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

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Spaceflights lead to dysregulation of the immune cell functionality affecting the expression of activation markers and cytokine production. Short oxidized multi-walled carbon nanotubes functionalized by 1,3-dipolar cycloaddition have been reported to activate immune cells. In this Communication we have performed surface marker assays and multiplex ELISA on primary monocytes and T cells under microgravity. We have discovered that carbon nanotubes, through their immunostimulatory properties, are able to fight spaceflight immune system dysregulations.

Gravity is the force of attraction by which terrestrial bodies tend to fall toward the Earth. In space, living organisms are confronted with two important factors: microgravity and cosmic radiation. A microgravity environment imparts to an object a lower acceleration compared to that produced by the Earth on its surface. Experiments conducted by American, Russian, and European investigators, in dedicated space missions as well as in simulations on Earth, have shown that mammalian cells are sensitive to gravitational changes.[1–8] It is well known that the constant influence of weightlessness leads to several modifications of many physiological cellular processes, such as proliferation, differentiation, growth, signal transduction, cytoskeletal architecture, motility and gene expression.[1–4] Moreover, immune cells are severely affected by microgravity.[5] T lymphocyte functions were found altered in more than 50% of crew members in space.[6] A severe inhibition of T cell activation under real and simulated microgravity conditions has been extensively demonstrated. Hashemi *et al.* reported a down regulation of CD25 and CD69 cell membrane activation markers in T cells after 24 hours under microgravity conditions.[7] These findings were recently confirmed by experiments on US Astronauts onboard the Space Shuttle.[8] CD25 and CD69 are important markers in the T cell mediated immune response. CD25 (alpha chain of the IL-2 receptor) is a late activation antigen. Activation mediated by the T-cell receptor (TCR) and costimulatory molecules induces an up-regulation of CD25 in T cells making them highly sensitive to IL-2, whereas CD69, a member of the C-type lectin superfamily (Leu-23), is one of the earliest cell surface antigens expressed by T cells following activation.

We reported that functionalized multi-walled carbon nanotubes (f-MWCNTs) lead to an up-regulation of CD25 and CD69 marker expression in human primary immune cells, in particular in monocytes.[9] Very recently, through a whole genome wide study we proposed functionalized CNTs (f-CNTs) as immunomodulator systems showing their potential as immune activators.[10] We would like to highlight that in contrast to other types of CNTs, different in functionalization and shape investigated in immune cells,[9–12] we found that oxidized MWCNTs, further functionalized by 1,3-dipolar cycloaddition, can act as immunomodulators.[9] No data are present in the literature regarding the interaction of CNTs and immune cells under microgravity conditions. Encouraged by our recent results, we wanted to evaluate the possibility of taking advantage of f-CNT immunostimulatory properties against spaceflight dysregulation of immune functions.

The high cost of experiments on board of spacecraft and space station facilities and the limited number of doubling experiments do not allow scientists to give continuity to the studies in real microgravity outside the Earth. Currently, thanks to different and advanced facilities, it is possible to carry out studies in microgravity simulating in part the spaceflight conditions. In this work, a tridimensional clinostat or Random Positioning Machine (RPM) was used to simulate microgravity

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(M; 0 × *g*) to evaluate whether f-CNTs are able to compensate immune microgravity induced dysregulation. Static control cell cultures were installed in the basement of the RPM.

We first assessed the possible impact of microgravity on the functionalization of CNTs by transmission electron microscopy (TEM) and the Kaiser test. To assess whether microgravity affects CNT uptake on primary human T cells and monocytes, we studied their internalization in microgravity by flow cytometry and confocal microscopy. Taking into consideration the results reported on activation markers by Hashemi *et al.*,[7] we then focused on CD25 expression on monocytes and T cells through peripheral blood mononuclear cell (PBMC) analysis and CD69, both on T cells present on PBMCs and isolated T lymphocytes, T-helper cells (CD4+) and cytotoxic T cells (CD8+). To gain a larger picture about the CNT effect on immune microgravity dysregulation, we also performed a multiplex cytokine assay on PBMCs (TNFα, IL6, IL10, IL13, IFNγ, and IL2) and on isolated T lymphocytes (IFNγ and IL2).

By screening a series of functionalized carbon nanotubes, we previously obtained the major effect on immune activation treating human primary cells with oxidized MWCNTs subsequently functionalized by 1,3-dipolar cycloaddition of azomethine ylides.[9,10] The present study was carried out with the same type of nanotube in terms of functionalization and shape (Fig. S1†). Characterization and functionalization methods of the MWCNTs have been previously described by our group.[13] Kawanami *et al.* focused on the effect of microgravity on CNT synthesis,[14] but no data are reported in the literature about the possible impact of microgravity on f-CNTs. We first assessed by TEM and the Kaiser test that MWCNTs (OX-MWCNT-NH_3^+) were not affected in their functionalization in microgravity. TEM images do not display differences in the morphology of the tubes under microgravity (Fig. 1), while the Kaiser test gave approximately the same values (within the uncertainty of this type of measurement) in terms of the amount of ammonium groups before (~40 μmol g^{-1}) and after (~50 μmol g^{-1}) treatment under microgravity. We previously showed a clear uptake on monocytes and T cells by the same type of nanotube.[9] To make sure that the equivalent internalization can be possible under microgravity conditions, we used the same fluorescently labeled nanotubes (Fig. S2†). As expected, data from flow cytometry showed a dose-dependent uptake of f-CNTs after 24 h. Interestingly, we did not detect significant difference in the internalization at 0 × *g* (Fig. S3A†). We confirmed our data at 100 μg ml^{-1} concentration for both the samples treated in static control or in microgravity by confocal microscopy (Fig. S3B†). The absence of difference in cell uptake prompted us to go ahead in understanding the potential of carbon nanotubes to modulate the immune spaceflight effects. Cellular uptake results led us to choose a working concentration of 100 μg ml^{-1} of f-CNTs. We fixed the time of incubation to be 24 h, the best time point to assess a possible immune response on primary human cells as used in the space mission and microgravity experiment on activation markers.[7]

To evaluate the ability of activator MWCNTs to counteract spaceflight immune suppression,[9] PBMCs were left untreated for 24 h or incubated with 100 μg ml^{-1} of OX-MWCNT-NH_3^+ in static controls and under microgravity conditions. Lipopolysaccharides (LPS) and concanavalin A (ConA), due to their well-known activation properties, were used as positive controls for monocytes (CD14+) and T cells (CD3+) respectively (Fig. 2). In addition to our previous data obtained under static conditions,[9] we here tested a possible synergic action of f-CNTs in the presence of traditional activators (LPS or ConA + OX-MWCNT-NH_3^+ samples). We first performed a functionality assay on CD14+ monocytes focusing on the CD25 marker (Fig. 2A). f-CNTs led to an increase of CD25 expression on monocytes, both in static controls and under microgravity conditions (M). This finding suggests that the ability of OX-MWCNT-NH_3^+ to act as monocyte activators[9,10] is not affected under microgravity conditions. We then evaluated CD25 expression on CD3+ T lymphocytes (Fig. 2B). f-CNTs showed a synergic effect with ConA on CD25 expression in static controls. In microgravity we noticed a down-regulation of CD25 on ConA activated samples due to the immune suppression as already reported.[7] Interestingly, in microgravity, samples treated with f-CNTs together with ConA did not show the down-regulation of CD25. Since a down-regulation of the CD69 early activation marker during clinorotation in spaceflight was also previously shown,[7] we decided to evaluate CD69 expression on T lymphocytes (Fig. 2C). Our results demonstrate again that f-CNTs have a clear synergic effect with ConA in static controls. The nanotubes counteract the down-regulation of CD69 due to the microgravity conditions. These findings show that the functional effect on T cells of f-CNTs in the presence of ConA is not deeply affected by microgravity in comparison to the treatment with only the activator. It is well known that the activation of T cells by ConA is inhibited under the 0 × *g* condition.[17]

The action of ConA on T cells is mediated by the G protein that induces phospholipase C and then triggers the activation cascade of IL2. Patching and capping of the ConA receptor are slightly retarded in microgravity. This action dramatically reduces IL2 secretion, and consequently the T cell activation.[17]

To thoroughly evaluate the CNT effect on CD69 expression in T cells in the absence of other cells present in PBMCs, T lymphocytes were isolated and incubated for 24 h with 100 μg ml^{-1} of f-MWCNTs alone or in the presence of ConA, and compared to untreated control cells, CD4+ and CD8+ subpopulations were analyzed by flow cytometry (Fig. 2D and E). We

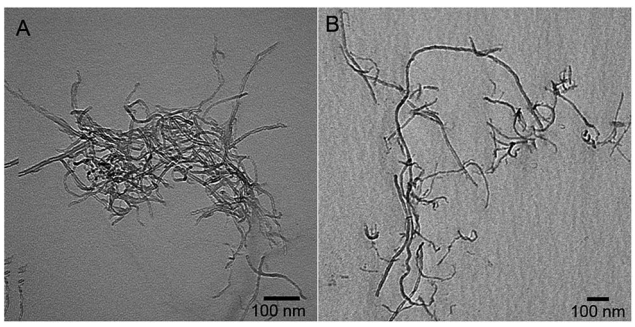

Fig. 1 TEM images of OX-MWCNT-NH_3^+ before (A) and after microgravity treatment (B).

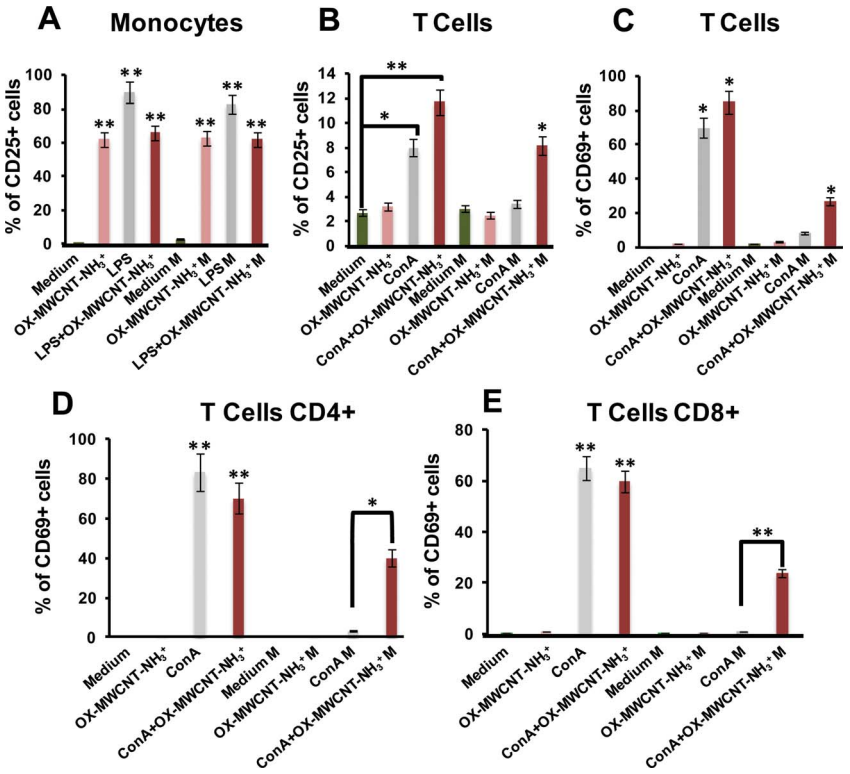

Fig. 2 Activation marker assay. Microgravity (M) was compared with static controls. Peripheral blood mononuclear cells and isolated human T lymphocytes were left untreated (medium) for 24 h or incubated with 100 μg ml^{-1} OX-MWCNT-NH_3^+ alone or in the presence of cell activators (LPS or ConA). (A) Monocytes: CD25 expression was assessed by flow cytometry on CD14+ monocytes present on PBMCs. LPS (2 μg ml^{-1}) was used as positive control. (B and C) T cells: CD25 (B) and CD69 (C) expression was assessed by flow cytometry on CD3+ T cells present on PBMCs. ConA (10 μg ml^{-1}) was used as positive control. (D and E) Isolated T lymphocytes from PBMCs: staining for CD4+ (D) was performed to identify T-helper cells on isolated T lymphocytes; staining of CD8+ (E) was performed to identify cytotoxic T cells. Difference in statistical significance (Student's *t*-test) is indicated by * = $p < 0.05$ and ** = $p < 0.01$. Bars indicate the compared samples under different conditions; no-asterisk marked samples are compared to controls (medium).

confirmed that f-CNTs with ConA in both subpopulations are able to contrast the effect of microgravity. Moreover, these results clearly show that f-CNTs can act on T lymphocytes even in the absence of monocyte interactions.

The altered immune cell functionalities have been reported in different studies looking also to the cytokine production.[8,15–19] Crucian *et al.* showed on Space Shuttle crew members that post-flight monocytes significantly reduced the production of IL-6, TNFα, and IL-10.[15] This reduction may impact overall immunocompetence. More recently, during the flight, a down-regulation of IFNγ, TNFα, IL10, IL4 and IL6 on whole blood samples of crew members after phorbol 12-myristate 13-acetate and ionomycin treatment has been shown.[8] The authors showed that a mitogenic stimulation led to a lower production of IFNγ and IL10, and to an up-regulation of TNFα and IL2.[8] Moreover, the strongly reduced T lymphocyte activation under spaceflight conditions has been reported not only for the CD25 marker but also for the production of IL2 after ConA stimulation.[17] To further confirm the ability of f-CNTs to act as immunomodulator systems and better investigate their interaction with immune cells under microgravity, we performed an assay on a wide range of cytokines. The samples were activated with ConA or left untreated in the presence of f-CNTs or without them. Fig. 3A illustrates the cytokine production by multiplex ELISA on PBMC supernatants. We confirmed the activator action of f-MWCNTs for the classical innate cytokines, TNFα and IL6, as previously reported,[9,20] and we found the synergic effect of f-CNTs with ConA in static controls and under microgravity. Interestingly, in microgravity, also f-CNTs alone are able to up-regulate the IL6 production as they do in static controls. For the other investigated cytokines (IL10, IL13, IFNγ and IL2) we found that f-CNTs alone did not boost their production but they seem to act in concert with ConA potentiating its stimulation both in static controls and under the $0 \times g$ condition. To better understand the interaction of f-MWCNTs with T lymphocytes, the major population affected by microgravity, we assessed the release of two classical adaptive cytokines, IFNγ and IL2, on the T isolated cell population (Fig. 3B). Cell functionality was inhibited by microgravity even in the presence of ConA stimulation. In static samples treated with ConA and nanotubes we found instead a statistically significant production of IFNγ and IL2 *versus* medium. Our experiments show that the effect of nanotubes appears to be particularly linked to an up-stimulation of the molecular effectors involved in the IL2 pathway. We assume that f-CNTs could promote the patching and capping of the ConA receptor. This action, together with IL2

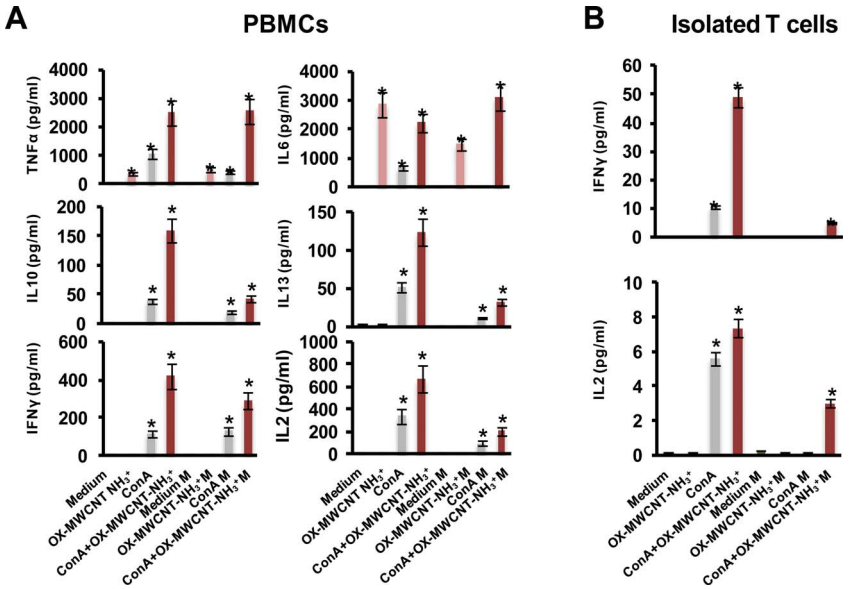

Fig. 3 Cytokine secretion assay. Cytokine production was assessed by multiplex ELISA on PBMCs (A) and on isolated human T lymphocytes (B). Microgravity (M) was compared with static controls. Peripheral blood mononuclear cells and isolated T cells were left untreated (medium) for 24 h or incubated with 100 μg ml^{-1} MWCNTs, alone or in addition with 10 μg ml^{-1} ConA. Values are expressed in pg ml^{-1}. The statistical significance of differences between the samples *versus* medium controls was calculated by Student's *t*-test (*, $p < 0.05$).

pathway stimulation, can explain the synergic effect between f-CNTs and ConA in 0 × *g*.

These findings, together with the surface marker data, open new perspectives on the capability of carbon nanotubes to act as immunomodulators proving this property by fighting immune function dysregulation under microgravity conditions, especially for T lymphocytes.

In conclusion, carbon nanotubes had attracted over the last few years huge interest from the scientific communities in a wide variety of biomedical applications.[21] Among other purposes, we and others focused on their possible relevance in biomedicine as drug carriers or contrast agents.[12,13,22,23]

Encouraged by our recent studies on functionalized carbon nanotubes we wanted to confirm the potential of specific functionalized carbon nanotubes as immunomodulators.[9,10] NASA is working to achieve the goal of returning humans to Moon by 2020 and put footprints on Mars by 2024 (http://www.nasa.gov.html last access 12/12/2013). Even if this is a dreaming scenario for humanity, the immune system suppression related to spaceflight should be considered more thoroughly before taking into consideration long space voyages. In this context nanotechnology may bring additional help for the future of humans on space missions.[24] Based on the results of this study together with further future possible investigations, we can envision an immune system pretreatment of space crew members with functionalized CNTs administered intravenously, once all issues about clinical applications of nanotubes are solved. We aim at reinforcing the concept that functionalized carbon nanotubes are able to stimulate immune cells having very interesting broad future applications in immunotherapy, as vaccine adjuvants and, with data here shown, as possible fighters to contrast spaceflight immune cell dysregulation.

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