Chapter 7

Natural Killer Cells Interaction with Carbon Nanoparticles

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Additional information is available at the end of the chapter

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Abstract

The increased use of nanomaterials for biomedical purposes has warranted the need to introspect their toxicological properties and assess their utility to human health, particularly the immune system. Natural killer (NK) cells hold a pivotal position in innate immunity and serve as first line of defense against foreign bodies. Acid functionalized Carbon nanotubes (CNTs) that easily polydisperse in aqueous solution and could be coupled with fluorescent molecules were used to study the effect of carbon nanoparticles on NK cells *in vitro* and *in vivo*. Flow cytometry-based assays were used to study the effect of CNTs on various physiological parameters of NK cells, such as cell recovery, apoptosis, cell cycle, and generation of reactive oxygen species. A downregulation of the cytotoxicity of IL-2-activated murine NK cells was observed in the presence of acid-functionalized CNTs. The mechanistic basis of this downregulation was studied by assessing markers of NK cell activation (CD69), generation (NLK1.1), degranulation (CD107a) and apoptosis (annexin V assay). This chapter provides a blueprint for assessing the effect of carbon nanoparticles on NK cells. The assays mentioned in this chapter can be extrapolated to study the effect of other nanoparticles on different cell types as well.

Keywords: NK cell cytotoxicity, carbon nanotube, flow cytometry, apoptosis, NK cell degranulation, YT-INDY

1. Introduction

Carbon nanoparticles (CNPs) have size less than 100 nm in at least one dimension and can be engineered in allotropic forms, such as nanodiamonds, fullerenes, nanobuds, and nanotubes. Each of these CNPs exhibits unique physicochemical properties and by virtue of their extremely low size can effectively interact with cells and tissues. Carbon nanoparticles, specifically carbon nanotubes (CNTs), are being tested for their potential use in the field of

nanomedicine including medicinal chemistry, imaging, vaccine delivery, etc. [1]. How these CNPs affect the living systems and the risks or benefits associated with their environmental, occupational, or therapeutic exposure is a matter of active research [2]. This chapter focuses on the interaction of an important component of innate immunity, the natural killer cells, with carbon nanotube.

1.1. Natural killer cells

Natural killer (NK) cells are important effector cells of the innate immune system and constitute about 5–15% of peripheral blood lymphocytes [3]. NK cells originate from lymphoid progenitors in the bone marrow and require IL-15-mediated signaling for development and survival [4]. NK cells can be identified through a set of markers, such as NK1.1 in mice or CD56 and CD16 in humans [5, 6]. The absence of CD3 on NK cells is a useful marker to differentiate between NK and natural killer T cells (NKT) [7].

NK cells kill syngeneic or allogeneic cells through mechanisms that require neither a prior sensitization with target cells nor the presentation of antigen in association with MHC-I. NK cell functions through an array of germline-encoded inhibitory or activating receptors that recognize MHC-I expressed in steady state on normal cells or altered ligands on dysregulated cells, respectively. Killer inhibitory receptor (KIR) in humans, the lectin-like Ly49 molecules in mice, and CD94/NKG2A heterodimers in both species detect MHC-I molecules on normal cells [8]. Perturbations in expression of MHC-I molecules on viral-infected cells or malignant transformed cells lead to loss of inhibitory signals causing activation of NK cells. Additionally, NK cells also require signaling through activating receptors, such as NKp30, NKp44, NKp46, NKp65, and NKp80 to trigger effector functions, such as cytokine production and cytolytic activity. Stimulation with inflammatory cytokines, such as IL-2, IL-15, IL-12, or IL-18 evokes differentiation into effector NK cells. CD16 or constant Fcγ-receptor IIIa (FcγRIIIa) exerts antibody-dependent cell-mediated cytotoxicity (ADCC) against various antibody coated cellular targets, leading to the exocytosis of perforin and granzyme-loaded vesicles. By integrating activating and inhibitory signals, NK cells contribute to the elimination of stressed cells expressing modified motifs while sparing healthy cells [9].

Upon recognition, target cells can get killed by NK cells through one of two pathways: either via the perforin and granzyme secretion pathway or via membrane-bound death receptors. NK cells store preformed perforin and granzyme in secretory vesicles that when triggered by activating signals causes formation of microtubule-organizing center (MTOC) which guides the vesicles, containing perforin and granzyme, in a directed way toward the target cell to prevent damage to bystander cells. Perforin forms pores in the target cell membrane, disrupts membrane integrity, and allows the entry of the apoptosis-inducing granzymes. The importance of the lytic perforin/granzyme pathway is evident in perforin knockout animals that exhibit lesser efficiency in ADCC or tumor control after transplantation of tumor cells. Perforin-/granzyme-containing cytoplasmic vesicles express CD107a or lysosomal-associated membrane protein (LAMP-1). Upon degranulation, CD107a is transferred transiently on the surface of NK cells and protects NK cells from damage from their own perforin or granzyme release [10].

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NK cytotoxicity via its membrane-bound death receptors occurs upon binding with ligands, expressed on target cells. Receptor-ligand pairs, such as Fas-FasL, TNFR-TNF, and TRAILR-TRAIL, induce recruitment of various adaptor proteins leading to the formation of the death-inducing signaling complex (DISC). Subsequently, the caspases 8 and 10 get activated via proteolysis and initiate apoptosis. In addition to the cytotoxic response, NK cells are important sources of various cytokines, such as IFN-γ, TNF-α, or IL-10 and of chemokines, such as CCL3/CCL4/CCL5 or CXCL8. Natural killer (NK) cells basally express high levels of the signal transducer and activator of transcription 4 (STAT4) and produce the cytokine gamma interferon (IFN-γ). Type 1 interferons could potentially activate STAT4 and promote IFN-γ expression; however, concurrent elevated expression of STAT1 negatively regulates access to this pathway. IFN-γ due to its pleiotropic functions is considered to be the signature cytokine of NK cells [11]. IFN-γ has been shown to have antiproliferative effects on tumor cells and exert anti-angiogenic activity. A combination of TNF-α and IFN-γ has been shown to trigger tumor senescence and activates macrophages and dendritic cells [12]. Cytokine secretion is mediated via recycling endosomes using a distinct pathway from cytolytic vesicles, which allows for differential regulation.

2. Carbon nanotube

Carbon nanotubes resemble rolled-up tubes of graphite sheet of sp^2 hybridized carbon atoms. A single cylindrical form of CNTs is designated as single-walled nanotubes (SWCNTs) that are 0.4–3.0 nm in diameter and up to 1000 nm in length. CNTs display a unique combination of extraordinary mechanical, thermal, and electronic properties. CNTs, by virtue of their exceptional high aspect ratio (surface area to volume ratio), can carry high amounts of ligands on the outer as well as in inner surface of their tubular backbone for tissue-/cell-specific drug delivery. These are therefore conceptualized as nano-bullets capable of carrying different drugs for targeting multiple microorganisms or diseases simultaneously. However, the use of CNTs in biological system poses practical problems due to their inherent tendency to form aggregate as a result of high intermolecular hydrophobic force arising out of unit graphene rings. Within the aqueous phase of the biological system, the hydrophobic behavior of CNTs could result not only in poor biodistribution but also pose a serious challenge due to agglomeration and cause tissue lesions or granulomas. In order to circumvent hydrophobicity of CNTs so that it is rendered biocompatible, their backbone can be chemically modified with functional groups or linkers that impart solvable properties to CNTs. This process is known as functionalization of CNTs.

2.1. Acid functionalization of SWCNT

SWCNTs may be acid functionalized by suspension in sulfuric acid and nitric acid and subjecting to high-pressure microwave as described before [13–15]. As a result the side walls of SWCNTs are decorated with high density of various oxygen-containing groups (mainly carboxyl groups). The carboxyl groups impart negative charge to SWCNTs, which facilitate the separation of nanotube bundles into individual tubes and enhance their dispersibility in

aqueous solutions. The carboxyl groups represent useful sites for covalent coupling of molecules, through the creation of amide or ester bonds, thereby facilitating further addition of wide range of bifunctional linker molecules, nucleic acids, peptides, or other nanoparticles, such as polyethylene glycol (PEG) [16].

The process of acid functionalization of CNTs is outlined in **Figure 1A**. Briefly, SWCNTs were suspended in equimolar ratio of nitric acid (HNO_3) and sulfuric acid (H_2SO_4) in high-pressure microwave digester that provide 450 W power for 3 min, resulting in an internal pressure of 20 ± 2 psi and temperature of 138–150°C. The suspension was cooled and dialyzed in excess milli-Q water till it attained neutral pH. Dialyzed suspensions of acid-functionalized SWCNTs (AF-SWCNTs) were lyophilized, weighed, and resuspended at the desired concentration in phosphate buffer saline (PBS) or water.

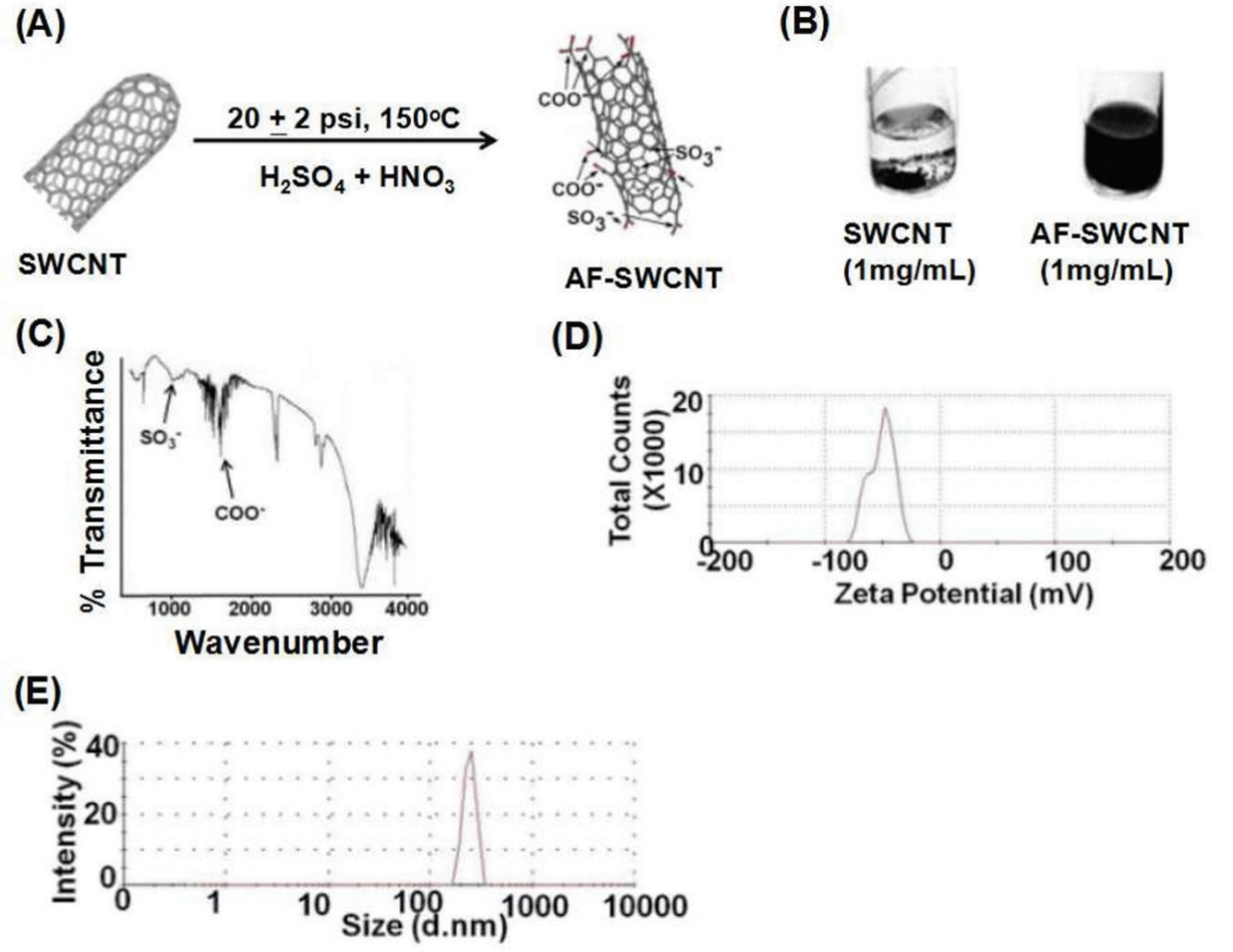


Figure 1. Functionalization of SWCNT and characterization of acid-functionalized SWCNT (AF-SWCNT): schematic overview of the process of acid functionalization of SWCNT is depicted in Panel A. Functionalization of SWCNT in the presence of equimolar H_2SO_4 and HNO_3 generates carboxyl and sulfonate groups on the backbone of SWCNT. Panel B shows that AF-SWCNT (1 mg/mL) homogenously dissolves in aqueous solution as compared to SWCNT. The physiochemical properties of AF-SWCNT were tested using fourier transmission infrared (FTIR) spectroscopy and Zetasizer. FTIR spectra of AF-SWCNT showed distinct bands in the range 1740 and 1350 cm^{-1}, characteristic of carboxyl and sulfonate groups, respectively (Panel C). These groups are negatively charged and affect the electrical properties of AF-SWCNT. Data obtained from Zetasizer showed that the mean charge and size of AF-SWCNT were −55 mV (Panel D) and 350 nm (Panel E), respectively.

In comparison to SWCNTs that agglomerate in aqueous solution, AF-SWCNTs (1 mg/mL) remain stably suspended. The physiochemical properties of AF-SWCNTs are influenced mainly by the duration of acid functionalization. Increasing the duration of functionalization reaction resulted in smaller sized particles and decreased recovery of functionalized nanotubes due to oxidative degradation. The recovery of AF-SWCNTs was relatively better with lower acid concentrations; the size and charge distribution of the resultant particles were comparable. Particles generated under constant temperature for different time durations did not differ significantly in their size or zeta potential. The zeta potential provides an estimate whether the particles within a liquid will tend to flocculate or not. A high positive or negative zeta potential of particle corresponds to greater dispersibility within the liquid, while values close to zero correspond to greater aggregation potential. Zeta potential of the AF-SWCNTs depends on the suspension media. AF-SWCNTs suspended in water had the higher zeta potential than PBS followed by culture media. Greater dispersion of AF-SWCNTs in water is due to formation of electrical double layer in which ionic accumulation of few-angstrom thick prevents particle aggregation. For details of physiochemical properties of AF-SWCNTs, see **Figure 1C–E**.

2.2. Attachment of fluorescent probes to AF-SWCNTs

CNTs, due to their nanosized structure, cannot be visualized using normal light microscope. The carboxylic groups generated on the AF-SWCNTs were exploited to attach fluorescent probes to the particles for the purpose of studying their uptake by cells [17]. **Figure 2A** shows the schematic overview of attachment of fluorescent probes to AF-SWCNTs. In our experiment, polydispersed AF-SWCNTs were treated with 1-ethyl 3-(3-dimethyl aminopropyl) carbodiimide (EDAC) and N-hydroxysuccinimide (NHS) in order to get succinimidyl intermediate. The mixture was continually shaken for 2 h and dialyzed in water using 3 kDa cutoff Centricon to remove excess NHS, EDAC, and urea by-product. AF-SWCNTs thus activated were incubated with Alexa Fluor 488/633 hydrazide in 1:1 ratio in the dark with continuous mixing for 12 h, followed by dialysis to remove free dye. Attachment of fluorescent probe to AF-SWCNTs can be confirmed using spectrophotometer or flow cytometer.

The interaction of fluorescently tagged AF-SWCNTs (FAF-SWCNTs) with cells could be easily quantified using flow cytometer (**Figure 2B**), and their localization within the cell can be visualized using confocal microscopy (**Figure 2C**). Although earlier studies on the uptake and localization of CNTs could be performed on fixed cells using transmission electron microscope (TEM), preparation of fluorescently tagged AF-SWCNTs was a significant step in this direction because it enabled us to directly observe the interaction of CNTs with live cells. Thus, FAF-SWCNTs in conjugation with organelle markers provided an important tool to observe the effect of CNTs on the cellular process like exocytosis, actin-myosin assembly, cell division, etc. For example, FAF-SWCNTs used in combination with LysoTracker and MitoTracker can be used to observe the actual interaction of CNTs with these organelles and the attendant effects on exocytosis and mitochondrial potential, respectively. Our studies showed that activated NK cells internalize higher amounts of AF-SWCNTs than resting NK cells. Our studies further indicated that internalized AF-SWCNTs are essentially localized in the cytoplasm of NK cells. Previous studies by our group on the uptake of diesel exhaust particles (another category of carbon nanoparticles) by macrophages and alveolar epithelial cells showed that

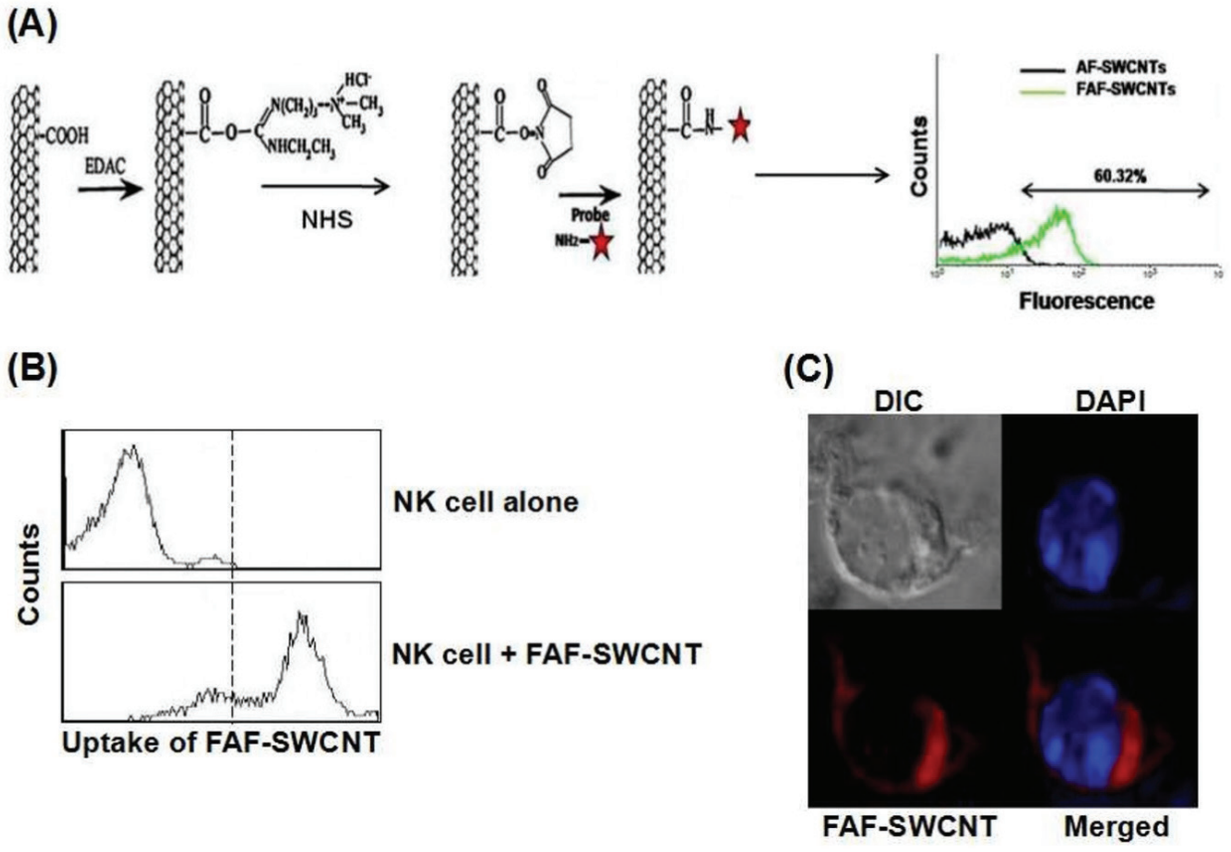


Figure 2. Attachment of fluorescent probe to AF-SWCNT. Panel A shows the schematic diagram of preparation of fluorescent-coupled AF-SWCNT (FAF-SWCNT). The carboxyl group provided a pivotal point to which amide containing fluorescent molecules could be attached. Flow cytometric data shows that 60% of AF-SWCNT particles got tagged with the fluorescence probe (denoted by green line). The interaction of NK1.1$^+$ cells with AF-SWCNT was studied by incubating the IL-2-activated splenocytes with FAF-SWCNT. Panel B shows that NK1.1$^+$ sorted cells sequestered FAF-SWCNT and generated positive signal for fluorescence. The localization of FAF-SWCNT in NK cells was visualized using confocal microscopy. NK1.1$^+$FAF-SWCNT$^+$ cells were isolated using fluorescence-activated cell sorter and cultured on poly-L-lysine coated cover slips. Cells were fixed using 4% paraformaldehyde, incubated with DAPI (0.2 mg/mL) and examined microscopically. Panel C shows the DIC, DAPI, and merged image of FAF-SWCNT in NK1.1$^+$ cells.

active uptake was blocked in the presence of Cytochalasin D, an inhibitor of actin-myosin assembly system [17]. Internalization of AF-SWCNTs has also been demonstrated in erythrocytes that lack membrane phagocytic functions [18]. Internalization of AF-SWCNTs in NK cells may therefore involve active transport, or by the virtue of their long needle-like structure, these nanoparticles may pierce cell membranes and enter into the cytoplasm.

3. Interaction of CNTs with NK cell

CNTs act as adjuvant or haptens and allow formation of protein bio-corona on their surface. When proteins unfold, they reveal hidden epitopes that may act as nanomaterial-associated molecular patterns (NAMPs) [19]. These molecular signatures are recognized by pattern recognition receptors (PRPs) present on the surface of innate immune cells. The activation of

PRPs may induce adaptive immune system causing inflammation, allergic reactions, complement activation, susceptibility to diseases, or autoimmune diseases [20, 21]. The prospects of CNTs as a magic bullet in cancer therapy are due to their ability to act passively or actively at site of tumor or for improving bioavailability of insoluble drugs. Besides, CNTs have unique anisotropic and spectroscopic properties that make them suitable for detection and radiation-guided ablation of tumors.

The ability of the immune system to successfully eliminate cancer cells or viral-infected cells is mediated mainly by cytotoxic T cells and NK cells. Therefore the inadvertent interaction of CNTs with NK cells in the body, particularly at site of tumor, cannot be ignored. This situation warrants extensive studies to assert that CNTs have minimal effect on the effector functions of NK cells so that the benefits achieved by the use of CNTs in cancer therapy are not outweighed by the toxic effect of CNTs on NK cells itself. The effects of nanoparticle on NK cell cytotoxicity should be examined to determine whether there is an association between NK cell activity and nanoparticle treatment. A decreased NK cell activity is often associated with chronic fatigue immune dysfunction syndrome (CFIDS), characterized by acute and chronic conditions that predispose individuals toward an immunocompromised state leading to AIDS [22].

Although the mechanistic details of NK-mediated killing of target cells is well established, little is known in literature about the interaction of NK cells with CNTs, and hence there is no consensus to evaluate immunomodulatory effects of CNTs on NK cells. We have established three models to assess the interaction of CNTs with NK cells and evaluate the effect of CNTs on effector function of NK cells *in vitro* and *in vivo*. *In vitro* assessment of the effect of CNTs on NK cells was performed using YT-INDY human NK cell line and IL-2-activated mouse spleen cells. *In vivo* effects of CNTs were studied in C57BL/6 inbred mice treated with poly I:C, a synthetic analogue of viral RNA. We have extensively used flow cytometry to assess these parameters because it provides accurate, reproducible, and quick quantitative estimation of cell subpopulations in the mixed culture of cells.

3.1. *In vitro* assessment of effect of CNTs on a NK cell line

YT-INDY, a NK cell line of human origin, proliferates continuously *in vitro* without being supplemented with cytokines or conditioned medium. *In vitro* assessment of the effect of CNTs on YT-INDY cells provides us preliminary estimation of dosage that may be deemed fit to explore on actual NK cells derived from primary cell cultures.

3.2. Cell viability assay

Cell viability assays are used for screening nanoparticles in order to determine if these affect cell proliferation or show direct cytotoxic effects causing cell death. Trypan Blue assay was used for staining dead cells, and viability was determined by counting the unstained cells microscopically. As is evident from **Figure 3A**, exposure of AF-SWCNTs resulted in decrease of cell recovery although the effect was marginally less in SWCNT treatment. YT-INDY, which has a doubling time of nearly 42 h, shows decreased proliferation rate in the presence of SWCNTs, the effect being more enhanced with AF-SWCNTs.

Trypan Blue staining does not distinguish between the healthy cells and the cells that are alive but losing cell functions. MTT is a water-soluble yellow dye that is readily cleaved and converted into insoluble purple formazan by the activity of mitochondrial dehydrogenase of living cells [23]. Results in **Figure 3B** clearly indicate that AF-SWCNTs above 25 μg/mL reduce cell viability, whereas SWCNTs show the same effect above 50 μg/mL.

Reduced cell viability in the presence of CNTs could be attributed not only to reduction in proliferation of cells but also due to accumulation of dead cells in the culture. The activity of cytoplasmic enzyme, such as lactate dehydrogenase (LDH), released upon plasma membrane damage, can be detected in culture supernatant and correlates with the proportion of lysed or dead cells [23]. Results in **Figure 3C** show that AF-SWCNTs caused increase in LDH release from YT-INDY cells. These results of LDH assay complement the data obtained from MTT assay.

3.3. Effect of CNTs on cell cycle

Increase in doubling time of YT-INDY cells in the presence of AF-SWCNTs pointed to a possible alteration in cell cycle. The effect of SWCNTs or AF-SWCNTs on cell cycle of YT-INDY

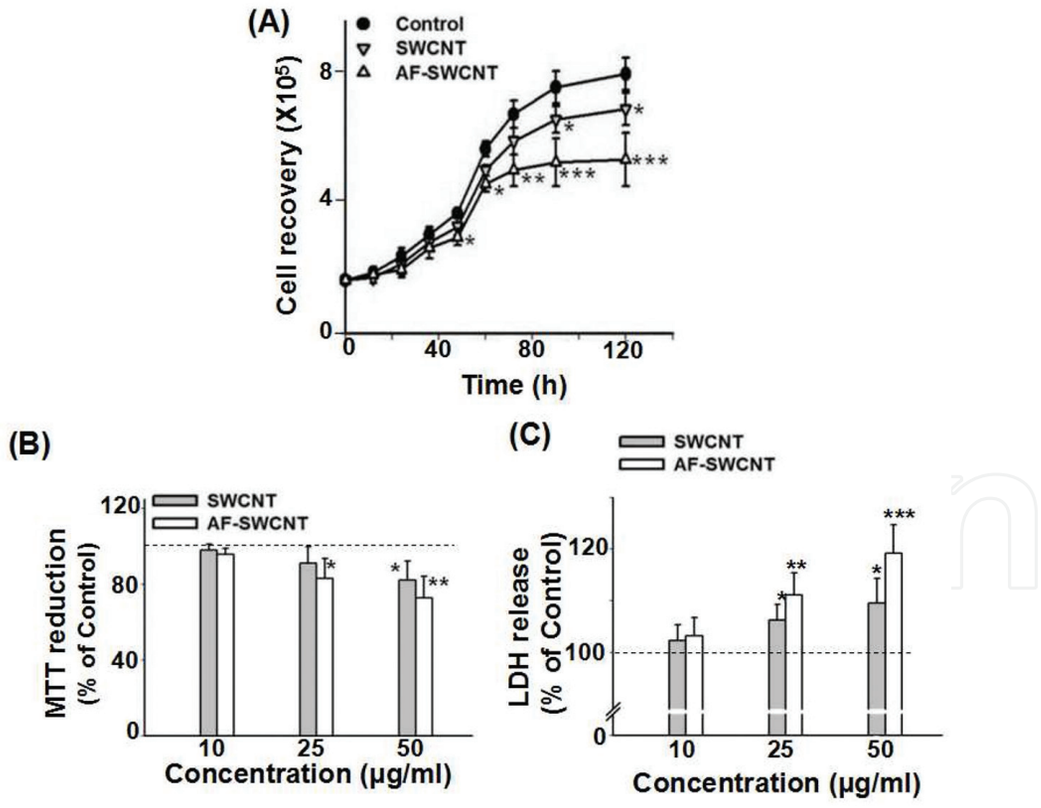


Figure 3. Effect of SWCNT and AF-SWCNT on viability of NK cells *in vitro*. YT-INDY cells were cultured in the presence of 10, 25, and 50 μg/mL of SWCNT or AF-SWCNT. Panel A shows the cell recovery of viable cells using Trypan Blue exclusion assay. Panel B shows the results of MTT assay in terms of percent cell recovery. Panel C depicts the percent LDH released from nonviable cells in cultures treated with 10, 25, and 50 μg/mL of SWCNT or AF-SWCNT. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$ by Student's *t* test.

cells was examined. A fourfold decrease in proportion of cells in S-phase indicated that AF-SWCNT treatment caused cell cycle arrest in YT-INDY cells (**Figure 4**).

3.4. Flow cytometric assessment of apoptosis using annexin V/7-AAD assay

Cellular toxicity evokes sequential steps leading to apoptosis and ultimately causing cell death. Apoptotic and necrotic response of YT-INDY to AF-SWCNTs was examined by using annexin V/7-AAD stainings described before [24]. Results in **Figure 5A** show that the treatment of YT-INDY cells with SWCNTs or AF-SWCNTs led to an increase in apoptotic cell by twofold and threefold, respectively.

Apoptosis can be brought about by a loss of calcium (Ca^{2+}) homeostatic control but can also be finely tuned, positively or negatively, by more subtle changes in Ca^{2+} distribution within intracellular compartments. An aberrant increase in intracellular calcium can trigger initiation of apoptosis. The levels of calcium in the cell have to be kept at an optimum level to maintain cellular homeostasis. Intracellular levels of calcium can be monitored using Fluo-3AM dye. The levels of calcium can be examined by flow cytometrically. Mean fluorescence intensity (MFI) values for Fluo-3AM signals gave an estimate of average levels of calcium per cell in the population of cells. AF-SWCNT treatment caused increased intracellular calcium indicating that they caused off of the signaling process that regulates apoptosis (**Figure 5B**).

3.5. Generation of reactive oxygen species

Reactive oxygen species (ROS) are continuously generated in living cells and play a key role in cellular homeostasis. ROS is principally produced and regulated in mitochondria and may affect the mitochondrial health and activity in a cell. The production of ROS in the cell is regulated as excessive ROS production leads to cascade of signaling processes that may trigger cell

Control	SWCNT	AF-SWCNT	
44	54	88	G1
38	33	9	S
18	13	3	G2/M

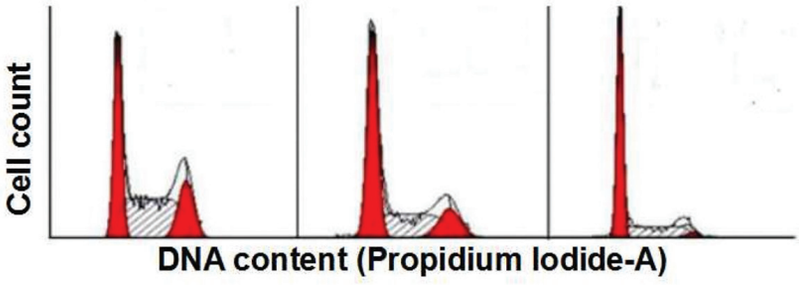
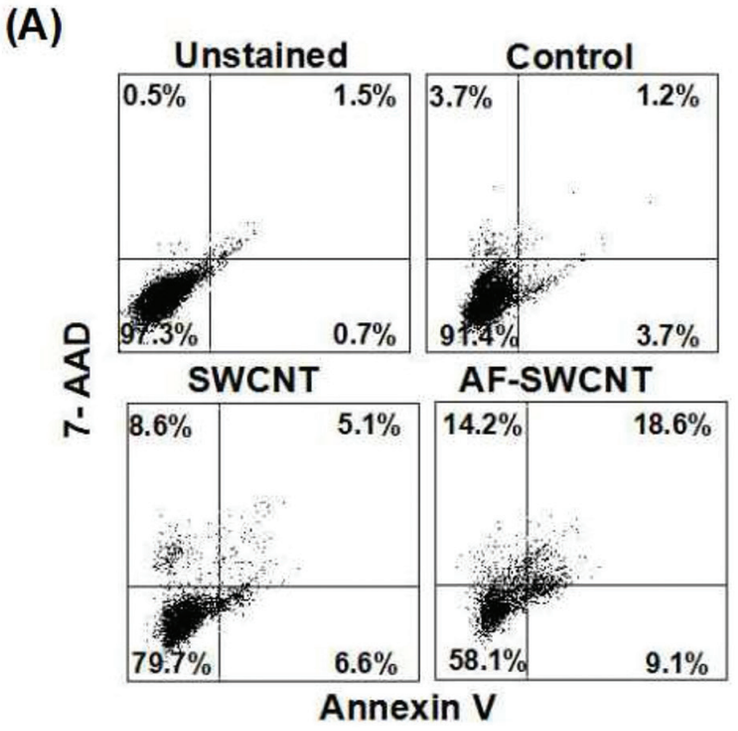


Figure 4. Effect of SWCNT and AF-SWCNT on cell cycle of YT-INDY cells. YT-INDY cells were cultured in the presence of SWCNT or AF-SWCNT. Cells were harvested, fixed with 70% ethanol, and treated with RNase. The fixed cells were stained with propidium iodide (2 μg/mL), and cell cycle was assessed flow cytometrically. Data shows percentage of cells in G1, S, and G2/M stages of cell cycle.



(B)

	Control	SWCNT	AF-SWCNT
Fluo-3AM (MFI)	100.6	114.7	120.1

Figure 5. Effect of SWCNT and AF-SWCNT on apoptotic response in YT-INDY cells. YT-INDY cells were cultured in the presence of 50 μg/mL of SWCNT or AF-SWCNT for 48 h. Cellular apoptosis was assessed flow cytometrically by staining cells with annexin V and 7-AAD dye. Percent dead, necrotic and apoptotic cells obtained from cultures treated with SWCNT or AF-SWCNT are shown in Panel A. Increased levels of calcium in cells as an initiator for apoptotic response are assessed using Fluo-3AM dye. Intracellular levels of calcium in YT-INDY cells treated with SWCNT or AF-SWCNT (50 μg/mL) are assessed flow cytometrically (Panel B).

to undergo apoptosis, cell cycle arrest, and modulation in cytokine production [25]. Results shown in **Figure 6** indicate that control cells produce basal levels of ROS (MFI 198). A 63% increase in ROS generation is observed in the presence of AF-SWCNTs as compared to a 21% increase in the presence of SWCNTs. Generation of ROS is an indication of cellular stress that corresponds to greater toxic potential of AF-SWCNTs as compared to SWCNTs.

3.6. Mitochondrial membrane potential

Generation of ROS causes disturbance in mitochondrial membrane potential ($\Delta\psi$). Mitochondrial potential is important for the proper functioning of mitochondria [26]. AF-SWCNTs being negatively charged could modulate the potential of mitochondria once it is taken up by cells. Effect of SWCNTs and AF-SWCNTs on mitochondrial potential in

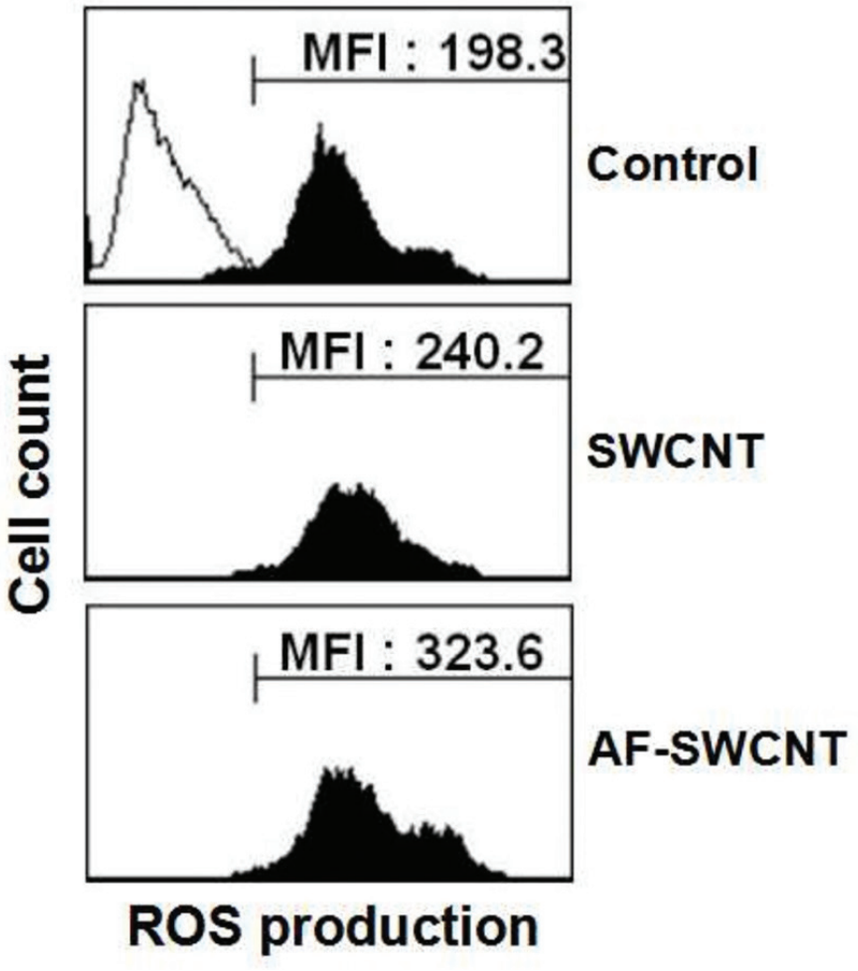
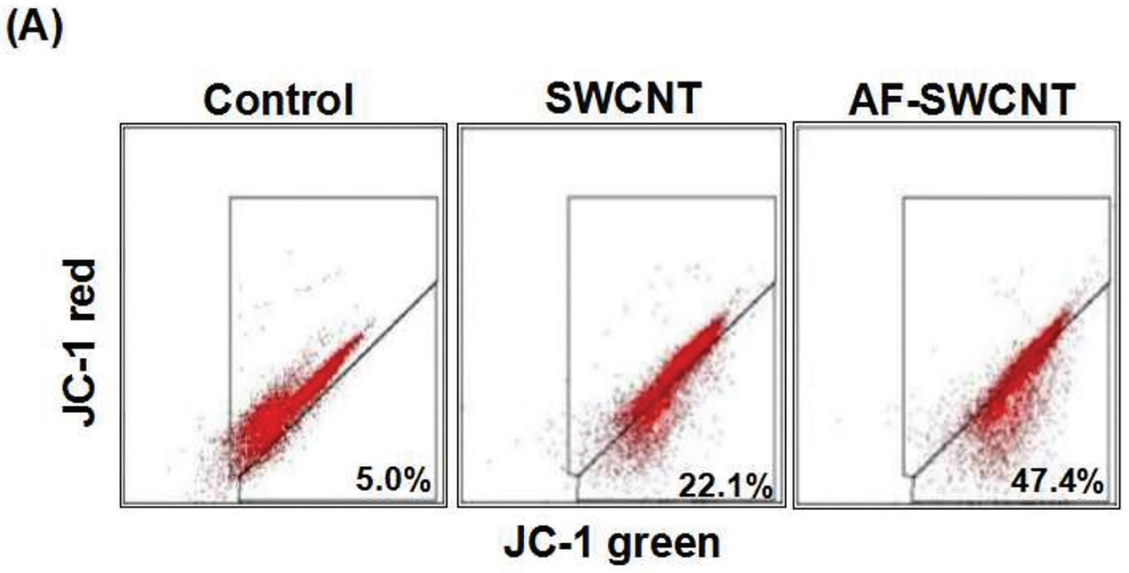


Figure 6. Generation of reactive oxygen species in YT-INDY cells treated with SWCNT and AF-SWCNT. YT-INDY cells were cultured with or without SWCNT or AF-SWCNT (50 μg/mL) for 24 h and ROS levels assessed by using H_2DCFDA dye. Fluorescent signals due to basal level of ROS in control cells are depicted as open histogram.

YT-INDY cells was examined using dyes: JC-1 and MitoTracker. JC-1 is a dimeric molecule which is converted to a monomeric form upon change in mitochondrial membrane potential. Change of JC-1 from dimeric form to monomeric form can be estimated by a shift from red fluorescence to green fluorescence. Increase in green fluorescence is an indicator for decrease in mitochondrial potential. MitoTracker red stain mitochondria in live cell and its accumulation are dependent upon membrane potential. The MFI of MitoTracker red emission therefore gives an estimate of the overall accumulation of MitoTracker red in live cells.

Results in **Figure 7** show that the treatment with SWCNTs or AF-SWCNTs resulted in an increase in JC-1 green fluorescence to 22 and 47%, respectively, indicating a significant decrease in mitochondrial potential. The corresponding reduction in MFI values of MitoTracker red fluorescence of SWCNTs and AF-SWCNTs culture were 20 and 54%, respectively.

Taken together, toxicity of SWCNTs and AF-SWCNTs was adjudged by a significant ($p < 0.05$) decrease in cell recovery, increased apoptosis, S-phase arrest in cell cycle, increased generation of ROS, poor mitochondrial health, and loss of cellular integrity.



(B)

	Control	SWCNT	AF-SWCNT
Mitotracker (MFI)	320.3	256.1	147.1

Figure 7. Estimation of mitochondrial potential in YT-INDY cells treated with SWCNT or AF-SWCNT. YT-INDY cells cultured with SWCNT or AF-SWCNT were harvested, centrifuged, and rinsed twice with PBS, resuspended in 5 mM JC-1 or 10 mM MitoTracker red in serum-free media. Fluorescence emission was analyzed flow cytometrically (JC-1 monomers, excitation wavelength 488 nm, emission filter 530/30 nm; JC-1 aggregates, excitation wavelength 488 nm, emission filter 585/42 nm). Decrease in red fluorescence or increase in green fluorescence is indicative of depolarization of mitochondria. Panel A shows the percent increase in green fluorescence of JC-1 in YT-INDY cells treated with SWCNT or AF-SWCNT. The uptake of mitochondrial dye, MitoTracker, depends on membrane potential. Data in Panel B shows the mean fluorescent intensity (MFI) of MitoTracker red in control and SWCNT- and AF-SWCNT-treated cells.

4. *In vitro* assessment of the effect of CNTs on splenic NK cells

Studies described above used NK cell lines for assessing the toxicity of various nanoparticles *in vitro*. This was followed by a detailed investigation into the effect of AF-SWCNTs on mouse spleen-derived NK cells.

4.1. NK cell cytotoxicity assay

While a basal level of NK cells exists in the mouse spleen, proliferation and activation of these cells can be induced *in vitro* by culturing with IL-2, IL-12, IL-15, or IL-18 [27]. NK cells achieve maximal activation after 3 days of activation with IL-2, and the activity gradually subsidizes

within 5 days [28, 29]. Effect of CNTs on basal as well as IL-2-induced NK response was examined *in vitro*.

Spleen cells (5×10^6/mL) obtained from C57BL/6 mice were treated with tris ammonium chloride buffer (ACK lysis buffer) to remove red blood cells (RBCs). Splenocytes, devoid of RBCs, were cultured with 500 U/mL IL-2 in complete medium (RPMI1640 + 10% FCS), with or without SWCNTs or AF-SWCNTs for 3 days. Control and activated spleen cells were washed, counted, and used as effector cells (E). YAC-1, a NK-sensitive murine lymphoma cell line, was used to examine the cytolytic potential of NK cells by using a 4 h ^{51}Cr release assay as described before [30, 31].

Lytic units (LU) per 10^7 effector cells were calculated from the E/T ratio versus percent lysis plots [32]. 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 one lytic unit.

Results in **Figure 8B** show that addition of SWCNTs or AF-SWCNTs during the chromium release assay had no significant effect on the killing of target cells by NK cells indicating that SWCNTs and AF-SWCNTs did not directly interfere with the E/T interaction in chromium release assay. A significant decrease in the NK activity by treatment with AF-SWCNTs is shown in **Figure 8A**.

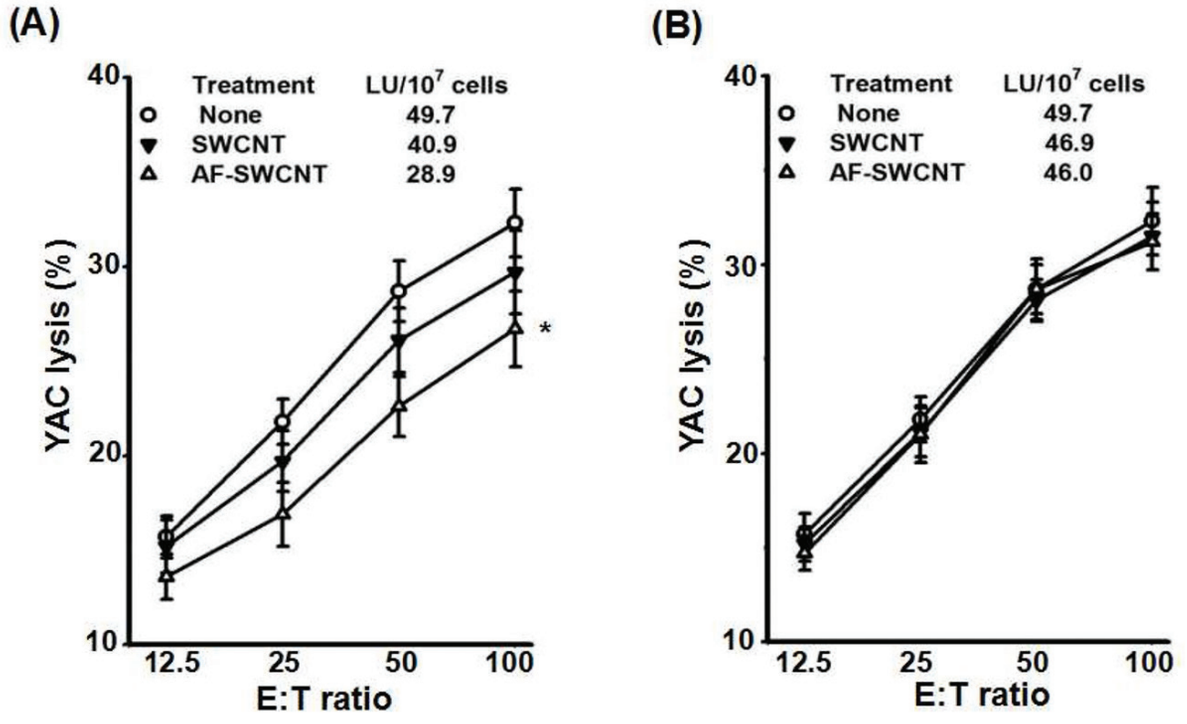


Figure 8. Effect of SWCNT and AF-SWCNT on the generation of NK cell response *in vitro*. NK cells were activated *in vitro* by culturing spleen cells with IL-2 in the presence or absence of SWCNT or AF-SWCNT (50 μg/mL). Anti-YAC-1 cytotoxic activity was assessed in a 4 h chromium release assay (CRA) at E/T ratios of 100, 50, 25, and 12.5 and lytic units/10^7 cells calculated (Panel A). Data in Panel B denoted the effect of addition of SWCNT and AF-SWCNT, during chromium release assay, on the cytotoxicity of NK cell response *in vitro*. *$p < 0.05$ by ANOVA.

4.2. Effect of CNTs on the recovery of IL-2-activated NK1.1⁺ cells *in vitro*

Suppression of IL-2-induced NK cell cytotoxicity by AF-SWCNTs could be due to a possible interference with the NK cell proliferation and/or activation process or a loss of NK cells due to toxic effect of AF-SWCNTs or both. In order to assess the possible toxic effect of SWCNTs and AF-SWCNTs on NK cells, recoveries of NK1.1⁺ cells from control and IL-2-activated spleen cultures were examined. Results in **Figure 9** indicate that the recovery of NK cells (percentage of NK 1.1⁺ cells by flow cytometry) in IL-2-activated spleen cell cultures declined by 17% if AF-SWCNTs were added to cultures.

4.3. Effect of SWCNTs and AF-SWCNTs on activated NK cells

Spleen cells express early activation marker CD69 on the surface as early as 12 h after *in vitro* stimulation with IL-2 [33]. Results in **Figure 10** show that in IL-2-treated spleen cell cultures, 53% of NK1.1⁺ cells expressed CD69 marker. Treatment with AF-SWCNTs significantly reduced the expression of CD69 on NK1.1⁺ cells by 23%.

Induction of apoptosis in NK cells activated by IL-2 in the presence of SWCNTs or AF-SWCNTs was also examined by using annexin V staining. Our results indicated that as compared to 11% apoptotic NK cells in control IL-2-activated spleen cells, the presence of SWCNTs and AF-SWCNTs increased the percentage of apoptotic cell to 17 and 22%, respectively (data not shown). Taken together, our results point to the possibility of decreased NK cell proliferation as well induction of apoptotic cell death resulting from exposure to AF-SWCNTs. In addition, expression of CD69, an early cell activation marker, was significantly lower in NK cells treated with AF-SWCNTs, indicating that the AF-SWCNTs interfered with NK cell activation process. Increased apoptosis of IL-2-activated NK cells indicates that activated NK cells are more prone to lysis than resting NK cells.

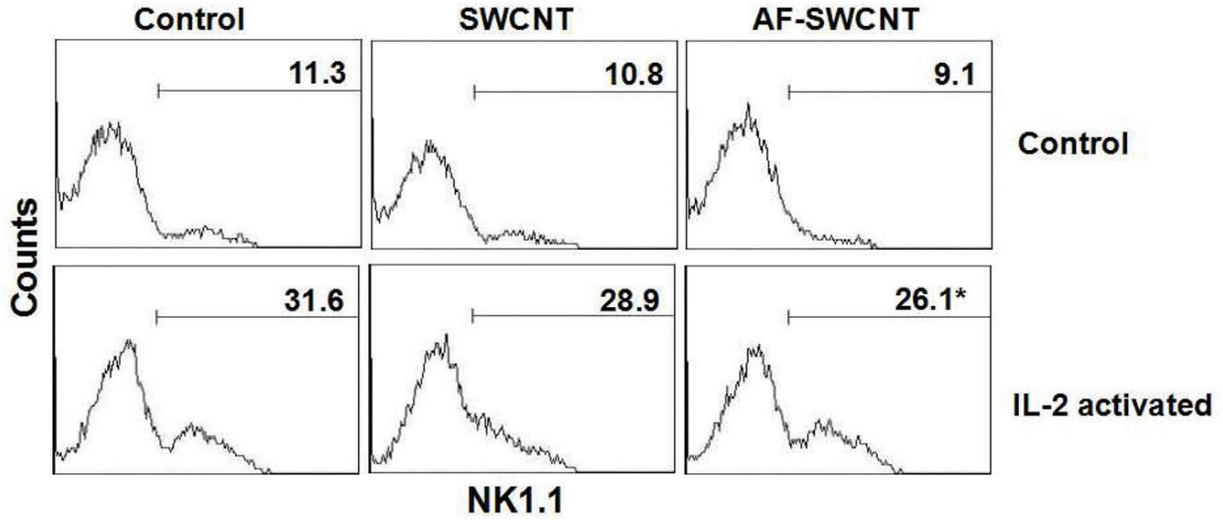


Figure 9. Effect of SWCNT and AF-SWCNT on recovery of NK1.1⁺ cells *in vitro*. Spleen cells activated by IL-2 in the presence and absence of 50 μg/mL of SWCNT or AF-SWCNT were stained with NK1.1 mAb and analyzed on flow cytometer. Illustrative flow cytometry histograms for percent NK cell recovery in control and activated NK cells have been shown. *$p<0.05$, by Student's *t* test.

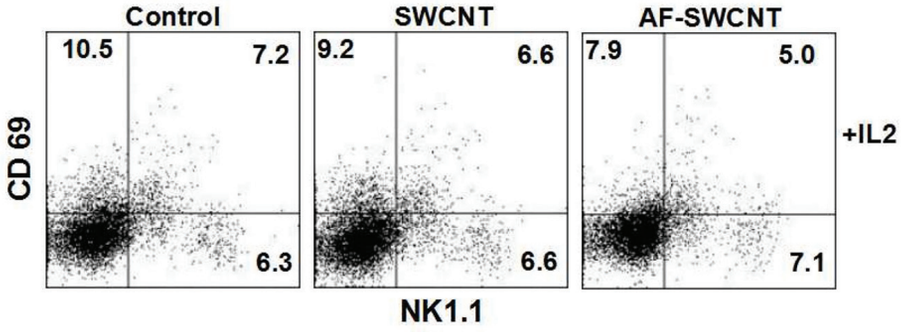


Figure 10. Effect of SWCNT and AF-SWCNT on the expression of CD69 activation marker on NK cells. Splenocytes cultured in the absence or presence of 500 U/mL of IL-2 were simultaneously treated with 50 μg/mL of SWCNT or AF-SWCNT. After 12 h, the splenocytes were harvested, double stained with antimouse NK1.1 and CD69 mAbs, and analyzed on a flow cytometer.

4.4. Effect of CNTs on effector functions of NK cells

NK cell cytotoxicity is mediated by release of cytotoxic granules like perforin or through Fas-FasL mechanism. Perforins are stored in preformed granules within the cytoplasm of NK cell and are released when NK cells are triggered by interaction with target cells. Granule release is correlated with the lysosomal marker CD107a (lysosomal-associated membrane protein or LAMP-1) [34].

Results in **Figure 11** show that addition of AF-SWCNTs to IL-2-activated culture resulted in downregulation in expression of CD107a on YAC cocultured $NK1.1^+$ cells from 57 to 44% (22% decline) indicating that AF-SWCNT treatment impaired the process of degranulation of activated NK cells, which may be a contributing factor in suppressing cell-mediated cytotoxicity seen in AF-SWCNT-treated NK cells.

FasL expression on IL-2-activated NK cells was also examined. Our results showed that 74% of IL-2-activated $NK1.1^+$ cells expressed FasL, and this significantly declined by 31% upon treatment with AF-SWCNTs (data not shown) (see **Figure 11**).

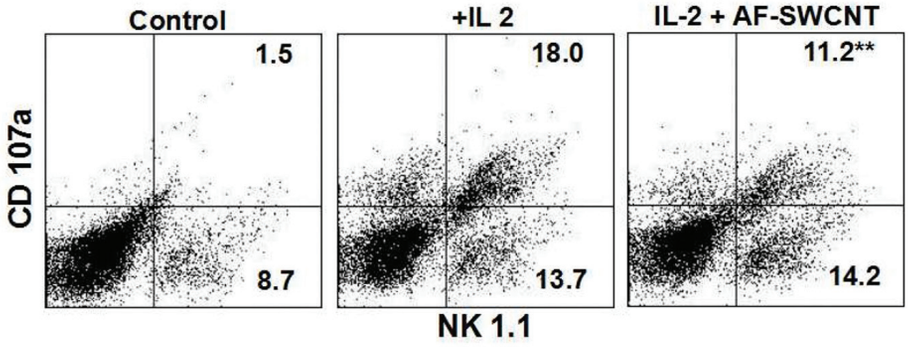


Figure 11. Effect of AF-SWCNT on NK cell degranulation. Splenocytes (2×10^6) cultured in the absence or presence of 500 U/mL of IL-2 were simultaneously treated with 50 μg/ml of AF-SWCNT. After 72 h, the activated splenocytes used as effector cells were harvested and cocultured with YAC-1 target cells to induce NK cell degranulation. The cells were stained with antimouse CD107a mAb and counterstained with antimouse NK1.1 mAb. **$p < 0.01$ by Student's *t* test.

5. *In vivo* assessment of the effect of CNTs on the NK cell in murine model

Effect of AF-SWCNTs was also examined on NK cell activation *in vivo*. For *in vivo* studies, mice were treated with poly I:C, a RNA analogue, which activates splenic NK cells. Poly I:C induces NK cell activation through the release of interferons [35–39]. The maximum activity of splenic NK cells upon stimulation with poly I:C occurs after 3 days of exposure [40, 41]. Intravenous treatment of AF-SWCNTs resulted in suppression of $NK1.1^+$ cells by 15% and reduction in NK cytotoxicity by 46% (data not shown). NK cell mediates cytolytic activity through release of cytokines-IFN-γ and TNF-α [42]. The effect of AF-SWCNTs was examined on expression of IFN-γ and TNF-α by coculturing splenocytes with YAC cells. Intracellular expression of IFN-γ and TNF-α in splenocytes obtained from mice treated with AF-SWCNTs was assessed flow cytometrically by coculturing with YAC cells *ex vivo*. Our results showed that treatment with AF-SWCNTs resulted in decline of IFN-γ and TNF-α in $NK1.1^+$ cells by 31 and 41%, respectively (**Figure 12**).

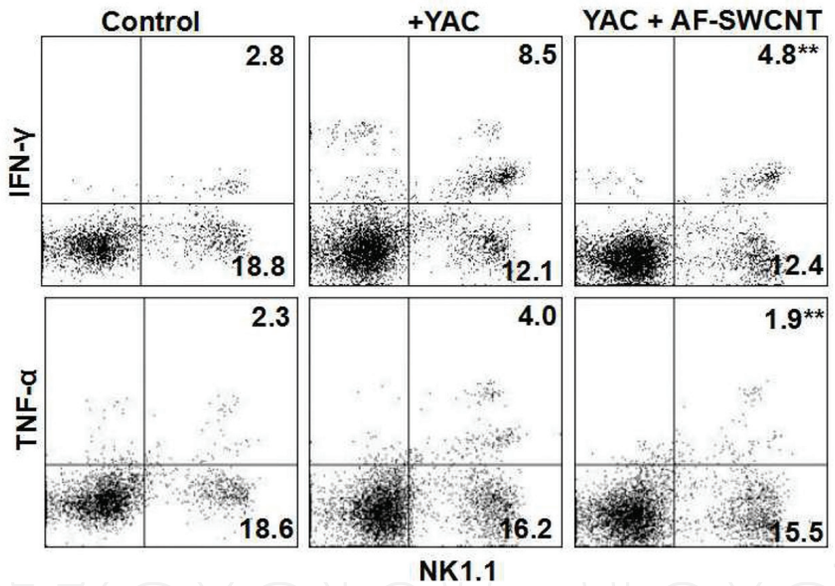


Figure 12. Intracellular expression levels of IFN-γ and TNF-α in NK cells. Splenocytes were obtained from mice administered with poly I:C and treated with AF-SWCNT. Splenocytes (1×10^6) were cocultured *ex vivo* with YAC cells (2×10^5) for 5 h and treated with brefeldin and monensin. Cells were stained with antimouse IFN-γ or antimouse TNF-α mAbs and counterstained with antimouse NK1.1 mAb. Percentages of $NK1.1^+$ cells expressing IFN-γ or TNF-α (upper right quadrant) in the presence and absence of YAC-1 target cells are shown. $^{**}p < 0.01$ by Student's *t* test.

6. Conclusions

NK cells possess inherent ability to kill tumor cells without requiring a prior sensitization. NK cells and cytotoxic T lymphocytes (CTLs) both exhibit cytolytic activity involving secretory (perforin and granzymes) and nonsecretory mechanisms (Fas-FasL interaction). SWCNTs as

such are insoluble and do not interact efficiently with cells. We have prepared an acid-derivatized form of SWCNTs by subjecting them to high pressure and temperature in the presence of concentrated sulfuric acid and nitric acid. Acid-functionalized SWCNTs (AF-SWCNTs) are not only polydispersed in aqueous solution but are also amenable to be attached with fluorescent ligands to their carboxyl groups created on the backbone of SWCNTs. As a result we could visualize the interaction of AF-SWCNTs with live NK cells. This was a significant step as it opened a vast arena to explore the activity of NK cells *in vitro* and *in vivo* without fixing them, as required for transmission electron microscopy. The various physiological parameters of NK cells, such as apoptosis, cell cycle, activation, generation, and degranulation have been studied using flow cytometry. This technique is superior to other conventional spectrometric techniques as the results obtained have higher reproducibility and even minor changes in subpopulation can be monitored.

AF-SWCNT treatment showed greater toxicity which was dose and time dependent. At higher dose of 50 μg/mL, AF-SWCNTs exerted toxic effects that led to decrease in cell proliferation and cell cycle arrest. Mechanistic details showed that AF-SWCNT treatment caused greater generation of ROS that led to fluctuations in mitochondrial potential and calcium concentration. These changes offset the homeostatic mechanisms of the cells, which led to their killing. Previous studies by our group had showed that AF-SWCNTs show significant inflammatory effects in mouse lungs induced anemia in mice and caused suppression of cytotoxic response *in vitro* and *in vivo* [43–46]. This chapter demonstrated the inhibitory effects of AF-SWCNTs on activated NK cells. AF-SWCNTs induced inhibition of NK activation by suppressing cellular proliferation, activation processes, and increased apoptosis. AF-SWCNT treatment led to decreased degranulation of NK cells, lower Fas-FasL interaction, and lower production of inflammatory cytokines, including IFN-γ and TNF-α. Taken together AF-SWCNT treatment led to downregulation of NK cell system and stipulates further research for their prospective use in autoimmune disorder or hypersensitive conditions.

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Abbreviations

7-AAD	7-Aminoactinomycin D
^{51}Cr	Radioactive chromium
ADCC	Antibody-dependent cell-mediated cytotoxicity
AF-SWCNT	Acid-functionalized single-walled carbon nanotube

AIDS	Acquired immune deficiency syndrome
ANOVA	Analysis of variance
CD	Cluster of differentiation
CTL	Cytotoxic T lymphocyte
DAPI	4′,6-Diamidino-2-phenylindole dye
DIC	Differential interference contrast microscopy
H2DCFDA	2′,7′-Dichlorodihydrofluorescein diacetate dye
IFN-γ	Interferon gamma
IL	Interleukin
mAb	Monoclonal antibody
MHC-I	Major histocompatibility complex class I
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye
RNA	Ribonucleic acid
SWCNT	Single-walled carbon nanotube
TNF-α	Tissue necrosis factor alpha
TNFR	Tissue necrosis factor alpha receptor
TRAIL	TNF-related apoptosis-inducing ligand
TRAILR	TNF-related apoptosis-inducing ligand receptor

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References

[1] Kumar S, Rani R, Dilbaghi N, Tankeshwar K, Kim KH. Carbon nanotubes: A novel material for multifaceted applications in human healthcare. Chemical Society Reviews. 2017;**46**(1):158-196

[2] Lam CW, James JT, McCluskey R, Arepalli S, Hunter RL. A review of carbon nanotube toxicity and assessment of potential occupational and environmental health risks. Critical Reviews in Toxicology. 2006;**36**(3):189-217

[3] Merkt W, Sturm P, Lasitschka F, Tretter T, Watzl C, Saure D, Hundemer M, Schwenger V, Blank N, Lorenz HM, Cerwenka A. Peripheral blood natural killer cell percentages in granulomatosis with polyangiitis correlate with disease inactivity and stage. Arthritis Research and Therapy. 2015;**17**:337

[4] Ranson T, Vosshenrich CA, Corcuff E, Richard O, Müller W, Di Santo JP. IL-15 is an essential mediator of peripheral NK-cell homeostasis. Blood. 2003;**101**(12):4887-4893

[5] Lai L, Alaverdi N, Maltais L, Morse HC 3rd. Mouse cell surface antigens: Nomenclature and immunophenotyping. The Journal of Immunology. 1998;**160**(8):3861-3868

[6] Bryceson YT, Fauriat C, Nunes JM, Wood SM, Björkström NK, Long EO, Ljunggren HG. Functional analysis of human NK cells by flow cytometry. Methods in Molecular Biology. 2010;**612**:335-352

[7] Lanier LL. NK cell receptors. Annual Review of Immunology. 1998;**16**:359-393

[8] Kim S, Iizuka K, Kang HS, Doken A, French AR, Greco S, Yokoyama WM. *In vivo* development of murine natural killer cell maturation. Nature Immunology. 2002;**3**:523-528

[9] Vivier E, Nunès JA & Vély F. Natural killer cell signaling pathways. Science. 2004;**306**: 1517-1519

[10] Cohnen A, Chiang SC, Stojanovic A, Schmidt H, Claus M, Saftig P, JanBen O, Cerwenka A, Bryceson YT, Watzl C. Surface CD107a/LAMP-1 protects natural killer cells from degranulation-associated damage. Blood. 2013;**122**(8):1411-1418

[11] Ascierto ML, Idowu MO, Zhao Y, Khalak H, Payne KK, Wang XY, Dumur CI, Bedognetti D, Tomei S, Ascierto PA, Shanker A, Bear HD, Wang E, Marincola FM, De Maria A, Manjili MH. Molecular signatures mostly associated with NK cells are predictive of relapse free survival in breast cancer patients. Journal of Translational Medicine. 2013;**11**:145

[12] Dunn GP, Koebel CM, Schreiber RD. Interferons, immunity and cancer immunoediting. Nature Reviews Immunology. 2006;**6**(11):836-848

[13] Wang Y, Iqbal Z, Mitra S. Rapidly functionalized, water-dispersed carbon nanotubes at high concentration. Journal of the American Chemical Society. 2006;**128**(1):95-99

[14] Saxena RK, Williams W, McGee JK, Daniels MJ, Boykin E, Gilmour MI. Enhanced *in vitro* and *in vivo* toxicity of poly-dispersed acid-functionalized single-wall carbon nanotubes. Nanotoxicology. 2007;**1**:291-300

[15] Tong H, McGee JK, Saxena RK, Kodavanti UP, Devlin RB, Gilmour MI. Influence of acid functionalization on the cardiopulmonary toxicity of carbon nanotubes and carbon black particles in mice. Toxicology and Applied Pharmacology. 2009;**239**(3):224-232

[16] Kaur S, Mehra NK, Jain K, Jain NK. Development and evaluation of targeting ligand-anchored CNTs as prospective targeted drug delivery system. Artificial Cells, Nanomedicine, and Biotechnology. 2017;**45**(2):242-250

[17] Kumari M, Sachar S, Saxena RK. Loss of proliferative and antigen presentation activity following internalization of poly-dispersed single-walled carbon nanotubes by primary lung epithelial cells. PLoS One. 2012;**7**(2):e31890

[18] Sachar S, Saxena RK. Cytotoxic effect of poly-dispersed single walled carbon nano-tubes on mouse erythrocytes. PLoS One. 2011;**6**(7):e22032

[19] Fahmy T. Pathogen associated molecular patterns (PAMPS) on nanomaterials. Paradigms for new vaccines. Trends in Biotechnology. 2011;**29**(6):294-306

[20] Bhattacharya K, Mukherjee SP, Gallud A, Burkert SC, Bistarelli S, Bellucci S, Bottini M, Star A, Fadeel B. Biological interactions of carbon-based nanomaterials: From coronation to degradation. Nanomedicine. 2016;**12**(2):333-351

[21] Orecchioni M, Bedognetti D, Sgarrella F, Marincola FM, Bianco A, Delogu LG. Impact of carbon nanotubes and graphene on immune cells. Journal of Translational Medicine. 2014;**12**:138

[22] Levine PH, Whiteside TL, Friberg D, Bryant J, Colclough G, Herberman RB. Dysfunction of natural killer activity in a family with chronic fatigue syndrome. Clinical Immunology and Immunopathology. 1998;**88**(1):96-104

[23] Kepp O, Galluzzi L, Lipinski M, Yuan J, Kroemer G. Cell death assays for drug discovery. Nature Reviews Drug Discovery. 2011;**10**(3):221-237

[24] Zembruski NC, Stache V, Haefeli WE, Weiss J. 7-Aminoactinomycin D for apoptosis staining in flow cytometry. Analytical Biochemistry. 2012;**429**(1):79-81

[25] Circu ML, Aw TY. Reactive oxygen species, cellular redox systems, and apoptosis. Free Radical Biology and Medicine. 2010;**48**(6):749-762

[26] Zorov DB, Juhaszova M, Sollott SJ. Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. Physiological Reviews. 2014;**94**(3):909-950

[27] Saxena RK, Saxena QB, Collins GD, Adler WH. Augmentation of spleen natural killer activity in mice treated with interleukin-2 preparation. Indian Journal of Experimental Biology. 1983;**21**:54-58

[28] Saxena RK, Saxena QB, Adler WH. Interleukin-2 induced activation of natural killer activity in spleen cells from old and young mice. Immunology. 1984;**51**:719-726

[29] Saxena RK, Adler WH. Modulation of natural cytotoxicity by alloantibodies. I. Alloantiserum enhancement of cytotoxicity of mouse spleen cells towards a human myeloid cell line. The Journal of Immunology. 1979;**123**:846-851

[30] Motzer SA, Tsuji J, Hertig V, Johnston SK, Scanlan J. Natural killer cell cytotoxicity: A methods analysis of 51 chromium release versus flow cytometry. Biological Research for Nursing. 2003;**5**(2):142-152

[31] Sarin A, Saxena RK. Interleukin-2 induced changes in the buoyant density of cytotoxic cells and its relationship with proliferative activity. Natural Immunity and Cell Growth Regulation. 1989;**8**:279-289

[32] Saxena RK, Saxena QB, Adler WH. Defective T-cell response in beige mutant mice. Nature. 1982;**295**:240-241

[33] Simms PE, Ellis TM. Utility of flow cytometric detection of CD69 expression as a rapid method for determining poly- and oligoclonal lymphocyte activation. Clinical and Diagnostic Laboratory Immunology. 1996;**3**(3):301-304

[34] Alter G, Malenfant JM, Altfeld M. CD107a as a functional marker for the identification of natural killer cell activity. Journal of Immunological Methods. 2004;**294**(1-2):15-22

[35] Saxena RK, Saxena QB, Adler WH. Regulation of natural killer activity *in vivo*. I. Loss of natural killer activity during starvation. Indian Journal of Experimental Biology. 1980;**18**:1383-1386

[36] Saxena QB, Saxena RK, Adler WH. Regulation of natural killer activity *in vivo*. IV. High natural killer activity in alcohol drinking mice. Indian Journal of Experimental Biology. 1981;**19**:1001-1006

[37] Saxena QB, Saxena RK, Adler WH. Regulation of natural killer activity *in vivo*. III. Effect of hypophysectomy and growth hormone treatment on the natural killer activity of the mouse spleen cell population. International Archives of Allergy and Applied Immunology. 1982;**67**:169-174

[38] Saxena QB, Saxena RK, Adler WH. Effect of protein caloric malnutrition on the level of natural and inducible cytotoxic activities in mouse spleen cells. Immunology. 1984;**51**:727-733

[39] Saxena QB, Saxena RK, Adler WH. Effect of feeding a diet with half of the recommended levels of all vitamins on the natural and inducible levels of cytotoxic activity in mouse spleen cells. Immunology. 1984;**52**:41-47

[40] Saxena RK, Saxena QB, Adler WH. Mechanism of loss of natural killer activity in P815 tumor bearing DBA/2 mice. Natural Immunity and Cell Growth Regulation. 1984;**3**:34-42

[41] Saxena RK, Adler WH, Nordin AA. Modulation of natural cytotoxicity by alloantibodies. IV. A comparative study of the activation of mouse spleen cell cytotoxicity by anti H-2 antisera, interferon and mitogens. Cellular Immunology. 1981;**63**:28-41

[42] Wang R, Jaw JJ, Stutzman NC, Zou Z, Sun PD. Natural killer cell-produced IFN-γ and TNF-α induce target cell cytolysis through up-regulation of ICAM-1. Journal of Leukocyte Biology. 2012;**91**(2):299-309

[43] Alam A, Sachar S, Puri N, Saxena RK. Interactions of poly-dispersed single walled Carbon Nanotubes with T cells resulting in down regulation of allogeneic CTL responses *in vitro* and *in vivo*. Nanotoxicology. 2013;**7**:1351-1360

[44] Abbas Z, Puri N, Saxena RK. Lipid antigen presentation through CD1d pathway in mouse lung epithelial cells, macrophages and dendritic cells and its suppression by poly-dispersed single-walled carbon nanotubes. Toxicology *in Vitro*. 2014;**29**:1275-1285

[45] Bhardwaj N, Saxena RK. Selective loss of younger erythrocytes from blood circulation and changes in erythropoietic patterns in bone marrow and spleen in mouse anemia induced by poly-dispersed single wall carbon nanotubes. Nanotoxicology. 2015;**9**(8):1032-1040

[46] Alam A, Puri N, Saxena RK. Uptake of poly-dispersed single-walled carbon nanotubes and decline of functions in mouse NK cells undergoing activation. Journal of Immunotoxicology. 2016;**13**(5):758-765