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Abstract
Addition of polydispersed acid functionalised single-walled carbon nanotubes (AF-SWCNTs) significantly suppressed alloimmune cytotoxic T cell (CTL) response generated in a mixed lymphocyte reaction (MLR) between spleen cells from C57BL/6 (H-2b) and BALB/c (H-2d) mice. AF-SWCNTs treatment also decreased CD69 expression, enhanced apoptotic response in T cells and reduced significantly the recovery of live CD4⁺ and CD8⁺ T cells from MLR cultures. A two to threefold increase was noticed in the binding/uptake of AF-SWCNTs by T cells in MLR cultures as compared with control cultured T cells. Confocal microscopy confirmed the internalization of AF-SWCNTs by live CD8⁺ T cells in MLR cultures. Administration of AF-SWCNTs suppressed the generation of anti-P815 CTL response in C57BL/6 mice and the recovery of T-cell populations from the spleens. The results demonstrate a suppressive effect of AF-SWCNTs on CTL response and provide an insight into the mechanism of this suppression.

Keywords: cytotoxic T cells, nanoparticles, apoptosis, CD69, confocal microscopy

Introduction
Due to their unique structures and remarkable electrical and mechanical properties, carbon nanotubes (CNTs) are finding wide applications in electronics, sensing, gas storage, aerospace, field-emission devices, catalytic supports, biomedical engineering and medical chemistry (Baughman et al. 2002; Bianco et al. 2003; Pantarotto et al. 2003). Structurally, single-walled carbon nanotubes (SWCNTs) resemble rolled up tubes of graphite sheet of sp² hybridised carbon atoms, having a diameter of about 1 nm (Lam et al. 2006). The nanodimension of SWCNTs coupled with high aspect ratio make them a suitable candidate for new class of transporting vehicles for high capacity of loading of ligands for therapeutic purposes (Pantarotto et al. 2004; Kam & Dai 2006; Delogu et al. 2009). Increasing potential applications of SWCNTs have also enhanced chances of environmental and occupational exposure to CNTs and necessitate the study of potential toxic effects of these materials, as they are easily inhaled and become airborne due to low density and small size (Cui et al. 2010).

Several studies on the toxicity of CNTs have been reported in literature. Lam et al. (2004) tested a variety of SWCNT samples with varying amounts of metal impurities and concluded that all SWCNT preparations induced dose-dependent lung granulomas in mice. Warheit et al. (2004) reported a mild and transient pulmonary inflammatory response in rats instilled intratracheally with SWCNTs, with subsequent development of multifocal granulomas in the lungs. Shvedova et al. (2005) found that the intratracheal instillation of pure SWCNTs resulted in granulomas, lung fibrosis and a significant elevation in markers of toxicity in broncho-alveolar lavage (BAL) fluid, and concluded that SWCNTs exerted greater toxicity on a mass basis than crystalline silica. The acid functionalised SWCNTs (AF-SWCNTs) that are de-bundled and dispersed easily in aqueous solution were found to be highly toxic to the mouse lung epithelial cell line LA4 and induced strong pulmonary inflammation in mice (Saxena et al. 2007). Tong et al. (2009) have shown that AF-SWCNTs instilled intratracheally could produce acute toxic effects in heart, suggesting that the nanotubes could traverse lung and reach heart. Effect of CNTs on immune system is not well documented and a clear consensus on the effect has yet to emerge. An immunosuppressive effect of SWCNTs leading to decreased bacterial clearance has been demonstrated (Shvedova et al. 2008). Bottini et al. (2006) showed that multi-walled CNTs induced apoptosis in Jurkat human T cell line, whereas Dumortier et al. (2006) found no significant effect of functionalised CNTs on the functionality of primary immune cells. Delogu et al (2009, 2012) also found no significant toxicity...
of multi-walled CNTs on human T cells in ex vivo studies. Mitchell et al. (2009) found an indirect suppressive effect of multi-walled CNTs on T- and B-cell response in vivo. By contrast, Greco et al. (2011) have recently demonstrated enhanced T- and B-cell activation in mice inoculated with multi-walled CNTs, resulting from increased cytokine production by antigen-presenting cells (APCs). Sun et al (2011) have recently shown that the growth inhibiting effect of cytotoxic T cells (CTLs) against H23 cell line was significantly lowered by carboxylated CNTs.

In the present study, the authors have examined the effect of polydisperse AF-SWCNTs on the generation of allogeneic CTL response in vitro (mixed lymphocyte reaction (MLR)) between spleen cells of C57BL/6 and BALB/c mice), and in vivo (CTL response to P815 tumour cells administered in C57BL/6 mice). The authors have shown that exposure to AF-SWCNTs could significantly reduce the generation of CTL response in vitro as well as in vivo. A significant drop in CD69 activation marker and enhanced apoptosis of CD8+ T cells was noticed along with a significantly lower recovery of live T cells. Using fluorescence-tagged AF-SWCNTs, a substantially greater uptake of AF-SWCNTs was observed in MLR-activated T-cell populations as compared with control cultured T cells. Confocal microscopy confirmed that the nanotubes were actually internalised by CD8 T cells. The results thus show that AF-SWCNTs can interfere with CTL response and provide insight into the mechanism of this effect.

Materials and methods

Animals

Inbred 8- to 12-week-old (20–24 g body weight) male C57BL/6 (H-2b haplotype) and male BALB/c (H-2d haplotype) mice obtained from National Institute of Nutrition, Hyderabad were used in this study. Mice were bred and maintained in the animal house facility in Jawaharlal Nehru University (JNU), New Delhi under pathogen-free conditions with a 12-h light/dark cycle. Both water and food were provided ab libitum. All protocols were approved by the Institutional Animal Ethics Committee, JNU.

Cells and reagents

The P815 murine mastocytoma and YAC-1 lymphoma cell lines were maintained in RPMI-1640 containing glutamine (2 mM), HEPES buffer (25 mM), pH 7.2 obtained from Sigma-Aldrich (India) and supplemented with 2 × 10^{-5} M 2-mercaptoethanol (Sigma-Aldrich), 60 μg/ml gentamycin, 10% fetal bovine serum (FBS) (Hyclone, South Logan, UT, USA), hereafter called the complete medium. All monoclonal antibodies (MAbs) against mouse CD3ε (fluorosothiocynate (FITC) or allophycocyanin (APC) conjugated), CD4 (FITC conjugated), CD8β (FITC or phycoerythrin (PE) conjugated), CD69 (FITC conjugated), Annexin V-FITC were obtained from BD Pharmingen (San Diego, CA, USA). DAPI used for nuclear staining was from Sigma-Aldrich.

SWCNTs used in the experiments reported in this manuscript were purchased from Sigma (catalogue no. 636797, amorphous carbon <3%), though similar results were obtained from SWCNTs procured from another source (purified HIPCO SWCNTs, ash <15%, from Carbon Nanotubes Inc., Houston, TX, USA). AF-SWCNTs were prepared by method described previously (Saxena et al. 2007). Briefly, 20 mg SWCNT were suspended in 20 ml of 1:1 mixture of concentrated HNO₃:H₂SO₄ at 20 ± 2 Psi for 3 min resulting in an internal temperature of 138–150°C. The suspensions were cooled and dialysed against water to remove acids. Resulting dilute suspension of AF-SWCNTs was lyophilised, weighed and known amounts resuspended in water. Detailed characterisation of AF-SWCNTs including elemental analysis, size and charge distribution, BET surface area and electron microscopic changes have been reported elsewhere (Saxena et al. 2007; Tong et al. 2009). In summary, particle size distributions of AF-SWCNT aqueous suspensions obtained from the zeta-sizer instrument showed that 95% of the particles were in the range between 22 and 138 nm. Mean zeta potential of AF-SWCNTs was -57.2 mV and BET surface area was reduces by one-third as compared with pristine SWCNTs. Basic tubular structure of AF-SWCNTs was intact though the sidewalls of nanotubes appeared roughened. In general, all changes were consistent with mild sulfonation/carboxylation of the nanotube sidewalls.

Control and acid functionalised CNTs suspensions were sonicated for 2 min in ice using a probe sonicator (Branson sonifier, VWR Scientific) prior to use for in vivo and in vitro studies. For chromium release cytotoxicity assays (CRA), target cells were labelled with sodium chromate (31Cr) obtained from the Board of Radiation and Isotope Technology, BARC, Mumbai.

Generation of CTL response in vitro and in vivo

In order to generate alloreceptive CTL in vitro, a MLR was performed as described previously (Saxena et al. 1982). Briefly, spleen cells (5 × 10^6/ml) from C57BL/6 mice were co-cultured with gamma-irradiated (1000 rads) spleen cells from BALB/c mice (1 × 10^6/ml) in complete medium, with or without SWCNT or AF-SWCNT for 5 days. Cells were washed, counted and their cytotoxic activity determined against P815 (H-2b) target tumour cells in a 4 h 51Cr release assay as described elsewhere (Saxena et al. 1982). For generating CTL response in vivo, P815 tumour cells (10 × 10^6) were administered i.p. to C57BL/6 mice and spleen cells isolated 11 days postimmunisation as described before (Saxena et al. 1982). Lytic units (LU)/10^7 effector cells were calculated from the E/T ratio versus percent lysis plots as described before (Cerottini & Brunner 1971; Cerottini et al. 1974). Briefly, E/T ratios corresponding to a 20% target lysis were determined from the E/T ratio versus percent lysis plots and the number of lymphocytes corresponding to this E/T ratio in the assay well was taken as 1 LU.

Immunophenotyping

Cells were stained with specific MAbs for flow cytometric analysis. Prior to staining, surface Fc receptors on cells were blocked using anti-mouse CD16/32 antibody (clone # 93, ebioscience) for 20 min on ice followed by treatment of the required MAbs. Cells stained with isotype control antibodies were used for setting flow cytometric gates. For assessing
apoptosis, cells were double stained with anti-mouse CD3-APC and Annexin V-FITC (BD Pharmingen). Each sample was fixed with 1% paraformaldehyde and analysed on a FACS-Calibur machine (BD Biosciences, Franklin Lakes, NJ, USA). Data on 10,000 live cells was accumulated and analysed using Cellquest software.

**Confocal microscopy of T cells**

Fluorescent probe (Alexa fluor 633, Molecular Probes, Carlsbad, CA, USA) was covalently attached to the COOH groups on AF-SWCNTs as previously described (Sachar & Saxena 2011; Kumari et al. 2012). Briefly, AF-SWCNTs were suspended in water and treated with 1-ethyl 3-(3-dimethylaminopropyl) carbodiimide (EDAC) and N-hydroxysuccinimide (NHS) to get a succinimidyl intermediate. The mixture was continually shaken for 2 h and dialysed extensively to remove excess NHS, EDAC and urea by-product. AF-SWCNTs thus activated were incubated with Alexa Fluor 488/633 hydrazide (Molecular Probes) in dark with continuous mixing, followed by dialysis to remove free dye. In order to study the uptake of AF-SWCNTs, MLR-activated C57BL/6 spleen cells were incubated with fluorescence-tagged AF-SWCNTs (5 μg/ml) for 24 h. Particles unbound/loosely bound to cells were removed by washing the cells two times with phosphate buffered saline (PBS). Cells were stained with anti-CD4 or anti-CD8 MAb and 7AAD. CD4^7AAD and CD8^7AAD population of cells representing live CD4^+ and CD8^+ T cells generated in MLR were sorted on a Fluorescence Activated Cell Sorter, Aria (Becton-Dickinson). These live cells were further incubated with DAPI (0.2 μg/ml) for 20 min on ice and thereafter examined under Confocal Microscope (Olympus FluoviewTM-FV1000) to assess the internalisation of fluorescence-tagged AF-SWCNTs.

**Statistical analysis**

Each experiment was repeated three to five times with reproducible results. Statistical analysis by two-way ANOVA and Student’s t-test were done using Sigmaplot and Sigmaplot software. In all experiments, p < 0.05 was considered as significant.

**Results**

**Effect of SWCNTs and AF-SWCNTs on the generation of CTL response in an MLR**

Alloimmune CTLs can be generated *in vitro* in an allogeneic MLR. In order to study the effect of CNTs on the generation of CTLs in MLR, spleen cells from C57BL/6 (H-2^b^) mice were co-cultured with gamma-irradiated BALB/c (H-2^d^) spleen cells in presence of different concentrations of SWCNTs or AF-SWCNTs, for 5 days. The H-2^b^ anti-H-2^d^ alloimmune CTL response generated in MLR was assessed by using P815 tumour target cells (H-2^d^) in a 4 h CRA of cytotoxicity. Results in Figure 1 (panel A) show that SWCNT preparation at 50, 25 or 10 μg/ml concentrations had no significant effect on the generation of CTLs in MLR. AF-SWCNT preparation however significantly lowered the CTL response at 50 and 25 μg/ml concentration, but not at 10 μg/ml concentration (panel B). LU/10⁷ effector cells were calculated from lysates versus E/T plot as described in Materials and methods. Maximum suppression of 34% was observed in presence of 50 μg/ml of AF-SWCNTs (p < 0.01).
Even though the MLR-generated CTL preparations were washed before testing their cytotoxic activity against P815 cells, some SWNCTs or AF-SWCNTs added in the MLR cultures could have been carried over to the CRA and potentially interfered with the cytotoxicity assay. To test this possibility, effect of directly adding SWCNTs and AF-SWCNTs during the CRA of CTL activity was also examined. Results in panel C of Figure 1 show that the presence of SWNCTs or AF-SWCNTs during the CRA had no significant effect on the killing of target cells by CTLs. Lower levels of MLR-generated CTL activity observed in presence of AF-SWCNTs may therefore result from actual suppression of the CTL activation process in MLR and is unlikely to be due to modulation of the effector–target interaction during the cytotoxicity assay.

Effect of SWCNTs and AF-SWCNTs on recovery of T-cell populations in an MLR

In order to assess a possible toxic effect of SWCNTs and AF-SWCNTs, recoveries of live T-cell subpopulations from control and MLR cultures were examined. Results in Table I show that the CD4⁺, CD8⁺ and CD3⁺ T cells were assessed by staining with the respective MAbs and flow cytometric analysis. Each value represents mean ± SEM of four observations (*p < 0.05, Student’s t-test) from four replicate wells; AF-SWCNTs, acid functionalised single-walled carbon nanotubes; MAb, monoclonal antibody; MLR, mixed lymphocyte reaction; SEM, standard error of mean.

Table I. Effect of SWCNTs and AF-SWCNTs on the relative recoveries of CD3, CD4 and CD8 T cells from control and MLR cultures.

<table>
<thead>
<tr>
<th>Cultured cells</th>
<th>Treatment</th>
<th>CD4⁺</th>
<th>CD8⁺</th>
<th>CD3⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (C57BL/6 spleen cells alone)</td>
<td>Control</td>
<td>15.8</td>
<td>12.6</td>
<td>29.1</td>
</tr>
<tr>
<td></td>
<td>+ SWCNT</td>
<td>16.3</td>
<td>11.6</td>
<td>27.3</td>
</tr>
<tr>
<td></td>
<td>+ AF-SWCNT</td>
<td>15.6</td>
<td>11.6</td>
<td>28.0</td>
</tr>
<tr>
<td>MLR (mixture of spleen cells from C57BL/6 + BALB/c mice)</td>
<td>Control</td>
<td>27.9</td>
<td>19.4</td>
<td>49.5</td>
</tr>
<tr>
<td></td>
<td>+ SWCNT</td>
<td>24.4</td>
<td>18.0</td>
<td>45.4</td>
</tr>
<tr>
<td></td>
<td>+ AF-SWCNT</td>
<td>21.4</td>
<td>17.0</td>
<td>41.3</td>
</tr>
</tbody>
</table>

MLR was carried out between spleen cells from C57BL/6 and BALB/c mice in presence of 50 μg/ml of SWCNTs or AF-SWCNTs. After 5 days of MLR, percentage of CD4⁺, CD8⁺ and CD3⁺ T cells were assessed by staining with the respective MAbs and flow cytometric analysis. Each value represents mean ± SEM of four observations (*p < 0.05, Student’s t-test) from four replicate wells; AF-SWCNTs, acid functionalised single-walled carbon nanotubes; MAb, monoclonal antibody; MLR, mixed lymphocyte reaction; SEM, standard error of mean.

Effect of SWCNTs and AF-SWCNTs on expression of CD69 activation marker on CD8⁺ T cells in an MLR culture. MLR was carried out between spleen cells from C57BL/6 and BALB/c mice in presence or absence of 50 μg/ml SWCNTs or AF-SWCNTs, as described in methods. After 3 days of MLR, cells were double stained with mouse CD8 and CD69 MAbs and analysed on a flow cytometer. Actual number of events (bold font) and percentage of events (normal font) are indicated in all quadrants. Representative data of one set of flow cytometry histograms has been shown.

For three replicate sets of histograms, values of mean ± SEM of CD8⁺CD69⁺ populations (top right quadrant) were: 1.0 ± 0.1 isotype control (panel A), 1.6 ± 0.1 control spleen cell culture (panel B), 10.2 ± 0.2 control MLR cells (panel C), 6.9 ± 0.2 MLR cells + SWCNTs (panel D) and 3.2 ± 0.1 (MLR cells + AF-SWCNTs (panel E).
these percentages were not significantly altered if SWCNTs or AF-SWCNTs were present for the duration of the culture. In MLR cultures, a marked increase ranging from 50% to 80% was observed in the proportions of CD4+, CD8+ and CD3+ T-cell populations at the end of 5-day culture (Table I). Treatment with SWCNT did not alter significantly the percentage of CD8+ T cell present in 5-day MLR cultures but a significant decrease of about 10% was observed in the percentage of CD4+ and CD3+ T-cell subpopulations ($p < 0.05$). Treatment with AF-SWCNT resulted in a more significant decline of 25%, 13% and 17%, respectively in CD4+, CD8+ and CD3+ cells ($p < 0.05$) (Table I). These results suggest that AF-SWCNTs may either suppress the generation of T cells or kill activated T cells in culture, or both.

Table II. Uptake of fluorescence-tagged AF-SWCNTs by live T-cell populations in control and MLR cultures.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Population</th>
<th>4 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control spleen cells</td>
<td>TCR+ live cells</td>
<td>14.7 ± 0.57</td>
<td>25.2 ± 1.73</td>
</tr>
<tr>
<td>Control spleen cells</td>
<td>CD4+ live cells</td>
<td>8.4 ± 1.18</td>
<td>18.2 ± 1.09</td>
</tr>
<tr>
<td>Control spleen cells</td>
<td>CD8+ live cells</td>
<td>13.5 ± 1.34</td>
<td>29.7 ± 1.01</td>
</tr>
<tr>
<td>MLR cells</td>
<td>TCR+ live cells</td>
<td>28.5 ± 2.4*</td>
<td>50.4 ± 1.04*</td>
</tr>
<tr>
<td>MLR cells</td>
<td>CD4+ live cells</td>
<td>21.9 ± 0.76*</td>
<td>43.1 ± 1.33*</td>
</tr>
<tr>
<td>MLR cells</td>
<td>CD8+ live cells</td>
<td>38.2 ± 3.3*</td>
<td>58.4 ± 2.4*</td>
</tr>
</tbody>
</table>

Splenocytes from C57BL/6 mice were cultured alone (top three rows) or with irradiated splenocytes from BALB/c mice (bottom three rows) as described in Materials and methods. Fluorescence-tagged AF-SWCNTs (5 μg/ml) were added to the culture 4 or 24 h before the 5-day time point. After 5 days of culture cells were harvested, washed and double stained with 7AAD and one of the T-cell marker MAbs (anti-TCR, CD4 or CD8). Live T cells (7AAD- and TCR+, CD4+ or CD8+ cell populations) were gated in flow cytometry and percentage of gated cells that were positive for AF-SWCNTs determined. All values represent mean ± SEM of percent AF-SWCNT positive live T-cell populations determined in three experiments. *$p < 0.005$ (differences between AF-SWCNT uptake in respective pairs of control spleen cells and MLR cells); AF-SWCNTs, acid functionalised single-walled carbon nanotubes; MAb, monoclonal antibody; MLR, mixed lymphocyte reaction; SEM, standard error of mean.

Figure 3. Effect of SWCNTs and AF-SWCNTs on the proportions of apoptotic T cells in an MLR culture. MLR was carried out between spleen cells from C57BL/6 and BALB/c mice in presence or absence of 50 μg/ml SWCNTs or AF-SWCNTs, as described in Materials and methods. After 5 days of MLR, cells were stained with mouse CD3 MAbs and Annexin V and analysed on a flow cytometer. Representative data of one set of flow cytometry histograms has been shown. For three replicate sets of histograms, values of mean ± SEM of apoptotic T cells (CD3+ Annexin V+ population, top right quadrangles) were: 0.8 ± 0.1 isotype control (panel A), 2.7 ± 0.4 control MLR cells (panel B), 6.7 ± 0.2 MLR cells + SWCNTs (panel C) and 12.5 ± 0.3 MLR cells + AF-SWCNTs (panel D).
membrane that may be assessed by staining of externalised phosphatidyl-serine with Annexin V (Koopman et al. 1994; Vermes et al. 1995). Apoptosis in CD3+ T cells in MLR cultures in presence of AF-SWCNTs was examined by Annexin V staining. Representative results in Figure 3 show that in control MLR cultures 2% cells were Annexin V positive. Treatment with SWCNT or AF-SWCNT increased the level of apoptotic cells to 5% ($p < 0.05$) and 11% ($p < 0.01$), respectively. These results indicate that both SWCNTs as well as AF-SWCNTs induced significant apoptotic response in T cells though the effect was more pronounced with AF-SWCNTs.

**Binding and uptake of AF-SWCNTs by resting and MLR-activated T-cell subpopulations**

Various effects of AF-SWCNTs on T cells could presumably be consequent to actual binding or uptake of AF-SWCNTs by these cells. To test for this possibility binding of AF-SWCNTs to T cells was examined by using fluorescence-tagged AF-SWCNT preparations. Control or MLR-activated spleen cells were incubated with fluorescence-tagged AF-SWCNTs for 4 or 24 h. Binding/uptake of AF-SWCNTs with the cells was examined by flow cytometry. Counterstaining of the cells for CD4, CD8 and TCR markers enabled the authors to assess the binding/uptake of AF-SWCNTs by whole population of T cells or CD4 and CD8 subpopulations. Summary of these results are given in Table II. These results show a certain basal level of AF-SWCNTs binding with control TCR+, CD4+ and CD8+ T-cell populations, which increased with time from 4 to 24 h. Interestingly, however, in all cases the binding of AF-SWCNTs with corresponding activated T-cell populations was substantially higher; the increase being from 90% to 180%. These results indicate that the binding/uptake of AF-SWCNTs is substantially greater for activated as compared with the resting T cells.

Actual internalisation of AF-SWCNTs by T cells was also examined by confocal microscopy. For this purpose, MLR-activated spleen cells were incubated with fluorescence-tagged AF-SWCNTs and then counterstained for CD4 and CD8 marker and 7AAD dye. Live CD4+ and CD8+ cells (CD4+7AAD− and CD8+7AAD− populations, respectively) were isolated by sorting on a fluorescence-activated cell sorter and examined using a confocal microscope for the presence of fluorescence-tagged AF-SWCNTs in the cytoplasm and nucleus. DAPI stain was used to define the nuclei in sorted cells. Results of a typical experiment in Figure 4 show that the fluorescent nanotubes were present in cytoplasm of both CD4+ and CD8+ cells. Some AF-SWCNT presence was also observed in nuclei but the presence was significantly lower as compared with the cytoplasm. These results indicate that AF-SWCNTs could traverse the cell membrane and reach the cytoplasmic space of T cells.

**In vivo effect of AF-SWCNT on the generation of CTLs**

Immunization of C57BL/6 mice with P815 (H-2b) tumour cells results in the generation of anti-P815 CTL activity that peaks on day 11 (Saxena et al. 1982). Effects of SWCNTs and AF-SWCNTs on in vivo CTL response were examined. Mice were immunised with a single intraperitoneal dose of $10^x$

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**Effect of SWCNTs and AF-SWCNTs on early activation of CD8+ T cells in an MLR**

CD69 expression is an early activation marker on CD8+ T cells (Simms & Ellis 1996; Lim et al. 1998). Effect of SWCNT and AF-SWCNT on CD69 expression on CD8+ T cells was studied on day 3 of the MLR. An earlier time point of 3 days was used because activation phase precedes the actual generation of CTLs. Representative results in Figure 2 show that in control cultures of C57BL/6 spleen cells less than 2% CD8+ cells expressed the CD69 activation marker whereas in activated MLR cultures, 9.9% of CD8+ cells expressed the CD69 marker. Treatment with SWCNTs and AF-SWCNTs resulted in a significant decline in CD69+CD8+ cells to 7.1% (38% decline) and 3.3% (67% decline), respectively (Figure 2). These results suggest that AF-SWCNTs may significantly downregulate the activation of CD8+ population of T cells.

**Induction of apoptosis by AF-SWCNT in CTLs generated in an MLR**

A direct toxic effect of AF-SWCNTs on activated T cells could contribute to reduced T-cell recoveries from MLR cultures. Cellular toxicity is generally preceded by apoptotic changes like the externalisation of phosphatidylserine in the cellular

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**Figure 4. Localization of AF-SWCNTs in CD4+ and CD8+ T cells by confocal microscopy.** MLR cultures were setup as described in legend to Figure 1. At the end of 4 days MLR, a preparation of fluorescence-tagged AF-SWCNT (5 μg/ml) was added to the culture medium and incubation continued for 24 h. Cells were washed three times to remove unbound/loosely attached AF-SWCNTs and double stained with 7AAD and CD4 or CD8 MAb. Live CD4+ and CD8+ T cells were isolated by cell sorting on fluorescence-activated cell sorter by selecting 7AAD−, CD4/CD8+ cells, and examined by confocal microscopy. Left and right panels show the images obtained with live CD4+ and CD8+ cells, respectively, panels A and E show DIC images, panels B and F show DAPI stained nucleus, panels C and G show distribution of AF-SWCNTs within the cells and panels D and H show a combined DAPI and AF-SWCNT fluorescence images of live CD4+ and CD8+ cells, respectively (magnification 100×).
10⁶ P815 cells and divided into three groups. Mice in the first group were further divided in subgroups that were administered single doses of 100, 50 or 10 μg SWCNTs or AF-SWCNTs i.v., 1 day after P815 immunisation. The second group of mice was similarly subdivided into subgroups that were administered single doses of 100, 50 or 10 μg SWCNT or AF-SWCNT i.v., 10 days after immunisation with P815 cells. Third control group of P815 immunised mice was not treated with SWCNTs or AF-SWCNTs. On 11th day, mice were sacrificed and anti-P815 CTL activity in spleen cells estimated in CRA using P815 cells as target. Results in Figure 5A and B show that SWCNTs treatment on day 1 or 10 at any dose had no significant effect on the CTL activity generated in response to P815 cells. In the group of immunised mice administered AF-SWCNTs on day 1 (Figure 5C), a significant decrease of 13% in CTL response was observed only at a dose of 100 μg/ml (p < 0.05), whereas lower doses of AF-SWCNTs were ineffective. Single doses of 100 or 50 μg of AF-SWCNTs on day 10 caused small yet significant decrease of 20% and 10%, respectively in CTL response (p < 0.01, Figure 5D). Lowest dose of AF-SWCNT (10 μg/ml) given on day 10 had no significant effect on the generation of CTLs (Figure 5D).

Effect of multiple doses of control and AF-SWCNTs on the generation of anti-P815 CTLs was also examined. For this purpose, P815 immunised mice received 25 μg of SWCNTs or AF-SWCNTs on every alternate day, that is, on day 1, 3, 5, 7 and 9, and spleen CTL activity determined on day 11. Results in Figure 6 show that multiple doses of 25 μg AF-SWCNTs caused 36% decline in CTL response.
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Figure 6. Effect of multiple doses of SWCNTs and AF-SWCNTs on CTL response in vivo. C57BL/6 mice were intraperitoneally administered 10⁷ P815 cells followed by multiple doses of 25 μg/ml of SWCNTs or AF-SWCNTs administered intraperitoneally on alternate days. After 11 days, anti-P815 cytotoxic activity spleen cells were assessed in a 4 h chromium release assay at E/T ratios of 100, 50, 25 and 12. Each value of percent target lysis denotes a mean ± SEM of lysis values from four replicate assay wells. *p < 0.05, **p < 0.01 by ANOVA.

(p < 0.001), while SWCNTs show a lesser yet significant decline of 7% in CTL response (p < 0.05).

Relative recoveries of CD3⁺, CD4⁺ and CD8⁺ T cells from spleens of P815 immunised mice, treated with multiple doses of SWCNTs or AF-SWCNTs, were also examined. Results in Table III show that treatment with multiple doses of AF-SWCNTs caused 14%, 25% and 14% decline, respectively in the recovery of CD3⁺, CD8⁺ and CD4⁺ cells (p < 0.01 in all cases). SWCNTs caused a lesser yet significant (p < 0.05) decline in recoveries of CD4⁺ and CD8⁺ cells. In control unimmunised mice also AF-SWCNT caused a marginal though statistically significant (p < 0.05) decline of CD3⁺ and CD8⁺ cells. SWCNT treatment had no effect on T-cell recoveries from control spleen cultures (Table III).

Table III. Effect of SWCNTs and AF-SWCNTs on the relative recoveries of CD4, CD8 and CD3 T-cell populations from spleens of C57BL/6 mice challenged with P815 tumour cells.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Treatment</th>
<th>CD4⁺ (%)</th>
<th>CD8⁺ (%)</th>
<th>CD3⁺ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6 alone</td>
<td>Control</td>
<td>19.4 ± 0.4</td>
<td>10.5 ± 0.4</td>
<td>31.1 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>+ SWCNT</td>
<td>19.4 ± 0.4</td>
<td>10.1 ± 0.3</td>
<td>30.5 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>+ AF-SWCNT</td>
<td>18.9 ± 1.0</td>
<td>9.7 ± 0.1*</td>
<td>28.9 ± 1.3*</td>
</tr>
<tr>
<td>C57BL/6 + P815</td>
<td>Control</td>
<td>22.0 ± 0.3</td>
<td>17.8 ± 0.3</td>
<td>40.4 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>+ SWCNT</td>
<td>21.3 ± 0.3*</td>
<td>15.5 ± 0.4*</td>
<td>38.8 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>+ AF-SWCNT</td>
<td>19.2 ± 0.6**</td>
<td>13.5 ± 0.4**</td>
<td>35.1 ± 1.0**</td>
</tr>
</tbody>
</table>

CTLs were generated in C57BL/6 mice by i.p. administration of 10⁷ P815 cells following which 25 μg/ml of SWCNTs or AF-SWCNTs or vehicle alone were administered on alternate days. After 11 days, splenocytes derived from control and SWCNT/AF-SWCNT-treated mice were labelled with anti-CD4, CD8 and CD3 MABs, as described in Materials and methods. Percent of T-cell subpopulations were analysed on flow cytometry. Each value represents mean ± SEM (*p < 0.05, **p < 0.01, Student’s t-test); AF-SWCNTs, acid functionalised single-walled carbon nanotubes; CTL, cytotoxic T cell; MAB, monoclonal antibody; MLR, mixed lymphocyte reaction; SEM, standard error of mean.

Discussion

CNTs as such are strongly hydrophobic in nature and exist in a highly agglomerated form. This agglomerated form is not suitable for biomedical applications, where a polydisperse hydrophilic form on nanotubes is required that can be optimally loaded with desired ligands and interact efficiently with cells. In the present study, the authors utilised an AF-SWCNT that has previously been shown to interact effectively with several biological systems (Saxena et al. 2007; Tong et al. 2009; Sachar & Saxena 2011). Information about the interactions of CNTs with the immune system is scarce in the literature and there are no reported studies specifically designed to study the effects of CNTs on the generation of CTLs. Such studies would be of interest not only from the toxicological point of view, but also for exploring the potential use of CNTs as an immune-modulatory reagent.

In the present study, the authors have found that AF-SWCNTs significantly inhibit the generation of alloimmune CTLs both in vitro as well as in vivo. For in vitro studies, they used the previously standardised system of H-2ᵇ anti H-2ᵈ MLR to generate anti-H-2ᵇ CTLs (Saxena et al. 1982). Addition of AF-SWCNTs during the MLR resulted in a significant decrease (maximum about 44%) in the levels of anti-P815 cytotoxicity generated. It was necessary for the AF-SWCNTs to be added during the induction phase of CTL generation because addition only at the time of cytotoxicity assay was not suppressive. These results suggest that the AF-SWCNTs may act during the activation phase of CTLs and may not interfere with the interactions of CTL effector cells with the target cells.

Activation of CTLs indicated by the expression of CD69 activation marker on CD8⁺ T cells on day 3 of MLR was substantially lower (about 80%) in presence of AF-SWCNTs. In addition, the percentage of T cells undergoing apoptosis was markedly up-regulated in the presence of AF-SWCNTs. Thus, the AF-SWCNTs may not only suppress activation of CD8⁺ T cells but may actually trigger cell death in T cells. This was confirmed by the data on the recovery of live T-cell populations from control and AF-SWCNT-treated MLR cultures. T-cell proportion in control spleen cell culture was about 29% that did not change by adding SWCNTs or AF-SWCNTs in the culture. In active MLR cultures however, there was a marked increase (70%) in the proportion of T cells; the increase being comparable...
for both CD4 and CD8 T-cell populations. A small yet significant decrease in T-cell recovery from MLR cultures was seen in presence of SWCNTs but the decrease was more pronounced in presence of AF-SWCNTs (Table I). While these results clearly show a significant downregulation of T-cell system by AF-SWCNTs, it is not clear if the effect of the AF-SWCNTs was directly mediated through some cellular and/or soluble mediators released in the culture. Studies using fluorescence-tagged AF-SWCNTs indicated that in unstimulated control spleen cell cultures there was a time-dependent increase in binding/uptake of AF-SWCNTs by CD4+ as well as CD8+ T-cell populations. The binding of AF-SWCNTs was markedly greater in T-cell populations in activated MLR cultures. These studies would not distinguish between surface binding or actual internalisation of AF-SWCNTs by T cells. Confocal microscopy experiments however confirmed the presence of AF-SWCNTs in the cytoplasm of both CD4+ and CD8+ T cells indicating that the AF-SWCNTs were internalised by these cells. Interestingly, AF-SWCNTs were predominantly present in the cellular cytoplasm though some presence of these particles was also seen in the nuclei. Mechanism of nanotubes internalization by T cells is not known. The previous studies have demonstrated internalisation of AF-SWCNTs by erythrocytes in vitro (Sachar & Saxena 2011) indicating that the entry of nanotubes in cells may not require phagocytic activity. T cells are not associated with phagocytic activity but can clearly internalise nanotubes through mechanism(s) that are not clear at present.

Significant increase in the uptake of AF-SWCNT by activated T cells is interesting as it suggests that activated T cells may be more susceptible to the effects of AF-SWCNTs. This is supported by the authors’ observation that in control spleen cell cultures AF-SWCNTs had no effect on the recovery of live T cells whereas in MLR cultures, a significant decline in T-cell recovery took place when AF-SWCNTs were added in the culture (Table I). Hanley et al. (2008) have previously shown that as compared with the resting T cells, activated T cells were more susceptible to ZnO nanoparticles. It may be speculated that activated cells may in general be more susceptible to the toxic effects of nanoparticles. This stipulation would require further confirmation and could be exploited for specifically targeting the nanoparticles to activated cell populations for therapeutic purposes.

In vivo administration of a single dose (100 μg) of AF-SWCNTs early (day 1) during the in vivo CTL activation phase induced only marginal suppression (13%) of the CTL response. Suppression was more (18.7%) if the AF-SWCNTs were administered as a single dose on day 10 of the activation reaction, that is, 1 day before harvesting the spleen CTLs. Maximum suppression (47%) was however obtained when AF-SWCNTs (25 μg/dose) were administered on alternate days throughout the activation phase of 11 days. These results coupled with results of the effect of AF-SWCNTs on in vitro CTL generation in MLR clearly show that AF-SWCNTs do not completely block the CTL response but only suppress it partially. Since the cytotoxic activity of effector CTLs is not inhibited by AF-SWCNTs, it is tempting to speculate that the presence of AF-SWCNTs may downregulate the process of activation and clonal expansion involved in CTL generation. Accordingly, a significant decline in the proportion of T-cell populations (especially CD8+ T cells) recovered from spleens of AF-SWCNT-treated mice was observed. These results would be concurrent with the suggestion that T cells during activation phase could be preferred target of AF-SWCNTs. It would be interesting to further examine if AF-SWCNTs could be used in vivo to downregulate T-cell activation process associated with autoimmunity and hypersensitivity.

Conclusion

Results of this study suggest that the exposure to AF-SWCNTs significantly suppresses the mouse CTL response in vivo as well as in vitro. AF-SWCNTs bind to T cells and are later detectable in the cellular cytoplasm by confocal microscopic studies. AF-SWCNTs lower the proportion of CD69+ activated T cells and induce apoptosis in MLR-activated T cells. A significantly reduced recovery of T cells is seen from AF-SWCNT-treated MLR cultures as well as from alloimmunised mice treated with AF-SWCNTs. Taken together, the study results suggest that AF-SWCNTs may interfere with the process of T-cell activation that precedes the generation of CTLs.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References


