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Activation of T and B lymphocytes Induces Increased Uptake of Poly-Dispersed Single-Walled Carbon Nanotubes and Enhanced Cytotoxicity

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Abstract

Uptake of poly-dispersed acid functionalized single-walled carbon nanotubes (AF-SWCNTs) in resting and activated B and T cells was studied by using fluorescence tagged AF-SWCNTs. Activated B and T cells (activated by LPS and staphylococcal enterotoxin B respectively), internalized substantially higher amounts of AF-SWCNTs as compared to the resting cells. The uptake of AF-SWCNTs was significantly elevated in both dividing and non-dividing T and B cells activated cultures but the increase was significantly more for dividing cells. Confocal microscopy indicated poor uptake of AF-SWCNTs by resting B and T cells whereas much higher uptake was seen in activated B and T cells. Effect of AF-SWCNTs on the kinetics of accumulation of live B and T cells in activated and proliferating cultures indicated that AF-SWCNTs exerted significantly higher cytotoxic effect on activated B and T cells as compared to resting control cells. Targeting of AF-SWCNTs specifically to activated cells in a mixture of resting and activated B and T cells was also demonstrated. Uptake of AF-SWCNTs was significantly lower at 4C as compared to 37C indicating that active uptake mechanisms were involved in AF-SWCNT uptake. Testing the effect of several known inhibitors of different pathways utilized by cells to internalize particles (chlorpromazine hydrochloride, cytochalasin D, wortmannin and filipin), indicated that these inhibitors had minimal effect on the AF-SWCNT uptake in resting B and T cells but significant effects were observed when activated B and T cells were used. Overall our results show that activated B and T lymphocytes internalize substantially more AF-SWCNTs as compared to resting cells and open up the possibility of specific targeting of activated lymphocytes to abrogate these cell populations in vivo

Keywords: SWCNT, AF-SWCNT, B cells, T cells, LPS, SEB, Flow Cytometry, Confocal Microscopy, Targeting Nanotubes

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Introduction

Carbon nanotubes (CNTs) were first synthesized about 25 years ago[1,2] and due to their unique physico-chemical properties, rapidly found potential usage in industry[3-7] as well as in bio- medical fields.[8-11] Pristine single walled carbon nanotubes (SWCNTs) are highly hydrophobic and agglomerate strongly to form insoluble clusters that do not interact efficiently with cells.[12] Acid functionalized SWCNTs (AF-SWCNTs) produced by treatment of SWCNTs with acids at high temperature and pressure, become hydrophilic, are easily dispersed in aqueous media and interact efficiently with cells.[13,14] Several studies in literature have focused on the interactions of SWCNTs with the immune system.[15-21] We have reported on the interactions of AF-SWCNTs with T cells and natural killer (NK) cells and found that in both cases, AF- SWCNTs down regulate the generation of cytotoxic T cell response and NK cell activation response in vitro and in vivo.[22,23] We have also found down regulation of antigen presentation by macrophages as well as primary lung epithelial cells upon exposure to AF-SWCNTs.[24]

In the present study, we have focused upon the changes in uptake of AF-SWCNTs upon activation of B and T lymphocytes by lipopolysaccharide (LPS) and T cell super-antigen staphylococcal enterotoxin B (SEB) respectively. We found that activated B and T cells internalize significantly greater amounts of AF-SWCNTs as compared to the resting B and T cells. Confocal microscopic studies indicated that while the AF-SWCNTs were essentially localized around the cell membrane of resting B and T cells, activated B and T cells actively internalized the AF-SWCNTs that were well dispersed within the activated B and T

cells. Significantly more uptake of AF-SWCNTs was noted in a mixture of resting and activated lymphocytes where AF-SWCNTs were found to be specifically targeted to activated cells. Effects of some inhibitors of various mechanisms of membrane transport were examined on AF-SWCNT uptake by resting and activated B and T cells in order to gain some insight into the mechanism of uptake. These results would help advance our understanding of interactions of carbon nanotubes with lymphocytes with special focus on differences between resting and activated lymphocytes and the possible use of AF-SWCNTs in targeted therapies in vivo. Further, these observations open up the possibility of in vivo depletion of activated T or B cells by AF-SWCNTs in clinical situations where abrogation of activated T or B cells may be beneficial.

Materials and Methods

Animals

Inbred C57BL/6 mice (8-15 weeks old, 20-25g body weight) were used throughout the study. Animals were maintained in the animal house facility at South Asian University (SAU), New Delhi, and obtained from the National Institute of Nutrition (NIN), Hyderabad. Animals were housed in positive pressure air-conditioned units (25°C, 50 % relative humidity) and kept on a 12 hour light/dark cycle. Water and chow were provided ad libitum. All the experimental protocols were conducted strictly in compliance with the guidelines notified by the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), Ministry of Environment and Forest (www.envfor.nic.in/divisions/awd/cpcsea_laboratory.pdf). The study was duly approved by SAU Institutional Animal Ethics Committee (IAEC project approval code: SAU/IAEC/2016/02).

Reagents and other supplies

Single-walled carbon nanotubes (Cat# 775535, > 95 % carbon basis), N-Hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC), 2-(N-morpholino) ethane sulfonic acid (MES), lipopolysaccharide (LPS) from Escherichia coli O26:B6, staphylococcal enterotoxin B (SEB) from Staphylococcus aureus were purchased from Sigma Aldrich (St. Louis, MO, USA). Chlorpromazine hydrochloride, cytochalasin D, wortmannin and filipin were purchased from Sigma Aldrich (India). Alexafluor 633 hydrazide was from Molecular Probes (Carlsbad, CA, USA). RPMI1640 medium (with 2mM glutamine, 1mM sodium pyruvate, 4.5 gm glucose/litre, 10mM HEPES, 1.5 gm/litre sodium bicarbonate and 20µg/ml gentamycin) and fetal bovine serum (FBS) were from Gibco (Carlsbad, CA, USA).Centricon 3kDa centrifugal filter device and 100kDa Dialysis tubing was ordered from Millipore (Billerica, MA, USA.). Anti- mouse CD16/32 purified, anti-mouse CD3, anti-mouse CD19, 7AAD and carboxy fluorescein succinimidyl ester (CFSE) were purchased from eBiosciences (San Diego, CA, USA). Propidium Iodide and RNase A were from Invitrogen (Carlsbad, CA, USA).

Acid functionalization of Single-walled carbon nanotubes:

Acid-functionalized single-walled carbon nanotubes (AF-SWCNTs) were prepared as described before[18]. Briefly, SWCNTs (20mg) were suspended in 1:1 ratio of concentrated H_2SO_4 and HNO_3 (20 ml) and subjected to high pressure microwave treatment where pressure was maintained at 20 ± 2 psi, power at 50% of 900W, and temperature 135-150°C for 3 minutes. After microwave treatment, suspension was cooled, diluted three times with water and dialyzed against deionized water till the pH was neutral. Dialyzed suspension was lyophilized and the powder weighed. Detailed characterization of AF-SWCNTs including size and charge distribution, BET surface area and electron microscopic changes, have been reported earlier. [26] Fluorescence tagged AF-SWCNTs (FAF-SWCNTs) were obtained by chemically tagging the AF-SWCNTs with the fluorochrome Alexa Fluor 633 by using the procedure described elsewhere.[25, 26]

Treatment of cells with transport inhibitors

Spleen cells were cultured without and with LPS/SEB (5 µg/ml) for 48 hours and 72 hours respectively and were treated with chlorpromazine hydrochloride (10 µg/ml),[27] cytochalasin D (4µM),[28] wortmannin (100 nM)[29, 30] and filipin (1 µg/ml)[31, 32] for 1 hour. After that, FAF- SWCNTs (10 µg/ml) were added to the culture and incubation continued for 4 hours. Cells were harvested, washed and stained with CD19 and CD3 antibodies and analyzed on a flow cytometer for FAF-SWCNT uptake.

Flow cytometric analysis

Cells to be analyzed were first incubated with Fc block (anti-mouse CD16/32 antibody, 0.5 (0.5 µg/10^6 cells in 100 µl PBS containing 2% FBS) for 30 minutes on ice to block Fc receptors present on cells. Cells were then stained with various antibodies or with 7AAD as described before.[33]

CFSE labeling of cells was done as described before.[34] Briefly, cells (100 x 10^6 in 1 ml PBS) were treated with 10µM carboxy fluorescein succinimidyl ester (CFSE) for 10 minutes on ice. Excess dye was removed by washing with PBS. Samples were analyzed on FACSverse and results analyzed using FACS Diva software.

Confocal Microscopy

For confocal microscopic analysis, cells were incubated with FAF-SWCNTs (10 µg/ml) for stated periods, stained with CD3/CD19 for labeling T and B cells respectively, counterstained with 7AAD and fixed with 4% paraformaldehyde. Purified B and T cells (99.5% pure) were obtained by cell sorting using FACS Aria III cell sorter. T or B cells were deposited on poly-L- Lysine (0.01%) coated coverslips and mounted onto glass slides using prolong gold antifade reagent as described before.[35] Images were acquired using A1R Laser Confocal Scanning Microscope (Nikon, Japan).

Statistical analysis

Each experiment was repeated three times at least. Statistical Analysis was performed using Sigma Plot software (Systat software, San Jose, CA). Student's t-test was used to calculate the significance level between groups and ANOVA was used to calculate significance between and within groups.

Results

Uptake of FAF-SWCNTs by resting and activated T and B lymphocytes

LPS is a polyclonal activator of B cells and induces B cell proliferation,[36] whereas SEB is a T cell super antigen that induces proliferation in a subset of T cells.[37] To study the effect of activation on the cellular uptake of AF-SWCNTs, we first studied the time kinetics and dose response of LPS and SEB activation. Due to the mitogenic effect of LPS and SEB, B and T cells respectively proliferate and accumulate in culture. Kinetics of accumulation of B and T cells in LPS and SEB treated cultures respectively indicated that maximum B cell accumulation in LPS (doses 1 and 5µg/ml) treated cultures was at 48 hours whereas for SEB (doses 1 and 5µg/ml) treated cultures, maximum T cell accumulation was at 72 hours (Figure 1).

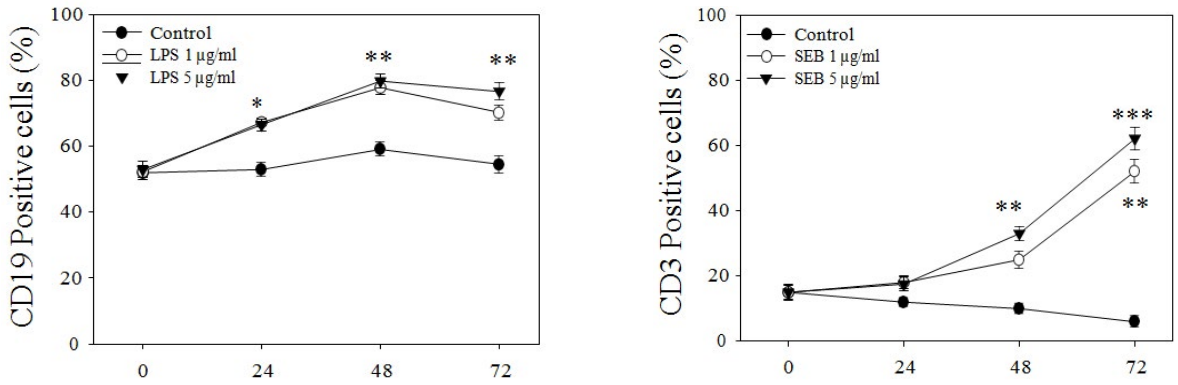

Figure 1: Activation of B and T lymphocytes by LPS and SEB. Splenocytes were isolated from C57BL/6 mice and were cultured in 24 well culture plate (1 X 106 cells/ml) with and without LPS/SEB (1 and 5µg/ml) for 24, 48 and 72 hours. Cells were harvested and stained with anti-mouse CD19 and anti-mouse CD3 antibody and counterstained with 7AAD to eliminate dead cell population. Samples were acquired using FACSverse for percentage of B/T cells. Panel A represents B lymphocytes percentage in absence (control) and in presence of LPS; Panel B represents percentage of T lymphocytes with and without SEB. Significance values were calculated using paired t-test. *p<0.05, **p<0.01, ***p<0.005.

To study the uptake of FAF-SWCNTs, spleen cells were activated with LPS and SEB for 48 and 72 hours respectively, washed and incubated with FAF-SWCNTs for 4 hours. Results of flow cytometric analysis of these cells are given in Figure 2. Counterstaining with CD3/CD19 and 7AAD allowed us to gate on live B and T cells and assess the FAF-SWCNT uptake in terms of percent FAF-SWCNT+ cells as well as the mean fluorescence intensity (MFI) of FAF-SWCNT+ cells. Results in the panel A and B of Figure 2 show the setting of gate for FAF-SWCNT uptake using control B and T cells respectively without exposure to FAF-SWCNTs. Data for the uptake of FAF-SWCNTs in control resting B and T cells are shown in panel C and D respectively. For activated B and T cells, the FAF-SWCNT uptake data is similarly shown in panels E and F respectively in Figure 2.

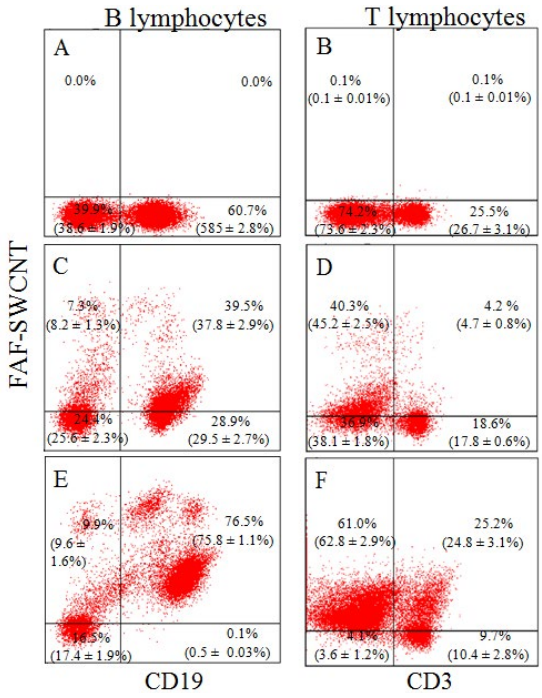

Figure 2: Uptake of FAF-SWCNTs by resting and LPS/SEB activated lymphocytes. Mouse spleen cells (1 x 106 cells/ml/well) were activated with LPS or SEB (5 µg/ml) for 48 hours and 72 hours to activate B and T cells respectively and incubated thereafter with FAF-SWCNTs (10 µg/ml) for 4 hours. Cells were harvested, washed and stained with anti-mouse CD19 or anti- mouse CD3 antibody and counterstained with 7AAD. Cells were analysed on flow cytometer where 50,000 events were recorded. 7AAD negative populations were gated and examined for the uptake of FAF-SWCNTs. Representative dot plots of data are shown. Values outside the bracket show percentage of cells of same dot plot and values in bracket represent Mean ± SEM of three replicate experiments.

Data of percentage of cells in each quadrangle of the histograms is provided. A marked increase in the uptake of FAF-SWCNTs by activated B as well as T cells is clearly seen from these results. Total B cells comprised 68.4% of all spleen cells [sum of percentages in UR and LR quadrangles in panel C], of which 57.7% [100 x 39.5% / (39.5% + 28.9%)] were positive for FAF-SWCNTs. In LPS activated spleen cell cultures, almost 100% of the B cells were FAF-SWCNT positive (panel E). Calculated the same way, the proportion of FAF-SWCNT+ T cells went up from 18.4% in resting T cells (panel D) to 72.2% in SEB activated T cells (panel F). Results of one representative experiment have been shown in also given in the figure (see data in brackets in quadrangles of different panels).

FAF-SWCNT uptake by dividing and non-dividing activated B and T cells.

Above results indicated that activated B and T cells on average took up substantially more FAF- SWCNTs as compared to resting B and T cells. Activated B and T cell preparations would have cells that have divided due to the mitogenic effects of LPS and SEB, as well as cells that are activated but have not yet divided. It was important to see the FAF-SWCNT uptake in these two sub-populations of activated cells since the process of cell division (comprising steps like nuclear division, cytokinesis and attendant membrane changes) itself may affect the uptake of FAF- SWCNTs. In order to clarify this issue, we labeled the spleen cells with CFSE dye and activated these cells with LPS and SEB. Cells that divide would have lower CFSE stain as the original stain on the mother cells gets diluted in the daughter cells. Dividing and non-dividing B and T cells in LPS and SEB treated cultures respectively could thus be gated based upon CFSE stain.

In Figure 3, the distribution of CFSE stain in control resting B cells (panel A) and T cells (panel C) is shown that was used to gate for non-dividing B and T cells. Post-activation, dividing and not-dividing B and T cells in activated spleen cell cultures could be gated based upon CFSE stain (panels B and D). FAF-SWCNT uptake in resting B cells, and non-dividing and dividing B cells in LPS activated cultures are shown in panels E, F and I respectively. Similar data for resting and activated T cells are shown in panels G, H and J. These results show that FAF-SWCNT uptake (i.e. percentage of FAF-SWCNT+ resting cells) was 37.4 ± 3.1 that went up to 65.8 ± 0.8 for non- dividing activated B cells and to 99.9 ± 0.8 in dividing activated B cells. Thus within the LPS activated cultures, dividing B cells took up significantly more FAF-SWCNTs than the non-dividing B cells. MFI values (values given in brackets in histograms in Figure 3) follow the same pattern. T cell data in panels G, H and J is very similar to what was seen for resting and activated B cells described above.

Comparison of panels B and D show that far bigger proportion of B cells divide in response to LPS as compared to the proportion of T cells that divide in response to SEB. This is understandable since LPS is a polyclonal B cell activator whereas SEB activates a subset of T cells that express TCR with an specific TCR-β chain.[37,38] Also, amongst the activated B and T cells (and in resting B and T cells to some extant) there seem to be a bi-phasic pattern of FAF- SWCNT uptake. A minor yet significant subpopulation of B and T cells seems to have a significantly higher uptake of FAF-SWCNTs (panels E, F and I for B cells and G, H and J for T cells).

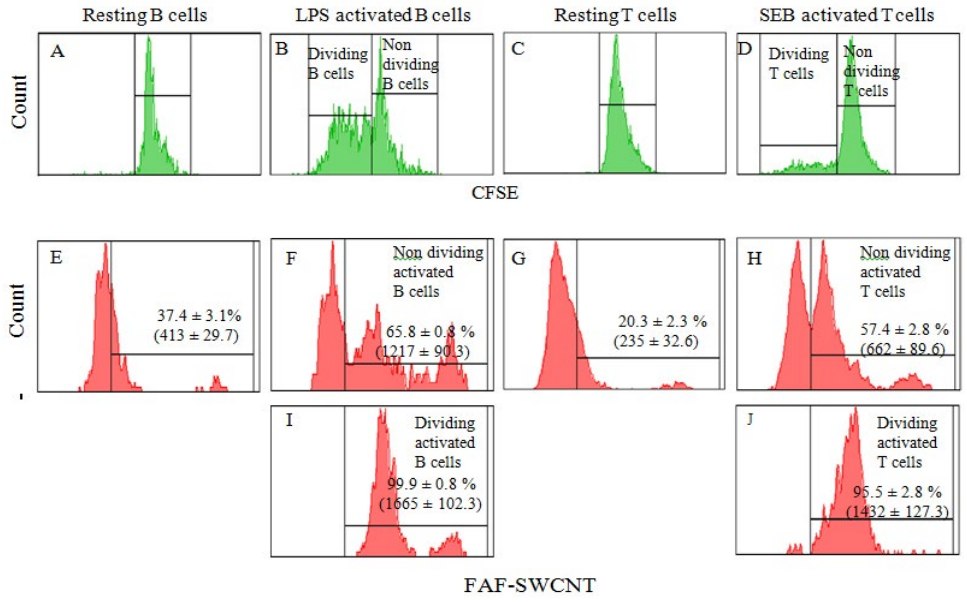

Figure 3: FAF-SWCNT uptake by dividing and non-dividing B and T cells. Spleen cells were labeled with CFSE dye (10 µM). CFSE labeled cells (1 x 106/ml) were treated with LPS or SEB (5 µg/ml) with and without FAF-SWCNT (10 µg/ml). Control spleen cells were also cultured with FAF-SWCNTs alone for 48 or 72 hours. Resting and activated cells were harvested after 48 h (LPS treatment) or 72 hours (SEB treatment), vwashed twice, stained with CD19 and CD3 antibodies, counterstained with 7AAD and analyzed on flow cytometer. Live B and T cells were gated on the basis of stain with CD19 / CD3 antibody while excluding 7AAD+ dead cells. Gated T and B cells were further analyzed for CFSE stain. Panel A shows the CFSE staining pattern of control B cells, Panel B: LPS activated B cells, Panel C: control T cells and Panel D: activated T cells. B and T cells with CFSE stain lower than the control cells were taken as dividing cells in activated cultures (panels B and D). Resting and activated (dividing and non-dividing) cell populations were further analyzed for FAF-SWCNTs (panels E to J). Percent cells positive for FAF-SWCNT stain in panel B, are indicated in each panel (mean ± SEM of three such experiments). Values in brackets represent the corresponding MFI data.

FAF-SWCNTs uptake by confocal microscopy

FAF-SWCNT uptake was also assessed by confocal microscopy. For this purpose, resting and activated spleen cells were pulsed with FAF-SWCNTs for 4 hours followed by staining with CD3 and CD19 antibodies. Pure (>99%) T and B cells were isolated using a BD aria-III cell sorter and were examined for FAF-SWCNT uptake under confocal microscope. Results in top panel of Figure 4A show the DIC and fluorescent images for control resting and LPS activated B cells. These results show that there was little uptake of FAF-SWCNTs by resting B cells but almost all activated B cells showed significant uptake of FAF-SWCNTs. Same data for resting and SEB activated T cells are shown in the lower panel of Figure 4A. Marked uptake of FAF- SWCNTs by activated T cells and relatively low uptake in resting T cells is clearly indicated by these results.

In order to assess the distribution of the FAF-SWCNTs internalized by resting and activated B and T cells, Z-sectioning of resting and activated B and T cells pre-incubated with FAF- SWCNTs was done on confocal microscope. Results in Figure 4B show the Z-sections of FAF- SWCNT treated resting (top panel) and LPS activated B cells (second panel from top). These results indicate that in resting B cells the FAF-SWCNTs were essentially localized around the cell membrane whereas in activated B cells FAF-SWCNTs were distributed throughout the interior of the cell. Similar Z-sectioning of resting and activated T cells pre-incubated with FAF-SWCNTs has also been shown in Figure 4B (lower two panels). In this case also, FAF-SWCNTs were localized near cellular membrane in resting T cells whereas in activated T cells FAF- SWCNTs were distributed throughout the cell.

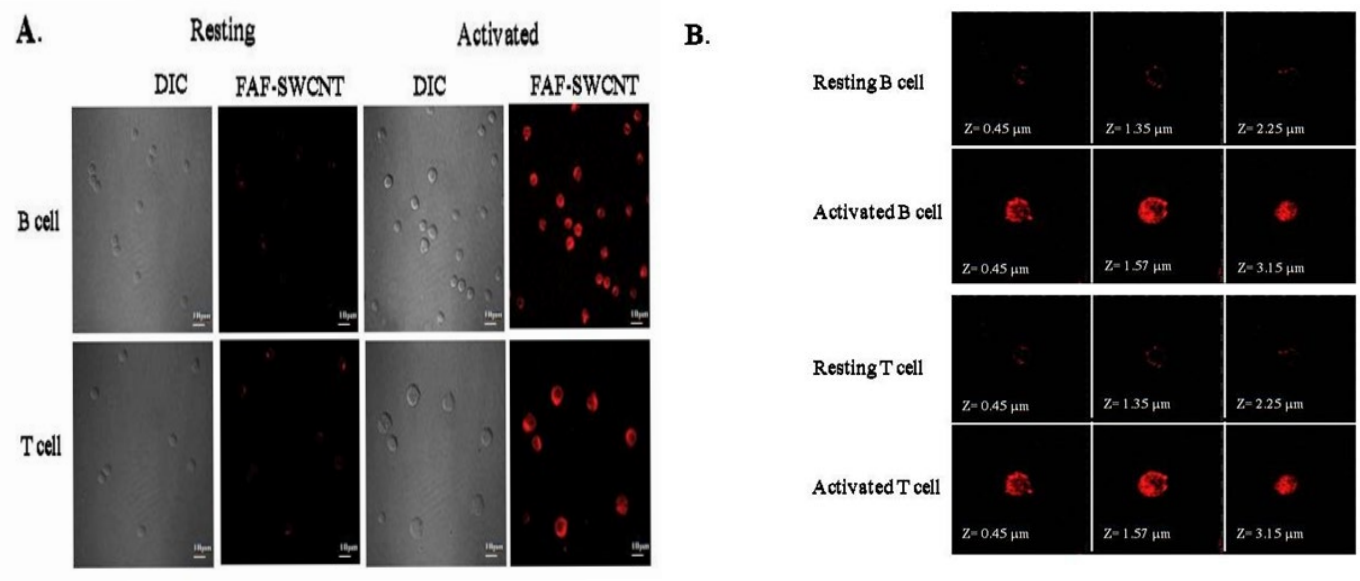

Figure 4: FAF-SWCNT uptake in resting and activated cells by confocal microscopy. Splenocytes were isolated and cultured with or without LPS or SEB (5 µg/ml). Control and activated cells were incubated with FAF-SWCNTs (10 µg/ml for 4 hours). Cells were harvested and stained with FITC anti-mouse CD19 or CD3 antibody. Pure preparations of B and T cells (7AAD negative) were isolated by using a FACSAria III cell sorter. Purified B / T cells (purity 99%) were adhered onto poly-l-lysine coated coverslip, dried and were mounted on glass slide using prolong gold antifade reagent and analyzed using Nikon A1R Laser Confocal Scanning Microscope. Panel A shows uptake/ binding of FAF-SWCNTs in resting and activated B and T lymphocytes. Panel B represents Z sectioning images showing localization of FAF-SWCNTs inside the cell. Magnification 100X.

Cytotoxicity of AF-SWCNTs towards resting and activated T and B cells

Confocal microscopy data revealed that AF-SWCNTs were internalized by activated cells but in resting cells they were localized around the cell membrane. We further evaluated whether this internalization of AF-SWCNTs results in cytotoxicity towards resting and activated cells. To assess this, spleen cells were activated with LPS or SEB and incubated with AF-SWCNTs (10 and 50 µg/ml) for 4, 12, 24 and 48 h. At each time point total live cell counts in cell cultures was determined using trypan blue staining. Cells were simultaneously stained with B and T cell markers and 7AAD (marker for dead cells) and percentages of live (7AAD negative) B and T cells in the cell preparations were determined on a flow cytometer (Figure 5). Knowing the total surviving cell in the cultures and the percentage of B and T cells, absolute counts of live B and T cells in cultures could be calculated. Results in Figure 5 show that while the exposure to AF- SWCNTs resulted in lower live B and T cell recoveries in both resting and activated cell cultures, the magnitude of cytotoxicity was substantially higher for activated B and T cells as compared to the controls. These results show that activated B and T cells that internalized significantly larger amounts of AF-SWCNTs also show a markedly higher cytotoxicity response than resting cells.

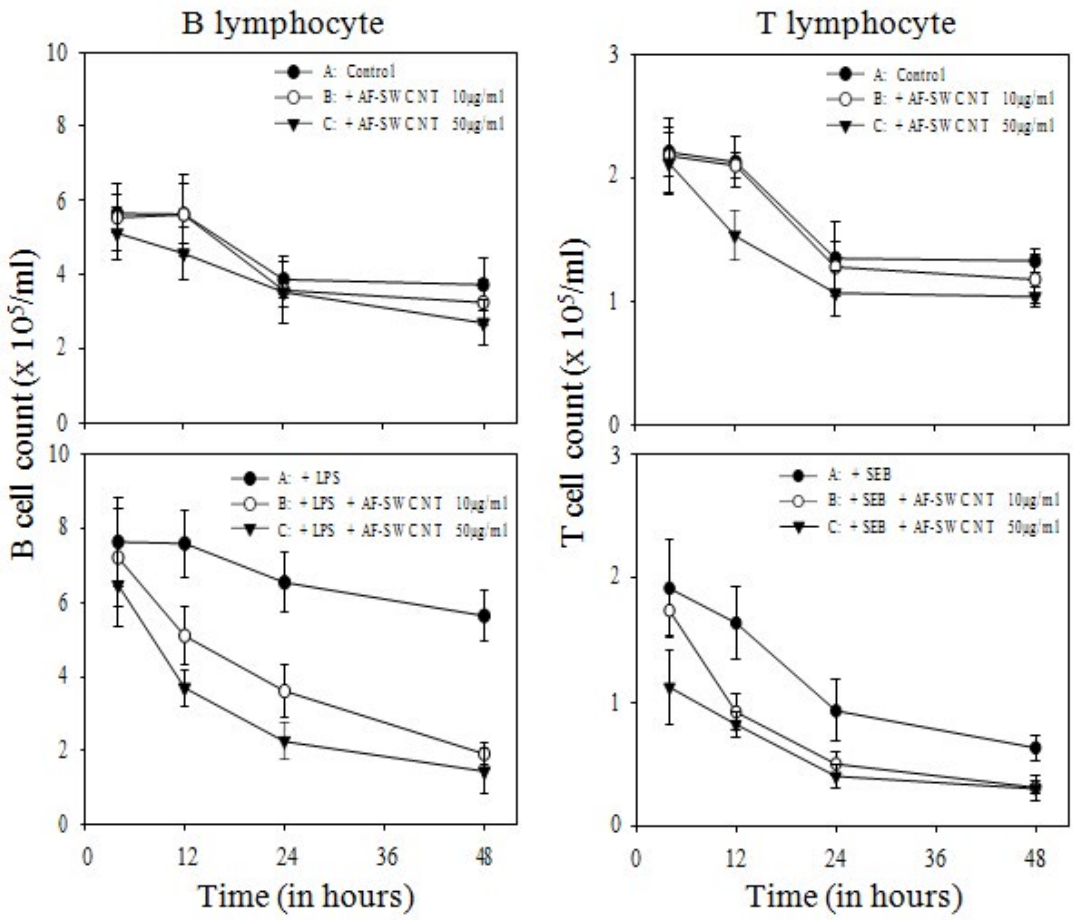

Figure 5: Absolute cell count of resting and activated B and T cells after treatment with AF- SWCNTs indicating cytotoxicity of AF-SWCNTs. Spleen cells (1.5 x 106 cells/ml) were seeded in 24 well culture plate in absence (resting cells) and in presence (activated cells) of LPS (5 µg/ml) and SEB (5 µg/ml). After 48 h of LPS incubation and 72 h of SEB incubation cells were treated with AF-SWCNTs (10 and 50 µg/ml) for 12, 24 and 48 h (In resting cells, AF-SWCNTs treatment was given at the time of seeding). Cells were harvested after respective time points and stain with 0.4 % trypan blue dye in 1:1 ratio and number of viable cells were counted. Simultaneously, at each time point, aliquots of cells were also stained with anti CD19 or CD3 antibody, counterstained with 7AAD and analyzed on FACS verse to determine the percentage of live B and T cells in the suspensions. Absolute recovery of live T and B cells in resting and activated cultures were determined. Each point in the graphs represents mean cell count ± SEM of triplicates. ANOVA was performed to calculate level of significance between curves for control and activated cells. Upper left panel (resting B cell), F ratio for A vs. B comparison =0.73 (not significant, NS); A vs. C comparison F ratio = 1.13 (NS). Upper right panel (activated B cells), F ratio A vs. B= 7.99 ($p<0.05$); F ratio A vs. C comparison = 22.63 ($p<0.01$). Lower left panel (resting T cells), F ratio A vs. B comparison = 0.04 (NS); F ratio A vs. C comparison =1.60 (NS). Lower right panel (activated T cells), F ratio A vs. B comparison = 6.32 ($p<0.05$); F ratio A vs. C comparison = 7.89 ($p<0.05$).

Specific targeting of FAF-SWCNTs to activated lymphocytes

Results so far indicate that activated B and T cells take up significantly greater amounts of FAF- SWCNTs as compared to control resting lymphocytes and the enhanced uptake using MFI parameter is more in activated B cells than in activated T cells. We further examined whether, in a mixed population of resting and activated lymphocytes, FAF-SWCNTs would be specifically targeted to activated cells. For this purpose, we activated spleen cells with LPS or SEB and mixed them with resting spleen cells (pre-labeled with a fluorescent tracking dye carboxy fluorescein succinimidyl ester (CFSE) in a 1:1 ratio. This mixtures of activated and resting cells were incubated with FAF-SWCNTs for 4 hours followed by flow cytometric analysis of FAF- SWCNT uptake in resting and activated B and T cells that could be gated based upon CFSE stain. As controls, resting and activated spleen cells were similarly incubated with FAF- SWCNTs separately also, so that FAF-SWCNT uptake could be compared in resting and activated cells in isolation as well as in mixtures. Results in Figure 6 essentially show that the uptake of FAF-SWCNTs in control and activated B and T cells were comparable whether the cells were exposed to FAF-SWCNTs alone or in mixtures. These results suggest that in a mixture of resting and activated lymphocytes, FAF-SWCNTs would be specifically targeted to activated B and T cells.

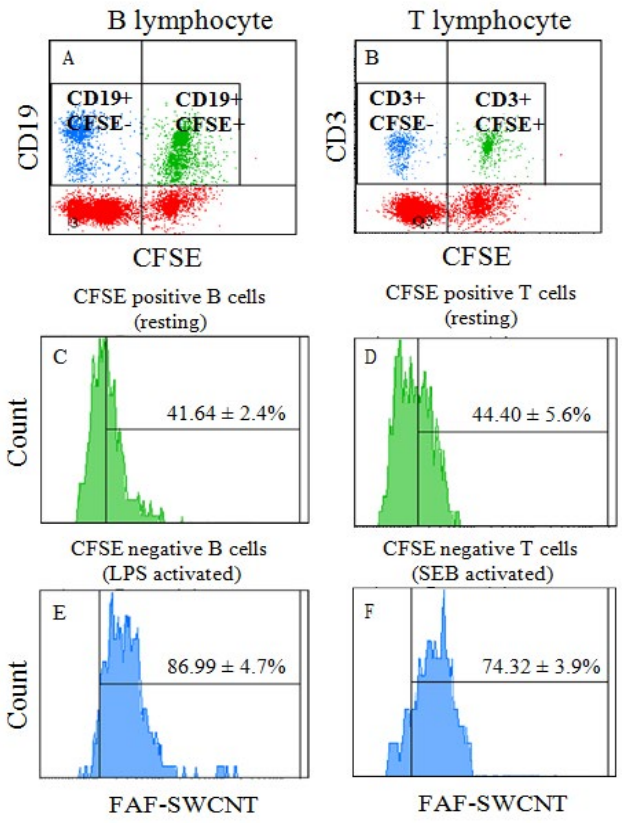

Figure 6: Targeting of AF-SWCNTs to activated B and T cells in a mixture of control and activated spleen cells. Fresh spleen cells (containing control resting B and T cells) were stained with CFSE. LPS/SEB activated cells (not labelled with CFSE) were mixed with resting CFSE labeled cells in a 1:1 ratio and incubated with FAF-SWCNTs (10µg/ml) for 4 hours. LPS and SEB activated spleen cell preparations were also separately incubated with FAF-SWCNTs (10 µg/ml). All cell preparations were stained with CD19 and CD3 antibodies and counterstained with 7AAD and analyzed on flow cytometer. Gated live B and T cells in all cell preparations were examined for the uptake of FAF-SWCNTs. Panel A and B shows the gating of CFSE positive (resting) and CFSE negative (activated) B/T cells. Panel C and D shows uptake of FAF-SWCNT in resting cells present in mixture and Panel E and F represent FAF-SWCNT uptake in activated cells present in mixture. Values here represent percentage of FAF-SWCNT uptake ± SEM of three replicate experiments.

Mechanism of FAF-SWCNT uptake in resting and activated B and T cells:

To gain an insight into the mechanism of uptake of FAF-SWCNTs in resting and activated lymphocytes, we studied the FAF-SWCNT uptake in resting and activated B and T cells at 4C and 37C. The idea being that if the internalization of FAF-SWCNT is an energy dependent process, it may slow down at 4C. Results in the panel A and B of in Figure 7 show that the uptake of FAF-SWCNTs by both control and LPS activated B cells was significantly lower at 4C. In case of T cells, uptake by control T cells was not affected at 4 C whereas for activated T cells, a significant decline was seen. These results indicate that the uptake of FAF-SWCNTs by resting and activated B cells and activated T cells may at least partially be an energy dependent process.

Further, we tested the effects of four inhibitors that interfere with different specific membrane transport mechanisms, on the FAF-SWCNT uptake in resting and activated B and T cells. The inhibitors we used were chlorpromazine hydrochloride (inhibits clathrin mediated endocytosis), cytochalasin D (inhibits phagocytosis), wortmannin (inhibits micro-pinocytosis) and filipin (inhibits caveolae mediated endocytosis). Results of these experiments are given in panels C to F of Figure 7. These results indicate that (a) FAF-SWCNT uptake in control B cells is mildly yet significantly inhibited by all four inhibitors; (b) in activated B cells none of the inhibitors had any effect on FAF-SWCNT uptake in LPS activated B cells; (c) in control T cells, all inhibitors had some inhibitory effect on FAF-SWCNT uptake; and, (d) in SEB activated T cells chloropromazine and wortmannin partially inhibited the uptake whereas cytochalasin D and filipin did not have a significant effect. Overall, these results indicate that several known mechanisms of cellular uptake may contribute in varying degrees, to the internalization of FAF- SWCNTs in resting and activated B and T cells.

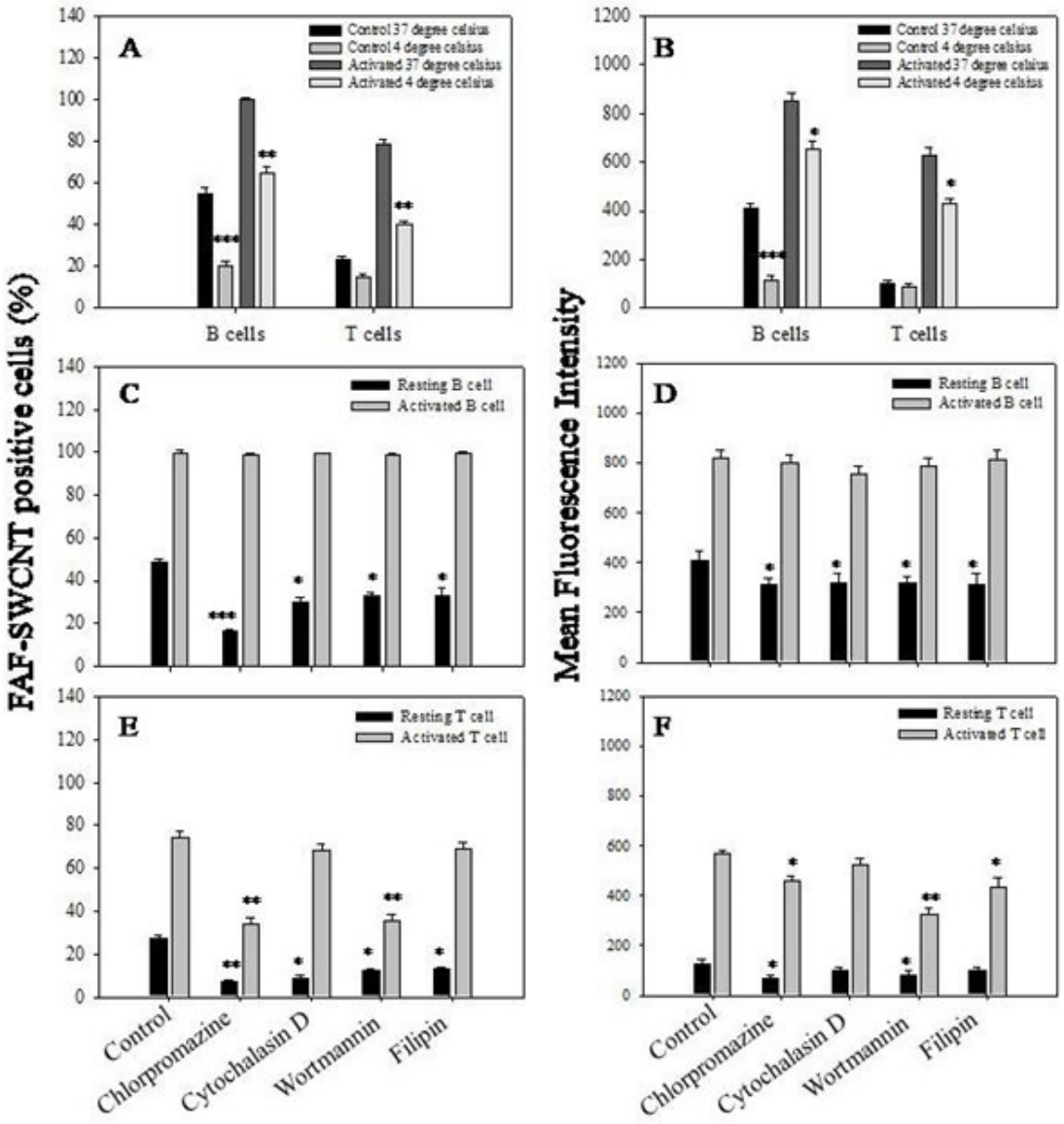

Figure 7: Effect of temperature and various transport inhibitors on the uptake of FAF- SWCNTs. Spleen cells (1x106 cells/ml) were cultured in two different set of plates with LPS/SEB at 37C for 48 and 72 hours respectively. Kept one of the plates containing activated cells at 4C to maintain the temperature. Thereafter, FAF-SWCNT (10 µg/ml) was added and incubation continued for 4 hours at 4C or 37C. Fresh spleen cells were taken as controls. Effect of low temperature on FAF-SWCNT uptake in terms of % FAF-SWCNT positive B and T cells (panel A) or MFI of FAF-SWCNT uptake (panel B) ± SEM are depicted in top two panels. In panels C to F, effect of various inhibitors of membrane uptake on FAF-SWCNT uptake is similarly depicted. Control and LPS/SEB activated spleen cells were treated with different inhibitors of cellular uptake [chlorpromazine hydrochloride (10 µg/ml), cytochalasin D (4 µM), wortmannin (100 nM) and filipin (1 µg/ml)] for 1 hour at 37C. Following which FAF-SWCNTs (10 µg/ml) were added to the cells and incubation continued for 4 hours at 37C. Cells were harvested, washed and stained with anti-mouse CD19 and anti-mouse CD3 antibody (for gating on B and T cells) and uptake of FAF-SWCNT by gated B and T cells was determined on flow cytometer. All data points represent mean ± SEM of three replicate experiments. Significance values were calculated for the effect of low temperature and transport inhibitors within control and activated B and T cell populations. *p<0.05, **p<0.01, ***p<0.005.

Discussion

T and B lymphocytes play a central role in adaptive immunity. Antigens presented in association with MHC molecules by antigen presenting cells, activate specific T-helper lymphocytes.[39,40] B cell receptors comprising antibody molecule bind to native antigen and for activation require a second signal through specific antigen reactive T helper cells.[41] In both cases, active proliferation and clonal expansion of T and B cells follow that is required for the generation of adaptive immune responses. T and B cells in lymphoid organs may thus exist either in a resting un-activated phase or as activated cells undergoing rapid proliferation. The current investigation was aimed at determining if and how the uptake of carbon nanotubes differs in resting and activated B and T lymphocytes. It is important to understand the interactions between resting and activated lymphocytes and the carbon nanotubes since the latter are increasingly being explored as carriers in drug targeting studies in vivo.[42-45] A clear insight in the differential uptake of AF-SWCNTs would help in devising strategies to target activated cell populations. In addition, activated lymphocytes have been likened to rapidly proliferating tumor cells[46-47] and data with respect to the interactions between activated lymphocytes and carbon nanotubes may provide useful insight into the interactions of carbon nanotubes with activated/proliferating cells in general.

Bacterial lipopolysaccharide (LPS) was used to activate B cell whereas staphylococcus enterotoxin B (SEB) was used to activate T cells. It should be noted that whereas LPS activates all B cells,[36] SEB is a super-antigen that activates a subpopulation of T cells that have a specific TCR β-chain capable of binding the SEB.[37] Nevertheless, both LPS and SEB induced marked proliferation in B and T cells respectively, and in both cases, activated cells took up much more AF-SWCNTs as compared to the control cells. It is important to ask whether the enhanced FAF-SWCNT uptake in activated B and T cells is just a consequence of cell division induced by LPS and SEB. Dividing B and T cells in activated cultures may pass through multiple stages of cell division that may be associated with enhanced membrane permeability. We used the tracking dye CFSE to gate on dividing and non-dividing B/T cells in activated cultures and found that dividing cells indeed had the maximum uptake of FAF-SWCNTs. Nonetheless, non-dividing cells in activated cultures also had FAF-SWCNT uptake that was significantly higher than that inresting cells. Thus enhanced FAF-SWCNT uptake in activated B and T cell preparations is not a consequence of cell division activity alone.

Confocal microscopy as well as z-sectioning studies further revealed that the localization of AF-SWCNTs was essentially confined to the cell membrane in case of resting B and T cells whereas for activated B and T cells the AF-SWCNTs were localized throughout the cellular cytoplasm. It seems therefore that in resting B and T cells, FAF-SWCNTs may predominantly adhere to cell membranes but active transport across the membrane may only be triggered by cell activation. Our results also clearly show that activated T and B cells not only have substantially higher intake of AF-SWCNTs but also cause significantly higher cytotoxicity in activated cells. Substantially higher cellular intake as well as cytotoxicity opens up the possibility of using AF- SWCNTs for targeting and killing activated B and T cells in vivo. AF-SWCNTs treatment could be beneficial in cases where a run-away immune activation may need to be curtailed, as in auto- immune diseases and in cases where transformed B/T cells result in actively proliferating leukemia. In such situations however, it is necessary to show that in a mixture of resting and activated lymphocytes (as would be the case in vivo) the AF-SWCNTs are specifically targeted to activated cells. Our results in Figure 6 clearly shows that in mixtures of resting and activated cells, AF-SWCNTs were specifically targeted to activated cells.

Mechanism of uptake of AF-SWCNTs in resting and activated B and T cells is not clear. Our results of a partial inhibition of uptake at low temperature suggests that active transport of the nanotubes may at least partially be responsible for internalization of AF-SWCNTs. In Figure 7, we have tested the effect of four different types of inhibitors of membrane transport on uptake of AF-SWCNTs by resting and activated B and T cells. A qualitative difference in the mechanism of AF-SWCNT uptake between activated B and T cells was suggested by the fact that none of the inhibitors we tried had any effect on enhanced uptake in activated B cells whereas, in activated T cells, Chlorpromazine (inhibitor of clathrin mediated transport) and Wortmannin (inhibitor of micro-pinocytosis) significantly reduced the uptake of AF-SWCNTs. In resting B and T cells a mild inhibition of uptake of AF-SWCNTs was seen with all inhibitors. It is likely that the uptake of carbon nanotubes may proceed through a variety of mechanisms. Further work would be needed to elucidate the mechanisms that participate in the uptake of carbon nanotubes by resting and activated lymphocytes. However, substantial enhancement in the uptake of AF-SWCNTs by activated B/T cells opens up the possibility of using this agent for targeting activated lymphocytes.

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