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Single-Walled Carbon Nanotubes Can Induce Pulmonary Injury in Mouse Model

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ABSTRACT

Carbon nanotubes are a nanomaterial that is extensively used in industry. The potential health risk of chronic carbon nanotubes exposure has been raised as of great public concern. In the present study, we have demonstrated that intratracheal instillation of 0.5 mg of single-walled carbon nanotubes (SWCNT) into male ICR mice (8 weeks old) induced alveolar macrophage activation, various chronic inflammatory responses, and severe pulmonary granuloma formation. We then used Affymetrix microarrays to investigate the molecular effects on the macrophages when exposed to SWCNT. A biological pathway analysis, a literature survey, and experimental validation suggest that the uptake of SWCNT into the macrophages is able to activate various transcription factors such as nuclear factor κB (NF-κB) and activator protein 1 (AP-1), and this leads to oxidative stress, the release of proinflammatory cytokines, the recruitment of leukocytes, the induction of protective and antiapoptotic gene expression, and the activation of T cells. The resulting innate and adaptive immune responses may explain the chronic pulmonary inflammation and granuloma formation in vivo caused by SWCNT.

Nanotechnology is advancing at an incredible pace such that it is has created a new industrial revolution over the past few years.[1] Along with this revolution, the use of engineered nanomaterials has been rapidly increasing in commercial applications. Carbon nanotubes (CNT) are one of the most attractive nanomaterials due to their unique physical and chemical characteristics.[2] Recently, they have been demonstrated to be an effective drug delivery vehicle[3−5] and a photosensitizer for cancer cells.[6] The increased use and production of CNT will exacerbate the possible exposure of individuals to them, and this has created a public concern about their potential environmental and health risks.[7−9]

A previous study has indicated that ultrafine carbon particles show greater lung penetration than larger particles and are able to cross the blood−brain barrier and impact on the central nervous system.[10] It has also been reported that there is an increased susceptibility to blood clotting in rabbits that have inhaled carbon nanospheres. This toxic effect showed up quickly after exposure and suggested that the carbon nanoparticle travel from the lungs to the bloodstream rather than release clotting agents from the lungs.[11] Additionally, because inhalation of asbestos fiber is known to induce asbestosis, lung cancer, and malignant mesothelioma of the pleura, there would seem to be a high probability that CNT are also likely to have significant toxic effects on human health; this is due to their structural resemblance to asbestos. Indeed many studies have indicated that in vitro, CNT exhibit substantial cytotoxicity, including the induction of oxidative stress, the inhibition of cellular proliferation, and the induction of apoptosis/necrosis; these effects have been detected in several types of cells, namely keratinocytes, fibroblasts, and macrophages.[12−20] The toxic effects are also reflected in vivo. In one case, Lam et al. investigated the impact of single-walled CNT (SWCNT) on lung tissue by instilling a suspension of SWCNT into the lungs of mice. They found that the SWCNT clumped together into bundles and produced pulmonary inflammation together with the

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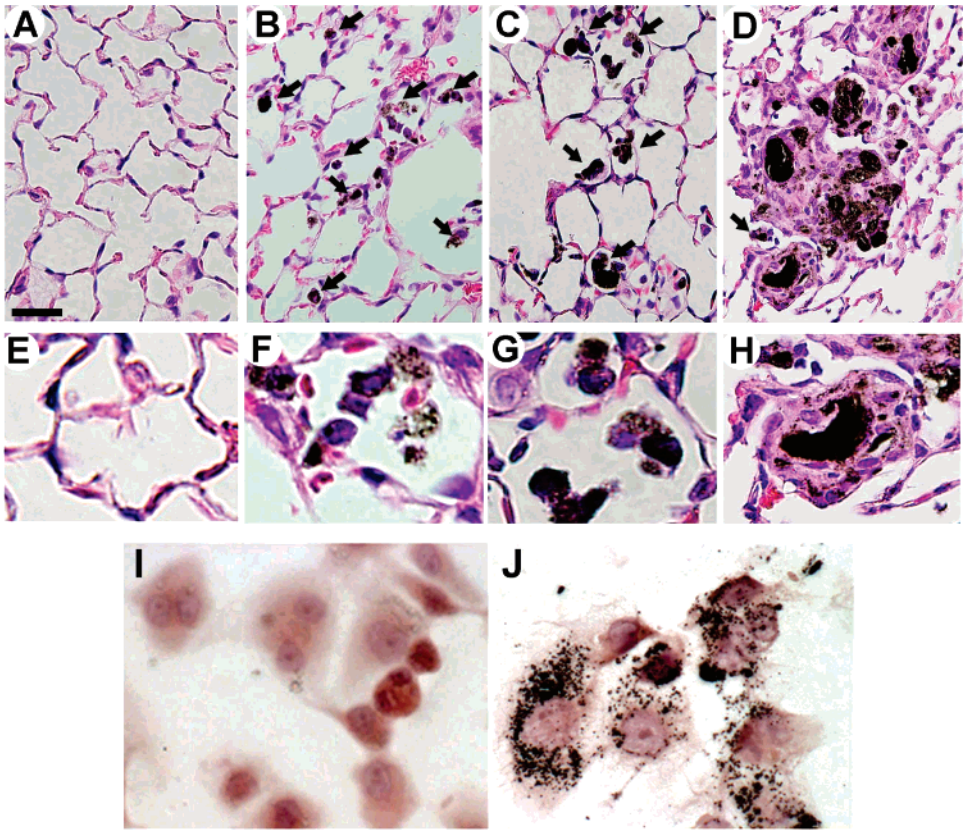

Figure 1. Hematoxylin and eosin staining of mouse lung tissue (A−H) and THP-1 derived macrophages (I,J). (A,E) Fluronic F-68-treated group acts as the solvent control. (B,F) Early response (3 days) of the mouse lung tissue to a single dose of 0.5 mg of SWCNT. (C,D,G,H) Two weeks response of the mouse lung tissue to a single dose of 0.5 mg of SWCNT. (F,G) SWCNT-loaded foamy-like macrophages in the alveolae; (H) multifocal macrophage-containing granuloma around the sites of SWCNT aggregates. (A−D) Original magnification ×100, bar = 100 μm; (E−H) ×400. The black arrows shown in panels B and C indicate the SWCNT-loaded foamy-like macrophages. (I) Fluronic F-68 control of the THP-1 derived macrophages. (J) The macrophages were treated with 0.05 mg/mL of SWCNT for 24 h.

subsequent formation of granulomas that entombed the SWCNT in the lungs.[21] Furthermore, a similar result involving lung granuloma formation as a reaction to SWCNT exposure has also been reported by Warheit et al., who instilled SWCNT into the trachea of rats.[22] Recently, a more comprehensive study showed that pharyngeal aspiration of SWCNT in mice elicited unusual pulmonary inflammation with the early onset of progressive fibrosis and granuloma.[23] The aforementioned studies all demonstrate that one of the common cytotoxic responses to SWCNT exposure in lung is granuloma formation. Currently, the mechanisms involved in granuloma formation are still unclear but need to be investigated further.

In the present study, the ICR mice were intratracheally instilled with or without a single dose of 0.5 mg of SWCNT/kg, and the mice were then examined at day 3 (for early responses) and day 14 (for late responses) after treatment. A significant inflammatory response and pathological changes were observed in the SWCNT-treated mice on histopathological examination of the lungs. At the early stage (day 3), the foamy-like macrophages loaded with SWCNT were found to have accumulated in alveolae (Figure 1B,F). The effects persisted and progressed to give rise to multifocal macrophage-containing granulomas around the sites of SWCNT aggregation at the 14-day postexposure (Figure 1C,D,G,H). This is consistent with the experimental results reported in the earlier literature.[22,23] The granulomas are mainly composed of aggregates of macrophages laden with SWCNT particles, and thereof an investigation of the genome-wide gene expression changes in macrophages exposed to SWCNT, associated with a detailed signaling pathway analysis, ought to provide molecular insights into this type of granuloma formation. To address this issue, we conducted in vitro experiments to investigate the effects of SWCNT on human macrophage-like cells differentiated from a human monocytic leukemia cell line THP-1. Exposure of the human THP-1 derived macrophages to 0.05 mg/mL of SWCNT resulted in the uptake of SWCNT to form the nanoparticle-loaded macrophages (Figure 1J), which is similar to our aforementioned in vivo observations in Figure 1F,G. Although differences in cytotoxic effects may exist between the in vivo alveolar macrophages and THP-1 derived macrophages in response to SWCNT exposure, in view of the formation of SWCNT-loaded macrophages with both types of macrophages, SWCNT-induced cytotoxicity in THP-1 derived macrophages is probably quite similar to that of their counterparts in vivo.

To elucidate the SWCNT-induced cytotoxicity at the molecular level, we measured the gene expression changes in human THP-1 derived macrophages exposed to SWCNT for 24 h using Affymatrix microarrays. To analyze the complicated genome-wide gene expression data, we intro-

Table 1. Potentially Affected Pathways Selected by the Pathway Scoring Method[a]

p-value	pathway title	website
0.0000	pertussis toxin-insensitive CCR5 signaling in macrophage	http://www.biocarta.com/pathfiles/h_ccr5Pathway.asp
0.0001	selective expression of chemokine receptors during T-cell polarization	http://www.biocarta.com/pathfiles/h_nktPathway.asp
0.0005	NF-kB activation by nontypeable *Hemophilus influenzae*	http://www.biocarta.com/pathfiles/h_nthiPathway.asp
0.0011	CD40L signaling pathway	http://www.biocarta.com/pathfiles/h_cd40Pathway.asp
0.0017	TNFR2 signaling pathway	http://www.biocarta.com/pathfiles/h_tnfr2Pathway.asp
0.0027	B lymphocyte cell surface molecules	http://www.biocarta.com/pathfiles/h_blymphocytePathway.asp
0.0075	cells and molecules involved in local acute inflammatory response	http://www.biocarta.com/pathfiles/h_lairPathway.asp
0.0082	adhesion and diapedesis of lymphocytes	http://www.biocarta.com/pathfiles/h_lymPathway.asp
0.0086	proepithelin conversion to epithelin and wound repair control	http://www.biocarta.com/pathfiles/h_pepiPathway.asp
0.0104	signal transduction through IL1R	http://www.biocarta.com/pathfiles/h_il1rPathway.asp
0.0105	adhesion and diapedesis of granulocytes	http://www.biocarta.com/pathfiles/h_granulocytesPathway.asp
0.0140	CXCR4 signaling pathway	http://www.biocarta.com/pathfiles/h_cxcr4Pathway.asp
0.0141	free radical induced apoptosis	http://www.biocarta.com/pathfiles/h_freePathway.asp
0.0164	Msp/Ron receptor signaling pathway	http://www.biocarta.com/pathfiles/h_mspPathway.asp
0.0165	erythropoietin mediated neuroprotection through NF-kB	http://www.biocarta.com/pathfiles/h_eponfkbPathway.asp
0.0206	IL-10 anti-inflammatory signaling pathway	http://www.biocarta.com/pathfiles/h_il10Pathway.asp
0.0216	inositol metabolism	http://www.genome.jp/dbget-bin/get_pathway?org_name=hsa&mapno=00031
0.0345	SODD/TNFR1 signaling pathway	http://www.biocarta.com/pathfiles/h_soddPathway.asp
0.0364	neutrophil and its surface molecules	http://www.biocarta.com/pathfiles/h_neutrophilPathway.asp
0.0410	neuroregulin receptor degredation protein-1 controls ErbB3 receptor recycling	http://www.biocarta.com/pathfiles/h_ErbB3Pathway.asp
0.0446	caspase cascade in apoptosis	http://www.biocarta.com/pathfiles/h_caspasePathway.asp
0.0455	adhesion molecules on lymphocyte	http://www.biocarta.com/pathfiles/h_lymphocytePathway.asp

[a] These have *p*-values <0.05 after exposure of THP-1 derived macrophages to SWCNT.

duced a computational method to decipher the molecular cytotoxic mechanisms induced by SWCNT exposure on the macrophages. The method weights each gene with its respective expression abundance change to evaluate and select the pathways that are most affected by transcriptional changes in genome-wide expression experiments (see Supporting Information for a detailed description). After using the aforementioned pathway scoring method to evaluate the statistical significance of gene expression, abundance changes were observed in 406 pathways archived in KEGG and BioCarta databases; a *p*-value threshold <0.05 was used to screen the potentially affected pathways (Table 1). On the basis of these results, an inferred gene network (Figure 2) responsible for SWCNT-mediated cytotoxicity in macrophages was derived from a comprehensive literature survey, which involved further data reduction aided at focusing the study on affected pathways that interconnected to each other through common intermediate genes (see Table S1 in Supporting Information). The network suggests that the uptake of SWCNT into macrophages is able to activate nuclear factor κB (NF-κB) and activator protein 1 (AP-1), and this leads to oxidative stress, the release of proinflammatory cytokines, the recruitment of leukocytes, an induction of protective and antiapoptotic gene expressions, and the activation of T cells. We then used the network as a guideline for subsequent experimental validation.

Uptake of SWCNT into Macrophages Induces Oxidative Stress in Mitochondria. After the SWCNT challenge, the activated macrophages phagocytosed the SWCNT (Figure 1J) and seemed to release a large amount of highly destructive reactive oxygen species (ROS), which was probably reflected in the significant increase in expressed mRNA (14-fold increase, Table 2) and the much higher protein level (Figure 3A) of the antioxidant enzyme, manganese superoxide dismutase (SOD2). SOD2 is located in the mitochondria and transforms reactive superoxide radicals into less toxic peroxides (H_2O_2) that in turn are converted into harmless water and oxygen by catalase or glutathione peroxidase. The remarkable increase in the SOD2 expression of the SWCNT-treated macrophages demonstrates that the elevated ROS levels are probably derived mainly from mitochondria respiration leakage. Moreover, the unchanged or undetectable expression of another major set of membrane-bound ROS producers, the NADPH-dependent oxidases (NOXs), further confirms this speculation (see Figure 3A and Table 2). Concomitantly, the expression of several key enzymes/scavengers responsible for H_2O_2 removal such as glutathione peroxidase 1 (GPX1), glutathione reductase (GSR), glutathione synthetase (GSS), catalase, and peroxiredoxin 5 (PRDX5) remained unaltered (Table 2). Thus, collectively, oxidative stress might continue to exist in the

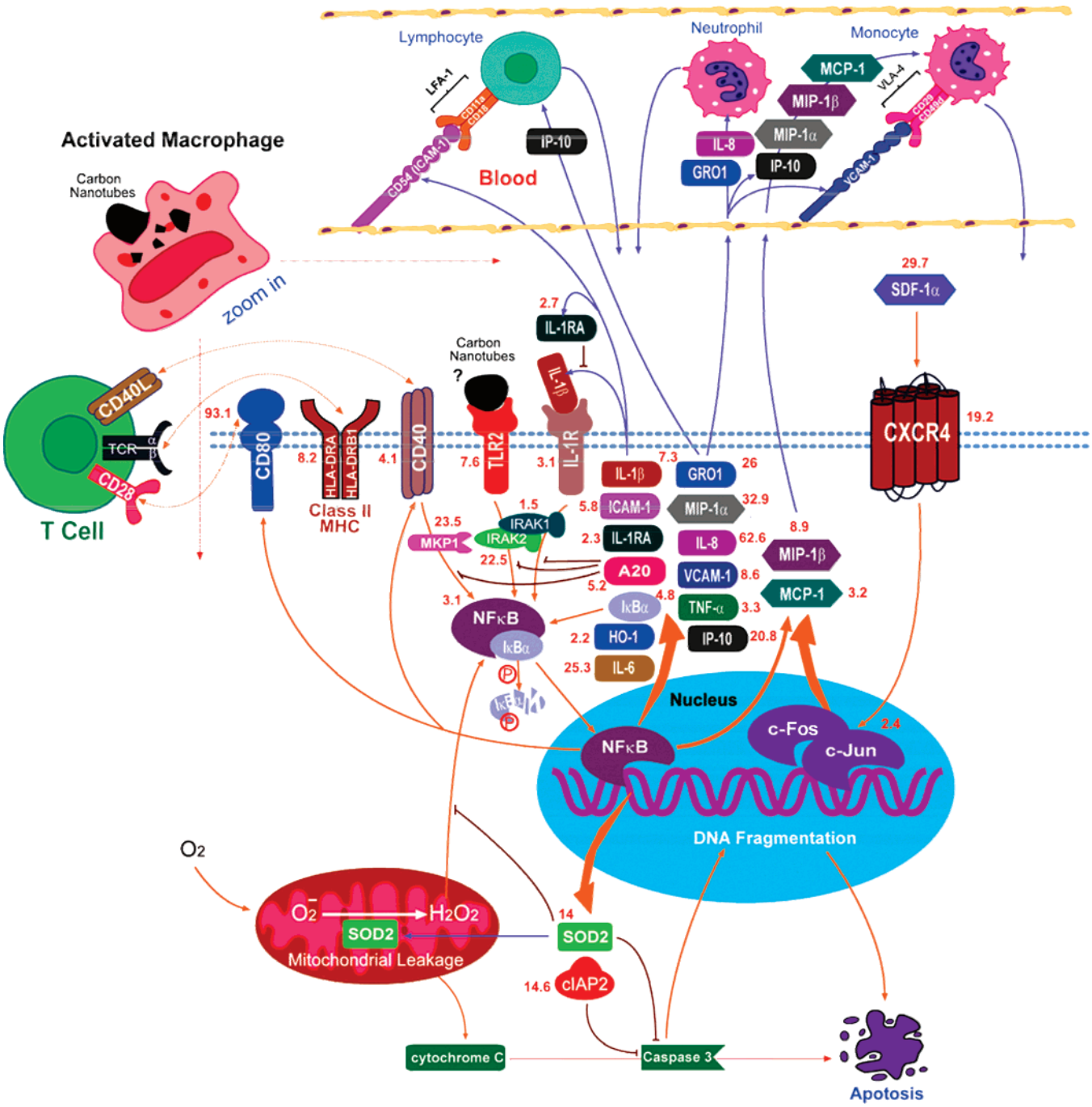

Figure 2. The inferred gene network that probably regulates SWCNT-associated cytotoxicity. An orange arrow directed from one gene (or event) to another represents that the gene (or its encoded protein) or the event can activate either the expression of another gene or another event. A T-ended brown line denotes that one gene (or event) function is inhibited by another. A blue-arrowed line signifies the movement of a gene product or cell from one site to another. A red number alongside a gene indicates a fold increase change in gene expression after the macrophages are exposed to SWCNT that has been confirmed by quantitative real-time polymerase chain reaction (PCR) measurements in duplicate.

SWCNT-treated macrophage due to H_2O_2 accumulation caused by the imbalance between ROS anabolism and catabolism.

Uptake of SWCNT into Macrophages Activates NF-κB and AP-1, Which Leads to Large Scale Production of Cytokines and Chemokines. Analysis by electrophoretic mobility shift assay (EMSA) and Western blot indicated that SWCNT uptake seems to increase the nuclear translocation of NF-κB (Figure 3B) and DNA binding of NF-κB (Figure 3C) and AP-1 (Figure 3D) in macrophages. To further determine whether the aforementioned DNA binding results from an increase in the DNA binding of NF-κB or AP-1 complexes, supershift experiments were performed using nuclear extracts from THP-1 macrophages 24 h following SWCNT treatment. As demonstrated in Figure 3F,G, p50/p65 and c-jun/c-fos proteins are present in the supershifted NF-κB and AP-1 complexes, respectively. These results suggest that SWCNT-induced activated NF-κB and AP-1 complexes are composed of p50/p65 and c-jun/c-fos proteins, respectively. The activation of these two transcription factors,

Table 2. Quantitative Real-Time PCR Primer Sequences and Measurements Expressed as Fold Increase Relative to the Control SWCNT Untreated Macrophages ± Standard Deviation (SD) from Two Experiments for the Genes Involving in SWCNT Associated Cytotoxicity

accession number	gene symbol	forward primer	reverse primer	average fold change	SD
NM_000201	ICAM-1	GAGCAATGTGCAAGAAGATAGC	ACCCGTTCTGGAGTCCAGTA	5.8	0.95
NM_001078	VCAM-1	CGCAAACACTTTATGTCAATGTTG	TGGCTCAAGCATGTCATATTCAC	8.6	2.01
NM_000594	TNF-α	CTCGAACCCCGAGTGACAA	AGCTGCCCCTCAGCTTGA	3.3	1.31
NM_000576	IL-1β	TGTACCCAGAGAGTCCTGTGC	TCCTCTTAGCACTACCCTAAGGC	7.3	0.20
NM_000877	IL-1R1	TCCAGTCACTAATTTCCAGAAGC	CCTGTACCAAAGCACAATGTCAA	3.1	0.09
NM_000600	IL-6	ATGCAATAACCACCCCTGAC	GAGGTGCCCATGCTACATTT	25.3	3.22
NM_000584	IL-8	CTGCAGCTCTGTGTGAAGGT	TAATTTCTGTGTTGGCGCAG	62.6	10.20
NM_002983	MIP-1α	AGAATTTCATAGCTGACTACTTTGAGA	TCGCTTGGTTAGGAAGATGAC	32.9	2.21
NM_002984	MIP-1β	TCCATGAGACACATCTCCTCC	ACAGTGGACCATCCCCATAG	8.9	1.45
NM_002982	MCP-1	GCACCAAGGGAAAAAGTGAA	CTAGAGGAGGAACGCAGGTG	3.2	0.25
NM_000609	SDF-1α	ATGCCCATGCCGATTCTTCG	GCCGGGCTACAATCTGAAGG	29.7	5.16
NM_001008540	CXCR4	TACACCGAGGAAATGGGCTCA	AGATGATGGAGTAGATGGTGGG	19.2	2.02
NM_003998	NF-κB	TGCCAACAGATGGCCCATAC	TGTTCTTTTCACTAGAGGCACCA	3.1	0.06
NM_020529	IκBα	CTCCGAGACTTTCGAGGAAATAC	GCCATTGTAGTTGGTAGCCTTCA	4.8	0.07
NM_002228	JUN	TGCCTCCAAGTGCCGAAAAA	TGACTTTCTGTTTAAGCTGTGCC	2.4	0.18
NM_000636	SOD2	AACCTCAGCCCTAACGGTG	AGCAGCAATTTGTAAGTGTCCC	14.0	0.35
NM_001165	cIAP2	TCCTGGATAGTCTACTAACTGCC	GCTTCTTGCAGAGAGTTTCTGAA	14.6	3.03
NM_006290	A20	AAGCTGTGAAGATACGGGAGA	CGATGAGGGCTTTGTGGATGAT	5.2	0.20
NM_001250	CD40	GCTACAGGGGTTTCTGATACCA	CTGTTGCACAACCAGGTCTTT	4.1	0.24
NM_003264	TLR2	ATCCTCCAATCAGGCTTCTCT	ACACCTCTGTAGGTCACTGTTG	1.6	0.09
NM_005191	CD80	CAAAGCTGACTTCCCTACACC	CTCCATTTTCCAACCAGGAGAG	93.1	4.30
NM_000577	IL1-RA	CATTGAGCCTCATGCTCTGTT	CGCTGTCTGAGCGGATGAA	2.7	0.28
NM_002133	HO-1	GTCTTCGCCCCTGTCTACTTC	CTGGGCAATCTTTTTGAGCAC	2.2	0.08
NM_001569	IRAK1	CCCGGAAGTTGCCATCCTC	GGCCTGGCTTGGTAGAAGAA	1.5	0.00
NM_001570	IRAK2	ATTCCTTGAGAAGCGACCTCC	ACGTCTGCCTCACTCCAGAA	22.5	1.84
NM_007199	IRAK3	AAGACCATCGGTGACCTTTTAC	TCTTCTCTGAAGGACTCAACACT	1.1	0.13
NM_019111	HLA-DRA	GGAGTCCCTGTGCTAGGATTT	ACATAAACTCGCCTGATTGGTC	8.2	0.06
NM_002124	HLA-DRB1	CGGGGTTGGTGAGAGCTTC	AACCACCTGACTTCAATGCTG	1.1	0.03
NM_000660	TGFB1	GGCCAGATCCTGTCCAAGC	GTGGGTTTCCACCATTAGCAC	1.1	0.06
NM_001565	IP-10	GTGGCATTCAAGGAGTACCTC	GCCTTCGATTCTGGATTCAGACA	20.8	2.10
NM_001752	catalase (CAT)	CGCAGAAAGCTGATGTCCTGA	TCATGTGTGACCTCAAAGTAGC	1.0	0.18
NM_000581	GPX1	TGCAACCAGTTTGGGCATCA	ACCGTTCACCTCGCACTTC	1.0	0.00
NM_000637	GSR	CTGATCGCCGGTGGTATGC	ACAGCAATGTATGCACCAACAA	1.0	0.33
NM_000178	GSS	CCCTGGCTGAGGGAGTATTG	TGCACAGCATAGGCTTGCTC	1.0	0.01
NM_007052	NOX1	GTTGTTTGGTTAGGGCTGAATGT	TTGCGACACACAGGAAGCAG	undetectable	
NM_000397	NOX2	CCCTCCTATGACTTGGAAATGGA	TGGTTTTGAAAGGGTGAGTGAC	1.2	0.08
NM_015718	NOX3	GTTATTTTGGGTTCAACACTGGC	CAGCTATCCCATAGGCGACC	undetectable	
NM_016931	NOX4	CATTTACCCTCACAATGTGTCCA	GCAGAGGCTGACCTCATAGTT	undetectable	
NM_024505	NOX5	GCTGTAGAGGCACCATGAGTG	TCAAATAGGGCAAAGAATCGCTC	undetectable	

especially NF-κB (see Figure 2), could trigger a cascade of inflammation responses including the significant release of a large number of proinflammatory cytokines and chemokines, the upregulation of cell-surface adhesion molecules (intercellular adhesion molecule 1, CD54 (ICAM-1), and vascular cell adhesion molecule 1, VCAM-1) and the induction of a panel of protective/survival genes. These inflammatory responses, in turn, stimulated the macrophages themselves and other immune and nonhematopoietic cells to amplify further the inflammatory cascades by positive-feedback regulation. The role of cytokines is multifaceted and is involved in a variety of activities. Highly induced interleukin-1β (IL-1β) (Figures 2 and 3H) can serve as an initiator of the further induction of inflammatory responses through binding with its receptor IL-1R by positive-feedback (see Figure 2). However, tumor necrosis factor-α (TNF-α) seems not to play a similar critical role to IL-1β in the initiation of the inflammatory responses based on the fact that there was unchanged expression of its receptor TNFR1. Interleukin 6 (IL-6) is a major proinflammatory cytokine released during acute inflammation, and the tremendous increase in expression (25-fold increase) would seem to reflect a significant inflammatory response in macrophages exposed to SWCNT. Furthermore, the activated macrophages induced a remarkable increase in the expression of chemokines that recruit leukocytes to the sites of inflammation. The high-level of expression of the NF-κB-regulated chemokines, Interleukin 8 (IL-8) (Figure 3E), and chemokine C−X−C motif ligand 1, CXCL1 (GRO1), would be able to attract neutrophils to the sites of inflammation. The expression of a panel of monocyte/macrophage chemoattractants including CXCL10 (IP-10), chemokine C−C motif ligand 2, CCL2 (MCP-1) (Figure 3H), CCL3 (MIP-1α) (Figure 3H), and

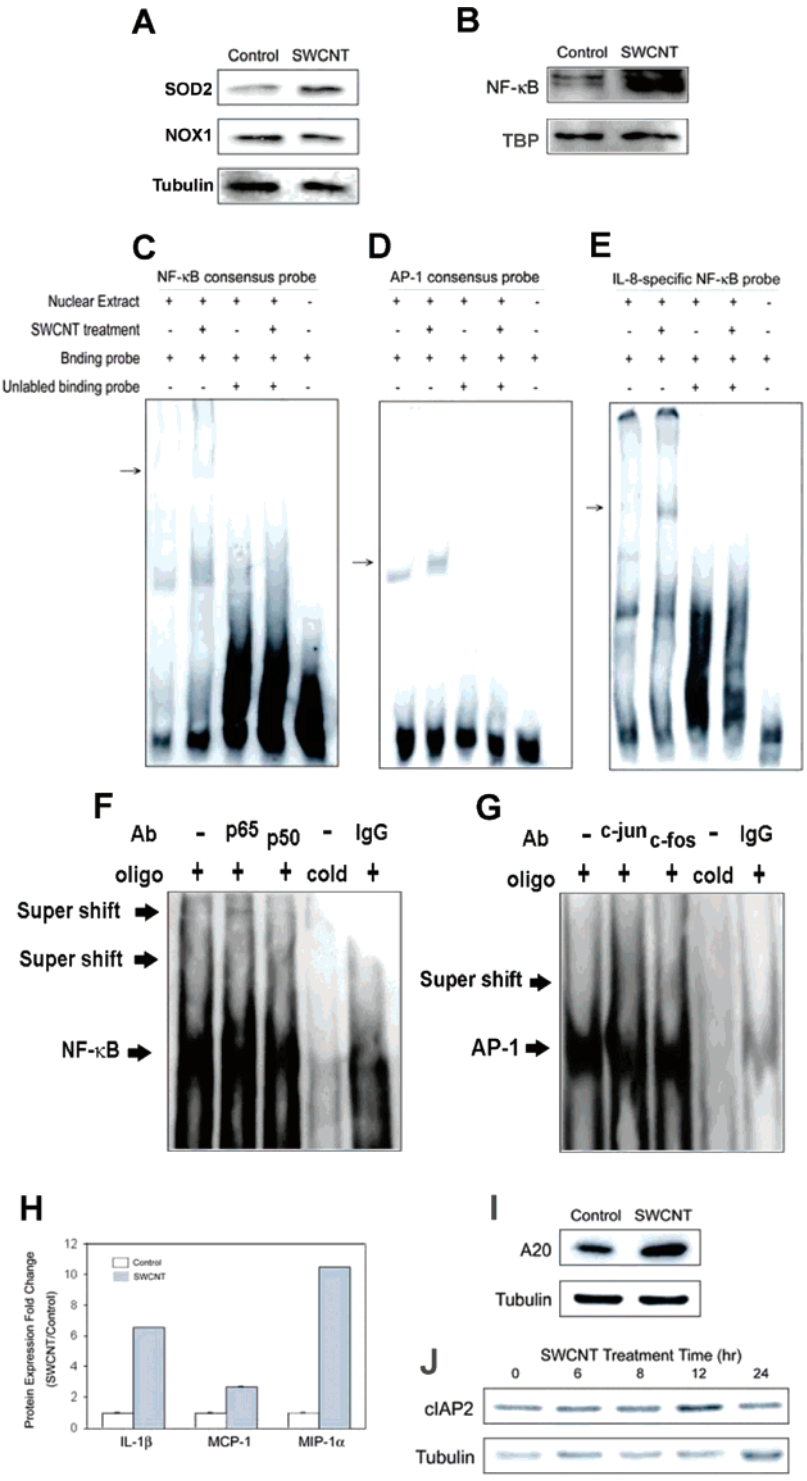

Figure 3. Experimental results for Western blotting, EMSA, super-shift assay, and ELISA. (A) The Western blotting results show an increased expression of SOD2 in SWCNT-treated THP-1 derived macrophages, whereas NOX1 protein level remains unchanged. Tubulin was the internal control. (B) NF-κB protein levels in the nuclear extracts of THP-1 derived macrophages before and after SWCNT treatment by Western blotting analysis. TATA box-binding protein (TBP) was the internal control for nuclear protein normalization. (C) EMSA results using NF-κB consensus oligonucleotides (four binding sites) as the binding probes. (D) EMSA results using AP-1 consensus oligonucleotides (one binding site) as the binding probe. (E) EMSA results using IL-8 specific NF-κB oligonucleotides (four binding sites) as the binding probes. (F) Supershift analysis with specific antibodies for NF-κB (p65 and p50) was performed using nuclear protein extracts from THP-1 derived macrophages 24 h following SWCNT treatment. The cold probe and rabbit IgG were used as the negative controls. (G) Supershift analysis with specific antibodies for AP-1 (c-jun and c-fos) was performed using nuclear protein extracts from THP-1 derived macrophages 24 h following SWCNT treatment. (H) Secreted cytokine levels of IL-1β, MCP-14, and MIP-1α in SWCNT-treated macrophage culture medium by ELISA assay. The results are expressed as a fold increase relative to the control (SWCNT-untreated macrophages) $\pm$ SD from three experiments (p-value <0.001 for all the measurements). (I) The Western blotting analysis shows that the SWCNT treatment induces A20 protein expression. (J) The expression of cIAP2 protein is induced in SWCNT-treated macrophages in a time-dependent manner as shown by Western blotting analysis.

CCL4 (MIP-1β) might be associated with the chronic inflammation and pulmonary granuloma formation.[24−28]

SWCNT Particles Induce the Expression of Protective and Antiapoptotic Genes. In parallel with the induction of the proinflammatory cytokines and chemokines, the SWCNT challenge to macrophages resulted in the upregulation of both protective and survival genes. The protective and survival genes serve to limit the proinflammatory response and thereby regulate the response to injury. For example, the naturally releasing cytokine IL-1 receptor antagonist IL1R1 (IL-1RA) inhibits the activity of IL-1β and modulates a variety of IL-1β-related immune and inflammatory responses (see Figure 2). In addition to SOD2, the induction of NF-κB-regulated human heme oxygenase 1 (HO-1)[29] is able to generate antioxidants that protect against inflammation. Furthermore, the induction of NF-κB inhibitor NFKBIA (IκBα), SOD2, and tumor necrosis factor alpha-induced protein 3, TNFAIP3 (A20), will inhibit the NF-κB system via negative feedback regulation. On low-level activation of NF-κB due to ROS attack, highly expressed IκBα and SOD2 alone are sufficient to suppress inappropriate induction of inflammatory responses. However, under a strong challenge A20 was significantly induced (Figure 3I) and acts as an inhibitor of NF-κB-dependent gene expression; this may have been activated by various stimuli including IL-1β and the activation of the macrophage surface receptors, Toll-like receptor 2 (TLR2) and CD40 (Figure 2). The stimulation of TLR2 and CD40 may be due to interaction between the phagocytosed SWCNT and T cells (see following paragraphs). The result suggests that A20 negatively regulates NF-κB activation and the follow-on inflammatory response to protect the activated macrophages against an excessive and harmful over-response to SWCNT exposure.[30,31]

The NF-κB also probably play a pivotal role in inducing genes that have an antiapoptotic function. In the present study, the NF-κB activation seems to remarkably enhance the mRNA expression of the antiapoptotic protein cIAP2, which was reported to directly suppress caspase-3 activation.[32] SWCNT stimulated the expression of cIAP2 protein in a time-dependent manner (Figure 3J). In addition, SOD2 also can inhibit apoptosis[33,34] and A20 has been reported to inhibit cytokine-mediated apoptosis.[35,36] Furthermore, we found that administration of SWCNT to macrophages did not change the expression of transforming growth factor-B1 (TGF-B1) (Table 2). NF-κB-regulated TGF-B1 release is thought to occur as an apoptotic cell is being phagocytosed by macrophage.[37] Therefore, the unchanged expression of TGF-B1 implies that there is substantial antiapoptosis happening in the SWCNT-stimulated macrophages. The above genes would seem to be able to safeguard the macrophage cells from apoptosis caused by factors such as the proinflammatory cytokines TNF-α and IL-1β and toxic ROS. Collectively, NF-κB probably induce or repress multiple genes, and this results in the blocking of apoptosis at multiple steps. The coordinate action of a panel of genes by NF-κB to suppress apoptosis may be important in preventing macrophage death in response to the proinflammatory signals associated with SWCNT exposure.

SWCNT Treatment of Macrophages Leads to the Activation of T Cells. T cell activation requires at least two signals, and these are antigen recognition and costimulation. The first signal is provided by the interaction of the T cell receptor (TCR) on the lymphocyte with major histocompatibility class (MHC) antigens on the antigen-presenting cell (APC) such as macrophages. The interaction of CD28 and CD40L on the T lymphocyte with the B7 (CD80 or CD86) and CD40 proteins, respectively, on the APC provides the necessary costimulatory second signal.[38,39] The present study showed that SWCNT exposure to macrophages induced significant expression of the lymphocyte-adhesive molecule ICAM-1, the T cell migration chemokine IP-10, and the MHC class II HLA-DRA molecule, which can then associate with its costimulatory molecules, CD40 and CD80 (Figure 2 and Table 2). These induced molecules, reported to be regulated by NF-κB,[40] can work together to direct T cells of the adaptive immune system to the inflammatory locations and cooperatively interact with the TCRs CD40L and CD28. This will result in the stimulation of T cell proliferation and the release of cytokines. In turn, the cytokines produced by T cells are able to further activate monocytes and macrophages. To our knowledge, the finding has not been reported by any earlier study.

Discussion and Conclusions. SWCNT particles possess fibrous structure with diameters about 1∼2 nm and lengths ranging from tens of nanometers to several micrometers. As mentioned above, this nanoparticle exhibits substantial cytotoxicity in vitro and in vivo and seems to be more toxic to cells or mice/rats than multiwalled carbon nanotubes (with diameters ranging from 10 to 20 nm),[13] multiwalled carbon nano-onions (with about 30 nm in diameter),[12] fullerene (C_{60}),[13] carbon black,[21] and graphite.[22] These nanoparticles are all made of carbon atoms but with distinct geometries and surface chemistries. In addition, SWCNT particles reveal more profound cytotoxicity than non-carbon nanoparticles such as quartz (SiO_2).[13,21] Collectively, the aforementioned experimental evidence reasonably suggest that the SWCNT-related cytotoxicity could be attributed to the geometry (fibrous structure) and surface chemistries (e.g., electrical properties) of them.[12,13,21] The present study is focused not on discovering what physical or chemical properties of SWCNT particles induce cytotoxicity in cells or animals exposed to them, but on exploring how SWCNT particles regulate cellular cytotoxicity at a molecular level. Thus, we used a nontoxic dispersion surfactant, Pluronic F-68 (cell culture grade), as the experimental controls in our in vitro and in vivo studies to differentiate the true cytotoxic mechanisms mediated by SWCNT particles themselves.

In the present study, the SWCNT-induced pathogenesis in the lungs of the ICR mice was examined in both early (less than 3 days) and late responses (14 days). During the early response phase, the histological data revealed an increase of macrophage infiltration and the formation of SWCNT-loaded foamy-like macrophages in the alveolar space, whereas there is no significant granuloma formation. The pulmonary granulomas could be identified mainly after one week SWCNT treatment and profound multifocal

granulomas were found after 2 weeks. Although the SWCNT-induced granulomas are composed mainly of SWCNT-laden macrophages, other cells including T lymphocytes, neutrophils, eosinophils, and other inflammatory cells need to be recruited further during different pathological phases for the granuloma development.[21] On the basis of the aforementioned observations, alveolar macrophages seem to initiate the SWCNT-induced pathogenesis through SWCNT phagocytosis, followed by the release of a variety of cytokines to recruit different immune cells, and the granuloma formation. To elucidate the original role of macrophages in the initiation of SWCNT-induced pathogenesis, a human macrophage-like THP-1 cell line was treated with SWCNT for 24 h to mimic the early response to SWCNT in vivo. As shown in Figure 1F,J, the THP-1 derived foamy-like macrophages, exposed to SWCNT for 24 h, best resemble their counterparts in vivo during the early response to SWCNT challenge.

However, one limitation of this study is that it has been conducted on cultured macrophage cells in vitro instead of isolated alveolar macrophages in vivo. That is because there are substantial difficulties in obtaining a large amount of isolated alveolar macrophages for microarray-based gene expression studies and the subsequent experimental validation. Therefore, the human THP-1 derived macrophages were used to investigate the SWCNT-induced cytotoxicity. Such an expedient may affect the outcome of the study. However, according to a earlier study on investigating the toxic effects of C_{60} (a carbon counterpart of SWCNT) on a human macrophage cell line and bovine alveolar macrophages,[41] about 60% decreases in cell viability and substantial increases of three inflammatory cytokines, TNF, IL-6, and IL-8, were observed in both types of macrophage cells after 4 and 20 h of incubation with this nanoparticle. The results demonstrated that both types of macrophages responded to the fullerene material exposure in a similar way. In addition, the SWCNT-treated THP-1 derived macrophages in the present study exhibit similar phagocytic responses to the mouse alveolar macrophages in vivo. In view of the aforementioned facts, the SWCNT-induced cytotoxicity may be probably similar in both macrophage cell types, although the cytotoxic effects of SWCNT on THP-1 derived macrophages may not accurately reflect in the real toxicological effects in alveolar macrophages in vivo.

It has been previously reported that SWCNT treatment induces ROS generation in mice[23] and human keratinoytes.[18,19] The present work confirms these results for SWCNT-treated macrophages but uses a different experimental methodology. By comparing the gene expression changes of the various antioxidative enzyme system such as SOD2, catalase, and glutathione peroxidase in macrophages under SWCNT treatment, the activity of these radical scavenging enzymes seems to be impaired as a result of the increase in local oxidative stress associated with H_2O_2 accumulation. The accumulation of H_2O_2 can lead to the production of hydroxyl radicals ($HO^{\cdot}$), which are highly damaging to cellular DNA, proteins, and lipids. H_2O_2 and hydroxyl radicals can trigger apoptosis[33] or stimulate the activation of redox-sensitive transcription factors such as AP-1 and NF-κB.[42] Consistent with a previous study,[23] our data also indicates that macrophages exposed to SWCNT do not undergo apoptosis, as demonstrated by the absence of active forms of Caspase-3, which is an apoptosis indicator, and a lack of DNA laddering (data not shown). Therefore, the aforementioned high pro-oxidant state might be continued throughout the activation of AP-1 or NF-κB.

The activation of NF-κB has already been observed in SWCNT-treated human keratinocytes.[18] However, our results further indicate that AP-1, in addition to NF-κB, is activated in SWCNT-treated macrophages. This stimulation of two redox-sensitive transcription factors would not seem to be through ROS attack only, but seems to involve other signaling pathways as well (see Figure 2). The result is a cascade of inflammatory responses involving the significant induction of a large number of proinflammatory genes and the recruitment of leukocytes. Because some target genes also serve as NF-κB/AP-1 activators and the activation of NF-κB/AP-1 can stimulate the macrophages themselves, the result is the recruitment of leukoytes, which trigger a proinflammatory signal amplification loop for further release of inflammatory mediators. Concomitantly, the induction of various protective and survival genes by NF-κB counteracts the inflammatory responses and protects the host from excessive cellular damage. The balance between the activation of these two contradictory panels of gene expression would seem to determine the subsequent immune responses. It has been reported that apoptosis is an important mechanism for the resolution of inflammation and that defects in leukocyte apoptosis are able to lead to inflammatory disease.[37] The defects in leukocyte apoptosis after exposure of macrophages to SWCNT may result in a persistent inflammatory reaction and chronic inflammation.

In this situation, the initially predominant innate immune system is unable to deal with the severe inflammatory reaction, and activation of an adaptive immune response becomes necessary. Indeed, this study shows that the SWCNT challenge endows macrophages (poor antigen-presenting cells) with an antigen-presenting function by increasing the expression level of HLA-DR, CD80, and CD40, which are able to interact with TCRs, CD28, and CD40L, respectively, on T cells resulting in T cell activation and proliferation. The cooperative interaction between the activated macrophages and T cells then results in the formation of pulmonary granulomas.[43] The capability of macrophages as antigen-presenting cells for T cell activation and the involvement of T cells in initiation, regulation, and resolution of granuloma formation[44,45] further support the aforementioned findings. The exceptional accumulation of macrophages within the granulomas may be attributed to the significant release of monocyte/macrophage chemoattractants such as MCP-1 and MIP-1α[24−28] by the activated macrophages. Granuloma formation around the activated macrophages may protect the surrounding host tissue from destructive chronic inflammation.

In conclusion, the present study's aim was to assess the genome-wide effects of SWCNT on macrophages and characterize nanoparticle-mediated cytotoxicity at a molec-

ular level. In following this goal, we have presented an inferred molecular mechanism describing the regulation of SWCNT-induced cytotoxicity in macrophages that is consistent with the literature and further experimental validation. Such a comprehensive and detailed molecular characterization of SWCNT-induced cytotoxicity, to our knowledge, has not been achieved previously, and this study provides an important insight into the molecular mechanisms involved in SWCNT-induced granulomas formation.

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Supporting Information Available: A detailed description of the experimental procedures, pathway scoring computation method, and gene expression and related information involved in the potentially affected pathways listed in Table 1. This material is available free of charge via the Internet at http://pubs.acs.org..

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