural characteristics of the nanotubes. The smaller diameter of MWCNTs 2 compared to MWCNTs 1 seems to facilitate cell penetration (Figure 3). Effect on cell complexity was more evident using the nanotubes with a larger diameter (MWCNTs 1), thus confirming the results shown in a recent work using the same type of nanotubes [35]. Raffa *et al.* compared the uptake of nonfunctionalized CNTs of different lengths [44]. CNT length clearly influenced cellular penetration, with CNTs shorter than 1 µm being more easily internalized. Strano and coworkers have developed a model to predict the cellular uptake and recycling (exocytosis) of SWCNTs and other nanoparticles, and to correlate the rate of internalization with the geometry of the nanomaterials [45]. The total uptake for SWCNTs and gold nanoparticles resulted in a maximum at a common diameter of 50 nm, which indicates that bundles of SWCNTs are able to penetrate into the cells. Sato *et al.* have studied the effect of CNT length on cell cytotoxicity indicating that the degree of inflammation was stronger for longer nanotubes [46]. Recently, the effect of the dispersion of different types of CNTs has been analyzed, varying in the dimensions only on cell viability, demonstrating that covalent functionalization stabilizes the dispersions and reduces the cytotoxic effects on cells [47].

We then investigated the impact on the percentage of the different cell populations. The effect of MWCNTs was clearly cell-type dependent. Indeed, we have observed a decrease in the percentage of $CD20^+$ B lymphocytes and an increase in the percentage of $CD16^+$ NK cells incubated with each type of MWCNTs. We did not find any toxic effect of MWCNTs on B cells, proving that the lower percentage of B cells treated with the nanotubes is clearly not correlated with their viability. The effect on the other types of cells was not significant. In a study on the pulmonary and systemic immune response to inhaled nonfunctionalized MWCNTs it has been oppositely found that the cellular function of NK cells decreased [48]. This is not surprising as the nanotubes were very long (5–15 µm) and accumulated into the lungs and also affected other cell types including T cells. Similarly, it was found that SWCNTs inhibited B-lymphocyte proliferation and reduced NK cell activity *in vitro* [49]. Besides CNTs, other types of nanoparticles can also affect the functions of immune cells. ZnO nanoparticles, for example, induce the production of proinflammatory cytokines and cytotoxicity on human immune cell populations, including B cells and NK cells [50]. Similarly, silver nanoparticles modulate the cytokine production on PBMCs in a dose-dependent manner, but show significant cytotoxic effects at high concentrations [51]. Whether there is a direct relationship between the increase of NK cells and a decrease of B cells treated with the different functionalized CNTs is not clear and is currently under study.

Analysis of the cytotoxic effects confirmed previous studies demonstrating that water soluble and appropriately functionalized CNTs are not toxic [21,22,30]. Functionalization by 1,3-dipolar cycloaddition makes CNTs nontoxic for different cell lines, in mouse animal models *ex vivo* and *in vivo* [20,52,53]. The results on the immune activation suggest that our MWCNTs are inert for T and B cells. On the other hand, we reported a strong effect on monocyte activation, particularly depending on the type of functionalization. We observed an increased activation only for the cells treated with OX-MWCNT-NH_3^+ 1 and 2. We confirmed these data looking at the secretion of IL-6 by ELISA on isolated monocytes. The type of functionalization could be just one of the explanations for the increased activation. A lower dose of OX-MWCNTs, which we observed to prevent the apoptosis on monocytes, may also cause an inflammatory response. An increased IL6 production, due to the presence of double-walled CNTs, was found by Crouzier *et al.* in an *in vivo* study on mice [54]. Pattani and coworkers investigated the effect of black chitosan focusing on different markers, among which was IL-6, they did not show any change of IL-6 secretion by PBMCs treated with nanoparticles

for 24 h [55]. These studies suggest that the levels of IL-6 produced depend on chemical-physical characteristics of the particles.

Our findings are in agreement with the data from other studies. Sato *et al.* reported that nonfunctionalized MWCNTs with different length provoked inflammation on monocytic THP1 cells [46]. Nygaard *et al.* showed that SWCNTs and MWCNTs promote immune response in mice and act as allergy adjuvant [24]. De Nicola *et al.* found a decrease in cell number for human monocytes treated with CNTs and explained this behavior as macrophage activation due to CNT exposure [38]. However, these studies were performed with pristine nonfunctionalized CNTs.

Our previous work on mouse macrophages showed an increase of TNF-α production by macrophages incubated with PEG-derivatized CNTs [20]. Similarly, macrophage activation was also observed using carbon nanohorns, an alternative form of CNTs [32]. Taken together, all these studies suggest that the identification of the best characteristics in terms of functionalization is fundamental to better understand which types of CNTs can be considered either as immunomodulator agents or drug delivery systems. Our results showed a relative expansion and a strong activation of NK cells treated with MWCNTs. However, these effects on NK cells seemed to be independent of the type of functionalization and physical properties of MWCNTs. A deep investigation of CNTs and their effect on NK cells is currently under study. Our data on NK cells are very promising for a possible use of CNTs for adoptive cell therapy with NK cells. Potervin *et al.* found that phosphonate-capped dendrimers inhibit the activation of T cells in IL-2 stimulated PBMCs, allowing a rapid enrichment of NK cells [56]. In our study, with MWCNTs, we found an enrichment of NK cells even without an inhibition effect on T cells. We would like to underline that the interesting variation on monocyte and NK cell activation did not correlate with any change on their viability. For this reason this finding may open new windows on future biomedical applications of functionalized CNTs. The new data presented here, in particular for monocytes and NK cells, reinforce and encourage the idea of the potential use of CNTs for several and new biomedical applications.

Conclusion

In the last years the nanotechnology field has emerged as one of the major hopes for medicine to treat and fight several types of diseases. Although CNTs harbor interesting biomedical applications, their potential biological activities and associated toxicity still need to be carefully evaluated. We and others previously demonstrated the absence of toxicity of well-purified and functionalized CNTs. However, to our knowledge no comprehensive study was carried out on human primary immune cells *ex vivo* using well-functionalized CNTs. In the present study, we have investigated the effect of water dispersible CNTs in a wide variety of immune cells isolated directly from human peripheral blood. We observed that different types of CNTs did not show any cytotoxic effect on these cells.

This finding gains particular value towards the biomedical applications of functionalized CNTs.

Future perspective

To date, CNTs have been considered one of the most important new tools for therapeutic and diagnostic applications in pharmacological and medical fields. CNTs act as exogenous agents with the potential capacity to stimulate the immune system of receiving organisms. In the last years emerging data showing the impact of different functionalized CNTs *in vitro* and in animal model were presented. In the future, it will be of fundamental importance to address the attention to the identification and production of CNTs with functionalizations able to render them inert or endowed of immunomodulating properties on humans for translation into clinical uses. Depending on the type of functionalization, CNTs playing an inert role for the immune system can be used as delivery carriers for small drugs, biomacromolecules, such as siRNA and DNA, into target cells or organs. On the other hand, CNTs with immonumodulating effect on the immune response may be a very promising research method for the development of antiviral, antibacterial vaccines or in immunostimulatory therapy protocols.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- The toxicity issues regarding carbon nanotubes (CNTs) remain to be elucidated on different cell types treated with a wide variety of functionalized CNTs.
- Previous studies demonstrated that water dispersible CNTs did not affect the mouse immune system.
- In this study, we have focused our attention on human primary immune cells. We carried out a series of experiments using different types of functionalized CNTs on a wide variety of cell subsets.
- We showed an increase of cell percentage for natural killer cells and a decrease for B cells treated with CNTs, while T cells and monocytes were not affected.
- None of the functionalized CNTs used in this study showed significant cytotoxic effects on the cell types analyzed.
- We detected an increased expression of CD25 surface marker and IL-6 production by monocytes incubated with cationic multiwalled CNTs.
- This finding strongly encourages performing additional studies on the interaction of appropriately functionalized CNTs and the human immune system.

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