Carbon Nanotubes Enhance Metastatic Growth of Lung Carcinoma Via Up-Regulation of Myeloid-Derived Suppressor Cells

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Supporting Information

Experimental Section

Preparation and characterization of SWCNT: SWCNT (CNI Inc., Houston, TX) were produced by the high pressure CO disproportionation process (HiPco) technique employing CO in a continuous-flow gas phase as the carbon feedstock and $Fe(CO)_5$ as the iron-containing catalyst precursor, and purified by acid treatment to remove metal contaminates. Chemical analysis trace metal (iron) in SWCNT was performed at the Chemical Exposure and Monitoring Branch (DART/NIOSH, Cincinnati, OH) using nitric acid dissolution and inductively coupled plasma-atomic emission spectrometry (ICP-AES). Analysis revealed that SWCNT comprised of 0.23 weight % iron. SWCNT were routinely tested for bacterial endotoxin (LPS) contamination using the endpoint chromogenic LAL method. The mean diameter and surface area of SWCNT were 1-4 nm and 1040 m^2/g. Surface area was determined by Brunauer, Emmett and Teller (BET) analysis, and diameter and length was measured by TEM.

The chemical cutting of SWCNT was performed as reported previously.[1] Purified SWCNT (10 mg) were dispersed in a mixture of sulfuric acid/nitric acid (20 mL H_2SO_4:HNO_3 (3:1) mixture) in a round-bottom flask and sonicated in a bath-sonicator (Branson 5510) for 3 hr 30 min at 40 °C. The resulting mixture was then diluted 10X with pure distilled water and then

filtered through a PTFE membrane filter (SterliTech, 0.2 µm, 25 mm). The collected sample was thoroughly washed with deionized water until a neutral pH was achieved. The obtained short SWCNT were dispersed in 25 mM HEPES buffer (pH 7.4; containing 150 mM NaCl) by sonication. For purity assessment and characterization of SWCNT, several standard analytical techniques were utilized. Zeta potential (−30.36 mV) was measured using Brookhaven Instruments PALS zeta potential analyzer applying the Smoluchowski model. Transmission electron microscopy (TEM) was employed to determine the length distribution of (228 ± 77) nm. Analysis was performed using an FEI Morgagni TEM with 80 keV electron beam. Atomic force microscopy (AFM) was done on a multimode scanning probe microscope (Veeco) in tapping mode. Raman spectroscopy was implemented to visualize the D and G bands. Raman spectra were excited with a 633 nm laser and measurements were performed using the Renishaw InVia Raman microscope. Diffuse reflectance infrared Fourier Transform spectroscopy (DRIFTS) was also performed using a Shimadzu IR-Prestige 21 Fourier transform spectrophotometer. Stock suspensions (1 mg/ml) were prepared before each experiment in PBS and pH adjusted to 7.0; suspensions were sonicated for 5 minutes and sterilized by autoclaving. Stock suspensions were diluted to achieve required concentrations and sonicated (three one-min cycles) before use.

Animals and SWCNT exposure: Animal studies were carried out under the experimental protocol (#11-AS-M-021) approved by the Institutional Animal Care and Use Committee at the National Institute for Occupational Safety and Health (NIOSH, Morgantown, WV, USA). Pathogen-free female C57BL6/J mice (7–8 wk old) were supplied by Jackson Labs (Bar Harbor, ME). Animals were individually housed in the NIOSH facilities approved by the American Association for Accreditation of Laboratory Animal Care (AAALAC) and acclimated for 1 wk. Sterile Beta Chip bedding (Northeastern Products, Warrensburg, NY) was changed weekly. Animals were supplied with water and food (Harlan Teklad, 7913, NIH-31 Modified Mouse/Rat

Diet, Irradiated; Harlan Teklad, Madison, WI) *ad libitum* and housed under controlled light, temperature, and humidity conditions. Experiments were conducted under a protocol approved by the Animal Care and Use Committee of NIOSH.

Animals were exposed to CNT by pharyngeal aspiration as described earlier.[2] In brief, after anesthesia with a mixture of ketamine and xylazine (Phoenix, St. Joseph, MO) (62.5 and 2.5 mg/kg subcutaneous in the abdominal area), each mouse was placed on a board in a near-vertical position, and the tongue was gently extended with lined forceps. A suspension of CNT (80 μg/mouse in saline) was placed posterior in the throat, and the tongue was held until the suspension was aspirated into the lungs. Six to seven animals per group were utilized for the *in vivo* assays. Eight mice per group were utilized for evaluation of metastases. All experiments have been independently repeated at least three times.

Tumor cell culture and administration: Lewis lung carcinoma cells (LLC) were obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 10% heat-inactivated FBS, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate (Invitrogen Life Technologies, Inc, Grand Island, NY).

Forty eight hours after CNT exposure, mice were inoculated with 3×10^5 LLC cells (300 μl PBS) via the tail vein. The injections were alternated between groups of animals in order to minimize variances during the injection. Twenty one days later, the animals were sacrificed by injection of sodium phenobarbital and the number of visible pulmonary metastases was determined using a dissection microscope. Lungs were weighted, and right lobes were fixed with 10% formaldehyde for histopathology evaluation of H&E stained specimens.

Evaluation of regulatory T cells, DC and MDSC subsets: For pulmonary DC and MDSC analysis (48h after CNT exposure and 21 days after tumor inoculation), mouse lungs were dispersed

using 2% collagenase A and 0.75% DNAse I (Roche Diagnostics GmbH, Mannheim, Germany) in RPMI 1640 with 10% FBS at 20°C for 1 h. Spleens were harvested into 5 ml of complete RPMI-1640 medium in a sterile culture dish, grounded, and cell suspensions were filtered through a 70 μm cell strainer. Bone marrow cells were flashed from tibia and femur in complete RPMI-1640 medium. Red blood cells in the lung, spleen and bone marrow preparations were lysed with lysing buffer (155 mM NH_4Cl in 10 mM Tris-HCl buffer pH 7.5, 25°C) for 3 min. After RBC lysis, cells were washed 2 times with FACS buffer (PBS containing 0.1% BSA and 0.05% sodium azide (Sigma, St. Louis, MO)). Cell suspensions were labeled with anti-CD11b, anti-CD11c, anti-Ly6G, anti-Ly6C, anti-Gr-1 and anti-CD45 antibodies (Biolegend Inc., San Diego, CA) directly conjugated to FITC, PE, PE/Cy7, APC/Cy7 or Alexa700. Stained cell suspensions were analyzed by flow cytometry (BD LSR II instrument, BD Biosciences, San Jose, CA). Data on MDSC and DC counts in the lung and spleen are presented as the percentage of total cells and CD45+ cells in the tested tissue.

Regulatory T cells (Treg) were assessed in the spleens of mice on day 21 after LLC cell injection. The spleen cells were stained with anti-CD4 and anti-CD25 antibodies followed by Anti mouse/rat/human FoxP3 Flow Kit (Biolegend Inc., San Diego, CA) according to manufacturer's instructions and analyzed by flow cytometry.

MDSC depletion: For the *in vivo* depletion of MDSC, mice were treated with LEAF™ Purified anti-mouse Ly-6G/Ly-6C (Gr-1) Ab (i.p., 50 μg/day, clone RB6-8C5, Biolegend Inc., San Diego, CA) 24 and 2 h before and 24 h after CNT exposure. The successful and selective depletion of MDSC was confirmed at 48 h post CNT administration in the spleen and lung of mice by assessment of MDSC subsets using flow cytometry.

Statistical analysis: Results were analyzed using one-way ANOVA and Student unpaired *t*-test with Welch's correction for unequal variances. All results are presented as means ± SEM. P values of < 0.05 were considered to be statistically significant.

References

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[2] A.V. Tkach, G.V. Shurin, M.R. Shurin, E.R. Kisin, A.R. Murray, S.H. Young, A. Star, B. Fadeel, V.E. Kagan, A.A. Shvedova, *ACS Nano* **2011**, *5*, 5755.

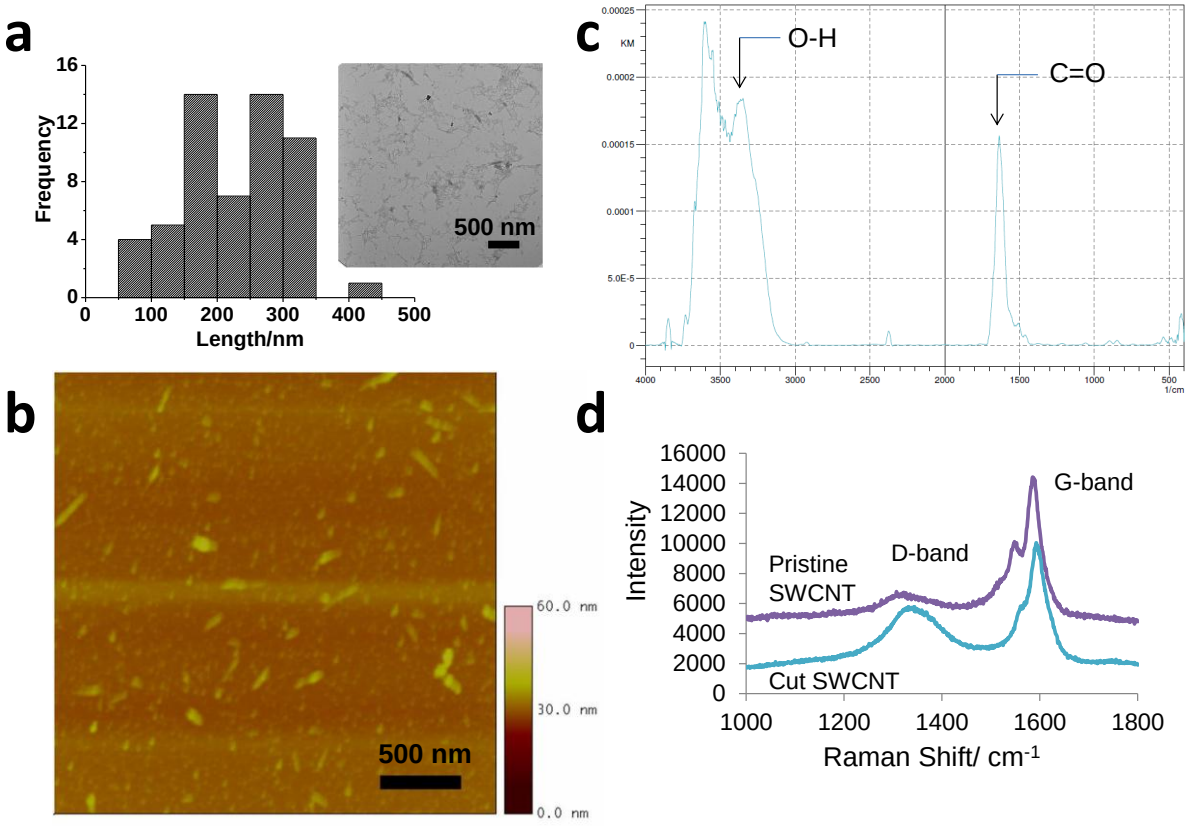

Figure S1

<u>Figure S1</u>. Characterization of SWCNT. SWCNT were chemically cut by ultrasonication treatment with sulfuric acid/nitric acid as described in Methods. **a.** Transmission electron microscope (TEM) image and histogram detailing the length distribution of SWCNT. The mean length was determined to be 228 ± 77 nm for the resulting short-cut SWCNT. **b**. Atomic force microscope (AFM) image indicating small bundles of short SWCNT (diameters 2 - 6 nm). **c**. Diffuse reflectance infrared Fourier Transform spectroscopy (DRIFTS) spectrum of the cut SWCNT sample indicating presence of carboxylic groups. The unit for the ordinate axis is Kubelka-Munk (KM). **d**. Raman spectra of pristine and short-cut SWCNT; the D- and G- bands are marked on the spectra. The observed increase in the D/G ratio of cut SWCNT is consistent with introduction defects during the chemical cutting procedure.

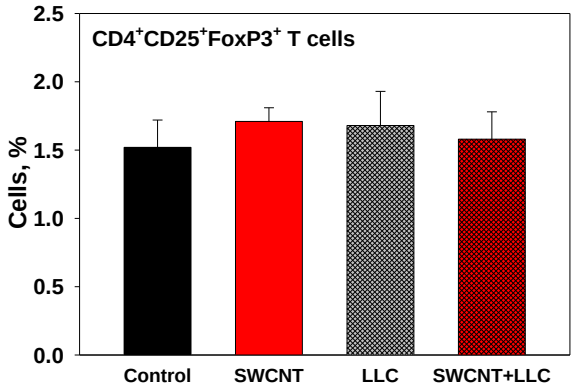

Figure S2

Figure S2. Analysis of regulatory T cells. Mice received SWCNT and LLC cells as described in Methods and the levels of $CD3^{+}CD25^{+}FoxP3^{+}$ Treg cells were assessed by flow cytometry in the spleen. The results of flow cytometry analysis of Treg cell alterations in tumor-free and tumor-bearing mice three weeks after LLC cell inoculation were analyzed from 3 independent experiments with 5-6 animals per group and are shown as the mean ± SEM.