Carbon nanotube scaffolds instruct human dendritic cells: modulating immune responses by contacts at the nanoscale

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Methods

MWCNT preparation

MWCNT with a diameter of 20-30 nm (Nanostructure & Amorphous Materials, Inc.), used as received, were functionalized using1,3-dipolar cycloaddition with heptanal and sarcosine at 130 °C for 120 h in N-N' dimethylformamide (DMF), as previously described[14]. TEM analysis was performed on a Philips EM 208 TEM, using an accelerating voltage of 100Kv. About 1 µg of compound was dispersed in 1 mL of DMF, then one drop of this solution was deposited on a TEM grid (200 mesh, nickel, carbon only). TGA-Q500 (TA instruments) was used to record TGA under

N_2 following a ramp of 10°C/min from 100 to 1000°C. In the case of de-functionalized MWCNTs the material was analyzed after exposing at 350°C under N_2 atmosphere.

Fabrication of the substrate

Functionalized MWCNTs were dispersed in DMF at a concentration of 0.01 mg/mL. The solution was drop casted on a glass substrate (slide coverslips, total area 10cm^2) and left to evaporate at 100°C to obtain a density of MWCNT film of $7x10^{-5}$ mg/mm^2. Finally the MWCNT were de-functionalized by an heating treatment at 350°C under nitrogen atmosphere. The integrity and the homogeneity of the scaffold was evaluated by SEM analysis.

Amorphous Carbon (AC) was purchased from Sigma-Aldrich (Carbon nanopowder ≥99%). The material was treated in the exact way as previously described for the functionalization of MWCNTs using AC as scaffold (see also[22]).

Atomic Force Microscopy (AFM)

AFM was used to acquire high resolution images of amorphous carbon on glass slides. Differently from other high resolution microscopy techniques AFM provides real three-dimensional reconstruction of sample surface, thus surface micro- or nano- roughness is determinable. Images were acquired using a commercially available microscope (NT-MDT Solver PRO, Moscow – Russia). Measurements were carried out in air at room temperature working in dynamic mode. Cantilevers, characterized by a resonant frequency of about 90 kHz (NSG03 tips from NT-MDT Co. - Moscow – Russia) were used working at low oscillation amplitudes with half free-amplitude set-point (512×512 pixels acquired at 1 lines/second scan speed).

Cells

DCs were generated from human monocytes of healthy donors as previously described[41]. Briefly, anti-CD14+ monocytes were positively sorted by magnetic microbeads (Miltenyi Biotec). Monocytes (at the density of $1x10^6$ cell/ml) were cultured for 6 days in medium (complete RPMI 10% FCS) supplemented with GM-CSF (1000U/mL, Labogen) and IL-4 (1000U/mL, Labogen), in the presence or absence (tissue culture polystyrene) of MWCNTs; then DCs were activated by 24 hours of incubation with LPS (1µg/mL, Sigma-Aldrich). The DC-MWCNT cultures were performed in 6 well plates (Grainer), each well containing a MWCNT slide. To determine vitality and adhesion the cultures were observed daily by phase contrast microscope (Zeiss); in this way, we distinguished cells adhering to MWCNT slides and cells floating in suspension over the MWCNTs. The floating cells were recovered every day from a different well, starting from day 1 of culture until day 6, counted in a Thoma chamber by trypan blue exclusion; at the same time also adhering cells were detached from MWCNTs by 15 min incubation with PBS/EDTA 2 mM and counted as above. The floating cells were plated again on a new MWCNT slide and 24 hours later the cells floating over this new MWCNT slide were harvested and counted.

In all experiments three population of cells were analysed: DCs differentiated and cultured in the absence of MWCNTs (controls), DCs cultured in the presence of MWCNTs, in this case the above mentioned two populations were distinguished: floating and adherent.

DC phenotype

DC phenotype was analyzed by Flow cytometry, we used a 4 colour EpicsXL cytometer (Beckman-Coulter), equipped with Expo 32 software. Cell surface markers were labeled with monoclonal antibodies (all from Immunotech) directed against the following antigens (the tags are given in parentheses): CD80 (FITC), CD86 (PE), HLA-DR (ECD), CD83 (PC5). Cell vitality was tested with propidium iodide (PI, Molecular Probes). The cells were labeled in PBS with 1% FCS for 15 min at room temperature (RT), washed twice and immediately analyzed. For each test at least 10000 events were acquired.

DC endocytosis test

Endocytosis in resting or LPS activated DCs was examined as described in a previous work[41]. Briefly, $1x10^5$ cells were re-suspended in RPMI 1640 with 10% FCS and equilibrated at 37°C or 0°C (negative control) for 10 min, then cells were pulsed with FITC-conjugated dextran (1 mg/ml, 40,000 Da, Molecular Probes) for 45 min. Cells were washed 4 times with cold PBS and analyzed by flow cytometry; PI was used to exclude dead cells. The labeling of cells pulsed at 0°C was subtracted from that of cells pulsed at 37°C to measure the endocytosed dextran.

Mixed lymphocyte reaction (MLR)

CD4+ T cells were negatively selected from peripheral blood mononuclear cells (PBMCs) using the T-cell isolation kit II from Miltenyi Biotec. Mixed Lymphocyte Reaction (MLR) was performed in 96 well U bottom plates (Nunc). $1x10^5$ CD4+ T cells were cultured for 5 days in RPMI with 10% FCS together with $1x10^4$- $1x\ 10^3$-$1x\ 10^2$ allogeneic DCs differentiated as usual or adherent to MWCNTs. Experiments were conducted in quadruplicate. At day 5, the proliferative response was measured by [3H]-Thymidine ([3H]-Thy, 1µCi/ml, Amersham) incorporation test. [3H]-Thy was added for the last 8 h of culture. Plates were then harvested (TomtecMacIII) on glass fiber filters (Perkin Elmer) and [3H]-Thy uptake was measured by liquid scintillation in a Microbeta 1450 Trimux counter (Wallac), proliferative response is reported as mean value of counts per minute (CPM).

RNA Extraction and Quantitative Real-Time RT/PCR

Total RNA was extracted using Quiazol (Quiagen). Real-time polymerase chain reaction (PCR) was performed with an ABI Prism 7900HT Sequence Detection System (Applied Biosystems), according to the manufacturer's instructions. All PCR amplifications were performed by MicroAmp optical 96well reaction plate with TaqMan Universal Master Mix and with Assay-on-Demand

(Applied Biosystems). Each assay was carried out in duplicate and included a no-template sample as negative control. Relative expression of mRNA levels was determined by comparing experimental levels with a standard curve generated with serial dilution of cDNA obtained from human PBMCs. β-Actin was used as a housekeeping gene for normalization. In each sample was evaluated mRNA level of: IL23p19; IL12p35; IL10; IL6;TNFα.

Video-microscopy

DCs were cultured in the presence of MWCNT coated coverslips and repeatedly imaged for one week during the differentiation process.

Time-lapse video-microscopy experiments run on an inverted Leica AM6000 microscope, equipped with a fully motorized stage and a micro-incubator with temperature, CO_2 and humidity control (Pecon, Italy).

In each recording session images were acquired with a long working-distance objective (40x 0.6NA, Leica Microsystem), every 30 s for 10 min, using oblique-illumination contrast. Image montage and video editing was performed with Fiji software.[1]

Confocal analysis of actin cytoskeleton

DCs cultured as described and adherent to MWCNTs were washed with PBS, fixed with 4% phosphate buffered paraformaldehyde for 10 min RT and permeabilized 30 min with 0.05% saponin (ICN Biomedicals) and 2% BSA (Sigma-Aldrich) in PBS; then cells were labeled with FITC conjugated phalloidin (0,1 μg/ml; Sigma) together with PE conjugated mouse monoclonal anti-CD11c (diluted 1:50 in PBS/0.05% saponin/2% BSA; BD Pharmingen) for 1 hour, RT in the dark. As a control, DCs cultured without MWNTs were plated on poli-L-lysine coated glass coverslip, fixed and stained as above. Confocal images were acquired in a Leica TCS SP5 microscope (Leica Microsystems, Mannheim, Germany) equipped with a He/Ne/Ar laser source, using a Leica Plan Apo x 63/1.40 NA oil immersion objective. Series of optical sections (1,024x1,024 pixels each;

pixel size 200 nm x 200 nm) were taken at intervals of 0.35 µm. Confocal images were processed using ImageJ 3D deconvolution software (National Institutes of Health [NIH], Bethesda, MD).

Transcriptional analysis

DCs were cultured as described. Floating DCs and MWCNTs attached ones were treated with TRIzol followed by RNA extractionand cDNA was synthesized from 1 mg of total RNA by reverse transcription using the Agilent kit (Quick Amp Labeling, Agilent), incorporating Cy3-dCTP or Cy5-dCTP (Quick Amp Labeling, Agilent) into each sampleof cDNA to be compared. The printed microarrays were provided by Agilent (*Agilent*-014850 Whole *Human* Genome Microarray *4x44K* Microarray kit). The analysis was performed on 4 different donors.

Microarray data have been submitted to the Array Express repository (experiment ID E-MEXP-3560, www.ebi.ac.uk/microarray-as/ae)

Differential expression, annotation, and visualization

Differential expression ratios were obtained from the average of the 4 different microarray experiments. To allow a direct comparison of the data obtained from each experiment, labeled cDNA corresponding to RNA from MWCNTs attached DCs was co-hybridized with RNA isolated from floating DCs. Microarrays were scanned with a GenePix 4000B scanner (Axon Instruments, Union City, CA). GenePix Pro 4.0 analysis software (Axon Instruments) was used to further process the data according to the manufacturer's instructions. Data analysis was accomplished using LIMMA Bioconductor package on R. Locally weighed linear regression (LOWESS) analysis was performed. Genes with $p<0.05$ were considered to be significantly differentially expressed with respect to the treatment condition. Data were expressed as Fold change in logaritmic scale (log_2FC). Differentially expressed gene (DEG) lists from the various data sets were uploaded in the Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov) to perform functional annotation. The up-regulated and down-regulated gene lists were annotated

separately. Gene Ontology enrichment over Biological Process was performed using the BINGO plug-in for the Cytoscape Desktop.

Pathway analysis

Pathway analysis was performed using the procedure by Beltrame et al (2009). Briefly, the Fisher's Exact Test was run over paired case-control ratios (attached vs floating DCs) calculated from the normalized data, using the public pathway set. p-values were then transformed into pathway signatures, either signed Binary Enrichment Factors (sBEFs) or Pathway Enrichment Factors (PEFs). sBEFs or PEFs were clustered, using boostrapping with support trees.[2] This computation was carried out with the TIGR Multiexperiment Viewer (TmeV) 4.4. Affected genes in the different pathway were calculated from the standard deviation from the median on all the $\log_2$ fold changes.

Transmission and scanning electron microscopy

DCs were grown for 7 days onto sterile glass slides coated with carbon nanotubes, as described. Then, the medium was removed and the adherent cells fixed in Karnowski's fixation fluid (PBS, 7% paraformaldehyde and 2.5% glutaraldehyde), post-fixed in 1% osmium tetroxide and embedded in Epon 812 epoxy-resin. After this treatment, the resin-embedded specimens were detached from glass slides by immersion in liquid nitrogen and cut with a LKB Nova ultramicrotome. Ultrathin sections were stained with uranyl acetate and alkaline bismuth subnitrate and examined under a JEM 1010 electron microscope (Jeol, Tokyo, Japan) at 80 kV.

References

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