Immunomodulatory properties of carbon nanotubes are able to compensate immune function dysregulation caused by microgravity conditions

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Electronic Supplementary Information

Material and Methods

Carbon nanotubes, TEM analysis and Kaiser test

Carbon nanotubes were obtained as purified material from Nanocyl (Sambreville, Belgium; Thin MWCNTs 95+% C purity, Nanocyl 3100®, batch n° 071119). The average diameter and length were 9.5 nm and 1.5 μm, respectively. Oxidation and functionalization of the nanotubes with the ammonium groups and a fluorescent probe (FITC) were performed as previously described.[1-3] TEM was performed using a Hitachi H600 microscope with an accelerating voltage of 75 kV. The samples were deposited onto a carbon-coated copper TEM grid (Formvar/Carbon 300 Mesh; Cu from Delta Microscopies). The amount of ammonium groups per gram of nanotubes was assessed using Kaiser test as described in reference 1. CNTs were homogeneously dispersed at 1 mg/ml in sterile ultrapure water, then they were sonicated 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 cell experiment.

Simulated microgravity conditions

Random Positioning Machine (RPM) is able to simulate microgravity very close to real spaceflight conditions (experiments carried out in space are from 1×10^{-6} to 1×10^{-4}xg, whereas the simulations with RPM are in the order of 1×10^{-3}xg). This apparatus has two rotating frames, driven by two separate motors. It rotates in such a way as to simulate weightlessness by removing the effects of gravity in any specific direction. As the rotation is autonomous and with random speeds and directions, it is termed Random Positioning Machine.

The Random Positioning Machine used in this work was developed by Fokker Space (Leiden, The Netherlands). It was located in a 37°C room and it was under the control of a computer with a dedicated program (RPM Control Software 14A, developed by Fokker Space, Leiden, The Netherlands). The rotation velocity of the frames was $60°xs^{-1}$. PBMCs and T lymphocytes were incubated under microgravity conditions for 24 h in 2.5 ml cryotubes (1×10^{6} cells/ml). Control cell cultures were installed in the basement of the RPM.

Cell cultures and isolation of human primary T lymphocytes

Experiments were performed using both PBMCs and isolated T lymphocytes. In both cases cells were obtained from informed healthy male donors (25-45 years old). At least three different donors were enrolled for each experiment. Cell separation was performed immediately after blood withdrawing. PBMCs were isolated from fresh heparinized blood by FicollePaque PLUS (GE Healthcare) density gradient centrifugation. For each experiment, cells were suspended at the concentration of $1x10^{6}$/ml in fresh medium. Cell viability was evaluated through a Trypan Blue exclusion test and cells were maintained in RPMI 1640 and supplemented with 10% (v/v) heat-inactivated FBS, 20 mM HEPES, 10 ml/L penicillin/streptomycin at 37°C.

T lymphocytes were purified from PBMCs using high affinity CD3+ T-cell enrichment columns (R&D Systems, Minneapolis; MN). Columns allow purification of human T cells via high affinity negative selection. Total CD3+ cell recovery ranges between 37% and 54% and the purity of recovered cells ranges

between 87% and 95%. Cell suspensions were stored for at least 12 h at 37°C to allow recovery from stress due to isolation procedure. Cells were suspended at the concentration of $1x10^6$/ml in fresh RPMI medium.

Uptake experiments

PBMCs were incubated in 2.5 ml cryotubes (1×10^6 cells/ml) with increasing amounts of OX-MWCNT-FITC (1, 10 and 100 μg/ml) for 24 h in microgravity or static conditions. Cells were then washed in phosphate buffered saline (PBS), centrifuged and stained with fluorochrome-conjugated monoclonal antibodies to identify major immune cell populations according to the expression of specific cell surface markers (clusters of differentiation [CD]). Phycoerythrin (PE)-, peridinin chlorophyll protein (PerCP)-, or allophycocyanin (APC)-conjugated anti-CD3 (SK7 clone), anti-CD14 (Mf9 clone), anti-CD25 (2A3 clone) were purchased from BD Biosciences (Mountain View, CA, USA). Flow cytometry measurements were performed using a FACSCalibur® (BD Biosciences). Ten-thousand and fifty-thousand events were collected.

Activation marker assays

Human primary cells were cultured in the presence or in the absence of *f*-MWCNTs, concanavalin A (ConA; 10 μg/ml) or bacterial endotoxin lipopolysaccharides (LPS; 1 μg/ml). ConA and LPS were purchased from Sigma-Aldrich. After 24 h incubation, cells were centrifuged and stained to identify immune cell populations of monocytes and T lymphocyte subpopulations and to analyze activation marker expression. Fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, peridinin chlorophyll protein (PerCP)-, or allophycocyanin (APC)- conjugated anti-CD3 (SK7 clone), anti-CD14 (Mf9 clone), anti-CD69 (L78 clone), anti-CD25 (2A3 clone), anti-CD4 (SK3 clone) and anti-CD8 (SK1 clone) were purchased from BD Biosciences (Mountain View, CA, USA). Staining was performed in the dark for 20 min. After washing, cells were then analyzed by flow cytometry.

Multiplex cytokine analysis

Cell culture supernatants from PBMCs and isolated T lymphocytes were used to quantify the production of cytokines using a MILLIPLEX MAP 5-plex Cytokine Kit (HCYTOMAG-60K -05, Millipore, Billerica, MA), according to manufacturer's protocol. The following human cytokines were measured: TNFα, IL6, IL10, IL13, IFNγ and IL2. Briefly, supernatants were centrifuged for 10 min to remove debris and 25 ml were added to 25 ml of assay buffer. Then 25 ml of magnetic beads coated with specific antibodies were added to this solution and incubated for 2 h under shaking. At the end of the incubation, the plate was washed twice in buffer and incubated for 1 h with 25 ml of a secondary biotinylated antibody at room temperature. Then, the plate was incubated for 30 min with Streptavidine Phycoerythrin, washed twice, and incubated with 150 ml of sheath fluid for 5 min. The plate was observed immediately on a Luminex_ 100_/200_platform (Luminex Corporation) with xPONENT 3.1 software. Standard curves for each cytokine were generated (in duplicate) by using the supplied reference cytokine concentrations. Cytokine/chemokine concentrations in the samples were determined with a 5-parameter logistic curve. Final concentrations were calculated from the mean fluorescence intensity and expressed in pg/ml. The assay was performed in a 96-

well plate, using all the assay components provided in the kit. All incubation steps were performed at room temperature and in the dark.

Statistical analysis

Statistical analyses were performed using Student's t-test. Data indicated with * and ** were considered statistically significant (p value *< 0.05; **<0.01). Data are presented as mean ± SD (n = 3). Incubations were performed in triplicate and experiments were carried out separately in cells from at least 3 different donors.

References

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SUPPORTING FIGURES

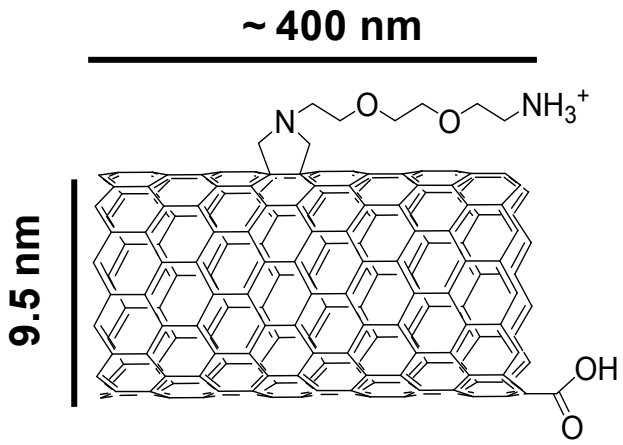

Figure S1. Structure of multi-walled carbon nanotubes functionalized by oxidation and 1,3-dipolar cycloaddition (OX-MWCNT-NH_3^+).

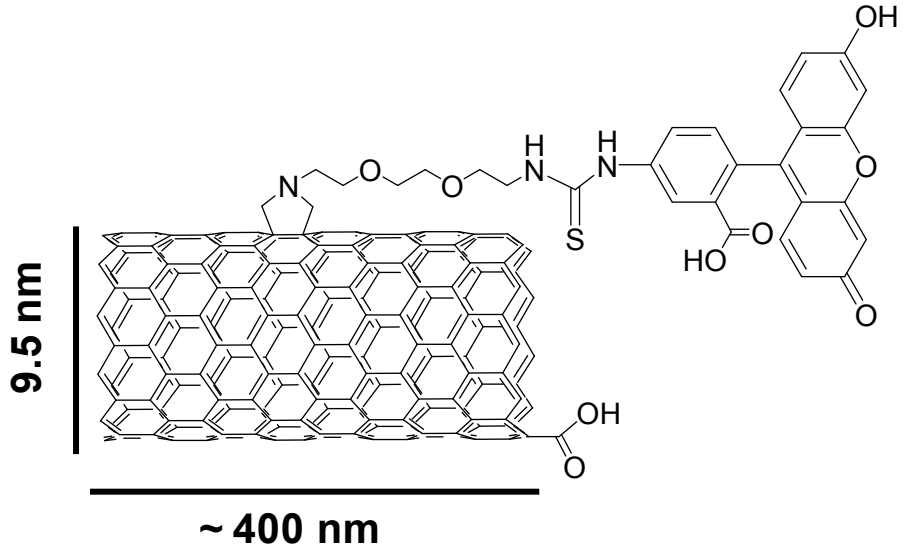

Figure S2. Structure of multi-walled carbon nanotubes functionalized with fluorescein isothiocyanate (OX-MWCNT-FITC).

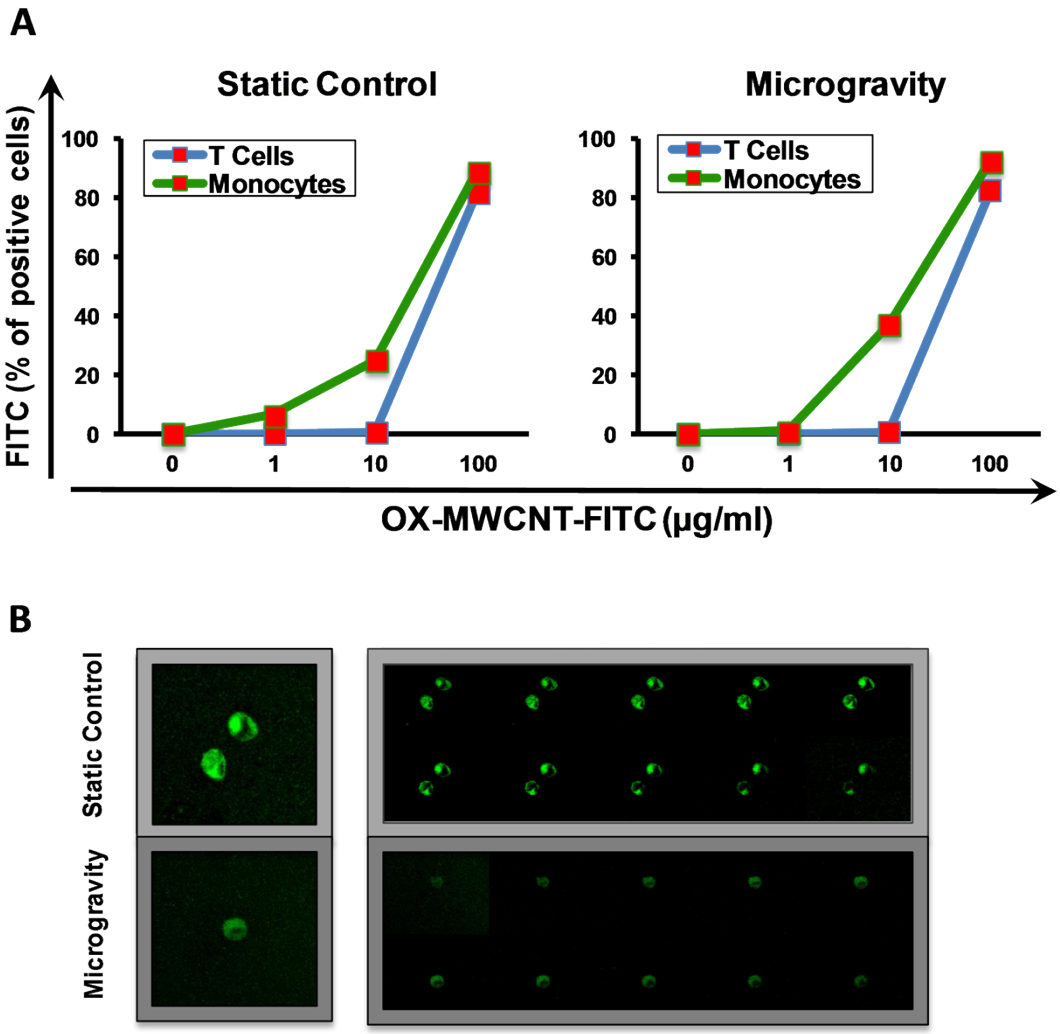

Figure S3. Uptake of OX-MWCNT-FITC by human primary immune cells. A) Peripheral blood mononuclear cells (PBMCs) were either left untreated or incubated for 24 h with 1, 10 and 100 µg/ml of OX-MWCNT-FITC. Uptake of increasing doses of fluorescent CNTs was investigated into T cells (CD3+) and monocytes (CD14+) after 24 h incubation in static controls on the left or in microgravity on the right. The highest uptake was detected at 100 µg/ml concentration in both cell types and conditions. B) *f*-CNT uptake was also assessed by confocal microscopy. PBMCs were incubated with 100 µg/ml of *f*-CNTs for 24 h and visualized by a Microradiance Nikon Eclipsa from Biorad (40 X). Microgravity conditions (bottom) were compared with static controls (top). Cells on left image are reported on the right as consecutive plans from the top to the bottom showing the presence of *f*-CNTs into the cytoplasm. Experiments were repeated three times with similar results.