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Cross-Talk between Lung and Systemic Circulation during Carbon Nanotube Respiratory Exposure. Potential Biomarkers

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ABSTRACT

Nanotechnology is an emerging field that demands urgent development of adequate toxicology and risk assessment. The previous experimental data on carbon nanotube respiratory exposure strongly suggest the need for complex evaluation of potential toxicity. Our work demonstrates that after carbon nanotube deposition in the lung, acute local and systemic responses are activated and characterized by a blood gene and protein expression signature. The approach described here will foster the development of biomarkers for application in human screening of nanoparticle exposure.

Engineered carbon nanomaterials, including carbon nanotubes, have elicited a great deal of interest due to their unique electronic and mechanical properties. Not surprisingly, the most technologically attractive features of these new materials, including their small size, large surface area, and high reactivity, are also the main factors for potential pulmonary and systemic toxicity.[1,2] Initial toxicological studies demonstrated that pulmonary deposition of single-walled or multiwalled carbon nanotubes (SWCNTs or MWCNTs) caused a lung accumulation of carbon nanotubes including aggregates as well as acute and chronic pulmonary toxicity in animal models.[3−9] Furthermore, we demonstrated that SWCNT-related pulmonary toxicity was associated with adverse cardiovascular effects including distressed aortic mitochondrial homeostasis and acceleration of atherogenesis in an ApoE−/− mouse model.[10] Also, an acute systemic prothrombotic response, a potential contributor to an adverse cardiovascular outcome, was shown following MWCNT-induced lung inflammation.[11] The lung insult, by release of inflammatory proteins, activation of circulating blood cells, and altered pulmonary function, can trigger systemic toxicities.[12] The initial toxicological data on CNT respiratory exposure strongly suggest the need for complex evaluation of both the local and systemic effects such as cardiovascular, neurologic, and immunologic toxicity. In this regard, the application of blood screening methods for evaluation of the systemic response to particle exposure of the lung will aid in predicting possible toxicity and, consequently, establish strategies for safe nanomaterial production and use.

Recently, studies have shown that, in addition to blood protein parameters, the gene expression analysis of circulating peripheral blood cells, which come into contact with all tissue and contribute to homeostasis, provides valuable information on the state of health or disease in the body. For example, human coronary artery disease and inflammation have been approached using blood cell gene expression array analysis.[13−15] Thus, we hypothesized that following a pulmonary exposure a rapid systemic response, which may affect the cardiovascular system, would result in an elevated series of measurable potential biomarkers in the blood. These biomarkers, including genes expressed in the circulating blood cells and/or soluble proteins released into the circulation, may not be unique to the type of particles but will represent the unity of local and systemic pathophysiological

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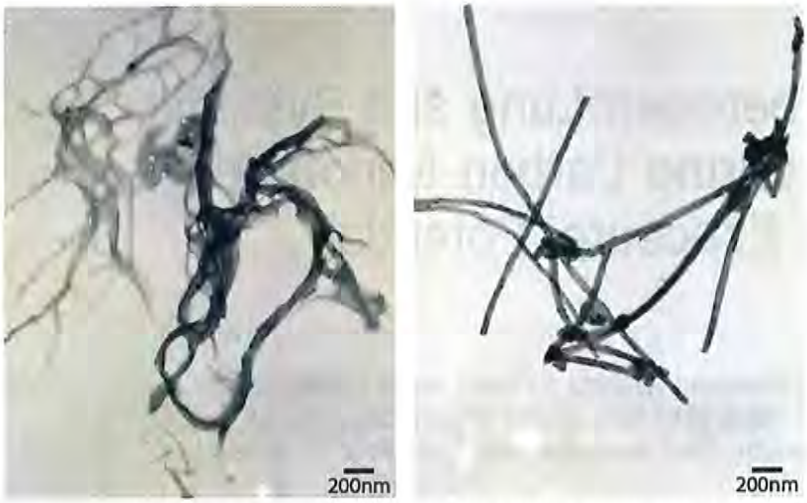

Figure 1. Representative transmission electron microscopy pictures of SWCNT (left panel) and MWCNT (right panel) preparations in dispersion media.

responses induced as a result of the exposure. To test the hypothesis, C57BL/6 mice were exposed by pharyngeal aspiration to vehicle, ultrafine carbon black, SWCNTs, or MWCNTs at a dose of 40 μg per mouse and sacrificed 4 h postexposure. We designed and applied a select panel of genes, known to play an important role in the molecular mechanisms of tissue injury such as inflammation, oxidative stress, and coagulation, to evaluate the simultaneous expression of these genes in the lung, circulating blood cells, and aorta upon pulmonary deposition of particles. A subset of genes found to be up-regulated in the aorta were evaluated in heart, liver, and kidney tissue samples. The gene expression response was correlated to changes in the levels of key inflammatory and prothrombotic blood proteins.

Briefly, male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) 10 weeks of age were exposed to UFCB (Printex 90, Degussa, Germany) or catalytically grown SWCNTs (Carbon Nanotechnologies, Inc., Houston, TX) or MWCNTs (Mitsui & CO., LTD).[16] Trace metal analysis by inductively coupled plasma optical emission spectrometry indicated iron content of SWCNTs at 8.8% by weight and 0.27% for MWCNTs. Particle size according to the manufacturer for UFCB was 14 nm in diameter and SWCNT was 0.8−1.2 nm in diameter and 0.1−1 μm in length. Characteristics of the MWCNTs (~80 nm in diameter and 10−20 μm in length) have been described in greater detail.[17,18] The particles were dispersed in a vehicle, dispersion media (DM), which contains mouse serum albumin (0.3 mg/mL final concentration) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DSPC; 5 μg/mL).[19] The particle suspensions were characterized by transmission electron microscopy (TEM) from the preparation used for dosing (Figure 1). The MWCNT appeared as better dispersed bundles with measurable sizes compared to the SWCNT.

Mice were exposed by pharyngeal aspiration[20] and sacrificed at 4 h postexposure, and blood was collected for serum or plasma antigen analysis and whole blood gene expression. The left lung was ligated and frozen in liquid nitrogen, and the right lung was used for bronchoalveolar lavage (BAL). The tissue and blood collection was performed as described before.[10] Aorta (from the aortic root to the iliac bifurcation), heart, liver, and kidney were dissected and immediately frozen in liquid nitrogen. All samples were stored at −80 °C prior to analysis. Gene expression changes in lung, aorta, and blood were measured using a custom designed TaqMan array which included parameters of inflammation (37%), oxidative stress (21%), growth factors (17%), tissue remodeling (12%), endothelial function (8%), and coagulation (5%) (Table S1). All of the genes found upregulated in the blood and aorta were confirmed by real-time RT-PCR. A subset of genes found to be up-regulated in the aorta were evaluated in heart, liver, and kidney by a conventional real-time RT-PCR. The lung response was characterized also by the conventional BAL evaluation. Blood protein profiling was performed by multiplex immunoassay. Total and active levels of plasminogen activator inhibitor-1 (PAI-1), a pro-coagulant acute phase, were measured in the lung and plasma by ELISA. A more detailed experimental procedure is available in the Supporting Information.

In vehicle-treated mice, alveolar macrophages represent the main cellular component, with neutrophils not exceeding more than 2−3% of the total BAL cell counts. In all particle treatment groups, the differential counts of BAL fluid demonstrated significant neutrophil (polymorphonuclear, PMN) influx with a milder response after UFCB as compared to CNT (Table 1). The microscopic analysis of BAL fluid also demonstrated different degrees of dispersion of the particles with the best dispersion achieved with the MWCNT, which is consistent with the TEM characterization of the material suspensions before the exposure (Figure 1). Lactate dehydrogenase (LDH) activity, a marker of cellular cytotoxicity, was increased in the BAL fluid but significantly only in the MWCNT-exposed group (DM 55 ± 8 U/L; UFCB 65 ± 8; SWCNT 71 ± 6; MWCNT 133 ± 9*; $*p < 0.05$).

Furthermore, CNT exposure induced gene upregulation of more than half of the tested genes in the lung related to inflammation, oxidative stress, coagulation, and remodeling (Figure 2A). MWCNT-exposed mice showed a greater

Table 1. Total and Differential (%) Cell Count of Bronchoalveolar Lavage (BAL) and Blood 4 h after Treatment with Vehicle (DM), UFCB, SWCNTs, or MWCNTs[a]

	cell no. (10^5)			
BAL	total cells	macrophages	neutrophils	eosinophils
DM	3.52 ± 0.44	3.49 ± 0.45 (99%)	0.03 ± 0.01 (1%)	0.00 ± 0.00 (0.00%)
UFCB	2.81 ± 0.51	2.40 ± 0.40[b] (87%[c])	0.40 ± 0.13 (13%[c])	0.01 ± 0.01 (0.42%)
SWCNT	3.95 ± 0.34	2.42 ± 0.19[b] (62%[c])	1.49 ± 0.20[c] (37%[c])	0.04 ± 0.00 (0.93%)
MWCNT	4.51 ± 0.44	1.93 ± 0.13[b] (44%[c])	2.51 ± 0.43[c] (55%[c])	0.08 ± 0.06 (1.42%)

	cell no. (10^6)				
blood	total cells	lymphocytes	monocytes	neutrophils	eosinophils
DM	2.56 ± 0.20	2.14 ± 0.19 (84%)	0.03 ± 0.01 (1.4%)	0.36 ± 0.04 (14%)	0.02 ± 0.01 (0.7%)
UFCB	3.03 ± 0.22	2.25 ± 0.25 (74%)	0.04 ± 0.02 (1.5%)	0.72 ± 0.12[c] (24%)	0.02 ± 0.01 (0.6%)
SWCNT	3.68 ± 0.23	2.44 ± 0.25 (66%[b])	0.03 ± 0.01 (0.9%)	1.15 ± 0.15[b] (32%[b])	0.06 ± 0.01 (1.7%)
MWCNT	2.47 ± 0.12	1.38 ± 0.12[c] (56%[c])	0.02 ± 0.01 (0.9%)	1.04 ± 0.06[b] (43%[c])	0.02 ± 0.01 (0.9%)

[a] Each value represents the mean ± standard error of five mice. [b] $p < 0.05$ vs DM. [c] $p < 0.05$ vs all groups.

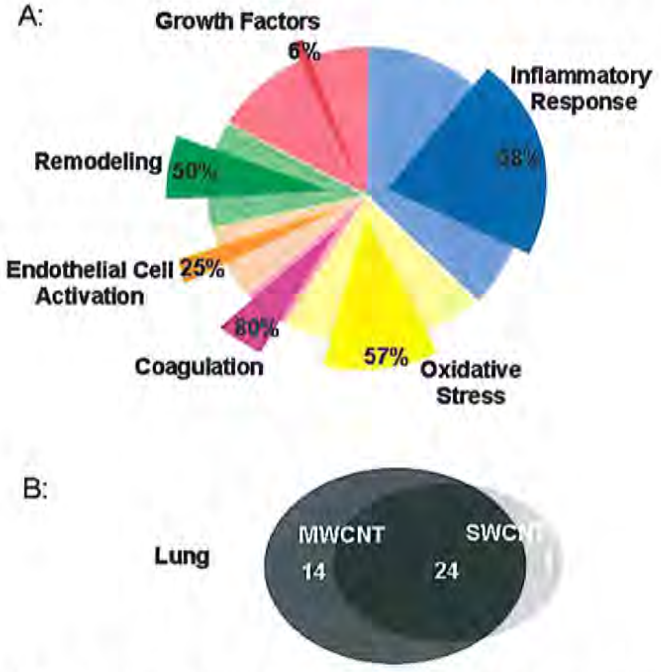

Figure 2. Effect of particle exposure on lung gene upregulation 4 h postexposure. (A) The pie chart shows a categorical breakdown of genes in the custom designed TaqMan array. The overlaid slices with inset percentages indicate genes that were induced by MWCNT exposure. (B) Comparison of the response between SWCNT and MWCNT for numbers of genes that were upregulated in the lung.

number of upregulated genes in the lung with qualitatively higher expression compared to the SWCNT-exposed group (Figure 2B and Table S2). As expected early after the lung insult, many genes involved in the recruitment and activation of inflammatory cells, including monocytes and neutrophils, as well as regulation of inflammation, were expressed in the CNT-exposed lungs (Table S2). This early lung response was also represented by activation of genes coding proteins of an acute stress response (e.g., metallothioneins (MT)) and coagulation (e.g., PAI-1, fibrinogen and tissue factor) (Table S2).

Simultaneous with increased lung gene expression, pulmonary exposure to CNT, primarily MWCNT, resulted in a significant increase in the circulating blood gene expression of several biomarkers of a neutrophil response, such as CXCL2 (MIP-2), S100a8, IL-8rβ, and Mac-1 (Figure 3, panels A and B, and Table S2). Consistently, the differential count demonstrated a significant increase in the number of neutrophils in the blood (Table 1).

Stress-response genes such as MT-1, hypoxia inducible factor 3 alpha (Hif-3α), matrix metalloproteinase 9 (MMP-9), and arginase II were also induced by MWCNT exposure in a fashion of shared expression between the lung and circulating blood cells (Figure 3B and Table S2). Furthermore, several genes were activated in the circulating blood cells but not in the lung at least at the 4 h time point after pulmonary exposure to MWCNT (Figure 3, panels A and C). Of the latter, osteopontin, colony stimulating factor-1 (CSF-1), and insulin growth factor receptor 1 (IGF-1R) might represent a blood macrophage response. The gene expression analysis of the lung and blood of UFCB-exposed mice, consistent with BAL data, indicated a milder response compared to the CNT-treated groups (representative lung gene expression as well as blood gene expression, Figure 3, panels D and E, respectively).

In addition to the gene expression analysis, blood samples were characterized by multiplex immunoassay technology to determine changes in circulating serum proteins, 69 in total, following CNT exposure. At 4 h postexposure several key inflammatory proteins were elevated in the serum as shown in Table S3; the effect was more pronounced in the MWCNT-treated mice. Both SWCNT and MWCNT exposure led to a marked serum increase of IL-6, a mediator of the acute phase response and bone marrow activation for release of leukocytes and platelets. Exposure to MWCNT also increased significantly the serum levels of the neutrophil chemoattractant CXCL1 (KC/GROα), a murine analogue of human IL-8, as well as several cytokines/chemokines, such as IL-5, CCL11 (eotaxin) and CCL22, mostly associated with a recruitment of lymphocytes and eosinophils.

Most of the elevated serum proteins reflected pulmonary gene expression rather than blood gene expression (Table 2). Similar to the MWCNT exposure, rapid and preferential release of CXCL1 (rat CINC) but not CXCL2 (MIP-2) into the systemic circulation was found following LPS inflammation.[21] Other inflammatory mediators, such as CXCL2 and IL-1β, were induced in both the blood and lung by MWCNT exposure, but without a corresponding serum protein increase at 4 h postexposure (Table 2). The serum levels of some

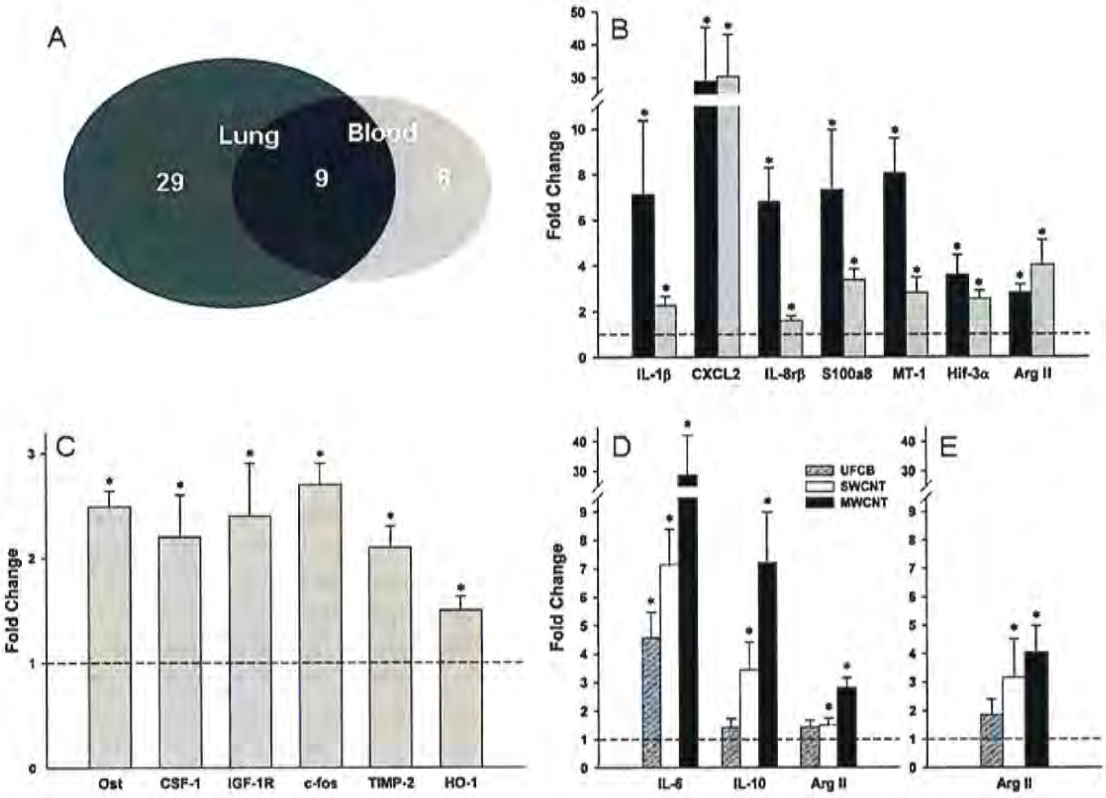

Figure 3. Effects of particle exposure on lung and blood gene expression 4 h postexposure. (A) Comparison of the response between the lung and blood for genes that were increased in mice exposed to MWCNT. (B) Representative genes expressed in lung (black bars) and whole blood cells (gray bars) from mice exposed to MWCNT. (C) Representative genes only in whole blood cells from mice exposed to MWCNT. (D) Comparison of the effects of UFCB, SWCNTs, and MWCNTs on lung gene expression (representative genes). (E) Comparison of the effects of UFCB, SWCNTs, and MWCNTs on blood arginase II gene expression.

Table 2. Changes in Serum Proteins and the Respective Lung and Whole Blood Cell Gene Expression (MWCNT Exposure)

name	lung RNA	blood RNA	serum proteins
CD40 (pg/mL)	↔	↓	↑
CCL11(pg/mL)	↑	↔	↑
IL-5 (ng/mL)	↑	↔	↑
IL-6 (pg/mL)	↑	↔	↑
CXCL1 (ng/mL)	↑	↔	↑
CCL22 (pg/mL)	↑	↔	↑
MMP-9 (ng/mL)	↑	↑	↑
Osteopontin (ng/mL)	↔	↑	↓
CXCL2 (pg/mL)	↑	↑	↔
Il-1β (ng/mL)	↑	↑	↔

proteins did not correspond to the respective lung or blood gene expression. Thus, the soluble CD40, a cell surface marker, might be increased in the serum as a result of its shedding from a variety of cells expressing it constitutively.[22] Osteopontin, a marker of macrophage activation, was slightly reduced in the serum at this time point, although this effect was accompanied by a minor increased expression in the blood cells. The link between gene expression and protein synthesis is a complex process with multiple steps of regulation which may vary for the different biomarkers. Therefore, these data strongly suggest that the simultaneous analysis of both blood cell gene expression and protein profiling helped to reveal in greater detail the CNT-induced response.

In addition to inflammatory mediators, total and active levels of PAI-1, a pro-coagulant acute-phase protein which is involved in inhibition of the fibrinolytic cascade, were measured in the lung and plasma. PAI-1 total protein levels were significantly increased in both the SWCNT- and MWCNT-exposed lungs (Figure 4A), which was consistent with the PAI-1 gene expression results (Figure 4B). Similar to the inflammatory response, the increased PAI-1 was greater in the MWCNT as compared to UFCB and SWCNT exposure. The lung PAI-1 activation was accompanied with an increase in the plasma levels of total and active PAI-1 (Figure 4, panels C and D, respectively) with a significant response only in the MWCNT-treated group. The blood PAI-1 response was not concomitant with activated gene expression in the circulating blood cells, which indicates that PAI-1 protein is most likely released from the particle-exposed lung into the systemic circulation.

The increase of serum inflammatory proteins and blood cell gene expression as a result of pulmonary exposure to CNT suggests a potential corresponding response by systemic tissues, including cardiovascular tissues. Indeed, gene expression analysis, using the custom-designed TaqMan arrays, demonstrated that several gene coding mediators of an acute stress response and inflammation were activated in the aortic tissue by the CNT pulmonary exposure (Figure 5 and Table S2). Consistent with the lung, pulmonary exposure to MWCNT (strong trend in SWCNT) increased gene expres-

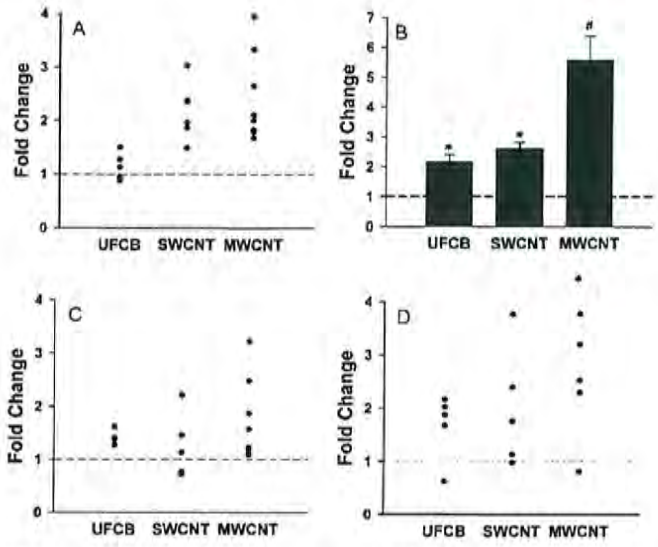

Figure 4. Effect of particle exposure on lung and plasma plasminogen activator inhibitor 1 (PAI-1) 4 h postexposure. (A) Total PAI-1 levels in lung homogenates from mice exposed to UFCB, SWCNT, or MWCNT. (B) Lung gene expression changes in PAI-1. (C) Total plasma PAI-1 levels following particle exposure. (D) Free (active) plasma PAI-1 levels following particle exposure.

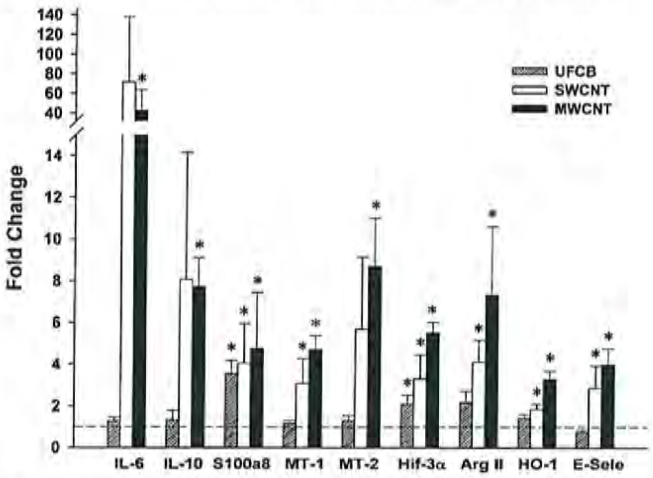

Figure 5. Effect of UFCB, SWCNT, and MWCNT exposure on aortic gene expression 4 h postexposure. $*p < 0.05$ vs vehicle-treated mice.

sion in the aorta of pro-inflammatory IL-6 and anti-inflammatory IL-10, as well as the inflammatory cell-tissue directing mediator S100a8. Additionally, the aorta responded to CNT pulmonary exposure with increased gene expression of MT-1 and -2, Hif-3α, arginase II, as well as HO-1, an oxidative stress surrogate biomarker (Figure 5 and Table S2). Only mild effects were found in UFCB-exposed mice (Figure 5). Some of these biomarkers, including MT-1, -2, and Hif-3α, were also induced in other systemic tissues such as heart, kidney, and liver as a result of the CNT exposure (Table 3). Although there was evidence of a generalized systemic response, only the aorta, in addition to the lung, demonstrated a significant induction of E-selectin gene expression as a result of CNT-exposure (Figure 5). E-selectin, a cell adhesion molecule expressed only on activated endothelial cells, plays an important role in the recruitment of leukocytes through the vessel wall.[23,24] The CNT exposure resulted in a trend of increased gene expression of aortic P-selectin (data not shown), another molecule involved in adhesion of leukocytes and platelets, which can be expressed on endothelial cells as well as platelets.

These studies demonstrated that 4 h after carbon nanotube deposition in the lung, local and systemic inflammatory as well as prothrombotic responses are activated, and the effect is portrayed by a complex pattern of gene expression and protein blood analyses (Figure 6).

In essence, the acute respiratory exposure to CNT resulted in the lung expression of many gene coding mediators of inflammation, oxidative stress, remodeling, and thrombosis. The lung response resulted in correspondent alterations in the systemic circulation including soluble biomarkers of inflammation and coagulation as well as activated blood cells. Interestingly, this response was paralleled by a representative systemic tissue response including the gene expression of arginase II, MT-1, and Hif-3α in aorta, heart, liver, and kidney. Although the simultaneous expression of these genes has not been studied, many factors, such as hypoxia, reactive oxygen species, and inflammation, can trigger transcriptional activation of each gene.[25−28] Furthermore, CNT-induced systemic distress at the level of aorta included the activation of E-selectin, an endothelial specific cell adhesion molecule that facilitates recruitment of leukocytes into the vessel wall. While the selectin expression in the lung is a part of the clearance mechanism, the expression in the systemic vasculature together with an impaired blood inflammatory and coagulation balance might contribute to endothelial dysfunction. If persistent, these alterations could trigger acceleration of atherosclerosis progression and/or precipitation of its complications.[29,30] In this respect, epidemiological and experimental studies have recently found a positive association between air pollution (including ultrafine particulates) and adverse cardiovascular outcomes, particularly in high risk individuals, such as those with preexisting chronic pulmonary or cardiovascular diseases.[31−33]

Pulmonary exposure to CNT triggered the induction of primary cytokines such as IL-6 and IL-1β, which regulate multiple pathways of the inflammatory cascade as well as secondary inflammatory mediators, chemokines (CCL2, 4, 19, 22; CXCL1, 2), which directly regulate leukocyte recruitment to the inflammatory site. The CC chemokines are mainly involved in the migration and activation of monocytes, macrophages, and lymphocytes while those of the CXC family attract neutrophils.[34] Chemokines exert their effects through specific seven transmembrane-spanning G protein-coupled receptors that are differentially expressed on leukocytes.[35] Consistent with an initial neutrophil influx during acute inflammation, the early response to CNT was associated with increased expression of the neutrophil-specific chemokine receptor CXCR2 (IL-8rβ) signifying the neutrophil presence in both the lung and blood. In addition, based on the CNT-induced CC-chemokine expression, it is obvious that the initial inflammatory cell response is likely to be followed by mononuclear leukocyte migration and activation. Coinciding with pulmonary inflammation, markers of coagulation were increased in both the lung and circula-

Table 3. Effect of SWCNT and MWCNT exposure on heart, liver, and kidney expression of selected genes, found to be up-regulated in the aorta[a]

	heart		liver		kidney	
gene	SWCNT	MWCNT	SWCNT	MWCNT	SWCNT	MWCNT
MT1	1.3 ± 0.2	2.3 ± 0.2^b	4.3 ± 2.5	15.2 ± 1.2^b	1.5 ± 0.3	1.7 ± 0.2^b
MT2	2.0 ± 1.0	5.8 ± 0.7^b	8.6 ± 5.9	37.5 ± 3.8^b	1.9 ± 0.4	2.6 ± 0.4^b
Hif-3α	2.1 ± 0.4^b	4.4 ± 0.6^b	2.8 ± 0.7^b	5.0 ± 1.2^b	3.8 ± 1.3^b	4.8 ± 0.4^b
Arg II	1.7 ± 0.5	4.7 ± 2.2^b	0.9 ± 0.1	1.2 ± 0.2	1.3 ± 0.2	1.3 ± 0.1
S100a8	3.4 ± 0.9^b	6.2 ± 2.6^b	1.0 ± 0.3	3.3 ± 1.0^b	1.8 ± 0.9	2.8 ± 0.7^b

[a] Data are represented as fold change from vehicle treated mice. [b] $p < 0.05$ vs vehicle-treated mice.

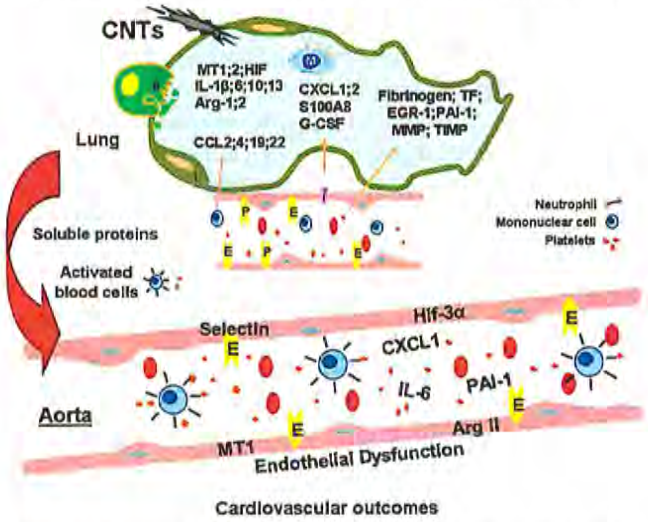

Figure 6. Summary of the findings and conclusions of these studies. The cartoon represents the cross-talk between lung and systemic circulation as well as the potential risk for cardiovascular adverse outcomes. Abbreviations include (I) alveolar type I cell; (II) alveolar type II cell; (M) alveolar macrophage; (MT) metallothionein; (HIF) hypoxia inducible factor; (IL) interleukin; (Arg) arginase; (G-CSF) granulocyte-colony stimulating factor; (TF) tissue factor; (EGR) early growth response; (PAI) plasminogen activator inhibitor; (MMP) matrix metalloproteinase; (TIMP) tissue inhibitor of metalloproteinase.

tion. Enhanced thrombotic activity has been associated with several pulmonary exposures including MWCNT.[11,36,37] Further, in vitro studies have shown that CNT can directly induce platelet aggregation while intravenous injection results in enhanced thrombosis implying a potential risk if CNTs extricate the lung.[38]

One protein involved in a procoagulant state is PAI-1.[39] To date, assessment of PAI-1, both lung and circulating levels, following pulmonary exposure to CNTs has not been done, although previously, pulmonary PAI-1 has been shown to be elevated following high-dose silica exposure (5 mg/mouse).[40] PAI-1 can be directly involved in the acute lung inflammation, more specifically in neutrophil recruitment to the alveolar compartment.[41] However, the primary role of PAI-1 is to inhibit fibrinolysis, the process of clot degradation, and has been shown as a risk factor in thrombotic associated diseases including cardiovascular disease.[39] Many clinical studies suggest PAI-1 as an early marker of an acute cardiovascular event such as myocardial infarction.[42] Recent epidemiological studies demonstrated a link between chronic occupational exposure to high concentrations of airborne particles and increased plasma PAI-1 levels.[43] Thus, increased circulating PAI-1 levels induced by CNT can be a potential cardiovascular risk factor.

Increased circulating PAI-1 as well as several inflammatory proteins most likely originated from the airspaces and that might be associated with CNT-induced alveolar epithelial damage and/or endothelial activation. It has been shown that both the cytotoxic and mutated noncytotoxic strains of *P. aeruginosa* induce the same degree of lung inflammation, but only the cytotoxic one induced marked cytokine release into the systemic circulation.[44] Recently, pulmonary exposure to diesel exhaust particles or carbon black nanoparticles, while alone had no measurable effect, aggravated LPS-induced systemic inflammation and coagulatory disturbances consistent with an increased leakage of LPS into the systemic circulation.[45] The cross-compartment signaling of the particle-induced lung inflammation and systemic consequences are not well understood. Particle/fiber characteristics which are associated with direct epithelial damage, such as sharp edges, rigid structures, poor phagocytosis, and different degrees of agglomeration may facilitate the release of proteins into the systemic circulation. Furthermore, high concentrations of iron in unpurified CNTs have been related to oxidative stress and inflammation.[1,12] Although having a lower iron content, MWCNT induced stronger inflammatory lung and systemic responses in the current studies which might be related to the more rigid and better dispersed MWCNT compared to the SWCNT.

The released soluble circulating inflammatory proteins, such as IL-6, may lead to activation of blood cell gene expression, and/or blood cells are activated during their transpass through the lung. Whole blood gene expression profiling, which can be global to identify multiple genes signatures or focused to identify pathophysiologically relevant molecular pathways, has the potential to be exceedingly informative.[13] In addition to many disease evaluations, blood gene expression studies have been applied in several environmental exposure assessments, such as cigarette smoking and arsenic exposure, and resulted in identification of "exposure-induced gene expression profiles".[46,47] We used the specific hypothesis-driven approach and demonstrated that whole blood cell expression closely represents the CNT-induced lung and systemic toxicity related to possible cardiovascular outcomes. Studies in progress, including varying time points and particle types, will determine the specificity of this response regarding exposure agents and pathophysiological outcomes. Thus, the combination of blood gene expression and circulating soluble protein analysis provides insight into the mechanisms of

particle toxicity and novel biomarkers which can find application in human screening as well as epidemiological studies.

In summary, single-walled and multiwalled CNTs deposited in the lung induced an acute lung and systemic effect, which was more pronounced in the MWCNT exposure. Overall, these studies demonstrated a close cross-talk between the pulmonary and systemic circulation. The systemic response, if it is chronic and persistent, may trigger or exacerbate cardiovascular dysfunction and disease, such as atherosclerosis. The methodological approach used in our current studies is a novel approach for evaluation of effects of pulmonary exposure to particles. This approach might foster the development of predictive tests for estimation of the potential toxicity of new nanomaterials based on their physiochemical characteristics and potential to induce oxidative stress, inflammation, and specific pulmonary and systemic toxicity.

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Supporting Information Available: A complete experimental methodology used for the study will be found in this section, and the genes contained within our Taqman array as well as the relative mRNA expression for lung, blood, and aorta are given in tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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Supporting Information:

METHODS:

Male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) 10 weeks of age were used in this study. All mice were provided food (Teklad 7913) and tap water *ad libitum* in ventilated cages in a controlled humidity and temperature environment with a 12hr light/dark cycle. Animal care and use procedures were conducted in accordance with the "PHS Policy on Human Care and Use of Laboratory Animals" and the "Guide for the Care and Use of Laboratory Animals" (NIH publication 86-23, 1996). These procedures were approved by the NIOSH Institutional Animal Care and Use Committee.

The mice were exposed to the following particles: UFCB (Printex 90, Degussa, Germany), catalytically grown-SWCNT (Carbon Nanotechnologies, Inc., Houston, TX) or -MWCNT (Mitsui & CO., LTD) [16]. The particles were dispersed as previously described [19] in mouse serum albumin (0.6mg/ml final concentration) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DSPC; 10µ/ml) then diluted 1:1 in PBS for dosing. A dilution of 1:1000 in distilled water from an 80µg stock of UFCB and CNT and 1:20 for silica was imaged by transmission electron microscopy (TEM). Mice (n=7 vehicle, n=5 UFCB, n=5 SWCNT and n=7 MWCNT) were dosed with 40µg of each material in a total of 50µl by pharyngeal aspiration [20]. Mice were sacrificed by carbon dioxide asphyxiation at 4hr post-exposure, and blood was collected for serum antigen analysis and whole blood mRNA expression. The left lung was ligated, removed and frozen in liquid nitrogen. The right lung was lavaged, and the bronchoalveolar lavage (BAL) was

used to determine cell counts and lactate dehydrogenase activity and levels. The tissue and blood collection was performed as described before [10]. Aorta (from the aortic root to the iliac bifurcation), heart, liver and kidney were dissected and immediately frozen in liquid nitrogen. A section of the liver was harvested and frozen in liquid nitrogen. All samples were stored at -80°C prior to analysis.

A second set of mice, treated exactly the same, were sacrificed and blood was collected into 3.2% sodium citrate at a 9 to 1 ratio, respectively. After centrifugation at 1500g for 12 minutes, plasma samples were collected and frozen for determination of plasminogen activator inhibitor 1 (PAI-1). The heart and aorta were harvested and snap frozen in liquid nitrogen and stored at -80 °C prior to RNA isolation.

For BAL of the right lung, the trachea was cannulated with a blunted 22 gauge needle and, while massaging the thorax, 0.3 ml of cold PBS was slowly instilled into the lung then withdrawn and placed into a 15 ml conical tube. This constituted the first fraction BAL fluid. Two subsequent lavages (0.5 ml/instillate) were collected into a separate tube which represented the second fraction. The BAL fluid was preserved on ice then centrifuged (500 x *g*, 10 min, 4°C). Aliquots of the first fraction BAL supernatant were used to assess lung injury. The supernatant of the second fraction was discarded. The cell pellets from both fractions were combined and centrifuged (500 x g, 6 minutes, 4°C) and the supernatant discarded. The final cell pellet was suspended in a known volume of PBS and used for cell enumeration (Coulter Counter) and differential staining.

Cells were plated onto glass slides using a Cytospin 3 centrifuge (Shandon Life Sciences International, Cheshire, England) set at 800 rpm for 5 minutes. Slides were stained using Leukostat stain (Fisher Scientific, Pittsburgh, PA). A minimum of 300 cells/slide consisting of alveolar macrophages/monocytes, lymphocytes or polymorphonuclear leukocytes (PMN) were identified using light microscopy. Slides from vehicle exposed mice (control) consisted typically of >98% alveolar macrophages.

For blood cell counts, blood was added to BD Unopette™ reservoirs at a dilution of 1:10 for 10 min to lyse the red blood cells. Cell counts were done using a hemocytometer. Whole blood (3µl) was placed on a glass slide; a blood smear was made and stained using Leukostat stain (Fisher Scientific, Pittsburgh, PA). A minimum of 200 leukocytes were counted per slide using light microscopy.

For real-time RT-PCR, lung and liver RNA was isolated using the RNeasy Mini Kit (Qiagen) according to manufacturer's directions. Heart and aorta were homogenized with a Qiagen Tissue Lyser and RNA was isolated using a standard Trizol protocol. Whole blood RNA was isolated using the Mouse RiboPure™ Blood RNA Isolation Kit (Ambion) according to manufacturer's directions. Evaluation of gene expression was determined by standard 96-well technology using the ABI 7900 (Applied Biosystems) with pre-designed Assays-on-Demand™ TaqMan® probes and primers (Applied Biosystems) and by a custom designed TaqMan® array (LDA). Using 96 well plates, one µg of total RNA was reverse transcribed using random hexamers (Applied Biosystems) and

Superscript II (Invitrogen). Five µl of cDNA (in duplicate for each gene) was then used for gene expression determination using pre-designed Assays-on-Demand™ TaqMan® probes and primers (Applied Biosystems). Ribosomal 18S was used as an internal reference and measured from 1/100 dilutions of the cDNA. For LDA, samples were run according to the Applied Biosystems TaqMan® array user bulletin. For our samples, a total of 1µg of total RNA converted to cDNA in a final volume of 200µl (96a format) was used. The housekeeping gene contained within the LDA used for normalization was hypoxanthine-guanine phosphoribosyltransferase (HPRT). Relative gene expression was calculated using the comparative threshold method (2-ΔΔCt) [48] with vehicle treated mice serving as the reference group.

Serum antigen measurements were determined by Rules Based Medicine (Austin, TX) by multiplex immunoassay RodentMAP v1.6. Plasminogen activator inhibitor 1 (PAI-1), both free and total, was determined using ELISA (Molecular Innovations). Plasma was collected and assayed according to manufacturer's directions. Lungs were homogenized (0.1% Triton Lysis containing final concentrations of 2mM pefabloc SC, 10µg/ml leupeptin and 2µg/ml pepstatin) using the Tissue Lyser from Qiagen. Homogenates were spun at 4°C for 10min at 5000RPM and the supernate collected. Total protein was determined using the Bradford assay and 100µl of a 5mg/ml sample was assayed.

All data are presented as means ± standard error. Analyses were performed using JMP® Statistical Discovery Software. Serum protein analysis and real-time RT-PCR confirmation of the TaqMan® arrays and any additional

genes were analyzed by one-way analysis of variance (ANOVA) generating a least squares mean table by Student's t-test. The TaqMan® arrays were done on n=4 per group while confirmation by the conventional real-time RT-PCR was done on complete sets (n=7) and analyzed by ANOVA. Conventional real-time RT-PCR confirmed TaqMan® arrays by 100%. Differences were considered statistically significant at $p<0.05$.

Table S1: Genes included in the custom designed TaqMan® array

Mm99999915_g1	Gapdh	glyceraldehyde-3-phosphate dehydrogenase
Mm01545399_m1	HPRT	hypoxanthine guanine phosphoribosyl transferase
Mm00439620_m1	Il1a	interleukin 1 alpha
Mm00434228_m1	Il1b	interleukin 1 beta
Mm00445259_m1	Il4	interleukin 4
Mm00446190_m1	Il6	interleukin 6
Mm00439616_m1	Il10	interleukin 10
Mm00434165_m1	Il12a	interleukin 12a
Mm00434204_m1	Il13	interleukin 13
Mm00801778_m1	Ifng	interferon gamma
Mm00443258_m1	Tnf	tumor necrosis factor
Mm00478374_m1	Ptgs2	prostaglandin-endoperoxide synthase 2
Mm00516023_m1	Icam1	intercellular adhesion molecule
Mm00475988_m1	Arg1	arginase I
Mm00477592_m1	Arg2	arginase II
Mm00434455_m1	Itgam	integrin alpha M
Mm00802529_m1	Emr-1	EGF module-containing mucin-like receptor 1
Mm00441891_m1	Cd40	CD40 antigen
Mm00440485_m1	Nos2	nitric oxide synthase 2, inducible, macrophage
Mm00441242_m1	Ccl2	chemokine (C-C motif) ligand 2
Mm00443111_m1	Ccl4	chemokine (C-C motif) ligand 4
Mm01302428_m1	Ccl5	chemokine (C-C motif) ligand 5
Mm99999051_gH	Ccr2	chemokine (C-C motif) receptor 2
Mm01963251_s1	Ccr5	chemokine (C-C motif) receptor 5

Mm00432688_m1	Csf1	colony stimulating factor 1 (macrophage)
Mm00438328_m1	Csf2	colony stimulating factor 2 (granulocyte-macrophage)
Mm00438334_m1	Csf3	colony stimulating factor 3 (granulocyte)
Mm00433859_m1	Cxcl1	chemokine (C-X-C motif) ligand 1
Mm00436450_m1	Cxcl2	chemokine (C-X-C motif) ligand 2
Mm00445552_m1	Cxcl12	chemokine (C-X-C motif) ligand 12
Mm99999055_m1	Cxcr4	chemokine (C-X-C motif) receptor 4
Mm99999117_s1	Il8rb	interleukin 8 receptor, beta
Mm00496696_g1	S100a8	S100 calcium binding protein A8 (calgranulin A)
Mm00447886_m1	Mpo	Myloperoxidase
Mm00436767_m1	Ssp1	secreted phosphoprotein 1
Mm00435860_m1	Serpine1	serine (or cysteine) peptidase inhibitor, clade E, 1
Mm00436753_m1	Serpine2	serine (or cysteine) peptidase inhibitor, clade E, 2
Mm01335418_m1	Thbs1	Thrombospondin-1
Mm00438853_m1	F3	Coagulation factor III
Mm00513575_m1	Fgg	fibrinogen, gamma polypeptide
Mm00438656_m1	Edn1	endothelin 1
Mm01243722_m1	Ednra	endothelin receptor type A
Mm00449197_m1	Vcam1	vascular cell adhesion molecule 1
Mm00441278_m1	E-Selectin	selectin, endothelial cell
Mm00476702_m1	Pecam1	platelet/endothelial cell adhesion molecule 1
Mm00435175_m1	Nos1	nitric oxide synthase 1, neuronal
Mm00435204_m1	Nos3	nitric oxide synthase 3, endothelial cell

Table S1 continued:

Mm00834384_g1	Hspb1	heat shock protein 1
Mm00658568_gH	Hspca	heat shock protein 1, alpha
Mm00468875_m1	Hif1a	hypoxia inducible factor 1, alpha subunit
Mm00621168_m1	Hif1an	hypoxia-inducible factor 1, alpha subunit inhibitor
Mm00469373_m1	Hif3a	hypoxia inducible factor 3, alpha subunit
Mm00656724_m1	Egr1	Early growth response 1
Mm00519268_m1	Bbc3	Bcl-2 binding component 3
Mm00438023_m1	Casp1	caspase 1
Mm00432307_m1	Casp4	caspase 4, apoptosis-related cysteine peptidase
Mm00432802_m1	GADD45	growth arrest and DNA-damage-inducible 45 alpha
Mm00660256_m1	Cryaa	crystallin, alpha A
Mm00515567_m1	Cryab	crystallin, alpha B
Mm00787254_s1	Dnaja1	DnaJ (Hsp40) homolog, subfamily A, member 1
Mm00444898_m1	Dnaja2	DnaJ (Hsp40) homolog, subfamily A, member 2
Mm00451053_m1	Rrad	Ras-related associated with diabetes
Mm00442773_m1	clu	Clusterin
Mm00487425_m1	c-fos	FBJ osteosarcoma oncogene
Mm00496660_g1	Mt1	metallothionein 1
Mm00809556_s1	Mt2	metallothionein 2
Mm00439508_m1	Mmp2	matrix metallopeptidase 2
Mm00442991_m1	Mmp9	matrix metallopeptidase 9
Mm00441818_m1	Timp1	tissue inhibitor of metalloproteinase 1
Mm00441825_m1	Timp2	tissue inhibitor of metalloproteinase 2

Mm00441826_m1	Timp3	tissue inhibitor of metalloproteinase 3
Mm00446568_m1	Timp4	tissue inhibitor of metalloproteinase 4
Mm00801666_g1	Col1a1	procollagen, type 1, alpha 1,
Mm00802331_m1	Col3a1	procollagen, type III, alpha 1,
Mm00801574_m1	Col4a4	procollagen, type IV, alpha 4,
Mm00802386_m1	Col4a2	procollagen, type IV, alpha 2
Mm00477827_m1	Nid1	nidogen 1
Mm00456212_m1	Nid2	nidogen 2
Mm00802831_m1	Igf1r	insulin-like growth factor I receptor
Mm00439565_g1	Igf2	insulin-like growth factor 2
Mm00439561_m1	Igf1	insulin-like growth factor 1
Mm00833447_m1	Igfbp1	insulin-like growth factor binding protein 1
Mm00437304_m1	Vegfa	vascular endothelial growth factor A
Mm00435540_m1	Pdgfa	platelet derived growth factor, alpha
Mm00440678_m1	Pdgfb	platelet derived growth factor, B polypeptide
Mm00480205_m1	Pdgfc	platelet-derived growth factor, C polypeptide
Mm00438923_m1	Fgfr1	fibroblast growth factor receptor 1
Mm00523992_g1	Frag1	FGF receptor activating protein 1
Mm00438906_m1	Fgf1	fibroblast growth factor 1
Mm00438696_m1	Egf	epidermal growth factor
Mm00515790_g1	Ctgf	connective tissue growth factor
Mm00558224_s1	Agtr1a, 1b	angiotensin II receptor, type 1a &1b
Mm00441724_m1	Tgfb1	transforming growth factor, beta 1
Mm00436971_m1	Tgfbr1	transforming growth factor, beta receptor I
Mm00498234_m1	Ltbp1	latent TGF beta binding protein 1

NOTE: Additional genes tested beyond the TaqMan® array include: Il2ra-Mm00434261_m1, Il5-Mm00439646_m1, CCL11-Mm00441238_m1, CCL19-Mm00839967_g1, CCL22-Mm00436439_m1, Bcl2l1-Mm00437783_m1, CD40 ligand-Mm00441911_m1, HO-1-Mm00516004_m1, SOD2-Mm00449726_m1, P-selectin-Mm00441295_m1, L-selectin-Mm00441291_m1, Fas-Mm00433237_m1.

Table S2: Effects of single-walled carbon nanotube (SWCNT) and multi-walled carbon nanotube (MWCNT) exposure on lung, blood and aorta gene expression. Data are represented as fold change from vehicle-treated mice and all values shown were significant ($p<0.05$). The asterisk (*) indicates genes that were confirmed from the TaqMan® array by individual gene real-time RT-PCR for the complete set of mice.

	Lung		**Blood**		**Aorta**	
	SWCNT	MWCNT	SWCNT	MWCNT	SWCNT	MWCNT
Inflammation						
IL-1β*		7.1±3.0		2.3±0.4		
IL-5	6.3±2.3	7.2±2.4				
IL-6*	7.1±1.2	28.6±13.5				42.7±20.9
IL-10*	3.4±1.0	7.2±1.8				4.7±1.4
IL-13		6.3±1.2				
CCL2 (MCP-1)*	2.7±0.8	5.4±1.5				
CCL4 (MIP-1β)	4.3±1.0	20.6±14.5				
CCL11 (Eotaxin)*	4.1±1.1	7.4±1.2				
CCL19 (MIP-3β)	2.1±0.2	3.1±1.0				
CCL22 (MDC)*	1.7±0.2	3.3±0.5				
CXCL1 (KC)*	2.8±0.5	12.5±4.4				

CXCL2 (MIP-2)*	3.2±0.6	28.9±15.2		30.4±11.8		
S100a8*		7.3±2.3		3.8±0.9	4.1±1.9	4.8±2.7
Osteopontin*	1.6±0.2			1.8±0.3		
CSF-3	19.7±2.5	184.0±103.2				
CSF-1				2.2±0.4		
ICAM-1		1.6±0.2				
Arginase I*		2.4±0.4				
Arginase II*	1.5±0.2	2.8±0.4	3.2±1.4	4.0±1.0	4.1±1.0	7.3±3.3
IL-8rβ*	3.1±0.7	6.8±1.5		1.6±0.2		
Itgam (Mac-1)		2.3±0.5		1.8±0.2		
Stress Related Genes						
MT1*	4.8±0.5	8.1±1.3		2.0±0.5	3.1±1.2	4.7±0.7
MT2	4.8±0.4	9.2±1.9				8.7±2.3
Hif-1α*		1.8±0.2				
Hif-3α*	3.9±1.4	3.6±0.7		1.7±0.2	3.3±1.2	5.5±0.5
COX-2		2.8±1.0				
EGR-1*		3.3±1.3				

Bcl2l1		2.3±0.2				
Casp4	1.7±0.2	2.0±0.3				
GADD45a		1.6±0.2				
HO-1*				1.5±0.1	1.9±0.2	3.3±0.4
Fas	1.7±0.3	2.1±0.2				
c-Fos				2.7±0.2		
Endothelial Cells						
E-Selectin*	3.9±1.1	8.3±2.3			2.9±1.1	4.0±0.8
P-Selectin*	2.5±0.5	5.6±1.7				
Coagulation						
PAI-1*	2.6±0.2	5.6±0.8				
Fibrinogen (γ chain)	2.3±0.2	2.1±0.4				
Tissue Factor*		3.0±0.5				
Remodeling						
MMP-9*		3.3±0.9		1.8±0.2		
TIMP-1	2.4±0.4	2.3±0.2				
TIMP-2				2.1±0.20		

CTGf (Fb)		1.6±0.2				
Growth Factor						
Igf1r				2.4±0.5		

Table S3: Changes in serum proteins 4hr following single-walled carbon nanotube (SWCNT) and multi-walled carbon nanotube (MWCNT) exposure compared to dispersion media (DM). *p<0.05 vs DM; #p<0.05 vs all groups.

	DM (n=4)	SWCNT (n=4)	MWCNT (n=5)
CD40 (pg/ml)	100±2	115±7	143±17*
CCL11 (pg/ml)	1171±153	963±57	2014±167#
IL-5 (ng/ml)	0.90±0.06	1.40±0.25	2.23±0.18#
IL-6 (pg/ml)	BLD	13±6	60±25
CXCL1 (pg/ml)	52±10	91±24	326±101#
CCL22 (pg/ml)	601±64	515±21	1112±203#
MMP-9 (ng/ml)	127±10	104±10	276±68#
Osteopontin (ng/ml)	380±34	411±17	287±23#