Pulmonary fibrosis is typically accompanied by inflammation, which is thought to play a role in its pathogenesis, and occurs with occupational exposure to particulates and metals, such as asbestos and vanadium pentoxide ($\text{V}_2\text{O}_5$). Lipopolysaccharide (LPS), a model of acute lung injury and inflammation, chronic bronchitis, and pulmonary fibrosis, upregulates platelet-derived growth factor receptor (PDGF-R$\alpha$) in rat lung fibroblasts (RLF). PDGF, a potent mitogen and chemoattractant for mesenchymal cells, is an important mediator in fibrotic lung diseases. This dissertation examines the effects of pre-existing inflammation, induced by LPS, on carbon nanotube (CNT)- and $\text{V}_2\text{O}_5$-induced pulmonary fibrosis in rats and the involvement of PDGF signaling. Rats were pretreated with 2.5 mg/kg LPS by intranasal aspiration, followed 24 hr later by 4 mg /kg CNT, carbon black (CB), or $\text{V}_2\text{O}_5$ administered by intratracheal instillation. Total and differential cell counts, lactate dehydrogenase (LDH), total protein, and PDGF and transforming growth factor-$\beta$ (TGF-$\beta$) protein levels (by ELISA) were examined in bronchoalveolar lavage (BAL) fluid of control and CB– and multi-walled carbon nanotubes (MWCNT) –exposed rats. Lungs from all animals were collected for histopathological analysis, immunohistochemistry, and RT-PCR of the PDGF-A, PDGF-C, PDGF-R$\alpha$, and TGF-$\beta$ genes. PDGF-A, PDGF-C, PDGF-R$\alpha$, TGF-$\beta$1, and COL1A2 gene expression was also measured in vitro in RLF and NR8383 rat alveolar macrophages in response to CNT or CB with and without LPS. In vivo, CNT and CB caused fibroproliferative, granulomatous lesions, which were located primarily in the alveolar ducts.
and alveoli. Pretreatment with LPS significantly increased collagen deposition associated with these lesions. In the BAL fluid, LPS pretreatment lead to increases in LDH, total protein, and PDGF-AA protein in rats exposed to MWCNT, and an increase in inflammatory cells in CB-exposed rats compared to controls. *In vitro*, LPS stimulated PDGF-Re gene expression in RLF, and LPS pretreatment followed by CB nanoparticles or CNT synergistically increased PDGF-A expression in NR8383 cells. Combined LPS/V2O5 exposure augmented V2O5-induced pulmonary inflammation, airway epithelial necrosis, and fibrosis and amplified *in vivo* collagen gene expression. The airway lesions were of particular interest because LPS pretreatment increased the incidence of bronchiolitis obliterans-like lesions, including subepithelial fibrosis and intraluminal fibrotic polyps. These data confirm that LPS pretreatment augments the fibrotic effects of CNT and V2O5 in rats, which likely involves enhanced PDGF signaling. This dissertation provides evidence that pre-existing pulmonary inflammation, as occurs with chronic obstructive pulmonary diseases or cigarette smoking, can enhance pulmonary fibrotic responses to environmental agents. Furthermore, exposure to environmental LPS may play a role in the pathogenesis of some fibrotic lung diseases.
Contribution of Bacterial Lipopolysaccharide to Carbon Nanotube- and Vanadium Pentoxide-Induced Pulmonary Fibrosis

by
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A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

Comparative Biomedical Sciences

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DEDICATION

I dedicate this dissertation to my wife, Claire, my children, Jack and Ellery, and my parents, Francis Cesta and Mary Elaine Cesta Peters.
BIOGRAPHY

Mark Francis Cesta was born December 21, 1965 at Fort Belvoir, Virginia to Frank and Elaine Cesta. At the age of two, Mark’s family moved to Bogota, Colombia before settling in Arizona 3 years later. Mark was raised in Tempe, Arizona and graduated from McClintock High School in 1983. Mark attended Arizona State University before earning his Doctor of Veterinary Medicine degree from Colorado State University in 1997. After working for 3 years as a private practitioner in and around Phoenix, Arizona, Mark and his wife moved to Cary, North Carolina where Mark enrolled as a graduate student at North Carolina State University and concurrently accepted a position as resident in the 3-year veterinary anatomic pathology residency program at North Carolina State University. Mark completed his residency in 2003 and was granted an Intramural Research Training Award by the Laboratory of Experimental Pathology at the National Institute of Environmental Health Sciences (NIEHS). Mark continued working on his graduate degree at The Hamner Institutes for Health Research while at NIEHS and also achieved board certification by the American College of Veterinary Pathologists in 2004. In 2005, Mark accepted a position as a toxicologic pathologist at Integrated Laboratory Systems, Inc. while continuing his graduate research on a part-time basis. In 2007, Mark accepted a position in the Cellular and Molecular Pathology Branch (formerly the Laboratory of Experimental Pathology) at the NIEHS as a staff scientist and pathologist for the National Toxicology Program where he continued to pursue his Ph.D. degree on a part-time basis.
ACKNOWLEDGEMENTS

I would like to thank my wife, Claire, for the constant love, support, and encouragement without which I could not have earned this degree. I would also like to thank my parents, Francis Cesta and Mary Elaine Cesta Peters, who taught me the importance of education, perseverance, and hard work. I would also like to thank my children, Jack and Ellery, whose innocent, smiling faces and unconditional love kept me going. I would like to thank the members of my committee, Drs. Phil Sannes, Jamie Bonner, Dave Malarkey, and Ken Adler for their guidance and support throughout this process. When no other labs had space or money, Dr. Sannes found both for me, for which I remain indebted to him. Without his efforts, I would not be writing this dissertation. Dr. Bonner, my principal investigator, provided ideas, guidance, direction, and a laboratory. I hope for the opportunity to collaborate with him for many years to come. Dr. Malarkey has guided me from the very beginning of my journey and continues to do so. He has been my primary mentor in science and pathology and, aside from my family and myself, he deserves the most credit for my being where I am today. I would also like to thank the many post-docs and lab mates who really taught me the nuts and bolts of research: Jody Khosla, Chengming Li, Donna Newman, Jennifer Ingram, Jimmy Mangum, Aurita Antao-Menezes, Liz Turpin, and Duncan Wallace, among others. Last, but by no means least, I am grateful to the Cellular and Molecular Pathology Branch and its chief, Dr. Robert Sills, of the NIEHS for giving me the opportunity to complete this work and to advance my career in so many ways.
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LIST OF SYMBOLS, ABBREVIATIONS, NOMENCLATURE

α₂M \( \alpha_2 \)-Macroglobulin

AAALAC Association for Assessment and Accreditation of Laboratory Animal Care

ACE Angiotensin converting enzyme

Al Aluminum

Al₂O₃ Aluminum oxide

ANOVA Analysis of variance

AP-1 Activator protein-1

ATF Activating transcription factor

BAL Bronchoalveolar lavage

BALF Bronchoalveolar lavage fluid

Be Beryllium

BET Backscatter electron transmission

BO Bronchiolitis obliterans

bp base pairs

BrdU Bromodeoxyuridine

Ca Cadmium

cAMP Cyclic adenosine monophosphate

CB Carbon black

CBD Chronic beryllium disease
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>CNT</td>
<td>Carbon nanotube</td>
</tr>
<tr>
<td>Co</td>
<td>Cobalt</td>
</tr>
<tr>
<td>Col1A2</td>
<td>Gene encoding type I procollagen</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclo-oxygenase</td>
</tr>
<tr>
<td>CtBP</td>
<td>C-terminal binding protein</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
</tr>
<tr>
<td>Cu</td>
<td>Copper</td>
</tr>
<tr>
<td>CVD</td>
<td>carbon vapor deposition</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3-diaminobenzidine</td>
</tr>
<tr>
<td>DEP</td>
<td>Diesel exhaust particle</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDX</td>
<td>Energy dispersive x-ray analysis</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGF-R</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EGR</td>
<td>Early growth response factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>ENA</td>
<td>Epithelial-neutrophil activating peptide</td>
</tr>
<tr>
<td>EndMT</td>
<td>Endothelial-mesenchymal transition</td>
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<tr>
<td>EPA</td>
<td>Environmental Protection Agency</td>
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<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ET</td>
<td>Endothelin</td>
</tr>
<tr>
<td>FA</td>
<td>Focal adhesion</td>
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<td>FAK</td>
<td>Focal adhesion kinase</td>
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<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>Fe</td>
<td>Iron</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<td>GAG</td>
<td>Glycosaminoglycan</td>
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<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
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<td>GAS-1</td>
<td>Growth arrest specific-1 gene</td>
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<tr>
<td>GCF2</td>
<td>GC factor 2</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte colony stimulating factor</td>
</tr>
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<td>GM-CSF</td>
<td>Granulocyte–Macrophage colony stimulating factor</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GTPase</td>
<td>Guanosine triphosphatase</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>Hg</td>
<td>Mercury</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
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<td>HOCL</td>
<td>Hypochlorous acid</td>
</tr>
<tr>
<td>HSPG</td>
<td>Heparan sulfate proteoglycan</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>ICP-AES</td>
<td>Inductively coupled plasma auger electron spectroscopy</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IκB</td>
<td>Inhibitor of NF-κB</td>
</tr>
<tr>
<td>IKK</td>
<td>Inhibitor of NF-κB kinase</td>
</tr>
<tr>
<td>IKKi</td>
<td>Inhibitor of NF-κB kinase inhibitor</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IRAK</td>
<td>IL-1R-associated kinase</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>IT</td>
<td>Intratracheal</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun amino-terminal kinase</td>
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<tr>
<td>KC</td>
<td>Keratinocyte chemoattractant</td>
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<td>KGF</td>
<td>Keratinocyte growth factor</td>
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<td>La</td>
<td>Lanthanum</td>
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<td>LAL</td>
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<td>LAP</td>
<td>Latency associated protein</td>
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<td>LBP</td>
<td>Lipopolysaccharide-binding protein</td>
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<td>LDH</td>
<td>Lactate dehydrogenase</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LT</td>
<td>Leukotriene</td>
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<td>LTBP</td>
<td>Latent TGF-β binding protein</td>
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<td>MAL</td>
<td>MyD88-adapter-like protein</td>
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<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<td>MCP</td>
<td>Macrophage chemotactic protein</td>
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<tr>
<td>M-CSF</td>
<td>Macrophage colony stimulating factor</td>
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<tr>
<td>MHCII</td>
<td>Major histocompatibility complex type II</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloprotease</td>
</tr>
<tr>
<td>Mo</td>
<td>Molybdenum</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MWCNT</td>
<td>Multi-walled carbon nanotube</td>
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<td>MyD88</td>
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</tr>
<tr>
<td>Ni</td>
<td>Nikel</td>
</tr>
<tr>
<td>NiO</td>
<td>Nikel oxide</td>
</tr>
<tr>
<td>NNI</td>
<td>National Nanotechnology Initiative</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NP</td>
<td>Nanoparticle</td>
</tr>
<tr>
<td>O$_2$$\cdot$$^-$</td>
<td>Superoxide radical</td>
</tr>
<tr>
<td>•OH</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>OXRI</td>
<td>Oxidative resistance-1 gene</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>Ca$^{2+}$- and Mg$^{2+}$-free phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth</td>
</tr>
<tr>
<td>PDGF-R</td>
<td>Platelet-derived growth factor receptor</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PI3-K</td>
<td>Phosphoinositol 3-kinase</td>
</tr>
<tr>
<td>PIPOX</td>
<td>Pipecolic acid oxidase</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC-$\gamma$</td>
<td>Phosphlipase C-$\gamma$</td>
</tr>
<tr>
<td>PM</td>
<td>Particulate matter</td>
</tr>
<tr>
<td>PM$_{10}$</td>
<td>Particulate matter with a diameter of less than 10 μm</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>-------------</td>
<td>------------</td>
</tr>
<tr>
<td>PM₂.₅</td>
<td>Particulate matter with a diameter of less than 2.5 μm</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear cell</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>PTP</td>
<td>Protein tyrosine phosphatase</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on activation, normal T expressed and secreted</td>
</tr>
<tr>
<td>REDOX</td>
<td>Reduction/Oxidation</td>
</tr>
<tr>
<td>RLF</td>
<td>Primary passage rat lung fibroblasts</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROFA</td>
<td>Residual oil fly ash</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription – polymerase chain reaction</td>
</tr>
<tr>
<td>SARM</td>
<td>Sterile α- and armadillo-motif-containing protein</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SMA</td>
<td>Smooth muscle actin</td>
</tr>
<tr>
<td>SARA</td>
<td>Smad anchor for receptor activation</td>
</tr>
<tr>
<td>SO₂</td>
<td>Sulfur dioxide</td>
</tr>
<tr>
<td>SOCS1</td>
<td>Suppressor of cytokine signaling 1</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Sp-1</td>
<td>Specificity protein-1</td>
</tr>
<tr>
<td>SPARC</td>
<td>Secreted protein, acidic and rich in cysteine</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>SUMO</td>
<td>Small ubiquitin-like modifier</td>
</tr>
<tr>
<td>SWCNT</td>
<td>Single-walled carbon nanotube</td>
</tr>
<tr>
<td>TAB2</td>
<td>TAK1 binding protein 2</td>
</tr>
<tr>
<td>TAK1</td>
<td>Transforming growth factor-β-activated kinase 1</td>
</tr>
<tr>
<td>TANK</td>
<td>TRAF family member-associated NF-κB activator</td>
</tr>
<tr>
<td>TARC</td>
<td>Thymus- and activation-regulated chemokine</td>
</tr>
<tr>
<td>TBK1</td>
<td>TANK binding kinase 1</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TGA</td>
<td>Thermogravimetric analysis</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>TGF-β RI</td>
<td>Transforming growth factor-β receptor type I</td>
</tr>
<tr>
<td>TGF-β RII</td>
<td>Transforming growth factor-β receptor type II</td>
</tr>
<tr>
<td>TGIF</td>
<td>Transforming growth interacting factor</td>
</tr>
<tr>
<td>Th1</td>
<td>T-helper type 1</td>
</tr>
<tr>
<td>Th2</td>
<td>T-helper type 2</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of matrix metalloprotease</td>
</tr>
<tr>
<td>TiO₂</td>
<td>Titanium dioxide</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll-interleukin-1 receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TRAF6</td>
<td>Tumor necrosis factor-receptor-associated factor 6</td>
</tr>
<tr>
<td>TRAM</td>
<td>TRIF-related adapter molecule</td>
</tr>
<tr>
<td>TRIAD3A</td>
<td>Triad domain-containing protein 3 variant A</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR-domain-containing adapter protein inducing IFN-β</td>
</tr>
<tr>
<td>UBC13</td>
<td>Ubiquitin-conjugating enzyme 13</td>
</tr>
<tr>
<td>UEV1A</td>
<td>Ubiquitin-conjugating enzyme E2 variant 1 isoform A</td>
</tr>
<tr>
<td>V</td>
<td>Vanadium</td>
</tr>
<tr>
<td>V$_2$O$_5$</td>
<td>Vanadium pentoxide</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WT1</td>
<td>Wilm’s tumor suppressor gene product</td>
</tr>
<tr>
<td>Y</td>
<td>Yttrium</td>
</tr>
</tbody>
</table>
CHAPTER ONE

GENERAL INTRODUCTION

Non-neoplastic lung disease is the third most common cause of death in the United States behind heart disease and cancer and is the cause of nearly 400,000 annual deaths (Stansfield & Jump, 2008). Unlike the death rates from heart disease and cancer, the death rates from lung disease are rising with a current, age-adjusted death rate of 135.5/100,000 (Stansfield & Jump, 2008). Direct health-care costs due to lung disease are currently estimated at $95 billion, while indirect costs may be as high as $59 billion (Stansfield & Jump, 2008). Finally, it is estimated that more than 35 million people in the U. S. currently suffer from chronic lung diseases (Stansfield & Jump, 2008). Thus, non-neoplastic lung diseases place a significant financial burden on the health care industry and contribute to the death and suffering of millions of people worldwide.

Chronic lung diseases are often classified as obstructive pulmonary diseases (chronic obstructive pulmonary disease or COPD), characterized by increased resistance to airflow through the airways, and restrictive pulmonary diseases, characterized by increased resistance to lung expansion. The main disorders within the COPD spectrum are emphysema, chronic bronchitis, bronchiectasis, and asthma (Husain & Kumar, 2005). Restrictive pulmonary diseases are divided into chest wall disorders and interstitial and infiltrative lung
diseases, which may be acute or chronic. There are many known causes of chronic interstitial lung disease, but there also many for which the cause is not known, such as sarcoidosis, Wegener’s granulomatosis, idiopathic pulmonary fibrosis, and those associated with the various collagen vascular diseases (e.g., scleroderma, rheumatoid arthritis, systemic lupus erythematosus, and others) (Husain & Kumar, 2005). Some of the known causes of interstitial lung diseases include autoimmune or allergic reactions, environmental or occupational exposures to such agents as airborne particulates (including ultrafine particles or nanoparticles) and other components of air pollution, organic matter, asbestos, silica, grain or cotton dust, mineral dusts, coal dust, beryllium, combustion products, metals and metal oxides (including vanadium pentoxide), irritant gases, infection by bacteria, fungi, or viruses, drugs such as bleomycin, cyclophosphamide, amiodarone, mitomycin C, phenytoin, etc., and radiation (Gulati & Redlich, 2008; Husain & Kumar, 2005; Pardo & Selman, 2002; Razzaque & Taguchi, 2003; Wilson & Wynn, 2009). Many of the environmental and occupational exposures also contribute to COPD (Balmes, 2005; Gulati & Redlich, 2008; Razzaque & Taguchi, 2003). Fibrosis, of the interstitium in the interstitial diseases or of the airways in the obstructive diseases, is a component of many of these disorders, and any of these diseases may progress to pulmonary fibrosis as an end-stage (Gulati & Redlich, 2008; Husain & Kumar, 2005).

Fibrosis is the accumulation of connective tissue. When this occurs in the interstitium of the lung, the alveoli and capillaries are permanently replaced by connective tissue, hampering the
diffusion of oxygen into the bloodstream. When around the airways, fibrosis can cause permanent narrowing of the airways due to contraction of the fibrotic tissue. The fibrotic remodeling results in a permanent loss of lung function and, usually, a terminal outcome (Pardo & Selman, 2002; Wilson & Wynn, 2009). Unfortunately, there is no effective treatment for pulmonary or airway fibrosis.

The pathogenesis of pulmonary fibrosis is not completely understood. In general terms, fibrosis occurs when there is a net increase in extracellular matrix (ECM) in the tissue due to an imbalance between ECM formation and degradation. Fibrosis is a chronic response to tissue injury that occurs when the damage is such that regeneration of the tissue is not possible, typically when there is damage to the tissue framework, or stroma (especially the basement membranes). As such, many fibrotic diseases are considered to be the result of an abnormal wound healing process. There are 3 phases of wound healing: injury, inflammation, and repair and contraction (Husain & Kumar, 2005; Wilson & Wynn, 2009). Tissue injury by any of the agents or events listed above induces an inflammatory response in which profibrotic chemokines are produced and secreted by inflammatory cells and damaged epithelial, endothelial, and other stromal cells. These chemokines are responsible for the recruitment and proliferation of fibroblasts and myofibroblasts that produce and secrete ECM proteins, which, if not removed by fibrolytic processes, results in fibrosis (Husain & Kumar, 2005; Razzaque & Taguchi, 2003; Wilson & Wynn, 2009; Wynn, 2008).
In this paradigm, inflammation is a common precursor to all forms of fibrosis. Not only does the inflammation create a pro-fibrotic environment, but the inflammatory process itself can also contribute to and exacerbate tissue damage. During the inflammatory process, leukocytes recruited to the site of injury release lysosomal enzymes (e.g., proteases and hydrolases), oxygen- or nitrogen-derived free radicals, and arachidonic acid metabolites (prostaglandins, leukotrienes, and lipoxins) (R. S. Cotran, H. Kumar, & T. Collins, eds., 1999b). These products, all of which are designed to neutralize the inciting agent, are passively released by dead or dying leukocytes or during phagocytosis of cellular debris, infectious agents, or inhaled material, or actively by exocytosis of leukocyte granules (Cotran et al., 1999b). These leukocyte products, however, also induce tissue damage, which increases the release of inflammatory and fibrotic mediators by damaged cells (Cotran et al., 1999b). It follows, therefore, that people with pre-existing inflammatory conditions, such as asthma or chronic bronchitis, may be more susceptible to the effects of pro-fibrotic agents. Alternatively, co-exposure to known inflammatory agents, such as endotoxin (lipopolysaccharide or LPS) or cigarette smoke, may amplify the effects of pro-fibrotic agents.

In this dissertation, I have explored the effects of pre-existing inflammation on the fibrogenic effects of fibrogenic agents in the lung. There are numerous animal models of pulmonary or airway inflammation, including SO₂, tobacco smoke, LPS, and serine protease (e.g. neutrophil elastase) exposure in rats (Nikula & Green, 2000). For the studies reported in this
dissertation, we used LPS to induce airway inflammation because it is ubiquitous in the environment and therefore highly relevant, it is well characterized and readily available, and it has been shown to upregulate the expression of platelet-derived growth factor receptor α (PDGF-Rα) in rat lung fibroblasts in vitro (Coin et al., 1996). We chose multi-walled carbon nanotubes (MWCNT) and V$_2$O$_5$ as our profibrotic agents. V$_2$O$_5$ is a known cause of pulmonary fibrosis in rats and its fibrotic mechanism is known to involve PDGF-Rα signaling (Bonner, Lindroos, Rice, Moomaw, & Morgan, 1998; Bonner, Rice, Moomaw, & Morgan, 2000; National Toxicology Program, 2002). The fibrotic effects of MWCNT are more controversial, however, but MWCNT are on the verge of mass production and will likely revolutionize the manufacturing industry. It is, therefore, critical to identify potential adverse effects that MWCNT may cause. Since LPS is ubiquitous in the environment, and most exposures to fibrotic agents arguably occur with concurrent LPS exposure, the effects of MWCNT and LPS is very relevant to the potential toxicity of MWCNT.
CHAPTER TWO

COMPARATIVE ANATOMY OF THE LUNG

The lung is a complex organ responsible for the exchange of gases between the blood and the air we breathe. In rodents, it is divided into the left side, which is comprised of a large, single lobe, and the right side, which is divided into 4 lobes, the cranial (anterior or apical), middle (cardiac), caudal (posterior), and accessory (median or azygous) lobes (Boorman & Eustis, 1990). In humans, the left side is divided into two lobes, and the right into three lobes (Husain & Kumar, 2005). The left and right lung lobes sit within the left and right pleural cavities, which are potential spaces within the thoracic cavity. The pressure within the pleural cavity is approximately -5 cm H2O in the resting position between inspiration and expiration (Dyce, Sack, & Wensing, 1987). This negative pressure causes the lungs to expand with the thoracic cavity, drawing in inspired air. The pleural cavities are bound by the ribcage, the diaphragm, the mediastinum, and the thoracic inlet, which are covered by the parietal pleura. The lungs are covered by the visceral pleura. The pleura is a membrane composed of mesothelial cells supported by connective tissue that contains varying amounts of elastic fibers (Plopper & Adams, 1993). The mesothelial cells produce pleural fluid, which lubricates the pleural surfaces, reducing the friction generated by the movement of the lungs across the thoracic wall during respiration. The connective tissue of the visceral pleura is continuous with the interlobular and interalveolar septa in the lung (Plopper & Adams, 1993;
Tyler & Julian, 1991). The thickness of the pleura and these septa varies among species. In humans, the pleura and septa are relatively thick while in laboratory animals, including macaques, there is much less connective tissue in the pleura septa (Tyler & Julian, 1991).

2.1 THE TRACHEOBRONCHIAL TREE

A dichotomous system of branching airways, the tracheobronchial tree, conducts the inspired air to the region of gas exchange in the lung. The first and largest airway, the trachea, originates at the larynx and splits into the primary bronchi within the thorax. The left primary bronchus then enters the left lung lobe, but the right primary bronchus gives rise to successive lobar bronchi, which enter the right lung lobes. The bronchi continue branching to form several generations of bronchi, which branch further to form the bronchioles. The last airway generation before the region of gas exchange is the terminal bronchiole. The number of airway generations and the number of airways, particularly terminal bronchioles, varies between species, but the precise number is difficult to estimate due to the asymmetry of the tracheobronchial tree. The average estimate of the number of terminal bronchioles in humans is around 25,000 and for rats is around 2500, but there is a great deal of variability in these estimates (McBride, 1991). The number of airway generations between the trachea and the terminal bronchiole is known to vary within the lung of a given individual (McBride, 1991). However, data on the variation of the number of airway generations and the number of terminal bronchioles within a species is very limited, further complicating comparisons.
between species (McBride, 1991). In primates, the branching pattern is largely symmetric, with each airway giving rise to two similarly sized, smaller airways that branch off at similar angles (Esch, Spektor, & Lippmann, 1988; Lippmann & Esch, 1988; McBride, 1991; Yeh, Schum, & Duggan, 1979). In rodents, the branching pattern is asymmetrical, or monopodial, each airway giving rise to two airways of unequal size with the larger branch coming off at a very small angle, forming a nearly straight trunk, and the smaller one branching off at a much larger angle (Esch et al., 1988; Lippmann & Esch, 1988; McBride, 1991; Yeh et al., 1979). The branching pattern varies by airway generation, with the more central (larger) airways branching in a more asymmetrical pattern and the peripheral (smaller) airways having a more symmetrical pattern (McBride, 1991). The branching pattern also varies between lobes. For example, in rats, the right cranial lung lobe has a more symmetrical branching pattern than the other lobes (McBride, 1991; Yeh et al., 1979).

In rodents, incomplete cartilage rings located in the airway walls support the trachea and extrapulmonary bronchi, but the intrapulmonary airways lack cartilage rings (Figure 2-1A). They also have submucosal glands within their walls, which contain goblet and serous cells (Kuhn III, 1985). In primates, the intrapulmonary bronchi also have cartilage and submucosal glands within their walls, though the cartilage forms discontinuous islands rather than rings (Figure 2-1B) (Kuhn III, 1985). The extrapulmonary airways of rodents are lined by pseudostratified columnar epithelium and the intrapulmonary airways are lined by simple epithelium (Mariassy, 1991). In primates, the majority of cells in the terminal bronchioles are
ciliated, whereas in rodents, the majority terminal bronchiolar cells are nonciliated (Plopper & Hyde, 1992).

The epithelium of the airways is composed of basal cells, ciliated cells, mucous cells, serous cells, Clara cells, neuroendocrine cells, brush cells, and small mucous granule cells (Hyde, Magliano, & Plopper, 1991). The basal cells are small, flattened to triangular-shaped cells that do not have a luminal surface (Hyde et al., 1991). They are anchored to the basement membrane by hemidesmosomes and to adjacent cells by desmosomes (Hyde et al., 1991; Mariassy, 1991). Their function is not certain, but their large numbers of desmosomes and hemidesmosomes and the lack of hemidesmosomes in other cell types suggest that they act to anchor the epithelium to the basement membrane (Mariassy, 1991). They may also be progenitor cells for replacing lost epithelial cells (Kuhn III, 1985). The goblet cells, serous cells, and ciliated cells are part of the mucociliary apparatus, which is one of the mechanisms by which the lungs are cleared of foreign material. The goblet cells secrete mucus, the serous cells secrete sulfated glycoconjugates, and the ciliated cells secrete glycoproteins, all of which form a layer of mucus lining the airway epithelium that traps foreign material, bacteria, etc. (Kuhn III, 1985). The cilia on the ciliated cells beat in a coordinated, wave-like fashion to propel the mucus toward the oropharynx where the mucus, and the entrapped material, is swallowed or expectorated (Kuhn III, 1985; Lopez, 2001). The serous cells also secrete a number of products, including lysozyme, lactoferrin, and peroxidase, which are important components of the innate immune system (Mariassy, 1991). The Clara cells are
found in the distal bronchioles and act as stem cells in bronchiolar epithelial repair (Kuhn III, 1985). They secrete Clara cell secretory protein, which is thought to have an immunomodulatory or anti-inflammatory function, and surfactant proteins (Ryerse, Hoffmann, Mahmoud, Nagel, & deMello, 2001). Clara cells also produce a number of P450 enzymes and are the main site of xenobiotic metabolism in the lung (Kuhn III, 1985; Plopper, Suverkropp, Morin, Nishio, & Buckpitt, 1992). The neuroendocrine cells, also called Kultschitzky cells, occur singly in the epithelium and in clusters called neuroepithelial bodies that are primarily located at airway bifurcations and are associated with fenestrated capillaries and afferent and efferent nerves (Kuhn III, 1985). Their function is unknown, but they have cytoplasmic granules that contain hormones including serotonin and calcitonin, as well as neuropeptides including bombesin and enkephalin (Kuhn III, 1985). The functions of the brush and small mucous granule cells are also unknown.

The cellular composition of the airways differs between species and between airway generations (Table 2-1). The trachea in rats and primates contains no Clara cells whereas in the mouse, approximately 50% of the cells are Clara cells (Hyde et al., 1991; Mariassy, 1991). Primates have a higher percentage of basal cells in the trachea than rats or mice, and in rats, approximately 40% of the tracheal cells are serous cells whereas mice and primates have no serous cells in the trachea (Hyde et al., 1991; Mariassy, 1991). In the terminal bronchiole, on the other hand, rodents lack basal cells, while in primates approximately 13.5% of the epithelial cells are basal cells (Hyde et al., 1991; Plopper & Hyde, 1992). In
primates, approximately 7.5% of the epithelial cells are goblet cells, while rodents completely lack goblet cells in the terminal bronchioles (Hyde et al., 1991; Plopper & Hyde, 1992). Rats have a higher percentage of ciliated cells in the terminal bronchioles (~55%) than do mice or primates (33% and 26%, respectively) (Hyde et al., 1991; Plopper & Hyde, 1992). Though interesting and probably functionally significant, the effects of these differences on lung function are unclear.

2.3 THE GAS EXCHANGE REGION

The conducting airway system gives rise to the gas exchange region of the lung. There are fewer differences in this region, but they may be significant in terms of extrapolating rodent data to humans. The gas exchange region is composed of alveolar ducts, alveolar sacs, and alveoli. The terminal bronchioles and all distal structures are often referred to as the centriacinar region (Boorman & Eustis, 1990). The main unit of gas exchange is the acinus, which includes all structures distal to one terminal bronchiole (Plopper & Adams, 1993). There is a great deal of variation in size among acini in a given lung (Mercer & Crapo, 1992). The transition zone between conducting airways (terminal bronchioles) and the gas exchange area (acini) varies between species. In general, primates and carnivores have a longer, more gradual transition zone in which there are several generations of respiratory bronchioles between a terminal bronchiole and the alveolar ducts distal to it (Figure 2-1C) (Hyde et al., 1991). A respiratory bronchiole is an alveolated airway in which the wall is interrupted by
alveolar outpocketings. In most laboratory animals (including mice, rats, gerbils, hamsters, rabbits, and guinea pigs) and larger domestic animals (including the horse, ox, pig, and sheep), the transition zone is much more abrupt. These species have very short or no respiratory bronchioles; the terminal bronchioles give rise directly to alveolar ducts (Figure 2-1D) (Hyde et al., 1991). This is an important distinction given the susceptibility of this region to injury.

Within the acinus, there are numerous generations of alveolar ducts, which lead to the alveoli. The average number of alveolar duct generations per acinar unit is fairly uniform across species (Mercer & Crapo, 1992). The alveoli are the main site of gas exchange in the lung. They are small, sac-like structures separated by thin septa (alveolar walls) that are covered by type I and type II alveolar epithelial cells (Figure 2-2). Oxygen from inspired air diffuses from the alveolar space, through the alveolar wall, and into the blood within the alveolar capillaries. To maximize the transfer of oxygen, the alveolar wall is very thin. In much of the alveoli, the wall is composed only of an endothelial cell, the fused basement membranes of the capillary and epithelium, and the alveolar type I epithelial cell, and the mean thickness of the air-blood barrier is $0.32 \pm 0.01 \, \mu m$ in mice ($M. \ musculus$), $0.34 \pm 0.01$ to $0.38 \pm 0.03 \, \mu m$ (depending on age and sex), and $0.62 \pm 0.04 \, \mu m$ in man (Pinkerton, Gehr, & Crapo, 1992). Thus, the air-blood barrier in man is approximately twice as thick as that of mice. The percentages (of total cells) of alveolar type I and type II cells in rodents and humans is similar, but rats have a higher percentage of endothelial cells, and humans have a
higher percentage of interstitial cells (fibroblasts, pericytes, interstitial macrophages, monocytes, and lymphocytes) and a thicker interstitium (Pinkerton et al., 1992). Humans also have more alveolar macrophages than rats, with approximately $6500 \times 10^9$ lavageable alveolar macrophages compared to $4.9 \times 10^9$ in the rat and $0.67 \times 10^9$ in the mouse (Valberg & Blanchard, 1992). This translates to approximately $22,000 \mu\text{m}^2$ of alveolar surface area per macrophage in humans and $140,000$ and $190,000 \mu\text{m}^2$ of alveolar surface area per macrophage in rats and mice, respectively (Valberg & Blanchard, 1992). However, alveolar macrophage numbers vary greatly.
Table 2-1. Distribution of cell types in the airways of primates and rodents.

<table>
<thead>
<tr>
<th>Location</th>
<th>Species</th>
<th>Basal Cells (%)</th>
<th>Ciliated Cells (%)</th>
<th>Clara Cells (%)</th>
<th>Goblet Cells (%)</th>
<th>Serous Cells (%)</th>
<th>Other (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trachea</td>
<td>Rhesus Monkeya</td>
<td>42.0</td>
<td>32.9</td>
<td>0</td>
<td>16.8</td>
<td>0</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td>Rата</td>
<td>13.4</td>
<td>40.6</td>
<td>0</td>
<td>0.5</td>
<td>39.2</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>Mouseа</td>
<td>7.7</td>
<td>35.8</td>
<td>52.1</td>
<td>0</td>
<td>0</td>
<td>12.0</td>
</tr>
<tr>
<td>Terminal</td>
<td>Rhesus Monkey</td>
<td>26.9</td>
<td>52.2</td>
<td>0</td>
<td>14.8</td>
<td>0</td>
<td>6.1</td>
</tr>
<tr>
<td>Bronchiole</td>
<td>(p.a.)а, b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rhesus Monkey</td>
<td>0</td>
<td>0</td>
<td>95.0</td>
<td>0</td>
<td>0</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>(o.s.)а, b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rата</td>
<td>-</td>
<td>55.0</td>
<td>37.0</td>
<td>0</td>
<td>-</td>
<td>8.0</td>
</tr>
<tr>
<td></td>
<td>Mouseа</td>
<td>0</td>
<td>32.8</td>
<td>67.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>


cp.a.–pulmonary artery side of terminal bronchiole; o.s.–opposite side of the same terminal bronchiole
Figure 2-1. Comparison of primate and rodent airways. (A) Intrapulmonary bronchiole of a mouse from the hilus (upper right) to the pleura. Note the complete lack of cartilage. H&E, 2X original magnification. (B) Intrapulmonary bronchus from a rhesus monkey. Numerous cartilage rings or irregular plates support the bronchial wall. H&E, 2X original magnification. (C) Respiratory bronchioles from a rhesus monkey. Note the numerous alveolar outpockets and branching from a single parent respiratory bronchiole to 2 daughter respiratory bronchioles. H&E, 10X original magnification. (D) Terminal and short respiratory bronchioles from a mouse Note the single alveolar outpocket of the respiratory bronchiole. H&E, 20X original magnification.
Figure 2-2. Normal alveolus from a mouse. Thin alveolar septa divide the lung parenchyma into alveoli. The septa contain capillaries (note the red blood cells) and are the location of gas exchange in the lung. Thin alveolar type I cells line the alveoli and the more cuboidal alveolar type II cells are found mainly in the corners of the alveoli. An unstimulated, binucleated alveolar macrophage can also be seen within the alveolus. H&E, 40X original magnification.
CHAPTER THREE
LITERATURE REVIEW

3.1 PULMONARY FIBROSIS

As stated in the introduction, fibrosis is the accumulation of connective tissue. This typically occurs when tissue damage involves not only the parenchymal cells, but also the stromal framework of the organ, and the normal tissue architecture cannot be restored (Strieter, 2008; Strieter & Mehrad, 2009). Damage to the stromal framework is characteristic of chronic inflammation, a component of nearly all fibrotic lung diseases (R. S. Cotran, H. Kumar, & T. Collins, 1999a). When this occurs in the lung, pulmonary function is irreversibly compromised.

Pulmonary fibrosis is the end-stage of interstitial lung disease (ILD) and also occurs in airway diseases such as COPD. Environmental exposure to particles or metals (or a number of other airborne agents) has been shown to cause a number of ILDs, and contributes to the development of other ILDs and COPD (Balmes, 2005; Gulati & Redlich, 2008). There also appears to be a genetic component. A familial form of idiopathic pulmonary fibrosis (IPF) has been identified and there is evidence of hereditary susceptibility to sarcoidosis and a number of other fibrotic lung diseases, such as those caused by pulmonary surfactant disorders (Steele & Brown, 2007). Mouse models also provide evidence of genetic
susceptibility to lung fibrosis. For example, the 129J strain of mice is resistant to asbestos- and transforming growth factor-β1 (TGF-β1)-induced pulmonary fibrosis whereas the C57BL/6 strain is very susceptible (Warshamana, Pociask, Sime, Schwartz, & Brody, 2002).

The mechanisms by which lung fibrosis develops are complex and varies with the type of disease or etiologic agent. The classical theory for the development of idiopathic pulmonary fibrosis (IPF) holds that repeated or sustained lung injury, induced by autoimmune reactions, allergic reactions, environmental particulates, pulmonary toxins, infection, or mechanical damage, gives rise to chronic inflammation in which there is continual production of chemokines, cytokines, growth factors and other mediators that result in increased numbers of activated myofibroblasts and a net increase in ECM components. There is an abundance of research supporting this theory, however, another theory has recently been proposed. The new theory proposes that the pulmonary fibrosis caused by injury to epithelial cells and an abnormal healing process and that the accompanying inflammation is simply a parallel process (Strieter & Mehrad, 2009). This new theory was developed due to the lack of correlation between inflammation and the severity or outcome of IPF and that the use of immunosuppressive or anti-inflammatory drugs do not seem to influence the progression of this disease (Strieter & Mehrad, 2009). This clinically-derived theory seems to separate the events involved in pulmonary fibrosis. The inflammatory response can exacerbate epithelial and endothelial damage, and inflammatory cells, themselves, are known to elaborate a number of pro-fibrotic mediators. Also, angiogenesis, which is closely connected to
inflammation, has been shown to be important in the progression of pulmonary fibrosis (Strieter, Gomperts, & Keane, 2007). Therefore, a more realistic hypothesis is one that incorporates the effects of the inflammatory response, epithelial-mesenchymal interactions, and possibly angiogenesis. In regard to particle-induced lung disease, inflammation is a key factor in the pathogenic effects of particles and arises in response to particle-induced oxidative stress (Donaldson et al., 2006; Donaldson & Tran, 2002; N. Li, Xia, & Nel, 2008). Metals, including vanadium pentoxide (V$_2$O$_5$), also induce oxidative stress and inflammation in the lung (Bonner et al., 2000; Dye, Adler, Richards, & Dreher, 1999; Ingram, Rice, Santos, Van Houten, & Bonner, 2003; Valko, Morris, & Cronin, 2005; Y. Z. Wang et al., 2003). Ergo, in the context of this dissertation, inflammation is likely a major factor in the development of lung fibrosis.

3.1.1 Cells Involved in Pulmonary Fibrosis

As noted above, the mechanisms involved in pulmonary fibrosis are complex. Though much of the process is still a mystery, some of the key players have been extensively studied and much is known regarding their roles in the fibrotic process. Following is a brief discussion of the main cell types involved in pulmonary fibrosis.

3.1.1.1 Myofibroblasts

The production and deposition of extracellular matrix proteins is carried out mainly by
myofibroblasts. Myofibroblasts are mesenchymal cells that exhibit features of both fibroblasts and smooth muscle cells. They are contractile cells containing α-smooth muscle actin (SMA) and localize to sites of active fibrosis (Hinz et al., 2007; Phan, 2002; Scotton & Chambers, 2007; Wilson & Wynn, 2009; Wynn, 2008). The expression of α-SMA in myofibroblasts may also influence signal transduction and gene expression, including that involved in ECM production (Hinz et al., 2007). Three possible origins of myofibroblasts have been proposed: 1) differentiation of resident lung fibroblasts, 2) transdifferentiation of other cell types into myofibroblasts, including epithelial cells, endothelial cells, smooth muscle cells, and pericytes, and 3) recruitment of circulating fibrocytes of bone marrow origin (Hinz et al., 2007; Scotton & Chambers, 2007; Strieter, Keeley, Hughes, Burdick, & Mehrad, 2009; Strieter & Mehrad, 2009; Wynn, 2008).

Though the exact contribution of each of these sources of myofibroblasts is unknown, the differentiation of resident fibroblasts into myofibroblasts seems to be the most common source (Hinz et al., 2007). The differentiation of fibroblasts into myofibroblasts is a complex process, but the accumulation of TGF-β1 and specialized ECM components, such as the fibronectin ED-A splice variant, and increased mechanical stress derived from alterations in the ECM that result from tissue damage and remodeling appear to be required (Hinz et al., 2007; Pardo & Selman, 2002). Fibroblasts form junctions with the extracellular matrix called focal adhesions (FAs). When the ECM is damaged, and the cellular substrate becomes less rigid, the FAs become “supermature” (i.e., they enlarge), which confers greater cytoskeletal
stress, which induces α-SMA production and the myofibroblast phenotype (Hinz et al., 2007). The role of TGF-β1 in fibrosis has been extensively studied and will be discussed in depth in section 3.1.2.2. One of its effects, however, is the differentiation of fibroblasts to myofibroblasts (Wynn, 2008). There is also mounting evidence that epithelial cells can transdifferentiate into myofibroblasts, a process termed epithelial-mesenchymal transition (EMT), under the influence of TGF-β1 or IL-4 (Phan, 2002; Strieter & Mehrad, 2009; Willis & Borok, 2007; Wynn, 2008; Yao, Xie, Chen, Deng, & Tang, 2004). Recently, endothelial-mesenchymal transition (EndMT) has also been shown to occur (Deissler, Lang, & Lang, 2006; Hashimoto et al., 2009; Kizu, Medici, & Kalluri, 2009; J. Li, Qu, & Bertram, 2009). Circulating fibrocytes of bone marrow origin that migrate to sites of lung fibrosis express markers of both fibroblasts (collagens I and III and fibronectin) and hematopoietic cells (CD45, MHCII, CD34) (Strieter et al., 2009; Strieter & Mehrad, 2009; Wynn, 2008). These cells have been shown to differentiate into ECM producing myofibroblasts that express α-SMA and decrease expression of CD34 (Quan, Cowper, & Bucala, 2006; Strieter et al., 2009; Strieter & Mehrad, 2009). This process is mediated or augmented by profibrotic mediators, including TGF-β1, endothelin-1, IL-13, and IL-4, and inhibited by antifibrotic mediators, such as interferon (IFN)-γ (Strieter et al., 2009; Strieter & Mehrad, 2009). Circulating CD14-positive monocytes may also be a source of fibrocytes (Quan et al., 2006). A number of other mediators are also involved in fibroblast differentiation into myofibroblasts. Pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), have been shown to activate fibroblasts through toll-like receptor (TLR) signaling in fibroblasts (Wynn, 2008).
Platelet-derived growth factor (PDGF) is a potent chemoattractant and mitogen for fibroblasts and myofibroblasts and will be discussed further in section 3.1.2.1 (Bonner, 2004, 2007; Wynn, 2008). IGF-1 and IL-6 are also fibroblast mitogens Wilson, 2009 #748} (Knight, Ernst, Anderson, Moodley, & Mutsaers, 2003; Kuwano, Hagimoto, & Hara, 2001; Moodley et al., 2003; Wynn, 2008).

Not only do myofibroblasts produce and secrete ECM components, they also secrete a number of proinflammatory and profibrotic cytokines. Fibrocytes, circulating myofibroblast precursors, have been shown to express TNF-α, IL-6, IL-8, IL-10, a number of chemokines, such as MIP1α/β, and matrix metalloproteinases (MMPs), such as MMP-9, under the influence of IL-1β (Quan et al., 2006). In wound healing studies, these mediators were generally produced early in the process, and the fibrocytes subsequently stopped producing them and began producing collagen and other ECM components (Quan et al., 2006).

Fibroblasts produce angiotensin II, TGF-β1, PDGF, tissue inhibitors of matrix metalloproteinases (TIMPs), prostaglandin (PG) E₂, connective tissue growth factor (CTGF), macrophage colony stimulating factor (m-CSF or CSF-1) and granulocyte/macrophage colony stimulating factor (GM-CSF), IL-1, TNF-α, endothelin (ET)-1, and others (Pardo & Selman, 2002; Razzaque & Taguchi, 2003; White, Lazar, & Thannickal, 2003; Wilson & Wynn, 2009; Wynn, 2008).

The cessation of ECM production in wound healing is achieved through apoptosis of
myofibroblasts. In fact, lung fibroblasts from patients with IPF seem to be resistant to apoptosis, despite upregulation of the apoptosis-inducing Fas ligand (Drakopanagiotakis, Xifteri, Polychronopoulos, & Bouros, 2008; Maeyama et al., 2001; Wilson & Wynn, 2009). Several mechanisms contributing to this resistance have been identified. It has been reported that X-chromosome-linked inhibitor of apoptosis (ILP) and FLICE-like inhibitor protein (FLIP$_L$) may be involved (Tanaka et al., 2002). TGF-β coordinately activates focal adhesion kinase (FAK) and AKT in human fetal lung fibroblasts, which confers apoptosis resistance (Horowitz et al., 2007). Also, fibroblasts in fibroblastic foci in IPF patients are largely Thy-1 negative and Thy-1 negativity has been associated with decreased apoptosis (Sanders, Kumbla, & Hagood, 2007). The mechanism by which Thy-1 negativity confers apoptosis resistance by involves TGF-β because Thy-1 negative rat lung fibroblasts have been shown to activate latent TGF-β (Zhou, Hagood, & Murphy-Ullrich, 2004).

### 3.1.1.2 Alveolar Macrophages

Alveolar macrophages are resident phagocytic and antigen presenting cells in the lung. They are replenished by circulating, bone marrow derived monocytes. Though alveolar macrophages secrete fibronectin, their main function is the phagocytosis and clearance of foreign material from the lung (Wilson & Wynn, 2009). They are the first inflammatory cell type to respond to particle, metal, or other pulmonary exposure, and they produce a number of mediators that promote inflammation and fibrosis, making them one of the primary
effector cells in the pulmonary fibrotic process. The mediators they secrete include CCL2 (macrophage chemotactic protein-1, or MCP-1), CCL3 (also known as macrophage inflammatory protein 1α, or MIP-1α), CXCL2 (also known as macrophage inflammatory protein-2, or MIP-2), TGF-β1, angiotensin II, arachadonic acid metabolites (including lipoxins, leukotrienes, and prostaglandins), IL-1β, IL-2, IL-4, IL-6, IL-8 (also known as CXCL8), IL-11, insulin-like growth factor (IGF)-I, PDGF, IFN-γ, and many others (Bonner, 2004; Goldstein, 1991; Knight et al., 2003; Kowal-Bielecka, Kowal, Distler, & Gay, 2007; Murugan & Peck, 2009; Strieter & Mehrad, 2009; White et al., 2003; Wynn, 2008). In fact, in bleomycin-induced lung fibrosis in mice and rats, alveolar macrophages are thought to produce nearly all the TGF-β1 that induces fibrosis (Wynn, 2008). They are also a known source of ROS in the lung (Kuwano et al., 2001; Razzaque & Taguchi, 2003).

Alveolar macrophages are normally quiescent, but become activated upon exposure to a number of agents, including infectious agents, environmental particulates, metals, and other foreign material. They may also become activated by exposure to mediators secreted by other cells, such as lipid mediators from alveolar epithelial cells (Martin, Rochelle, Fischer, Krunkosky, & Adler, 1997). Recent studies have identified two types of activated alveolar macrophages, classically activated or M1 macrophages and alternatively activated (AA) or M2 macrophages (Strieter, 2008; Wilson & Wynn, 2009). The M1 type macrophage arises through exposure to Th1 cytokines, TNF-α, LPS, fungal wall components, and degraded ECM, while the M2 type arises from exposure to Th2 cytokines, TGF-β, or apoptotic cells.
3.1.1.3 Epithelial cells

Lung epithelial cells have recently garnered much attention in the pathogenesis of fibrotic lung diseases. They are the first line of defense in pulmonary exposure to environmental or occupational agents of lung disease. The classical theory of the pathogenesis of pulmonary fibrosis in humans holds that persistent exposure to an irritant (known or unknown) causes chronic inflammation that drives the fibrotic response. However, because of poor correlation between inflammation and the severity or outcome of pulmonary fibrosis and the ineffectiveness of immunosuppressive or anti-inflammatory drugs to affect the fibrotic process, a new theory has recently been proposed (Strieter & Mehrad, 2009). This new theory asserts that epithelial damage with an abnormal wound-healing response drives the fibrotic process and that the inflammation is an independent process (Strieter & Mehrad, 2009).
Though it seems simplistic to dismiss the role of inflammation in the fibrotic process, there is ample evidence that the respiratory epithelium plays a significant role in pulmonary fibrogenesis.

The pulmonary epithelium may contribute to the fibrotic process in several ways: They secrete a number of proinflammatory and profibrotic mediators, they have been shown to undergo EMT and are a source of myofibroblasts, and apoptosis of alveolar type II cells has been associated with pulmonary fibrosis, though the mechanism(s) involved have yet to be identified (Corvol, Flamein, Epaud, Clement, & Guillot, 2009; Thannickal & Horowitz, 2006). In response to exogenous stimuli, the alveolar epithelium elaborates a number of mediators, including arachidonic acid metabolites (i.e., cyclooxygenase and lipoxygenase products), ROS, TNF-α, PDGF, TGF-β, ET-1, IGF-1, and IL-4, all of which have been shown to play a role in the fibrotic process (Martin, Rochelle et al., 1997; Pardo & Selman, 2002). Alveolar type II epithelial cells produce surfactant proteins. To date, four proteins have been identified, surfactant protein-A (SP-A), SP-B, SP-C, and SP-D. Abnormalities in SP-C have been associated with IPF, but SP-C abnormalities are relatively rare in humans and are not involved in the majority of cases (Lawson et al., 2004).

The transdifferentiation of alveolar type II cells into fibroblasts and myofibroblasts, or EMT, has been shown to occur in the lungs of patients suffering from IPF (Strieter & Mehrad, 2009). During the process of EMT, the epithelial cells lose polarity, epithelial cell markers
(e.g., cytokeratins, zonula occludens-1, and E-cadherin), and tight junctions, undergo
cytoskeletal reorganization and change to a spindle-shaped morphology, and acquire
mesenchymal cell markers (Corvol et al., 2009; Willis & Borok, 2007). TGF-β1 is thought to
play a significant role in EMT, mainly through a Smad-dependent pathway (Corvol et al.,
2009; Willis & Borok, 2007). The mechanism involved may include TGF-β1-induced
expression of MMPs, which induce EMT by disrupting integrin–matrix interactions (Willis
& Borok, 2007). Other mediators, individually or in combination, have also been shown to
induce EMT under various conditions, including ET-1, fibroblast growth factor (FGF)-2,
epidermal growth factor (EGF), CTGF, IGF-II, IL-1, hepatocyte growth factor (HGF), and
Wnt ligands (Jain, Shaul, Borok, & Willis, 2007; Willis & Borok, 2007). Additionally, the
ECM itself appears to be involved in regulating EMT. Alveolar epithelial cells grown on
fibrin or fibronectin in vitro undergo EMT through integrin-mediated activation of
endogenous latent TGF-β1, whereas alveolar epithelial cells grown on a mixture laminin and
collagen did not undergo EMT, and when exposed to TGF-β1, undergo apoptosis (K. K. Kim
et al., 2006).

Evidence suggests that epithelial cell apoptosis plays a role in the pathogenesis of pulmonary
fibrosis. Apoptotic epithelial cells have been identified overlying fibroblastic foci in lung
biopsies from IPF patients, an area which is also often denuded (Pardo & Selman, 2002;
Sisson et al., 2010; Willis & Borok, 2007). The cause of epithelial cell apoptosis in
pulmonary fibrosis is not entirely clear, however, evidence suggests the involvement of
myofibroblasts. Myofibroblasts from IPF patients produce angiotensin I and/or II, which can induce apoptosis in epithelial cells (Pardo & Selman, 2002). Myofibroblasts also produce ROS, which can cause DNA damage in epithelial cells resulting in p53-mediated apoptosis (Corvol et al., 2009). Alveolar type II cells from patient with interstitial lung disease have been shown to have high levels of p53 (Corvol et al., 2009). TGF-β1, which induces promotes epithelial cell apoptosis, may also play a role (Corvol et al., 2009). Apoptotic cells produce TGF-β1 and induce TGF-β1 production in neighboring, nonapoptotic epithelial cells (Corvol et al., 2009). This engenders the notion of a continuous cycle whereby epithelial cell apoptosis increases the amount of TGF-β1, which contributes to fibrosis and induces further epithelial cell apoptosis, which further stimulates the production of TGF-β1, etc. (Corvol et al., 2009).

3.1.1.4 Leukocytes and other cells

Other cells, particularly leukocytes, particularly neutrophils, eosinophils, and lymphocytes, have been implicated in the pathogenesis of pulmonary fibrosis. Neutrophils are among the first circulating inflammatory cells to respond to lung injury. They migrate to sites of injury in response to the CXC chemokines CXCL8 (IL-8) and epithelial-neutrophil activating peptide-78 (ENA-78, also known as CXCL5), as well as IL-17, CCL2 (macrophage chemotactic protein-1, or MCP-1), CCL3 (MIP-1α), platelet activating factor (PAF), and granulocyte colony stimulating factor (G-CSF) among others (R. S. Cotran, H. Kumar, & T.
Collins, eds., 1999c; Kuwano et al., 2001; White et al., 2003; Wilson & Wynn, 2009).

Eosinophils are also early responders that play a role in some forms of pulmonary fibrosis and migrate to sites of injury in response to IL-5, CCL11 (eotaxin), TNF-α, and PAF (Cotran et al., 1999c; Kuwano et al., 2001; Wilson & Wynn, 2009; Wynn, 2008). Once at the site of injury, these cells produce a number of mediators, including TGF-β1, IL-13 (Wilson & Wynn, 2009; Wynn, 2008). Eosinophil-derived major basic protein and eosinophil peroxidase induce the production of ET-1, TGF-β1, PDGF, EGF, MMP-9, fibronectin, and tenascin by bronchial epithelial cells \textit{in vitro} (Pegorier, Wagner, Gleich, & Pretolani, 2006).

Neutrophils are a significant source of ROS and produce myeloperoxidase, which converts the relatively weak oxidant H$_2$O$_2$ to HOCL, a more potent oxidant (Cotran et al., 1999b; White et al., 2003). Furthermore, these ROS can activate pro-MMPs, and both the ROS and MMPs can exacerbate tissue and cell damage (White et al., 2003).

Lymphocytes, which migrate to sites of injury later in the process, mark the transition from acute inflammation to chronic inflammation and are important in the fibrotic process.

Lymphocytes include T-lymphocytes, B-lymphocytes, and natural killer (NK) cells. T-lymphocytes are subdivided into CD8+ cytotoxic T cells and CD4+ T-helper cells, which are further subdivided into T-helper type 1 (Th1) and T-helper type 2 (Th2) cells. This is an important distinction because Th1 cells generally secrete cytokines that are considered proinflammatory, including IFN-γ, IL-2, IL-12, and TNF-α, while Th2 cells secrete cytokines that are consistent with a wound-healing response, including IL-4, IL-5, IL-13, and
IL-21 (Pardo & Selman, 2002; Wilson & Wynn, 2009; Wynn, 2008). Th1 cytokine profiles are generally considered antifibrotic and Th2 cytokine profiles are generally considered profibrotic (Pardo & Selman, 2002; Wilson & Wynn, 2009; Wynn, 2008). Other cells have similar subclassifications. For example, the M1 macrophages secrete a largely Th1-type cytokine profile and M2 macrophages secrete a largely Th2-type cytokine profile (Pardo & Selman, 2002; Strieter, 2008).

Endothelial cells have also been implicated in the fibrotic process. They are the initial barrier between the blood and the lung parenchyma. Loss of endothelial cells in alveolar injury results in loss of normal lung architecture and pulmonary fibrosis (Strieter, 2008). Endothelial cells are central to the process of inflammation in that they regulate vascular permeability and leukocyte adhesion and transcytosis (Cotran et al., 1999b). They regulate transcytosis of leukocytes and fibrocytes to the lung through expression of adhesion molecules and MMPs that disrupt the basement membrane, allowing transmigration of these cells (Wynn, 2008). Endothelial cells also secrete a number of proinflammatory and profibrotic mediators, including interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), connective tissue growth factor (CTGF), endothelin-1 (ET-1), insulin-like growth factor (IGF-1), fibroblast growth factor-2 (FGF-2), and a number of CC and CXC chemokines (Keane, Strieter, & Belperio, 2005). They are central to the coagulation cascade and hemostasis. During hemorrhage and through vascular leakage during inflammation, fibrin is deposited in
the tissues at sites of injury. The fibrin acts as a scaffold for the migration of fibroblasts in response to PDGF and other chemoattractants (Wynn, 2008). The role of vascular remodeling, the generation and degradation of blood vessels, in the pathogenesis of pulmonary fibrosis is unclear. Vascular remodeling occurs at sites of fibrogenesis and is necessary for fibroproliferation, but whether it plays a lead role or supporting role in the pathogenesis of pulmonary fibrosis remains to be determined (Tzouvelekis, Anevitis, & Bouros, 2006; Voelkel, Douglas, & Nicolls, 2007; Wynn, 2008).

Other cell types may also play a role in the pathogenesis of pulmonary fibrosis. Mast cells are typically found in connective tissue adjacent to blood vessels and are known to be involved in the pathogenesis of allergic diseases (S. W. Wang et al., 2005). They produce a number of inflammatory mediators, but can also be polarized to produce a Th1-type cytokine pattern or a Th2-type cytokine pattern (Pardo & Selman, 2002). They also express many profibrotic mediators in response to IgE cross-linking, including PDGF-A, PDGF-C, TGF-β1, HB-EGF, vascular endothelial growth factor (VEGF), IL-5, IL-8, IL-13, MIP-1α, MIP-1β, and MCP-1 (S. W. Wang et al., 2005). Many of these have also been found to be upregulated in pulmonary fibrogenesis (Bonner, 2007; Holgate et al., 2010; Ingram et al., 2003; Pardo & Selman, 2002; Razzaque & Taguchi, 2003; Strieter & Mehrad, 2009; White et al., 2003; Wilson & Wynn, 2009; Wynn, 2008). Mast cells produce fibroblast growth factor-2 (FGF-2) and chymase, which reportedly increases TGF-β activity (Allen & Spiteri, 2002;
3.1.2 Mediators of Pulmonary Fibrosis

As is evident from the previous sections, there are many mediators involved in the pathogenesis of pulmonary fibrosis. In this section, those that are most important, or that are most relevant to this dissertation will be discussed.

3.1.2.1 Platelet-Derived Growth Factor

The PDGFs are among the most potent mitogens and chemotactic agents for mesenchymal cells, including smooth muscle cells, fibroblasts, and myofibroblasts and act in a paracrine or autocrine manner (Andrae, Gallini, & Betsholtz, 2008; Trojanowska, 2008). PDGFs are also involved in angiogenesis, which is also important in the fibrotic process (Cotran et al., 1999a; Wynn, 2008). The involvement of PDGFs in fibrotic diseases of the lung is well established. It is expressed in alveolar macrophages, fibroblasts, alveolar type II cells, endothelial cells, and smooth muscle cells in patients with early stage IPF (Homma et al., 1995). In fact, alveolar macrophages recovered from IPF patients have been shown to release 4 times more PDGF than macrophages recovered from individuals without lung disease (Martinet, Rom, Grotendorst, Martin, & Crystal, 1987). In patients with scleroderma, a systemic disease characterized by fibrosis of the skin, lungs, and other organs, antibodies that stimulate the PDGF receptor (PDGF-R) have been identified (Baroni et al., 2006). In animal models of
pulmonary fibrosis, PDGF levels are increased in alveolar macrophages and epithelial cells at
the bronchoalveolar junction in response to asbestos (Bonner & Brody, 1991; J. Y. Liu et al.,
1997). In rats, V2O5 induces PDGF in vivo, as well as an increase in cell proliferation as
measured by BrdU immunohistochemistry (IHC) (Mangum et al., 2006). Bleomycin also
induces PDGF expression in alveolar macrophages in vivo (H. Li, He, Que, & Weng, 1996).

In vitro, V2O5 has been shown to increase PDGF expression in rat lung fibroblasts and/or
alveolar macrophages in response to asbestos, V2O5, LPS, or carbon nanotubes (CNT) (Cesta
et al., 2009; Lasky et al., 1995). While it is generally accepted that PDGF’s main contribution
to the fibrotic process are its chemotactic and mitogenic effects on mesenchymal cells, it may
also be a cofactor in TGF-β1-induced collagen production by smooth muscle cells (SMC)
(Halloran, So, & Baxter, 1996). Pharmacologic inhibition of PDGF-Rs have been shown to
ameliorate the fibrotic effects of V2O5 in rats and of thoracic irradiation in mice (Abdollahi et
al., 2005; Rice, Moomaw, Morgan, & Bonner, 1999). Lastly, transgenic mice overexpressing
PDGF in the lung develop lung fibrosis, inflammation, and emphysema (Hoyle et al., 1999).

There are four PDGF genes that encode four PDGF subunits, PDGF-A, -B, -C, and -D
chains, which dimerize to form four homodimers (PDGF-AA, -BB, -CC, and -DD) and a
single heterodimer (PDGF-AB). The relatively recently discovered PDGF-CC and -DD
isoforms are secreted in a latent form requiring cleavage of the C1r/C1s, Urchin EGF-like
protein and bone morphogenic protein 1 (CUB) which prevents binding to PDGF-Rs (X. Li
& Eriksson, 2003; Reigstad, Varhaug, & Lillehaug, 2005; Zhao, Liu, Liu, Nilsson, & Nister,
Interestingly, the CUB domain from PDGF-CC reportedly has mitogenic activity for human coronary artery cells *in vitro* (Reigstad et al., 2005). The five PDGF ligands bind to two receptors, PDGF-Rα and PDGF-Rβ, which dimerize upon ligand binding to form three active isoforms, PDGF-Rαα, PDGF-Rαβ, and PDGF-Rββ. PDGF-BB binds to all three receptor isoforms and PDGF-AB and -CC bind to PDGF-αα and PDGF-Rαβ, but PDGF-AA binds exclusively to PDGF-Rαα and PDGF-DD binds almost exclusively to PDGF-Rββ (under certain circumstances PDGF-DD may bind to PDGF-Rαβ) (Tallquist & Kazlauskas, 2004).

The PDGF receptors are receptor tyrosine kinases (RTKs) that autophosphorylate at specific intracellular tyrosine residues upon ligand binding and dimerization (Claesson-Welsh, 1996; Schmahl, Raymond, & Soriano, 2007; Tallquist & Kazlauskas, 2004). To activate the receptor, however, a second event is required: suppression of protein tyrosine phosphatase (PTP) activity (Sundaresan, Yu, Ferrans, Irani, & Finkel, 1995; Svegliati et al., 2005; Tallquist & Kazlauskas, 2004). PTPs dephosphorylate the tyrosine residues on RTKs, thereby inactivating them. In cells that express PDGF-Rs, the inhibition of PTPs involves PDGF-dependent generation of H₂O₂, which inactivates PTPs (Tallquist & Kazlauskas, 2004). This mechanism appears to be mediated by phosphoinositol 3-kinase (PI3-K) (Tallquist & Kazlauskas, 2004).

Activation of PDGF-R initiates a number of signaling cascades. A number of signal
transduction molecules bind to and are activated by activated PDGF-Rs, including Src family proteins, PI3K, phospholipase C-γ (PLC-γ), SHP-2, GTPase activating protein (GAP), Shc, Grb2, Grb7, Nck, Crk, and signal transducer and activator of transcription (STAT)-1, STAT-3, STAT-5, and STAT-6 (Deb, Zamanian-Daryoush, Xu, Kadereit, & Williams, 2001; Heldin & Westermark, 1999; Tallquist & Kazlauskas, 2004). Activation of these signal transducers initiates a number of intracellular signaling cascades. PI3-K, which converts phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂] to phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P₃], mediates numerous cellular responses including cell migration and chemotaxis and cell growth and survival (Andrae et al., 2008; Heldin & Westermark, 1999). The downstream effectors of PI3-K include the Rho family of GTPases, AKT (also known as protein kinase B, or PKB), Jun amino-terminal kinase (JNK), and some protein kinase C (PKC) family members (Andrae et al., 2008; Heldin & Westermark, 1999; Vanhaesebroeck, Leevers, Panayotou, & Waterfield, 1997). Interestingly, Src family member inhibit PDGF-Rα-induced, PI3-K/AKT/Forkhead gene-mediated antiapoptotic effects by activating a ubiquitin ligase (c-Cbl) required for degradation of the activated PDGF-Rα (Vantler et al., 2006). PI3-K appears to be particularly important in PDGF-Rα signaling (Tallquist & Kazlauskas, 2004). Phospholipase C-γ (PLC-γ), which converts PI(4,5)P₂ to PI(1,4,5)P₃ and diacylglycerol (DAG), mobilizes intracellular Ca²⁺ and activates PKC family members, the effects of which include increased cell growth and motility (Andrae et al., 2008; Heldin & Westermark, 1999). PDGF-R activation also results in activation of the Ras-MAPK cascade through the binding of the Grb2 and Shc binding proteins (Andrae et al., 2008; Heldin &
The MAPK cascade is involved in cell growth, proliferation, migration, and differentiation (Andrae et al., 2008; Heldin & Westermark, 1999). However, PDGF activation of the p38 MAPK provides negative feedback in rat lung myofibroblasts by inhibiting PDGF-stimulated growth of these cells through a direct interaction of p38 with ERK-2 (Rice, Ingram, & Bonner, 2002). The end product of some of these pathways is the induction of immediate early genes (e.g., Jun and Fos family members and EGR-1), which may mediate many of the PDGF-mediated effects (Schmahl et al., 2007).

Control of PDGF signaling occurs at multiple levels, including transcription of the ligand and of the receptor, post-transcriptional mechanisms, translation, and inhibition of ligand binding (Kaetzel, 2003). The PDGF-A gene’s start site is 24-34 bp downstream of the Goldberg-Hogness box (a consensus TATAA sequence) (Kaetzel, 2003). The proximal promoter region, a GC-rich region 200 bp upstream of the TATA box, contains binding sites for the transcription factors specificity protein 1 (Sp1) and related factors, early growth response factor 1 (EGR-1), GC factor 2 (GCF2), nuclear factor 1-X (NF1-X), and Wilm’s tumor suppressor gene product (WT1) (Heldin & Westermark, 1999; Kaetzel, 2003). A number of mediators have been shown to increase PDGF expression or secretion. IL-13 induces PDGF-A expression in lung fibroblasts through a Stat6/Egr-1 mediated mechanism that is inhibited by activated Stat1 (Ingram et al., 2006). IFN-γ is another inducer of PDGF expression, and the mechanism may involve a Stat1-independent pathway (Bonner, 2004). IL-1β, TGF-β1, angiotensin II, thrombin, and even PDGF itself also increase PDGF expression (Bonner,
2004; Kaetzel, 2003; Kaetzel, Coyne, & Fenstermaker, 1993). Additionally, PDGF-A gene transcription can be enhanced by vitamin D, though the importance of this mechanism in pulmonary fibrosis is unclear (Pedigo, Zhang, Koszewski, & Kaetzel, 2003). In the lung, the anticoagulant protein C inhibits the expression of PDGF in lung epithelial cells and macrophage-differentiated THP-1 cells in vitro, but the mechanisms involved have not been elucidated (Shimizu et al., 2003). TGF-β1 also increases PDGF-AA production in connective tissue cells (Battegay, Raines, Seifert, Bowen-Pope, & Ross, 1990; Seifert, Coats, Raines, Ross, & Bowen-Pope, 1994).

Post-transcriptionally, PDGF binding to ECM components can regulate its activity. PDGF binds to a number of ECM proteins, including heparan sulfate, chondroitin sulfate, dermatan sulfate, and numerous collagen types (Andrae et al., 2008; Sasaki et al., 2000; Silber, Walenga, Fareed, & Kovacs, 1993; Somasundaram & Schuppan, 1996; Zafiropoulos, Fthenou, Chatzinikolaou, & Tzanakakis, 2008). Binding of PDGF to chondroitin sulfate, dermatan sulfate, heparan sulfate, or heparin inhibited PDGF-mediated cell proliferation (Andrae et al., 2008; Sasaki et al., 2000; Silber et al., 1993; Zafiropoulos et al., 2008). The binding of PDGFs to heparan sulfate proteoglycans (HSPGs) appears to be mediated by N- and O- sulfation of HSPGs (Andrae et al., 2008). In contrast, binding of PDGF-AA to collagens I, III, IV, and VI—with binding specificity in the order of III, I, VI, IV—increased PDGF-mediated proliferation of human fibroblasts (Somasundaram & Schuppan, 1996). The mechanism through which various ECM components affects PDGF activity likely involves
integrin binding. PDGF-R activation is maximized when they are associated with integrins (Giancotti & Ruoslahti, 1999; Miyamoto, Teramoto, Gutkind, & Yamada, 1996). PDGF-Rs have been reported to associate with $\alpha_\nu\beta_3$ integrin in fibroblasts and with $\alpha_2\beta_1$ integrin in smooth muscle cells (SMC) and this association enhanced proliferation and migration of these cells (Chung, Lin, Chang, Peng, & Huang, 2009; Hollenbeck et al., 2004; Schneller, Vuori, & Ruoslahti, 1997; Woodard et al., 1998). Furthermore, the tumor suppressor PTEN inhibits integrin and growth factor activation of the MAPK cascade, but not activation of AKT by PDGF (Gu, Tamura, & Yamada, 1998). PDGFs have also been shown to bind to SPARC (secreted protein, acidic and rich in cysteine) and $\alpha_2$-macroglobulin ($\alpha_2$M), which prevents binding to PDGF-Rs (Bonner, 1994; Raines, Lane, Iruela-Arispe, Ross, & Sage, 1992). $\alpha_2$M and ECM components may also act as a source of PDGF through release of PDGF from these molecules (Bonner, 1994; Dirks & Bloemers, 1995-1996).

Regulation of PDGF-R is also very complex. PDGF-R$\alpha$ is expressed mainly in mesenchymal cells and is induced or suppressed by many factors, whereas expression of PDGF-R$\beta$ is constitutive and tends not to vary in response to these factors (Andrae et al., 2008; Bonner, 2004). A number of mediators have been shown to upregulate PDGF-R$\alpha$, but the most potent is IL-1$\beta$ through effects on both transcription and translation of PDGF-R$\alpha$ (Bonner, 2004). The effects of IL-1$\beta$ on PDGF-R$\alpha$ expression may involve NF-$\kappa$B, though NF-$\kappa$B appears to play a minor role if any, but does not involve activator protein-1 (AP-1) (Lindroos, Rice,
Furthermore, IL-1β upregulation of PDGF-Rα is negatively regulated by the MEK/ERK pathway (Lindroos et al., 1998). α2M also negatively regulates IL-1β-mediated upregulation of PDGF-Rα by binding to IL-1β (Lindroos, Coin, Osornio-Vargas, & Bonner, 1995). In rat lung fibroblasts, LPS induces PDGF Rα expression through both a direct mechanism that likely involves toll-like receptor 4 (TLR4), the main receptor for LPS, and through an IL-1β mediated mechanism (Bonner, 2004; Coin et al., 1996). In vascular smooth muscle cells, fibroblast growth factor-2 (FGF-2) induces upregulation of PDGF Rα (Bonner, Badgett, Lindroos, & Coin, 1996; Schollmann et al., 1992). The ECM may also affect PDGF-Rα expression. Culture with heparan sulfate increased PDGF-Rα and -Rβ expression in human lung fibroblasts (Malmstrom & Westergren-Thorsson, 1998).

Lipopolysaccharide (LPS) is an exogenous inducer of PDGF-Rα expression in rat lung myofibroblasts, which occurs through a direct mechanism that likely involves TLR4 activation, but this effect has not been shown in other species (Coin et al., 1996). TGF-β1, on the other hand downregulates PDGF-Rα expression in lung fibroblasts at relatively high doses, but has no effect at lower doses that increase PDGF-AA production (Battegay et al., 1990; Bonner, Badgett, Lindroos, & Osornio-Vargas, 1995; Seifert et al., 1994). Cyclooxygenase 2 (COX-2) is an enzyme involved in the conversion of arachadonic acid to prostaglandin E2 (PGE2). PGE2 down-regulates PDGF-Rα expression and thus may have antifibrotic effects (Bonner, 2004; Bonner et al., 2002).
3.1.2.2 Transforming Growth Factor-β

TGF-β1 is perhaps the most widely studied and well-characterized mediator of fibrosis and is known to induce the production and secretion of ECM proteins in many fibrotic diseases, including pulmonary fibrosis. There are three mammalian TGF-β isoforms: TGF-β1, TGF-β2, and TGF-β3. TGF-β2 and TGF-β3 are constitutively expressed in the lung and are not thought to play a major role in pulmonary fibrosis (Lasky & Brody, 2000; Wynn, 2008). TGF-β1 is the most prevalent and best characterized of the three. It is considered the most active of the three isoforms in promoting fibrosis and will be the focus of this review (Gharaee-Kermani, Hu, Phan, & Gyetko, 2009; Wynn, 2008). TGF-β1 induces the production and secretion of ECM proteins by mesenchymal cells and the EMT, but it can also induce growth arrest and apoptosis, suppress the immune system, and play a role in cell migration in a number of cell types (Heldin, Landstrom, & Moustakas, 2009; Ross & Hill, 2008).

TGF-β1 activates the transmembrane TGF-β receptors, which activates a number of intracellular signaling cascades. The TGF-β receptors are a family of serine/threonine kinase receptors, of which there are two types: TGF-β receptor type I (TGF-β RI), which is also known as Alk5, and TGF-β receptor type II (TGF-β RII) (Attisano & Wrana, 2002; Heldin et al., 2009; Massague, Seoane, & Wotton, 2005; Miyazawa, Shinozaki, Hara, Furuya, & Miyazono, 2002; Rahimi & Leof, 2007; Ross & Hill, 2008; ten Dijke & Hill, 2004). TGF-β1
binds to TGF-β RII at the membrane surface (de Caestecker, 2004; Heldin et al., 2009). Once this occurs, TGF-β RI binds to the TGF-β RII/TGF-β complex and TGF-β RII phosphorylates TGF-β RI, activating the receptor (Attisano & Wrana, 2002; de Caestecker, 2004; Heldin et al., 2009; Massague et al., 2005; Miyazawa et al., 2002; Rahimi & Leof, 2007; Ross & Hill, 2008; ten Dijke & Hill, 2004).

The activated TGF-β RI initiates a number of signaling cascades. TGF-β activates the Smads, the Rho family of GTPases, MAPKs, including ERK, p38, and JNK, PI3-K, AKT, and Src (Attisano & Wrana, 2002; Heldin et al., 2009). The most important of these, however, are the Smad-mediated signaling pathways. There are eight Smad proteins, which can be divided into three groups: the R-Smads (Smad1, Smad2, Smad3, Smad5, and Smad8), the Co-Smad (Smad4, which is the only member of this group), and the I-Smads (Smad6 and Smad7) (Attisano & Wrana, 2002; Heldin et al., 2009; Massague et al., 2005; Miyazawa et al., 2002; Rahimi & Leof, 2007; Ross & Hill, 2008; ten Dijke & Hill, 2004). Activated TGF-β RI directly phosphorylates the R-Smads, Smad2 and Smad3, which requires the interaction of SARA (Smad anchor for receptor activation), an intracellular, membrane associated protein that recruits Smad2/3 to TGF-β RI (Attisano & Wrana, 2002; Massague et al., 2005; Ross & Hill, 2008). Smad2/3 then enter the nucleus, a process that occurs without the need for nuclear transport proteins because Smads can interact directly with nucleoporins in the nuclear membrane, where they are found as complexes with Smad4 (Attisano & Wrana,
This complex can directly bind to DNA promoters, but various DNA binding partners and transcriptional coactivators, such as activating transcription factor 3 (ATF3), FoxH1 and FoxO, and E2F4/5, direct DNA binding, thus regulating gene transcription (Attisano & Wrana, 2002; Massague et al., 2005; Ross & Hill, 2008).

Negative regulation of Smad signaling is very complex and occurs through multiple mechanisms. The I-Smads (Smad 6 and Smad7) are inhibitory Smads that antagonize Smad signaling, but are also induced by Smad signaling (Heldin et al., 2009). The I-Smads act through a number of mechanisms. They bind to the activated TGF-β RI, inhibiting R-Smad activation (ten Dijke & Hill, 2004). They recruit E3-ubiquitin ligases, such as Smad ubiquitin regulatory factor (Smurf) 1/2 and protein phosphatase 1, to TGF-β RI, targeting it for degradation (Itoh & ten Dijke, 2007; Ross & Hill, 2008; ten Dijke & Hill, 2004). The I-Smads also interfere with R-Smad/Co-Smad interactions as well as Smad–coactivator and Smad–DNA interactions (Massague et al., 2005; Ross & Hill, 2008; ten Dijke & Hill, 2004). Negative regulation also occurs through binding of transcriptional corepressors, including transforming growth interacting factor (TGIF), C-terminal binding protein (CtBP), Evi-1, SnoN, and Ski, which compete with coactivators for Smad binding (Massague et al., 2005; Ross & Hill, 2008). Some of these corepressors inhibit transcription of specific genes through association with histone deacetylases (Itoh & ten Dijke, 2007). Small ubiquitin-like modifier (SUMO) binding to Smad4 has been shown to have an inhibitory effect on Smad-induced gene transcription, however, some studies have also shown an activating effect of SUMO on TGF-
β-induced gene expression (Massague et al., 2005; Ross & Hill, 2008). Recently, it has been shown that sumoylation of the TGF-β RI enhances Smad activation (Kang, Saunier, Akhurst, & Derynck, 2008; Miyazono, Kamiya, & Miyazawa, 2008). These observed differences likely reflect cell-type or context specific variation in SUMO effects. MAPKs and cyclin-dependent kinases (CDKs) are able to phosphorylate Smads, but the residues they phosphorylate differ from those phosphorylated by TGF-β RI, and the effects of this phosphorylation has been shown to have both activating and inhibitory effects, which, again, is likely cell-type and context specific (Ross & Hill, 2008).

TGF-β is secreted in an inactive form. TGF-β is produced as a homodimeric propeptide (pro TGF-β) that dimerizes to form the latency associated protein (LAP) (Todorovic et al., 2005). The LAP then forms a complex with one of three latent TGF-β binding proteins (LTBP), LTBP-1, -3, and -4 (Todorovic et al., 2005). LTBP anchors the LAP to the ECM. The three LTBP isoforms may have differential binding properties for both TGF-β and the ECM and may have differing sensitivities to proteolytic cleavage (Rifkin, 2005; Todorovic et al., 2005). TGF-β is activated by proteolytic cleavage of the active form from the LAP–LTBP complex. Several activators of TGF-β have been identified, including MMPs 2 and 9, thrombospondin-1, plasmin, the αvβ6 integrin, and ROS (Annes, Munger, & Rifkin, 2003; Leask & Abraham, 2004; Rifkin, 2005).
The effects of TGF-β, ECM production and deposition, growth arrest, apoptosis, angiogenesis, immune suppression, and others, are mediated by a number of cytokines and other secreted proteins. TGF-β-induced collagen production by myofibroblasts is a direct effect of TFG-β: Signaling through the TGF-β activator protein induces expression of the collagen 1A1 gene and signaling through Smad3 induces expression of the collagen 1A2 gene (Gharaee-Kermani et al., 2009). TGF-β also inhibits ECM degradation by inhibiting production of MMPs and plasminogen activators and by increasing production of TIMPs (Gharaee-Kermani et al., 2009). TGF-β also stimulates fibroblasts to secrete a number of proinflammatory and profibrotic cytokines, such as TNF-α, PDGF, IL-1β, and IL-13 (Gharaee-Kermani et al., 2009). Interestingly, while TGF-β induces the production of PDGF by fibroblasts, at higher concentrations, it inhibits fibroblast expression of PDGF-R (Battegay et al., 1990). TGF-β also induces the production of angiotensin II, a profibrotic and proangiogenic factor, by alveolar epithelial and endothelial cells (Gharaee-Kermani et al., 2009). Connective tissue growth factor (CTGF) is induced by TGF-β, stimulates fibroblast formation and matrix production, and may be a downstream effector of some of the effects of TGF-β (Leask & Abraham, 2004). In lung epithelial cells, TGF-β enhances apoptosis through downregulation of p21, increasing expression of proapoptotic BCL family members Bax and Bid, and interaction of TGF-β RII with Daxx, a proapoptotic Fas receptor associated (Gharaee-Kermani et al., 2009).
3.1.2.3 Other Mediators

Though PDGF and TGF-β are among the most important growth factors in the pathogenesis of pulmonary fibrosis, a number of other growth factors, as well as cytokines and chemokines are thought to play a significant role. The Th2 cytokines IL-4 and IL-13 have, perhaps, received the most attention. Interleukins are secreted mainly by lymphocytes recruited to the lung during inflammation. IL-4 receptors are present on many subtypes of mouse and human fibroblasts and in vitro exposure of these cells to IL-4 induced production of ECM proteins (Wilson & Wynn, 2009; Wynn, 2008). IL-4 also has the ability to promote the differentiation of T-lymphocytes into Th2 lymphocytes and of macrophages into M2 macrophages, which results in the release of more Th2 cytokines (Strieter, 2008; Wilson & Wynn, 2009). Additionally, IL-4 can induce the expression of TGF-β in fibroblasts and plays a role in TGF-β production by eosinophils (Kodera, McGaha, Phelps, Paul, & Bona, 2002).

IL-13 has also been associated with pulmonary fibrosis and has been shown to induce fibrosis in animal models (Strieter & Mehrad, 2009; Wilson & Wynn, 2009; Wynn, 2008). IL-13 is similar to IL-4 in that they both activate Stat6 by binding to the type II IL-4 receptor, which is a heterodimer of IL-4Rα and IL-13Rα (Wills-Karp & Finkelman, 2008). IL-13 can also stimulate M2 macrophage differentiation, but not Th2 lymphocyte differentiation (Wills-Karp & Finkelman, 2008; Wilson & Wynn, 2009; Wynn, 2008). IL-13 also binds to the IL-13Rα2 receptor, which, through an AP-1 mediated mechanism, increases expression of the TGF-β1 gene (Fichtner-Feigl, Strober, Kawakami, Puri, & Kitani, 2006). Additionally, IL-13
stimulates MMP and cathepsin production, which play a role in tissue remodeling and can activate latent TGF-β (Lanone et al., 2002; Wynn, 2008). It is thought that many of the fibrotic effects of IL-13 are mediated by TGF-β (Strieter & Mehrad, 2009; Wilson & Wynn, 2009; Wynn, 2008). IL-5 is another cytokine that has been linked to pulmonary fibrosis. IL-5 recruits eosinophils to the injured lung, which produce TGF-β in response to IL-4 (Wilson & Wynn, 2009). IL-5 can also increase IL-13 production (Wilson & Wynn, 2009). IL-8, another important interleukin in pulmonary fibrosis is a potent neutrophil chemoattractant. As noted above, neutrophils produce a number of factors that epithelial and endothelial cell damage and mediate pulmonary fibrosis. Increased levels of IL-8 have been identified in IPF and cystic fibrosis patients (Keane et al., 2005; Wilson & Wynn, 2009). IL-1β is a major mediator of lung inflammation and contributes to pulmonary fibrosis. It is released by alveolar macrophages in response to V₂O₅ or LPS and has been shown to induce the expression of PDGF-Rα in lung fibroblasts (Boyle et al., 1999; Ingram, Rice, Geisenhoffer, Madtes, & Bonner, 2004; Y. Z. Wang, Zhang, Rice, & Bonner, 2000; Wewers, Winnard, & Dare, 1999). It has also been shown to induce MMP-1 activity and, in combination with PDGF, MMP-9 production by lung fibroblasts (Sasaki et al., 2000). Thus, IL-1β may enhance PDGF-induced fibroblast proliferation and chemotaxis. Additionally, IL-1β administration in mice has been shown to induce pulmonary fibrosis, likely through the induction of TGF-β (Bonnaud et al., 2005). However, IL-1β also induces expression of MMPs, plasminogen activator, and PGE₂, which play a role in matrix degradation and, in the
case of PGE2, inhibition of fibroblast proliferation (Keane et al., 2005). IL-6 is mitogenic for fibroblasts from patients with cryptogenic fibrosing alveolitis and systemic scleroderma, but not for normal fibroblasts (Hasegawa, Fujimoto, Takehara, & Sato, 2005; Knight et al., 2003). It also promotes the Th2 polarization of T-lymphocytes (Diehl & Rincon, 2002).

A number of chemokines in the CC and CXC families and their receptors have been implicated in the pathogenesis of pulmonary fibrosis. Chemokines are produced by a number of cell types, including alveolar macrophages, epithelial cells, endothelial cells, lymphocytes, neutrophils, eosinophils, and mast cells, and are chemoattractant molecules important in recruiting cells, mainly leukocytes, to sites of injury (Keane et al., 2005). They are produced in response to a number of factors, including IL-1, TNF-α, complement components, leukotriene B4 (LTB4), and IFNs. CCL2 (also known as monocyte chemoattractant protein-1, or MCP-1) and CCL3 (also known as macrophage inflammatory protein-1α, or MIP-1α), which bind to CCR2 and CCR1, respectively, are chemotactic for macrophages and have been associated with lung fibrosis (Keane et al., 2005; Wynn, 2008). They are produced mainly by macrophages and epithelial cells and may affect the levels of IL-4 or IL-13 (Keane et al., 2005; Wynn, 2008). In addition to recruiting leukocytes to the lung, chemokines are also chemoattractants for fibrocytes, which express the receptors CCR3, CCR5, CCR7, and CXCR4 in humans and CCR2, CCR7, and CXCR4 in mice, and migrate in response to CXCL12 and may respond to CCL19 and CCL21 (Strieter et al., 2007). The CXC chemokines also play a significant role in angiogenesis (Strieter et al., 2007). CXCL8 binds
to CXCR1 and CXCR2 and is important in pulmonary fibrosis as discussed above (Keane et al., 2005). The ELR motif is a sequence of three amino acids (glutamate–leucine–arginine) found just before the first cysteine residue at the amino terminus of several of the CXC family chemokines (Strieter et al., 2007; Wilson & Wynn, 2009). ELR+ CXC chemokines, CXCL1, 2, 3, 5, and 8, bind to CXCR2 and are angiogenic, and the ELR– CXC chemokines, CXCL4, 9, 10, and 11, bind to CXCR3 and are angiostatic (Strieter et al., 2007; Wilson & Wynn, 2009). The ELR+ CXC chemokines are considered profibrotic because they provide an avenue for fibrocytes to enter areas of active fibrosis and supply the fibrotic tissue, which is metabolically active, with the required oxygen, nutrients, and other metabolic substrates (Strieter et al., 2007).

In addition to PDGF and TGF-β, a number of other growth factors are considered profibrotic, including FGFs, IGF-1, CTGF, VEGF, keratinocyte growth factor (KGF), and HGF. FGF-2 is chemotactic and mitogenic for mesenchymal cells, including smooth muscle cells (SMC) and myofibroblasts and is increased in the BAL fluid from IPF patients (Y. Inoue, King, Barker, Daniloff, & Newman, 2002; Keane et al., 2005). FGF-2, upon tissue injury, is released from the ECM (Sannes, Khosla, Johnson et al., 1996). This release may be mediated by TGF-β and may be the mechanism through which TGF-β exerts its proliferative effects on myofibroblasts (Khalil, Xu, O'Connor, & Duronio, 2005). Additionally, FGFs affect alveolar type II cell DNA synthesis in vitro, which is modified by the degree of sulfation of the ECM (Sannes, Khosla, & Cheng, 1996). IGF-1, which is increased in IPF patients as well as
patients in the fibroproliferative phase of acute respiratory distress syndrome (ARDS),
enhances PDGF-induced fibroblast proliferation (Keane et al., 2005). In the normal lung,
IGF-1 is produced alveolar macrophages, but in the lungs of IPF patients, IGF-1 is produced
by alveolar and interstitial macrophages and epithelial cells (Kuwano et al., 2001). VEGF is a
major angiogenic factor and may contribute to pulmonary fibrosis by promoting
angiogenesis, similar to the ELR+ CXC chemokines. VEGF is chemotactic for mast cells,
which are discussed above (Kuwano et al., 2001). VEGF also induces the expression of
CTGF in retinal vascular cells via a TGF-β-independent pathway, though this has not been
shown to occur in the lung (Suzuma et al., 2000). CTGF, also known as CCN2, is increased
in IPF patients and promotes ECM production and myofibroblast differentiation (Allen &
Spiteri, 2002; Keane et al., 2005; Scotton & Chambers, 2007). CTGF is thought to be a
downstream effector of TGF-β1, but is induced by other factors as well, including thrombin
(Scotton & Chambers, 2007). CTGF is produced by fibroblasts, vascular smooth muscle
cells, and alveolar type II cells (Keane et al., 2005; Scotton & Chambers, 2007). KGF and
HGF are both increased in IPF patients, are mitogenic for epithelial cells, and have been
shown to be protective against pulmonary fibrosis in animal models (Kuwano et al., 2001).

Renin catalyzes the conversion of angiotensinogen to angiotensin I, which is then converted
to angiotensin II by angiotensin converting enzyme (ACE) (Verlander, 1992). Angiotensin II
has been incriminated in the pathogenesis of lung fibrosis by virtue of its ability to stimulate
TGF-β1 production, SMAD 2 production, and SMAD 3 activation (Wynn, 2008). It is also 
promotes fibrosis through it’s angiogenic properties.

In addition to the many profibrotic mediators, there are a number of mediators with both 
antifibrotic and profibrotic properties that are secreted in inflammatory and/or fibrotic lung 
diseases, including IL-10, TNF-α, and MMPs. IL-10 is a Th2 cytokine with anti-
inflammatory activity that is generally considered to be protective against pulmonary 
fibrosis, but this is somewhat controversial. It inhibits the production of several 
proinflammatory mediators, including IFN-γ, IL-1, TNF-α, CXC and CC chemokines, and 
free radicals (Garantziotis et al., 2006; Keane et al., 2005). IL-10 overexpression in airway 
epithelial cells reduced LPS-induced subepithelial fibrosis in a mouse model (Garantziotis et 
al., 2006). Introduction of the IL-10 gene in mice using a liposomal vector inhibited 
bleomycin-induced lung fibrosis (Arai et al., 2000). In IL-10 knockout mice exposed to 
silica, however, showed evidence of increased cell damage and inflammation, but decreased 
fibrosis, suggesting anti-inflammatory but profibrotic functions for IL-10 (Kuwano et al., 
2001).

Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine produced mainly by 
mononuclear phagocytes whose effects often overlap with those of IL-1β. Overexpression of 
TNF-α did not result in significant lung fibrosis in one study, in contrast to overexpression of
IL-1β, but it is considered to be a mediator of fibrosis (Gauldie, Bonniaud, Sime, Ask, & Kolb, 2007; Wilson & Wynn, 2009). TNF-α receptor knockout mice are protected from the fibroproliferative effects of inhaled asbestos fibers (J. Y. Liu, Brass, Hoyle, & Brody, 1998). TNF-α is increased in BAL fluid from IPF patients and has several effects on fibroblasts. It induces the expression of TGF-β in lung fibroblasts and the production of PDGF by fibroblasts and other cells, thus promoting ECM production and fibroblast proliferation (Keane et al., 2005; Sullivan, Ferris, Pociask, & Brody, 2005, 2008). It also amplifies fibroblast production of CXC and CC chemokines, IL-1, IL-6, GM-CSF, and glycosaminoglycans (GAGs) (Keane et al., 2005). TNF-α induces the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), which are important for leukocyte extravasation from blood vessels (Vancheri, Mastruzzo, Sortino, & Crimi, 2004). However, TNF-α also increases fibroblast production of MMP-1 (collagenase), gelatinase, PGE2, and decreases procollagen type I production in fibroblasts (Keane et al., 2005). It is clear that the relationship of TNF-α to pulmonary fibrosis is complex.

The role of matrix metalloproteinases in the fibrotic process is also complex. On the one hand, they play a major role in the degradation of the ECM, which is required for removal of excess ECM produced during lung injury. On the other hand, through degradation of the ECM, they disrupt basement membranes allowing inflammatory cells and
fibroblasts/fibrocytes to enter and migrate through the lung, they clear a path for new blood vessels during angiogenesis, and they liberate ECM-bound fibrogenic growth factors, effecting their activation (Keane et al., 2005; Winkler & Fowlkes, 2002). MMPs are regulated, in part, by tissue inhibitors of matrix metalloproteinases (TIMPs). TGF-β inhibits the expression of a number of MMPs and promotes the production of TIMPs (Kuwano et al., 2001). Lung fibroblasts and alveolar macrophages from IPF patients express MMP-9, which they normally do not express (Winkler & Fowlkes, 2002). Furthermore, fibroblasts from IPF patients express high levels of TIMPs, resulting in an imbalance of MMPs and TIMPs (Winkler & Fowlkes, 2002). This causes the abnormal ECM remodeling seen in IPF lungs (Winkler & Fowlkes, 2002).

Interferon-γ (IFN-γ) is an important Th1 cytokine and is considered to be antifibrotic. It is also a major effector of the innate immune response (Keane et al., 2005). It is produced by Th1 lymphocytes and natural killer cells and promotes inflammation through upregulation of ICAM-1 and interferon-inducible chemokines, thus recruiting inflammatory cells. The antifibrotic effects of IFN-γ include inhibition of fibroblast synthesis and collagen production, presumably through downregulation of TGF-β and PDGF, and decreased production of the profibrotic ELR⁺ CXC chemokines and CTGF (Keane et al., 2005; Kuwano et al., 2001; Razzaque & Taguchi, 2003). IFN-γ, particularly when combined with prednisolone, is proving to be relatively successful the treatment of IPF in clinical trials (Kuwano et al., 2001; Pardo & Selman, 2002; Razzaque & Taguchi, 2003). IL-12, another
Th1 cytokine with antifibrotic properties, is a potent inducer of IFN-\(\gamma\) (Keane et al., 2005; Kuwano et al., 2001).

Arachadonic acid metabolites generated by cyclo-oxygenase and lipoxygenase include prostacyclin, prostaglandins, thromboxanes, and leukotrienes. These lipid mediators have a diverse array of effects and play a significant role in inflammation. In many organs, PGE\(_2\) has proinflammatory effects, but in the lung, where it is produced by alveolar macrophages, epithelial cells, fibroblasts, and smooth muscle cells, PGE\(_2\) has anti-inflammatory and antifibrotic effects (Charbeneau & Peters-Golden, 2005; Keane et al., 2005; Vancheri et al., 2004). Its anti-inflammatory effects include inhibition of adhesion molecule expression (e.g., ICAM-1 and VCAM-1), leukocyte chemotaxis, and lymphocyte function, thus inhibiting leukocyte recruitment (Charbeneau & Peters-Golden, 2005). PGE\(_2\) also inhibits the production of many proinflammatory and profibrotic mediators, such as ROS, IL-4, IL-5, IL-8, TNF-\(\alpha\), FGF, ET-1, and NF-\(\kappa\)B (Charbeneau & Peters-Golden, 2005; Vancheri et al., 2004). Many of these effects are mediated by PGE\(_2\)-induced production of IL-10 (Charbeneau & Peters-Golden, 2005; Vancheri et al., 2004). In lung fibroblasts, PGE\(_2\) inhibits fibroblast migration, proliferation, collagen synthesis, and myofibroblast differentiation, and promotes myofibroblast apoptosis (S. Huang, Wettlaufer, Hogaboam, Aronoff, & Peters-Golden, 2007; S. K. Huang, Wettlaufer, Chung, & Peters-Golden, 2008; S. K. Huang et al., 2009; Kohyama et al., 2001; Kolodsiick et al., 2003; Moore et al., 2003; White et al., 2005). The mechanism by which PGE\(_2\) inhibits fibroblast proliferation likely
involves its antagonism of IL-1β-induced upregulation of PDGF-Rα, and inhibition of CTGF expression (Allen & Spiteri, 2002; Boyle et al., 1999). PGE₂ also inhibits Smad signaling, abrogating some of the effects of TGF-β (Strieter, 2008). PGE₂ levels are decreased in the BAL fluid of IPF patients, and fibroblasts from patients with usual interstitial pneumonia are less responsive to PGE₂, which may be due to decreased E prostanoid receptor 2 (one of the PGE₂ receptors) levels, or a decrease in PKA activity, which results in decreased cAMP and decreased E prostanoid receptor signaling (Charbeneau & Peters-Golden, 2005; S. K. Huang, Wettlaufer, Hogaboam et al., 2008; White et al., 2003).

3.1.3 Bronchiolitis Obliterans

Bronchiolitis obliterans (BO) is a form of pulmonary fibrosis in which the small airways are specifically affected. These airways become obstructed due to subepithelial fibrosis, which, in some airways, results in complete occlusion of the lumen (Elssner & Vogelmeier, 2001; Nicod, 2006). BO can occur as a result of pulmonary exposure to toxicants, severe lower respiratory infections, particularly in individuals with connective tissue disorders, radiation exposure, or lung or bone marrow transplantation (Cosgrove & Schwarz, 2008; Nicod, 2006). There are two morphologic forms of BO: bronchiolitis obliterans organizing pneumonia (BOOP), which is typically reversible and is characterized by the growth of fibrous polyps into the bronchioles, and constrictive bronchiolitis, which is typically not reversible and is characterized by concentric fibrous proliferation from the wall of the bronchiole that results
in narrowing and, eventually, complete obliteration of the lumen (Cosgrove & Schwarz, 2008; King & Kinder, 2008). As with most fibrotic disorders, BO is typically accompanied by inflammation (Elssner & Vogelmeier, 2001; Grossman & Shilling, 2009; Hertz et al., 1992). BO also occurs in domestic animals after viral or parasitic lung infections or inhalation of toxic gases (Dungworth, 1993). In both humans and animals, the lesions are thought to stem from severe injury and necrosis of the airway epithelium (Dungworth, 1993; King & Kinder, 2008). Though the pathogenesis is not completely understood, studies have identified a role for PDGF, PDGF-A in particular, and TGF-β, which, as discussed above, are important mediators in the fibrotic process (Alho, Maasilta, Vainikka, & Salminen, 2007; Hertz et al., 1992; Kallio, Koskinen, Aavik, Buchdunger, & Lemstrom, 1999).

3.2 PARTICLE-INDUCED LUNG DISEASE

Epidemiological studies from around the world have shown a correlation between increased levels of airborne particulate matter (PM) of various sizes and compositions and mortality or hospitalization for respiratory disease of people of various ages (Andersen et al., 2008; Anderson, Bremner, Atkinson, Harrison, & Walters, 2001; Arena et al., 2006; Atkinson et al., 1999; Bell et al., 2008; Bennett et al., 2007; Braun-Fahrlander et al., 1997; L. Chen, Mengersen, & Tong, 2007; L. Chen, Yang, Jennison, & Omaye, 2000; Daniels, Dominici, Zeger, & Samet, 2004; Dominici, McDermott, Zeger, & Samet, 2003; Dominici et al., 2006; Eftim, Samet, Janes, McDermott, & Dominici, 2008; Fung, Luginaah, & Gorey, 2007; Fusco et al., 2001; Goldberg et al., 2001; Granados-Canal, Chardon, Lefranc, & Gremy, 2005; Gwynn, Burnett, & Thurston, 2000; Host et al., 2008; Hruba, Fabianova, Koppova, &
From these studies, it is estimated that for every 10 μg/m³ increase in the annual concentration of PM$_{2.5}$ particles (particles that are 2.5 μm in diameter and smaller), mortality increases by 1.4% (Borm, 2002). The same increase in PM$_{10}$ particles (particles that are 10 μm in diameter and smaller) results in a mortality increase of 0.5% to 1.5% (Valavanidis, Fiotakis, & Vlachogianni, 2008). In a recent article, Pope, et al. report that a 10 μg/m³ reduction in fine particulates is associated with an increase in life expectancy of 0.61±0.2 years (Pope, Ezzati, & Dockery, 2009). These estimates vary, of course, depending on the region examined, the age distribution of the population examined, and a host of other factors.
The composition of airborne PM is not uniform. There is a large size distribution and a high degree of variation in shape and chemical composition. Airborne PM has many constituents from a variety of sources. These include primary particles, such as those produced by combustion (e.g., smoke and automobile exhaust) or natural particles (e.g., dust, pollen, spores, sea salt), as well as secondary particles such as those forming from the condensation of vaporized materials (e.g., nitrates and sulfates) or oxidation of atmospheric gases (Dockery, 2009). Organic chemicals and various metals can also be found in airborne PM (U.S. Environmental Protection Agency, 2008a).

There is a wide range of particle sizes of atmospheric PM. The EPA divides airborne PM into 4 categories based on their aerodynamic diameter: supercoarse (particle sizes > 10 μm), coarse (particle sizes > 2.5 μm and ≤ 10 μm), fine (particle sizes > 0.1 μm and ≤ 2.5 μm), and ultrafine (particle sizes ≤ 0.1 μm) (U.S. Environmental Protection Agency, 2008b). Adverse health effects have been associated with particles with an aerodynamic diameter of less than 10 μm, the inhalable fraction. Particles larger than 10 μm are deposited in the nose and oral pharynx (Witschi, Pinkerton, Van Winkle, & Last, 2008). While coarse particles are largely deposited in the airways of the tracheobronchial tree, a significant portion of inhaled fine and ultrafine particles are deposited in the alveoli (Hoover, Stefaniak, Day, & Geraci, 2007; Oberdorster, 1996; Schmid et al., 2009; Witschi et al., 2008). Furthermore, ultrafine particles are more readily taken up by epithelial cells and translocate to the interstitial space where they remain for relatively long periods of time, and macrophages are less efficient at
phagocytizing ultrafine particles compared to larger particles (J. S. Brown, Zeman, & Bennett, 2002; Moller et al., 2008; Oberdorster, 1996; Schmid et al., 2009; Tabata & Ikada, 1988; Wiebert et al., 2006). Consequently, ultrafine particles (or nanoparticles) remain in the lungs longer than larger particles.

The lung response to inhaled particles depends on many factors: some are attributable to the host, others are inherent characteristics of the particles, and others are related to the exposure conditions. Particle characteristics that influence the lung’s response include shape, size, biopersistence, composition, surface area, and surface reactivity (Oberdorster, 1996; Oberdorster, Ferin, & Lehnert, 1994; Oberdorster, Oberdorster, & Oberdorster, 2005). For example, asbestos fibers, which are known to cause significant lung disease including fibrosis and cancer, have a high aspect ratio (i.e., they are long, thin particles) and persist in the lungs for relatively long periods of time (Heintz, Janssen-Heininger, & Mossman, 2010; Kane & Hurt, 2008; Poland et al., 2008; Shukla, Ramos-Nino, & Mossman, 2003). On the other hand, the toxicity of silica, another known cause of lung fibrosis and, possibly, cancer, is more dependent on the surface area and surface reactivity of the particles (Peretz, Checkoway, Kaufman, Trajber, & Lerman, 2006; Rimal, Greenberg, & Rom, 2005; Steenland et al., 2001). Thus, different particles may induce similar diseases, but through different mechanisms.

In the most general terms, particle inhalation induces lung injury. Initially, the lung epithelial
cells are injured, but if the injury is severe enough, other cells and tissues may also be
damaged. The mechanism by which particles cause damage may vary and has not been fully
elucidated, but common to almost all particle exposures is the generation of reactive oxygen
species (ROS), which plays a central role particle-induced toxicity (Donaldson & Tran, 2002;
Martin, Krunkosky et al., 1997). This lung injury induces an acute inflammatory response,
driven by the innate immune system, in which there is an influx of neutrophils and
macrophages and fewer numbers of eosinophils and mast cells that release a number of
cytotoxic compounds and induce further damage to the epithelium and other tissues (Husain
& Kumar, 2005). Alveolar macrophages and type II alveolar epithelial cells, the latter having
the capability to replace dead or damaged type I alveolar epithelial cells by proliferating and
differentiating into type I alveolar epithelial cells, produce chemokines that further drive the
inflammatory response. They are also known to produce a number of cytokines and growth
factors that promote lung fibrosis. With persistent injury, the inflammation becomes chronic
and mononuclear inflammatory cells (e.g., T- and B-lymphocytes) become more numerous.
In chronic inflammation, the adaptive immune system becomes more active and T-cells, B-
cells, and other cells, produce Th1 or Th2 cytokines, depending the circumstances
surrounding the particle exposure, which modulate the lung response to the particle exposure
(Bonner, 2007; Wynn, 2008). One of the consequences of the ongoing inflammation,
particularly when the lymphocyte response is shifted toward the release of Th2 cytokines, is
the release of mediators that promote myofibroblast accumulation and production of ECM
components by these cells (Bonner, 2007; Wynn, 2008). Continued deposition of collagen and other ECM components with a lack of compensatory ECM degradation by collagenases and other proteases, such as matrix metalloproteinases (MMPs), results in pulmonary fibrosis.

3.3 METAL-INDUCED LUNG DISEASE

3.3.1 Exposure, Associated Diseases, and General Mechanisms

Pulmonary exposure to metals is a long-standing occupational health issue and is known to cause COPD, interstitial lung diseases, and cancer (Fontenot & Amicosante, 2008; Kelleher, Pacheco, & Newman, 2000). More recently, however, pulmonary exposure to metals in airborne PM has also been implicated in respiratory diseases. While it is clear that metals have the capacity to cause significant pulmonary disease, the ability metals at exposure concentrations found in ambient air to induce lung disease is less clear due to a lack of studies that specifically examine the effects of metals at these concentrations (L. C. Chen & Lippmann, 2009; Schlesinger, Kunzli, Hidy, Gotschi, & Jerrett, 2006). Overall, however, the body of data supports a role for metals in the toxicity of ambient PM (Lippmann & Chen, 2009; Schwarze et al., 2006).

Many metals have the capacity to induce lung disease, including aluminum (Al), beryllium (Be), cadmium (Ca), cobalt (Co), copper (Cu), iron (Fe), mercury (Hg), nickel (Ni), and
vanadium (V) (Fontenot & Amicosante, 2008; Kelleher et al., 2000; Valko et al., 2005). The histopathological lesions caused by metals vary with the type of metal. For example, Be is a well-known cause of granulomatous inflammation and interstitial fibrosis, and also causes acute pneumonitis, tracheitis, and bronchitis (Fontenot & Amicosante, 2008; Kelleher et al., 2000). Al and Co also cause granulomatous inflammation, but far less commonly than Be (Fontenot & Amicosante, 2008; Kelleher et al., 2000). Al mainly causes interstitial fibrosis, while Co mainly causes giant cell interstitial pneumonia, desquamative interstitial pneumonitis, and interstitial fibrosis (Fontenot & Amicosante, 2008; Kelleher et al., 2000).

The mechanisms by which inhaled metals induce lung disease are not completely understood, but, as with particles, ROS are thought to play a central role (Lippmann & Chen, 2009; Schwarze et al., 2006; Valko et al., 2005). Some transition metals, such as V, Ni, Fe, and Cu, are able to participate in Fenton-like reactions, generating the hydroxyl radical (•OH), which subsequently produces the superoxide radical (O2•−) and hydrogen peroxide (H2O2) (Valko et al., 2005). In the pathogenesis of Be-induced lung disease (chronic beryllium disease, or CBD), exposed individual become sensitized to Be and macrophages present Be to CD4+ T-cells, which then secrete Th1 cytokines (e.g., IL-2, TNF-α, and IFN-γ), which leads to the formation of granulomas and eventual interstitial fibrosis (Fontenot & Amicosante, 2008; Kelleher et al., 2000). It has also been shown that certain polymorphisms in the gene encoding the major histocompatibility complex class II (MHCII) molecule are associated with CBD susceptibility, suggesting that these polymorphisms confer increased ability to
present Be to T-cells (Fontenot & Amicosante, 2008; Kelleher et al., 2000). A similar immune mechanism is thought to play a role in the pathogenesis of Co-induced lung disease (hard metal lung disease) (Fontenot & Amicosante, 2008; Kelleher et al., 2000). Ni also causes lung disease through a delayed-type hypersensitivity response, but the mechanism by which this occurs is not yet known (Fontenot & Amicosante, 2008; Kelleher et al., 2000).

3.3.2 Vanadium Pentoxide-Induced Lung Disease

Vanadium is rather abundant in the earth’s crust and is used extensively in the steel and chemical industries. Vanadium pentoxide \((V_2O_5)\) is one of the most commonly used forms of V and occupational exposure occurs during mining and processing of the ore, manufacturing of products that contain V, and in cleaning and repair of oil-fired boilers (Barceloux, 1999; Ehrlich et al., 2008; Pierce, Alessandrini, Godleski, & Paulauskis, 1996). Environmental exposure may also occur in areas where large amounts of fossil fuels are burned (i.e., in urban areas or near power plants that burn residual fuel oil) (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2006). In fact, V is considered a marker of air pollution from the burning of residual oil and coal (Dundar, 2006; C.-F. Wang, Chang, & Men, 1999).

\(V_2O_5\) is a known occupational health hazard and causes irritation of the upper respiratory tract, decreased lung function, chronic bronchitis, and occupational asthma (Irsigler, Visser, & Spangenberg, 1999; Kiviluoto, 1980; Woodin et al., 1998; Woodin, Liu, Hauser, Smith, &
Christiani, 1999; Woodin et al., 2000; Zenz & Berg, 1967). It is also found in high concentrations in residual oil fly ash (ROFA) and is largely responsible for the toxicity of ROFA in experimental animals (Dye et al., 1999; Woodin et al., 2000). Cynomolgous monkeys exposed to 5 mg/m$^3$ V$_2$O$_5$ by whole body inhalation for 1 week developed pulmonary function deficits that were accompanied by pulmonary inflammation characterized by an influx of PMNs (Knecht, Moorman, Clark, Lynch, & Lewis, 1985). In laboratory rats and mice, V$_2$O$_5$-exposure by intratracheal instillation or whole body inhalation causes pulmonary inflammation and fibrosis (Bonner et al., 2000; National Toxicology Program, 2002). In rats, V$_2$O$_5$ exposure also caused airway remodeling similar to that seen in human asthma patients, with peribronchiolar fibrosis, smooth muscle hypertrophy, and goblet cell hyperplasia (Bonner et al., 2000).

In general, the toxicity of vanadium increases as its oxidation state increases, thus, vanadium (V), the most oxidized form, is the most toxic form (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2006). The most common oxidation states of V are +2 (II), +3 (III), +4 (IV), and +5 (V). In V$_2$O$_5$, vanadium is in the +5 state (vanadate), the most oxidized form. In extracellular fluids, the predominant form of vanadium is vanadate, the +5 state, which is readily transported across cell membranes (Cantley & Aisen, 1979; Nechay, Nanninga, & Nechay, 1986; Rubinson, 1981). In the cytoplasm, it is reduced to the +4 state (vanadyl) by glutathione and other reducing agents and is stabilized in this form by binding to intracellular proteins (Nechay et al., 1986; Rubinson, 1981).
The mechanisms of V$_2$O$_5$ toxicity are incompletely understood, but current evidence suggests that the generation of ROS is a key event (Dye, Adler, Richards, & Dreher, 1997; Dye et al., 1999; Ghio, Silbajoris, Carson, & Samet, 2002; Stohs & Bagchi, 1995; Valko et al., 2005). *In vitro* exposure of rat tracheal epithelial cells to ROFA, V, or Ni plus V, but not Ni or Fe alone, caused cell damage, increased epithelial permeability, and decreased cellular GSH (Dye et al., 1999). The addition of dimethylthiourea, a free radical scavenger, inhibited these effects (Dye et al., 1999). In addition, V has been shown to activate or increase gene expression of a number of intracellular signaling molecules, including p53, activating protein (AP)-1, signal transducer and activator of transcription (STAT)-1, NF-κB, and the mitogen activated protein kinases (MAPK) extracellular signal regulated kinases (ERK), p38, and c-Jun N-terminal kinases (JNK) in a number of cell types (Ingram et al., 2003; Valko et al., 2005). The majority of these appear to be activated through V-induced generation of ROS (Ingram et al., 2003; Valko et al., 2005). ROS have been shown to induce phosphorylation of protein tyrosine residues and reversibly inhibit protein tyrosine phosphatases (Hecht & Zick, 1992; Samet et al., 1997; Zick & Sagi-Eisenberg, 1990; Zor et al., 1993). Through this mechanism, V$_2$O$_5$ is able to activate receptor tyrosine kinases, such as the epidermal growth factor receptor (EGFR) and PDGF-R$\alpha$, in a ligand-independent manner (Bonner, 2007).

In lung fibroblast cultures, Ingram et al. have shown that V$_2$O$_5$ exposure affects the expression of more than 1400 genes, increasing expression of 300 genes and decreasing expression of over 1100 genes (Ingram et al., 2007). Many of these genes are important
mediators of inflammation, fibrosis, oxidative stress, and the cell cycle (Ingram et al., 2007). Of these >1400 genes, the increased expression of the genes encoding IL-8, CXCL9, CXCL10, heparin-binding epidermal growth factor (HB-EGF), VEGF, HGF, CTGF, SOD2, pipecolic acid oxidase (PIPOX), oxidative resistance 1 (OXR1), STAT-1, and growth arrest specific (GAS)-1 were confirmed by RT-PCR (Ingram et al., 2007). In other studies, V₂O₅ has been shown to activate or increase expression of ERK 1/2, p38 MAPK, PDGF-A, PDGF-B, PDGF-Rα, and TGF-β, and to activate the epidermal growth factor receptor (EGFR), all of which are involved in cell proliferation or fibrosis (Bonner, Lindroos et al., 1998; Cesta et al., 2009; Ingram et al., 2003; Mangum et al., 2006; Y. Z. Wang et al., 2003). Vanadium has also been shown to induce secretion of IL-1β by alveolar macrophages in rats, which, in turn, induces PDGF-Rα expression in rat lung fibroblasts (Bonner, Rice et al., 1998). Other cytokines induced in the lung by V₂O₅ include macrophage inflammatory protein (MIP)-2, keratinocyte chemoattractant (KC, a functional murine homologue of human IL-8), prostaglandins (PG) E₂ and F₂α, IL-6, and iNOS (Chong, Lin et al., 2000; Chong, Shi, Love, Christiani, & Paulauskis, 2000; Dye et al., 1999; Ghio et al., 2002; Pierce et al., 1996).

Other potential mechanisms by which vanadium may induce toxicity have been investigated. In vitro, vanadate concentrations of 5 mM or less stimulates adenyl cyclase while higher vanadate concentrations inhibit adenyl cyclase (Nechay, 1984). Thus, V₂O₅ may affect intracellular levels of cyclic adenosine monophosphate (cAMP). Cyclic AMP is a mediator of PGE₂ inhibition of fibroblast proliferation and matrix protein production (S. K. Huang,
Wettlaufer, Chung et al., 2008). Cyclic AMP also plays a regulatory role in the activity of the cystic fibrosis transmembrane conductance regulator (CFTR), an ion channel in epithelial cell membranes (Bradbury, 2001). Mutations in the gene encoding CFTR have been implicated in cystic fibrosis. Mutations in the CFTR gene have been associated with abnormalities in glutathione transport and dysregulation of the inflammatory response of the lung, suggesting a secondary mechanism by which vanadium may induce oxidative stress in the lungs (Gao, Kim, Yankaskas, & Forman, 1999; Linsdell & Hanrahan, 1998; Pitt, 2001). Vanadate has also been shown to decrease rennin secretion in rat kidney slices and in volume-expanded dogs (Churchill & Churchill, 1980; Lopez Novoa, Garcia, Cruz-Soto, Benabe, & Martinez-Maldonado, 1982). However, this would result in a decrease in angiotensin II levels and would be expected to have antifibrotic effects, so the connection between the effects of vanadium on rennin secretion and pulmonary fibrosis is unclear.

Another possible mechanism of vanadate-induced toxicity is through the ability of vanadyl to bind to intracellular proteins, including amino acids, nucleic acids, phosphates, phospholipids, glutathione, citrate, oxalate, lactate, ascorbate and many others (Nechay et al., 1986). This may result in inactivation or alteration of these proteins. There is in vitro evidence that vanadate acts as an inhibitor of various ATPases (e.g., Na⁺/K⁺ ATPase) and it has been speculated that this may contribute to the toxicity induced by vanadium (Nechay, 1984; Nechay et al., 1986). When tested in cultures of whole cells, however, the Na⁺/K⁺ ATPase has been found to be less sensitive to vanadate inhibition, possibly due to the intracellular conversion of vanadate to vanadyl, which is a less potent inhibitor of the Na⁺/K⁺
ATPase (Cantley & Aisen, 1979; Cantley & Josephson, 1976). It remains unclear whether or not this mechanism plays a significant role \textit{in vivo}. There is evidence that vanadium is genotoxic. Studies have shown that vanadium induces DNA damage in cultured human fibroblasts and red blood cells, and adversely affects DNA repair in workers from a vanadium plant (Ehrlich et al., 2008; Ivancsits, Pilger, Diem, Schaffer, & Rudiger, 2002; Sakurai, 1994). As with most other effects of vanadium, this is thought to be due to oxidative damage. Lastly, there is evidence that vanadium has adverse effects on the immune system and may induce immunosuppression in exposed individuals (Cohen, McManus et al., 1996; Cohen, Parsons, Schlesinger, & Zelikoff, 1993; Cohen, Schlesinger, & Zelikoff, 1993; Cohen, Yang, Zelikoff, & Schlesinger, 1996; Pinon-Zarate et al., 2008). This may explain the increased susceptibility of workers chronically exposed to vanadium to pulmonary infections and could contribute to lung damage and fibrosis (Cohen, Yang et al., 1996).

3.4 NANOPARTICLES

Human exposure to nanoscale particles, or nanoparticles (NP), has occurred since the dawn of man. Sources of naturally produced NP include erosion and dust, volcanoes, the oceans, and even plants and animals (Buzea, Blandino, & Robbie, 2007; Fiorito, Serafino, Andreola, Togna, & Togna, 2006; Oberdorster et al., 2005). The onset of the industrial revolution saw an increase in anthropogenic sources of NP and a related increase in human exposure due to their production by combustion and other industrial processes. These are the so-called
incidental NP. More recently, rapid advances in nanotechnology have led to the production of engineered NP and the imminent mass production of these nanomaterials will provide an additional source of human exposure to NP. This manuscript will focus on the engineered NP and the term NP will refer only to engineered NP. In 2001, President Clinton established the National Nanotechnology Initiative (NNI), a federal program to coordinate nanotechnology research and development. Since its inception, the NNI budget has steadily increased from $464 million in 2001 to over $1.5 billion in 2009, reflecting the broad support of the U. S. government for nanotechnology research (National Nanotechnology Coordinating Office, 2008).

The NNI defines nanotechnology as “the understanding and control of matter at dimensions between approximately 1 and 100 nanometers” (National Nanotechnology Coordinating Office, 2008). Thus, any particle that is less than or equal to 100 nm in at least one dimension is generally considered a nanoparticle (Borm et al., 2006; Donaldson et al., 2006; National Nanotechnology Coordinating Office, 2008; Stone, Johnston, & Clift, 2007; Warheit, 2008). Nanoscale materials have unique physical and chemical properties that differ considerably from larger forms of the same material (Borm et al., 2006; Fiorito et al., 2006; National Nanotechnology Coordinating Office, 2008; Warheit, 2008). Some of these properties include increased strength, increased electrical conductance, increased chemical reactivity, and differing thermal and optical properties (Ding et al., 2005; Fiorito et al., 2006; Lam, James, McCluskey, Arepalli, & Hunter, 2006; Warheit, 2008). These characteristics are very
desirable for, among other things, manufacturing, electronics, and medical and scientific imaging. In fact, the Project on Emerging Nanotechnologies at the Woodrow Wilson International Center for Scholars lists on their website over 800 currently available consumer products that contain nanomaterials, including home appliances, electronics, cosmetics, clothing, sporting goods, food products, and children’s’ toys (Woodrow Wilson International Center for Scholars, 2008).

There are many types of engineered NP. They can be made of many different materials, such as metals, metal oxides, carbon, and various polymers. They can be made in many different shapes, including nanospheres, nanotubes, nanowires, nanoneedles, nanofibers, nanoshells, and nanorings. The shape and composition of an NP, as well as a number of other physicochemical characteristics such as their small size, high surface area to volume ratio, altered surface chemistry, and altered surface charge convey the unique properties of nanoparticles (Buzea et al., 2007; Fiorito et al., 2006; Hoet, Bruske-Hohlfeld, & Salata, 2004; Oberdorster et al., 2005).

3.4.1 Nanoparticle Toxicity

The physicochemical characteristics of NP that give them their unique properties also raise concerns over their safety. To date, there is a single report linking nanoparticle exposure to pulmonary disease in female workers in China, two of whom died, were occupationally exposed to polyacrylate spray that presumably contained NP (Song, Li, & Du, 2009).
However, the conclusion that nanoparticles were the causative agent was based on circumstantial evidence. The workplace air was not analyzed for NP, nor were the NP found in the lungs of the workers analyzed to confirm their workplace origin. Consequently, there are no confirmed cases of NP-induced lung diseases.

Because testing on NP is still in its infancy the body of literature is far smaller than for other types of particles, and the potential of NP to cause disease is still unclear. There are a number of factors that may affect the toxicity of NP. Among these are their size, shape, surface charge, solubility, surface chemistry (oxidation state and REDOX potential), aggregation state, and composition, including impurities such as residual catalysts from the manufacturing process (Donaldson et al., 2006; Oberdorster et al., 2005). Due to the increased surface area to volume ratio of NP, more of the particles’ molecules are exposed and can interact with their surroundings compared to larger particles.

Evidence suggests that NP may be more toxic to cells than larger particles composed of the same material. Cobalt-chromium alloy (CoCr) NP, when compared to micron-sized CoCr particles, induce more DNA damage and cytotoxicity in primary human dermal fibroblasts, and generate more free radicals in an acellular environment (Papageorgiou et al., 2007). Similarly, instillation of nanosized nickel oxide (NiO) particles into the lungs of Wistar rats resulted in greater inflammation than instillation of micron-sized NiO particles (Ogami et al.,
Several independent studies have shown that ultrafine (i.e., nanoscale) titanium dioxide (TiO$_2$) particles elicit greater inflammatory responses \textit{in vivo} and \textit{in vitro} than fine (i.e., $\leq$ 2.5 $\mu$m) TiO$_2$ particles (Hohr et al., 2002; Oberdorster et al., 1994; Sager, Kommineni, & Castranova, 2008; Singh et al., 2007). There is mounting evidence that ultrafine particulate matter (PM) also induces greater inflammatory responses than fine PM (D. M. Brown, Wilson, MacNee, Stone, & Donaldson, 2001; J. Y. Kim et al., 2006; Valavanidis et al., 2008). The increased toxicity of ultrafine particles relative to fine particles is likely a consequence of the greater surface area per mass of the smaller particles (D. M. Brown et al., 2001; Hohr et al., 2002; Oberdorster et al., 1994; Sager et al., 2008; Singh et al., 2007). Additionally, their smaller size allows for increased intracellular translocation and localization to mitochondria and mitochondrial damage, potentially leading to increased generation of ROS and greater oxidative stress (N. Li et al., 2003; Xia et al., 2004). \textit{In vivo}, additional reasons for the increased toxicity of ultrafine particles include decreased clearance of smaller particles, increased translocation to the interstitium (Oberdorster et al., 1994). However, chemicals adsorbed onto the particles, the composition of the particles, and the surface chemistry and reactivity of the particles are also likely to have an effect on particle toxicity (Oberdorster et al., 2005; Warheit, Webb, Colvin, Reed, & Sayes, 2007; Warheit, Webb, Reed, Frerichs, & Sayes, 2007; Xia et al., 2004).

The shape of NP may also impact their toxicity. In 1981, Stanton, et al. proposed that the fibrous shape of asbestos was largely responsible for their carcinogenic activity (Stanton et
al., 1981). Since then, it has become clear that the high aspect ratio (i.e., fibrous shape) of asbestos plays a role in the pathogenesis of asbestos-induced diseases—though it has also become clear that other factors, especially biopersistence and surface chemistry, also play a role (Robledo & Mossman, 1999; Shukla et al., 2003). Since their discovery, CNT, which have a shape similar to that of asbestos, have been analogized to asbestos (Kane & Hurt, 2008; Service, 1998). This comparison, and the impact of shape on CNT toxicity, is discussed further in the next section.

Another factor that may affect NP toxicity is their aggregation state. NP have a propensity to aggregate due to a number of factors, including high surface energy, low stability coefficient, and, for some types of NP, hydrophobicity (Hyung, Fortner, Hughes, & Kim, 2007; Ju-Nam & Lead, 2008; Kallay & Zalac, 2002). In fact, NP aggregate faster than larger particles at the same mass concentration (Kallay & Zalac, 2002). Aggregation states include aggregation, agglomeration, and coagulation (Sayes, 2008). All of these terms refer to the self-assembly of NP into larger particles. Aggregation is reversible and aggregated NP can disaggregate; agglomeration is an irreversible process in which the NP begin to fuse together; and coagulation refers to NP that have agglomerated to the point that they have formed a single, solid mass (Sayes, 2008). A host of factors can influence the aggregation state of NP, including the temperature, humidity, type and concentration of electrolytes, pH, presence and concentration of natural organic matter (e.g., in the environment), presence and concentration of serum proteins, presence and concentration of surfactants, and NP size, concentration, and
surface chemistry (Hyung et al., 2007; Ju-Nam & Lead, 2008; Kallay & Zalac, 2002; Saleh, Pfefferle, & Elimelech, 2008; Sayes, 2008).

3.4.2 Carbon Nanotubes

3.4.2.1 Carbon Nanotube Structure, Production, and Physical Characteristics

Carbon nanotubes (CNT) are one of the most important forms of NP. CNT are a crystalline form of carbon in which the individual carbon atoms are joined by sp² bonds (Kolosnjaj, Szwarc, & Moussa, 2007). Essentially, they are sheets of graphene rolled into tubes (Donaldson et al., 2006; Ju-Nam & Lead, 2008; Kolosnjaj et al., 2007). There are two types of CNT: single-walled carbon nanotubes (SWCNT) and multi-walled carbon nanotubes (MWCNT). SWCNT can be thought of as are single graphene sheets rolled into a tube, whereas MWCNT are multiple, layered graphene sheets rolled into a tube so that there are a series of concentrically arranged tubes in a single MWCNT. The diameter of SWCNT ranges from 0.7 nm to 20 nm (Kolosnjaj et al., 2007). MWCNT are, naturally, larger with a diameters ranging from 1.4 nm to 100 nm and the distance between layers is generally around 0.34 nm (Kolosnjaj et al., 2007). The lengths of CNT vary greatly but are typically on the order of tens of microns (Donaldson et al., 2006; Kolosnjaj et al., 2007). CNT are extremely strong and stiff. SWCNT, for example, can be up to 10 times stronger than steel and 1.2 times as stiff as diamond (Donaldson et al., 2006). Depending on their structure, they can have differing thermal qualities and can have either metallic or semi-conductive
characteristics (Kolosnjaj et al., 2007).

There are currently three methods that are commonly used for producing CNT: arc discharge, chemical vapor deposition, and laser ablation. Various metals are used as catalysts in the production of CNT, the most common being Fe, Co, Ni, and Mo (Donaldson et al., 2006). Additionally, various materials, commonly aluminates, silicates, or magnesium oxide, are used to support the catalyst and the growing nanotube during the production process (Donaldson et al., 2006). Raw CNT typically contain impurities such as amorphous carbon (‘soot’), residual metal catalysts (up to 30%), or residual support materials (Donaldson et al., 2006; Kolosnjaj et al., 2007). Several methods have been employed to remove these impurities such as treatment with strong acids, which tend to cut the CNT into shorter lengths and generates carboxylic acid and hydroxyl group residues attached to the CNT (Donaldson et al., 2006; Kolosnjaj et al., 2007).

3.4.2.2 Carbon Nanotube Toxicity

The potential toxicity of CNT has been a subject of much debate. MWCNT, and other forms of manufactured nanoparticles, are on the verge of revolutionizing the manufacturing industry. There are already numerous products containing nanomaterials available to the public, and more are designed and produced every year. The global market for carbon nanotubes was worth $50.9 million at the end of 2006, and is expected to reach $807.3 million by the end of 2011 (Oliver, 2007). With the increasing use and impending mass
production of nanomaterials, human exposure is almost a certainty. Human exposures are most likely to occur in occupational settings where nanomaterials are manufactured or are used in the manufacture of other products (Maynard et al., 2006; Wiesner, Lowry, Alvarez, Dionysiou, & Biswas, 2006). Due to difficulties in detecting airborne CNT, there are few reports on human occupational exposure or release of CNT during handling or manufacturing. The few reports that have been published confirm the potential for occupational exposure to CNT (J. H. Han et al., 2008; Maynard et al., 2004; Tsai et al., 2009). The potential for exposure of the general public from damage to or normal wear of consumer products containing nanomaterials also exists (Card, Zeldin, Bonner, & Nestmann, 2008; Donaldson et al., 2006). Though there is currently no published human toxicity data on CNT, but the potential for human exposure, results of toxicity testing in animal models, and extrapolation from the toxicity of other types of nanoparticles suggest that CNT have the potential to cause disease in exposed human beings.

The unique properties of CNT, as with other types of NP (discussed above), may endow them with the capacity to induce significant toxicity. CNT also have structural properties that may heighten their toxic potential. One such feature of CNT that is unique to CNT is their chirality. The chiral angle, or chirality, of a nanotube is the angle between the lengthwise dimension and the axis of rolling of the graphene sheet (Bachilo et al., 2003; S. N. Kim, Rusling, & Papadimitrakopoulos, 2007). The number of different chiral angles within a sample of SWCNTs increases as the diameter and distribution of diameters of the SWCNT
increases (S. N. Kim et al., 2007). Furthermore, the chirality of a nanotube determines whether it has metallic properties or semiconductor properties (S. N. Kim et al., 2007). The chirality of the nanotubes may affect their toxicity, though no such studies have been reported for CNT.

Other features of CNT that may enhance their toxicity include their high aspect ratio (length to diameter ratio) and biopersistence. These features have engendered comparisons to asbestos, a known cause of pulmonary fibrosis and cancer. Asbestos is known to be genotoxic, and, several studies have provided evidence that CNT may also be genotoxic, though negative genotoxicity studies have also been reported (Gonzalez, Lison, & Kirsch-Volders, 2008; Kisin et al., 2007; Muller et al., 2008; Sargent et al., 2009; Schins, 2002; Szendi & Varga, 2008; Yang, Liu, Yang, Zhang, & Xi, 2009). CNT have also been reported to activate the complement cascade in vitro (Salvador-Morales et al., 2006). CNT injected into the peritoneal cavity of mice has been shown to induce granulomatous inflammation of the peritoneal lining after 7 days, a reaction that is analogous to the initial response of the mesothelial lining of the lung to asbestos (Poland et al., 2008). Additionally, several studies have shown that CNT can reach the pleura and induce pleural changes (D. W. Porter et al., 2009; Ryman-Rasmussen, Cesta et al., 2009). However, there are differences between CNT and asbestos, such as surface chemistry and reactivity that leave the validity of such a comparison in question (Kane & Hurt, 2008).
In vitro studies of the effects of CNT have shown a range of adverse effects with implications regarding their potential to cause lung disease. SWCNT or MWCNT have been shown to be cytotoxic to A549 cells, FE1-Muta mouse lung epithelial cells, rat lung epithelial cells, RAW 264.7 macrophages, NR8383 cells, MSTO-211H mesothelioma cells, rat aortic smooth muscle cells, human dermal fibroblasts, BJ foreskin cells, HEK293 cells, and human keratinocytes (Cui, Tian, Ozkan, Wang, & Gao, 2005; Davoren et al., 2007; Jacobsen et al., 2008; Jia et al., 2005; Kagan et al., 2006; J. P. Kaiser, Wick, Manser, Spohn, & Bruinink, 2008; Karlsson, Cronholm, Gustafsson, & Moller, 2008; Manna et al., 2005; Raja et al., 2007; Sarkar et al., 2007; Sharma et al., 2007; Simon-Deckers et al., 2008; Tabet et al., 2009; Tian, Cui, Schwarz, Estrada, & Kobayashi, 2006; Wick et al., 2007). The mechanism for this cytotoxicity is not entirely clear, but SWCNT have also been shown to induce oxidative stress in A549 cells, FE1-Muta mouse lung epithelial cells, rat lung epithelial cells, RAW 264.7 macrophages, NR8383 cells, HEK293 cells, BJ foreskin cells, and human keratinocytes by production of reactive oxygen species (ROS) and depletion of glutathione (GSH) and superoxide dismutase (SOD) (Jacobsen et al., 2008; Kagan et al., 2006; Manna et al., 2005; Pulsamp, Diabate, & Krug, 2007; Sarkar et al., 2007; Sharma et al., 2007). It has been shown that CNT can pass through cell and nuclear membranes and are found free in the cytoplasm and in the nucleus, which has been correlated with cell death in a human monocyte-derived cell line (A. E. Porter et al., 2007; Simon-Deckers et al., 2008). The mechanism by which free cytoplasmic and nuclear CNT may cause cell death has not been elucidated, but they may be able to interact directly with nucleic acids, lipids, and proteins,
affecting the function of proteins or organelles (A. E. Porter et al., 2007). CNT have been identified within mitochondria and may affect mitochondrial function, which could lead to the generation of ROS (Jia et al., 2005; Simon-Deckers et al., 2008; Unfried et al., 2007). In one study, MWCNT and SWCNT were shown to disrupt lipid rafts in the cell membrane of RAW 264.7 macrophages, increasing their antigen presenting capabilities, increased their production of IFN-γ and IL-13, and increased their production of IL-1β and IL-12 and decreased their production of TNF-α in response to antigen stimulation (Hamilton et al., 2007). These effects on the plasma membrane of macrophages were largely confirmed in an independent study by Hirano, et al. (Hirano, Kanno, & Furuyama, 2008). In another study, SWCNT exposure of RAW 264.7 macrophages increased production of TGF-β and, to a lesser extent, TNF-α and IL-1β (Shvedova et al., 2005). Interestingly, CNT were shown to decrease the phagocytic ability of macrophages in vitro (Jia et al., 2005; X. Wang et al., 2009). Exposure of A549 cells to MWCNT or rat lung epithelial cells to SWCNT induces the production of ROS (Sharma et al., 2007; Ye, Wu, Hou, & Zhang, 2009). In the A549 cells, it was further shown that MWCNT exposure induced IL-8 production, in part through the ROS-mediated increase in NF-κB activation (Ye et al., 2009).

There are relatively few inhalation studies on the toxicity of carbon nanotubes. This is partly due to difficulties encountered in aerosolizing the particles for inhalation experiments because of their propensity to aggregate. Therefore, in the majority of in vivo studies on the toxicity of CNT in the lungs, the CNT have been administered via intratracheal instillation or
pharyngeal aspiration. The predominant effect of instilled CNT, regardless of their source, manufacturing method, physical characteristics, or metal content, in mice and rats is granuloma formation in the bronchioles and parenchyma of the lungs (Cesta et al., 2009; Lam, James, McCluskey, & Hunter, 2004; J.-G. Li et al., 2007; Mangum et al., 2006; Muller et al., 2005; D. W. Porter et al., 2009; Shvedova, Kisin, Murray, Johnson et al., 2008; Shvedova et al., 2005; Shvedova et al., 2007; Shvedova, Kisin, Murray, Kommineni et al., 2008; Warheit et al., 2004). These lesions are characterized by large, CNT-laden macrophages in the bronchiolar lumen or alveoli and bronchiolar or alveolar wall with deposition of collagen in the surrounding tissues and scattered lymphocytes (Figure 4-2E, F, G, H). Granuloma formation was also the principal finding in another study examining the intraperitoneal injection of CNT in mice (Poland et al., 2008). In several of the pulmonary studies, a concurrent interstitial fibrosis was also reported (Mangum et al., 2006; Muller et al., 2005; D. W. Porter et al., 2009; Shvedova, Kisin, Murray, Johnson et al., 2008; Shvedova et al., 2005; Shvedova et al., 2007; Shvedova, Kisin, Murray, Kommineni et al., 2008). In a subset of these, supportive data showed that the CNT also caused increased levels of proinflammatory or profibrotic mediators either in vitro or in vivo, including IL-1β, TNF-α, TGF-β, PDGF-A, and PDGF-C (Mangum et al., 2006; Muller et al., 2005; Shvedova, Kisin, Murray, Johnson et al., 2008; Shvedova et al., 2005; Shvedova et al., 2007; Shvedova, Kisin, Murray, Kommineni et al., 2008). In a single study by Elgrabli, et al., no granulomas or evidence of other inflammation, fibrosis, or functional respiratory deficits were found after IT instillation of MWCNT in rats (Elgrabli et al., 2008). The authors suggest that the
granulomas found in previous studies were due to the fact that the MWCNT aggregates were not of respirable size (Elgrabli et al., 2008). However, in several of the previous studies, histopathology confirmed the presence of CNT in the alveoli (Cesta et al., 2009; Mangum et al., 2006; Muller et al., 2005; Shvedova et al., 2005; Shvedova et al., 2007; Shvedova, Kisin, Murray, Kommineni et al., 2008). Furthermore, one of these studies was a comparison of instillation and inhalation of SWCNT, and inhalation resulted in the same types of lesions (Shvedova, Kisin, Murray, Johnson et al., 2008). In the study by Elgrabli, et al., apoptosis of the alveolar macrophages was the only finding (Elgrabli et al., 2008).

Two of these studies compared the effects of unpurified and purified CNT (Kagan et al., 2006; Lam et al., 2004). Kagan, et al. investigated the role of residual Fe (26% in unpurified SWCNT and 0.23% in purified SWCNT) on SWCNT-induced oxidative stress in RAW 264.7 macrophages in vitro (Kagan et al., 2006). In this study, the unpurified SWCNT had a greater effect on the conversion of superoxide radicals to hydroxyl radicals, loss of intracellular antioxidants (GSH), and accumulation of lipid hydroperoxides in zymosan-stimulated RAW 264.7 macrophages (Kagan et al., 2006). In the other study, Lam, et al. examined the in vivo effects of three different SWCNT samples in the lungs of mice after IT instillation (Lam et al., 2004). They tested an unpurified sample containing 26.9% Fe and 0.78% Ni, a purified sample containing 2.14% Fe and no Ni, and an unpurified sample from a different source containing 0.53% Fe, 25.99% Ni, and 5.01% Y (Lam et al., 2004). All three samples induced similar lesions, mainly interstitial granulomas, with no difference in
severity, suggesting that the adverse effects are due to the SWCNT and the residual metals are of little consequence (Lam et al., 2004).

Several studies have examined the effects of CNT inhalation in rodents. Six of these are short term studies (1 to 15 days of exposure) utilizing C57BL/6 mice, Kunming mice, or Wistar rats; one study used SWCNT and four used MWCNT (Ellinger-Ziegelbauer & Pauluhn, 2009; J.-G. Li et al., 2007; Mitchell et al., 2007; Ryman-Rasmussen, Cesta et al., 2009; Ryman-Rasmussen, Tewksbury et al., 2009; Shvedova, Kisin, Murray, Johnson et al., 2008). Two are subchronic studies (90 days of exposure) utilizing Wistar rats and MWCNT (Ma-Hock et al., 2009; Pauluhn, 2010). In five of these studies, including the two subchronic studies, fibrosis was reported (Ma-Hock et al., 2009; Pauluhn, 2010; Ryman-Rasmussen, Cesta et al., 2009; Ryman-Rasmussen, Tewksbury et al., 2009; Shvedova, Kisin, Murray, Johnson et al., 2008). Ryman-Rasmussen, et al. reported airway fibrosis in OVA-challenged mice exposed to MWCNT for 6 hrs in one study, and subpleural fibrosis in another study in mice exposed to MWCNT for 6 hrs (Ryman-Rasmussen, Cesta et al., 2009; Ryman-Rasmussen, Tewksbury et al., 2009). The latter study also reported pleural mononuclear aggregates with MWCNT in exposed mice, indicating that MWCNT have the potential to affect the pleura (Ryman-Rasmussen, Cesta et al., 2009). Pauluhn reported neutrophilic inflammation, increased interstitial collagen, bronchoalveolar epithelial hyperplasia, and granulomatous changes in Wistar rats, and Ma-Hock, et al. reported inflammation consisting of alveolar macrophages with fewer neutrophils and granulomas with collagen deposition,
but no interstitial fibrosis, both after 90 day exposure to MWCNT (Ma-Hock et al., 2009; Pauluhn, 2010). Ellinger-Ziegelbauer, et al. reported acute inflammation and interstitial fibrosis 3 months after a single 6 hr inhalation exposure to MWCNT in Wistar rats (Ellinger-Ziegelbauer & Pauluhn, 2009). They also reported that the MWCNT-exposed rats had a gene expression profile similar that of quartz-exposed rats (Ellinger-Ziegelbauer & Pauluhn, 2009). In one of these studies, Mitchell, et al. reported a lack of lung lesions in mice, including changes in BAL fluid parameters and gene expression changes, after 7 or 14 days of inhalation exposure to MWCNT (Mitchell et al., 2007). They did, however, report systemic immunosuppressive effects in these mice, manifested as a decreased splenic T-cell antigen response to sheep erythrocytes, decreased T-cell proliferative ability, and decreased natural killer cell function (Mitchell et al., 2007).

Two studies have compared the effects inhalation and instillation or aspiration of CNT on the lung, both in mice. Shvedova, et al. compared the effects of inhaled versus aspirated SWCNT in C57BL/6 mice (Shvedova, Kisin, Murray, Johnson et al., 2008). Inhalation exposure resulted in an increased numbers of PMNs and TNF-α and IL-6 one day post-exposure, and increased TGF-β levels 1 day and 7 days post-exposure in the BAL fluid (Shvedova, Kisin, Murray, Johnson et al., 2008). The mice exposed via inhalation also had increased lung collagen levels at 7 and 28 days post-exposure compared to aspiration (Shvedova, Kisin, Murray, Johnson et al., 2008). They also reported granulomas in exposed mice (Shvedova, Kisin, Murray, Johnson et al., 2008). In another study, Li, et al. compared
the histopathological lesions in the lungs of Kunming mice after IT instillation or inhalation of MWCNT (J.-G. Li et al., 2007). In this study, compared to inhalation exposure, IT instillation resulted in larger aggregates of MWCNT that were not as widely distributed into the alveoli (J.-G. Li et al., 2007). The granulomatous lesions appeared to be similar in both groups, but were smaller in the inhalation group (J.-G. Li et al., 2007).

Recently, CNT have been implicated as modulators of the immune system, but the results of relevant studies are mixed. On the one hand, CNT have been reported to induce or enhance allergic reactions (Dobrovolskaia & McNeil, 2007). In mice, in the presence of allergens, CNT increase lung expression of IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IFN-γ, eotaxin, regulated on activation, normal T expressed and secreted (RANTES), and MCP-1, and thymus- and activation-regulated chemokine (TARC), GM-CSF, MIP-1α in the presence or absence of antigen (Dwivedi, Misra, Shanker, & Das, 2009; K. Inoue et al., 2009; K. Inoue, Takano, Yanagisawa, Ichinose et al., 2006; K. Inoue et al., 2005; K. I. Inoue, Yanagisawa, Koike, Nishikawa, & Takano, 2010; Nygaard et al., 2009; Ryman-Rasmussen, Tewksbury et al., 2009). Additionally, antigen plus CNT induced greater IgE and antigen-specific IgG1 levels, and greater oxidative stress compared to antigen or CNT alone (K. Inoue et al., 2005; Nygaard et al., 2009). Lastly, CNT have been shown to activate dendritic cells, increasing their expression of MHC class II and CD86 and their ability to induce proliferation of T-cells (K. Inoue et al., 2009; K. I. Inoue et al., 2010). On the other hand, CNT have been reported to induce immunosuppression. MWCNT induce apoptosis in T-cells in vitro (Bottini et al.,
More importantly, MWCNT, after inhalation exposure, cause systemic immune suppression manifested as deficits in splenic T-cell function and proliferative ability (Mitchell et al., 2007; Mitchell, Lauer, Burchiel, & McDonald, 2009). This was proposed to occur through a TGF-β mediated mechanism whereby TGF-β secreted by alveolar macrophages in the lung in response to MWCNT, entered the bloodstream and stimulated COX-2 production of PGE₂ and IL-10 in the spleen, which have immunosuppressive effects on T-cells (Mitchell et al., 2009). Clearly, the effects of CNT on the immune system require further study, but CNT induction of proinflammatory and profibrotic cytokines in the lung, such as IL-4, IL-5, IL-6, IL-13, and MCP-1 are likely to affect the fibrotic response to CNT.

Unfortunately, the data regarding the capacity of CNT to cause lung fibrosis are inconclusive. The majority of animal studies, however, have found some degree of pulmonary fibrosis in response to CNT. Therefore, the concern over the potential of CNT to cause pulmonary fibrosis in humans is warranted and justify continued, more intensive research in this area.

### 3.5 LIPOPOLYSACCHARIDE

Endotoxin is an important component of the wall of Gram-negative bacteria and is ubiquitous in the environment. It is a known cause of pulmonary inflammation and has been implicated as the causative agent in occupational several lung diseases associated with organic dust
exposure, including grain dust-induced lung disease and byssinosisis (cotton dust-induced lung
disease), among others (Lane, Nicholls, & Sewell, 2004; D. A. Schwartz et al., 1995).
Exposure to LPS in house dust is also a risk and enhancing factor for asthma (Michel, 2003;
Thorne et al., 2005). It is commonly used in experimental animals as a model of acute lung
injury and chronic bronchitis (Nikula & Green, 2000). It has also been used to study
subepithelial airway fibrosis and airway remodeling and interstitial fibrosis (Brass, Savov,
Gavett, Haykal-Coates, & Schwartz, 2003; Brass et al., 2008; Corbel et al., 2001;
Garantziotis et al., 2006; H. Li et al., 2009).

Sensitivity to endotoxin varies greatly among animal species and strains, and the sensitivity
can vary by route of administration. For example, sheep are more sensitive to pulmonary
endotoxin exposure, developing lung injury and respiratory failure at relatively low doses,
but are less sensitive to intravenous (IV) exposure with higher doses required to induce shock
(Brigham & Meyrick, 1986). In contrast, dogs develop shock at much lower IV doses than
that required to induce lung injury via pulmonary exposure (Brigham & Meyrick, 1986).
Recent studies have shown variation in the response to pulmonary LPS exposure in eight
strains of mice based on measurements of acute lung injury and cytokine production (Alm,
Li, Chen et al., 2010; Alm, Li, Yang et al., 2010). Additionally, several mouse strains,
including C3H/HeJ, C57BL/10ScCr, and BXD29, have mutations in the toll-like receptor 4
(TLR4) gene—TLR4 is the principal receptor to which LPS binds and exerts it effects—and
are resistant to the effects of LPS (Cook et al., 2006; Poltorak et al., 1998). In humans,
polymorphisms in the TLR4 gene modulate the response to infectious agents and the outcome of infectious diseases (Balistreri et al., 2007; Bhide et al., 2009; Faber et al., 2009; Hishida et al., 2009; Hodgkinson, Patel, & Ye, 2008; LeVan et al., 2005; Michel et al., 2003; Shalhub et al., 2009).

Lipopolysaccharide (LPS) is the active component of endotoxin. It is composed of a polysaccharide moiety and a lipid component, lipid A (Heine, Rietschel, & Ulmer, 2001). The polysaccharide moiety may be further subdivided into the O-chain and the core region (Heine et al., 2001). The O-chain is composed of identical, repeating polysaccharide units composed of two to eight sugar monomers (Heine et al., 2001). The O-chain is unique to the bacterial serotype in which it is found (Heine et al., 2001). The lipid A component is highly conserved and its general structure is a biphosphorylated β-(1→6)-linked glucosamine disaccharide with ester-linked fatty acids at the 3 and 3’ positions and amide-linked fatty acids at the 2 and 2’ positions (Caroff & Karibian, 2003; Heine et al., 2001). The lipid A portion is responsible for the majority of the biologic effects of LPS, but the polysaccharide portion enhances the effects of the lipid A moiety and is required for mitogenicity of B-lymphocytes and activation of human macrophage cell lines (Caroff & Karibian, 2003).

LPS is known to modulate the immune system. LPS is an example of a pathogen-associated molecular pattern (PAMP), which are conserved microbial structures essential for microbial survival that are recognized by pattern recognition receptors (PRRs) (Martinon, Mayor, &
Tschopp, 2009). Toll-like receptors (TLRs), one of which, TLR4, is responsible for the majority of the effects of LPS, are perhaps the most well-studied of the PRRs (Martinon et al., 2009). Other examples of PRRs in the lung include the collectins surfactant protein-A (SP-A) and SP-D and nod-like receptors, which are cytosolic receptors involved in activation of the inflammasome (Martinon et al., 2009; Petrilli, Dostert, Muruve, & Tschopp, 2007; Sano & Kuroki, 2005). LPS activation of TLR4 and other receptors stimulates the innate immune system characterized by production of IFN-γ, TNF-α, IL-1β, and other mediators, the influx of neutrophils, activation of resident macrophages, and antimicrobial activity (e.g., production of ROS, proteases, etc.). LPS contributes to the activation of the Nacht domain-, LRR-, and PYD-containing protein 3 (NALP3) inflammasome, which induces expression and activation of IL-1β and IL-18, further stimulating the innate immune system (Palm & Medzhitov, 2009; Petrilli et al., 2007). LPS also has effects on the adaptive immune response. These effects are primarily mediated by effects on dendritic cells, but LPS binding to TLR4 also enhances B-cell migration to lymph nodes, B-cell proliferation, and the generation of memory B-cells and plasma cells (Hwang, Park, Harrison, & Kehrl, 2009; Pasare & Medzhitov, 2004). In dendritic cells, LPS induces the maturation and the upregulation of MHC and production of IL-12 (Iwasaki & Medzhitov, 2004; Pasare & Medzhitov, 2004). IL-12 promotes a Th1-type immune response, however, humans exposed to endotoxin exhibit a response consistent with the Th2 pattern (Lauw et al., 2000; Zimmer et al., 1996).
3.5.1 Pulmonary Effects of Lipopolysaccharide

In the lung, LPS has a number of effects that result in decreased lung function, acute lung injury and inflammation, and, with longer exposures, fibrosis (Thorn, 2001). The initial response to pulmonary LPS exposure (IV infusion also causes acute lung injury, but the pathogenesis differs in that endothelial cells are the initial site of injury) is dependent on the dose and may include a fibrinuous alveolar exudate, and alveolar septal edema, and numerous intra-alveolar neutrophils and large, foamy (activated) macrophages (alveolitis) (Harkema & Hotchkiss, 1992). The lesions are typically characterized by thickened alveolar septa due to alveolar type II cell hyperplasia; mild fibrosis and an influx of mononuclear leukocytes and neutrophils may be present in the interstitium (interstitial pneumonia) (Harkema & Hotchkiss, 1992; Harkema et al., 2004). Perivenular cuffing by mononuclear leukocytes and neutrophils may also be present (Harkema & Hotchkiss, 1992; Nikula & Green, 2000; H. M. Wang, Bodenstein, & Markstaller, 2008). The terminal and preterminal bronchioles may have a hyperplastic epithelium (Harkema & Hotchkiss, 1992). These bronchioles and the associated pulmonary arteries may have interstitial edema with mononuclear leukocyte and neutrophil accumulation (Harkema & Hotchkiss, 1992). In the larger airways, there typically is an increase in acid mucosubstances on the epithelial surface, an increase in mucous cell numbers with a corresponding decrease in serous cells, and peribronchial/peribronchiolar edema and inflammation (bronchiolitis) (Harkema & Hotchkiss, 1993). In airways at all levels, there may be multifocal clumps of catarrhal exudate and hyperplasia of the airway epithelium (Harkema & Hotchkiss, 1992; Harkema et al., 2004). In guinea pigs exposed to
LPS by inhalation, there is evidence of alveolar type I epithelial cell damage (Johnson, Reynolds, & Toward, 2005). With IV administration of LPS, there is injury and apoptosis of endothelial cells but there are fewer neutrophils in the lungs than is seen after pulmonary exposure (Matute-Bello, Frevert, & Martin, 2008). After a single intratracheal exposure to LPS, these effects may resolve completely within 48 hours (Janardhan et al., 2006; Wohlford-Lenane, Deetz, & Schwartz, 1999).

Pulmonary TLR4 is expressed in numerous cell types, including alveolar macrophages, airway epithelial cells, alveolar type II epithelial cells, fibroblasts, endothelial cells, vascular smooth muscle cells, neutrophils, eosinophils, T-cells, mast cells, and some dendritic cell subtypes (Chaudhuri, Dower, Whyte, & Sabroe, 2005; Chaudhuri & Sabroe, 2008; de la Barrera, Aleman, & Sasiain Mdel, 2006; Iwamura & Nakayama, 2008; Janardhan et al., 2006). Macrophages and epithelial cell are the main targets of LPS, but macrophages are much more sensitive to LPS than epithelial cells (Gon, 2008; Thorn, 2001). LPS induces alveolar macrophages to produce a number of cytokines, including IL-1α, IL-1β, IL-6, IL-8, IFN-γ, TNF-α, eotaxin, MIP-1α, MIP-1β, MIP-2, metallothionein, and IP-10 (Dentener, Bazil, Von Asmuth, Ceska, & Buurman, 1993; Johnston, Finkelstein, Gelein, & Oberdorster, 1998; Thorn, 2001). LPS also induces macrophage production of ROS and MMPs and promotes macrophage migration (S. Y. Kim et al., 2009). Another LPS-mediated effect is the induction of macrophage apoptosis, which is augmented by IFN-γ and diminished by IL-4, IL-10, and TGF-β, and LPS may also enhance macrophage phagocytosis (Bingisser et al.,
The battery of mediators produced by alveolar type II epithelial cells in response to LPS is similar to that produced by alveolar macrophages, but is lower in quantity (Kanj, Kang, & Castranova, 2005). Additionally, ROS production by LPS-exposed alveolar type II epithelial cells was not detected in vitro, whereas alveolar macrophages produced ROS in a dose-dependent manner (Kanj et al., 2005). In bronchial or bronchiolar epithelial cells, LPS promotes the production of a number of cytokines, including TNF-α, IL-6, IL-8, MIP-1β, MIP-2, MCP-1, and IP-10, and the innate immune mediator β defensin, and increases expression of ICAM-1 (Basu & Fenton, 2004; Guillot et al., 2004; Khair, Davies, & Devalia, 1996; Neff et al., 2006; Pace et al., 2008). In endothelial cells, LPS upregulates the expression of adhesion molecules, such as ICAM-1, VCAM-1 and E- and P-selectin, and induces the production of cytokines, including IL-1, IL-6, IL-8, MCP-1, GM-CSF, CXCL1 (also known as Gro-α), and RANTES (regulated on activation, normal T expressed and secreted), and ET-1 (Grandel & Grimminger, 2003; Khair et al., 1996). LPS also causes endothelial gap formation and disruption of the endothelial barrier (Bannerman & Goldblum, 1997; Goldblum, Ding, Brann, & Campbell-Washington, 1993).

### 3.5.2 Signaling by Lipopolysaccharide

The effects of LPS are mediated primarily by TLR4, but the binding of LPS to TLR4 is not a simple affair. Prior to TLR4 recognition of LPS, LPS binds to LPS-binding protein (LBP),
which is produced in the liver and circulates in the bloodstream (Lu, Yeh, & Ohashi, 2008; Palsson-McDermott & O'Neill, 2004). LBP aids in the binding of LPS to CD14, which, in myeloid cells, including macrophages, is bound to the cell membrane via a glycosylphosphatidylinositol tail (Dauphinee & Karsan, 2006; Lu et al., 2008; Palsson-McDermott & O'Neill, 2004; Triantafilou & Triantafilou, 2005). There is also a soluble form of CD14 that is utilized by cells lacking the membrane bound form of CD14, such as endothelial and epithelial cells (Dauphinee & Karsan, 2006; Lu et al., 2008; Palsson-McDermott & O'Neill, 2004). LPS is then transferred to the MD-2/TLR4 complex (Lu et al., 2008; Palsson-McDermott & O'Neill, 2004). MD-2 is required for LPS-induced TLR4 signaling and binds to the lipid A portion of LPS (Palsson-McDermott & O'Neill, 2004). Once LPS is transferred to the MD-2/TLR4 complex, TLR4 aggregates to form an oligomer, which initiates the signaling cascade (Lu et al., 2008; Palsson-McDermott & O'Neill, 2004).

TLR4 signaling pathways have been divided into myeloid differentiating primary response gene 88 (MyD88)-dependent and –independent pathways (Dauphinee & Karsan, 2006; Lu et al., 2008; O'Neill & Bowie, 2007). Oligomerization of TLR4 brings the Toll/interleukin-1 receptor (TIR) domains on the cytosolic side of the TLR4 molecules into closer proximity (O'Neill & Bowie, 2007). Signaling by TLR4 involves recruitment of the TIR domain-containing adapter proteins, which bind to the TIR domains (Dauphinee & Karsan, 2006; Lu et al., 2008; O'Neill & Bowie, 2007). There are five TIR domain-containing adapter proteins in humans: MyD88, MyD88-adapt...
domain-containing adapter protein inducing IFN-β (TRIF, also known as TICAM1), TRIF-related adapter molecule (TRAM, also known as TICAM2), and sterile α- and armadillo-motif-containing protein (SRAM).

In the MyD88-dependent pathway, MAL binds to the TLR4 TIR domain, which then recruits MyD88 (Dauphinee & Karsan, 2006; Lu et al., 2008; O'Neill & Bowie, 2007). IL-1R-associated kinase 4 (IRAK4) then binds to MyD88 and recruits IRAK1 (Dauphinee & Karsan, 2006; Lu et al., 2008; O'Neill & Bowie, 2007). IRAK-2 may also be involved in TLR4 signaling (Lu et al., 2008; O'Neill & Bowie, 2007). The downstream target of IRAK1 is thought to be tumor necrosis factor-receptor-associated factor 6 (TRAF6), which binds with ubiquitin-conjugating enzyme E2 variant 1 isoform A (UEV1A) and ubiquitin-conjugating enzyme 13 (UBC13), transforming growth factor-β-activated kinase 1 (TAK1), and TAK1 binding protein 2 (TAB2) (Dauphinee & Karsan, 2006; Lu et al., 2008; O'Neill & Bowie, 2007). Ultimately, this activates the inhibitor of NF-κB kinase (IKK), which activates NF-κB, and the MAPKs p38 and JUN N-terminal kinase (JNK) (Lu et al., 2008; O'Neill & Bowie, 2007). Activation of the MAPKs leads to activation of the transcription factor AP-1 (Lu et al., 2008). Activation of AP-1 and NF-κB leads to rapid induction of proinflammatory cytokines. Through the MyD88-dependent pathway, the inhibitor of NF-κB ζ (IκBζ) and interferon regulatory factor 5 (IRF5) are also activated and may be involved in LPS-induced IL-6, IL-12, and TNF-α expression (Lu et al., 2008). TNF-α expression occurring through
this mechanism is delayed relative to its expression induced via NF-κB (O'Neill & Bowie, 2007).

The MyD88-independent signaling pathway involves the binding of TRAM to the TLR4 TIR domain, which then recruits TRIF (Lu et al., 2008; O'Neill & Bowie, 2007; Watters, Kenny, & O'Neill, 2007). TRIF then activates TRAF3 and receptor interacting protein 1 (RIP1) (O'Neill & Bowie, 2007; Watters et al., 2007). RIP1 then activates the MAPKs and AP-1, as well as IKK and NF-κB (Lu et al., 2008; O'Neill & Bowie, 2007; Watters et al., 2007). TRAF3 complexes with TRAF family member-associated NF-κB activator (TANK), TANK binding kinase 1 (TBK1), and IKK inhibitor (IKKi) to activate IRF3, which induces the expression of type 1 interferons and interferon-inducible genes (Lu et al., 2008; O'Neill & Bowie, 2007; Watters et al., 2007).

Inhibition of the MyD88-dependent pathway occurs at multiple levels. A splice variant of MyD88 that lacks the region that binds to IRAK4 inhibits IRAK4 activation (Lu et al., 2008; O'Neill & Bowie, 2007). The suppressor of cytokine signaling 1 (SOCS1) and triad domain-containing protein 3 variant A (TRIAD3A) are E3 ubiquitin ligases that inhibit TLR4 signaling (Lu et al., 2008; O'Neill & Bowie, 2007). SOCS1 induces the ubiquitination and degradation of MAL, and TRIAD3A induces the degradation of MAL, TRIF, RIP1, and TLR4 itself (Lu et al., 2008; O'Neill & Bowie, 2007). IRAK-M is an inhibitory IRAK family member that lacks kinase activity and IRAK-2c is an IRAK-2 splice variant and both have
been implicated in inhibition of IRAK (Lu et al., 2008; O'Neill & Bowie, 2007). TGF-β also inhibits the MyD88-dependent pathway by inducing the ubiquitination of MyD88, which is one mechanism by which TGF-β exerts its anti-inflammatory effects (Watters et al., 2007). Inhibition of the MyD88-independent pathway also occurs, naturally. TRAF1 and TRAF4 inhibit TRIF (TRAF4 also inhibits TRAF6 in the MyD88-dependent pathway) (Lu et al., 2008; O'Neill & Bowie, 2007). SARM also inhibits TRIF, likely through competitive binding for TRIF effector proteins (O'Neill & Bowie, 2007).

3.5.3 Effects of Lipopolysaccharide and Particle Exposure

Numerous studies have examined the impact of exposure to airborne particulates—most studies using diesel exhaust particles (DEP)—on the pulmonary effects of LPS. The results of the majority of these studies suggest that LPS can augment the effects of inhaled particles, and vice versa. In most of these studies, the particles and the LPS were administered concurrently, but in two, the LPS was administered prior to the particles. In these two studies, one using human alveolar macrophages and the other using Sprague-Dawley rats (effects were examined in vivo and in alveolar macrophages from the BAL fluid ex vivo), DEP were found to inhibit DEP-stimulated alveolar macrophage cytokine release (K. Inoue, Takano, Yanagisawa, Sakurai et al., 2006; Mundandhara, Becker, & Madden, 2006).

In most of the studies in which the particles and LPS were administered concurrently, the combination of particles and LPS resulted in exacerbation of the particle effects. In four
studies in which mice or rats were exposed to DEP and LPS by intratracheal (IT) instillation, the combined exposure resulted in exacerbation of acute lung injury (assessed by lung water content [pulmonary edema], hemorrhage [determined by histopathologic examination], increased expression of IL-1β, MIP-1α, MCP-1, KC, ICAM-1, or COX-2, and increased production of free radicals (Arimoto, Kadiiska, Sato, Corbett, & Mason, 2005; K. Inoue et al., 2004; Takano et al., 2002; Yanagisawa et al., 2003). Two studies examined the effects of concurrent LPS and NP (nano-sized CB or CNT) intratracheally instilled in mice. In these studies, concurrent LPS exposure was found to augment pulmonary inflammation assessed by lung water content (pulmonary edema), histopathologic examination, BAL fluid cell counts and differentials, and expression of IL-1β, MIP-1α, MCP-1, MIP-2, and KC (K. Inoue, Takano, Yanagisawa, Hirano, Sakurai et al., 2006; K. I. Inoue et al., 2008). In a third study in which mice were exposed to LPS by IT instillation followed immediately by inhalation exposure to DEP, lung inflammation was found to be decreased using the same parameters listed above (K. Inoue, Takano, Yanagisawa, Sakurai et al., 2006).

Four studies examined the effects of pretreatment with LPS on particle-induced lung changes, two in vivo and two in vitro. Both in vitro studies utilized alveolar macrophages collected from CD rats that were pretreated with LPS for 3 hours and one utilized primary human alveolar macrophages as well, but these were pretreated with LPS for 20 hours (Imrich, Ning, Koziel, Coull, & Kobzik, 1999; Long et al., 2001). In both studies, LPS pretreatment increased TNF-α production in response to particle exposure (Imrich et al.,
1999; Long et al., 2001). Additionally, one study found that the LPS-retreated rat macrophages were more sensitive to the toxic effects of ROFA (Imrich et al., 1999). In the *in vivo* studies, F344 rats were pre-exposed to LPS. In one study, LPS exposure by IT instillation was followed 48 hours later by inhalation exposure to concentrated air particles (CAPs). In this study, LPS pre-exposure had no effect on the lungs, including BAL fluid parameters (total cells, differential cell counts, measurements of secreted mucins, total protein, elastase, β-glucuronidase, and alkaline and acid phosphatases), morphometric and light microscopic airway epithelial changes, and *Muc5ac* gene expression by airway epithelial cells. In the other study, LPS exposure by IT instillation was followed 30 minutes later by inhalation exposure to ozone and/or ultrafine CB particles. In this study, LPS pre-exposure resulted in increased neutrophils in the BAL fluid and increased ROS generation by BAL macrophages in response to phorbol myristate acetate (PMA) stimulation (Elder, Gelein, Finkelstein, Cox, & Oberdorster, 2000).
CHAPTER FOUR
MANUSCRIPT ONE

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BACTERIAL LIPOPOLYSACCHARIDE ENHANCES PDGF SIGNALING AND PULMONARY FIBROSIS IN RAT EXPOSED TO CARBON NANOTUBES

4.1 ABSTRACT

Engineered multi-walled carbon nanotubes (MWCNT) represent a possible health risk for pulmonary fibrosis due to their fiber-like shape and potential for persistence in the lung. We postulated that bacterial lipopolysaccharide (LPS), a ubiquitous agent in the environment that causes lung inflammation, would enhance fibrosis caused by MWCNT. Rats were exposed to LPS and then intratracheally instilled with MWCNT or carbon black (CB) nanoparticles 24 hrs later. Pulmonary fibrosis was observed 21 days post-MWCNT exposure, but not with CB. LPS alone caused no fibrosis but enhanced MWCNT-induced fibrosis. LPS plus CB did not significantly increase fibrosis. MWCNT increased platelet-derived growth factor-AA (PDGF-AA), a major mediator of fibrosis. PDGF-AA production in response to MWCNT, but not CB, was synergistically enhanced by LPS. Immunostaining showed PDGF-AA in bronchiolar epithelial cells and macrophages. Since macrophages engulfed MWCNT, were positive for PDGF-AA, and mediate fibroblast responses, experiments were performed with
rat lung macrophages (NR8383 cells) and rat lung fibroblasts in vitro. LPS exposure increased PDGF-A mRNA levels in NR8383 cells and enhanced MWCNT-induced PDGF-A mRNA levels. Moreover, LPS increased MWCNT- or CB-induced PDGF receptor-α (PDGF-Rα) mRNA in fibroblasts. Our data suggest that LPS exacerbates MWCNT-induced lung fibrosis by amplifying production of PDGF-AA in macrophages and epithelial cells, and by increasing PDGF-Rα on pulmonary fibroblasts. Our findings also suggest that individuals with pre-existing pulmonary inflammation are at greater risk for the potential adverse effects of MWCNT.

4.2 INTRODUCTION

Carbon nanotubes (CNT) are engineered graphene tubes with unique properties that are anticipated to revolutionize the fields of electronics, structural engineering, and medicine (Avouris, Chen, & Perebeinos, 2007; Baughman, Zakhidov, & de Heer, 2002; Bianco, Kostarelos, & Prato, 2005). The increasing production of single-walled carbon nanotubes (SWCNT) and multi-walled carbon nanotubes (MWCNT) is also accompanied by an increase in the potential for human exposure, mainly in occupational settings, but conceivably in the general population as well (Card et al., 2008; Donaldson et al., 2006). Unfortunately, some of the physical properties of MWCNT that make them desirable for electronics, engineering, and medicine may augment their toxic potential relative to larger particles or even other types of nanoparticles. Some of these properties include altered
surface chemistry compared to larger particles of the same material, a high surface area to
volume ratio, a high length to width aspect ratio, and a high degree of biopersistence
(Donaldson et al., 2006).

A number of studies have addressed the potential of CNTs to cause pulmonary fibrosis, the
scarring of lung tissue caused by an increase in fibroblasts and their collagen deposits
(Wynn, 2008). For example, SWCNT or MWCNT administered by intratracheal instillation
or pharyngeal aspiration in mice or rats induce pulmonary granulomas and interstitial lung
fibrosis (Lam et al., 2004; Muller et al., 2005; Shvedova et al., 2005; Warheit et al., 2004).
Fibrotic reactions in the lungs of mice exposed to SWCNT were associated with increased
levels of TGF-β1, a primary mediator of collagen deposition during fibrogenesis (Shvedova
et al., 2005). We previously reported that SWCNT administered into the lungs of rats by
pharyngeal aspiration caused interstitial lung fibrosis that was associated with increased
levels of platelet-derived growth factor-AA (PDGF-AA) (Mangum et al., 2006). Most
recently, we reported increased airway fibrosis and elevated PDGF-AA in the lungs of mice
that inhaled MWCNT with ovalbumin allergen challenge (Ryman-Rasmussen, Tewksbury et
al., 2009).

PDGF is a potent mitogen and chemoattractant for fibroblasts and is thought to play a pivotal
role in the progression of fibrotic diseases (Bonner, 2004). There are four PDGF genes,
designated A–D, that encode four homodimeric protein isoforms (PDGF-AA, -BB, -CC, and
and one heterodimeric isoform (PDGF-AB). There are also two PDGF receptors, PDGF-R\(\alpha\) and PDGF-R\(\beta\), which dimerize upon ligand binding, forming three isoforms (PDGF-R\(\alpha\alpha\), -R\(\alpha\beta\), and -R\(\beta\beta\)). PDGF-AA binds exclusively to PDGF-R\(\alpha\). The cell-surface PDGF-R\(\alpha\) is an inducible receptor that regulates fibroblast mitogenic and chemotactic responses to secreted PDGF following particle or fiber exposure (Lindroos, Coin, Badgett, Morgan, & Bonner, 1997). During lung fibrogenesis in rats, PDGF-R\(\alpha\) levels become markedly upregulated during the fibroblast proliferative stage of the disease (Bonner, Lindroos et al., 1998).

Bacterial lipopolysaccharide (LPS), a component of gram-negative bacterial cell walls, is a potent exogenous inducer of increased fibroblast levels of PDGF-R\(\alpha\) in the lungs of rats (Coin et al., 1996). LPS is ubiquitous in the environment and has been implicated in a number of occupational lung diseases in humans, most of which are associated with organic dust exposure (Thorn, 2001). In experimental animal models, LPS causes airway inflammation with acute exposures and airway remodeling and hyperreactivity with subchronic exposures. The effects of subchronic pulmonary exposure to LPS are similar to chronic bronchitis, and so subchronic treatment of rodents with LPS is used to model this disease (Brass et al., 2003; Nikula & Green, 2000). The lung levels of a variety of secreted proinflammatory mediators or their cognate receptors are increased by LPS through activation of toll-like receptor-4 (TLR-4) (Schroder, Sweet, & Hume, 2006). For example, LPS stimulates the release of a number of inflammatory mediators from alveolar
macrophages, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-8 (Becker, Mundandhara, Devlin, & Madden, 2005; Nagaoka, Trapnell, & Crystal, 1990). LPS also upregulates the expression of both PDGFA and PDGF-B genes in alveolar macrophages (Nagaoka et al., 1990). These studies suggest that LPS could exacerbate pulmonary fibrosis by “priming” pulmonary macrophages and fibroblasts for enhanced production of cytokines, growth factors, and their receptors.

Little is known regarding the pulmonary fibrotic response to CNTs in the context of pre-existing inflammation. This is an important issue since individuals with bronchitis, chronic obstructive pulmonary disease, asthma, or exposure to high levels of environmental LPS could be particularly sensitive to the adverse effects of CNTs. The purpose of this study was to determine whether pre-exposure to LPS would exacerbate the fibrotic response to MWCNT by amplifying expression of the PDGF-AA and PDGF-Rα genes in the lungs of rats.

### 4.3 MATERIALS AND METHODS

#### 4.3.1 Animals

Male, pathogen-free Sprague-Dawley rats, 6 to 8 weeks old, were purchased from Charles River Breeding Laboratories (Kingston, NY) and housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility that was
humidity and temperature controlled. Rats were housed in microisolator cages on Alphadri cellulose bedding and supplied water and cereal-based diet NIH07 (Zeigler Brothers, Gardners, PA) ad libitum. The animal studies were approved by The Hamner Institutes for Health Research Institutional Animal Care and Use Committee.

4.3.2 General Experimental Design

Animals were randomly assigned to six treatment groups of five rats each and acclimated for 1 week prior to exposure. Rats from three of the treatment groups were exposed to 2.5 mg/kg LPS from *E. coli* (Serotype 026:B6, Sigma, St. Louis, MO) by nasal aspiration and the rats from the other three treatment groups were exposed to Ca\(^{2+}\) and Mg\(^{2+}\)-free phosphate-buffered saline (PBS). Twenty-four hrs later, rats were exposed to 4 mg/kg of CB or MWCNT by intratracheal instillation. Negative control animals were treated with PBS. Animals were killed at 1 and 20 days following particle exposure. The lungs were lavaged with PBS and the right lung lobes were minced and placed in RNALater solution (Qiagen, Valencia, CA). Lungs were stored at –20°C and used for RNA isolation. The left lung lobe was collected for histopathology.

4.3.3 Characterization of Bulk Carbon Nanotubes

MWCNT were purchased from Helix Material Solutions, Inc., Richardson, TX. These MWCNT were synthesized by carbon vapor deposition (CVD) with nickel and lanthanum
catalysts. Characterization of the size, purity, surface area and elemental composition of the MWCNT provided by the manufacturer and verified by independent analysis (Millennium Research Laboratories Inc., Woburn, MA) have been previously reported by our laboratory (Ryman-Rasmussen, Tewksbury et al., 2009). Size was characterized by transmission electron microscopy (TEM), purity was determined by thermogravimetric analysis (TGA), elemental analysis was performed by energy dispersive x-ray analysis (EDX) and inductively coupled plasma auger electron spectroscopy (ICP-AES), and the specific surface area was determined by BET analysis.

4.3.4 Assay for Lipopolysaccharide Contamination

Lipopolysaccharide (LPS) was measured by a Limulus amebocyte lysate (LAL) assay kit according to manufacturer's specifications (Associates of Cape Cod, East Falmouth, MA). MWCNT were sonicated in vehicle (0.1% Pluronic surfactant in PBS) for 60 minutes at RT prior to performing LAL. The maximum sensitivity of this assay is 0.005 EU/ml. MWCNT suspensions were negative for endotoxin within this detection limit.

4.3.5 Preparation and Instillation of LPS and Nanoparticles

Rats were administered E. Coli LPS by intranasal aspiration and particles by intratracheal instillation. In brief, animals were anesthetized with isoflurane and ~50 μl volume LPS (dose concentration equivalent of 2.5 mg of LPS per kg of bodyweight) or PBS (for control
animals) was pipetted into the antrum of the nasal cavity. Twenty-four hrs later, the animals were again anesthetized with isoflurane and ~100 μl volume of either CB or MWCNT (dose concentration equivalent of 4 mg of particles per kg of bodyweight) was instilled into the trachea using an 18 gauge catheter attached to a 1.0 ml syringe. The MWCNT suspensions were prepared by first milling the dry particles in a Retsch Mixer Mill (Retsch Inc., Newtown, PA) for 5 minutes at 30 cycles per second. Just prior to instillation, the milled nanoparticles were suspended in 1% Pluronic F68 (BASF Corp., Florham Park, NJ), a biocompatible, nonionic surfactant, in PBS and wet milled for an additional 5 minutes, then further diluted with PBS to achieve the desired final dosing concentration suspended in 0.1% Pluronic F68. Carbon black (CB) nanoparticles (Raven 5000 Ultra II) were obtained from Columbian Chemicals Company (Marietta, GA) and treated in an identical manner to MWCNT for all experiments. All particles were sterilized prior to instillation. Control rats were administered PBS with 0.1% Pluronic F68 surfactant (vehicle).

4.3.6 Bronchoalveolar Lavage

Rats were euthanized by pentobarbital overdose and exsanguination via the abdominal aorta and lungs were lavaged 3 times with 5-ml volumes of PBS. Bronchoalveolar lavage fluid (BALF) collected from the second and third lavages was pooled (the fluid from the first lavage was kept separate) and all BALF was placed on ice. BAL cells collected by centrifugation were resuspended in culture medium and enumerated using an automated cell counter (Model ZM, Coulter, Marietta, GA). Cytospins were prepared with 25,000 cells per
Differential cell counts were performed on HEMA-3 (Fisher Scientific, Pittsburgh, PA) stained cytocentrifuge slide preparations. Cell numbers were quantified by light microscopy using the 40X objective and 500 cells per animal were counted. Total protein and LDH in cell-free BALF from the first two pooled lavages were analyzed spectrophotometrically using a COBAS FARA II (Roche Diagnostic Systems Inc., Montclair, NJ) and assay kits for LDH (LD Liquid Reagent, Pointe Scientific, Canton, MI) and total protein (Coomassie Plus Protein Assay Reagent, Pierce/ThermoFisher).

### 4.3.7 Lung Fixation and Histopathology

One hr prior to euthanasia, rats received a single intraperitoneal injection of 50 mg/kg body weight of bromodeoxyuridine (BrdU; Sigma-Aldrich). At necropsy, the left lungs were pressure-infused intratracheally at 30 cm H\(_2\)O with 10% neutral buffered formalin. Lungs were fixed for approximately 48 hrs and then transferred to 70% ethanol. Three cross-sectional portions of the left lung were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E), Masson’s trichrome, or immunostained for BrdU by established methods.

### 4.3.8 Pathology Scoring of Lungs

Three sections of lung, one each from the cranial, middle, and caudal portions of the left lung lobe, from each rat were evaluated in a blinded fashion. Those from 24-hr time point were
scored for inflammation and those from the 21-day time point for inflammation and fibrosis. The inflammation scores reflect the averages of the scores for the number of polymorphonuclear cells, the number and size of intra-alveolar macrophage aggregates (these macrophages were typically laden with nanoparticles), and alveolar wall thickening. This average score was then adjusted for the number of nanoparticles present by dividing the average score by the relative score of the number of nanoparticles present to give an adjusted average score. Only the lungs from the 21-day time point were scored for fibrosis. The lungs were scored for the amount of collagen present (based on Masson’s trichrome-stained sections), the thickness of the alveolar walls, and the number of fibroblast-like cells associated with the particle-associated lesions. These scores were averaged and the average scores were then divided by the relative scores for the amount of nanoparticles present in the lungs to give an adjusted average score for each animal. All scores were relative scores on a scale from 0-4, with zero representing the levels of these parameters in the PBS control group, 1 representing minimal change, 2 representing mild change, 3 representing moderate change, and 4 representing marked change.

4.3.9 Immunohistochemistry for PDGF-A

Formalin fixed, paraffin embedded rat tissue sections were deparaffinized in xylene and rehydrated through graded ethanols. Endogenous peroxidase was blocked with 3% hydrogen peroxide. Antigen retrieval was performed with the Decloaker® pressure cooker and 6.0M citrate buffer (Biocare Medical, CA). Protein blocking was performed with 10% normal
donkey serum (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) for 20 minutes, followed by the Avidin-Biotin Blocking Kit (Vector Laboratories, Burlingame, CA). Primary antibody, PDGF-A (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), was applied at a 1:25 dilution for 60 minutes at room temperature. For negative controls, the primary antibody was replaced with rabbit non-immune IgG serum diluted to match the antibody protein concentration and was also applied at 1:25. The slides were incubated in donkey anti-rabbit secondary antibody (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) for 30 minutes at a 1:500 dilution. Label incubation was performed for 30 minutes using the Vectastain Standard Elite Kit (Vector Laboratories, Burlingame, CA). Slides were developed with DAB, counterstained with hematoxylin, dehydrated, cleared and cover-slipped.

4.3.10 In Vitro Experiments with Cultured Lung Macrophages and Fibroblasts

Primary rat lung fibroblasts (RLFs) were isolated from rats by established methods and cryopreserved (Mangum et al., 2006). Thawed cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B in a humidified atmosphere with 5% CO₂ at 37°C. NR8383 cells (ATCC, Manassas, VA, Cat. No. CRL-2192) were grown in F-12K nutrient mixture supplemented with 15% FBS, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B in a humidified atmosphere with 5% CO₂ at 37°C. Upon reaching confluence, 1 μg/ml LPS was added to the
culture medium. Twenty-four hrs later, the LPS/serum-containing medium was replaced with serum-free medium (F-12 nutrient mixture supplemented with 0.25% bovine serum albumin [BSA], 1 ml Insulin/Transferrin/Selenium [Bio-Whittaker, Walkersville, MD], 100 units/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B) containing 1 μg/ml LPS. Twenty-four hrs later, the medium was removed and the cells were rinsed with warm PBS and treated with 10 μg/cm² MWCNT or CB in serum-free medium for 1 hr. The medium was then removed, the cells rinsed with warm PBS, and the medium was replaced with fresh serum-free medium. After 4 or 24 hrs, the cells were collected and stored at -80°C for later RNA isolation using the Qiagen RNeasy Mini kit (Qiagen, Valencia, CA).

4.3.11 Taqman Real Time Quantitative RT-PCR

The lungs were removed from the RNALater solution, blotted dry, and homogenized in buffer RLT (from Qiagen RNeasy Mini kit) with 10 μl/ml β-mercaptoethanol added. Total RNA from right anterior lungs or from cultured cell pellets was isolated using the Qiagen RNeasy Mini kit and converted to cDNA with the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Thirty nanograms of cDNA was amplified using Taqman Gene Expression Assays specific for β-actin (Rn_00667869), PDGF-A (Rn_00709363), PDGF-C (Rn_00579958), PDGF-Rα (Rn_01399472), TGF-β1 (Rn_00572010), and Type I procollagen (COL1A2) (Rn_00584426) on the Applied Biosystems 7900 Prism® Sequence Detection System (Applied Biosystems, Foster City,
CA). The PCR conditions and data analysis were performed according to the manufacturer’s protocol described in User Bulletin No. 2, Applied Biosystems Prism 7900 Sequence Detection System. Gene expression was measured by the quantitation of cDNA converted from mRNA corresponding to the target genes relative to the vehicle-treated control groups and normalized to β-actin reference endogenous control. Relative quantitation values ($2^{-\Delta\Delta CT}$) were expressed as fold-change over controls.

4.3.12 ELISAs

Quantikine ELISA kits (R&D Systems, Minneapolis, MN) were used to assay BALF for total TGF-β1 (active and inactive forms) 24 hrs after particle exposure and PDGF-AA 24 hrs and 21 days after particle exposure. Duplicate samples using 50 μl BALF were used for each animal. Assays were performed according to kit instructions and absorbances were read on a Multiskan EX microplate spectrophotometer (ThermoFisher Scientific) microplate reader. Values were normalized relative to total protein in BALF and expressed as pg/ml.

4.3.13 Data and Statistical Analysis

All graphs were constructed and statistical analyses performed using GraphPad Prism® software v. 5.00 (GraphPad Software, Inc., San Diego, CA). A one-way ANOVA with a post-hoc Tukey test, Bonferroni test, or Dunnett’s test was used to identify significant differences among treatment groups. Significance was set at p<0.05 unless otherwise stated.
4.4 RESULTS

4.4.1 Characterization of MWCNT

We have previously reported the physical and chemical properties of the MWCNT used in this study (Ryman-Rasmussen, Tewksbury et al., 2009). Data from the manufacturer was verified and compared to data from a contracted independent analysis. Residual nickel and lanthanum, catalysts used in the MWCNT manufacturing process, were detected by independent analysis using ICP-AES at 0.34% and 0.03%, respectively. EDX was also used to measure these metals and yielded value of 0.12% for nickel but not detectable levels of lanthanum. MWCNT purity measured by thermogravimetric analysis was >94%, the specific surface area measured by BET analysis was 109 m²/g, and size ranges of the MWCNT measured by TEM were 10-30 nm width and 0.3-50 μm length. Representative TEM images of the MWCNT along with a typical EDX elemental analysis are shown in Figure 4-1.

4.4.2 LPS Enhances MWCNT-Induced Pulmonary Interstitial Fibrosis

In this study, LPS was delivered 24 hrs prior to the intratracheal instillation of MWCNT, CB nanoparticle or vehicle (saline/0.1% Pluronic F68). The lungs of rats receiving intranasal saline then IT saline vehicle or LPS intranasal then vehicle had no significant lesions at 21 days post-exposure (Figure 4-2A, -2B). MWCNT caused significant fibrosis in the interstitium associated with nanoparticle-induced fibroproliferative lesions in the lungs of rats at 21 days, whereas CB did not (Figure 4-2C, -2D). These lesions were present in all rats.
exposed to nanoparticles (CB or MWCNT) with or without LPS and were often present near bronchiolar/alveolar duct junctions, though some appeared to be more distally located. They were more numerous in MWCNT-exposed rats than CB-exposed rats. In rats pre-exposed to LPS, the lesions were larger and more fibrotic. The lesions were characterized by thickening of the alveolar and alveolar duct walls by spindloid cells, macrophages laden with nanoparticles, and extracellular matrix, some of which was identified as collagen upon examination of Mason’s trichrome-stained sections. Some of these lesions, particularly in the MWCNT- and MWCNT/LPS-exposed groups, though to a lesser extent in the LPS/CB-exposed rats as well, also exhibited minimal hyperplasia of the epithelium of the terminal bronchioles. Both MWCNT and CB had similar spatial deposition patterns and were localized to the centroacinar region of the lung. Nanoparticles were found both within the cytoplasm of alveolar macrophages and free in the proximal alveolar ducts and adjacent alveoli at 24 hrs, and nearly all the nanoparticles were found within alveolar macrophages at 21 days. MWCNT or CB exposure, with or without LPS pre-exposure, caused a significant increase in neutrophils retrieved by bronchoalveolar lavage (Table 4-1). LPS pre-exposure significantly enhanced interstitial fibrosis associated with MWCNT-induced lesions (Figure 4-2F, -2H). The combination of LPS and CB caused a mild but not significant fibroproliferative response (Figure 4-2E, -2G), and CB alone or LPS alone caused no fibrosis. Quantitative pathology scoring showed that only the combination of LPS and MWCNT caused a significant increase in the mean fibrosis score at 21 days post-exposure (Figure 4-3).
4.4.3 LPS Enhances MWCNT-Induced LDH and Total Lung Protein Levels

The level of lactate dehydrogenase (LDH), a marker of cellular injury, in the BAL fluid from the lungs of rats was increased by MWCNT or CB approximately 2-fold compared to vehicle at 24 hrs post-exposure (Figure 4-4A). LPS exposure alone did not increase LDH levels compared to vehicle control. However, LPS pre-exposure enhanced MWCNT-induced LDH levels in BAL fluid to a level that was statistically different from vehicle controls. The LDH levels for CB-exposed, CB-exposed/LPS pre-exposed, and MWCNT-exposed rats were elevated compared to controls, but were not statistically different from vehicle controls. Similar to LDH levels, the total protein level in BAL fluid, a marker of lung injury and inflammation, was increased by MWCNT or CB exposure (Figure 4-4B). LPS did not increase total protein above vehicle control. MWCNT or CB elevated total protein levels nearly 2-fold above vehicle. LPS pre-exposure enhanced MWCNT-induced total protein to a level that was statistically different from vehicle, but did not enhance CB-induced total protein levels.

4.4.4 Particles Induce Acute Lung Inflammation Which Is Enhanced by LPS Pre-Exposure in Carbon Black-Exposed but Not MWCNT-Exposed Rats

In all particle-exposed groups (with and without LPS pre-exposure) at 24 hrs post-particle instillation, the percentage of polymorphonuclear leukocytes (PMNs) in the BALF was significantly increased compared to PBS controls and the LPS only group (which was similar
to PBS controls) (Table 4-1). There was a corresponding decrease in the percentage of macrophages and no change in the percentage of lymphocytes.

4.4.5 LPS Enhances MWCNT-Induced PDGF-AA Secretion in Rat Lungs

We next evaluated whether PDGF-AA, a potent chemoattractant and stimulator of fibroblast replication that plays a major role in pulmonary fibrosis, would be elevated in the lungs of rats exposed to MWCNT with or without LPS. PDGF-AA protein levels were measured in BAL fluid samples by ELISA. MWCNT or LPS increased PDGF-AA levels 3- to 4-fold at 24 hrs, but levels returned to baseline by 21 days post-exposure (Figure 4-5). LPS pre-exposure synergistically enhanced MWCNT-induced PDGF-AA levels in BAL fluid at 24 hrs, but this effect was not observed at 21 days post-exposure. In contrast, CB exposure did not increase PDGF-AA levels above control, nor did the combination of LPS and CB increase PDGF-AA. In addition to PDGF-AA, we also measured BAL fluid levels of TGF-β1, which is the key regulator of collagen production during fibrogenesis. TGF-β1 was detectable only in rats pre-exposed to LPS and then exposed to MWCNT, and relatively low levels (5 to 10 pg/ml TGF-β1) were measured (data not shown).

4.4.6 PDGF Immunostaining in the Lungs of Rats Exposed to LPS and MWCNT

Immunohistochemistry was performed using an antibody selective for PDGF-AA. In control animals, PDGF-AA immunoreactivity was most prominent in the airway epithelial cells and
alveolar macrophages (Figures 4-6 and 4-7). There was less prominent immunostaining in endothelial cells, perivascular cardiac muscle cells, mast cell granules, and some peribronchial and perivascular smooth muscle cells (particularly around larger vessels). In the airways, PDGF-AA immunoreactivity was specific to ciliated epithelial cells; Clara cells, goblet cells, and other non-ciliated epithelial cell types were negative. At 1 day post-exposure, additional PDGF-AA immunostaining was observed in the lungs of rats exposed to the combination of LPS and MWCNT in occasional fibroblast-like cells (elongated, spindle-shaped cells) in thickened alveolar septa and occasional epithelial cells at alveolar duct bifurcations in areas associated with MWCNT-induced inflammation (Figure 4-6). Similarly, at 21 days post-exposure, additional PDGF-AA immunostaining was noted in the hyperplastic epithelium of the terminal bronchioles in animals exposed to MWCNT or to the combination of CB and MWCNT and LPS (Figure 4-7). Though nanoparticles obscured PDGF-AA immunostaining in many alveolar and interstitial macrophages, positive immunoreactivity could be detected in macrophages that contained fewer nanoparticles. The increased numbers of macrophages in treated groups are the most likely source of increased PDGF-AA, but fibroblasts and airway epithelium, including hyperplastic terminal bronchiolar epithelium, may have contributed to PDGF-AA levels in the lungs of these animals.
4.4.7 LPS Enhances MWCNT-Induced PDGF-A Chain and PDGF-Rα mRNA Expression in Cultured Lung Macrophages and Fibroblasts In Vitro

Since macrophages engulfed the majority of MWCNT in the lung and because they are a major source of PDGF, we investigated the effect of LPS and MWCNT on PDGF and PDGF receptor (PDGF-Rα) mRNA expression levels in a rat lung macrophage cell line (NR8383). Taqman quantitative real-time RT-PCR was used to evaluate mRNA levels for PDGF and PDGF-Rα. MWCNT and CB treatment (10 μg/cm²) stimulated a marginal, albeit not statistically significant, approximately 2-fold increase in PDGF-A and PDGF-Rα mRNA levels in NR8383 macrophages at 4 hrs that returned to vehicle control levels by 24 hrs (Figure 4-8A, -8B). LPS alone stimulated 7- to 10-fold increase in PDGF-A mRNA at 4 and 24 hrs, and increased PDGF-Rα mRNA approximately 5-fold at 4 hrs and returned to control levels by 24 hrs. LPS pre-exposure synergistically increased MWCNT or CB-induced PDGF-A and PDGF-Rα mRNA levels (Figure 4-8A, -8B). We also evaluated the effect of LPS and MWCNT on PDGF and PDGF-Rα mRNA levels in primary passage rat lung fibroblasts (RLF), since these cells are the central target cell type that proliferate to PDGF signals and produce collagen during fibrogenesis. MWCNT or CB, with or without LPS, did not affect PDGF-A mRNA levels in RLF (Figure 4-9A). MWCNT or CB also did not increase PDGF-Rα mRNA levels in RLF, but LPS caused a statistically significant increase in PDGF-Rα mRNA, yet this increase was not further increased by MWCNT or CB (Figure 4-9B).
MWCNT have been reported to cause lung fibrosis in mice or rats, yet little is known about the effect of pre-existing inflammation on the fibrogenic activity of MWCNT (J.-G. Li et al., 2007; Muller et al., 2005). This is an important issue, since the most susceptible individuals at greatest risk for environmental or occupational exposure to carbon nanotubes would likely be those with pre-existing respiratory disease. In this study we investigated whether pre-existing lung inflammation caused by bacterial LPS (i.e., endotoxin), a ubiquitous environmental contaminant, would enhance interstitial lung fibrosis caused by MWCNT exposure. LPS pre-exposure enhanced MWCNT-induced lung fibrosis, increased MWCNT-induced lung injury as measured by LDH release and total protein levels, and synergistically elevated MWCNT-induced production of PDGF-AA, a central mediator of fibrosis (Bonner, 2004). Therefore, the data presented here support our hypothesis that pre-existing respiratory inflammation resulting from LPS pre-exposure exacerbates the lung fibrotic response to carbon nanotubes.

LPS is a well-established stimulus of the lung inflammatory response and promotes acute inflammation characterized by the infiltration of circulating neutrophils into the lung, which play a critical role the development of LPS-induced airway disease in chronic exposures (Brass et al., 2003; Savov, Gavett, Brass, Costa, & Schwartz, 2002). In the current study, we did not see an increase in PMNs in the BAL fluid of rats 48 hrs after exposure to LPS alone,
though there was a slight increase in the total cell numbers. This can be attributed to the short duration of the effects of a single exposure to LPS, which have been shown to resolve completely within 48 hrs of exposure (Wohlford-Lenane et al., 1999). Many environmental and occupational lung diseases are caused by LPS that adheres to inhaled particles or fibers. For example, grain dust and cotton worker’s lung disease are largely attributed to LPS (D. A. Schwartz, 2001). Also, many ambient air pollution particles cause lung inflammation due to the presence of LPS, which contributes to a complex mixture of organic and inorganic components that comprise these particles (Becker, Fenton, & Soukup, 2002; Bonner, Lindroos et al., 1998). Pristine carbon nanotubes contain little to no LPS since they are synthesized at very high temperatures. We were unable to detect LPS in our nanotube stock by a commercially available endotoxin assay, but the possibility exists that MWCNT could become contaminated with LPS after the manufacturing process depending upon storage and use conditions. This is because LPS is nearly ubiquitous in the environment and therefore is a common factor that could exacerbate respiratory challenges from other inhaled substances. The mechanism whereby LPS exacerbates the lung fibrotic response to carbon nanotubes is not yet known, but our findings suggest that amplification of PDGF-AA and its receptor could play a role in this interactive process. It is well-established that LPS mediates pro-inflammatory effects by binding and activating the transmembrane toll-like receptor 4 (TLR4), which then signals intracellular signaling pathways that culminate in the production of cytokines such as tumor necrosis factor-α (TNF-α) and interleukins (IL)-1, -6, and -8 (Lu et al., 2008). In addition, LPS docking to the cell surface and coordinated binding to TLR4 is
facilitated by the membrane CD14 receptor. We previously reported that LPS, acting through a CD14-dependent mechanism, increased the numbers of cell-surface PDGF receptors on rat lung fibroblasts (Coin et al., 1996). Upregulation of PDGF receptor levels increases the growth and chemotactic responses of fibroblasts to PDGF-AA secreted by macrophages and other lung cell types (Bonner, 2004). In the present study, we found that LPS exposure to the lungs of rats increased PDGF-AA protein levels in BAL fluid at 1 day post-exposure but not at 21 days post-exposure. However, we detected PDGF-AA protein by immunohistochemistry at both 1 and 21 days. The reason for our inability to detect an increase in secreted PDGF-AA protein in BAL fluid at 21 days in mice treated with MWCNT and/or LPS is unclear. Since the PDGF-AA detected by immunohistochemistry most likely represents a membrane-tethered form and the PDGF-AA detected in BAL fluid by ELISA most likely represents PDGF-AA that has been cleaved from the cell membrane by proteolytic activity, it is conceivable that at 21 days, the amount of the cleaved form was decreased to a level below our detection limits, reflecting a decrease in PDGF-AA activity at 21 days post-exposure. We also showed that LPS increased PDGF-Rα mRNA levels in primary rat lung fibroblasts and NR8383 cells, a rat lung macrophage cell line. MWCNT exposure to the lungs of rats in vivo or to cultured NR8383 cells in vitro weakly stimulated PDGF-AA production, but this effect was synergistically amplified by LPS pre-exposure. Therefore, our data show that LPS could enhance MWCNT-induced lung fibrosis by amplifying MWCNT-induced PDGF-AA production in macrophages and epithelial cells and by increasing PDGF receptor levels on fibroblasts, which respond to macrophage- and
epithelial-derived PDGF-AA.

Other investigations have shown that bacteria or bacterial-derived products modify the toxicity of carbon nanotubes. For example, the pharyngeal aspiration of SWCNT into the lungs of mice followed by bacterial infection with *Listeria monocytogenes* 3 days later amplified lung inflammation and collagen formation, and decreased phagocytosis of bacteria by macrophages and bacterial clearance from the lungs of mice (Shvedova, Fabisiak et al., 2008). This study suggested that enhanced acute inflammation and pulmonary injury with delayed bacterial clearance after SWCNT exposure may lead to increased susceptibility to lung infection in exposed populations. In another study, mice that were exposed to SWCNT or MWCNT by intratracheal instillation at the same dose used in our present study (4 mg/kg) developed lung inflammation within 24 hrs that was enhanced with LPS co-exposure (K. I. Inoue et al., 2008). Moreover, CNTs tended to enhance expression of pro-inflammatory cytokines (TNF-α, IL-1β) in the lung and circulation in the presence of LPS, as well as in cultured mononuclear cells. These results suggested that LPS can facilitate CNT-induced systemic inflammation and possibly affect coagulation, at least in part, via the activation of mononuclear cells.

We recently reported that pre-existing allergic lung inflammation caused by ovalbumin challenge enhanced airway fibrosis in the lungs of C57BL/6 mice exposed to MWCNT by inhalation (Ryman-Rasmussen, Tewksbury et al., 2009). That study also showed that inhaled
MWCNT (the same source used in the present study) caused increased production of PDGF-AA in the lungs of mice but MWCNT alone did not cause airway fibrosis. Likewise, ovalbumin allergen challenge alone did not cause airway fibrosis but significantly increased the level of lung TGF-β1. However, the combination of ovalbumin allergen pre-exposure and MWCNT inhalation caused significant airway fibrosis and was accompanied by increases in both PDGF-AA and TGF-β1 (Ryman-Rasmussen, Tewksbury et al., 2009). These findings support the idea that both PDGF-AA (a stimulator of fibroblast replication) and TGF-β1 (the primary stimulator of fibroblast collagen synthesis) are required and sufficient for airway fibrosis. Moreover, since the mouse ovalbumin model is a well-established model of allergic airway disease, this study suggested that individuals with asthma might be at greater risk for the development of chronic airway disease if exposed to carbon nanotubes.

The dose and administration methodology used to assess CNT toxicity and to determine the potential of CNTs to cause disease are important considerations. Initial studies of CNTs on rats and mice used intratracheal instillation or pharyngeal aspiration techniques to deliver a bolus of material to the lung (Lam et al., 2004; Mangum et al., 2006; Muller et al., 2005; Shvedova et al., 2005; Warheit et al., 2004). The doses administered in these studies ranged from 1 to 4 mg/kg CNT. The dose of 4 mg/kg used in the present study is therefore consistent with these earlier studies. The concern with instillation or aspiration methodologies is that they do not produce deposition patterns similar to inhaled particles (K. I. Inoue et al., 2008; Ryman-Rasmussen, Tewksbury et al., 2009). However, others have shown that inhalation
exposure to SWCNT produce very similar fibrogenic effects to those seen after a pharyngeal exposure route (Shvedova, Kisin, Murray, Johnson et al., 2008). While our methodology in the present studies achieved a well-dispersed dose of MWCNT in the lungs of rats, further study should address the effect LPS pre-exposure on inhaled MWCNT. Our findings with MWCNT in the absence of LPS in the present study share some similarities and differences to a previous study in which we intratracheally instilled SWCNT into rats, which provides a comparison of the pulmonary effects of MWCNT and SWCNT (Mangum et al., 2006). In our previous study, rats exposed to SWCNT also developed interstitial fibrotic lesions in the same anatomic regions of the lung (terminal bronchiolar and alveolar regions) and had increased PDGF-AA (Mangum et al., 2006). The fibrotic lesions were relatively diffuse and localized to areas of CNT deposition, and therefore we did not detect a significant increase in total lung collagen content. For this reason, in the present study we did not measure total lung collagen by hydroxyproline or Sircol assay, but instead utilized a histopathologic scoring method. The trace metal content of MWCNT (nickel and lanthanum) differed from the trace metal content of SWCNT (cobalt and molybdenum). The lengths of both the SWCNT and MWCNT exceeded 10 μm, however, the MWCNT were 30 to 50 nm in width, whereas the SWCNT were 1 to 3 nm in width and the MWCNT. Most importantly, SWCNT caused the formation of many unique bridge structures between alveolar macrophages in the lungs of rats, whereas no such bridge structures were observed in the lungs of rats that were exposed to MWCNT (Mangum et al., 2006).
The mechanism through which MWCNT or SWCNT increased PDGF-AA production is unknown. CNTs have some features in common with asbestos fibers (e.g., high aspect ratio, residual metals), which are also known to increase PDGF by macrophages and fibroblasts (Bonner, Osornio-Vargas, Badgett, & Brody, 1991; Lasky et al., 1995). However, metals in asbestos fibers (e.g., iron, magnesium) are naturally occurring, whereas CNTs are manufactured using metal catalysts such as iron, nickel and cobalt. Nickel was a catalyst present in the MWCNT used in the present study, and nickel has been reported to increase PDGF production by human macrophages and cause pulmonary fibrosis in experimental animals and in humans exposed occupationally (Kelleher et al., 2000; Kuwabara et al., 1995). Therefore, we speculate that the fibrogenic potential of MWCNT is due at least in part to residual nickel catalyst.

In our hands, SWCNT or MWCNT are weak inducers of TGF-β1 production in the lung. Our previous work with SWCNT showed no significant increase in lung TGF-β1 mRNA in rats exposed by pharyngeal aspiration (Mangum et al., 2006). In contrast, other work has shown that SWCNT increased TGF-β1 levels in the lungs of mice (Shvedova et al., 2005). TGF-β1 levels in BAL fluid peaked at 7 days post-exposure in these mice and then returned to near baseline levels by 28 days post-exposure. Therefore, it is possible that in our studies with rats, which were evaluated either 1 or 21 days post-exposure to SWCNT or MWCNT in the present study, we simply missed the window of TGF-β1 expression (Mangum et al., 2006).
We did observe low levels of TGF-β1 protein in the BAL fluid from rats exposed to LPS and MWCNT, which indicates that this important stimulator of collagen production is expressed under conditions that result in maximal fibrosis. A caveat is that we measured TGF-β1 by ELISA and such colorimetric assays are prone to some interference of protein binding and/or enzymatic activity by carbonaceous particles. While we were able to measure MWCNT-induced increases in the level of PDGF by ELISA, we cannot rule out some interference of nanoparticles in ELISA assays for measuring growth factor levels.

In summary, we report that pre-exposure to bacterial LPS enhances the fibrogenic effect of MWCNT delivered to the lungs of rats. Exacerbation of MWCNT-induced fibrosis by LPS is accompanied by enhanced production of PDGF-AA and its receptor in the lungs of rats, as well as in cultured rat lung macrophages and fibroblasts. Given the importance of the PDGF system in fibrotic diseases, our data indicate a possible mechanism of action whereby LPS increases MWCNT-induced fibrosis in two ways. First, LPS synergistically elevates macrophage production of PDGF-AA by MWCNT. Second, LPS upregulates the receptor to which PDGF-AA binds on fibroblasts, thereby amplifying growth and chemotactic responses. In general, our data support the hypothesis that pre-existing inflammation exacerbates the fibrogenic response of the lungs to carbon nanotubes.
Table 4-1. Differential counts of inflammatory cells in bronchoalveolar lavage (BAL) fluid collected from rats 24 hrs after exposure to MWCNT or CB nanoparticles, with or without a 24-hr LPS pre-exposure.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Total BAL Cells (x 10³)</th>
<th>Macrophages (% total cells)</th>
<th>PMNs (% total cells)</th>
<th>Lymphocytes (% total cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>997 ±58</td>
<td>92.44 ±1.94</td>
<td>4.72 ±1.23</td>
<td>2.84 ±0.8</td>
</tr>
<tr>
<td>LPS</td>
<td>1729 ±281</td>
<td>93.25 ±2.05</td>
<td>4.30 ±1.88</td>
<td>2.45 ±0.21</td>
</tr>
<tr>
<td>CB</td>
<td>3210 ±653</td>
<td>48.07 ±1.80b</td>
<td>49.07 ±1.62b</td>
<td>2.87 ±0.41</td>
</tr>
<tr>
<td>MWCNT</td>
<td>1054 ±258</td>
<td>51.33 ±2.01b</td>
<td>45.87 ±2.03b</td>
<td>2.80 ±0.12</td>
</tr>
<tr>
<td>LPS/CB</td>
<td>12,906 ±3,391b</td>
<td>47.70 ±0.71b</td>
<td>49.90 ±1.14b</td>
<td>2.40 ±0.80</td>
</tr>
<tr>
<td>LPS/MWCNT</td>
<td>947 ±173</td>
<td>42.95 ±4.00a</td>
<td>53.40 ±4.51a</td>
<td>3.65 ±0.90</td>
</tr>
</tbody>
</table>

Data are expressed as percent of total cells retrieved from bronchoalveolar lavage. \(^a\)p < 0.01 or \(^b\)p < 0.001 compared to control as determined by one-way ANOVA with post Tukey’s test. CB – Carbon black; MWCNT – Multi-walled carbon nanotubes; LPS – Lipopolysaccharide; PMN – Polymorphonuclear cell; BAL – Bronchoalveolar lavage
Figure 4-1. Transmission electron microscopic (TEM) characterization of bulk MWCNT. (A) Low magnification electromicrograph of MWCNT (bar = 2 microns). (B) Intermediate magnification TEM image of MWCNT (bar = 0.2 microns). (C) High magnification TEM electromicrograph of MWCNT (bar = 20 nm). (D) Energy dispersive X-ray (EDX) analysis of MWCNT.
Figure 4-2. Lung histopathology in rats 21 days postexposure. Blue staining indicates collagen. (A) Saline control. (B) LPS alone. (C) CB nanoparticles alone. (D) CB nanoparticles with LPS pre-exposure. (E) MWCNT alone. (F) MWCNT with LPS pre-exposure. (G) Higher magnification of inset from panel E. (H) Higher magnification of inset from panel F. All panels 10X original magnification, except G and H, 40X original magnification.
Figure 4-3. Lung pathology scoring in rats 21 days after exposure. The lungs were scored for the amount of collagen present (based on Masson’s trichrome-stained sections), the thickness of the alveolar walls, and the number of fibroblasts associated with the particle-associated lesions. These average scores were then divided by the relative scores for the amount of nanoparticles present in the lungs to give an adjusted average score (see Methods). *P<0.05 and **P<0.01 compared to control or LPS as determined by one-way ANOVA with post Dunnett’s multiple comparison test.
Figure 4-4. Markers of lung injury in BAL fluid. (A) Lactate dehydrogenase (LDH) and (B) total protein in the lungs of rats 24 hrs after exposure to MWCNT or CB nanoparticles, with or without a 24-hr LPS pre-exposure. *P<0.05 and **P<0.01 compared to control or LPS as determined by one-way ANOVA with post Tukey’s test or Bonferroni’s test.
Figure 4-5. PDGF-AA protein levels in bronchoalveolar lavage (BAL) fluid collected from rats 1 or 21 days after exposure to 4 mg/kg MWCNT or CB nanoparticles with or without a 24-hr LPS pre-exposure. PDGF-AA was measured by ELISA. *P<0.05 and **P<0.01 compared to control as determined by one-way ANOVA with post Tukey’s test or Bonferroni’s test.
Figure 4-6. Immunohistochemistry for PDGF-AA at 24 hrs postexposure. Brown staining indicates PDGF-AA protein. Open arrows indicate PDGF-positive epithelium. Solid arrows indicate PDGF-positive macrophages, some containing CB or MWCNT. (A) Saline control. (B) LPS alone. (C) CB nanoparticles alone. (D) CB nanoparticles with LPS pre-exposure. (E) MWCNT alone. (F) MWCNT with LPS pre-exposure. (G) Higher magnification of inset from panel E. (H) Higher magnification of inset from panel F. Hematoxylin counterstain (light blue). 20X original magnification, except panels G and H, which are 40X original magnification. Bars = 100 μm.
Figure 4-7. Immunohistochemistry for PDGF-AA at 21 days postexposure. Brown staining indicates PDGF-AA protein. Open arrows indicate PDGF-positive epithelium. Solid arrows indicate PDGF-positive macrophages, some containing CB or MWCNT. (A) Saline control. (B) LPS alone. (C) CB nanoparticles alone. (D) CB nanoparticles with LPS pre-exposure. (E) MWCNT alone. (F) MWCNT with LPS pre-exposure. (G) Higher magnification of inset from panel E. (H) Higher magnification of inset from panel F. Hematoxylin counterstain (light blue). 20X original magnification, except panels G and H, which are 40X original magnification. Bars = 100 μm.
Figure 4-8. PDGF-A and PDGF-Rα gene expression in NR8383 cells. (A) PDGF-A and (B) PDGF-Rα mRNA levels in rat alveolar macrophages (NR8383 cells) exposed to MWCNT for 4 or 24 hrs with or without 1-hr LPS pre-exposure. Nonadherent cultures of NR8383 cells were grown to near confluence in 10% FBS-DMEM, then washed and rendered quiescent in serum-free defined medium, and treated with 10 μg/cm² MWCNT or CB for 4 or 24 hrs prior to collecting RNA. PDGF-A and PDGF-Rα mRNA levels were measured by real-time RT-PCR as described in Methods. *P<0.05, **P<0.01, and ***P<0.001 compared to controls as determined by one-way ANOVA with post Tukey’s test or Bonferroni’s test.
Figure 4-9. PDGF-A and PDGF-Rα gene expression in RLF. (A) PDGF-A and (B) PDGF-Rα mRNA levels in early passage rat lung fibroblasts (RLF) exposed to MWCNT for 4 or 24 hrs with or without 1-hr LPS pre-exposure. Confluent quiescent cultures of RLF were treated with 10 μg/cm² MWCNT or CB for 4 or 24 hrs prior to collecting RNA. PDGF-A and PDGF-Rα mRNA levels were measured by Taqman quantitative real-time RT-PCR as described in Methods. **P<0.01 and ***P<0.001 compared to controls as determined by one-way ANOVA with post Tukey’s test or Bonferroni’s test.
5.1 ABSTRACT

Vanadium pentoxide (V$_2$O$_5$) is a known cause of occupational lung disease and causes inflammation and fibrosis in the lungs of rats. Lipopolysaccharide (LPS) is recognized as an inducer of acute lung injury and inflammation. In this report, we show that LPS pre-exposure exacerbates the lung fibrotic effects of V$_2$O$_5$. Rats were treated with saline (PBS) or LPS, followed by PBS or V$_2$O$_5$ 24 hrs later and were sacrificed 24 hrs or 7 days post-exposure. Rats treated with V$_2$O$_5$ with and without LPS had similar lesions, consistent with bronchiolitis obliterans, chronic inflammation, fibrosis, and airway epithelial injury. However, in rats pre-treated with LPS, the lesions were more severe and widespread. Additionally, there were significantly increased numbers of inflammatory cells in the bronchoalveolar lavage fluid (BALF) of rats exposed to LPS and V$_2$O$_5$ compared to rats treated with V$_2$O$_5$ alone at 24 hrs post-exposure. At 7 days post-exposure, rats treated with LPS and V$_2$O$_5$ had a significant increase in COL1A2 gene expression in whole lung
compared to rats treated with V$_2$O$_5$ alone. TGF-β1 protein was elevated in the BALF at 24 hrs and PDGF-AA and PDGF-Rα were detected by immunohistochemistry at 24 hrs and 7 days, however, there was no detectable difference between the rats treated with LPS and V$_2$O$_5$ and those treated with V$_2$O$_5$ alone. These data suggest that pre-existing inflammation in the lungs, such as that associated with asthma or chronic bronchitis, may exacerbate the adverse pulmonary effects of occupational exposure to V$_2$O$_5$.

5.2 INTRODUCTION

Fibrotic disorders are those in which normal parenchymal tissue is replaced by extracellular matrix. They are typically preceded by inflammation and are characterized by an increase in the number of lung fibroblasts, or myofibroblasts, that produce extracellular matrix proteins, including collagen (Wynn, 2008). Platelet-derived growth factor (PDGF) is a potent mitogen and chemoattractant for fibroblasts and myofibroblasts and is therefore thought to play an important role in the pathogenesis of many fibrotic diseases, including those of the lung (Bonner, 2004; Ingram & Bonner, 2006; Trojanowska, 2008). There are five PDGF isoforms (PDGF-AA, -BB, -AB, -CC, and -DD) composed proteins encoded by four PDGF genes, designated A-D. The two PDGF receptors, PDGF-Rα and PDGF-Rβ, are tyrosine kinase receptors that dimerize upon ligand binding forming three isoforms (PDGF-Rαα, -Rαβ, and -Rββ). PDGF-AA binds exclusively to PDGF-Rα. PDGF-Rα regulates fibroblast mitogenic and chemotactic responses to secreted PDGF. PDGF-Rα expression can be upregulated by
exposure to a number of agents, including particles and fibers (Bonner, Rice et al., 1998; Lindroos et al., 1997). During lung fibrogenesis in rats, PDGF-Rα levels become markedly upregulated in fibroblasts and myofibroblasts during fibroblast proliferative stages of the disease (Bonner, Lindroos et al., 1998). Furthermore, PDGF-A and -B and PDGF-Rα expression are known to be upregulated in rats exposed to bacterial lipopolysaccharide (LPS) and vanadium pentoxide (V₂O₅) (Coin et al., 1996; Mangum et al., 2006).

Vanadium pentoxide (V₂O₅) the most commercially important compound of vanadium (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2006; National Toxicology Program, 2002). Its primary use is as an alloying agent in the steel industry, but is also used as a catalyst and in the ceramics and textiles industries. Occupational exposure to V₂O₅ has been associated with occupational lung disease, causing irritation of the upper and lower respiratory tracts and bronchitis in exposed human beings (Barceloux, 1999; IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2006; J. Y. Kim et al., 2003; Y. Liu et al., 2005; National Toxicology Program, 2002; Woodin et al., 1998; Woodin et al., 1999; Woodin et al., 2000). Furthermore, evidence suggests that V₂O₅ may induce bronchial hyper-responsiveness and asthma in exposed individuals (Irsigler et al., 1999). More recently, there has been increased concern regarding V₂O₅ as an air pollutant, particularly in urban areas and areas where coal or residual oil are burned (Bell, Ebisu, Peng, Samet, & Dominici, 2009; Fortoul et al., 2002; IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2006).
The effects of V$_2$O$_5$ on the respiratory system of laboratory animals are similar to that observed in humans. In cynomolgus macaques, V$_2$O$_5$ has been shown to induce pulmonary inflammation and impaired pulmonary function after acute and subchronic inhalation exposure (Knecht et al., 1992; Knecht et al., 1985). In laboratory mice and rats, intratracheal (IT) instillation of V$_2$O$_5$ causes lung inflammation and fibrosis (Bonner, Lindroos et al., 1998; Bonner et al., 2000; IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2006; National Toxicology Program, 2002; Toya, Fukuda, Takaya, & Arito, 2001). Airway remodeling, similar to that observed in humans with asthma, has been described in rats exposed to V$_2$O$_5$ (Bonner et al., 2000). In rats, the mechanism by which V$_2$O$_5$ causes lung fibrosis has been shown to involve platelet-derived growth factor (PDGF) signaling (Bonner, Lindroos et al., 1998).

LPS is present in organic dusts and air pollution and has been implicated in a number of occupational lung diseases in humans (Becker et al., 2005; Garantziotis et al., 2006; S. L. Huang, Cheng, Lee, Huang, & Chan, 2002; Laitinen, Kangas, Husman, & Susitaival, 2001; Soukup & Becker, 2001; Thorn, 2001). In experimental animals, pulmonary exposure to LPS is commonly used to model acute lung injury and chronic bronchitis. IT instillation of LPS in rats and mice causes inflammation and goblet (mucous) cell hyperplasia (Nikula & Green, 2000). Brass, et al. reported that subchronic inhalation exposure to LPS causes airway remodeling and hyperreactivity in C3HeB/FeJ mice, but not C3H/HeJ mice, which are reportedly LPS resistant (Brass et al., 2003). Activation of toll-like receptor-4 (TLR4) by
LPS results in increased lung levels of a variety of secreted pro-inflammatory mediators or their cognate receptors, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-8 release from alveolar macrophages (Becker et al., 2005; Nagaoka et al., 1990; Schroder et al., 2006; Thorn, 2001). LPS also up-regulates the expression of both PDGF-A and PDGF-B genes in alveolar macrophages and PDGF-Rα in rat lung fibroblasts (Coin et al., 1996; Nagaoka et al., 1990). These studies suggest that LPS could exacerbate pulmonary fibrosis by “priming” pulmonary macrophages and fibroblasts for enhanced production of profibrotic cytokines, growth factors, and their receptors.

A number of studies have examined the effects of LPS on pulmonary inflammation or acute lung injury induced by various agents (or vice versa) (refs.), but few have examined the effect of LPS on experimentally induced pulmonary fibrosis. We have previously shown that LPS pre-exposure exacerbates intratracheally instilled multi-walled carbon nanotube-induced pulmonary fibrosis (Cesta et al., 2009). In this study, we show that LPS pre-exposure exacerbates V2O5-induced COL1A2 gene expression, lung inflammation, pulmonary fibrosis, and airway epithelial damage in rats, possibly by increasing susceptibility of the lung tissues to V2O5-induced damage.
5.3 MATERIALS AND METHODS

5.3.1 Animals

Male, pathogen-free Sprague-Dawley rats, 6 to 8 weeks old, were purchased from Charles River Breeding Laboratories (Kingston, NY) and housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility that was humidity and temperature controlled. Rats were housed in microisolator cages on Alphadri cellulose bedding and supplied water and cereal-based diet NIH07 (Zeigler Brothers, Gardners, PA) ad libitum. The animal studies were approved by The Hamner Institutes for Health Research Institutional Animal Care and Use Committee.

5.3.2 General Experimental Design

Animals were randomly assigned to four treatment groups of five rats each and acclimated for 1 week prior to exposure. Rats from two of the treatment groups were exposed to 2.5 mg/kg LPS from *E. coli* by nasal aspiration and the rats from the other two treatment groups were exposed to Ca²⁺- and Mg²⁺-free phosphate-buffered saline (PBS). Twenty-four hrs later, rats were exposed to 4 mg/kg of V₂O₅ by IT instillation. Negative control animals were treated with PBS. Animals were killed at 1 and 7 days following particle exposure. The lungs from the rats of the 1-day time point were lavaged with PBS. The right lung lobes from the rats of both time points were minced and placed in RNALater solution (Qiagen, Valencia, CA). Lungs were stored at -20°C and used for RNA isolation. The left lung lobes from the
rats of both time points were collected for histopathology.

**5.3.3 Preparation and Instillation of LPS and Vanadium Pentoxide**

Rats were administered LPS (Serotype 026:B6, Sigma, St. Louis, MO) by intranasal aspiration and V$_2$O$_5$ by IT instillation. In brief, animals were anesthetized with isoflurane and ~50 μl volume LPS (dose concentration equivalent of 2.5 mg of LPS per kg of bodyweight) or PBS (for control animals) was pipetted into the antrum of the nasal cavity. Twenty-four hrs later, the animals were again anesthetized with isoflurane and ~100 μl volume of V$_2$O$_5$ (dose concentration equivalent of 4 mg per kg of bodyweight) was instilled into the trachea using an 18 gauge catheter attached to a 1.0 ml syringe. The V$_2$O$_5$ solutions were prepared by first milling the dry V$_2$O$_5$ in a Retsch Mixer Mill (Retsch Inc., Newtown, PA) for 5 min. at 30 cycles per second. Just prior to instillation, the milled V$_2$O$_5$ was added to a solution of 1% Pluronic F68 (BASF Corp., Florham Park, NJ), a biocompatible, nonionic surfactant, in PBS and wet milled for an additional 5 min., then further diluted with PBS to achieve the desired final dosing concentration suspended in 0.1% Pluronic F68. Control rats were administered PBS with 0.1% Pluronic F68 surfactant (vehicle).

**5.3.4 Bronchoalveolar Lavage**

Rats were euthanized by pentobarbital overdose and exsanguination via the abdominal aorta and lungs of the rats from the 1-day time point were lavaged 3 times with 5-ml volumes of
PBS. Bronchoalveolar lavage fluid (BALF) collected from the second and third lavages was pooled (the fluid from the first lavage was kept separate) and all BALF was placed on ice. The BALF was centrifuged and the collected cells were resuspended in culture medium and enumerated using an automated cell counter (Model ZM, Coulter, Marietta, GA). Cytospins were prepared with 25,000 cells per slide. Differential cell counts were performed on HEMA-3 (Fisher Scientific, Pittsburgh, PA) stained cytocentrifuge slide preparations. Cell numbers were quantified by light microscopy using the 40X objective and 500 cells per animal were counted. Total protein and LDH in cell-free BALF from the first two pooled lavages were analyzed spectrophotometrically using a COBAS FARA II (Roche Diagnostic Systems Inc., Montclair, NJ) and assay kits for LDH (LD Liquid Reagent, Pointe Scientific, Canton, MI) and total protein (Coomassie Plus Protein Assay Reagent, Pierce/ThermoFisher).

5.3.5 Lung Fixation and Histopathology

At necropsy, the left lungs were pressure-infused intratracheally at 30 cm H₂O with 10% neutral-buffered formalin. Lungs were fixed for approximately 48 hrs and then transferred to 70% ethanol. Three cross-sectional portions of the left lung were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E) or Masson’s trichrome by established methods.
5.3.6 Pathology Scoring of Lungs

Three sections of lung, one each from the cranial, middle, and caudal portions of the left lung lobe, from each rat were evaluated in a blinded fashion. The lung sections from the 24-hr time point were scored for inflammation and airway epithelial necrosis using the H&E-stained tissue sections. The lung sections from the 7-day time point were scored for the number of fibroblasts, the amount of collagen, chronic inflammation, alveolar type II cell hyperplasia, alveolar epithelial necrosis, and goblet cell hyperplasia in the airway epithelium using H&E- or Masson’s trichrome-stained tissue sections. The inflammation scores reflect a subjective assessment of the number of inflammatory cells present and the degree and amount of intra-alveolar fluid, alveolar wall thickening, and perivascular edema present. All scoring was based on the relative severity and extent of the lesions seen in this study. The scores were assigned on a scale from 0-4, with zero representing the absence of the lesion, 1 representing minimal change, 2 representing mild change, 3 representing moderate change, and 4 representing marked change.

5.3.7 Immunohistochemistry for PDGF-AA and PDGF-Rα

Formalin-fixed paraffin-embedded lung tissue sections (5μm thickness) were deparaffinized in xylene and rehydrated through graded ethanols. Endogenous peroxidase activity was quenched with 3% H2O2 followed by antigen retrieval using a Decloaking Chamber (Biocare Medical, Concord, CA) for 5 minutes at 120°C with citrate buffer, pH 6.0 (PDGF-A), or with
0.1% trypsin (PDGF-Rα). Non-specific binding was blocked with 10% normal donkey serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 20 minutes alone (PDGF-A) or followed by the Avidin/Biotin blocking kit (Vector Laboratories, Burlingame, CA) (PDGF-Rα). Tissues were incubated with rabbit anti-PDGF-A antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:25 dilution or rabbit anti-PDGF-Rα antibody (RB-9027, NeoMarkers, Fremont, CA) at a dilution of 1:500 for 60 minutes at room temperature. For negative controls, the primary antibody was replaced with non-immune rabbit IgG serum (Jackson ImmunoResearch) at the same concentration and dilution as the primary antibody. Incubation with biotinylated donkey anti-rabbit IgG secondary antibody (Jackson ImmunoResearch) at 1:500 for 30 m at room temperature followed. Signal was detected using VECTASTAIN Elite ABC kit (Vector Laboratories, Inc., Burlingame, CA) and DAB (Dako, Carpinteria, CA). Slides were then counterstained with hematoxylin, dehydrated, and cover-slipped.

5.3.8 Taqman Real Time Quantitative RT-PCR

The lungs were removed from the RNALater solution, blotted dry, and homogenized in buffer RLT (from Qiagen RNeasy Mini kit) with 10 μl/ml β-mercaptoethanol added. Total RNA was isolated using the Qiagen RNeasy Mini kit and converted to cDNA with the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Thirty nanograms of cDNA was amplified using Taqman Gene Expression Assays specific for β-actin.
(Rn_00667869), PDGF-A (Rn_00709363), PDGF-C (Rn_00579958), PDGF-Rα (Rn_01399472), TGF-β1 (Rn_00572010), and Type I procollagen (COL1A2) (Rn_00584426) on the Applied Biosystems 7900 Prism® Sequence Detection System (Applied Biosystems, Foster City, CA). The PCR conditions and data analysis were performed according to the manufacturer’s protocol described in User Bulletin No. 2, Applied Biosystems Prism 7900 Sequence Detection System. Gene expression was measured by the quantitation of cDNA converted from mRNA corresponding to the target genes relative to the vehicle-treated control groups and normalized to β-actin reference endogenous control. Relative quantitation values (2^{-ΔΔCT}) were expressed as fold-change over controls.

5.3.9 ELISA

Quantikine ELISA kits (R&D Systems, Minneapolis, MN) were used to assay BALF for total TGF-β1 (active and inactive forms) 24 hrs and 7 days after V₂O₅ exposure and PDGF-AA 24 hrs and 7 days after V₂O₅ exposure. Duplicate samples using 50 μl BALF were used for each animal. Assays were performed according to kit instructions and absorbances were read on a Multiskan EX microplate spectrophotometer (ThermoFisher Scientific) microplate reader. Values were normalized relative to total protein in BALF and expressed as pg/ml.

5.3.10 Data and Statistical Analyses

Statistical analyses of cell counts and ELISA data in the BAL fluid and RT-PCR in whole
lung were performed using GraphPad Prism® software v. 5.00 (GraphPad Software, Inc., San Diego, CA). A one-way ANOVA with a post-hoc Tukey test, Bonferroni test, or Dunnett’s test was used to identify significant differences among treatment groups. Because histopathology scores were not normally distributed, nonparametric methods were used to compare groups. Kruskal-Wallis analysis of variance indicated significant differences across the four groups for each of the endpoints, so pairs of groups were compared using one-sided exact Mann-Whitney tests (Conover, 1971). Because the lesions from one of the animals in the PBS/V2O5 group (animal no. 70) were inordinately severe, suggesting that it may be an outlier (there were not enough animals for us to test this statistically), statistical analyses were performed both with and without this animal. The results of both analyses are presented in Table 5-2. Significance was set at p<0.05 unless otherwise stated.

5.4 RESULTS

5.4.1 LPS Enhances V2O5-Induced Pulmonary Inflammation as Determined by Total BALF Cells

LPS or PBS was delivered by intranasal aspiration 24 hrs before the IT instillation of V2O5 or PBS. At 24 hrs, the lungs of V2O5-treated rats (with and without LPS pretreatment) had increased numbers of inflammatory cells (macrophages, polymorphonuclear cells, and lymphocytes) in the BALF compared to the lungs of rats receiving PBS or LPS only (Table 5-1). The total cell number in the BALF of the LPS/V2O5 group was >5-fold higher than in
the PBS/V$_2$O$_5$ group. The distribution of cells in the BAL fluid from the PBS/PBS and LPS/PBS groups was >90% macrophages with occasional polymorphonuclear cells (PMNs) and lymphocytes. In both the PBS/V$_2$O$_5$ and LPS/V$_2$O$_5$ groups, the distribution of cells in the BAL fluid indicated a very neutrophilic response with approximately 80% PMNs, approximately 20% macrophages (usually large, foamy, activated macrophages), and a small number of lymphocytes (Table 5-1). Intranasal PBS or LPS followed by IT V$_2$O$_5$ caused a significant increase in LDH and total protein levels compared to the PBS/PBS and LPS/PBS control groups, but there was no significant difference between the two V$_2$O$_5$-treated groups (Figure 5-1A, -1B). While PDGF-A chain protein levels were not detected in the BALF by ELISA 24 hrs post-V$_2$O$_5$ exposure, comparably increased levels of TGF-β1 were detected in the BALF from the PBS/V$_2$O$_5$ and LPS/V$_2$O$_5$ groups. The levels of TGF-β1 in the BAL fluid from the V$_2$O$_5$-treated groups were 30-40 fold higher than those of the PBS/PBS and LPS/PBS groups (Figure 5-2).

Histopathological examination of the lungs at 24 hrs (Figure 5-3A, -3B) revealed that the inflammatory lesions in the V$_2$O$_5$ and LPS/V$_2$O$_5$ groups were similar. In the PBS/PBS and LPS/PBS groups, there was minimal, acute inflammation composed of a few, small, perivascular collections of neutrophils. In the V$_2$O$_5$ and LPS/V$_2$O$_5$ groups, there was mild to marked, suppurative inflammation characterized by perivascular edema and abundant peribronchial/peribronchiolar, interstitial, and intra-alveolar neutrophils and macrophages with fewer eosinophils and occasional large granular lymphocytes. Many airways were

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surrounded by small amounts of edema with fibrin and numerous inflammatory cells. There were variable numbers of necrotic epithelial cells in the airways (particularly the small to medium sized airways). In some instances, there were denuded airway segments or segments with flattened epithelium. The epithelium was infiltrated by neutrophils. In the more severely affected animals, there was segmental sloughing of the airway epithelium with an intraluminal exudate and segmental destruction of the airway wall with loss of the smooth muscle layer. In some cases, there were suppurative lesions centered on terminal bronchioles or alveolar ducts with associated hyperplasia of the alveolar type II pneumocytes and increased numbers of elongated cells that resembled fibroblasts, reactive endothelial cells, or alveolar type I cells. The alveolar septa were thickened by edema, fibrin, and inflammatory cells, which were also observed within the alveoli and around vessels.

5.4.2 LPS Enhances V2O5-Induced Bronchiolitis Obliterans, Interstitial Fibrosis, Chronic Inflammation, and Airway Epithelial Damage 7 Days Post-Instillation

Four of five rats from the LPS/V2O5 group and only one from the PBS/V2O5 group had lesions consistent with bronchiolitis obliterans (BO). These lesions were characterized by intrabronchiolar fibrosis, of which there were two forms. In one form, polypoid masses of fibroproliferative tissue projected into and occluded the airway lumen. These polypoid masses of fibroproliferative tissue were composed of large, spindloid, fibroblastic cells amid abundant extracellular matrix (ECM). They could be seen extending from the bronchiolar
wall into the airway lumen and their surfaces were often covered by flattened and elongated bronchiolar epithelial cells, particularly at their point of origin (Figure 5-3C, -3D). In the other form, abundant subepithelial fibroproliferation and chronic inflammation caused narrowing of the airway lumen. In some instances, within the confines of a single bronchiole as identified by the ring of smooth muscle, there were multiple, small, epithelium-lined airway lumina amid loosely arranged fibroproliferative tissue (Figure 5-3E). Rarely, the airway lumen was completely replaced by fibroproliferative tissue and inflammatory cells, the smooth muscle being the only evidence of the presence of a bronchiole. Airways of all sizes were affected. None of the PBS/PBS or LPS/PBS rats had intrabronchiolar fibrosis.

LPS pre-exposure also resulted in increases in V₂O₅-induced BO, interstitial fibrosis, chronic inflammation, and airway epithelial damage 7-days post-exposure. A number of lesions were either quantitatively or semi quantitatively scored (Table 5-2). The scores for all lesions evaluated, except goblet cell hyperplasia, were higher in the LPS/V₂O₅ group compared to the PBS/V₂O₅ group; however, none of the increases was statistically significant. Measures of interstitial fibrosis included the number of fibroblasts (or myofibroblasts), the number of fibroblastic foci, the number of airways with intraluminal fibrosis, amount and intensity of collagen staining in Masson’s Trichrome-stained lung sections, and alveolar septal thickening. Measures of chronic inflammation included chronic inflammation (relative numbers of mononuclear inflammatory cells), alveolar type II cell hyperplasia, and alveolar septal thickening. Measures of airway epithelial damage included airway epithelial necrosis.
and hyperplasia. The lesions from one animal in the PBS/V2O5 group (animal number 70) were much more severe than those of any other animal, including those from the LPS/V2O5 group. When this animal is omitted from the statistical analyses, a number of lesions become statistically significant, including the percent lung affected, the number of fibroblastic foci, alveolar wall thickening, and alveolar type II hyperplasia (Table 5-2).

Histopathologically, in addition to the BO lesions, the rats from the LPS/V2O5 and PBS/V2O5 groups had mild to marked tissue destruction, chronic inflammation, and fibroproliferative lesions (Figure 5-3F, -3G, -3H). In some areas, in the more severely affected rats, the alveolar septa were destroyed. These areas contained high numbers of loosely arranged spindloid cells (fibroblasts), new blood vessels, and inflammatory cells. Numerous small airways lined by cuboidal epithelium suggested that air spaces in these regions were collapsed. In other areas, there was chronic inflammation that was predominantly centered on terminal bronchioles and alveolar ducts. In the most severe cases, large regions of the lung parenchyma were consolidated and devoid of airspaces. The inflammation was characterized by thickened alveolar septa, perivascular edema, increased numbers of alveolar macrophages, neutrophils, and lymphocytes with fewer eosinophils, mast cells, and occasional large granular lymphocytes. The alveolar septa were thickened by inflammatory cells, ECM, and elongated cells representing fibroblasts or reactive endothelial cells. The alveoli contained abundant alveolar macrophages with fewer neutrophils and lymphocytes. In severe cases, the alveoli were completely occluded by proteinaceous fluid and macrophages, macrophages
alone, or macrophages and spindloid to stellate cells (fibroblasts). Airway and alveolar epithelial cells were frequently necrotic, manifested as large amounts of pyknotic and cellular debris. There were increased numbers of goblet cells in the airway epithelium, which was often hyperplastic. The smooth muscle surrounding the airways was often thickened, and there were increased numbers of fibroblasts surrounding the smooth muscle. Numerous alveoli around affected terminal bronchioles and alveolar ducts were lined by cuboidal epithelium resembling bronchiolar epithelium (bronchiolar metaplasia of alveolar epithelium). Scattered throughout the lungs, were variably sized fibroblastic foci similar in appearance to the intraluminal bronchiolar fibrosis.

5.4.3 COL1A2 Gene Expression in the Lungs of Rats 7-Days Post-V2O5 Exposure Is Increased by LPS Pre-Exposure

Gene expression of PDGF-A, PDGF-C, PDGF-Rα, TGF-β1, and COL1A2 was measured in whole lung tissue by RT-PCR 24 hrs and 7 days post-exposure. At 7 days, expression of the COL1A2 gene was increased compared to PBS/PBS, LPS/PBS, and PBS/V2O5 groups (Figure 5-4). Expression of the PDGF-A, PDGF-Rα, PDGF-C, and TGF-β1 genes were not found to be increased.
5.4.4 PDGF-AA and PDGF-Rα Immunostaining in the Lungs of Rats Exposed to V₂O₅ with or without LPS

Immunohistochemistry was performed using antibodies selective for PDGF-AA or PDGF-Rα (Figure 5-5). For both antibodies, there was no detectable difference in immunoreactivity between the V₂O₅ and LPS/V₂O₅ groups. PDGF-AA immunoreactivity was most prominent in the airway epithelial cells, alveolar macrophages, and endothelial cells. There was less prominent immunostaining in perivascular cardiac muscle cells, mast cell granules, and some peribronchial and perivascular smooth muscle cells (particularly around larger vessels). In the airways, PDGF-AA immunoreactivity was specific to ciliated epithelial cells; Clara cells, goblet cells, and other non-ciliated epithelial cell types were negative. At 1 day post-exposure, the types of cells positive for PDGF-AA were similar in all groups, but in the lungs of rats treated with V₂O₅, there were increased numbers of PDGF-AA-positive perivascular and peribronchiolar cells. Morphologically, these cells were consistent with macrophages (round cells with round to oval, often indented nuclei and abundant cytoplasm) and fibroblasts (spindloid to stellate cells). In areas of intense inflammation, the alveolar septa exhibited increased staining. This may represent increased PDGF-AA production by interstitial endothelial cells. In some instances, the staining appeared to involve alveolar type I cells, as well, though this was not consistent. PDGF-AA immunoreactivity was similar at 7 days post-exposure. There was PDGF-AA immunopositivity in elongated cells (fibroblasts, myofibroblasts, and/or smooth muscle cells) in areas of intraluminal fibrosis. The
multinucleated giant cells were also immunoreactive for PDGF-AA.

PDGF-Rα immunostaining was similar in distribution to PDGF-AA with the exception of the airway epithelium where staining was absent. PDGF-Rα immunoreactivity was most prominent in smooth and cardiac muscle with slightly weaker staining in alveolar macrophages and endothelial cells. In the V₂O₅-treated animals (with and without LPS) at both time points, PDGF-Rα positivity was similar to controls, but there were numerous immunopositive cells (morphologically consistent with macrophages and fibroblasts) around blood vessels and bronchioles and in regions of parenchymal destruction at 7 days post-exposure. There were also lightly staining cells in the intraluminal fibrotic masses.

5.5 DISCUSSION

V₂O₅ has previously been shown to cause airway and interstitial fibrosis in a number of species (Bonner et al., 2000; National Toxicology Program, 2002). Intratracheal instillation of 1 mg/kg body weight of V₂O₅ in rats causes airway remodeling and interstitial fibrosis. The airway remodeling consisted of thickening of the airway smooth muscle layer, peribronchiolar myofibroblast accumulation and collagen deposition, and goblet cell hyperplasia of the airway epithelium and is similar to that seen in human asthma patients, (Bonner et al., 2000). Similar changes were seen in the present study in the PBS/V₂O₅ and LPS/V₂O₅ groups, though the severity of lesions was generally higher in the later group.
However, the 4 mg/kg V2O5 dose exceeded the maximum tolerated dose. Beginning at 4-5 days all V2O5-exposed rats developed respiratory distress that progressed to the point that all these animals had to be euthanized on Day 7.

In this study, we have shown that LPS pre-exposure significantly increases V2O5-induced lung inflammation and COL1A2 gene expression at 24 hrs and 7 days post-exposure, respectively. In addition, LPS pre-exposure resulted in increases in a number of V2O5-induced histopathologic changes at 7 days post-exposure, including BO, and lesions consistent with interstitial fibrosis, chronic inflammation, and airway epithelial damage, though none of these increases were statistically significant (Table 5-2). We used five animals per treatment group, but one of the animals in the 7-day PBS/V2O5 group died on Day 1. The low number of animals per group provided less than 50% statistical power to detect a difference of 1 in the mean lesion grades (e.g., the difference between minimal and mild or mild and moderate) and less than 75% power to detect a difference of 1.5. There was also less than 30% power to statistically identify the difference in the incidence of BO between the PBS/V2O5 and LPS/V2O5 groups. This degree of statistical power made the identification of biologically significant differences in the severities of lesions between the PBS/V2O5 and LPS/V2O5 groups unlikely. Additionally, the lung lesions in one of the animals in the 7-day PBS/V2O5 group (animal no. 70) were inordinately severe compared to the lesions in the other rats of the same group. In fact, the lesions in this animal were more severe than those of the rats in the LPS/V2O5 group as well. While we cannot rule out
experimental error, this animal may have been particularly sensitive to the effects of instilled
\(V_2O_5\). When this animal was omitted from the statistical analyses, several lesions become
statistically significant (percent lung affected, number of fibroblastic foci, alveolar septal
thickening, and alveolar type II hyperplasia). Also, the differences between the PBS/\(V_2O_5\)
and LPS/\(V_2O_5\) groups in the severities of all lesions (except goblet cell hyperplasia)
increased however, the statistical power was still too low for these differences to be
identified as significant.

LPS, a ubiquitous environmental contaminant derived from the outer membrane of Gram-
negative bacteria, is a well-known effector of airway inflammation and is commonly used as
a model of acute lung inflammation in laboratory animals. LPS primarily activates Toll-like
receptor-4 (TLR4), which results in recruitment of polymorphonuclear cells (PMNs) to the
lung and increases in lung levels of mediators of inflammation such as IL-1\(\beta\), IL-8, TNF-\(\alpha\),
and TGF-\(\beta\) (Savov, Brass, Berman, McElvania, & Schwartz, 2003; Thorn, 2001). In the
present study, there was no detectable increase in PMNs in the BAL fluid of rats 48 hrs after
exposure to LPS, alone as might be expected. This is likely due to the short duration of the
effects of a single exposure to LPS, which have been shown to resolve completely within 48
hrs of exposure (Janardhan et al., 2006; Wohlford-Lenane et al., 1999). Many occupational
lung diseases are thought to be attributable mainly to LPS, particularly those associated with
organic dust exposure such as grain fever, byssinosis, mill fever, and bagassosis (Christiani
& Wang, 2003; Lane et al., 2004; Rylander, 2006; D. A. Schwartz, 2001). Several studies
have examined the combined effects of LPS and other agents of pulmonary disease. We recently reported that LPS pre-exposure enhanced multi-walled carbon nanotube-induced lung fibrosis in rats, in part, through increased lung levels of PDGF-AA and upregulation of PDGF-Rα on lung fibroblasts (Cesta et al., 2009). Similarly, pharyngeal aspiration of SWCNT into the lungs of mice followed by bacterial infection with *Listeria monocytogenes* 3 days later amplified lung inflammation and collagen formation, and decreased phagocytosis of bacteria by macrophages and bacterial clearance from the lungs of mice (Shvedova, Fabisiak et al., 2008). Inoue, et al. reported that carbon black nanoparticle exposure augmented LPS-induced lung inflammation in mice, increasing the lung levels of a number of inflammatory mediators including IL-1β, macrophage inflammatory protein-1α (MIP-1α), MIP-2, and macrophage chemoattractant protein (K. Inoue, Takano, Yanagisawa, Hirano, Sakurai et al., 2006). LPS has also been shown to augment the adverse respiratory effects of ozone in rats and mice co-exposed to these agents (Johnston, Oberdorster, Gelein, & Finkelstein, 2002; Wagner, Hotchkiss, & Harkema, 2001; Wagner, Van Dyken, Hotchkiss, & Harkema, 2001). A number of studies have demonstrated that LPS amplifies the production of inflammatory mediators by alveolar macrophages in response to ambient air particles, which contain a significant amount of vanadium (Bonner, Rice et al., 1998; S. L. Huang et al., 2002; Imrich et al., 1999; Ning, Imrich, Goldsmith, Qin, & Kobzik, 2000; Riley, Boesewetter, Kim, & Sirvent, 2003). Treatment of alveolar macrophages with pentavalent vanadium and LPS resulted in decreased the amount of secreted TNF-α and IL-1 (Cohen, Parsons et al., 1993; Cohen, Schlesinger et al., 1993). *In vitro*, LPS and Zn co-exposure of
RLE-6TN cells, a rat alveolar type II alveolar epithelial cell line, results in greater cytotoxicity than Zn alone (Riley et al., 2003). These data indicate that LPS has the capacity to exacerbate the inflammatory effects of other agents of pulmonary disease by recruiting neutrophils and increasing macrophage production of inflammatory mediators in response to other agents.

In the present study, we provide evidence that LPS exacerbates V₂O₅-induced lung fibrosis. The mechanism by which this occurs has yet to be elucidated, but the LPS-mediated increase of the inflammatory response in the lung likely plays a role since inflammation is a known precursor to fibrosis. In this study, there were increased levels of TGF-β1 in the BAL fluid and increased numbers of cells immunoreactive for PDGF-AA or PDGF-Rα in V₂O₅-treated rats. Though we did not detect differences in PDGF-AA or PDGF-Rα levels between the PBS/V₂O₅ and LPS/V₂O₅ groups, there is ample evidence to suggest that PDGF signaling plays a role in LPS-mediated enhancement of fibrotic responses in rats. LPS and V₂O₅ have been shown to upregulate PDGF-Rα expression in rat lung myofibroblasts and to increase PDGF-A production by alveolar macrophages (Bonner, Lindroos et al., 1998; Bonner, Rice et al., 1998; Coin et al., 1996; Mangum et al., 2006; Nagaoka et al., 1990). Additionally, vanadium ions and ROS have been shown to induce phosphorylation of protein tyrosine residues and inhibit protein tyrosine phosphatases (Hecht & Zick, 1992; Samet et al., 1997; Zick & Sagi-Eisenberg, 1990; Zor et al., 1993). Through this mechanism, LPS and V₂O₅ could increase PDGF-Rα signaling without increasing PDGF-Rα gene expression. The
differential fibrotic responses in the two V$_2$O$_5$-treated groups may also have been affected by LPS-induced differences in the levels of other mediators of fibrosis that were not examined in this study, such as the CC- and CXC-chemokine receptor families, connective tissue growth factor (CTGF), and IL-4 (Wynn, 2008).

The BO lesions in both large and small bronchioles were an unusual finding. The polypoid, intrabronchiolar fibroproliferative masses resembled bronchiolitis obliterans organizing pneumonia (BOOP) seen in a number of pulmonary disorders in humans, though in humans, BOOP lesions are restricted to respiratory bronchioles, alveolar ducts, and alveoli (King & Kinder, 2008). The subepithelial fibrotic lesions resembled constrictive bronchiolitis in humans. These types of lesions are occur when there is severe damage to the airway epithelium (Dungworth, 1993; King & Kinder, 2008). Accumulation of neutrophils at the site of injury also plays a role in the pathogenesis of these lesions by causing additional damage to the airway epithelium and matrix through release of inflammatory mediators (King & Kinder, 2008). The role of LPS in the development of these lesions is supported by four observations. First, in the smaller polypoid lesions where the fibrotic tissue did not extend beyond the confines of its base, which likely represent early BO lesions, the epithelium overlying the polyp was thin and the cells elongated, consistent with regenerating epithelium. Second, the BO lesions were more prevalent in the LPS/V$_2$O$_5$ group than the PBS/V$_2$O$_5$ group. Third, the airway epithelial necrosis was more severe in the LPS/V$_2$O$_5$ group than the PBS/V$_2$O$_5$ group. Fourth, the rats pre-exposed to LPS had higher numbers of neutrophils
recovered in the BAL fluid. These findings support our hypothesis that LPS pre-exposure exacerbates airway epithelial damage and necrosis and the development of BO lesions.

In summary, we report that LPS pre-exposure augments $V_2O_5$-induced lung inflammation, COL1A2 gene expression, interstitial and intrabronchiolar fibrosis, and airway epithelial damage in rats. The mechanism by which LPS exerts this effect is unclear, but may involve PDGF or TGF-β signaling. The finding of BO is unusual and is likely associated with an LPS-mediated increase in airway epithelial damage. Further experiments with lower doses of $V_2O_5$ that are better tolerated by the rats will need to be conducted to determine the utility of the combination of LPS and $V_2O_5$ exposure as a model for human BO and to confirm the histopathological findings in these rats. These results support our hypothesis that pre-existing inflammation exacerbates the fibrogenic response of the lungs to vanadium pentoxide.
Table 5-1. Differential counts of inflammatory cells in bronchoalveolar lavage (BAL) fluid collected from rats 24 hrs after exposure to V$_2$O$_5$ with or without a 24-hr LPS pre-exposure.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Total BAL Cells (X $10^3$)</th>
<th>Macrophages (% total cells)</th>
<th>PMNs (% total cells)</th>
<th>Lymphocytes (% total cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS/PBS</td>
<td>996.9 ± 58.3</td>
<td>92.4 ± 1.9</td>
<td>4.7 ± 1.2</td>
<td>2.8 ± 0.8</td>
</tr>
<tr>
<td>LPS/PBS</td>
<td>1,729.0 ± 281.4</td>
<td>93.3 ± 2.1</td>
<td>4.3 ± 1.9</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>PBS/V$_2$O$_5$</td>
<td>3,748.0 ± 1209.0$^a$</td>
<td>20.1 ± 5.1$^b$</td>
<td>77.1 ± 5.6$^b$</td>
<td>2.9 ± 0.7</td>
</tr>
<tr>
<td>LPS/V$_2$O$_5$</td>
<td>19,759.2 ± 4114.2$^{c,d}$</td>
<td>18.6 ± 2.3$^b$</td>
<td>80.1 ± 2.4$^b$</td>
<td>1.3 ± 0.2</td>
</tr>
</tbody>
</table>

Differential data are expressed as percent of total cells retrieved from bronchoalveolar lavage. $^a$p<0.01 compared to controls, $^b$p<0.001 compared to controls, $^c$p<0.001 compared to controls, or $^d$p<0.001 compared to PBS/V$_2$O$_5$ as determined by one-way ANOVA with post Tukey’s test. PBS – Phosphate Buffered Saline; LPS – Lipopolysaccharide; PMN – Polymorphonuclear Cell; V$_2$O$_5$ – Vanadium Pentoxide
**Table 5-2.** Mean histopathologic lesion scores 7 days after V₂O₅-exposure.

<table>
<thead>
<tr>
<th>Lesion</th>
<th>PBS/PBS</th>
<th>LPS/PBS</th>
<th>PBS/V₂O₅</th>
<th>LPS/V₂O₅</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Lung Affected</td>
<td>0.67</td>
<td>0</td>
<td>36.0 (16.9)</td>
<td>50.3*</td>
</tr>
<tr>
<td>Bronchiolitis Obliterans</td>
<td>0</td>
<td>0</td>
<td>1.0 (0)</td>
<td>4.0</td>
</tr>
<tr>
<td>Fibroblast Foci</td>
<td>0</td>
<td>0</td>
<td>8.8 (2.7)</td>
<td>14.0*</td>
</tr>
<tr>
<td>Number of Fibroblasts</td>
<td>0</td>
<td>0</td>
<td>2.0 (1.3)</td>
<td>3.2</td>
</tr>
<tr>
<td>Collagen</td>
<td>0</td>
<td>0</td>
<td>2.0 (1.3)</td>
<td>2.8</td>
</tr>
<tr>
<td>Chronic Inflammation</td>
<td>0</td>
<td>0</td>
<td>2.0 (1.3)</td>
<td>3.0</td>
</tr>
<tr>
<td>Alveolar Type II Cell Hyperplasia</td>
<td>0</td>
<td>0</td>
<td>2.0 (1.3)</td>
<td>3.0*</td>
</tr>
<tr>
<td>Alveolar Epithelial Necrosis</td>
<td>0</td>
<td>0</td>
<td>2.3 (1.7)</td>
<td>3.0</td>
</tr>
<tr>
<td>Goblet Cell Hyperplasia</td>
<td>0</td>
<td>0</td>
<td>2.5 (2.7)</td>
<td>2.4</td>
</tr>
</tbody>
</table>

All lesions were subjectively scored on a relative scale of 0-4 (0 – no lesion, 1 – minimal, 2 – mild, 3 – moderate, 4 – marked) using H&E stained lung sections unless otherwise indicated. Numbers in parentheses represent the mean scores for the PBS/V₂O₅ group with animal #70 excluded. *p<0.05 compared to PBS/V₂O₅ group with animal #70 excluded. aMeasured as the percentage of total lung area examined, bMeasured as the number of animals per group with at least one BO lesion, cMeasured as the total number of fibroblastic foci per animal, dEstimated using Mason’s trichrome-stained lung sections. PBS–Phosphate Buffered Saline; LPS–Lipopolysaccharide
Figure 5-1. Markers of lung injury in BAL fluid. LDH (A) and total protein (B) levels in BAL fluid of rats 24 hours post-V_2O_5 exposure. Both are increased in rats exposed to V_2O_5 compared to controls, but there is no difference between the PBS/V_2O_5 and LPS/V_2O_5 groups.
Figure 5-2. TGF-β1 protein levels in the BAL fluid 24 hrs post-V₂O₅ exposure. TGF-β1 protein, as measured by ELISA, is increased in the BAL fluid from rats exposed to V₂O₅ with and without LPS pretreatment, but there is no difference between the PBS/V₂O₅ and LPS/V₂O₅ groups.
**Figure 5-3.** Lung histopathology from V$_2$O$_5$-treated animals with and without LPS pretreatment 24 hrs or 7 days postexposure. (A) LPS/V$_2$O$_5$-treated rat 24 hrs postexposure. Marked suppurative bronchiolitis with sloughing of the epithelium (arrows) and segmental disruption of the airway wall (asterisk). H&E, Bar = 150 μm. (B) LPS/V$_2$O$_5$-treated rat 24 hrs post-exposure. Small bronchiole epithelial necrosis. Note the necrotic epithelial cells on the surface (arrows) and neutrophilic inflammation. H&E, Bar = 50 μm. (C) LPS/V$_2$O$_5$-treated rat 7 days postexposure. Bronchiolitis obliterans. An intraluminal fibrotic polyp obstructs the airway. H&E, Bar = 100 μm. (D) Masson’s trichrome-stained section of C showing the collagen (blue) in the intraluminal polyp. Bar = 100 μm. (E) LPS/V$_2$O$_5$-treated rat 7 days post-exposure. Bronchiolitis obliterans. In this form, subepithelial fibrosis causes narrowing of the airway lumen. The smooth muscle of the airway (arrowheads) indicates the airway borders. Note the multiple, small airway lumina and increased collagen (blue). Masson’s trichrome stain, Bar = 100 μm. (F) LPS/V$_2$O$_5$-treated rat 7 days postexposure. Fibroblastic focus (arrowheads) lined by hyperplastic ATI pneumocytes. Note the thickening of adjacent alveolar septa. H&E, Bar = 50 μm. (G) PBS/V$_2$O$_5$-treated rat 7 days postexposure. Bronchiolar epithelial hyperplasia and minimal airway fibrosis with a patent lumen. H&E, Bar = 100 μm. (H) PBS/V$_2$O$_5$-treated rat 7 days post-exposure. Chronic interstitial inflammation (left) and inter-
stitial remodeling (right). On the left, the alveolar septa are thickened by spondloid cells, ECM, inflammatory cells, and hyperplastic ATII pneumocytes. On the right, there is destruction of the parenchyma with replacement by inflammatory cells, ECM, neovascularization, and scattered spindloid cells. H&E, Bar = 50 μm.
**Figure 5-4.** Col1a2 gene expression in whole lung 7 days post-\(V_2O_5\) exposure. mRNA levels measured by RT-PCR. Col1a2 gene expression is significantly increased in the LPS/\(V_2O_5\) group compared to PBS/PBS and LPS/PBS controls and to the PBS/\(V_2O_5\) group. a - \(P<0.01\) compared to the PBS/PBS group, b - \(P<0.05\) compared to the LPS/PBS and LPS/\(V_2O_5\) groups.
Figure 5-5. Immunohistochemistry for PDGF-AA and PDGF-Rα in the lungs of V₂O₅-treated rats 7 days postexposure. (A) PDGF-A immunoreactivity in a rat treated with V₂O₅ after pre-exposure to LPS. The main sources of PDGF-AA protein include the bronchiolar epithelium (open arrowheads), bronchiolar smooth muscle, (arrowheads), endothelium and vascular smooth muscle (open arrow), and alveolar macrophages (arrows). Hematoxylin counterstain, Bar = 50 μm. (B) Negative control of the lung section in A. Hematoxylin counterstain, Bar = 50 μm. (C) PDGF-Rα immunoreactivity in a rat treated with V₂O₅ without pre-exposure to LPS. Cells expressing PDGF-Rα include bronchiolar smooth muscle (arrowhead), alveolar macrophages (arrows), and peribronchiolar fibroblasts (open arrows). Hematoxylin counterstain, Bar = 50 μm. (D) Negative control of the lung section in C. Hematoxylin counterstain, Bar = 50 μm.
CHAPTER SIX

GENERAL SUMMARY AND CONCLUSIONS

This dissertation has examined the relationship between pre-existing inflammation and the fibrotic effects of MWCNT or $V_2O_5$. The involvement of PDGF-AA and its cognate receptor, PDGF-Rα, in this relationship were also examined. My hypothesis was that pre-existing inflammation would exacerbate pulmonary fibrogenesis caused by exposure to known fibrotic agents, in part, through increased signaling of PDGF-Rα. Numerous in vitro experiments and two in vivo studies in male Sprague-Dawley rats were conducted to generate supportive data for this hypothesis.

MWCNT and SWCNT have been shown to cause interstitial lung fibrosis while $V_2O_5$ has been shown to cause fibrosis of the airways and of the interstitium (Bonner et al., 2000; Lam et al., 2004; Mangum et al., 2006; Muller et al., 2005; National Toxicology Program, 2002; Shvedova et al., 2005). Furthermore, it has been shown that the mechanism by which $V_2O_5$ induces pulmonary fibrosis in rats involves PDGF-Rα signaling (Bonner, Lindroos et al., 1998; Bonner, Rice et al., 1998). In this dissertation, evidence is presented that the mechanism by which LPS and MWCNT induce fibrosis also involves PDGF-Rα signaling (Cesta et al., 2009). We show that, in vitro, pre-exposure to LPS followed by exposure to MWCNT or CB nanoparticles has a synergistic effect on PDGF-A gene expression by
NR8383 cells, a rat alveolar macrophage cell line (Cesta et al., 2009). MWCNT and CB nanoparticles alone increased PDGF-A mRNA levels approximately 2-fold and LPS alone increased PDGF-A mRNA levels approximately 10-fold at 4 hrs compared to controls (Cesta et al., 2009). However, LPS pre-exposure followed by MWCNT or CB nanoparticles increased PDGF-A mRNA 18- to 20-fold compared to controls at the same time-point, approximately double the levels induced by LPS alone (Cesta et al., 2009). In the same study, PDGF-AA protein levels were shown to be increased in the BAL fluid of rats pre-exposed to LPS followed by MWCNT 1 day post-exposure and the fibrosis caused by the combination of LPS and MWCNT was greater than that caused by MWCNT alone (Cesta et al., 2009).

In the V2O5 study, there is evidence that LPS exacerbates V2O5-induced interstitial lung fibrosis and BO, a severe form of airway fibrosis in which the airways become occluded by subepithelial fibrosis (constrictive bronchiolitis) or intraluminal fibrotic polyps. BO is a frequent and life-threatening complication of lung transplantation (Andersson-Sjoland, Erjefalt, Bjmer, Eriksson, & Westergren-Thorsson, 2009; Elssner & Vogelmeier, 2001; Grossman & Shilling, 2009; Nicod, 2006; Sato, Keshavjee, & Liu, 2009). It is thought that, in both animals and humans, severe airway epithelial damage leads to the excessive fibroproliferation characteristic of bronchiolitis obliterans (Dungworth, 1993; Elssner & Vogelmeier, 2001; Grossman & Shilling, 2009; Nicod, 2006). The damage to the epithelium may be due to any number of causes, such as exposure to toxins, allograft rejection after lung transplantation, or severe viral or bacterial infection, and may be amplified by neutrophil-
mediated damage (Dungworth, 1993; Elssner & Vogelmeier, 2001; Grossman & Shilling, 2009; Nicod, 2006). In this study, there is evidence that LPS pre-exposure amplified the bronchiolar epithelial damage induced by V$_2$O$_5$. There is also evidence that LPS pre-exposure worsened V$_2$O$_5$-induced interstitial fibrosis. The mechanisms through which LPS exerts these effects are not clear. There was no detectable difference in PDGF-AA or TGF-β1 protein levels in the BAL fluid, or PDGF-A, PDGF-C, PDGF-Rα, or TGF-β1 gene expression in whole lung samples.

In these studies, we tested one type of MWCNT from one source. MWCNT differ in size distribution (length and diameter), surface area, purity, amount and type of residual metal catalyst, chirality, shape (some may have more bends or coils than others), and other parameters depending on their source and method of production. Their aggregation states and degree and type of functionalization also vary, so our results may not apply to other MWCNT or even the same MWCNT tested under different conditions. Two inhalation studies examined the effects of a 3-month exposure to MWCNT (Ma-Hock et al., 2009; Pauluhn, 2010). The MWCNT used in these studies were from different sources; one sample was produced by Bayer MaterialScience, Leverkusen, Germany and the other was produced by Nanocyl S. A., Sambreville, Belgium. The Bayer sample contained approximately 0.5% Co and were 200-300 μm in length, and the Nanocyl sample contained approximately 9.6% Al$_2$O$_3$ and trace amounts of Co and Fe and were 0.1 – 10 μm in length (all other parameters were similar) (Ma-Hock et al., 2009; Pauluhn, 2010). The results, however, were similar and
were seen at similar doses. In preliminary studies, we found that the effects SWCNT (Helix Material Solutions, Richardson, TX) with and without LPS pretreatment on PDGF-A and PDGF-Rα gene expression in RLF and NR8383 cells in vitro (Appendix B) were similar to those of MWCNT with and without LPS pretreatment (Cesta et al., 2009). This suggests that CNT with differing physicochemical characteristics tested under similar conditions could yield consistent results.

Many metals are known to cause lung disease in humans and laboratory rodents, including Al, Co, Fe, Ni, all of which have been identified as residual metal catalysts in CNT (Fontenot & Amicosante, 2008; Kelleher et al., 2000; Nemery, 1990). The amounts of these metals in the CNT are typically very low, and whether or not these metals are released from the CNT after inhalation is unclear. Thus, the contribution of residual metal catalysts to CNT-induced lung disease has been a topic of controversy. The MWCNT used in the studies reported in this dissertation contained Ni (5.53 ± 3.92%) and small amounts of La (0.06%) (Ryman-Rasmussen, Tewksbury et al., 2009). Due to the design of these studies, we cannot determine the contribution of these metals (Ni is of particular concern) to the development of the MWCNT-induced lung lesions or to the in vitro increase in PDGF-A and PDGF-Rα gene expression. Other studies have examined the effects of purified CNT, CNT treated to remove the residual metals, both in vitro and in vivo, and the purified CNT induced pro-inflammatory and/or pro-fibrotic responses in all cases (Kagan et al., 2006; Lam et al., 2004; Shvedova, Fabisiak et al., 2008; Shvedova et al., 2005; Shvedova et al., 2007; Shvedova, Kisin, Murray,
Kommineni et al., 2008; Simon-Deckers et al., 2008; Ye et al., 2009). It is, however, extremely difficult to completely remove residual metals from CNT, regardless of the method used, and in all of these studies, the purified CNT still contained small amounts of metals, mainly Fe (Kagan et al., 2006; Lam et al., 2004; Shvedova, Fabisiak et al., 2008; Shvedova et al., 2005; Shvedova et al., 2007; Shvedova, Kisin, Murray, Kommineni et al., 2008; Simon-Deckers et al., 2008; Ye et al., 2009). In the two studies comparing the effects of purified and unpurified SWCNT, one found that the Fe within the SWCNT had a greater effect on the redox status of stimulated RAW 264.7 macrophages in vitro, and the other found no effect of the residual metals on SWCNT-induced mouse lung lesions in vivo. This apparent discrepancy brings into question the relevance of in vitro studies using monocultures of primary cells or cell lines, in which the complex interactions of multiple cell types and extracellular components found in vivo are lacking. Overall, these studies suggest that CNT themselves have the capacity to induce lung toxicity regardless of their metal content, but modification of these effects by residual metals must remain a consideration.

An obvious limitation to the V2O5 study is that the dose V2O5 (4 mg/kg) used was too high. All the rats exposed to V2O5, both with and without LPS pre-exposure, developed severe respiratory distress 2-4 days after exposure. One of these animals was found dead 4 days post-exposure and the remaining animals had to be euthanized 7 days post-exposure due to worsening of the respiratory distress. The effects of such an excessive dose of V2O5 on the results of this study are not clear, but could have overwhelmed the innate immune system of
these rats, masking any effects LPS may have had on growth factor production. Also, their euthanasia at 7 days may have been too soon to realize any obvious effects on collagen deposition, though there were other indications of pulmonary fibrosis.

Another limitation of the V$_2$O$_5$ study is the lack of statistical significance of the histopathologic lesions in the lungs of the LPS/V$_2$O$_5$ group compared to the PBS/V$_2$O$_5$ group. Though not statistically significant, the increases in the scores for lesions consistent with pulmonary fibrosis, including BO, in the LPS/V$_2$O$_5$ group compared to all other groups, including the PBS/V$_2$O$_5$ group, were notable. The high number of lesions that increased in severity suggests that they are biologically significant. The low number of animals per group in this study, however, did not provide enough statistical power to be able to detect these changes as significant (see chapter 4). Additionally, the lesions in one of the rats in the PBS/V$_2$O$_5$ group (animal #70) were so severe as to suggest that this animal may have been a statistical outlier, which, unfortunately, cannot be tested because of the low number of animals per group. When animal #70 was excluded from the statistical analyses, several lesions consistent with chronic inflammation and pulmonary fibrosis were found to be statistically significant (% lung affected, number of fibroblastic foci, alveolar septal thickening, and alveolar type II pneumocytes hyperplasia).

In the *in vivo* studies presented here, IT instillation was the exposure method used. IT instillation and pharyngeal aspiration, a variant of IT instillation where the test agent is
placed at the back of the throat and the tongue held until the material is aspirated, has been criticized as an exposure method because it does not mimic actual, environmental or occupational exposure conditions. IT instillation and pharyngeal aspiration have some advantages. They are easier, cheaper, and faster than inhalation exposure, and bypass the upper respiratory tract. However, with IT instillation or pharyngeal aspiration, a high, single dose of test material is administered as a bolus. This may be particularly troublesome in studies on CNT because of their propensity to aggregate, which would alter the effective size of the particles being studied. Additionally, the lung distribution of test materials and the clearance and retention of particles, as well as the pattern of injury and BAL fluid parameters that result from IT instillation or pharyngeal aspiration often differs from that of inhalation exposure (Driscoll et al., 2000; Osier, Baggs, & Oberdorster, 1997; Osier & Oberdorster, 1997). The two studies described in chapter three in which the effects of CNT administered by inhalation and IT instillation of pharyngeal aspiration were compared, however, showed similar lesions for the two exposure techniques, differing mainly in severity and distribution (J.-G. Li et al., 2007; Shvedova, Kisin, Murray, Johnson et al., 2008). The goal of our studies was to compare the effects of MWCNT or V_2O_5 with and without LPS and to explore the involvement of PDGF-Rα signaling in the pathogenesis of the lesions. Since we were not focusing on the risk of exposure in humans, and used the same exposure method for all treatment groups, our use of IT instillation is justified and does not jeopardize the validity of our conclusions.
This dissertation shows that pre-existing inflammation (induced in these studies by LPS pre-exposure) exacerbates the pulmonary fibrotic effects of MWCNT and V₂O₅ and that PDGF-Rα signaling is likely to be involved, at least in rats. The upregulation of PDGF-Rα expression by LPS has been shown to occur in rats, but has not been shown to occur in other species (Coin et al., 1996). In rats, LPS can upregulate PDGF-Rα gene expression in RLF both directly and through induction of IL-1β secretion (Boyle et al., 1999; Brass et al., 2007; Ingram et al., 2004). Furthermore, particles, including CNT, and vanadium induce PDGF-A secretion by epithelial cells and macrophages (Cesta et al., 2009; Mangum et al., 2006). PDGF-A is chemotactic and a potent mitogen for fibroblasts. However, TGF-β1 is also central to the fibrotic process. TGF-β1 promotes collagen gene expression in mesenchymal cells and has also been shown to induce epithelial-mesenchymal transition (EMT) of alveolar type I cells (Willis & Borok, 2007). In the second study, TGF-β1 protein levels were increased in V₂O₅-treated animals, but there was no difference between the LPS pre-treated group and the non-pre-treated group. V₂O₅ was clearly involved in the fibrotic response to V₂O₅, but its role in the exacerbation of the fibrosis by LPS is unclear. Numerous other growth factors, chemokines, and cytokines are also involved in the fibrotic process (see chapter 3). ROS are also thought to be central mediators of lung injury and fibrosis in a number of diseases and have been shown to be generated in the lungs in response to LPS, particles, or V₂O₅ exposure (Donaldson & Tran, 2002; Dye et al., 1999; Germain, Corbel, Belleguic, Boichot, & Lagente, 2001; Hsu & Wen, 2002; Valko et al., 2005). In fact, oxidative stress is the central mechanism through which particles and metals exert their toxic
effects (Donaldson et al., 2006; Donaldson & Tran, 2002). One would expect that ROS play a significant role in the exacerbation of pulmonary fibrosis by LPS. The role of ROS, RNS, and other growth factors and cytokines, such as CTGF, CC and CXC chemokines and their receptors, IL-4, IL-13, etc., in the exacerbation of the fibrotic response to MWCNT or V$_2$O$_5$ were not examined in the experiments reported in this dissertation.

Additional studies examining the roles of other mediators and signaling pathways on the exacerbating effect of LPS pre-exposure on MWCNT- or V$_2$O$_5$-induced lung fibrosis could yield important information regarding the pathogenesis of pulmonary fibrotic diseases in humans and aid in the identification of subpopulations that may more susceptible to metal or particle-induced lung disease. I would be particularly interested in assessing the role of ROS in this process. Most effects of LPS are mediated by TLR4, but other receptors or pathways may also be involved. Examining the role of TLR4 would also provide useful information, particularly for identifying susceptible subpopulations where TLR4 polymorphisms may play a role. Studies to examine the involvement of TLR4 could include genetically modified mice (or rats) in which the TLR4 gene has been deleted or inactivated. However, C3H/HeJ and C57BL/10ScCr mice are resistant to the effects of LPS due to mutations in the TLR4 gene and would be well suited to such studies (Poltorak et al., 1998). Another, perhaps more obvious additional study would be to repeat the second study examining the effects of LPS on V$_2$O$_5$-induced lung fibrosis using a lower and better tolerated dose of V$_2$O$_5$ and more animals per group. This would allow the rats time to develop more mature fibrotic lesions.
and would avoid the complications generated by the excessive dose and the low statistical power in this study. It would also be useful to explore the utility of the LPS/V2O5 treatment regimen that we used in this experiment as a potential animal model for BO. Lastly, it would be of interest to repeat these studies using other models of lung inflammation, such as tobacco smoke, nitric acid, or SO2 to confirm that the exacerbation of MWCNT- and V2O5-induced lung fibrosis was due to pre-existing inflammation rather than some other property or activity specific to LPS.

It is clear that human exposure to CNT is nearly inescapable and that inhalation is a likely route. What is not clear, however, is whether or not inhalation of CNT will result in pulmonary disease in exposed human beings. Current knowledge, including the results presented in this dissertation, suggests the potential for CNT-related toxic effects in humans, but there is no human toxicity data to support this premise. Furthermore, in vivo inhalation studies of MWCNT in laboratory animals have largely produced relatively minimal lung lesions that did not result in significant clinical signs of disease or toxicity (Ellinger-Ziegelbauer & Pauluhn, 2009; Ma-Hock et al., 2009; Mitchell et al., 2007; Pauluhn, 2010; Ryman-Rasmussen, Cesta et al., 2009; Ryman-Rasmussen, Tewksbury et al., 2009). However, in the one study that examined the effects of inhaled MWCNT in the face of pre-existing inflammation, ovalbumin sensitization and challenge plus MWCNT resulted in airway fibrosis whereas MWCNT alone did not. In all studies, in vitro or in vivo using intratracheal instillation or pharyngeal aspiration, that examined the combined effects of
particles, including NP, and an agent of pulmonary inflammation (LPS, ozone, cigarette smoke, etc.), including those in this dissertation, the combined exposure resulted in more severe lung lesions, increases in BAL fluid parameters, increased cytotoxicity, or increased production of chemokines (Arimoto et al., 2005; Cesta et al., 2009; Han, Andrews, Gairola, & Bhalla, 2008; H. Inoue et al., 2009; K. Inoue, Takano, Yanagisawa, Hirano, Ichinose et al., 2006; K. Inoue et al., 2007; K. Inoue, Takano, Yanagisawa, Hirano, Sakurai et al., 2006; K. Inoue et al., 2004; K. Inoue, Takano, Yanagisawa, Sakurai et al., 2006; Pace et al., 2008; Ryman-Rasmussen, Tewksbury et al., 2009; Takano et al., 2002; Wagner, Hotchkiss et al., 2001; Wagner, Van Dyken et al., 2001; Yanagisawa et al., 2003).

In conclusion, the research reported in this dissertation supports my hypothesis that pre-existing inflammation has the capacity to exacerbate the deleterious pulmonary effects of agents that cause pulmonary fibrosis. This is an important concept for two reasons. First, it will help to identify human subpopulations, such as those with chronic bronchitis, asthma, or cigarette smokers (including those without COPD) that are more susceptible to the effects of occupational or environmental exposure to airborne particles, metals, or other fibrogenic materials. This may lead to increased efforts to prevent occupational exposures in these subpopulations, thereby decreasing the overall risk and financial impact of occupational lung diseases. Second, some ubiquitous environmental contaminants or pollutants (e.g., LPS and ozone) have been shown to induce lung inflammation, and these results suggest a role for these agents in the pathogenesis of pulmonary fibrosis.


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APPENDICES
APPENDIX A

ASSESSMENT OF COLLAGEN DEPOSITION IN THE LUNGS OF RATS EXPOSED TO MWCNT OR CB WITH OR WITHOUT LPS PRETREATMENT BY IMAGE ANALYSIS

Collagen deposition in the lungs of rats exposed to MWCNT or CB, with and without pretreatment with LPS, as determined by histopathologic assessment, was confirmed by image analysis. This data shows an increase in collagen deposition in the lungs of the MWCNT-exposed, LPS pretreated rats compared to the other groups (Figure A1). Statistical analysis of this data was not performed, however, the amount of collagen measured in this group is nearly double that of the MWCNT only group.

For image analysis, lung sections were stained with picrosirius red and scanned at 40X magnification using an Aperio Scanscope XT digital slide scanner (Aperio Technologies, Vista, CA). Five regions of roughly equal size from each lung containing nanoparticles and fibroproliferative lesions were chosen at 40X magnification. In rats that did not receive nanoparticles, five regions of the lung containing similar lung structures were chosen randomly. Regions containing large airways and blood vessels were not selected for analysis due to the increased collagen associated with these structures. The image analysis was performed on the selected regions using the Aperio Color Deconvolution Algorithm. This
software counted the number of pixels in the areas that contained stained tissue (all tissue) and the number of pixels that contained red-stained tissue (collagen). From this data, the total area of red-stained tissue, or collagen, was determined and reported as the total area of collagen staining per total area of analysis.

Picrosirius red stains the large and small collagen fibers, including those associated with basement membranes. Consequently, picrosirius red stains the basal laminae of airway and parenchymal epithelia and of the endothelium as well as the collagen deposited during fibrosis. As a result, quantitation of the fibrosis using the image analysis technique would include basal laminae. However, this is not expected to affect the results because collagen deposition was confirmed using the Mason’s trichrome stain (reported in chapter 4 of this dissertation), which does not stain basal laminae. Also, while the basal laminae may be thickened after particle exposure, they would likely represent a small percentage of the picrosirius red-stained tissue. Furthermore, basement membrane production is associated with epithelial damage and is involved in remodeling of the ECM. Thus, an increase in the amount of basal laminae or basal lamellar proteins may be considered a part of the fibrotic process, and inclusion of these components in the assessment of tissue fibrosis (by staining with picrosirius red) is appropriate.
Figure A1. Assessment of collagen deposition in the lungs of rats exposed to MWCNT or CB with or without LPS by image analysis. The data is consistent with the results presented in chapter 4, confirming the increase in collagen deposition in the LPS pretreated, MWCNT-exposed rats compared to controls. PBS—phosphate buffered saline; LPS—lipopolysaccharide; CB—carbon black nanoparticles; MWCNT—multi-walled carbon nanotubes.
In addition to the experiments presented in the body of this dissertation, additional experiments were performed examining the \textit{in vitro} effects of single-walled carbon nanotubes (SWCNT) with and without LPS pretreatment on primary rat lung fibroblasts (RLF) and NR8383 rat alveolar macrophages. In these experiments, expression of genes for PDGF-A, PDGF-C, PDGF-R\(\alpha\), TGF-\(\beta\), CTGF, and COL1A2 was evaluated by RT-PCR. These experiments were performed in exactly the same manner as those presented in chapter 4 of this dissertation, so for a description of the materials and methods used, please refer to pages 100-102.

The physical characteristics of the SWCNT were provided by the manufacturer (Helix Material Solutions, Richardson, TX), which was the same company that provided the MWCNT for the experiments reported in chapter 4. Unlike the MWCNT, the characteristics of the SWCNT were not confirmed by an independent analysis. The SWCNT were approximately 1.3 nm in diameter (measured using transmission electron microscopy), 0.5-40 micrometers in length (measured using transmission and scanning electron microscopy),
had a surface area of 300-600 m\textsuperscript{2}/g (measured using backscatter electron transmission), and contained 2.6% Co and 1.7% Mo as residual catalysts.

The results of these experiments are similar to the results of the in vitro experiments on MWCNT. In NR8383 rat alveolar macrophages, LPS induced the expression of the PDGF-A and PDGF-R\(\alpha\) genes at 4 hrs—approximately 35-fold for PDGF-A and 21-fold for PDGF-R\(\alpha\)—but not of the TGF-\(\beta\) gene (Figure B1). Treatment with SWCNT alone had no effect on the expression of these genes. Pretreatment with LPS resulted in a synergistic increase in PDGF-A expression, which increased to approximately 95-fold, but had no effect on PDGF-R\(\alpha\) expression. At 24 hrs post-exposure, the effects on PDGF-A expression persisted, but PDGF-R\(\alpha\) expression returned to control levels (Figure B2). In RLF, LPS induced the expression of PDGF-R\(\alpha\) by 4 hrs post-exposure, which persisted to 24 hrs post-exposure, but SWCNT, either alone or after LPS pretreatment, had no effect on PDGF-R\(\alpha\) gene expression (Figures B3 and B4).

Though no statistical analysis was performed on these data, the increases in PDGF-A and PDGF-R\(\alpha\) gene expression are clearly treatment-related and are clearly greater than controls. These results suggest that LPS pretreatment would have the same \textit{in vivo} effects on SWCNT-induced lung lesions as it does on MWCNT-induced lung lesions.
Figure B1. Effect of LPS pretreatment on SWCNT-induced profibrotic gene expression in NR 8383 rat alveolar macrophages 4 hrs post-exposure in vitro. LPS induces PDGF-A expression, but SWCNT or CB nanoparticles do not. However, LPS pretreatment followed by SWCNT or CB nanoparticles results in a synergistic increase in PDGF-A gene expression. LPS also induced the expression of PDGF-Rα gene expression, but neither SWCNT nor CB nanoparticles had an effect. TGF-β gene expression is not affected at four hrs. LPS–lipopolysaccharide; CB–carbon black nanoparticles; SWCNT–single walled carbon nanotubes. CB–carbon black nanoparticles; LPS–lipopolysaccharide; PDGF–Platelet-derived growth factor; PDGF-Rα–Platelet-derived growth factor receptor α; SWCNT–single walled carbon nanotubes; TGF–Transforming growth factor.
Figure B2. Effect of LPS pretreatment on SWCNT-induced profibrotic gene expression in NR 8383 rat alveolar macrophages 24 hrs post-exposure \textit{in vitro}. The effect of LPS and LPS followed by SWCNT or CB nanoparticles on DGF-A gene expression persists to the 24 hr time point, but the effect on PDGF-Rα expression does not. TGF-β gene expression is not affected at 24 hrs. LPS–lipopolysaccharide; CB–carbon black nanoparticles; SWCNT–single walled carbon nanotubes. CB–carbon black nanoparticles; LPS–lipopolysaccharide; PDGF–Platelet-derived growth factor; PDGF-Rα–Platelet-derived growth factor receptor α; SWCNT–single walled carbon nanotubes; TGF–Transforming growth factor.
**Figure B3.** Effect of LPS pretreatment on SWCNT-induced profibrotic gene expression in primary rat lung fibroblasts 4 hrs post-exposure *in vitro*. LPS induces PDGF-Rα expression, but SWCNT or CB nanoparticles do not. SWCNT or CB nanoparticles have no effect on *Pdgf-α* gene expression either with or without LPS. PDGF-A, PDGF-C, TGF-β, CTGF, and COL1A2 gene expression do not appear to be affected at four hrs. CB—carbon black nanoparticles; COL1A2—collagen 1A2; CTGF—connective tissue growth factor; LPS—lipopolysaccharide; PDGF—platelet-derived growth factor; PDGF-Rα—Platelet-derived growth factor receptor α; SWCNT—single walled carbon nanotubes; TGF—Transforming growth factor.
**Figure B4.** Effect of LPS pretreatment on SWCNT-induced profibrotic gene expression in primary rat lung fibroblasts 24 hrs post-exposure *in vitro*. LPS induces PDGF-Rα expression, but SWCNT nanoparticles do not. SWCNT or CB nanoparticles have no effect on PDGF-Rα gene expression either with or without LPS. PDGF-A, PDGF-C, TG-β, CTGF, and COL1A2 gene expression do not appear to be affected at four hrs. Note–in this experiment, RT-PCR on the SWCNT alone and LPS/CB groups did not work. The reason was never identified, but the data on the other groups are consistent with the data presented above as well as in chapter 4. CB–carbon black nanoparticles; COL1A2–collagen 1A2; CTGF–connective tissue growth factor; LPS–lipopolysaccharide; PDGF–Platelet-derived growth factor; PDGF-Rα–Platelet-derived growth factor receptor α; SWCNT–single walled carbon nanotubes; TGF–Transforming growth factor.