

ORIGINAL ARTICLE

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-walled carbon nanotubes

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Abstract

Administration of poly-dispersed acid-functionalized single-walled carbon nanotubes (AF-SWCNTs, 10 µg intravenously on alternate days) induced a sustained anemia in mice. Using a modified double *in vivo* biotinylation (DIB) technique, blood counts of reticulocytes and erythrocytes of different age groups were simultaneously enumerated in control and AF-SWCNT treated mice. A sustained reticulocytosis was observed in AF-SWCNT treated mice. Young erythrocytes (up to 7 days old) that are normally resistant to elimination in control mice were eliminated at a significant rate in AF-SWCNT treated mice. Old erythrocytes, however, accumulated in circulation indicating that younger erythrocytes were selectively eliminated from the blood circulation of AF-SWCNT treated mice. Cells representing various stages of erythroid differentiation in bone marrow and spleen were enumerated flow cytometrically by double staining with anti-Ter119 and anti-transferrin receptor (CD71) monoclonal antibodies. Proportion of erythroid cells was significantly reduced (up to 27%) in bone marrow (BM) indicating a fall in erythropoietic activity. A concomitant increase in the spleen erythroid population was however observed that could be a compensatory response. Changes in erythroid populations in bone marrow and spleen correlated with changes in erythroblast-A population in these organs that represent an early stage of erythroid differentiation. Uptake of intravenously administered fluorescence tagged AF-SWCNTs (FAF-SWCNTs) was relatively low (3–4%) in erythroid cells in bone marrow and spleen. A significantly higher proportion of pro-erythroblasts and erythroblast-A (early stages of erythroid differentiation) took-up FAF-SWCNTs. Uptake of AF-SWCNTs by early precursors of erythroid differentiation with toxic consequences may be a contributing factor in AF-SWCNT induced anemia.

Keywords

Anemia, DIB technique, erythropoiesis, flow cytometry, reticulocytes

History

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Introduction

Due to their unique structural and remarkable electronic, mechanical and chemical properties, engineered nanomaterials are finding wide applications in molecular electronics, micro devices, gas storage, catalytic supports, aerospace, automobile, atomic force microscopy and in biological applications like biosensors, drug delivery and new promising therapies (Baughman et al., 2002; Bianco et al., 2005; Kam et al., 2005; Liu et al., 2005). Single-walled carbon nanotubes (SWCNTs) are a class of engineered nanomaterials that represent rolled up tubes of graphite sheet of sp² hybridized carbon atoms, having a diameter of about 1 nm. The nano-dimension of SWCNTs coupled with high aspect ratio makes it a suitable candidate for new class of transport vehicles for high capacity loading of ligands for therapeutic purposes (Delogu et al., 2009;

Kam et al., 2006; Pantarotto et al., 2004). The widespread application and usage have increased the chances of environmental and occupational exposure to SWCNTs in humans, animals, microbes and plant life forms and raised the issue of potential toxic effects of these materials.

Nanoparticles interaction with the body is dependent on their size, chemical composition, surface structure, solubility and shape (Chithrani et al., 2006; Jiang et al., 2008; Sohaebuddin et al., 2010). Studies on toxicity of nanoparticles indicate that some of those products may enter the human body and become toxic at the cellular level in various tissues and organs (Hsieh et al., 2012; Love et al., 2012). Several studies have demonstrated the toxicity of SWCNT to different type of cells *in vitro* (Davoren et al., 2007; Helland et al., 2007; Saxena et al., 2007), and *in vivo* (Lam et al., 2004; Shvedova et al., 2005). Treatment with acid-functionalized SWCNTs (AF-SWCNTs) increased apoptosis and inhibited proliferation on LA4 lung epithelial cell line and primary lung epithelial cells in culture (Kumari et al., 2012; Saxena et al., 2007). AF-SWCNTs have also been shown to suppress T-cell activation response (Alam et al., 2013) and induce cardiac damage (Tong et al., 2009) in mice.

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We have previously reported that AF-SWCNTs treatment induces membrane damage and phosphatidyl-serine externalization in erythrocytes (Sachar & Saxena, 2011). Administration of a single dose of 100 µg of AF-SWCNTs induces transient anemia in mice (Sachar & Saxena, 2011). These results point to a toxic effect of AF-SWCNTs on the erythroid system. The present study was designed to further investigate the nature of overall interaction of AF-SWCNTs not only with erythrocytes of different age groups in blood circulation but also with reticulocytes and the erythroid precursors in bone marrow and spleen.

We have developed a double *in vivo* biotinylation (DIB) technique (Khandelwal & Saxena, 2006, 2008; Saxena et al., 2012) that has extensively been used to study the *in vivo* turnover patterns of circulating erythrocytes of different age groups (Bhardwaj & Saxena, 2014; Khandelwal et al., 2007; Sachar & Saxena, 2011). In the present communication, we have modified the DIB protocol to further differentiate between reticulocytes and matured young erythrocytes in blood circulation. Using this modified technique, we have demonstrated that administration of 10 µg AF-SWCNTs on alternate days induces sustained anemia accompanied with reticulocytosis and also a significant elimination of young erythrocytes from blood circulation. We have also examined the changes in the relative proportions of cell populations at different stages of erythroid differentiation in bone marrow and spleen of AF-SWCNT treated mice, by using a flow cytometric technique reported earlier (Bhardwaj & Saxena, 2014; Kalfa et al., 2010; Marinkovic et al., 2007). Uptake of fluorescence tagged AF-SWCNTs (FAF-SWCNTs) by erythroid subpopulations in bone marrow and spleen was also examined. This data and its interpretation for understanding the mechanism of AF-SWCNT induced anemia have been discussed.

Materials and methods

Animals

Inbred C57BL/6 mice (8–12 weeks old, 20–25 g body weight) were used throughout this study. Animals were bred and maintained in the animal house facility at Jawaharlal Nehru University (JNU), New Delhi, or obtained from the National Institute of Nutrition, Hyderabad. The animals were housed in positive-pressure air conditioned units (25 °C, 50% relative humidity) and kept on a 12-h light/dark cycle. Water and mouse 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, Government of India (CPCSEA guidelines www.envfor.nic.in/divisions/awd/cpcsea_laboratory.pdf). The study was duly approved by the JNU Institutional Animal Ethics Committee (IAEC Approved Project Code: 5/2010).

Reagents and other supplies

Biotin-X-NHS (N-hydroxysuccinimide ester of biotin) was obtained from Calbiochem (La Jolla, CA). Streptavidin Allophycocyanin (SAV-APC), rat anti-mouse Ter-119-APC, anti-mouse CD16/CD32 was from BD Biosciences (San Diego, CA). Anti-mouse CD71-PE, anti-mouse CD71-FITC, anti-mouse IgG1κ-PE, anti-mouse IgG1κ-FITC, anti-mouse IgG2bκ-APC, were procured from e-biosciences (San Diego, CA). Alexa fluor 488/633 hydrazide, 5 (and 6) was purchased from Molecular Probes (Eugene, OR). Fetal bovine serum (FBS) was obtained from Hyclone (South Logan, UT). RPMI, HEPES, Dimethylformamide

(DMF), MES buffer, 1-ethyl 3-(3-dimethyl aminopropyl) carbodiimide (EDAC), N-hydroxysuccinimide (NHS) and other analytical reagents were obtained from Sigma-Aldrich (India). Single-walled carbon nanotubes (Catalogue No. 775533, >95% carbon basis) were procured from Sigma-Aldrich (St. Louis, MO).

Acid functionalization of carbon nanotubes

AF-SWCNTs were prepared by method described earlier (Sachar & Saxena, 2011; Saxena et al., 2007; Tong et al., 2009). Briefly, SWCNTs were suspended in 1:1 mixture of concentrated H₂SO₄ and HNO₃ and heated in high-pressure vessel in a microwave oven (138–150 °C, 20 ± 2 psi) for 3 min. Suspension was dialyzed extensively against deionized water, lyophilized and weighed. Detailed characterization of AF-SWCNTs including elemental analysis, size and charged distribution, BET surface area and electron microscopic changes have been reported earlier (Saxena et al., 2007; Tong et al., 2009). In summary, particle size distributions of AF-SWCNTs aqueous suspensions obtained from zeta-sizer instrument showed that 95% particles were between 22 and 138 nm in size. Mean zeta potential of AF-SWCNTs was –57.2 mV and BET surface area was reduced by one-third as compared to pristine SWCNTs. Basic tubular structure of AF-SWCNTs remained intact though the sidewalls of nanotubes appeared roughened. In general, all changes were consistent with mild sulfonation/carboxylation of the nanotube sidewalls while leaving the basic nanotube structure unchanged. The AF-SWCNTs were dispersed in phosphate-buffered saline (PBS) and sonicated for 2 min in ice using a probe sonicator (Branson sonicator, VWR Scientific, Thane, India) prior to use.

Attachment of fluorescence probe to AF-SWCNTs

Fluorescence probe was covalently attached to the carboxylic group on AF-SWCNTs as reported earlier (Sachar & Saxena, 2011). Briefly, AF-SWCNTs were suspended in MES buffer and treated with 1-ethyl 3-(3-dimethyl aminopropyl) carbodiimide (EDAC) and N-hydroxy succinimide (NHS) to generate a succinimidyl intermediate. The mixture was continuously shaken for 2 h and dialyzed extensively to remove excess NHS, EDAC and Urea byproduct. Activated AF-SWCNTs were incubated with Alexa fluor 488/633 (1:1 ratio, 50 µg each in 200 µl volume) in dark with continuous shaking followed by dialysis to remove free dye (Sachar & Saxena, 2011).

Double *in vivo* biotinylation technique for demarcation of reticulocytes and three discrete populations of erythrocytes

Rationale and details of the double *in vivo* biotinylation (DIB) technique and its use in studying erythrocyte turnover and age-related changes in erythrocytes *in vivo*, have been described elsewhere (Bhardwaj & Saxena, 2014; Khandelwal & Saxena, 2006, 2007, 2008; Khandelwal et al., 2007; Sachar & Saxena, 2011; Saxena et al., 2012). For *in vivo* biotin labeling mice were given three daily i.v. injections through tail vein of 1 mg of biotin-X-NHS Ester (BXN) dissolved in 20 µl of dimethylformamide (DMF) and 250 µl of PBS. For the second biotinylation step, mice were given intravenously 0.6 mg of BXN dissolved in 12 µl of DMF and 250 µl of PBS. Five days after the second biotinylation step, AF-SWCNT administration was started on alternate days. For demarcation of reticulocytes and different age groups of erythrocytes, blood cells isolated from a DIB labeled mouse at different given time points, were stained with anti-mouse CD71 monoclonal antibody (transferrin receptor) and fluorescence conjugated streptavidin monoclonal antibody followed by flow cytometry.

Acid-functionalized single-walled carbon nanotubes treatment and sample collection

For *in vivo* toxicity studies, mice were administrated i.v. 10 µg AF-SWCNTs on alternate days, and blood samples (25–50 µl) were collected at different time points from tail vein in PBS containing 5 mM EDTA. Erythrocyte numbers and hemoglobin (Hb) levels were estimated by using an electronic hematology particle counter (Melet Scholesing, MSE4 Laboratories, Osny, France). Bone marrow (BM) cells were flushed out of femur and tibia using a 25-gauge needle and resuspended in RPMI medium with 10% FBS. Single cell suspensions of spleen cells were made by gently teasing the spleen in a small volume of PBS. Splenic and BM cells were strained through a fine sieve, pelleted by centrifugation, washed and suspended at desired concentration in RPMI medium with 10% FBS. For *in vivo* uptake experiment, a single dose of 75 µg fluorescence conjugated AF-SWCNTs (FAF-SWCNTs) was administered i.v. and mice were sacrificed 12 h later to harvest bone marrow and spleen cells.

Measurement of intracellular reactive oxygen species

Intercellular ROS levels were assessed as described before (Bhardwaj & Saxena, 2014; Marinkovic et al., 2007). Briefly, erythrocytes were washed and resuspended in pre-warmed PBS supplemented with 2% FBS and incubated with CM-H₂DCFDA stain (5 µM) in the dark for 30 min at 37 °C in an atmosphere of 5% CO₂ in air. The oxidative conversion of CM-H₂DCFDA to its fluorescent product by ROS was measured immediately by flow cytometry.

Flow cytometric analysis

Mouse blood was collected in PBS containing 5 mM EDTA and washed three times with ice cold saline containing HEPES buffer (10 mM, pH 7.4) and 1% FBS. Staining of erythrocytes for identifying biotin^{low}, biotin^{-ve} and biotin^{high} populations has been described elsewhere (Khandelwal & Saxena, 2006, 2007). For enumerating erythroid cells at different stages of differentiation in bone marrow and spleen, freshly prepared single cell suspensions from bone marrow or spleen were first incubated with anti-CD16/32 antibody (Fc block, 1 µg/10⁶ cells in 50 µL of PBS + 2%FBS) for 10 min followed by staining with anti-CD71-PE and anti Ter-119-APC for 20 min at 4 °C (Bhardwaj & Saxena, 2014; Kalfa et al., 2010; Marinkovic et al., 2007). After incubation, cells were washed and analyzed on a BD FACS Verse flow cytometer using FACSuite software for acquisition and analysis (BD Biosciences, Gurgaon, India).

Statistical analysis

Statistical analysis was carried out by using Sigma Plot software (Systat Software, San Jose, CA). Standard *t*-tests as well as the one way ANOVA were used to calculate the significant levels between groups, and *p* < 0.05 was considered significant.

Results

Modified double *in vivo* biotinylation protocol for simultaneous enumeration of reticulocytes and three discrete erythrocyte populations in blood

Basic principle of DIB protocol is shown in Figure 1(A). In this protocol, after first biotinylation step, mice were rested for 5 days before second low-intensity biotinylation step was carried out. At various time points after the second biotinylation step, blood cells isolated from a DIB-labeled mouse were stained with anti-CD71 (transferrin receptor) and fluorescence conjugated streptavidin monoclonal antibody followed by flow cytometry (Figure 1B).

Results in Figures 1(B) show that at various time points after the second biotinylation step, four discrete erythrocyte populations could be identified in the blood based upon the intensity of CD71 and biotin labeling. Cells could be categorized in four discrete groups, namely CD71^{+ve}/biotin^{-ve} (reticulocytes, box a), CD71^{low}/biotin^{-ve} (youngest erythrocytes, box b), biotin^{low} (cohort of erythrocytes released in the blood between the first and the second biotinylation steps, box c) and biotin^{high} (oldest age group present in blood at the time of first biotinylation step, box d) (Figure 1B). Results in Figure 1(B) show that after 2 days of second biotinylation step, approximately 3.65% new erythrocytes entered circulation comprising 1.65% reticulocytes (Box-a, Figure 1B), and 2.00% youngest erythrocytes released over the two-day period (Box-b, Figure 1B). Furthermore, at each of the later time points, new reticulocytes that freshly entered circulation could be enumerated in box-a of the respective panel (Figure 1B). Representative histograms in Figure 1(B) showed that the proportion of reticulocytes in blood ranged between 1.5 and 2.85% at different time points. Proportion of biotin low erythrocytes (box c) that represented the cohort of erythrocytes released between the first and the second biotinylation steps remained constant (range 12.42–9.83%), till 35-day time point but decreased thereafter till it was 4.65% on day 49-time point. As expected, proportion of erythrocytes that were present in blood at the time of first biotinylation step (biotin^{high}, box d) declined, whereas the proportion of erythrocytes released in blood circulation after the second biotinylation step (box b) increased with the passage of time.

Changes in relative proportions of reticulocytes and three age defined subpopulations of erythrocytes in AF-SWCNT treated mice

Mice were administered AF-SWCNTs (10 µg, intravenously) on alternate days for 35 days. On days 7, 14, 21, 28 and 35, blood samples were collected and erythrocyte counts as well as the blood levels of Hb were estimated. Significant decline in erythrocyte count as well as Hb levels was observed at different time points during the treatment with AF-SWCNTs. Results in Figure 2(A) show that erythrocyte count and Hb levels decreased by 10–22% and 13–25%, respectively at different time points. These results indicate that repeated administration of AF-SWCNTs induced a sustained anemia in mice.

Changes in the relative proportions of the four cellular subpopulations defined by the DIB technique, described in Figure 1, were examined in control and AF-SWCNT treated mice on 19th day time point. The relative proportions of reticulocytes (box a), and other three subpopulations of erythrocytes (boxes b, c and d) in control mice were 2.23:33.85:15.80:48.02, respectively (Figure 2C). In AF-SWCNT treated mice these proportions changed to 5.32:26.44:15.07:53.05 (Figure 2D). Thus, the AF-SWCNT treatment resulted in an increase in the percentage of blood reticulocyte from 2.23 to 5.32%, and the proportion of younger erythrocytes (box b) decreased while the proportion of oldest erythrocytes (box d) increased in the blood.

Time kinetics of changes in the proportion of different age groups of erythrocytes is shown in Figure 3. Results were shown as percent change in the relative proportions of reticulocytes and the three erythrocyte subpopulations in AF-SWCNT treated mice, taking the corresponding values in the control mice as hundred. A sustained surge of blood reticulocytes in AF-SWCNT treated mice was noticed and the reticulocyte counts remained significantly higher (about 50 to 75% higher than control on 14th day and later time points, Figure 3A). A sustained and significant fall was noticed in the relative proportions of younger

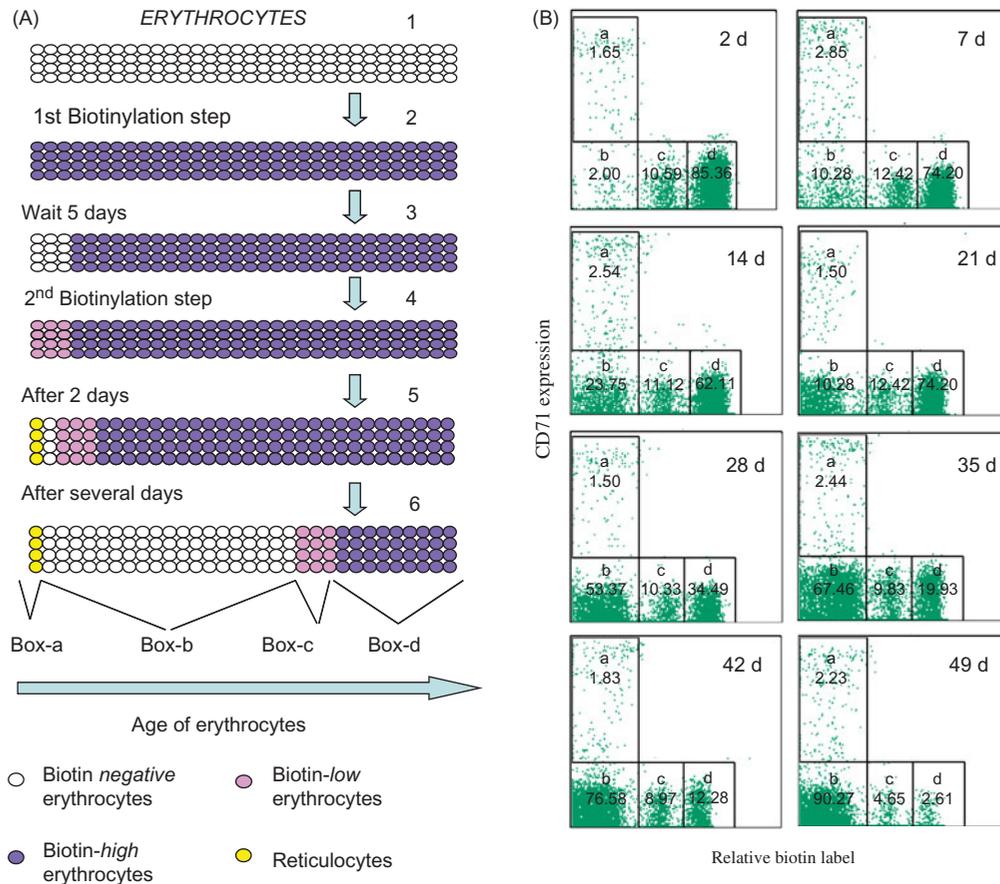


Figure 1. Modified DIB technique to simultaneously enumerate reticulocytes and erythrocytes of different age groups in blood circulation. (A) Mice were DIB labeled according to DIB protocol. 1 – Normal circulating erythrocytes are biotin negative. 2 – After three daily intravenous injections of BXN (Biotin-X-NHS 1 mg/mouse/day) all erythrocytes become biotin labeled. 3 – After 5 days, about 10% newly generated erythrocytes are biotin negative. 4 – A second *in vivo* biotinylation step with a single injection of 0.6 mg BXN results in low intensity of biotin labeling on freshly produced band of erythrocytes. 5 – After 2 days, CD71⁺/biotin⁻ cells are reticulocytes and CD71⁻/biotin⁻ populations are youngest erythrocytes. 6 – at all later time points, CD71⁺ populations are reticulocytes, biotin negative population represents the young erythrocyte population in circulation, biotin low population represents a cohort of erythrocytes produced during a limited time window (5 days in example) between first and second steps of biotinylation. Biotin-high population represents the oldest cells having the high intensity of biotin label. (B) Blood was collected at different time points (2, 7, 14, 21, 28, 35, 42 and 49 days of second biotinylation step) and erythrocytes were stained with anti-mouse CD71-PE antibody along with streptavidin APC followed by flow cytometric analysis. Cells were gated based on CD71 expression and different intensity of biotin label. Four different populations: reticulocytes (CD71⁺/biotin⁻, box-a), young erythrocytes (CD71/biotin⁻, box-b), Intermediate (biotin^{low}, box-c) and old (biotin^{high}, box-d) aged erythrocytes have been identified. Values in each panel represent percentages of cells.

erythrocytes (Figure 3B), whereas the relative proportions of oldest erythrocytes doubled over the control values on 35 day time point (Figure 3D). Cohort of erythrocytes released during the 5-day between the first and the second biotinylation steps declined steeply by about 20% on 7th day time point of AF-SWCNT treatment, remained low till 21-day time point then increased thereafter till it was significantly higher than control on the 35th day (Figure 3C).

A significant fall in the young erythrocyte cohort in AF-SWCNT treated mice on 7 to 21 day time points is especially significant in control mice because young erythrocytes are resistant to elimination during this earlier time span (Figure 1B and Saxena et al., 2012). These results clearly show that the anemia in AF-SWCNT treated mice was not due to a uniform decline of erythrocytes irrespective of their age, but was due to a significant elimination of younger erythrocytes.

Erythropoietic activity in AF-SWCNT-treated mice

Bone marrow and spleen are two prime erythropoietic sites in adult mice (Marinkovic et al., 2007; Paulson et al., 2011). Erythroid cells in bone marrow and spleen are characterized by

the expression of Ter119 and CD71 markers and based upon the levels of expression of these two markers, four distinct stages of erythroid differentiation may be identified; these being, early pro-erythroblast (Ter^{med} CD71^{high}), early basophilic erythroblast (Ter^{high} CD71^{high}) (erythroblast A), late basophilic, polychromatophilic erythroblast and orthochromatophilic erythroblast (Ter^{high} CD71^{med}) (erythroblast B) and orthochromatophilic erythroblast with mature erythrocytes (Ter^{high} CD71^{low}) (erythroblast C) (Bhardwaj & Saxena, 2014; Marinkovic et al., 2007; Paulson et al., 2011). Total erythroid population in bone marrow can be enumerated by setting an inverted L-shaped gate in flow cytometric histograms that include all the above-mentioned four stages of erythroid differentiation (Bhardwaj & Saxena, 2014; Kalfa et al., 2010; Marinkovic et al., 2007).

To investigate the effect of AF-SWCNTs treatment on erythropoietic activity in bone marrow and spleen, proportions of all erythroid cells in bone marrow and spleen of control and AF-SWCNT treated mice were examined. Results of a representative experiment showed the presence of 39.36% erythroid cells within the inverted-L-shaped gate in control bone marrow (sum of percentage values in boxes p, q, r and s, in Figure 4A). In AF-SWCNTs treated mice, the proportion of erythroid cells fell

Figure 2. Induction of sustained anemia by multiple intravenous administrations of AF-SWCNTs and changes in relative proportions of reticulocytes and erythrocytes of different age groups. Mice were administered at repeated doses (10 μg , i.v.) of AF-SWCNTs on alternate days. Blood was collected and erythrocyte counts and hemoglobin levels were measured at different time points. Panels A and B show the changes in erythrocytes count and hemoglobin level, respectively, after AF-SWCNT treatments. All values represent mean \pm SEM of data from 5 to 7 mice (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$ by t -test at individual time points. $p < 0.05$ for effect of AF-SWCNTs on erythrocyte count as well as hemoglobin levels by one way ANOVA). Relative proportions of reticulocytes and three different age groups of erythrocytes were determined by DIB protocol as describe in legends of Figure 1. Blood was collected from control and AF-SWCNTs treated mice (19 days post-second-step biotinylation) and erythrocytes were double stained with anti-mouse CD71-PE along with streptavidin APC followed by flow cytometric analysis. Panels C (control) and D (AF-SWCNTs treated) illustrate the proportions of different aged define subpopulations of erythrocytes after 14 days of AF-SWCNTs treatment, i.e. 19 days after the second biotinylation step. Erythrocyte populations in boxes a, b, c and d represent CD71⁺ve/biotin⁻ve (reticulocytes), biotin⁻ve (age <19 days), biotin^{low} (age 19 to 24 days), biotin^{high} (age >24 days) population of erythrocytes. Values in different boxes represent percentage of cells.

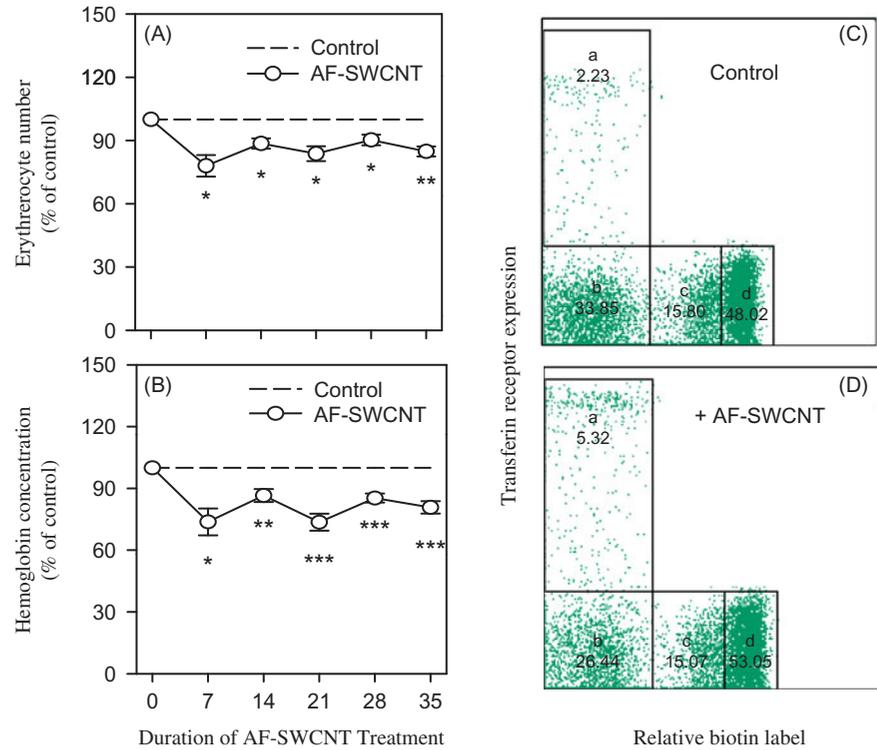


Figure 3. Time kinetics of alterations in relative proportions of reticulocytes and erythrocytes of different age groups in AF-SWCNT treated mice. Mice were DIB labeled and exposed to AF-SWCNTs on alternate days. Blood was collected at different time points (7, 14, 21, 28 and 35 days of second biotinylation step) and erythrocytes were stained with anti-mouse CD71-PE and streptavidin APC antibody followed by flow cytometric analysis. Proportion of reticulocytes (CD71⁺ve, population A), young (biotin⁻ve, population B), Intermediate (biotin^{low}, population C) and old (biotin^{high}, population D) erythrocytes in blood after AF-SWCNTs treatments have been shown. Data are represented as mean \pm SEM; $n = 5$ in control and 6–8 in treated groups. * $p < 0.05$, ** $p < 0.005$ and *** $p < 0.0005$ by t -test at individual time points. $p < 0.05$ for effect of AF-SWCNTs in all four panels by one way ANOVA).

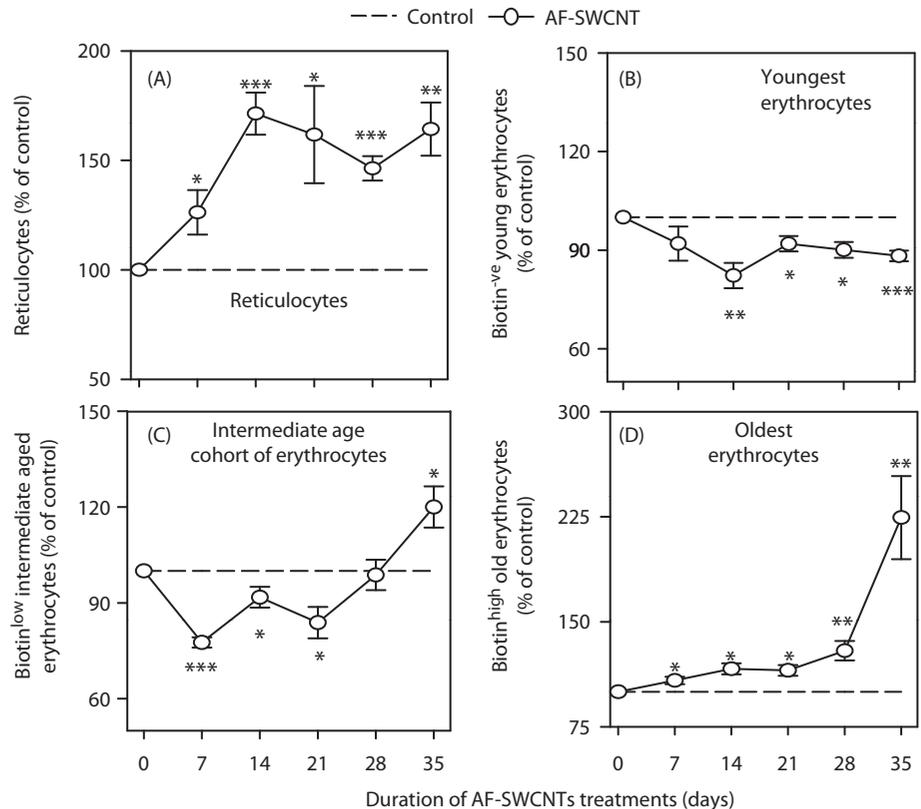
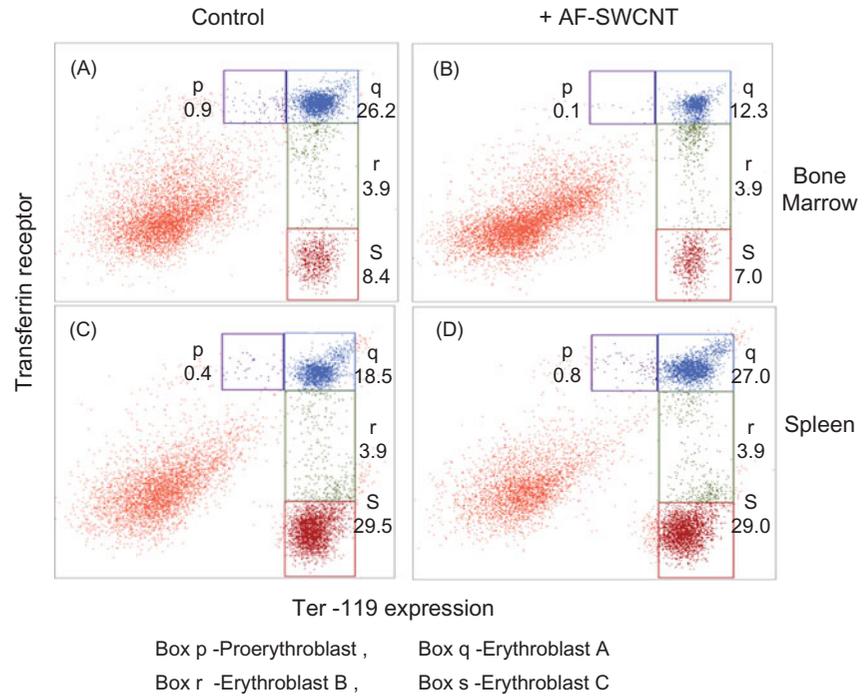


Figure 4. AF-SWCNT treatment induced changes in the relative proportions of cells in different stages of erythroid differentiation in bone marrow and spleen of mice. Mice were treated with repeated doses of AF-SWCNTs on alternate days. Bone marrow and spleen cells were harvested after 21 days of AF-SWCNT treatment. Proportion of erythroid cells in bone marrow and spleen was determined by staining with anti-mouse Ter-119-APC and anti-mouse CD71-PE antibody followed by flow cytometry. Four distinct stages (p, q, r and s) in erythroid differentiation were determined based on Ter-119/CD71 staining as described in the text. Panels A, B and C, D show Typical gating for delineating the four stages of erythroid differentiation *viz* pro-erythroblast (population-p), erythroblast A (population-q), erythroblast B (population-r) and erythroblast C (population s), have been shown for bone marrow (panels A, B) and spleen (panels C, D) of control (panels A, C) and AF-SWCNT treated (panels B, D) mice. Values in parentheses next to the gated cells represent the percentage values of different subpopulations of erythroid as percentage of all bone marrow cells.



to 23.43% (Figure 4B), suggesting a significant fall in erythropoietic activity in bone marrow. In contrast, the erythroid population in spleen expanded from 52.29% in control mice to 60.78% of spleen cells in AF-SWCNTs treated mice (Figure 4C and D).

Since there are four well-defined stages of erythroid differentiation in bone marrow and spleen, AF-SWCNTs induced alterations in relative proportions of these four erythroid subpopulations were examined. Flow cytometric diagrams in Figure 4 illustrate the gates used for the enumeration of the four stages of erythroid differentiation (pro-erythroblast, box-p; erythroblast-A, box-q; erythroblast-B, box-r; erythroblast-C, box-s). Decreased erythropoietic activity in bone marrow of AF-SWCNT treated mice is explained by a substantial fall in the relative proportions of erythroblast-A subpopulations in box-q, that constitute early stages of erythroid differentiation (Figure 4A and B). Similarly, increased erythropoietic activity in spleen of AF-SWCNT treated mice corresponded with an increased proportion of erythroblast-A stage of erythroid differentiation (Figure 4C and D).

Results in Figure 4 show representative data from a single experiment. Combined results of five experiments showing the mean relative proportions of different erythroid subpopulations \pm SEM, in bone marrow and spleen are shown in Table 1. These results show that a significant decline occurred in the relative proportion of pro-erythroblasts, erythroblast-A and C subpopulations in AF-SWCNT treated mice. In the spleen of AF-SWCNT treated mice, erythroblast-A and B subpopulations were significantly raised. Overall, increase or decrease in proportions of all erythroid cells correlated well with changes in the major early differentiation stage of erythroblast-A. Erythroblast-C population that represents the terminal stage of erythroid differentiation in bone marrow and spleen, declined significantly in both organs in AF-SWCNT treated mice. This could be due to the release of these cells into the blood circulation as a result of increased demand due to anemia.

Uptake of AF-SWCNTs by erythroid cells in bone marrow and spleen

AF-SWCNT induced changes in the relative proportions of erythroid subpopulations in bone marrow and spleen could be an

indirect effect of AF-SWCNTs or a direct consequence of interaction/uptake of AF-SWCNTs by the erythroid cells in these organs. In order to determine if AF-SWCNTs are actually taken up by the bone marrow and spleen erythroid cells, a fluorescence-tagged AF-SWCNT preparation (FAF-SWCNT) was administered intravenously in mice and the uptake of FAF-SWCNTs was assessed by flow cytometric analysis. Since the Alexa 488/633 dye used to tag the AF-SWCNT has fluorescence output distinct from the PE and APC fluorochrome used for anti-Ter119 and anti-CD71 antibodies, the erythroid subpopulations in bone marrow and spleen cells could be gated as in Figure 4, and FAF-SWCNT uptake could be examined for all erythroid cells as well as separately for each of the four erythroid subpopulations comprising various stages of differentiation of erythroid cells. Since two subpopulations (pro-erythroblasts and erythroblast-B) were present in very low proportions, 50 000 events were analyzed in these flow cytometric analyses to get reliable information about the uptake of FAF-SWCNTs, instead of the norm of 10 000 events. Results of three experiments are summarized in Table 2. These results show that the uptake of FAF-SWCNTs was rather low (3 to 4%) for whole erythroid cell population in both bone marrow and spleen. However, in the pro-erythroblast subpopulation that is very low in bone marrow and spleen, there was a relatively high uptake of FAF-SWCNTs (average 32 and 83% in bone marrow and spleen, respectively). Uptake of FAF-SWCNTs declined in erythroblast A and B subpopulations and was negligible in the more mature erythroblast-C subpopulation. These results point out to the possibility of a direct interaction of AF-SWCNTs with the early stages of differentiation of erythroid cells in bone marrow and spleen.

Discussion

We have previously shown that a single dose of 100 μ g of AF-SWCNTs induce significant yet transient anemia in mice (Sachar & Saxena, 2011). In the present study, we have shown that multiple doses (10 μ g) of AF-SWCNTs given on alternate days, induced a significant and sustained fall in blood erythrocyte count as well as blood Hb levels. A DIB technique was used to assess whether the elimination of erythrocyte in AF-SWCNT induced

Table 1. Effect of AF-SWCNT treatment on the relative proportions of cells in different stages of erythroid differentiation in bone marrow and spleen of mice.

Organ	Subpopulation	Relative proportion of the subpopulation in the organ (Mean \pm SEM)		
		Control	+AF-SWCNT	Change (%)
Bone marrow	All erythroid	42.88 \pm 2.92	31.56 \pm 4.71*	-26.4
	Pro-erythroblast	0.99 \pm 0.11	0.57 \pm 0.26	-42.4
	Erythroblast A	26.41 \pm 1.65	19.19 \pm 3.13*	-27.3
	Erythroblast B	3.35 \pm 0.20	4.80 \pm 1.20	+43.2
Spleen	Erythroblast C	12.09 \pm 1.05	6.79 \pm 1.17*	-43.8
	All erythroid	46.55 \pm 2.45	58.75 \pm 5.23*	+26.2
	Pro-erythroblast	0.75 \pm 0.23	0.66 \pm 0.24	-12.0
	Erythroblast A	18.20 \pm 1.04	28.45 \pm 4.10*	+56.3
	Erythroblast B	3.91 \pm 1.02	6.02 \pm 0.62	+53.9
	Erythroblast C	32.33 \pm 1.55	23.22 \pm 1.98*	-28.1

Mice were treated with repeated doses of AF-SWCNTs (10 μ g, *iv*) on alternate days. Bone marrow and spleen cells were harvested after 21 days of AF-SWCNT treatment. Proportion of erythroid cells in bone marrow and spleen was determined by staining with anti-mouse Ter 119-APC and anti-mouse CD71-PE antibody followed by flow cytometry (see Figure 4). Four distinct stages in erythroid differentiation Pro-erythroblast, Erythroblast A, Erythroblast B, and Erythroblast C were determined based on Ter 119/CD71 staining in bone marrow and spleen of control and AF-SWCNT treated mice.

All values represent mean \pm SEM; $n = 5$ in each groups. * $p < 0.05$.

Table 2. Uptake of AF-SWCNTs by cells of erythroid line of differentiation in bone marrow and spleen.

Subpopulation	FAF-SWCNT positive cells (%)	
	Bone marrow	Spleen
All erythroid	3.72 \pm 0.19	4.06 \pm 0.06
Pro-erythroblast	32.41 \pm 1.59	83.79 \pm 1.71
Erythroblast A	4.18 \pm 0.10	17.21 \pm 0.88
Erythroblast B	10.32 \pm 1.88	10.43 \pm 0.81
Erythroblast C	0.71 \pm 0.08	0.18 \pm 0.02

Mice were administered fluorescence tagged AF-SWCNTs (FAF-SWCNTs, single dose of 75 μ g intravenously). BM and spleen cells were isolated after 12 h of treatment and stained with anti-Ter119 and anti-CD71 monoclonal antibodies followed by flow cytometric analysis. Cells were gated based on Ter119/CD71 expression and FAF-SWCNT uptake was measured in the whole erythroid cell population as well as in gated subpopulations (p, q, r and s boxes in Figure 4). Since some of the erythroid differentiation stages have relatively fewer cells, a total of 50 000 events were accumulated on flow cytometer rather than the usual 10 000. The tabulated data represent mean \pm SEM of three different independent experiments.

anemia occurred uniformly irrespective of the age of the circulating erythrocytes or restricted a specific age group of erythrocytes in blood. DIB technique while identifying the young erythrocyte population in blood, did not separately enumerate blood reticulocytes that constitute a subpopulation of the youngest erythroid cells released into the blood circulation and mature into young erythrocytes within a period of 1–2 days (Koury et al., 2005). A modification of the DIB technique that we have used in this study involved additional staining with CD71 antibody (stains transferrin receptors present specifically on reticulocytes), and allowed us to further subdivide the youngest erythrocytes into reticulocytes and young erythrocytes other than reticulocytes (Figure 1). Time kinetics of changes in the relative proportions of reticulocytes clearly indicated that multiple doses of AF-SWCNTs resulted in a sustained reticulocytosis (Figure 3). On the earliest time point of 7th day after the commencement of AF-SWCNT treatment, there was a sharp decrease (about 20%) in the proportion of the biotin^{low} cohort of youngest erythrocytes

(Figure 3c). As seen from the data in Figure 1, cohort of erythrocytes released into the blood circulation of untreated control mice that remains stable and is resistant to elimination on earlier time points and there was no loss of the cohort population on 7 to 21 day time points. Significant elimination of erythrocytes from blood circulations occurred only at later time points after 21 days (see also Saxena et al., 2012). Significant decline in the proportion of youngest erythrocytes up to the age of 7 days in circulation in AF-SWCNT treated mice clearly indicates that youngest group of erythrocytes acquire susceptibility to elimination in mice treated with AF-SWCNTs. Throughout the course of AF-SWCNT treatment, the biotin^{low} population of younger erythrocyte remained significantly depressed in AF-SWCNT treated mice (Figure 3b). In contrast, the relative proportion of old erythrocytes (biotin^{high} population) increased progressively in blood circulation and doubled at 35 day time point (Figure 3d). These results suggest that in anemia induced by AF-SWCNTs, the loss of erythrocytes was not generalized but was restricted to younger erythrocytes.

Pristine SWCNTs are highly hydrophobic and have a tendency to agglomerate strongly into large aggregates that do not interact with cells. Acid-functionalization un-bundles the SWCNTs so that they can effectively interact with cells. We have previously shown that poly-dispersed preparations of ultra-fine carbon particles with carboxyl and sulfonate groups obtained by acid-functionalized do not have the cardio-vascular toxicity associated with AF-SWCNTs preparations (Tong et al., 2009), indicating that the addition of carboxyl and sulfonate groups on nanoparticles by itself is not a sufficient condition to render the particles cytotoxic. The unique shape of carbon nanotubes may therefore have a role in the toxicity displayed by their acid-functionalized derivative.

We have previously shown that the administration of AF-SWCNTs through intra-tracheal and gavage routes also exert systemic toxic effects including anemia in mice (Sachar & Saxena, 2011; Tong et al., 2009), indicating that the AF-SWCNTs may diffuse in the system irrespective of the route of administration. In the present study, we used the intravenous route for administering the AF-SWCNT preparations that would result in quick dissipation of the particles and a direct exposure of blood elements including erythrocytes to AF-SWCNTs. In light of our earlier results, effects of administration of AF-SWCNTs through

other routes is also likely to be effective in inducing anemia though the extent of effect may depend upon factors that determine the diffusibility of AF-SWCNTs in the system.

Similar results were also recently reported in anemia induced by exposure to paraquat, a toxic herbicide (Bhardwaj & Saxena, 2014), and the differential susceptibility of young and old erythrocytes in that system was linked to their ability to produce reactive oxygen species (ROS) (Bhardwaj & Saxena, 2014). In the AF-SWCNT induced anemia, we did not find a significant alteration in ROS production in young or old erythrocytes (Supplementary Table 1). Further, while paraquat had no direct toxic effect on erythrocytes *in vitro* (Bhardwaj & Saxena, 2014), AF-SWCNTs are taken up by erythrocytes and exert a direct toxic effect on mouse erythrocytes (Sachar & Saxena, 2011). Mechanisms of induction of anemia by AF-SWCNTs and paraquat may therefore be different.

Examination of the proportions of subpopulations of erythroid cells in different stages of differentiation in the bone marrow and spleen of control and AF-SWCNT treated mice indicated that while the erythroid population was significantly depressed in the bone marrow of AF-SWCNT treated mice, there was a concomitant increase in the proportion of erythroid cells in spleens. These results suggest that the erythropoietic activity may be shifting or expanding to spleen in AF-SWCNT treated mice. In both organs, the changes were closely correlated with changes in the erythroblast-A population that represents an early differentiation step of erythroid cells (Figure 4). Substantial fall in the erythroid population, indicative of lower erythropoietic activity could be a contributory factor in AF-SWCNT induced anemia in mice. We have previously demonstrated through confocal microscopic studies that fluorescence tagged AF-SWCNTs (FAF-SWCNTs) could enter mature erythrocytes. It was important to examine if intravenously administered AF-SWCNTs could reach bone marrow and directly interact with the cells of the erythroid lineage. Our results indicated that only 3 to 4% of Ter-119⁺ erythroid cells in bone marrow and spleen were positive for FAF-SWCNTs after 12 h of a single 75 µg dose of FAF-SWCNTs. Interestingly, however, a much larger proportions (32.4% in bone marrow and 83.8% in spleen) of the small population of the earliest erythroid stage of differentiation i.e. pro-erythroblasts were positive for FAF-SWCNTs. Next stage of differentiation i.e. erythroblast-A was also relatively heavily stained (17.2% positive) with FAF-SWCNTs in spleen. Only 4.18% of the erythroblast-A population was FAF-SWCNT positive in bone marrow. It is possible that erythroblast-A population in bone marrow is more susceptible to lysis upon exposure to AF-SWCNTs than the corresponding population in spleen, though this proposition needs further investigation. In general, the first three stages of erythroid differentiation had a significantly higher proportion of FAF-SWCNT stained cells. This observation would be supportive of the suggestion that the cells in the earlier stages of erythroid differentiation may take up more AF-SWCNTs and the resulting toxicity may be one of the important factors in AF-SWCNT induced anemia in mice.

Conclusion

In conclusion, we have demonstrated a sustained anemia with a significant and sustained reticulocytosis in mice exposed to multiple doses of AF-SWCNTs. Two factors that may contribute to the anemia induced by AF-SWCNTs, are (a) direct elimination of young erythrocytes in blood circulation, and (b) enhanced uptake of AF-SWCNTs by precursor cells belonging to earlier stages of erythroid differentiation, and a resulting suppression of bone marrow erythropoietic activity. A compensatory augmentation of erythropoietic activity may occur in spleens of

AF-SWCNT treated mice. This study represents first detailed investigation of the interactions of carbon nanotubes with the cells of the erythroid lineage in blood circulation as well as in spleen and bone marrow.

Declaration of interest

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

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References

- Alam A, Sachar S, Puri N, Saxena RK. 2013. Interactions of polydispersed single-walled carbon nanotubes with T cells resulting in downregulation of allogeneic CTL responses *in vitro* and *in vivo*. *Nanotoxicology* 7:1351–60.
- Baughman RH, Zakhidov AA, de Heer WA. 2002. Carbon nanotubes – the route toward applications. *Science* 297:787–92.
- Bhardwaj N, Saxena RK. 2014. Elimination of young erythrocytes from blood circulation and altered erythropoietic patterns during paraquat induced anemic phase in mice. *PLoS One* 9:e99364.
- Bianco A, Kostarelos K, Prato M. 2005. Applications of carbon nanotubes in drug delivery. *Curr Opin Chem Biol* 9:674–9.
- Chithrani BD, Ghazani AA, Chan WC. 2006. Determining the size and shape dependence of gold nanoparticle uptake into mammalian cells. *Nano Lett* 6:662–8.
- Davoren M, Herzog E, Casey A, Cottineau B, Chambers G, Byrne HJ, Lyng FM. 2007. *In vitro* toxicity evaluation of single walled carbon nanotubes on human A549 lung cells. *Toxicol In Vitro* 21: 438–48.
- Delogu LG, Magrini A, Bergamaschi A, Rosato N, Dawson MI, Bottini N, Bottini M. 2009. Conjugation of antisense oligonucleotides to PEGylated carbon nanotubes enables efficient knockdown of PTPN22 in T lymphocytes. *Bioconjug Chem* 20:427–31.
- Helland A, Wick P, Koehler A, Schmid K, Som C. 2007. Reviewing the environmental and human health knowledge base of carbon nanotubes. *Environ Health Perspect* 115:1125–31.
- Hsieh WY, Chou CC, Ho CC, Yu SL, Chen HY, Chou HY, et al. 2012. Single-walled carbon nanotubes induce airway hyperreactivity and parenchymal injury in mice. *Am J Respir Cell Mol Biol* 46:257–67.
- Jiang J, Oberdorster G, Elder A, Gelein R, Mercer P, Biswas P. 2008. Does nanoparticle activity depend upon size and crystal phase? *Nanotoxicology* 2:33–42.
- Kalfa TA, Pushkaran S, Zhang X, Johnson JF, Pan D, Daria D, et al. 2010. Rac1 and Rac2 GTPases are necessary for early erythropoietic expansion in the bone marrow but not in the spleen. *Haematologica* 95:27–35.
- Kam NW, Liu Z, Dai H. 2006. Carbon nanotubes as intracellular transporters for proteins and DNA: an investigation of the uptake mechanism and pathway. *Angew Chem Int Ed Engl* 45:577–81.
- Kam NW, O'Connell M, Wisdom JA, Dai H. 2005. Carbon nanotubes as multifunctional biological transporters and near-infrared agents for selective cancer cell destruction. *Proc Natl Acad Sci USA* 102: 11600–5.
- Khandelwal S, Saxena RK. 2006. Assessment of survival of aging erythrocyte in circulation and attendant changes in size and CD147 expression by a novel two step biotinylation method. *Exp Gerontol* 41: 855–61.
- Khandelwal S, Saxena RK. 2007. Age-dependent increase in green autofluorescence of blood erythrocytes. *J Biosci* 32:1139–45.
- Khandelwal S, Saxena RK. 2008. A role of phosphatidylserine externalization in clearance of erythrocytes exposed to stress but not in eliminating aging populations of erythrocyte in mice. *Exp Gerontol* 43: 764–70.
- Khandelwal S, van Rooijen N, Saxena RK. 2007. Reduced expression of CD47 during murine red blood cell (RBC) senescence and its role in RBC clearance from the circulation. *Transfusion* 47:1725–32.
- Koury MJ, Koury ST, Kopsombut P, Bondurant MC. 2005. *In vitro* maturation of nascent reticulocytes to erythrocytes. *Blood* 105: 2168–74.

- Kumari M, Sachar S, Saxena RK. 2012. Loss of proliferation and antigen presentation activity following internalization of polydispersed carbon nanotubes by primary lung epithelial cells. *PLoS One* 7:e31890.
- Lam CW, James JT, McCluskey R, Hunter RL. 2004. Pulmonary toxicity of single-wall carbon nanotubes in mice 7 and 90 days after intratracheal instillation. *Toxicol Sci* 77:126–34.
- Liu Y, Wang M, Zhao F, Xu Z, Dong S. 2005. The direct electron transfer of glucose oxidase and glucose biosensor based on carbon nanotubes/chitosan matrix. *Biosens Bioelectron* 21:984–8.
- Love SA, Maurer-Jones MA, Thompson JW, Lin YS, Haynes CL. 2012. Assessing nanoparticle toxicity. *Annu Rev Anal Chem (Palo Alto Calif)* 5:181–205.
- Marinkovic D, Zhang X, Yalcin S, Luciano JP, Brugnara C, Huber T, Ghaffari S. 2007. Foxo3 is required for the regulation of oxidative stress in erythropoiesis. *J Clin Invest* 117:2133–44.
- Pantarotto D, Singh R, McCarthy D, Erhardt M, Briand JP, Prato M, et al. 2004. Functionalized carbon nanotubes for plasmid DNA gene delivery. *Angew Chem Int Ed Engl* 43:5242–6.
- Paulson RF, Shi L, Wu DC. 2011. Stress erythropoiesis: new signals and new stress progenitor cells. *Curr Opin Hematol* 18:139–45.
- Sachar S, Saxena RK. 2011. Cytotoxic effect of poly-dispersed single walled carbon nanotubes on erythrocytes in vitro and in vivo. *PLoS One* 6:e22032.
- Saxena RK, Williams W, Mcgee JK, Daniels MJ, Boykin E, Gilmour MI. 2007. Enhanced *in vitro* and *in vivo* toxicity of poly-dispersed acid-functionalized single-wall carbon nanotubes. *Nanotoxicology* 4291:300.
- Saxena RK, Bhardwaj N, Sachar S, Puri N, Khandelwal S. 2012. A double in vivo biotinylation technique for objective assessment of aging and clearance of mouse erythrocytes in blood circulation. *Transfus Med Hemother* 39:335–41.
- Shvedova AA, Kisin E, Murray AR, Johnson VJ, Gorelik O, Arepalli S, et al. 2005. Unusual inflammatory and fibrogenic pulmonary responses to single-walled carbon nanotubes in mice. *Am J Physiol Lung Cell Mol Physiol* 289:L698–708.
- Sohaebuddin SK, Thevenot PT, Baker D, Eaton JW, Tang L. 2010. Nanomaterial cytotoxicity is composition, size, and cell type dependent. *Part Fibre Toxicol* 7:22–39.
- Tong H, McGee JK, Saxena RK, Kodavanti UP, Devlin RB, Gilmour MI. 2009. Influence of acid functionalization on the cardiopulmonary toxicity of carbon nanotubes and carbon black particles in mice. *Toxicol Appl Pharmacol* 239:224–32.

Supplementary material available online
Supplementary Table 1.