PULMONARY PATHOLOGIES FOLLOWING INHALATION OF MULTI-WALLED CARBON NANOTUBES AT OCCUPATIONAL LEVELS

By

Marshal Anthony Phipps, Jr.

Director of Thesis: Dianne M. Walters, Ph.D.

Home Department: Department of Biomedical Science

Multi-walled carbon nanotubes (MWCNT) are a nanomaterial that is growing in use and popularity. The health effects of occupational pulmonary exposure to MWCNT are currently unknown. The goals of this study were to build a dust generator capable of producing occupational levels of MWNCT and to examine pulmonary effects of occupational levels of inhaled dry MWCNT. We designed, built, and tested a dust generator capable of producing MWCNT concentrations in the occupational exposure range (25,000 to 50,000 particles/cm^3). In a time course study, C57BL/6J male mice were exposed to either a dust of MWCNT at a daily average of approximately 37,000 particles/cm^3 and a daily peak of about 50,000 particles/cm^3 or to air alone. Six mice per group were exposed for 4 hours per day for 5 days a week for 2 weeks and sacrificed 1, 3, 7, 10, 14, 28, and 84 days post-exposure. In a strain comparison study DBA/2J and A/J mice underwent the same exposure and were sacrificed at 14 days post-exposure. For both studies bronchoalveolar lavage (BAL) fluid was collected to assess cellular profile and protein content. The right lung was used for collagen measurement. The left lung was used for histological evaluation. Total protein, a measure of lung permeability, did not change significantly after MWCNT exposure compared to air controls at any time point. Likewise, eosinophil and neutrophil cell counts were not significantly different

between air and MWCNT-exposed mice. However, compared to air controls, MWCNT-exposed mice had increased numbers of total cells and macrophages at 28 days post exposure and increased monocytes from 10 to 14 days exposure. The presence of MWCNT was visually noted in BAL cell pellets, histology slides, and lung homogenate membrane pellets at all of the time points. Dark field microscopy showed MWCNT in BAL cells at all of the time points in MWCNT exposed mice. Collagen levels were not different between exposure groups at any time point. The strain comparison found that C57BL/6J mice had increases in monocytes and lymphocytes at 14 days post exposure and A/J mice showed a trend towards an increase in lung protein levels in MWCNT exposed mice at 14 days post exposure. MWCNT were visually detected in lung homogenate membrane pellets and in the histology slides from all strains. Although short-term inhalational exposure to occupationally relevant levels of dry dusts of MWCNTs did not elicit significant increases in measures of lung injury or fibrogenesis, increases in mononuclear cells and lack of particle clearance may indicate an altered host-defense capacity which could lead to disease with further particle accumulation or subsequent pathogen exposure.

PULMONARY PATHOLOGIES FOLLOWING INHALATION OF MULTI-WALLED CARBON NANOTUBES AT OCCUPATIONAL LEVELS

A Thesis

Presented To the Faculty of the Department of Biomedical Science

Brody School of Medicine at East Carolina University

In Partial Fulfillment of the Requirements for the Degree

Master of Science in Biomedical Science

By

Marshal Anthony Phipps, Jr.

August, 2015

PULMONARY PATHOLOGIES FOLLOWING INHALATION OF MULTI-WALLED CARBON NANOTUBES AT OCCUPATIONAL LEVELS

By

Marshal Anthony Phipps, Jr.

APPROVED BY:

DIRECTOR OF THESIS _____

Dianne M. Walters, Ph.D.

COMMITTEE MEMBER _____

Kymberly Gowdy, Ph.D.

COMMITTEE MEMBER _____

Richard Franklin, Ph.D.

COMMITTEE MEMBER _____

Mary Jane Thomassen, Ph.D.

COMMITTEE MEMBER _____

Alexander Murashov, MD, Ph.D.

DIRECTOR OF THE BIOMEDICAL SCIENCE MASTERS PROGRAM _____

Richard Franklin, Ph.D.

DEAN OF THE GRADUATE SCHOOL _____

Paul Gemperline, Ph.D.

ACKNOWLEDGEMENTS

I would like to acknowledge the help and support of my thesis director Dianne M. Walters. Dr. Walters taught me the bench research methods that I was not experienced with and gave me the opportunity to fully participate in this study. I would like to thank the members of my committee: Dr. Richard Franklin, Dr. Kymberly Gowdy, Dr. Mary Jane Thomassen, and Dr. Alexander Murashov. I would also like to thank Cameron Worthington for his work in the laboratory; Jonathan Shanahan and Jared Brown who performed the dark field imaging; Dr. James Bonner of North Carolina State University who provided the MWCNT we used; and Gene Oakley who built the dust generator. Finally, I would like to thank the North Carolina Biotechnology Center (Award # 2013-BRG-1202) who provided the grant that funded this study.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF SYMBOLS/ABBREVIATIONS

AB-PAS	Alcian Blue periodic acid-Schiff
APS	Aerodynamic particle sizer
BAL	Bronchoalveolar lavage
BSA	Bovine serum albumin
CNT	Carbon nanotube(s)
CPC	Condensation particle counter
CuO	Cooper oxide
DNA	Deoxyribonucleic acid
ECU	East Carolina University
HBSS	Hank’s Balanced Salt Solution
LPS	Lipopolysaccharide
MN	Micronuclei
MWCNT	Multi-walled carbon nanotubes
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NEAT	Nanoparticle emission assessment technique
NIOSH	National Institute for Occupational Safety and Health

NOAEL	No observed adverse effect level
OEL	Occupational exposure limit
PCR	Polymerase chain reaction
REL	Recommended exposure limit
RIPA	Radioimmunoprecipitation assay buffer
ROS	Reactive oxygen species
SWCNT	Single walled carbon nanotubes
TGF-β	Transforming growth factor-beta

CHAPTER 1

INTRODUCTION

1.1 PROBLEM AND APPROACH

Carbon nanotubes (CNT) are a nanomaterial being used, or proposed for use, in a wide variety of commercial and biomedical applications due to their unique properties such as small size, chemical reactivity, strength, biocompatibility, and electrical, thermal, and magnetic capabilities. Because of their extensive applicability, human exposure to CNT is inevitable, yet little is known about their safety. Even though to date there have been no reports of human disease associated with exposure to CNT, their toxicity to humans is unknown. Pulmonary toxicity is particularly worrisome since CNT have a structure similar to that of asbestos, which is known to result in lung diseases in humans and can present decades after the initial exposure. Given that nanotubes were only recognized in 1991 [1], there may not have been adequate time for the human health impact to have been recognized. While the full effects of CNT on human pulmonary tissue are unknown, animal studies to date indicate that pulmonary exposure to CNT can induce inflammation [2-5], granulomas [6-8], and tumors [9]. However, most studies on the impact of CNT have used non-physiological delivery methods. Additionally, the doses administered to date have been markedly higher than doses that are encountered ambiently or in occupational settings. Thus, there is a need for studies that evaluate the health effects of multi-walled carbon nanotubes (MWCMT) within an occupationally relevant range. In this study we designed, developed, and tested a nanotube dust generation system capable of delivering occupational levels of nanomaterials to evaluate the impact of MWCNT on the lungs of mice. Validation of the

delivery system was conducted to enable the second aim of identifying MWCNT-induced lung pathologies and investigating the mechanisms through which lung disease may be induced by inhalation of occupational levels of MWCNT.

1.2 BACKGROUND

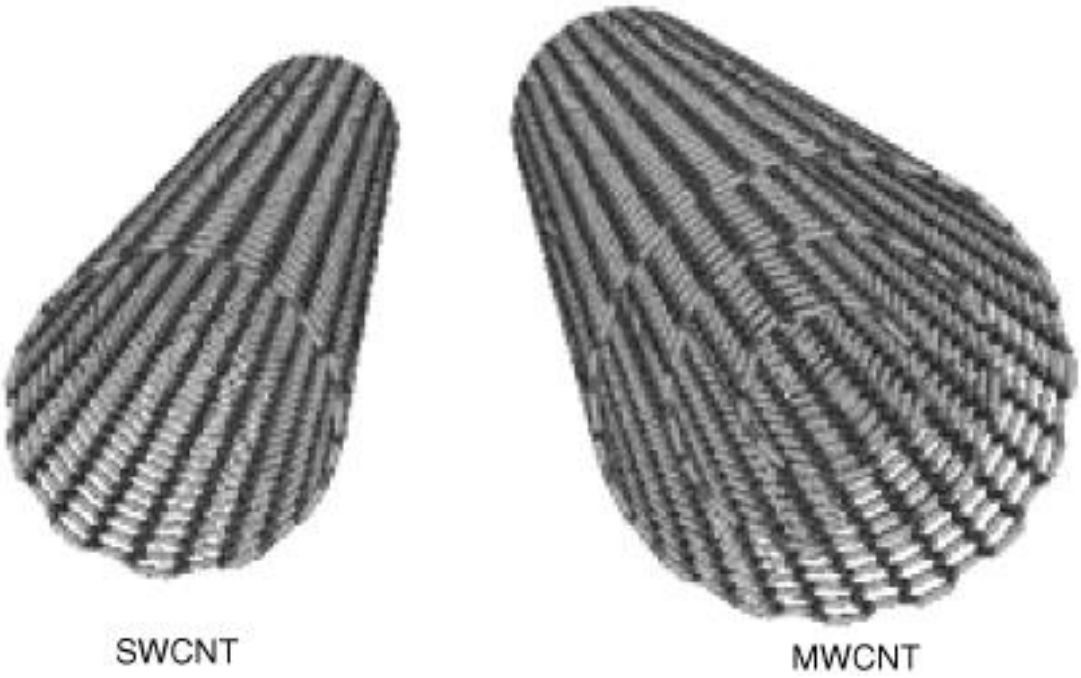
1.2.1 CARBON NANOTUBES

CNT are a type of nanoparticle, which are also called ultrafine particles or nanosized particles. Ultrafine particles are nanoparticles that occur naturally while nanoparticles typically refer to engineered particles. By definition, these particles have dimensions smaller than 100 nanometers (nm) in one dimension. The small size of nanoparticles results in both a large surface area compared to their mass/volume and high particle concentration rates. Because of their small size, nanoparticles can enter the human body via inhalation, ingestion, or absorption and can translocate into the blood or lymphatic system [2, 10]. Once in the blood or lymph, nanoparticles can distribute to other organs such as the heart, spleen, and central nervous system. The size, surface area (which increases as the size decreases), and bioactivity give nanoparticles properties which can result in either negative or positive biological effects including inflammation, oxidative stress, antioxidant activity, the ability to penetrate cell membranes and thus deliver drugs, etc. The activity could also include a combination of positive and negative effects [2]. Nanoparticles can occur naturally from volcanoes or fires, unintentionally from drilling or welding, or from manufacturing or engineering processes intended to create them for use in various products. Created or engineered nanomaterials can be manufactured in a variety of formats and as such offer attractive development potential [11]. Manufactured nanoparticles are generally identified based on their shape and include nanotubes, nanorings, nanofibers, nanowires, etc. [2] Fiber-

like nanoparticles can occur singly or in ropes or clumps and may be partially rigid, partially flexible, or rigid [12]. Engineered nanoparticles can be made of elements such as carbon but also of lipids, chitosan, silica, lactic acid, metals. The source material impacts the properties of the nanoparticles [13].

Carbon nanotubes are the specific focus of this study. They are an allotrope of carbon from the fullerene family typically characterized as either single walled carbon nanotubes (SWCNT) or multi-walled carbon nanotubes (MWCNT). The tubes are graphene cylinders with either one or multiple walls [12] as shown in Figure 1.1.

Figure 1.1: Carbon Nanotube Structure. Diagram of a single-walled carbon nanotube (SWCNT) and a multi-walled carbon nanotube (MWCNT) demonstrating the cylindrical structures with a single wall in SWCNT and the concentric walls in MWCNT [14].



SWCNT

MWCNT

Carbon nanotubes were first observed in the late 1950s by Roger Bacon while working with carbon fiber and again in the 1970s by Morinobu Endo while working with a gas phase process. In 1991, Sumio Iijima found multiwall carbon nanotubes in a carbon arc discharge. Two years later he and Donald Bethune independently observed single walled carbon nanotubes (buckytubes) [1, 15] after which research in the field rapidly expanded. Carbon nanotubes are stronger than steel, can function as metals or semi-conductors, are thermally conductive, can be functionalized, and are considered to be one dimensional.

Given the wide array of potential uses for carbon nanotubes, multiple methods to produce them arose. Each method has advantages and disadvantages and produces products with varying degrees of purity [16]. Manufacturing of carbon nanotubes can be done via physical processes (arc discharge or laser ablation), chemical processes (chemical vapor deposition, high pressure carbon monoxide reaction also known as HiPco®, or CoMoCAT®) or miscellaneous processes (helium arc discharge, electrolysis, flame synthesis). The three most common methods are arc discharge, laser ablation, and chemical vapor deposition. Key considerations for the manufacturing method chosen include cost on a per unit basis, the availability of raw materials, the amount of energy needed, the design of the reactor, the rate at which production can occur, the product purity, the product yields, the amount of post-production processing that is required, and the type of processing that can be done, i.e. batch versus continuous. All three methods can produce pure carbon nanotubes. Both laser ablation and chemical vapor deposition can both produce relatively high yield. However when taking all factors into consideration chemical vapor deposition best meets the previous

considerations, i.e. low cost per unit, no extensive post production refinement needed, easy availability of raw materials, etc. [17]. In spite of the advantages chemical vapor deposition does produce contaminants that have to be removed to ensure purity. This removal can be costly and uses processes that can shorten the CNT and/or introduce defects in the sidewalls of the CNT. Improvement on current production methods, as well as development of new methods of production and alteration of CNT, is the subject of intense research and commercial interest. Production in 2013 exceeded several thousand tons of nanotubes per year [18, 19] and is expected to grow over the decade beyond 2013 [20].

1.2.2 USES OF CARBON NANOTUBES

Nanotubes, both SWCNT and MWCNT, have current and proposed uses in both commerce and healthcare with higher current use in commercial areas than in health care. Due to their high aspect ratio and strength carbon nanotubes are used as fillers in plastics, in composite materials, in thin films, and in resins. Their conductivity makes them useful in the automotive industry for electrostatic painting and in fuel lines to deplete electrical charges. MWCNT are added to polymers and resins as strengthening components, and are particularly useful in boat hulls and turbine blades due to their stiffness. Because of the material damping features of MWCNT they are used in tennis rackets, bicycles, and baseball bats where oscillation is a negative feature. Due to their conductivity, carbon nanotubes are commonly used in lithium ion batteries for laptops and cell phones as well as in transistors and semiconductors. The structure of carbon nanotubes may also mean they can be used for water purification by taking advantage of tangled

CNT to electrostatically remove bacteria, viruses, and other contaminates [18].

A key feature of CNT for biomedical applications is that they can be functionalized which can remove bioactivity [21] and, in conjunction with their small size, allow for uses such as delivery of medications to precise locations including the lungs. They have many other potential uses in medical settings, such as in rehabilitative medicine for engineering tissues, imaging, diagnosing diseases, and/or texturing surfaces [22]. They can be used in the monitoring and treatment of diseases such cancer and/or infections [23] and as a means of transporting genetic material or peptides to cells [24]. Furthermore, CNT can be used for tissue regeneration (particularly bone tissue through scaffolding), for cardiovascular applications, and for chemotherapy both by way of drug delivery and by way of phototherapy [25]. Finally, they can also be used for biomedical sensors from both the standpoint of their size and chemical compatibility [18].

The goal of functionalization is to allow utilization of carbon nanotubes in practical ways while minimizing any potential toxic impacts of the very properties that allow for their use in biomedical applications. Functionalization methods include binding proteins to the CNT, covalent purification, non-covalent surface modifications, attachment of DNA, polyethylene glycol, chitosan, or surfactants, or combinations of these methods [25]. Functionalization can also be used to mimic the properties of naturally occurring micro-tubes which share multiple properties with carbon nanotubes and are simultaneously stiff and resilient and which provide essential cell functionality including transport and motility [26]. Massive amounts of research and investment have

been targeted at practical uses of carbon nanotubes, however a number factors that make nanotubes unique (aspect ratio, biopersistence, and residual metal content), and therefore allow for this type of functionalization, may also lead to toxicity thus requiring careful attention to determining the toxicity of carbon nanotubes and minimizing the potential negative impacts [22]. A first step in minimizing adverse health effects is to understand the toxicity of CNT through the expected routes of exposure such as inhalation.

1.2.3 OCCUPATIONAL EXPOSURE TO CARBON NANOTUBES

Inhaled dust is a serious occupational risk that can result in injury and chronic lung disease [27]. The risk to workers for occupational exposure to CNT occurs during production and/or handling of the materials. Exposure can also occur when nanotubes are prepared for use or actually used for drug delivery [28]. Even though a field study of exposure rates showed both lower concentrations of particles in the air and on gloves than anticipated based on laboratory studies [29], workers are exposed to CNT. In 2011 sixty-one companies were manufacturing carbon nanotubes in the United States employing 620 workers with a predicted annual growth rate of 15-17% [30].

Given the potential for negative health effects resulting from exposure to MWCNT, it is important to study this in order to implement safety guidelines. Wide ranges of values have been found for occupational exposures that depend on the factory where they are made as well as the manufacturing processes used. A National Institute for Occupational Safety and Health (NIOSH) team visited multiple manufacturing plants and developed a tool to assess exposure. The nanoparticle emission assessment technique (NEAT) was used to determine the inhalation exposure

via air samples while not accounting for incidental exposures [31]. A follow up study by NIOSH used this technique at twelve sites making a variety of nanomaterials including both SWCNT and MWCNT and found that in some conditions workers could be exposed to over 42,000 particles/cm^3 below 1000 nm but also found that existing engineering controls could minimize exposure [32]. A study conducted at a Swiss plant that manufactures nanoparticles found an average concentration of nearly 60,000 particles/cm^3 and a peak of 136,000 particles/cm^3. Of note this study took all phases of manufacturing into consideration, i.e. producing, maintaining, and handling the nanomaterials [33]. Monitoring by a variety of methods at a manufacturing and processing plant for carbon nanofibers found elevated particle concentrations related to ultrafine particles released during a thermal treatment but did find short term increases in particles that could be inspired at a rate of 1.1 mg/m^3 during handling of the carbon nanofibers [34].

Based on available research, NIOSH proposed a recommended exposure limit (REL) for CNT at a level of 7 µg/m^3 of elemental carbon for respirable particles. This is an 8 hour time weighted average using elemental carbon as a marker for exposure to CNT. This limit was exceeded in two of three plants tested [35]. Occupational monitoring showed exposure to carbon nanotubes most commonly occurred when opening the chemical vapor deposition cover and when working with various catalysts [36]. Implementing environmental control measures at a laboratory using MWCNT significantly decreased the numbers found in samples [37]. Control methods other than natural ventilation also reduced particles at locations where handling CNT occurred [36, 38].

Of particular concern with nanotubes in occupational settings is that their length may increase the toxicity, the needle-like shape may mimic the impact of asbestos fibers, and they are biopersistent. Although CNT are typically purified after manufacture, they may still contain metals, organic, and/or other support materials. The common metals used are copper, iron, nickel, and molybdenum or a mix of these metals. CNT are frequently chemically modified to accomplish goals such as increased solubility. CNT can also be coated. The toxic impact of the carbon nanotubes is a product of the surface area and the toxicity of the surface. Of concern in the workplace are things that can be inhaled (< 10 μm aerodynamic diameter) meaning they can enter the human respiratory tract and things that can be respired (< 4.5 μm aerodynamic diameter) meaning they can get beyond the ciliated airways. Of additional concern is the need to determine methods to measure these very small particles [12]. Another study that exposed mice with the goal of mirroring the exposure rates found in eight manufacturing plants showed variation in deposition rates but limited inflammatory response [39].

1.2.4 TOXICOLOGICAL PROPERTIES OF CARBON NANOTUBES

In addition to the many current and proposed uses for CNT, there are an equal number of concerns about their safety and possible toxicity. As manufacturing and use of carbon nanotubes increases, so does the potential for human exposure. Key concerns related to the potential toxicity of carbon nanotubes include their physical properties (small size, large surface area, aspect ratio, etc.) and their chemical properties, as well as any contaminants from either manufacturing or functionalization processes. Other toxicological factors include route of exposure (dermal, inhalation,

ingestion, or injection), dose and duration of exposure, biopersistence, and aggregation of the nanotubes [40-42].

Multiple studies have discussed the similarities of CNT to asbestos in which pulmonary retention of fibers results in injury and inflammation followed by chronic lung pathology, specifically mesothelioma and fibrosis. Pertinent toxicity factors include the long length of the asbestos fibers so that they cannot be consumed completely by alveolar macrophages, the thin diameter allowing movement beyond the airways and lung tissue, and their biopersistence leading to dose accumulation. Similar to asbestos fibers, inhalation of CNT, which can occur as tangles or longer fibers, can lead to incomplete consumption by macrophages resulting in inflammation [43], oxidative stress to the lungs [40], granulomas in the presence of long MWCNT, and in some cases a systemic response which could impact the cardiovascular system [44]. Furthermore, similar to asbestos, carbon nanotubes can be translocated into other organs and tissues via either the lymphatic system or cellular migration. Asbestos and carbon nanotubes both demonstrated cellular toxicity, DNA damage, fibrosis, and malignant mesothelioma via peritoneal exposure [6, 45]. Additionally, both fibers induced expression of genes related to mesothelioma and to lung cancer [46].

Of additional concern is the long lag time from asbestos exposure to disease, which can be 15 to 40 years. The disease can not only impact those who mine asbestos but those who work with it when it is used as a fire retardant or in construction, those who contact things contaminated by asbestos, and even spouses of people who work with asbestos. Smoking and genetics also play a role in asbestos related diseases [47]. At this time it is unknown whether exposure to CNT will result in the same outcome as

asbestos exposure, but given the observed similarities between CNT and asbestos, it is a possibility. Therefore, there is a need to understand CNT toxicity so that adverse outcomes can be prevented, whether through engineering safer nanotubes or through protective strategies, regulation of nanomaterials, and guidelines for exposure limits. Key concerns related to the potential toxicity of carbon nanotubes that need to be addressed include their physical properties (small size, large surface area compared to their size, aspect ratio, etc.) and their chemical properties as well as any contaminants that may be present from either the manufacturing or the functionalization process. Other factors include realistic exposure scenarios, the route of exposure (dermal, inhalation, ingestion, or injection), the dose and duration of the exposure, the biopersistence of the nanotubes, and the aggregation of the nanotubes [40-42].

Many rodent studies have been done with instillation or aspiration of MWCNT, but occupational exposure is far more likely to occur via inhalation which was shown in mice to result in different lung pathologies attributed to different sizes, different diameters, and distribution patterns of aggregation of the MWCNT [48-50]. The dustiness of different MWCNT may be an important occupational factor since exposure of mice to inhaled, low dust forming MWCNT showed minimal granuloma inflammation and only slight neutrophilia [51]. Volumetric lung burden correlated with toxicity and following species adjustments suggests an occupational exposure limit (OEL) of 0.05 mg Baytubes/m(3) for humans [52]. A two week study using whole body inhalation with rats demonstrated deposition of MWCNT in both the nasal cavity and lungs with clearance shown from the nasal cavity. Like multiple other studies this study also showed dose dependence, migration of MWCNT via the lymphatic system, granuloma

formation at the highest doses, and persistence of the carbon nanotubes. This study established a no observed adverse effect level (NOAEL) for rats of 0.2 mg/m(3) for two weeks however the biopersistence could have led to problems even at this dose had the study gone on longer [53]. Purity also matters as a study of long term pristine MWCNT exposure in A549 human cells showed no major long term effects or induced adaptive mechanisms [54]. As described above many studies have been done looking at the impact of carbon nanotubes, however many of these studies were done using aspiration or tracheal installation, neither of which is a physiological mechanism.

In addition to inflammation and fibrosis, studies also showed biopersistence of carbon nanotubes. Rats administered MCWCNT via whole body inhalation showed a decrease in the amount of retained MWCNT at three months with dose dependent half times occurring at 51 and 54 days [55]. Even sub-chronic doses of MWCNT demonstrated biopersistence when given to rats via nose only exposure and was dose dependent, showed translocation to lymph nodes at the two highest doses, and also demonstrated that MWCNT aggregated into bundles known as assemblages. The assemblages, which aggregated in the alveoli and were consistently retained in the alveolar macrophages, are intertwined and coil like structures that could then form clumps. The assemblages themselves appeared to result in the toxicity as opposed to some other factor. When compared with carbon black, both carbon black and MWCNT resulted in the same type of triggering response but the MWCNT based on BAL analysis were more toxic which was believed to be due to the high displacement volume caused by the assemblages of MWCNT [56]. A study using this same type of MWCNT delivered by both dry and wet dispersion to rat lungs showed biopersistence at three

months for both, but with wet dispersion being at levels three times higher than dry dispersion when analyzed by both dosimetry and electron microscopy [57]. Exposure of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-deficient mice to SWCNT showed bioperistence but also decreased fibrosis thus indicating that NADPH oxidase is necessary to regulate apoptosis and allow for a move away from acute inflammation [58]. When unrespirable agglomerates of MWCNT were administered via instillation, there was evidence of apoptosis but not of other toxic effects [59]. In another study, MWCNT caused oxidative stress in rat epithelial cells along with apoptosis [60].

Multiple studies compared the effect of carbon nanotubes prepared in various ways and with various properties, doses, and the inhalation of other materials. Rats exposed one time via inhalation to either air, quartz (a positive control known to be toxic), or MWCNT demonstrated dose dependence not impacted by residual cobalt thus indicating the toxic impact was from the assemblage of the carbon nanotubes and not due to any impurities inherent in the MWCNT [61]. Toxicity in mice lungs was shown to be dose dependent using low, medium, and high occupational inhalation doses resulting in persistent inflammation and cell toxicity at the high dose, transient cell toxicity at the medium dose, and low-grade inflammation at the low dose [39]. Toxicity as measured by inflammation and cytokine release in mice pulmonary tissue increased with the diameter and length of MWCNT which impacted lung retention as well as dose, but were not significantly impacted by either purification or functionalization [21, 62]. In addition histopathology of mouse nose tissue following MWCNT administration also showed toxic effects including hyaline droplet formation and neutrophilic rhinitis [63]. Even at sub-chronic inhalation rates carbon nanotubes resulted in pulmonary

inflammation and fibrosis in rats [64]. MWCNT also could both penetrate and persist in alveolar macrophages, the alveolar wall, and pleura [65] for a prolonged period after exposure [66]. Furthermore, MWCNT could be found not only in lung tissues but also in the parietal pleura, respiratory muscles, heart, kidney, and brain almost a year after exposure [67]. Pleural presence was also demonstrated in prolonged exposure to MWCNT [10]. In human lung epithelial cells a study with various metal oxides showed variations across multiple oxides but a significant difference with copper oxide (CuO) as well as toxicity attributed to MWCNT [68]. Another study found no acute toxicity but did find time and dose dependent ROS and a decrease in the mitochondrial membrane with unpurified SWCNT thought to be the result of trace metals [69]. Nose exposure of rats showed no toxicity from similar lung volumes of carbon black or graphite nanoplatelets but did demonstrate toxicity via BAL analysis and microscopic evaluations from graphene and MWCNT with the MWCNT inducing toxicity at a significantly lower dose than the graphene. Given the insufficiency of any impurities to induce these effects, the toxicity was attributed to either a combination of factors or other unknown factors [70].

1.2.5 PATHOLOGY RELATED TO CARBON NANOTUBES

Introduction

Although CNT exposure has not been associated with human disease to date, animal studies suggest that CNT exposure can produce adverse health effects. Specific studies showed that exposure of mouse mesothelial lining of the abdominal cavity to long MWCNT produced inflammation and granulomas similar to that produced by asbestos [71]. MWCNT administered intraperitoneally to mice in different doses produced mesotheliomas [72] at a higher rate and with worsening severity with

increased doses; however even mice given the lowest of three doses resulted in microscopic precursors to mesothelioma when followed long term [73]. Asbestosis is known to worsen among workers with longer exposures whether from asbestos manufacturing, from asbestos removal, and/or exposure concomitant with smoking [74, 75]. Production of both carbon nanotubes and carbon nanofibers may result in similar worker exposure via inhalation during multiple phases of production including making, purifying, handling and transporting nanomaterials. Asbestos is a natural silicate, while the nanomaterial of concern (MWCNT) is artificially produced. Nevertheless, these materials have a similar shape, size, and biopersistence (the presence of fibers retained in the lungs). Parallels between asbestos and carbon nanotube exposures in mice have been drawn in terms of their biological effects on macrophages, and additional cells such as lymphocytes, eosinophils, multinucleated giant cells, and neutrophils, and granuloma formation. Asbestos exposure can result in plaque formation, asbestosis, mesothelioma, and/or lung cancer. Multiple studies have shown a clear correlation between asbestos exposure and the development of lung disease with sex (male), age (older), smoking, and length of exposure increasing the risk [76, 77]. While mouse models have raised concern that carbon nanotubes can similarly induce fibrosis and potentially lung cancer, some studies have failed to show toxicity [42].

Multiple studies demonstrated that the size of the nanotubes, particularly the length, affects toxicity. Direct injection of long carbon nanotubes or asbestos into the pleural space of mice resulted in increased granulocytes, increased protein, increased mesothelial cells, and fibrosis as opposed to short tubes. The same study investigated the injection of beads greater than 10 μm and found that they failed to exit via the

stomata and led to inflammation, whereas smaller beads exited the stomata and did not elicit inflammation [78]. A related study using aspiration of carbon nanotubes also demonstrated length dependent inflammation in that inflammation occurred with long carbon nanotubes but not short or tangled carbon nanotubes [3]. Nagai, et al, demonstrated that thin, rigid carbon nanotubes' needle-like structure could penetrate the membrane of mesothelial cells (believed to be a different mechanism than asbestos) but showing the same deletion of tumor suppressing genes as asbestos [79].

Carbon nanotubes with smaller diameters demonstrated more cytotoxicity related to cell internalization of the carbon nanotube whether the nanotube was inhaled, ingested, or passed through the skin [80]. Others found that fibers that were long and thin could not be disposed of via phagocytosis and when implanted in rats for as long as a year resulted in malignancies [81]. The length of the MWCNT determined the type of bioactivity in both rodent and human alveoli [82]. SWCNT instilled by way of the trachea showed more toxicity than carbon black and in some cases more than quartz, a known fibrogenic material. The biopersistence and high aspect ratio determined the degree of toxicity [83]. MWCNT also showed genetic toxicity via the induction of micronuclei (MN) in lung cells via both DNA breaks and chromosomal loss, possibly as their size allowed for interaction with molecules dimensionally similar to DNA [84].

Induction of Inflammation

Multiple studies with carbon nanotubes showed that they can induce pulmonary inflammation. Studies measured inflammation in a variety of ways including cytokines, differential cell counts, etc., however with various interpretations including that some inflammation offered some protection [4] and that release of cytokines led to decreased

oxidative stress [85]. Other studies showed a range of reactions including a single exposure to MWCNT resulting in inflammation and oxidative stress [5], inflammation that progressed over time [63], and not only lung inflammation but possible systemic inflammation [86]. In contrast, biodegraded SWCNT aspirated into mice lungs did not cause inflammation [87] while non-biodegraded double walled carbon nanotubes invoked an inflammatory response very similar to asbestos [88]. Both purified ground carbon nanotubes and MWCNT resulted in inflammation, fibromas, and the production of TNF-α, a cytokine involved in acute systemic inflammatory reactions [89]. Increased doses of MWCNT showed increased inflammation, fibrosis, and even the rare translocation of the MWCNT to the lymph system [10]. Mice exposed only to nose only inhaled SWCNT and MWCNT developed inflammation, fibrosis, changes in both oxidant and antioxidant levels, and apoptosis proteins [90]. A comparison of pristine and functionalized MWCNT injected intra-tracheally in rats showed inflammation and toxic effects for both [91].

Pulmonary Fibrosis

As noted, pulmonary fibrosis is a common feature of particle deposition and is impacted by both the size and composition of the particles. This fibrosis can lead to other pathology and/or can worsen existing problems such as asthma. Smaller particles have a larger surface area, an increased ability to generate reactive oxygen species (ROS), and an increased ability to cause inflammation. The presence of other compounds in particles, particularly transition metals such as vanadium or copper, can induce cell signaling and therefore oxidative stress [92]. MWCNT can result in early lung fibrosis that is dependent upon the dose and time as well as being notable for increased

neutrophils, macrophages, cytokines, and growth factors related to fibrogenesis [93]. Human lung cell cultures exposed to MWCNT showed ROS production, cytokines which are inflammatory markers, and myofibroblast changes believed to be necessary for the development of fibrosis [94]. A study of rats given one aspiration dose of SWCNT did not show inflammation but did show fibrotic alveolar lesions [95].

As previously noted, carbon nanotubes can cause ROS which can activate transcription factors and signaling pathways that result in fibrosis. Both short and long SWCNT induced oxidative stress but long SWCNT resulted in a more pronounced ROS than short SWCNT did, possibly due to the length of the tubes resulting in the phagocytes being unable to completely consume the SWCNT (frustrated phagocytosis). Both short and long SWCNT resulted in activation of transforming growth factor-beta (TGF-β) which stimulates the production of collagen by the fibroblasts but the long SWCNT resulted in a significantly stronger response [96]. Aspirated MWCNT were seen with the distribution decreasing from the alveolar macrophages to the alveolar septa to the sub-pleural tissues resulting in fibrosis [97]. Pleural distribution of MWCNT was also seen when mice were given four different doses of MWCNT and then tested with bronchoalveolar lavage (BAL) at four different intervals. Inflammation and damage was seen in all cases but was dose dependent with rapid onset of fibrosis, persistent inflammation, and MWCNT in the pleura [98]. Aspirated SWCNT in mice demonstrated similar inflammation and fibrosis but also showed early granulomas, elevation of cytokines, production of TGF-β1, alveolar thickening, respiratory deficits, and a decrease in bacterial clearance in the lungs but no engulfment of the SWCNT similar to other studies with SWCNT [7].

Carcinogenic Effects of Carbon Nanotubes

Carcinogen related effects of carbon nanotubes include demonstrated DNA damage from SWCNT in as little as three hours after exposure [99] including the induction of DNA destabilization [100] or modification [101], and potential carcinogenicity [102]. Carcinogenicity related to MWCNT may be modified by the tube diameter, tube length, tube rigidity, and/or the presence of any surface modifications of the tubes [103]. Other carcinogenic impacts include mitotic spindle disruption at occupational levels of exposure [104] with the potential to pass that disruption to daughter cells [105], centrosome disruption with aneuploidy which is characteristic in cancer progression [106-108], sub pleural fibrosis [109], and lung adenomas. Adenomas were found at a 90.5% tumor rate and a 5.5% metastasis rate for mice receiving an initiator followed by MWCNT exposure, indicating that the MWCNT are acting as promotors [9]. Both MWCNT, nanosized titanium oxide, and soot induced micronuclei [110], as well as ROS generation and apoptosis in human lung epithelial A549 cells [111-114] . Of note, heating reduced the genotoxicity of MWCNT while grinding the heated material restored its genotoxicity [84]. Additionally, dispersion of SWCNT in dipalmitoylphosphatidylcholine, a major component of pulmonary surfactant, resulted in increased ROS while fetal calf serum decreased the oxidative stress [115]. Alternately, a longitudinal study failed to demonstrate a carcinogenic response. In this study, rats received peritoneal injections of either MWCNT with or without known defects, or of a known carcinogen, or of a negative control substance. After two years, the rats injected with the known carcinogen developed mesothelioma but none of rats injected with other materials did at a rate that could be explained by those materials.

These somewhat surprising results were attributed to the relatively short MWCNT that were used, the non-sustained inflammatory reaction, and/or to the lack of free radicals produced by the nanotubes that were used [116].

Other Impacts of Carbon Nanotubes

Additional impacts of MWCNT include granuloma formation with T cell and macrophage infiltration, an increase in osteopontin [117], and the induction of *Twist*1 expression in mouse BAL cells, similar to that seen in human samples obtained from sarcoidosis patients [118] with risk factors such as exposure to wood fires, firefighting and/or fireplaces which may contain environmental carbon nanotubes [119].

Cardiovascular impacts following intra-tracheal instillation of MWCNT included elevated heart rate and abdominal arterial lesions in hypertensive mice [120] and increased susceptibility to cardiac ischemia or reperfusion injury even in the absence of lung inflammation [44]. Other studies found an increase in serum cytokines and inflammatory gene expression in blood [121, 122]. Inhalation of MWCNT had a persistent impact on the coronary arterioles with impairment of endothelium-mediated vessel dilation.

Suppression of the systemic immune response persisted for a month following exposure to MWCNT [123]. This occurred in the absence of lung changes, but with evidence of splenic involvement which may indicate that MWCNT can reach the circulatory system [124, 125]. [126]. Impaired lymphatic clearance is dependent upon the size and shape of the nanomaterial. Fibers with a diameter less than 3 μm or with a length greater than 15 μm showed biopersistence and therefore had the most potential for toxic effects [127]. MWCNT delivered to rat lungs via installation also impacted the

liver and kidneys resulting tubular necrosis and interstitial nephritis [128]. Dermal exposure to MWCNT at varying doses and exposure times showed their presence in epithelial cells [129]. MWCNT in human skin cells also resulted in cell pathway disruption, cell cycle arrest, and apoptosis [133].

MWCNT delivered to mouse lungs via aspiration resulted in impaired pulmonary function including decreased lung compliance [130]. Length of exposure also mattered as a 30 day exposure in mice showed no obvious pathology but a 60 day exposure resulted in significant pathology [131]. A multi laboratory comparison study showed similar potency and effects using mice and rats and both aspiration and instillation although there was variability in neutrophilia [132].

The wide range of problems caused by nanomaterials are attributed to their size, which by definition must have one dimension less than 100 nanometers (nm), but also may be a result of other properties including the surface area, generation free radicals, crystal structure, coatings, preparation method, purity, and/or ability to aggregate [28, 134, 135]. A comparison of pristine MWCNT to acid treated MWCNT showed that both induced reversible granulomas, but the pristine MWCNT granulomas took longer to resolve [8].

1.3 SIGNIFICANCE/IMPACT

In spite of massive amounts of research and development in the field of nanomaterials, there remains much that is unknown about the safety of exposure to MWCNT. Most toxicity studies to date have been conducted using exposure methods that do not occur in occupational settings, i.e. instillation or aspiration of CNT as opposed to inhalation. These methods bypass the natural protective mechanisms of the

respiratory tract and also deliver a larger bolus dose (massively larger in many studies) than would occur at a given time in the workplace. The majority of studies to date have also been done using a single strain of mice, thus any effects of different genetic makeup will not be seen in those studies. This study was designed to address several questions left unanswered by others, including development of pulmonary toxicity associated with physiological inhalation exposure to occupational concentrations. This study will also provide data that may be used to set regulatory guidelines related to MWCNT exposure in order to prevent later public/occupational health outcomes as occurred with the widespread use of asbestos.

1.4 HYPOTHESIS AND AIMS

Based on the physical and chemical properties of MWCNT there is concern about adverse health effects with human inhalational exposure. Workers who are exposed to a dry dust of MWCNT may be at risk for toxic effects similar to those of asbestos. Studies of the pulmonary effects of MWCNT in rodents have found negative health effects; however, most of these studies have not been done at occupational levels or through a physiological route. For this reason, study of occupational levels through natural inhalation exposure is warranted.

1.4.1 HYPOTHESIS

Inhalation exposure to occupational levels of dry MWCNT will have a negative impact on lung health. This hypothesis will be addressed in the following specific aims.

1.4.2 AIM 1

Design, develop, and test a dust generator capable of creating occupational levels of dry MWCNT. Key features of a nanotube dust generator for investigation of the

health effects of inhaled MWCNT in rodents should include 1) the ability to reproducibly produce an occupational concentration of MWNCT; 2) the capacity to maintain airborne MWCNT concentrations within the target range for at least 4 hours a day; 3) the ability to generate a respirable dust from a mass of commercially available MWCNT without damaging the nanotubes; and 4) the incorporation of safety measures to protect the operator from nanotube exposure.

1.4.3 AIM 2

Develop a mouse model to investigate occupational exposure to MWCNT in order to investigate pulmonary toxicity and potential mechanisms of MWCNT-induced lung disease.

CHAPTER 2

METHODS AND MATERIALS

2.1 EXPERIMENTAL DESIGN

In order to assess the pulmonary toxicity of occupational levels of inhaled MWCNT in mice, we first designed, developed and tested a dust generator constructed on the ECU campus. This generator was then used to expose mice to either a dry dust of MWCNT at a concentration of 37,000 particles/cm^3 via nose-only exposure or to air only for four hours per day, five days a week, for two weeks. The mice were euthanized at 1, 3, 7, 10, 14, 28, and 84 days post-exposure for time course studies or at 14 days post-exposure for the strain comparison. Multiple specimens were collected for analysis, including bronchoalveolar lavage (BAL) fluid from the right lung to determine changes in lung cellularity and protein levels as an index of lung injury. The right lung was also collected in order to measure collagen levels. The left lung was fixed for histological assessment.

2.2 ANIMALS

Four week old male C57BL/6 (B6), DBA/2J (D2), and A/J mice (Jackson Laboratories, Bar Harbor, ME) were provided with standard mouse chow and water *ad libitum*. Mice were housed three to a cage with a 12 hour light/dark cycle and acclimated for 5 days in the ECU vivarium prior to any experimentation. Mice were randomly assigned to experimental or control groups and conditioned in the exposure restraints for increasing times each day the week before the study began. All procedures and animal handling methods were approved by the East Carolina University (ECU) Institutional Animal Care and Use Committee (IACUC).

2.3 MWCNT DUST GENERATION AND EXPOSURE

The MWCNT used were a kind gift from Dr. James C. Bonner of North Carolina State University, originally obtained from Helix Material Solutions [109]. Physical and chemical analysis of the MWCNT were provided by the manufacturer. MWCNT dusts were produced with a dust generator built in-house using a starting mass of approximately 0.75 mg MWCNT with addition of about 0.25 mg daily to achieve a daily average airborne concentration of 37,000 particles/cm^3 over four hours. Particle concentration is an essential dose metric for nanomaterial exposure since it can be measured relatively easily and tracked over time [136]. Mice were exposed to either medical grade breathing air from a tank as controls or MWCNT suspended in medical grade breathing air at a rate of 2L/min per day for four hours per day, five days per week for two weeks. At the conclusion of the exposure period, mice were removed from the restraints, observed carefully for any signs of distress and returned to their cages and the vivarium with free access to food and water.

Particle concentration was measured with equipment from TSI (Shoreview, MN) using an aerodynamic particle sizer (APS) (model 3321) which measures particles larger than 500nm, an electrostatic classifier (model 3080) which is used in conjunction with a nano water-based condensation particle counter (CPC) (model 3788) to measure particles smaller than 500 nm. The particles first pass an impactor that removes any large particles. Then, any existing charge is removed from the particles and a new charge is applied that scales with the size of the particle where larger particles are more highly charged. These charged particles can then be separated by charge such that

only particles of a known size are able to leave the classifier. These particles then enter the CPC where water is condensed around the particles so that they can be detected by a laser and counted. This information about particle size and the number of particles is then combined by a computer program to give a count of how many particles are found in the sample for each size. The APS measures particle sizes from 0.5 μm to 20 μm. The APS uses a time of flight method to measure aerodynamic particle size and counts the number of particle for each size. The vast majority of particles counted in this study were below 0.5 μm so the APS data was not used.

2.4 TISSUE COLLECTION AND PHENOTYPING

Collection of tissues

Following exposure, the mice were anesthetized with a weight based intraperitoneal injection (0.02 ml/gram of body mass) of tribromoethanol and euthanized via thoracotomy and exsanguination at 1, 3, 7, 10, 14, 28, and 84 days post-exposure for time course studies or 14 days post-exposure for strain comparison studies. Blood was drawn from the right ventricle and collected in serum separator tubes to obtain serum samples. Bronchoalveolar lavage (BAL) was conducted on the right lung only by clamping the left bronchus to prevent inflation of the left lung, followed by insertion of an 18 gauge angiocath into the trachea. The right lung was lavaged four times with separate aliquots of 26.25 ml/kg cold Hank's Balanced Salt Solution (HBSS). The first BAL return was kept separate from the subsequent returns for protein analysis. All BAL fluid was kept on ice until and during processing. The right lung was removed and snap frozen in liquid nitrogen for later measurement of lung collagen content. The left bronchus was then unclamped and wedged with an 18 gauge angiocath to inflate and

fix the left lung with formalin. Additional organs including the heart, liver, kidneys, and spleen were snap frozen in liquid nitrogen, but were not analyzed in this study.

Measurement of Lung Permeability

Total protein was measured in BAL fluid from the first return as an index of lung permeability and injury using a Bradford assay (Bio-Rad, Hercules, CA) according to manufacturer's instructions. All BAL fluid was centrifuged at 500 * g for 10 minutes to pellet the BAL cells. The supernatant from the first BAL return was drawn off and frozen at -70C until protein measurements were made. Briefly, Protein Assay solution was diluted 1:5 in ultrapure water. 50 µL of BAL fluid was added to 2.5 mL working solution of protein assay reagent in 4.5 mL cuvettes. The cuvettes were inverted to mix the sample and reagent, allowed to incubate at room temperature for 15 minutes, and then read at 595 nm on a spectrophotometer (Beckman Coulter DU 730, Pasadena, CA). A set of bovine serum albumin (BSA) standard protein concentrations at 0, 62.5, 125, 250, 500, 1000 µg/mL was run concurrently and the standard curve generated was used to calculate sample protein concentrations.

Bronchoalveolar Lavage Cell Counts

The cellular profile of bronchoalveolar lavage was used as a measure of lung inflammation. After centrifugation, supernatant from BAL returns 2-4 were discarded and cells from all BAL returns were pooled for each animal in 1 ml of HBSS. The total number of cells recovered were counted on a hemocytometer using phase microscopy (Leica DM 4000 B, Wetzlar, Germany). A volume of the cell suspension from each animal containing 20,000 cells was pipetted in to a cytofunnel, placed in a cytofuge (Shandon Cytospin3, Thermo Fisher Scientific; Waltham, MA) and spun onto slides.

The slides were allowed to dry overnight, then stained with a three step stain set (Richard-Allan, Kalamazoo, MI) for differential cell counts. 300 cell per slide/sample were differentiated based on standard morphological criteria using bright field microscopy.

Lung Collagen Content

The right lung was used to measure soluble collagen as an indication of structural remodeling within the lungs. The right lung was homogenized in 2 ml of Radioimmunoprecipitation assay (RIPA) buffer on ice. The lung slurry was centrifuged at 10,000 * g at 4 degrees C to remove cell membranes. 100 µL of the lung homogenate supernatant was added to 500 µL Sircol dye (Biocolor, Carrickfergus, UK) and incubated at room temperature for 30 minutes on a shaker platform to allow dye binding to any soluble collagen. The samples were centrifuged at 10,000 * g to pellet collagen-bound dye. Excess dye was removed from the pellets and the dye was released by adding 1.0 mL NaOH (0.5N) and vortexing. The samples were then transferred to 1.5 mL cuvettes and read at 540 nm on a spectrophotometer (Beckman Coulter DU 730, Pasadena, CA). A set of known standard concentrations were made using rat tail collagen (Biocolor, Carrickfergus, UK) and assayed with the samples. Lung collagen content of samples was determined from the standard curve.

Lung Histology

The left lung was fixed in formalin for 72 hours before being cross-sectioned into 3 pieces of approximately 5mm thickness. These pieces were placed in labelled cassettes and stored in 70% ethanol at 4 degrees C until standard histological processing was conducted. The three lung sections were embedded in paraffin and two

five-micron sections were cut and placed per slide. Slides were deparaffinized and rehydrated through an ethanol gradient before being stained with Masson's trichrome stain to evaluate collagen or Alcian Blue - Periodic Acid-Schiff (AB-PAS) to evaluate airway mucus content. Slides were cover slipped and allowed to dry before examination using bright field light microscopy. Scale bars and other markings were added using Leica Application Suite v3.8 (Wetzlar, Germany).

Dark Field Microscopy

In addition to making differential cell counts, BAL cells were imaged utilizing enhanced dark field microscopy (Cytoviva, Auburn, AL), at a magnification of 100x. Scale bars were added using ImageJ (Bethesda, MD). Dark field microscopy is able to image MWCNT more clearly than bright field microscopy due to differences in light scattering for MWCNT as compared to tissue.

Statistics

Data are presented as mean ± SEM. Two-way analysis of variance (ANOVA) was used to analyze differences between treatment groups and times or between treatment groups and strains as appropriate, with post hoc comparisons using the Bonferonni method (GraphPad Prism, San Diego, CA). Significance was assumed at $p < 0.05$. N=5-6 mice for all groups.

CHAPTER 3

DUST GENERATOR

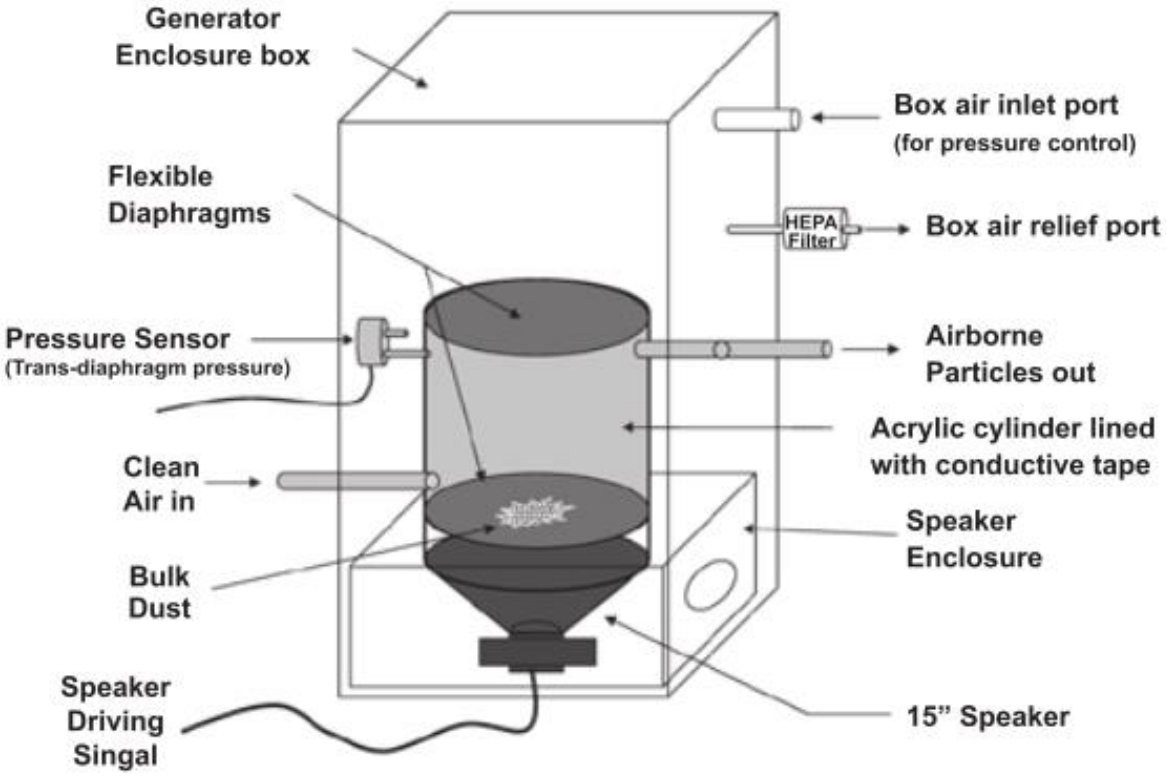
3.1 INTRODUCTION

Numerous toxicological studies of MWCNT in animals indicate a strong potential for pulmonary toxicity. Most toxicology studies to date have used high doses of MWCNT administered as a bolus, usually by way of tracheal instillation or aspiration, but these exposure methods produce different deposition patterns and may lead to distinctive pathologies as compared to natural inhalation. The ability to generate realistic occupational exposure scenarios is important for development of relevant animal models to investigate health effects of MWCNT. A primary objective of this project was to determine whether natural inhalation of dry MWCNT at occupational concentrations would result in pulmonary toxicity. Other researchers have created computer controlled dust generators for inhalation research including Mitchell, et.al. [124], Baron [137], et.al. and McKinney, et.al. for NIOSH in 2008 [138]. Commercial dust generators are available but none of them met our parameters primarily since the available options use a grinding motion which could physically alter the MWCNT thereby rendering them different from the MWCNT to which occupational workers are exposed. Since commercially available dust generators did not meet our requirement of not damaging the MWCNT, our first specific aim was to design, build, and test a dust generator that could reproducibly generate occupational levels of MWCNT without damaging them at an average concentration of 37,000 particles/cm^3. This target concentration was selected based on the literature about occupation concentrations in nanomaterial production facilities [32, 33].

3.2 DUST GENERATOR DESIGN AND BUILD

The NIOSH dust generator mirrored our parameters closer than any other options and so our design was based on this version. As shown in Figure 3.1 the NIOSH dust generator uses an acoustical system along with computerized feedback loops to produce an airborne dust for exposing animals to consistent concentrations of MWCNT. This system was tested and shown to produce particles at a desired concentration for long periods of time with little variance from the target concentration. These particles mirrored the characteristics of particles found in an occupational setting and could produce a range of reproducible concentrations while requiring minimal intervention by the operator [138].

Figure 3.1: NIOSH Dust Generator. Diagram illustrating the dust generator used by NIOSH including acrylic enclosure box containing the cylindrical dust chamber, flexible latex membranes, a speaker, and bulk dust. Air enters from the left and the speaker generates acoustical pressure to create dust in the dust chamber allowing the air flow to carry the MWCNT to the exposure area [138].


Generator
Enclosure box
Box air inlet port
(for pressure control)
Flexible
Diaphragms
HEPA
Filter
Box air relief port
Pressure Sensor
(Trans-diaphragm pressure)
Airborne
Particles out
Acrylic cylinder lined
with conductive tape
Clean
Air in
Speaker
Enclosure
Bulk
Dust
Speaker
Driving
Singal
15" Speaker

The ECU dust generator was built with the goal of generating an occupational concentration of dry MWCNT and exposing mice. Requirements for the build included not damaging the MWCNT in order to replicate occupational exposure since the length of MWCNT may be associated with their toxicity. The dust generator also needed to be able to operate for 4 hours per day and reproducibly generate occupational concentrations of airborne dust from day to day. The dust generator must also be able to safely contain the MWCNT in order to prevent exposure to the operator.

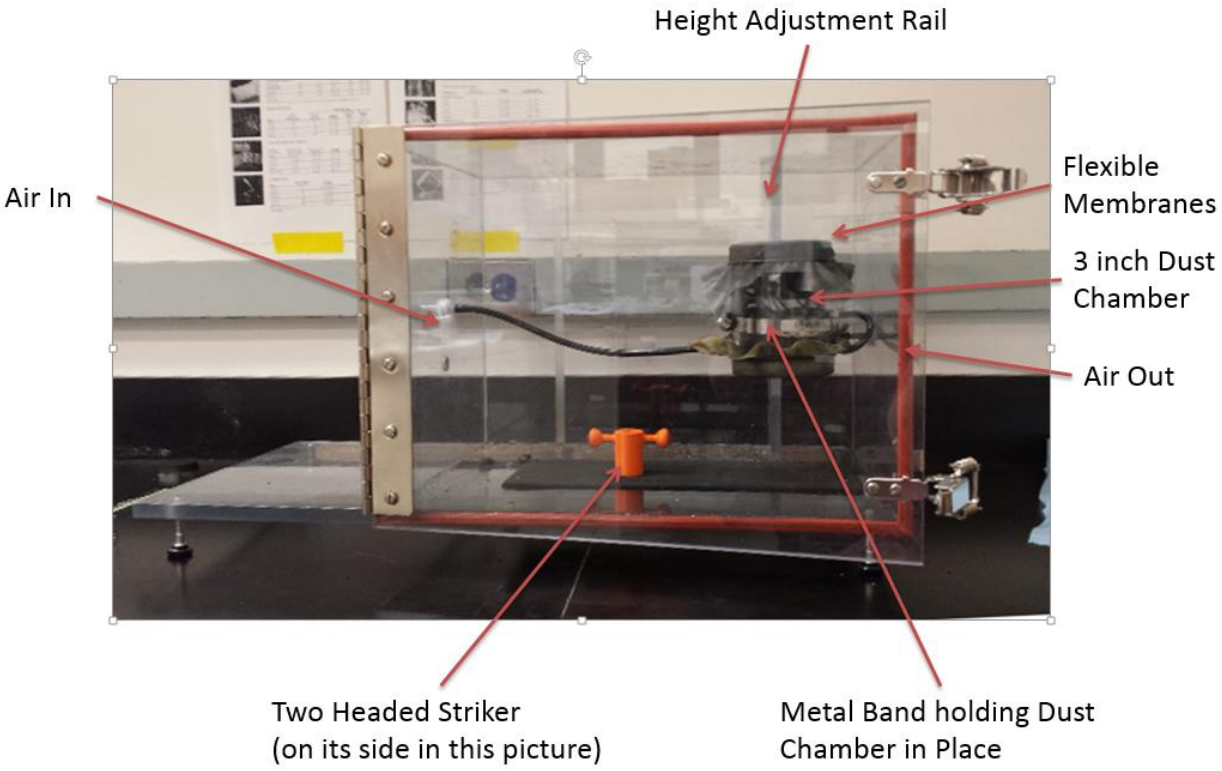
The overall design of the exposure apparatus allowed air to flow from a pressurized tank, through a flow meter, to the control animal tower, or through a separate flow meter into the dust generator device where MWCNT would be entrained in the airflow before delivery to an animal tower. The first version of the dust generator (Figure 3.2) had a three inch diameter dust chamber held in place with a metal band. This design used a two-arm striker that spun on the shaft of an electric motor to hit the membrane on the bottom of the dust chamber twice per revolution of the motor, thus resulting in dust being entrained in the air flow through the chamber. However, the spinning action of the striker dragged across the membrane and tore the membrane. Thus, the design was changed to use a piston which minimized wear on the membrane and resulted in increased membrane life.

Another design issue encountered with the original version was the ability to adjust the height of the dust chamber above the piston. Because placement of the chamber and adjustment of height above the piston (and therefore deformation of the membrane) could not be easily accomplished, we were unable to consistently deliver an

occupational concentration. Dust chamber height adjustment was necessary in order to change the concentration of MWCNT produced while the generator was in operation.

Further design modifications were done in order to correct for deficiencies in the ability to generate and maintain occupational levels of airborne MWCNT. These changes included increasing the diameter of the dust chamber from 3 inches to 6 inches in order to increase the surface area of the membrane and increase the concentration of MWCNT, as well as changing the material for piston sleeve from titanium to nylon to increase durability. This last change resulted in reduced wear and noise and gave us the ability to run the motor at higher speeds.

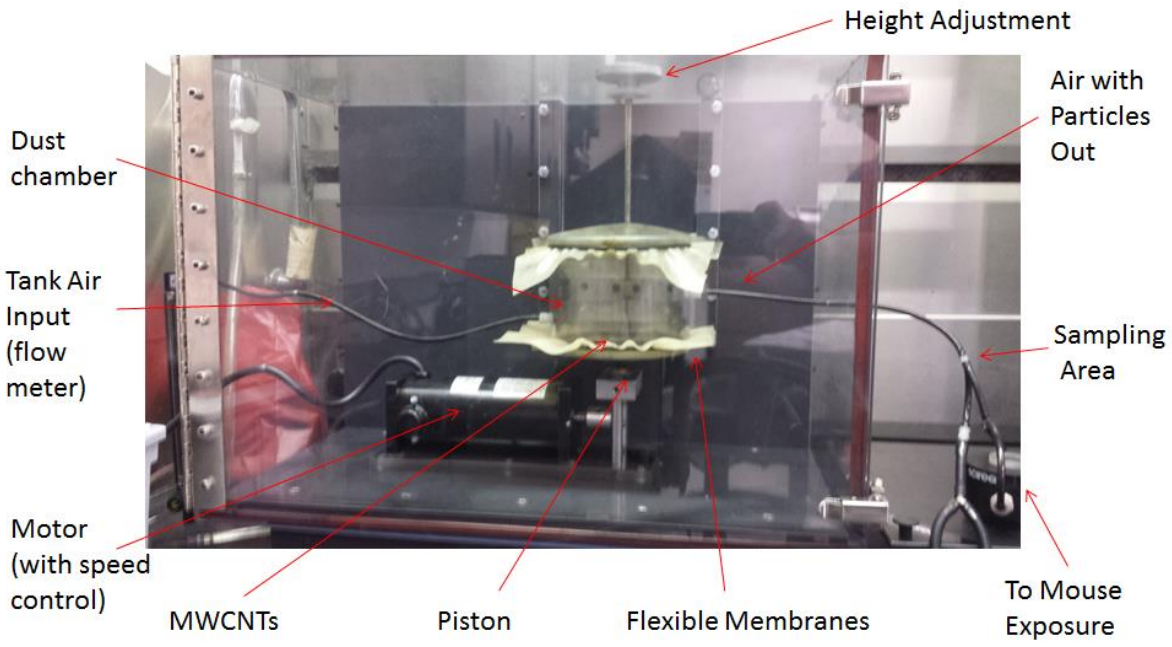
Figure 3.2: ECU Dust Generator, Version 1. Original version of the ECU dust generator showing the two-arm striker, 3 inch dust chamber, rail for height adjustment, metal band and membranes. The two arm striker led to issues with tearing of the bottom membrane when in use. The rail for height adjustment and the metal band holding the dust chamber in place did not allow for accurate adjustments to be made from day to day or during operation of the dust chamber.


Height Adjustment Rail
Air In
Flexible Membranes
3 inch Dust Chamber
Air Out
Two Headed Striker
(on its side in this picture)
Metal Band holding Dust Chamber in Place

As shown in Figures 3.2-3 the ECU dust generator was built by Gene Oakley of the Physics Department at ECU with the help of a group of undergraduate engineering students. An outer containment box of clear polycarbonate backed with PVC is sealed with weather stripping to prevent the MWCNT from escaping (thus meeting our criteria to protect the operator) and contains a motor, metal piston, and dust chamber. The dust chamber is a clear acrylic cylinder that has flexible latex rubber membranes on either end held in place by rubber O-rings. The top membrane can be easily removed in order to add MWCNT to the chamber. The chamber is mounted on a metal rail that allows height adjustments to be made to the dust chamber during operation. Beneath the dust chamber is the piston. The motor has a speed controller with 10 major increments that can be adjusted to increase or decrease the speed of the piston. Air flows from a compressed air tank into a Y connector and then to the control animal exposure tower and to the dust chamber. This design allows for simultaneous delivery of air to both the control and experimental exposure towers meaning that both the experimental and control mice are exposed to air from the same tank. A flow meter measures air flow into the control animal tower and is maintained at 2 L/minute. Another flow meter measures the air entering the containment chamber and is maintained at 2.6 L/minute. This higher flow rate allows for removal of 0.6L/minute just outside of the containment chamber in order to sample the concentration of the MWCNT. Air entering the containment box via the tubing is fed through the dust chamber which is filled with MWCNT. The piston, with its speed controlled by the variable speed motor, repetitively strikes the bottom membrane of the dust chamber. This striking of the membrane causes the bulk MWCNT material to become airborne. The dust is then entrained in the airflow, exiting the dust

chamber and containment box through tubing and is delivered to the experimental animal exposure tower.

Figure 3.3: ECU Dust Generator, final design. The two-arm striker was replaced with a metal piston the stroke of which is guided by a nylon sleeve. A larger, 6 inch diameter cylinder was used for the dust chamber. The dust chamber was held securely in place by attaching it to a metal plate that is mounted on a threaded post that produces a 1 mm change in height per revolution. This modification to the height control mechanism allowed consistent placement and fine adjustment of the dust cylinder. Clean air from a tank enters the dust chamber through a regulator. The piston creates a dust within the dust chamber from the bulk MWCNT and the dust is then entrained in the air stream and carried out of the exit port to a sampling point, then to a nose only exposure tower.


Height Adjustment
Air with Particles Out
Dust chamber
Tank Air Input (flow meter)
Sampling Area
Motor (with speed control)
MWCNTs
Piston
Flexible Membranes
To Mouse Exposure

3.3 DUST GENERATOR TESTING

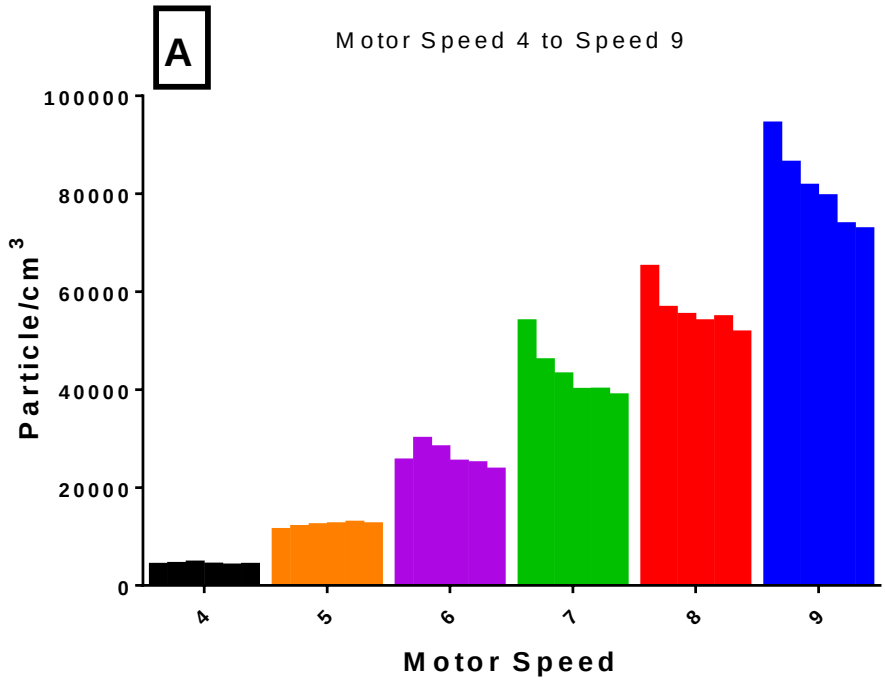
Testing was performed to determine the capacity of the dust generator to produce a consistent occupational level of dry MWCNT dust. Airflow, amount of material, dust chamber height, and motor speed could all be adjusted with this system. Airflow needed to be at a rate high enough to allow proper ventilation for up to twelve animals but not so high that the animals would become dehydrated from dry air moving past their noses for four hours. Thus, airflow was set to approximately 2L/minute for both the control and experimental animals. The air entering the dust generation system was set at 2.6 L/minute with 0.6 L/minute being sampled after dust generation, but before delivery to the animal exposure tower, to measure the concentration of MWCNT in the air. The starting material was difficult to adjust during dust generation as it involved removing at least one membrane to add material and removing the whole dust chamber to remove material and was only changed at the beginning of the day unless serious problems arose such as the membrane coming loose or a very high or low concentration developed during operation.

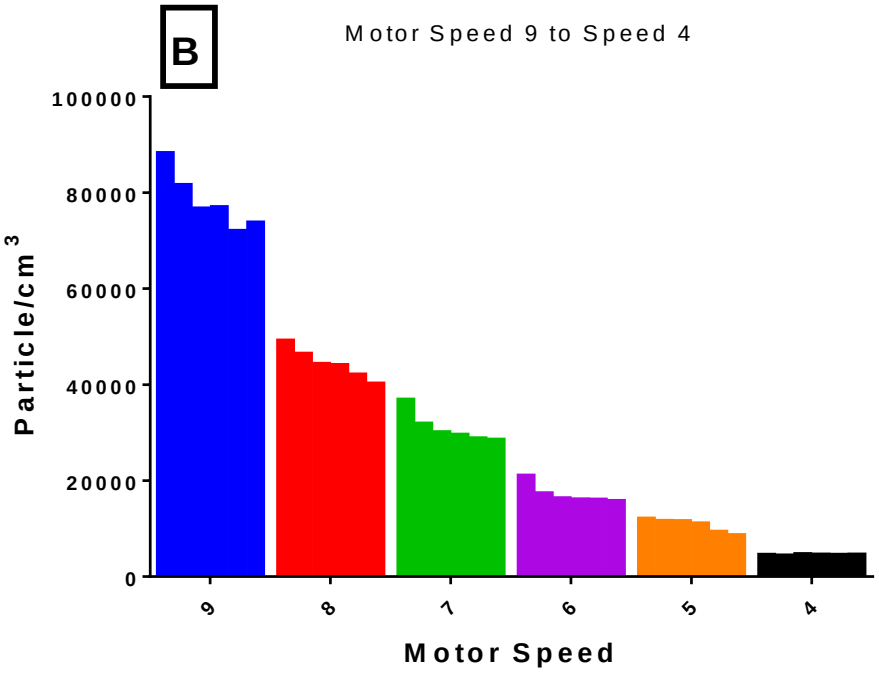
A starting material load of approximately 0.75 grams of MWCNTs was found to be effective to achieve our target particle concentration of 37,000 particles/cm^3. Approximately 0.25 grams of MWCNT were added daily to maintain the average concentration. The MWCNT needed to be totally replaced every four or five days to prevent agglomeration of the MWCNT and maintain airborne concentrations of nano-sized particles. Both height and motor speed could easily be adjusted while the dust generator was operating in order to alter the concentration of MWCNT being produced. Effective starting values for both the height and motor speed were determined.

The height of the dust generator was systematically adjusted during testing and a mark was made at an approximate position for maintaining our average particle concentration. The height was then adjusted before each exposure to achieve the target particle concentration. Small adjustments could quickly be performed during the exposure to maintain the concentration within the desired range.

Motor speed changes also provided a means to alter the concentration of the MWCNT. The motor control unit has markings from 0 to 10 to control the speed at which the piston cycles. Increasing the motor speed leads to an increase in the concentration of MWCNT. In testing, the motor ran for 30 minute increments with samples collected every 5 minutes. As demonstrated in Figure 3.3 there was a linear increase from motor speed 4 to motor speed 9. This test was repeated going from motor speed 9 down to motor speed 4 to ensure that the effect was due to motor speed alone and not influenced by the amount of time the generator had been running and the particle mass within the dust chamber. As seen in the following graphs consistent concentrations were achieved with both an increase and a decrease in motor speed.

Figure 3.4: Effect of Motor Speed on Particle Concentration. Motor speed tests were performed to assess the effectiveness of changing motor speed on particle concentration. A: The motor was started at speed setting 4 and the output of the dust generator was measured every 5 minutes for a 30 minute period. The motor speed was then adjusted to setting 5 and the measurements were made. This process was repeated until motor speed 9 was tested. B: The same test was performed but testing was started at motor speed 9 and the speed was adjusted down to setting 4.


A
Motor Speed 4 to Speed 9
100000
80000
60000
40000
20000
0
Particle/cm 3
4
5
6
7
8
9
Motor Speed


B
Motor Speed 9 to Speed 4
100000
80000
60000
40000
20000
0
Particle/cm 3
9
8
7
6
5
4
Motor Speed

3.4 DUST GENERATOR PRODUCTION OF OCCUPATIONAL LEVELS

By making adjustments as needed, an occupational concentration can be consistently created with the ECU dust generator. Using a predetermined starting particle mass of 0.75 grams the concentration typically starts around 50,000 particles/cm^3 and slowly decays to approximately 25,000 particles/cm^3 at the end of four hours (Figure 3.4). The average concentration for the ten days of exposure is approximately 37,000 particles/cm^3 and can be attained across multiple experiments as shown in Figure 3.5.

Figure 3.5: Day to Day Variation in Particle Concentration Over Time. Particle concentration over four hours of continuous exposure is shown using a representative two week study showing number of particles/cm^3 by minutes. Each curve on the graph represents one day of exposure with minutes shown on the X axis and particles/cm^3 shown on the Y axis. The first run measured room air which was typically around 1500 particles/cm^3 and the second run measured the tank air and was typically below 100 particles/cm^3. Samples with the motor turned on typically started around 50,000 particles/cm^3 and slowly decrease to around 25,000 particles/cm^3 after 4 hours. Once the motor was turned off particle concentration returned to baseline within 5 minutes. Troughs and peaks can be seen where adjustments were made due to lower than expected concentrations at that time in the exposure.

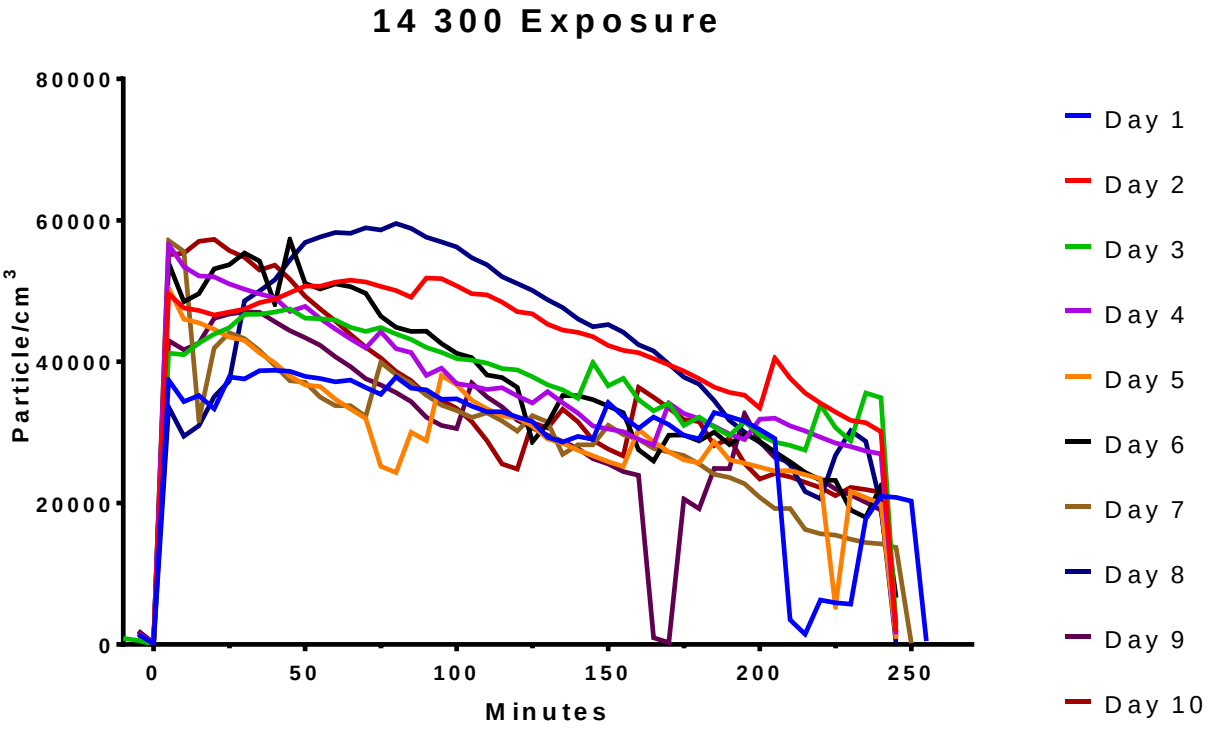
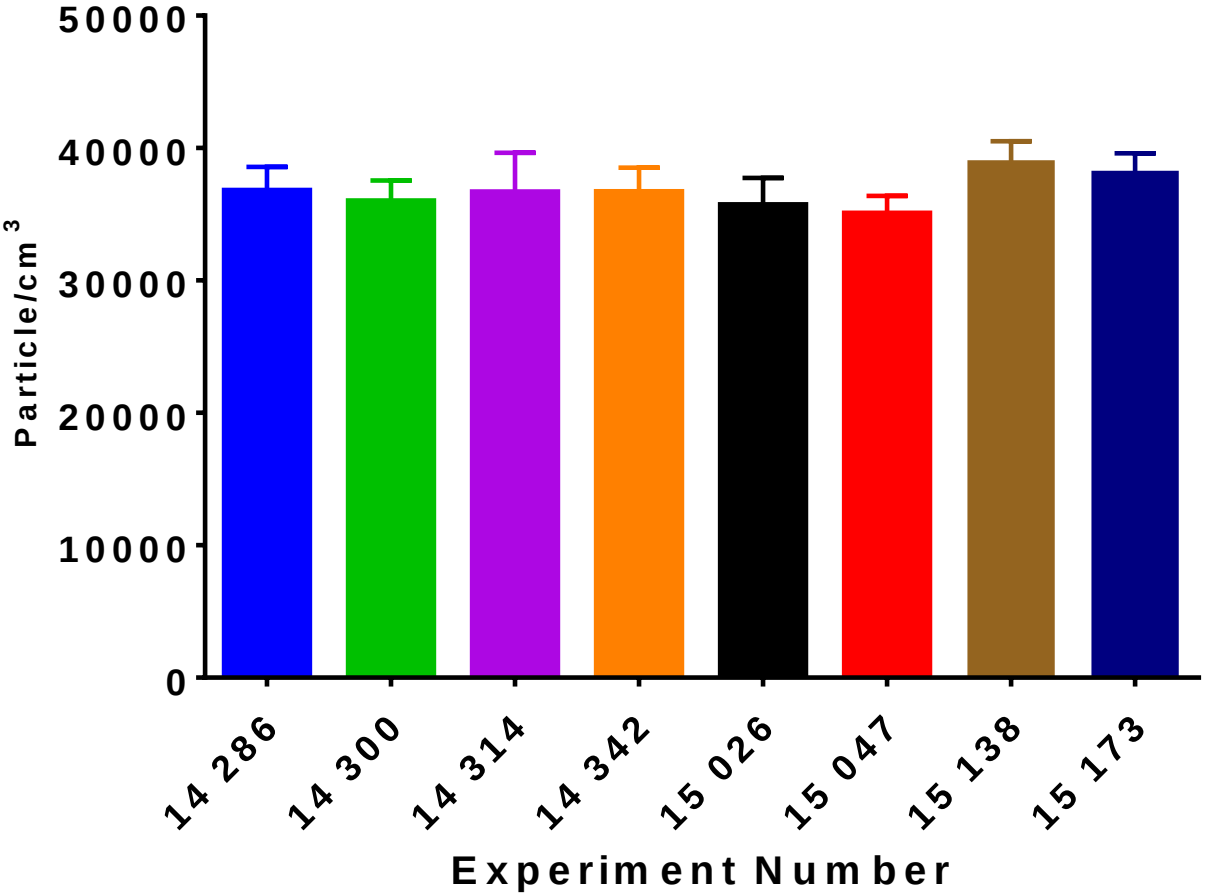

14 300 Exposure
80000
60000
40000
20000
0
Particle/cm 3
0
50
100
150
200
250
Minutes
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
Day 9
Day 10

Figure 3.6: Reproducibility of Average Particle Numbers. Particle number reproducibility is shown as particles/cm^3 on the Y axis for each two week exposure on the X axis. We were able to consistently achieve average concentrations of approximately 37,000 particles/cm^3 for each two week run.


50000
40000
30000
20000
10000
0
Particle/cm3
14 286
14 300
14 314
14 342
15 026
15 047
15 138
15 173
Experiment Number

3.5 DISCUSSION

While many animal studies indicate that MWCNT are toxic when delivered to the respiratory system, few have used occupationally relevant concentrations and routes of exposure. Other dust generators for inhalation research exist [124, 137, 138]; however, most were inappropriate for our purposes because they alter the physical properties of nanotubes. Specifically, the Mitchell version is believed to alter the physical properties of the CNT [138] and uses whole body exposure which may cause skin or fur contamination [139]. The Baron version was designed for use with SWCNT, but chops the particles up which is thought to reduce toxicity. Of the commercial dust generators, most use a grinding motion which could shorten nanotube length and diminish toxicity [138]. Since commercially available dust generators did not meet our requirement of not damaging the MWCNT and are expensive, we chose to design our own by altering and simplifying the NIOSH design. In order to address pulmonary toxicity of MWCNT in a realistic occupational scenario we designed, built, and tested a dust generator that met the criteria of being able to reproducibly generate occupational levels of MWCNT without causing damage to the nanotubes, while protecting the operator from exposure to high concentrations of MWCNT.

We found that the ECU dust generator is capable of producing MWCNT concentrations in our target range by adjusting the height of the dust chamber or speed of the piston. The motor/piston speed correlates to changes in particle concentration in a nearly linear fashion. Another possible technique to control particle concentration is by altering airflow. We chose to use a rate of 2 L/min based on common practice in the field of inhalation toxicology; however, an estimate of the ventilatory requirements for 12 mice indicates that 2L/min is well in excess of that requirement. Reduced airflow may

allow more particles to build up in the chamber and produce increased particle concentrations for delivery to the animal chamber.

Compared to the NIOSH dust generator the ECU dust generator lacks computer feedback loops meaning that it was less expensive to build but also that it requires significantly more operator attention and intervention in order to produce these desired concentrations. Unlike the NIOSH dust generator which maintains desired concentrations more consistently, the ECU concentrations decay over time apparently due to loss of particle mass. Although we did not add particles during the daily exposure period because of the necessity of stopping the exposure, we did observe that adding a relatively greater mass before each daily exposure resulted in higher particle concentrations. Thus, it is possible that a design alteration that allows for easier addition of particles during exposure could lead to more consistent exposure levels. Alternatively, more frequent or greater operator adjustments would likely increase consistency. On the other hand, workers in an occupational setting are unlikely to be exposed to a consistent concentration of MWCNT. Rather, particle concentration varies with the specific process or activity in an occupational setting [32]. We therefore chose to allow the particle concentration to decay over time to more closely mimic occupational exposure variation.

CHAPTER 4

TIME COURSE AND STRAIN COMPARISON

4.1 INTRODUCTION

Human health effects associated with exposure to MWCNT have not been documented to date. However, concerns about potential adverse health effects due to similarities between MWCNT and asbestos have been raised, and numerous animal studies support these concerns. As previously discussed, most animal studies on pulmonary responses to MWCNT exposure have used high bolus doses and/or non-physiological routes such as intratracheal instillation or aspiration. Not surprisingly, many of these studies found various pathologies including inflammation and granulomas. Yet such pathology may be a result of the delivery route and high dose. In order to assess risks associated with occupational exposures, it is important to use realistic exposure scenarios.

Therefore, we developed a mouse model to investigate pulmonary toxicity of MWCNT using the dust generator we developed to deliver a dry dust of MWCNT at an occupational concentration for inhalation. First, a time course study was conducted to determine the time of peak pulmonary effects induced by inhalational exposure to MWCNT. For this experiment, C57BL/6J male mice were used because it is the most commonly used strain in the literature and allowed us to compare our findings with other studies on the pulmonary effects of MWCNT. Mice were exposed to air only (control) or to an average concentration of 37,000 particles/cm^3 of dry, inhaled MWCNT for 4 hours/day, 5 consecutive days/week, for two weeks. The mice were sacrificed 1, 3, 7, 10, 14, 28, and 84 days after the last exposure and measures of lung injury (BALF

protein), inflammation (BAL differential cell counts), fibrosis (lung collagen content), and other lung pathologies (histological examination), as well as particle retention (dark field microscopy) were made.

As most environmentally induced lung diseases also have a genetic susceptibility component, we also compared three diverse strains of mice to determine if there may be a genetic basis for differential responses to inhaled MWCNT. For this set of experiments, we exposed A/J and DBA/2J male mice to dusts of MWCNT as described for C57BL/6J mice. Mice were euthanized 14 days post-exposure and the same end points were examined.

4.2 RESULTS

4.2.1 TIME COURSE

BALF protein levels, a measure of lung vascular permeability indicating lung injury, were not significantly different between MWCNT-exposed and air control mice at any time point (Figure 4.1), nor were there significant differences between time points. Total cell numbers recovered from BALF were significantly elevated at 28 days and showed a trend towards increased numbers at 1 day post exposure in MWCNT exposed mice compared to air controls (Figure 4.2). However, there was considerable variation in cell numbers in both air and MWCNT-exposed mice between time points. Similarly, alveolar macrophages were elevated at 28 days post exposure in MWCNT exposed mice with a trend toward increases at one day (Figure 4.3A). Macrophages made up the majority (78-98%) of the cell numbers and thus parallel total cell numbers. Monocytes were significantly elevated at 10 and 14 days post exposure in MWCNT exposed mice compared to air control mice (Figure 4.3B), and while there was variation

in numbers across time points, absolute numbers were less than a tenth of absolute macrophage numbers. Significant increases in recruited leukocytes (neutrophils, eosinophils, and lymphocytes) were not observed in response to MWCNT exposure at any time point. (Figures 4.3 D-F). Both neutrophils and eosinophils were present in low numbers in both MWCNT and air-exposed mice intermittently, particularly during the first 10 post-exposure days. Again there was substantial variation in numbers across time points. Lymphocytes were present more consistently and showed trends toward elevation in numbers with MWCNT exposure at 10, 14, and 28 days post exposure. Lung soluble collagen levels were consistent between both treatments and time points and did not show any significant differences between air controls and MWCNT-exposed mice at any time point (Figure 4.4).

Figure 4.1: Time course of BALF protein. BALF protein was measured using the Bradford method. BALF protein was not elevated in MWCNT exposed mice compared to air control mice when tested at 1, 3, 7, 10, 14, 28, and 84 days post exposure. Blue bars represent the air exposed animals and red bars represent the MWCNT exposed animals. n = 5 for air exposed mice at 14 day post exposure and n = 6 for all other groups.

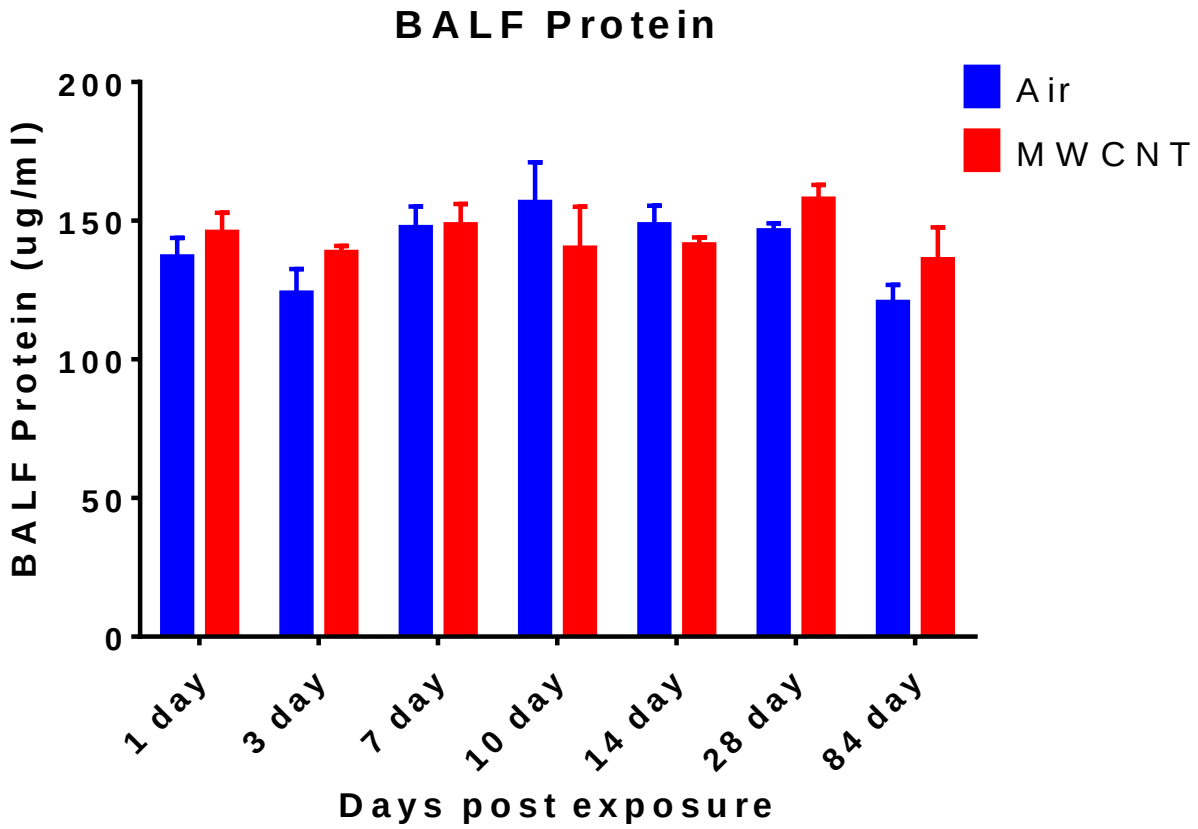

BALF Protein
BALF Protein (ug/ml)
200
150
100
50
0
Air
MWCNT
1 day
3 day
7 day
10 day
14 day
28 day
84 day
Days post exposure

Figure 4.2: Time course of total BALF cell numbers. Total cells expressed as number of cells/ml of recovered lavage fluid increased at 28 days post exposure in MWCNT exposed mice compared to air exposed mice. Blue bars represent the air exposed animals and red bars represent the MWCNT exposed animals. n = 5 for air exposed mice at 14 day post exposure and n = 6 for all other groups. * indicates $p < 0.05$ compared to air controls within the time point.

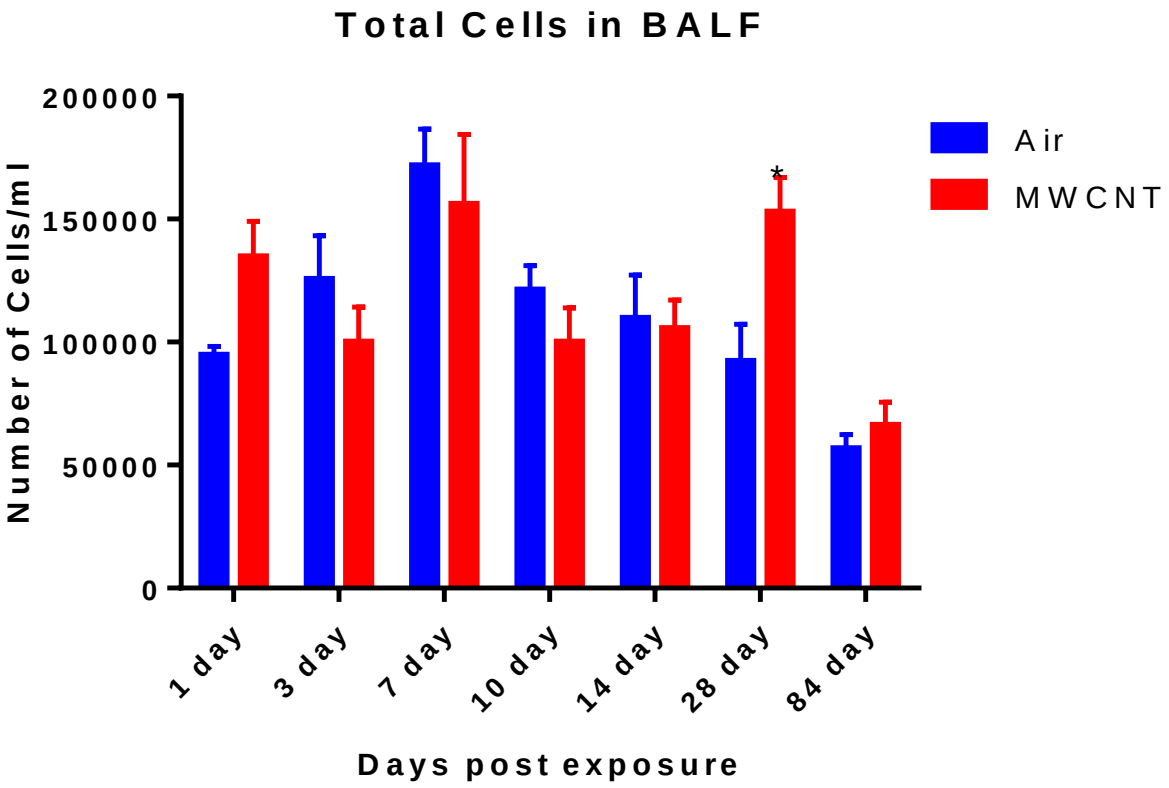
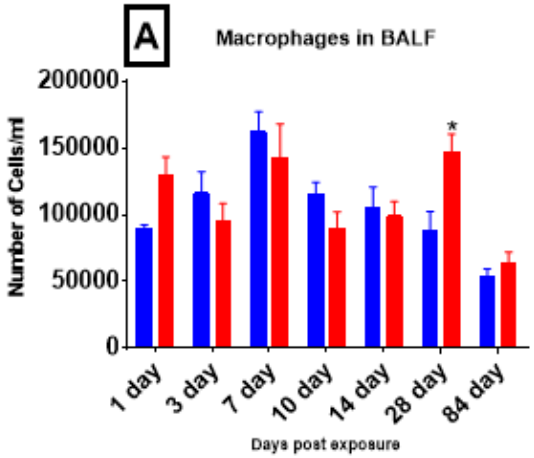
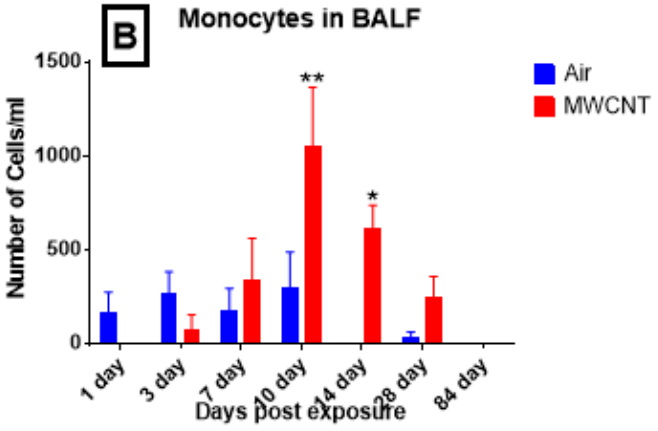
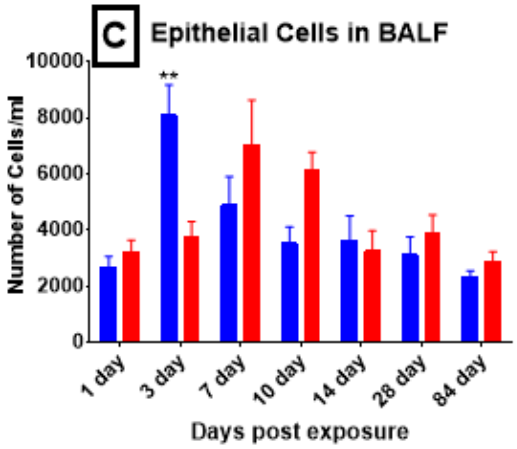
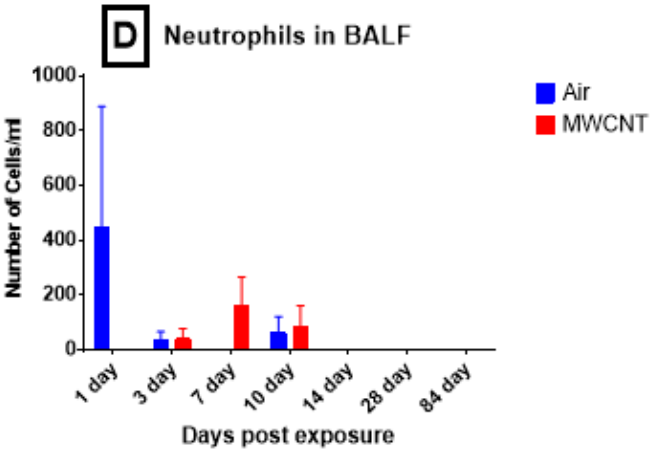

Total Cells in BALF
Number of Cells/ml
200000
150000
100000
50000
0
1 day
3 day
7 day
10 day
14 day
28 day
84 day
Days post exposure
Air
MWCNT
*

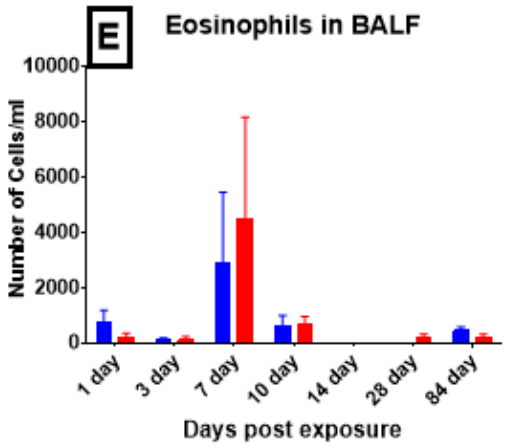
Figure 4.3: Time course of BAL Cellular Profile. For all graphs, cell numbers are expressed per ml of recovered lavage fluid. A) Macrophages increased at 28 days post exposure in MWCNT exposed mice compared to air control mice. B) Monocytes were significantly increased at 10 and 14 days post exposure for MWCNT exposed mice compared to air controls. C) Epithelial cells were increased at 3 days post exposure in air control mice compared to the MWCNT exposed mice. D) Neutrophil numbers were low and did not significantly change with regard to treatment or time point. E) Eosinophil numbers did not show any significant changes. F) Lymphocytes numbers tended to be elevated in MWCNT-exposed mice compared to air control, but these changes did not reach statistical significance. Blue bars represent the air exposed animals and red bars represent the MWCNT exposed animals. n = 5 for air exposed mice at 14 day post exposure and n = 6 for all other groups. * indicates $p < 0.05$ compared to air controls within time point. ** indicates $p < 0.01$ compared to MWCNT-exposed within time point.


A
Macrophages in BALF
Number of Cells/ml
200000
150000
100000
50000
0
*
1 day
3 day
7 day
10 day
14 day
28 day
84 day
Days post exposure


B
Monocytes in BALF
Number of Cells/ml
1500
1000
500
0
**
*
1 day
3 day
7 day
10 day
14 day
28 day
84 day
Days post exposure
Air
MWCNT


C
Epithelial Cells in BALF
Number of Cells/ml
10000
8000
6000
4000
2000
0
**
1 day
3 day
7 day
10 day
14 day
28 day
84 day
Days post exposure


D
Neutrophils in BALF
Number of Cells/ml
1000
800
600
400
200
0
1 day
3 day
7 day
10 day
14 day
28 day
84 day
Days post exposure
Air
MWCNT


E
Eosinophils in BALF
Number of Cells/ml
10000
8000
6000
4000
2000
0
1 day
3 day
7 day
10 day
14 day
28 day
84 day
Days post exposure

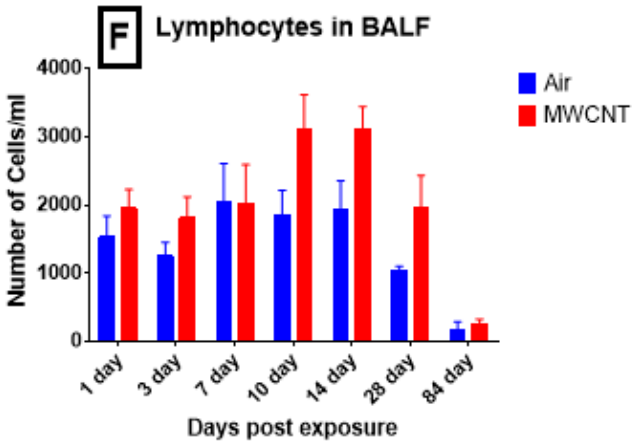
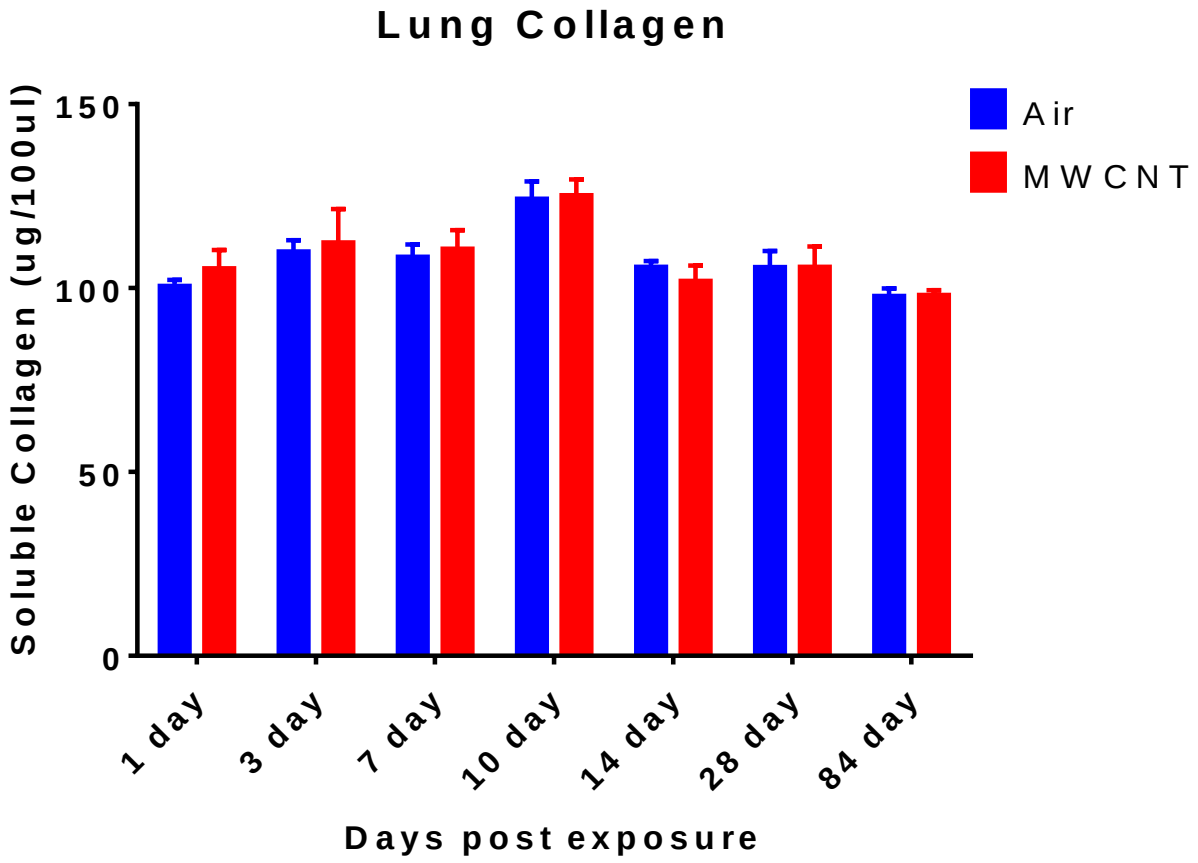

F
Lymphocytes in BALF
Number of Cells/ml
4000
3000
2000
1000
0
1 day
3 day
7 day
10 day
14 day
28 day
84 day
Days post exposure
Air
MWCNT

Figure 4.4: Time course of lung collagen content. Soluble lung collagen was measured in homogenized right lung using the Sircol Assay. Lung collagen content was not increased at any time point and did not change with treatment. n = 5 for air exposed mice at 14 day post-exposure and n = 6 for all other groups.

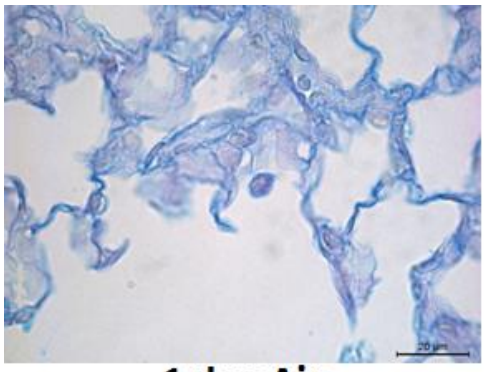

Lung Collagen
Soluble Collagen (ug/100ul)
150
100
50
0
Air
MWCNT
1 day
3 day
7 day
10 day
14 day
28 day
84 day
Days post exposure

Despite a lack of collagen response to MWCNT exposure, when the lung was homogenized for the collagen assay, the MWCNT exposed animals had a visible gray layer in the membrane pellet at every time point.

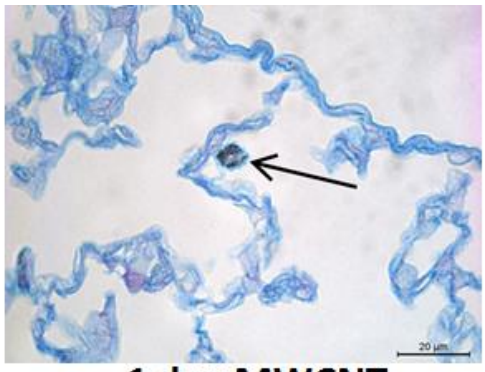
Histological lung sections were stained with Masson's Trichrome to allow visualization of collagen (blue), or Alcian Blue-Periodic Acid Schiff (AB-PAS) to allow visualization of mucus containing cells. Overt differences between air- and MWCNT-exposed mice were not observed at any time point. In fact, all sections appeared to have normal airway and parenchymal lung structure and cellular content. However, all sections from MWCNT-exposed mice had numerous alveolar macrophages containing black particles, while such particles were not seen in any air control lung sections (Figure 4.5).

The observation that MWCNT may have accumulated in alveolar macrophages in both BAL cells and histological sections, led us to have BAL cells imaged with dark field microscopy to validate the presence of MWCNT. As seen in Figure 4.6 nanotubes were present in macrophages recovered in BALF from MWCNT exposed animals for all time points including out to 84 days post exposure. Histological sections were also imaged using dark field microscopy, but nanotubes were not observed in lung tissues other than alveolar macrophages (data not shown).

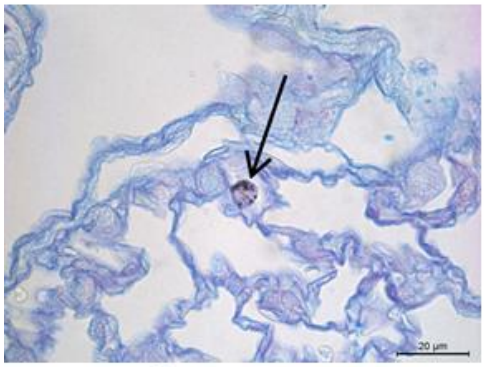
Figure 4.5: Lung Histology. Representative images from formalin fixed, paraffin embedded left lung sections show MWCNT-laden alveolar macrophages (arrows) at 1, 3, 7, 10, 14, 28, and 84 days post exposure to MWCNT. Alveolar macrophages in air exposed mice did not contain any visible particles at any time point (1 day post air exposed lung shown on top left). All slides shown are stained with AB-PAS. Scale bar is 20 microns. Images taken using 100x objective with oil.



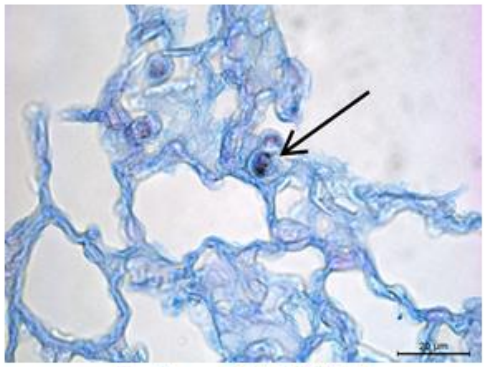
1 day Air



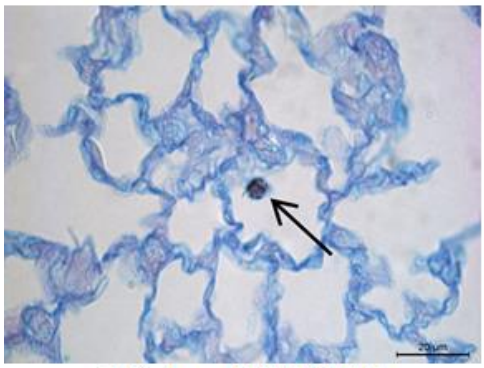
1 day MWCNT



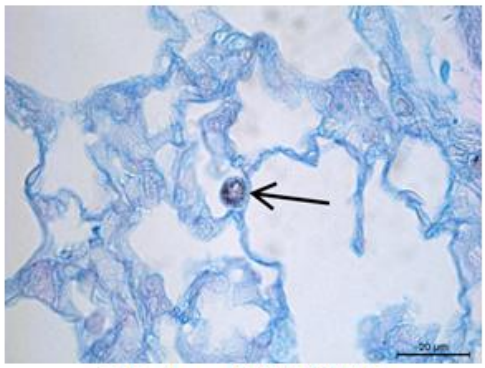
3 day MWCNT



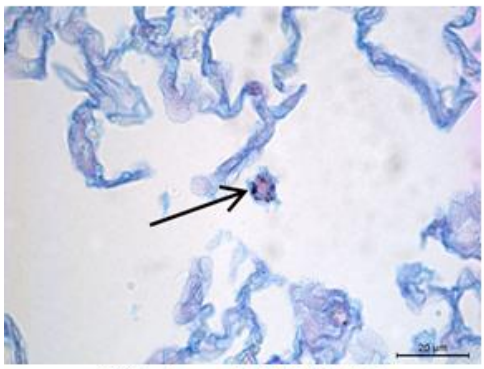
7 day MWCNT



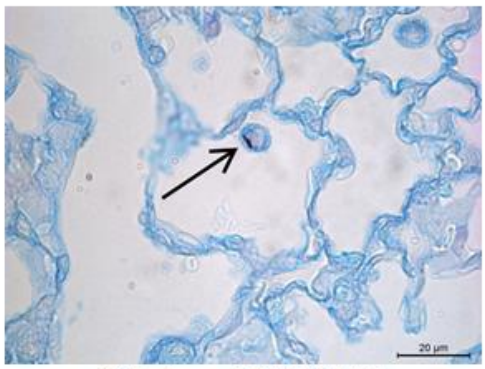
10 day MWCNT



14 day MWCNT

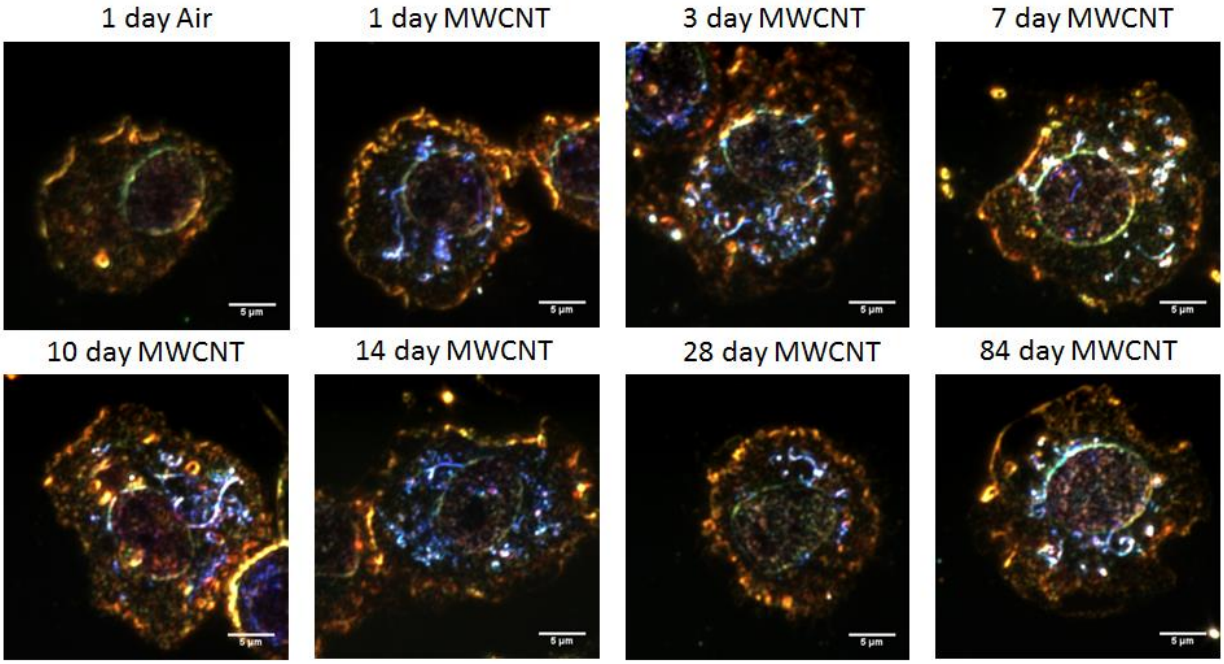


28 day MWCNT



84 day MWCNT

Figure 4.6: Dark field microscopy of BAL cells. Dark field images demonstrate that MWCNT are present in alveolar macrophages recovered from BALF at all time points post MWCNT exposure, but not in air controls. MWCNT appear as blue/white fibers and particles that are retained in alveolar macrophages for at least 84 days post exposure. A 1 day post exposure air control image is shown (top left) for comparison. Scale bars are 5 microns.


1 day Air
1 day MWCNT
3 day MWCNT
7 day MWCNT
10 day MWCNT
14 day MWCNT
28 day MWCNT
84 day MWCNT

4.2.2 STRAIN COMPARISON

Numerous studies have shown that susceptibility to environmentally induced lung disease phenotypes can be strain dependent. For example, fibrotic responses are known to differ between strains after exposure to V_2O_5 [140], asbestos [141], bleomycin [142], and silica [143]. In fact, the C57BL/6J strain of mice is known to be susceptible to bleomycin- and asbestos-induced fibrotic responses, but is resistant to V_2O_5-induced pulmonary fibrosis. In order to determine whether the lack of pulmonary responses to inhaled MWCNT that we observed in the time course study described above may have been because C57BL/6J mice are genetically resistant, two additional strains of mice, DBA/2J and A/J, were then exposed to MWCNT at the same average concentration as previously described and sacrificed at 14 days post exposure. Data from C57BL/6J mice at the 14 day time point above was compared to A/J and DBA/2J data. The 14 day time point was chosen because it is a time point at which fibrotic, as well as some cellular responses have been observed after nanotube exposure [98], plus we observed an elevation of monocytes at this time. A/J and DBA/2J strains were chosen because they are genetically very different from the C57BL/6J mice and from each other, which should allow detection of differential genetic susceptibilities. Additionally, DBA/2J mice are highly susceptible to V_2O_5-induced pulmonary fibrosis [140], while A/J mice are known to be predisposed to Th2-like responses which are thought to contribute to fibrotic responses [144].

Similar to the responses seen in C57BL/6J at 14 days post exposure, A/J and DBA/2J mice did not exhibit significant differences in protein levels, total cells, or collagen levels between exposure groups; however, strain and treatment dependent

trends were observed. As seen in Figure 4.6, BALF protein was not significantly increased in MWCNT exposed mice compared to air control mice at 14 days post exposure in DBA/2J or A/J mice. However, in A/J mice MWCNT exposure induced a trend towards elevated BALF protein levels compared to the air controls, although A/J mice had lower protein levels than C57BL/6J or DBA/2J mice in general. Total BAL cells were not significantly increased in any strain of mice exposed to MWCNT (Figure 4.7). Yet in contrast to protein levels, total cell counts (Figure 4.7) and several specific cell types (Figure 4.8) were elevated in DBA/2J mice after MWCNT exposure, although these increases were not statistically significant. Specifically, DBA/2J mice exposed to MWCNT showed a trend of increasing macrophages that was not seen in C57BL/6J and A/J mice (Figure 4.8A). Monocytes were significantly increased at 14 days post exposure for C57BL/6J MWCNT exposed mice compared to air controls and were slightly elevated in DBA/2J mice (Figure 4.8B). Epithelial cells, neutrophils, and eosinophils did not significantly increase in MWCNT exposed mice compared to air controls in any of the three strains yet DBA/2J mice had a non-statistically significant increase in neutrophils, and eosinophils were observed in both air and MWCNT-exposed mice of the A/J and DBA/2J strains (Figure 4.8C-E). Lymphocyte numbers in MWCNT-exposed C57BL/6J mice were significantly elevated, however, C57BL/6J mice had a higher number of lymphocytes in air-exposed mice compared to the other strains as well (Figure 4.8F). Finally, there were no differences in lung collagen content with regard to treatment or strain (Figure 4.9).

Figure 4.7: BALF protein in three strains of mice. C57BL/6J (B6), DBA/2J (D2), and A/J strains of mice exposed to inhaled MWCNT or air did not have statistically significant differences in BALF protein levels at 14 days post-exposure. However, A/J mice showed a trend towards increased BALF protein in MWCNT-exposed mice that nearly reach significance. BALF protein was measured using the Bradford method. n = 5 for air exposed B6 mice and n = 6 for all other groups.

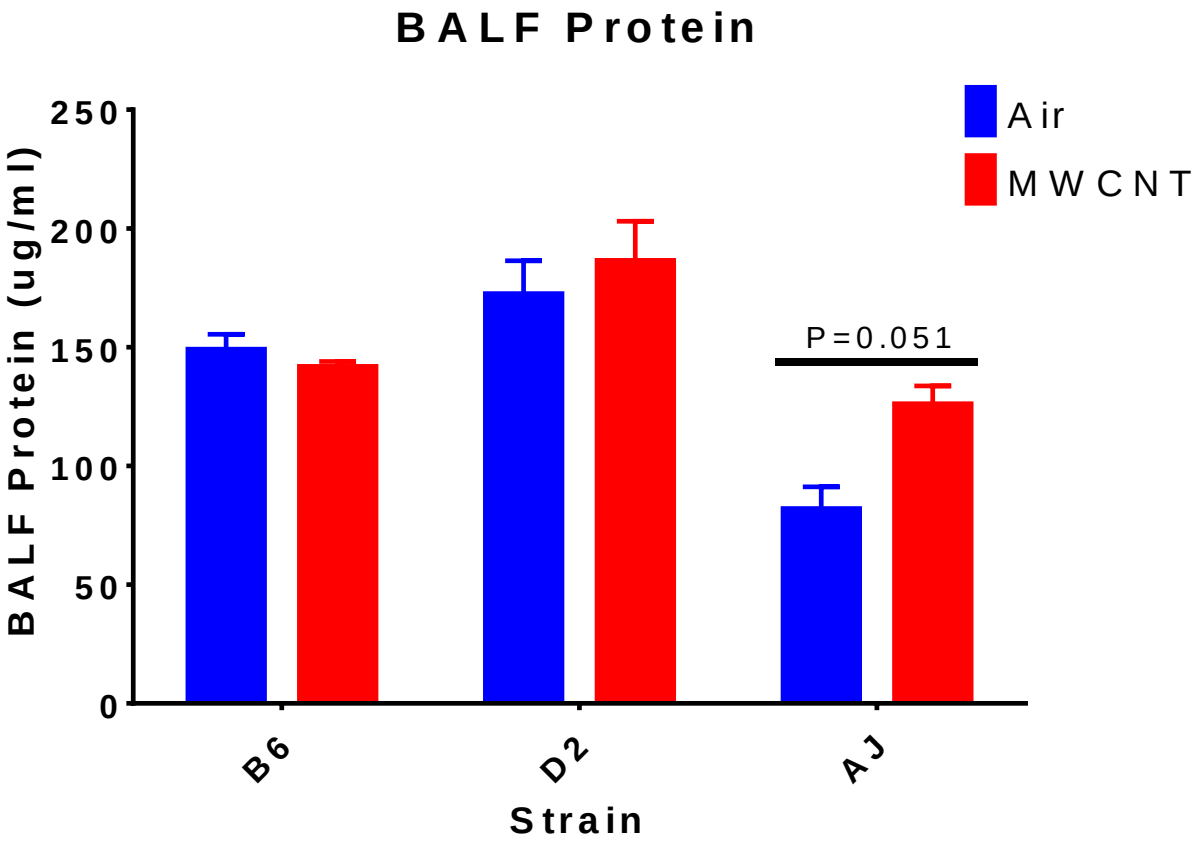

BALF Protein
Air
MWCNT
BALF Protein (ug/ml)
250
200
150
100
50
0
P=0.051
B6
D2
AJ
Strain

Figure 4.8: Comparison of total BAL cells across three strains of mice. Total cell numbers were not significantly increased at 14 days post MWCNT exposure compared to air controls in C57BL/6J (B6), DBA/2J (D2), or A/J mice. Yet, D2 mice exposed to MWCNT did show a trend of increasing cells as compared to the air exposed mice. n = 5 for air exposed B6 mice and n = 6 for all other groups.

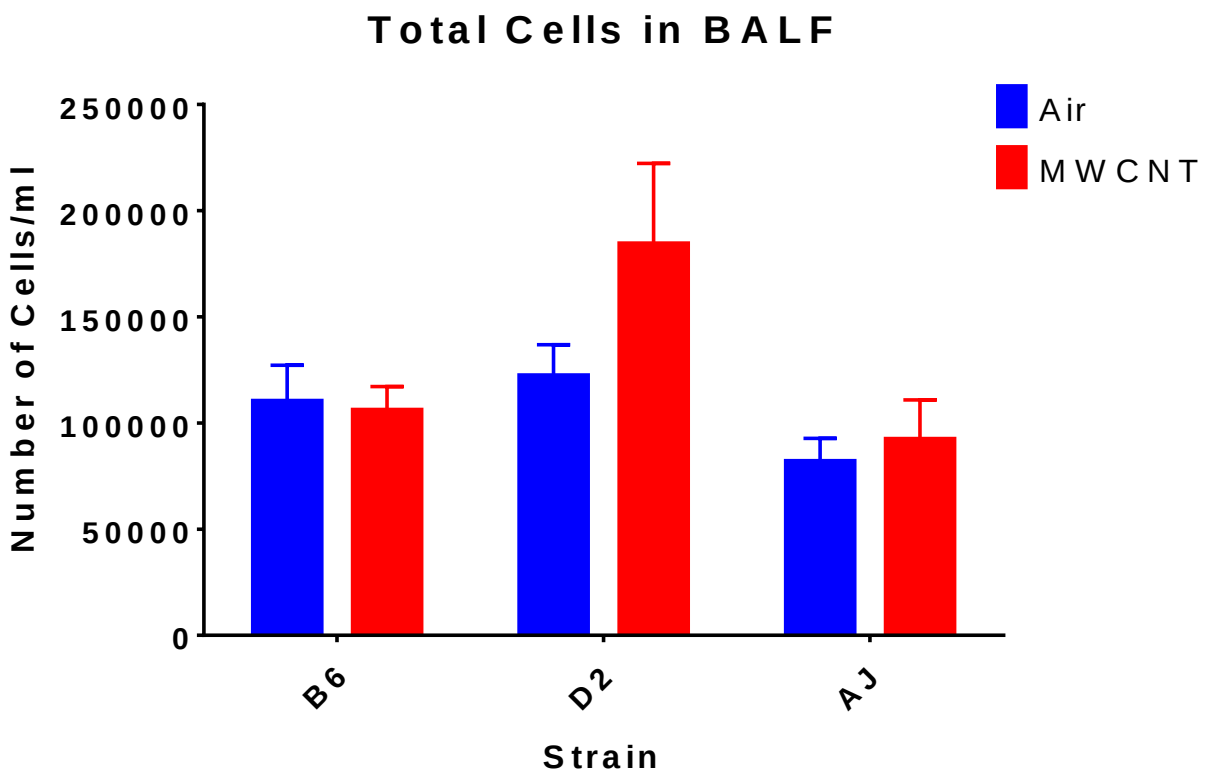

Total Cells in BALF
Number of Cells/ml
250000
200000
150000
100000
50000
0
B6
D2
AJ
Strain
Air
MWCNT

Figure 4.9: Differential BALF cell counts in three mouse strains. All graphs (A-F) represent cell numbers recovered from BALF at 14 days post exposure in C57BL/6J (B6), DBA/2J (D2), and A/J mice. A) Although alveolar macrophage numbers did not significantly increase in MWCNT exposed mice compared to air control mice in any of the three strains, there was a trend toward increased numbers in D2 mice exposed to MWCNT. B) Monocytes were significantly increased at 14 days post MWCNT exposure in B6 mice compared to air controls. C) Epithelial cell numbers varied somewhat by strain, but were not significantly altered by exposure in any strain. D) Neutrophil numbers were very low and were observed sporadically in D2 MWCNT-exposed mice and A/J air-exposed mice. E) Eosinophil numbers were also low and observed sporadically in D2 and A/J mice. F) Lymphocyte numbers were significantly elevated in MWCNT-exposed B6 mice compared to air control mice, while D2 mice showed a similar trend with lower numbers, but A/J mice did not. n = 5 for air exposed B6 mice and n = 6 for all other groups. ** indicates $p < 0.01$ compared to air controls within strain. **** represents $p < 0.0001$ compared to air controls within strain.

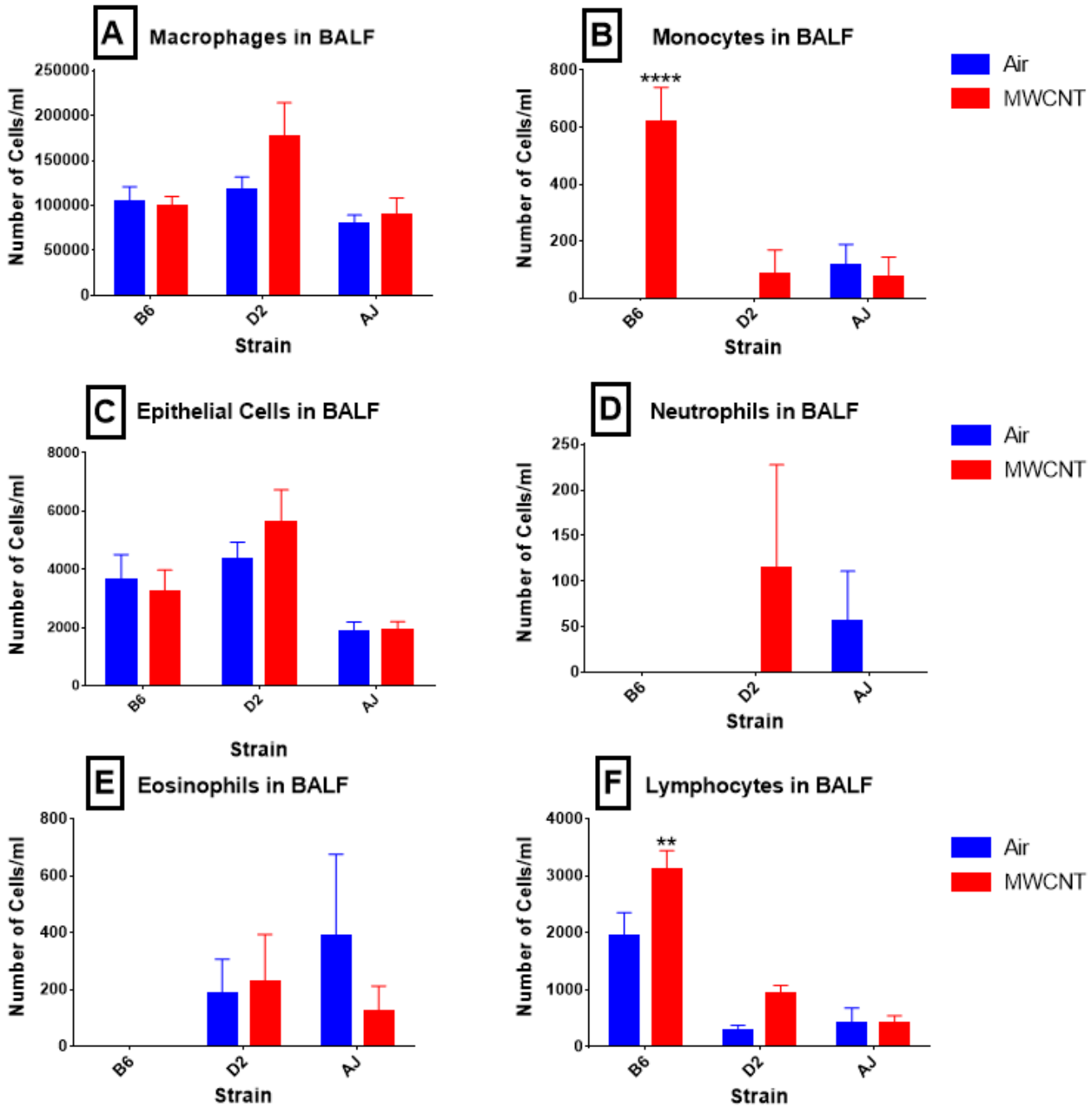
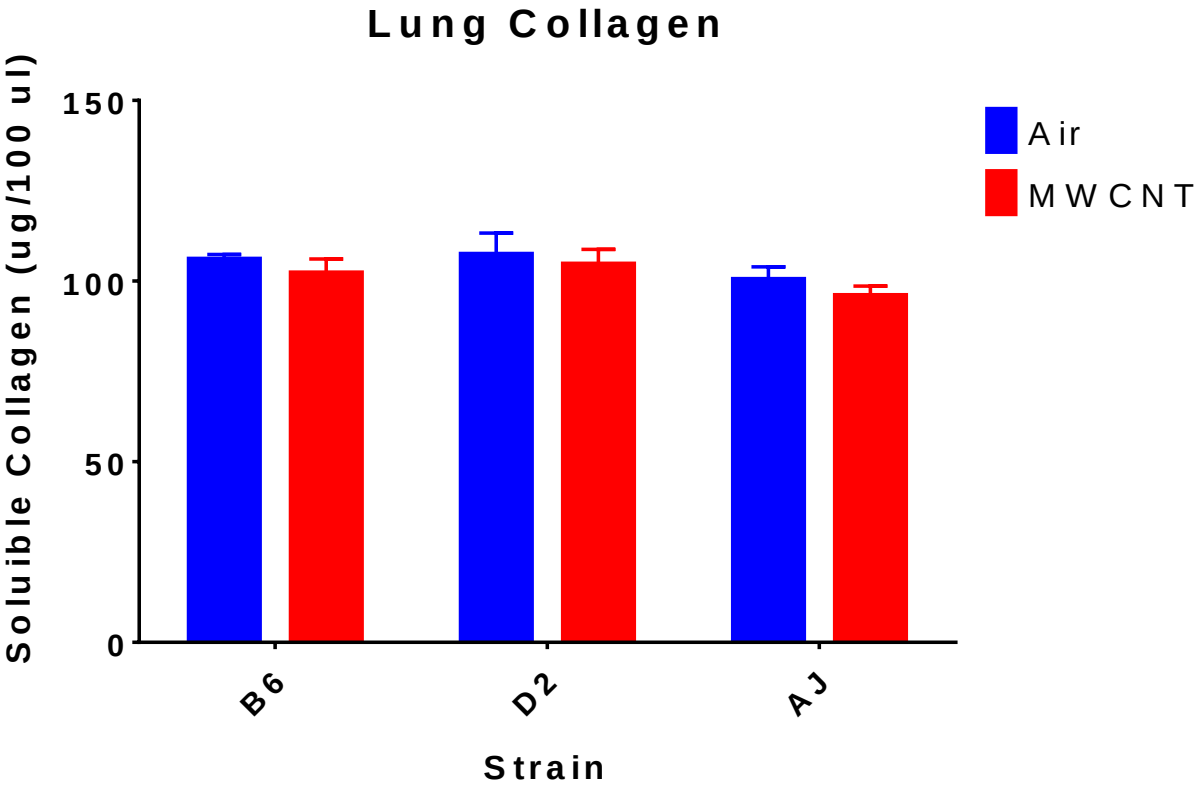
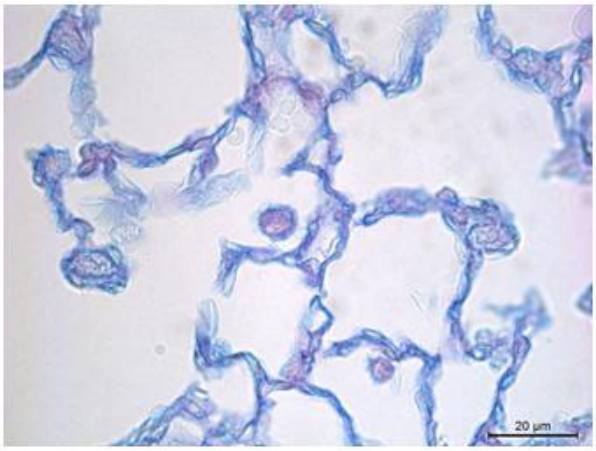

A
Macrophages in BALF
Number of Cells/ml
250000
200000
150000
100000
50000
0
B6
D2
AJ
Strain
B
Monocytes in BALF
****
800
600
400
200
0
Air
MWCNT
C
Epithelial Cells in BALF
8000
6000
4000
2000
D
Neutrophils in BALF
250
200
150
100
50
E
Eosinophils in BALF
F
Lymphocytes in BALF
**
4000
3000
2000
1000

Figure 4.10: Strain comparison of lung collagen content. Soluble lung collagen levels were not changed at 14 days post exposure with regard to exposure or strain. [C57BL/6J (B6), DBA/2J (D2), or A/J mice]. n = 5 for air exposed B6 mice and n = 6 for all other groups.

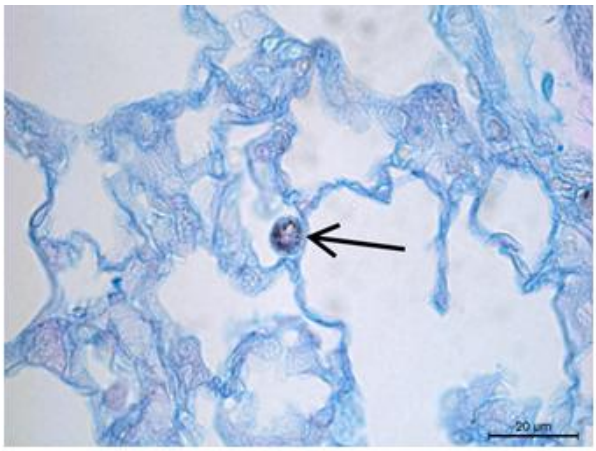

Lung Collagen
Soluible Collagen (ug/100 ul)
150
100
50
0
B6
D2
AJ
Strain
Air
MWCNT

Although no dark field microscopy was performed on these strains, alveolar macrophages were observed to contain MWCNT both histologically and in cell pellets recovered in BALF. Likewise, a gray layer in the membrane pellet of homogenized lung tissue was present in all strains. As in C57BL/6J mice, histological lung sections from both air and MWCNT-exposed A/J and DBA/2J mice had normal structure and showed no evidence of inflammation, fibrosis, or increased mucus production at 14 days post-exposure; however, MWCNT were visible within alveolar macrophages at high magnification (Figure 4.11).

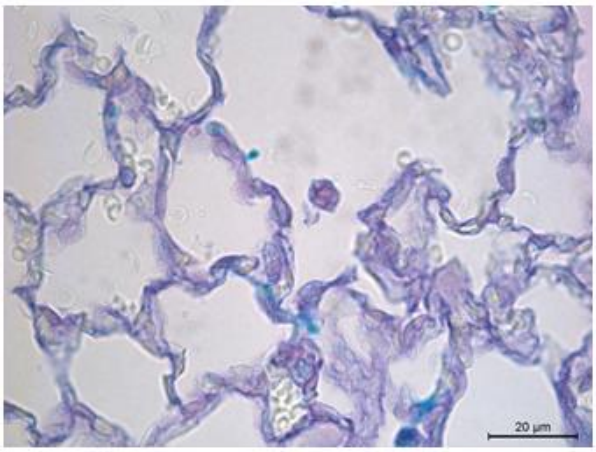
Figure 4.11: Histological lung sections from three strains of mice. Histology images showing dark MWCNT in alveolar macrophages (arrows) of C57BL/6J (B6), DBA/2J (D2), and A/J mice 14 days after MWCNT exposure, but not after air exposure. Tissue stained with AB-PAS. Scale bar is 20 microns.



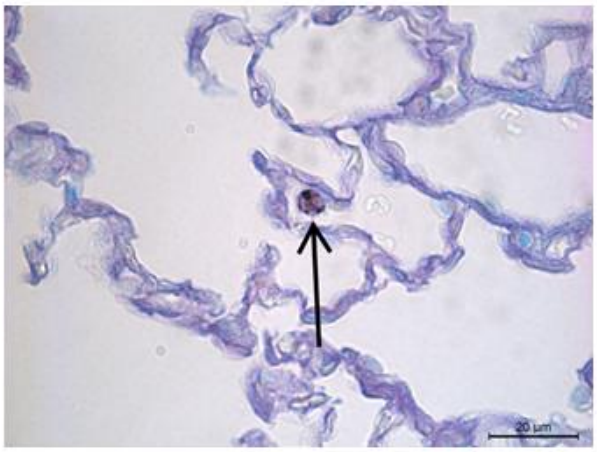
B6 Air



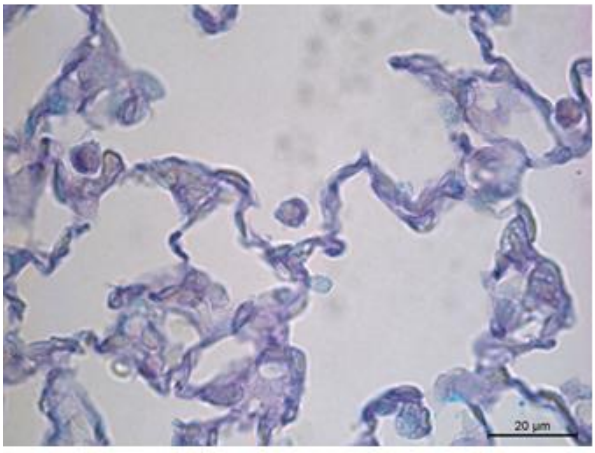
B6 MWCNT



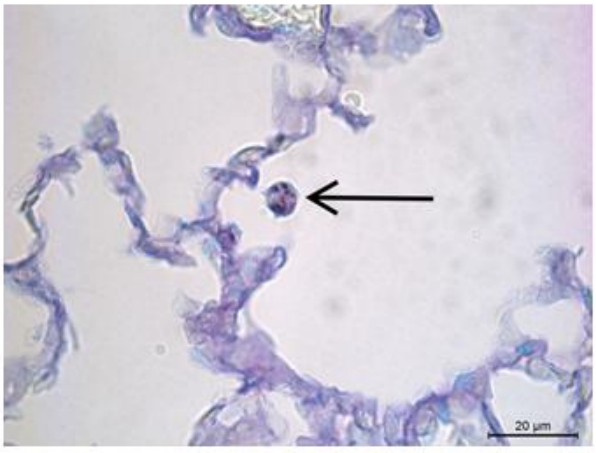
D2 Air



D2 MWCNT



AJ Air



AJ MWCNT

4.3 DISCUSSION

Pulmonary exposure to MWCNT has been reported to result in several pathological phenotypes in mice. However, many of these reports employed high-dose, bolus techniques to deliver the nanotubes. Use of these unrealistic exposure scenarios can produce biological responses that are vastly different from responses to more realistic exposures. Because several thousand tons of MWCNT are being produced yearly to meet the wide variety of applications, occupational exposure presents a potential health risk for workers. Thus, one of the goals of this project was to determine whether exposure to occupational levels of MWCNT through inhalation of a dry dust results in pulmonary toxicity.

We therefore developed a mouse model to examine pulmonary outcomes over a three-month time course after exposure to an intermediate occupational concentration of MWCNT. Our data suggests that at the concentrations and short duration of exposure we used, there are minimal acute effects on the respiratory system. Specifically, the lack of increases in protein levels indicates that lung permeability was not increased by MWCNT exposure and the epithelial-endothelial barrier in the lungs remained intact. Furthermore, significant increases in recruited inflammatory cells (neutrophils, eosinophils and lymphocytes) were not observed in the time course. However, in the strain screen, lymphocytes and monocytes were increased in C57BL/6J mice which may be the result of a mild immune response to inhaled MWCNT. Other studies found a variation of lung immune responses depending on the nanoparticle inhaled [145], a modulation of the immune response when using inhaled MWCNT in allergic rats [146], and systemic immune alterations even in the absence of significant

lung inflammation [124]. In the time course, we observed a significant increase in epithelial cells at 3 days post exposure in air control mice. This cause of this anomalous increase is unknown but may have resulted from unknown exposures in the vivarium, a response to dry air during exposure, or inconsistencies in the lavage technique.

Histological examination of lung tissue did not show any overt effects of MWCNT, except the presence of particle-laden alveolar macrophages. This may be interpreted as the macrophages acting to defend the lungs from foreign materials by phagocytosing them. However, the lack of macrophage clearance of CNT, as evidenced by their presence out to 84 days post-exposure, may indicate a MWCNT-induced effect on macrophage function. This may have important ramifications for secondary exposures after exposure to MWCNT.

While the same data were used for C57BL/6J mice in the time course and strain screen, the p values for the cell counts changed due to changes in overall power and variance of the data sets. Thus, in the time course, lymphocytes were not significantly elevated for MWCNT exposed C57BL/6J mice at 14 days, yet significance was reached when analyzing them with the A/J and DBA/2J data.

One of our initial objectives was to use genetic mapping to identify susceptibility factors and potential mechanisms of observed pulmonary phenotypes. Unfortunately, under the conditions used for the current studies we did not observe a significant phenotype suitable for genetic mapping and thus did not pursue mapping objectives. None-the-less, we did see exposure-dependent trends in different strains of mice that may suggest genetic susceptibility to specific phenotypes (different strain-dependent

effects). Most studies of respiratory exposure to MWCNT have been conducted in C57BL/6 mice or outbred strains, thus our observation of differential responses, although not statistically significant, may indicate that genetic background plays an important role in responses to inhaled MWCNT. The use of higher concentrations or more prolonged exposures in future studies could produce significant inter-strain differences.

In summary, we did not find evidence of acute pulmonary effects from MWCNT at the concentration and duration of exposures used, with the exception of retention of MWCNT in alveolar macrophages. On the other hand, we did observe some trends that may suggest genetic susceptibility to MWCNT-induced pulmonary deficiencies with longer exposures or higher concentrations of MWCNT.

CHAPTER 5

DISCUSSION

5.1 SUMMARY OF STUDY

This study was designed to investigate the pulmonary toxicity of inhalation exposure to occupational levels of MWCNT in mice. To accomplish this objective, a dust generator capable of reproducibly delivering occupational levels of dry MWCNT was successfully built and used to expose mice. At the exposure concentration and duration that was used, there were no signs of overt toxicity. However, there were minor changes in the cellular profile recovered in lavage fluid, which varied over time and with strain suggesting that multiple factors likely influence pulmonary responses to MWCNT including physicochemical properties of the nanotubes, as wells as dose, duration of exposure, and genetic or host factors. Notably, the persistence of MWCNT in alveolar macrophages to three months post-exposure suggests that even at low doses, MWCNT can alter macrophage function and reduce clearance which could have an important impact on innate host-defense mechanisms.

5.2 DISCUSSION

This study designed, built, tested, and deployed a dust generator capable of producing dusts with consistent levels of MWCNT to facilitate the study of pulmonary effects of inhaled MWCNT in mice. Using this dust generator we found no overt signs of toxicity in mouse pulmonary tissue other than evidence of retained nanotubes in alveolar macrophages as far out as 84 days post exposure and across several strains of mice.

Toxicity is a complex process with multiple factors including physical and chemical properties of the agent; characteristics of the exposure including the route, dose/concentration, and duration; biopersistence of the agent; aggregation of the agent; and multiple host factors. Of particular concern with nanomaterials are the physical and chemical properties of the material including size, surface area, agglomeration, shape, surface chemistry, chemical composition, rigidity, etc. In terms of pulmonary pathology, key considerations include deposition, biopersistence, exposure duration, and interaction with other body systems [136]. MWCNT toxicity appears to be a result of deposition and retention [147]. The cellular dose received is a function of multiple properties including the particle size, shape, density, and charge however additional factors such as metabolism may confound the cellular dose. Unfortunately, measurement of the cellular dose is difficult, costly, and generally not possible for most studies [148].

Our mild results could be due to several factors. First, the duration of exposure we used was 10 days at a rate of 5 days per week which is considerably shorter than that used by others who observed more severe effects. Our exposure time of two weeks is about 2% of a mouse's life span and may have simply been inadequate to observe more significant effects. Pauluhn found that inhalation exposure over a 1 month period (using 11 and 241 mg/m^3) with a post exposure period of 3 months may be necessary in order to elicit effects specific to the tested nanoparticle [149]. Although our post-exposure time of 84 days was close to three months, the exposure duration we used was less than half of the 1 month exposure period described by Pauluhn. The effects of chronic exposure may be important as many workers could be exposed over a period of

years or decades. Our finding that MWCNT were retained in the mouse lung tissue for at least 84 days, suggests that repeated or chronic exposure could lead to accumulation of material and long-term pulmonary effects such as impaired lung clearance or deficits in host defense mechanisms.

Pulmonary toxicity in rats exposed to MWCNT by inhalation was shown to be a product of both time and concentration [61]. Our chosen concentration was well within the occupational range measured in production and processing facilities [32]. As previously mentioned, numerous studies that have reported lung pathology used drastically higher particle concentrations. A higher airborne concentration would have likely elicited greater responses; however, our objective was to determine effects of exposure to occupational levels of MWCNT, not to demonstrate toxicity without regard to dose/concentration.

The physicochemical properties of MWCNT may also influence toxicity [21, 132, 150, 151]. Effects could be due to chemical functionalization or residual metal contamination from manufacturing of the MWCNT, or to different physical properties such as length and rigidity [79, 152]. One possible explanation for our mild results is that the nanotubes we used had only low levels of chemical contaminants such as metals. Studies have found that MWCNT with iron impurities induced ROS while MWCNT without iron did not [153] as did nickel contamination [154]. . Nickle oxide nanoparticles induced oxidative stress in rat lungs while titanium oxide nano and fine particles did not, thus appearing to suggest that the presence of nickel is a factor in oxidative stress that could lead to inflammation and cellular damage [152, 159]. Previous studies using the same batch of MWCNT as we used reported that nickel accounted for 5.5% of the

chemical composition; however, a different measurement methodology found a much lower percentage [155]. An in vitro study of lung epithelial cells showed that pristine MWCNT can demonstrate biopersistence in these cells without having long term consequences [54].

The length, diameter, and rigidity of nanotubes all appear to be factors in causing inflammation [9, 21, 156]. Nagai, et al., reported that the rigidity of MWCNT plays a crucial role in determining mesothelial injury [79]. However, as seen in the dark field images of alveolar macrophages that have phagocytosed MWCNT (Figure 4.6), the longer fibers appear to be curved and flexible. Additionally, many of the ingested particles appear to be short, although this may be an artifact of processing. The MWCNT we used were reported to range from 0.3 – 50μm in length; however, we did not have the particles analyzed post-dust generation, so it is possible that our system selectively generated dusts of only shorter particles or broke the longer nanotubes, although the latter seems unlikely. Certainly, the dust we generated was within a respirable size range as we observed gray material in the BAL cell pellets (presumably in the macrophages) from MWCNT-exposed mice, as well as in the membrane pellets of homogenized lung tissue.

Deposition and clearance of particles plays an important role in pulmonary responses to exposure. We exposed mice to dry MWCNT, which were previously shown to have a deposition rate three times less than that of wet MWCNT although the dry MWCNT also had a clearance rate that was slower than for wet MWCNT (87 days versus 46 days) [57]. These studies support our observation of biopersistence to 84 days post MWCNT exposure. Another factor in deposition and clearance of particles

and subsequent lung pathology is agglomeration. Li, et al compared MWCNT inhalation with instillation in mice. The inhalation group had different patterns of MWCNT aggregates than were observed in mice instilled with MWCNT. Furthermore, mice that inhaled MWCNT did not show the expected pathology that was seen in MWCNT instilled mice [48]. MWCNT instilled in rats with BSA as a dispersing agent were seen in the macrophages but without inflammatory markers. The lack of an inflammatory response could have been a result of protein interactions with MWCNT to induce phagocytosis or due to the smaller agglomerates because of the dispersing agent [59]. Similarly, we observed MWCNT in macrophages, but no cellular inflammation, suggesting that inhalation of dry MWCNT may result in improved dispersion of the MWCNT or smaller agglomerates that produce less inflammation and associated pathology. Deposition of inhaled MWCNT in the concentration range of 0.3-5mg/m^3 for 6 hours per day for either 7 or 14 days resulted in deposition weights of MWCNT of 0.2 to 2.7 mg/kg in mice, but did not produce lung pathology [124]. Our study used a concentration which corresponds to a mass that is smaller than the highest concentrations referenced above, thus, it is expected that we also had a lower deposited mass.

Finally, the lack of any signs of lung injury or inflammation after inhalation of occupational levels of MWCNT may be due to immunosuppressive effects of stress on the mice. A study of mice exposed to acute inescapable foot shocks found depressed antibody response, inhibited lymphocyte reactions, and attenuation of immune function [157]. Although soft restraints were used which allow animals to move their limbs and prevent overheating were used, it is possible that the time in restraints during the

exposure stressed the mice and limited host defense and immune responses to MWCNT.

5.3 LIMITATIONS

This study had several limitations. First, our overall objective was to determine pulmonary effects of occupational levels of inhaled MWCNT. Therefore, our dust generator was designed to produce occupational concentrations of MWCNT. The initial design did not consider the potential need for higher concentrations. However, exposure to a much higher concentration of MWCNT might have resulted in obvious pathologies and identification of significant phenotypes on which genetic mapping could have been conducted. Once defined phenotypes were identified, a more focused experiment could have looked for early signs of these effects at occupational concentrations. Our study also dispersed only dry MWCNT as opposed to wet MWCNT which have been shown to have a significantly higher deposition rate than dry MWCNT [57]. This may have limited the negative impacts but is a closer model to occupational exposure in which the MWCNT become airborne during manufacturing, handling, transporting, etc. [20]. A study of occupational exposure rates showed that 2 of 6 sites tested were above 7 $\mu g/m^3$ which was the NIOSH REL at that time [35]. NIOSH currently recommends exposure to no more than 1 $\mu g/m^3$ of air which is the lowest measurable airborne concentration and is intended to protect workers long term [160]. We are not confident in the particle mass measurements we obtained since our equipment measures aerodynamic diameter which assumes spherical particles while MWCNT are cylindrical particles, thus making it difficult to compare our results with the results of studies that used mass concentrations rather than number concentrations.

Additionally, only short-term exposure was tested in this study. Given the observation that MWCNT are retained in alveolar macrophages, a longer duration of exposure may have resulted in further accumulation of particles and potentially pulmonary pathology. Additionally, we were not able to quantitate the pulmonary deposition and retention of MWCNT nor could we determine how similar the deposition and clearance/retention is between mice and humans for inhaled MWCNT under the conditions we used. The MWCNT we used are likely to be low in toxicity for a number of reasons including low levels of metal contaminants, flexibility of the fibers, and possibly a high proportion of relatively short tubes. We did not try to validate that the MWCNT exiting the containment chamber were of the same length that we started with. Use of electron microscopy to examine the MWCNT before and after the dust generator could have addressed this limitation.

We were interested in MWCNT since it is one of the most widely used nanomaterials; however, other nanomaterial may have greater toxicity. Furthermore, we only used one type of MWCNT and did not explore other MWCNT which may vary greatly in length, rigidity, diameter, surface area, metal contaminants, or chemical modifications.

5.4 FUTURE STUDIES

There are many questions left to answer regarding the pulmonary toxicity of MWCNT and other nanomaterials. Our studies are a starting point to address the biological effects of realistic occupational exposure scenarios. Use of a different nanotube/nanomaterial and/or concentration is a possible area of future study. We would not vary using inhalation but previous studies found variations in both toxicological and biological responses to CNT exposure with variations dependent upon

the type of CNT, the dose of CNT, and the route by which the CNT were administered [124], so repeat studies using MWCNT with different properties and/or a different concentration may have different results. If another nanomaterial is commonly made, known to be dangerous at high concentrations, and is an occupational risk, we can test occupational levels of those materials for toxicity. Further, smaller diameters and increased rigidity are known to increase the toxic effects of MWCNT [161] so study of MWCNT that meet those criteria may find different effects at occupational levels than we found with our MWCNT. Since dose is a key part of toxicity, longer exposures but still at occupational concentration could elicit different results. Future studies could also be done using mice with pre-existing pulmonary pathology. Asthma is known to increase the effect of MWCNT on the pulmonary system [155] and other lung pathologies may also increase the effect of MWCNT in the lung. Asbestos exposure and tobacco smoke are known to interact [162, 163] and it is possible that a similar effect will occur with MWCNT. Similarly, secondary exposure is an area that needs to be studied. There are inconclusive data that suggest bacterial lipopolysaccharide (LPS) combined with nanotubes may worsen the effects compared to nanotubes alone and appear to indicate that pre-existing inflammation increases risk [86, 164]. Further study of LPS exposure as a secondary exposure or to other substances is warranted for occupational concerns because workers who have already been exposed to nanotubes may also be exposed to other things that cause toxicity. Because we found that MWCNT are retained in the lung, and in macrophages in particular, secondary insults to the lung may not be able to be cleared as easily and their effects may be compounded.

Further study of macrophages themselves is an important area to study because the presence of MWCNT may alter or hinder their activity. Assessing macrophage phenotype is possible through PCR looking for markers of M1 or M2. Phagocytosis can be assayed using flow cytometry to determine how well macrophages are able to phagocytose particles. *In vitro* work specifically related to macrophages is also an area for future work.

5.5 CONCLUSION

The results of this study might seem like good news for people working with MWCNT since we did not find evidence of overt pulmonary pathology. Unfortunately, this may not be the case since we did find biopersistence to the limits of our study. Had we extended the length of our exposures we may have seen pathology as a result of a lag time between exposure and pathology similar to the lag time between exposure to asbestos and diseases related to asbestos exposure. We did test at a level well above the current NIOSH recommendation of an exposure rate of no more than 1 $\mu g/m^3$ of air which is the lowest measurable airborne concentration and is intended to protect workers long term [160]. We successfully built and tested a dust generator that can be used for future studies looking at longer exposures, different MWCNT, alternate concentrations, and co-morbidities that might result in the earlier development of pulmonary pathologies. Given the wide spread and growing use of MWCNT a clear understanding of the possible risks from exposure to MWCNT, particularly via inhalation, is a crucial public health issue that needs ongoing investigation.

REFERENCES

1. Iijima, S., *Helical microtubules of graphitic carbon.* Nature, 1991. **354**(6348): p. 56-58.

2. Oberdörster, G., E. Oberdörster, and J. Oberdörster, *Nanotoxicology: An Emerging Discipline Evolving from Studies of Ultrafine Particles.* Environmental Health Perspectives, 2005. **113**(7): p. 823-839.

3. Murphy, F.A., et al., *Length-dependent pleural inflammation and parietal pleural responses after deposition of carbon nanotubes in the pulmonary airspaces of mice.* Nanotoxicology, 2013. **7**(6): p. 1157-67.

4. Bhattacharya, K., et al., *Mechanisms of carbon nanotube-induced toxicity: focus on pulmonary inflammation.* Adv Drug Deliv Rev, 2013. **65**(15): p. 2087-97.

5. Han, S.G., R. Andrews, and C.G. Gairola, *Acute pulmonary response of mice to multi-wall carbon nanotubes.* Inhal Toxicol, 2010. **22**(4): p. 340-7.

6. Jaurand, M.C., A. Renier, and J. Daubriac, *Mesothelioma: Do asbestos and carbon nanotubes pose the same health risk?* Part Fibre Toxicol, 2009. **6**: p. 16.

7. Shvedova, A.A., et al., *Unusual inflammatory and fibrogenic pulmonary responses to single-walled carbon nanotubes in mice.* Am J Physiol Lung Cell

8. Kim, J.E., et al., *Toxicity and clearance of intratracheally administered multiwalled carbon nanotubes from murine lung.* J Toxicol Environ Health A, 2010. **73**(21-22): p. 1530-43.

9. Sargent, L.M., et al., *Promotion of lung adenocarcinoma following inhalation exposure to multi-walled carbon nanotubes.* Part Fibre Toxicol, 2014. **11**: p. 3.

10. Porter, D.W., et al., *Acute pulmonary dose-responses to inhaled multi-walled carbon nanotubes.* Nanotoxicology, 2013. **7**(7): p. 1179-94.

11. Bonner, J.C., *Nanoparticles as a potential cause of pleural and interstitial lung disease.* Proc Am Thorac Soc, 2010. **7**(2): p. 138-41.

12. Donaldson, K., et al., *Carbon nanotubes: a review of their properties in relation to pulmonary toxicology and workplace safety.* Toxicol Sci, 2006. **92**: p. 5 - 22.

13. De Jong, W.H. and P.J.A. Borm, *Drug delivery and nanoparticles: Applications and hazards.* International Journal of Nanomedicine, 2008. **3**(2): p. 133-149.

14. Scarselli, M., P. Castrucci, and M.D. Crescenzi, *Electronic and optoelectronic nano-devices based on carbon nanotubes.* Journal of Physics: Condensed Matter, 2012. **24**(31): p. 313202.

15. *Carbon Nanotubes - History and Development of Carbon Nanotubes (Buckytubes)*. 2013 6/11/2013 [cited 2015 7/5/2015]; Available from: http://www.azonano.com/article.aspx?ArticleID=982.

16. Nessim, G.D., *Properties, synthesis, and growth mechanisms of carbon nanotubes with special focus on thermal chemical vapor deposition.* Nanoscale, 2010. **2**(8): p. 1306-23.

17. Rafique, M.M.A. and J. Iqbal, *Production of Carbon Nanotubes by Different Routes-A Review.* Journal of Encapsulation and Adsorption Sciences, 2011. **Vol.01No.02**: p. 6.

18. De Volder, M.F., et al., *Carbon nanotubes: present and future commercial applications.* Science, 2013. **339**(6119): p. 535-9.

19. Kumar, M. and Y. Ando, *Chemical vapor deposition of carbon nanotubes: a review on growth mechanism and mass production.* J Nanosci Nanotechnol, 2010. **10**(6): p. 3739-58.

20. NIOSH, *Current intelligence bulletin 65. Occupational Exposure to Carbon Nanotubes and Nanofibers*, C.f.D.C.a.P. US Department of Health and Human Services, National Institute for Occupational Safety and Health, Editor. 2013: Cincinnati, Ohio.

21. Hamilton, R.F., et al., *Effect of MWCNT size, carboxylation, and purification on in vitro and in vivo toxicity, inflammation and lung pathology.* Particle and Fibre Toxicology, 2013. **10**: p. 57-57.

22. Gordon, A.T., et al., *Introduction to nanotechnology: potential applications in physical medicine and rehabilitation.* Am J Phys Med Rehabil, 2007. **86**(3): p. 225-41.

23. Shvedova, A.A., et al., *Mechanisms of pulmonary toxicity and medical applications of carbon nanotubes: Two faces of Janus?* Pharmacol Ther, 2009. **121**(2): p. 192-204.

24. Yang, W., et al., *Carbon nanotubes for biological and biomedical applications.* Nanotechnology, 2007. **18**: p. 412001.

25. Vardharajula, S., et al., *Functionalized carbon nanotubes: biomedical applications.* Int J Nanomedicine, 2012. **7**: p. 5361-74.

26. Pampaloni, F. and E.L. Florin, *Microtubule architecture: inspiration for novel carbon nanotube-based biomimetic materials.* Trends Biotechnol, 2008. **26**(6): p. 302-10.

27. Sirajuddin, A. and J.P. Kanne, *Occupational lung disease.* J Thorac Imaging, 2009. **24**(4): p. 310-20.

28. Kayat, J., et al., *Pulmonary toxicity of carbon nanotubes: a systematic report.* Nanomedicine, 2011. **7**(1): p. 40-9.

29. Maynard, A.D., et al., *Exposure to carbon nanotube material: aerosol release during the handling of unrefined single-walled carbon nanotube material.* J Toxicol Environ Health A, 2004. **67**(1): p. 87-107.

30. Schubauer-Berigan, M.K., M.M. Dahm, and M.S. Yencken, *Engineered carbonaceous nanomaterials manufacturers in the United States: workforce size, characteristics, and feasibility of epidemiologic studies.* J Occup Environ Med, 2011. **53**(6 Suppl): p. S62-7.

31. Methner, M., L. Hodson, and C. Geraci, *Nanoparticle Emission Assessment Technique (NEAT) for the Identification and Measurement of Potential Inhalation Exposure to Engineered Nanomaterials — Part A.* Journal of Occupational and Environmental Hygiene, 2009. **7**(3): p. 127-132.

32. Methner, M., et al., *Nanoparticle Emission Assessment Technique (NEAT) for the identification and measurement of potential inhalation exposure to engineered nanomaterials--Part B: Results from 12 field studies.* J Occup Environ Hyg, 2010. **7**(3): p. 163-76.

33. Demou, E., P. Peter, and S. Hellweg, *Exposure to Manufactured Nanostructured Particles in an Industrial Pilot Plant.* Annals of Occupational Hygiene, 2008. **52**(8): p. 695-706.

34. Evans, D.E., et al., *Aerosol monitoring during carbon nanofiber production: mobile direct-reading sampling.* Ann Occup Hyg, 2010. **54**(5): p. 514-31.

35. Dahm, M.M., et al., *Occupational exposure assessment in carbon nanotube and nanofiber primary and secondary manufacturers.* Ann Occup Hyg, 2012. **56**(5): p. 542-56.

36. Lee, J.H., et al., *Exposure assessment of carbon nanotube manufacturing workplaces.* Inhal Toxicol, 2010. **22**(5): p. 369-81.

37. Han, J.H., et al., *Monitoring multiwalled carbon nanotube exposure in carbon nanotube research facility.* Inhal Toxicol, 2008. **20**(8): p. 741-9.

38. Yeganeh, B., et al., *Characterization of airborne particles during production of carbonaceous nanomaterials.* Environ Sci Technol, 2008. **42**(12): p. 4600-6.

39. Erdely, A., et al., *Carbon nanotube dosimetry: from workplace exposure assessment to inhalation toxicology.* Part Fibre Toxicol, 2013. **10**(1): p. 53.

40. Donaldson, K., et al., *Pulmonary toxicity of carbon nanotubes and asbestos - similarities and differences.* Adv Drug Deliv Rev, 2013. **65**(15): p. 2078-86.

41. Madani, S.Y., A. Mandel, and A.M. Seifalian, *A concise review of carbon nanotube's toxicology.* Nano Reviews, 2013. **4**: p. 10.3402/nano.v4i0.21521.

42. Sanchez, V.C., et al., *Biopersistence and potential adverse health impacts of fibrous nanomaterials: what have we learned from asbestos?* Wiley Interdiscip Rev Nanomed Nanobiotechnol, 2009. **1**(5): p. 511-29.

43. Donaldson, K., et al., *Asbestos, carbon nanotubes and the pleural mesothelium: a review of the hypothesis regarding the role of long fibre retention in the parietal pleura, inflammation and mesothelioma.* Part Fibre Toxicol, 2010. **7**: p. 5.

44. Urankar, R.N., et al., *Expansion of cardiac ischemia/reperfusion injury after instillation of three forms of multi-walled carbon nanotubes.* Part Fibre Toxicol, 2012. **9**: p. 38.

45. Pacurari, M., V. Castranova, and V. Vallyathan, *Single- and multi-wall carbon nanotubes versus asbestos: are the carbon nanotubes a new health risk to humans?* J Toxicol Environ Health A, 2010. **73**(5): p. 378-95.

46. Kim, J.S., et al., *Toxicogenomic comparison of multi-wall carbon nanotubes (MWCNTs) and asbestos.* Arch Toxicol, 2012. **86**(4): p. 553-62.

47. Luus, K., *Asbestos: mining exposure, health effects and policy implications.* McGill Journal of Medicine : MJM, 2007. **10**(2): p. 121-126.

48. Li, J.G., et al., *Comparative study of pathological lesions induced by multiwalled carbon nanotubes in lungs of mice by intratracheal instillation and inhalation.* Environ Toxicol, 2007. **22**(4): p. 415-21.

49. Morimoto, Y., et al., *Pulmonary toxicity of well-dispersed multi-wall carbon nanotubes following inhalation and intratracheal instillation.* Nanotoxicology, 2012. **6**(6): p. 587-99.

50. Morimoto, Y., et al., *Inhalation toxicity assessment of carbon-based nanoparticles.* Acc Chem Res, 2013. **46**(3): p. 770-81.

51. Ma-Hock, L., et al., *Inhalation toxicity of multiwall carbon nanotubes in rats exposed for 3 months.* Toxicol Sci, 2009. **112**(2): p. 468-81.

52. Pauluhn, J., *Multi-walled carbon nanotubes (Baytubes): approach for derivation of occupational exposure limit.* Regul Toxicol Pharmacol, 2010. **57**(1): p. 78-89.

53. Umeda, Y., et al., *Two-week Toxicity of Multi-walled Carbon Nanotubes by Whole-body Inhalation Exposure in Rats.* J Toxicol Pathol, 2013. **26**(2): p. 131-40.

54. Thurnherr, T., et al., *A comparison of acute and long-term effects of industrial multiwalled carbon nanotubes on human lung and immune cells in vitro.* Toxicol Lett, 2011. **200**(3): p. 176-86.

55. Oyabu, T., et al., *Biopersistence of inhaled MWCNT in rat lungs in a 4-week well-characterized exposure.* Inhal Toxicol, 2011. **23**(13): p. 784-91.

56. Pauluhn, J., *Subchronic 13-week inhalation exposure of rats to multiwalled carbon nanotubes: toxic effects are determined by density of agglomerate structures, not fibrillar structures.* Toxicol Sci, 2010. **113**: p. 226 - 242.

57. Pauluhn, J. and M. Rosenbruch, *Lung burdens and kinetics of multi-walled carbon nanotubes (Baytubes) are highly dependent on the disaggregation of aerosolized MWCNT.* Nanotoxicology, 2015. **9**(2): p. 242-52.

58. Shvedova, A.A., et al., *Increased accumulation of neutrophils and decreased fibrosis in the lung of NADPH oxidase-deficient C57BL/6 mice exposed to carbon nanotubes.* Toxicol Appl Pharmacol, 2008. **231**(2): p. 235-40.

59. Elgrabli, D., et al., *Induction of apoptosis and absence of inflammation in rat lung after intratracheal instillation of multiwalled carbon nanotubes.* Toxicology, 2008. **253**(1-3): p. 131-6.

60. Ravichandran, P., et al., *Induction of apoptosis in rat lung epithelial cells by multiwalled carbon nanotubes.* J Biochem Mol Toxicol, 2009. **23**(5): p. 333-44.

61. Ellinger-Ziegelbauer, H. and J. Pauluhn, *Pulmonary toxicity of multi-walled carbon nanotubes (Baytubes) relative to alpha-quartz following a single 6h inhalation exposure of rats and a 3 months post-exposure period.* Toxicology, 2009. **266**(1-3): p. 16-29.

62. Hirano, S., *A current overview of health effect research on nanoparticles.* Environmental Health and Preventive Medicine, 2009. **14**(4): p. 223-225.

63. Hubbs, A., et al., *Pulmonary inflammation, epithelial hyperplasia, and lymph node translocation after multi-walled carbon nanotube inhalation.* Toxicol Sci, 2011. **120**: p. 11.

64. Kuempel, E.D., *Carbon nanotube risk assessment: implications for exposure and medical monitoring.* J Occup Environ Med, 2011. **53**(6 Suppl): p. S91-7.

65. Mercer, R.R., et al., *Distribution and persistence of pleural penetrations by multi-walled carbon nanotubes.* Part Fibre Toxicol, 2010. **7**: p. 28.

66. Mercer, R.R., et al., *Distribution and fibrotic response following inhalation exposure to multi-walled carbon nanotubes.* Part Fibre Toxicol, 2013. **10**: p. 33.

67. Mercer, R.R., et al., *Extrapulmonary transport of MWCNT following inhalation exposure.* Part Fibre Toxicol, 2013. **10**: p. 38.

68. Karlsson, H.L., et al., *Copper oxide nanoparticles are highly toxic: a comparison between metal oxide nanoparticles and carbon nanotubes.* Chem Res Toxicol, 2008. **21**(9): p. 1726-32.

69. Pulskamp, K., S. Diabate, and H.F. Krug, *Carbon nanotubes show no sign of acute toxicity but induce intracellular reactive oxygen species in dependence on contaminants.* Toxicol Lett, 2007. **168**(1): p. 58-74.

70. Ma-Hock, L., et al., *Comparative inhalation toxicity of multi-wall carbon nanotubes, graphene, graphite nanoplatelets and low surface carbon black.* Part Fibre Toxicol, 2013. **10**: p. 23.

71. Poland, C., et al., *Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study.* Nat Nanotechnol, 2008. **3**: p. 423 - 428.

72. Takagi, A., et al., *Induction of mesothelioma in p53+/- mouse by intraperitoneal application of multi-wall carbon nanotube.* J Toxicol Sci, 2008. **33**: p. 105 - 116.

73. Takagi, A., et al., *Dose-dependent mesothelioma induction by intraperitoneal administration of multi-wall carbon nanotubes in p53 heterozygous mice.* Cancer Sci, 2012. **103**(8): p. 1440-4.

74. Frost, G., et al., *Occupational exposure to asbestos and mortality among asbestos removal workers: a Poisson regression analysis.* Br J Cancer, 2008. **99**: p. 822 - 829.

75. Frost, G., A. Darnton, and A.H. Harding, *The effect of smoking on the risk of lung cancer mortality for asbestos workers in Great Britain (1971-2005).* Ann Occup Hyg, 2011. **55**(3): p. 239-47.

76. Klebe, S., et al., *Sarcomatoid mesothelioma: a clinical-pathologic correlation of 326 cases.* Mod Pathol, 2010. **23**(3): p. 470-9.

77. Metintas, S., et al., *Environmental asbestos exposure in rural Turkey and risk of lung cancer.* Int J Environ Health Res, 2012. **22**(5): p. 468-79.

78. Murphy, F.A., et al., *Length-dependent retention of carbon nanotubes in the pleural space of mice initiates sustained inflammation and progressive fibrosis on the parietal pleura.* Am J Pathol, 2011. **178**(6): p. 2587-600.

79. Nagai, H., et al., *Diameter and rigidity of multiwalled carbon nanotubes are critical factors in mesothelial injury and carcinogenesis.* Proc Natl Acad Sci U S A, 2011. **108**(49): p. E1330-8.

80. Shang, L., K. Nienhaus, and G.U. Nienhaus, *Engineered nanoparticles interacting with cells: size matters.* J Nanobiotechnology, 2014. **12**: p. 5.

81. Stanton, M.F., et al., *Relation of particle dimension to carcinogenicity in amphibole asbestoses and other fibrous minerals.* J Natl Cancer Inst, 1981. **67**(5): p. 965-75.

82. Sweeney, S., et al., *Multi-walled carbon nanotube length as a critical determinant of bioreactivity with primary human pulmonary alveolar cells.* Carbon, 2014. **78**(0): p. 26-37.

83. Lam, C.W., et al., *Pulmonary toxicity of single-wall carbon nanotubes in mice 7 and 90 days after intratracheal instillation.* Toxicol Sci, 2004. **77**(1): p. 126-34.

84. Muller, J., et al., *Structural defects play a major role in the acute lung toxicity of multiwall carbon nanotubes: toxicological aspects.* Chem Res Toxicol, 2008. **21**(9): p. 1698-705.

85. Crouzier, D., et al., *Carbon nanotubes induce inflammation but decrease the production of reactive oxygen species in lung.* Toxicology, 2010. **272**(1-3): p. 39-45.

86. Inoue, K., et al., *Effects of pulmonary exposure to carbon nanotubes on lung and systemic inflammation with coagulatory disturbance induced by lipopolysaccharide in mice.* Exp Biol Med (Maywood), 2008. **233**(12): p. 1583-90.

87. Kagan, V.E., et al., *Carbon nanotubes degraded by neutrophil myeloperoxidase induce less pulmonary inflammation.* Nat Nanotechnol, 2010. **5**(5): p. 354-9.

88. Meunier, E., et al., *Double-walled carbon nanotubes trigger IL-1β release in human monocytes through Nlrp3 inflammasome activation.* Nanomedicine: Nanotechnology, Biology and Medicine, 2012. **8**(6): p. 987-995.

89. Muller, J., et al., *Respiratory toxicity of multi-wall carbon nanotubes.* Toxicol Appl Pharmacol, 2005. **207**(3): p. 221-31.

90. Ravichandran, P., et al., *Pulmonary biocompatibility assessment of inhaled single-wall and multiwall carbon nanotubes in BALB/c mice.* J Biol Chem, 2011. **286**(34): p. 29725-33.

91. Roda, E., et al., *Comparative pulmonary toxicity assessment of pristine and functionalized multi-walled carbon nanotubes intratracheally instilled in rats: morphohistochemical evaluations.* Histol Histopathol, 2011. **26**(3): p. 357-67.

92. Bonner, J.C., *Lung fibrotic responses to particle exposure.* Toxicol Pathol, 2007. **35**(1): p. 148-53.

93. Dong, J., et al., *Pathologic and molecular profiling of rapid-onset fibrosis and inflammation induced by multi-walled carbon nanotubes.* Arch Toxicol, 2015. **89**(4): p. 621-33.

94. He, X., et al., *Multiwalled carbon nanotubes induce a fibrogenic response by stimulating reactive oxygen species production, activating NF-kappaB signaling, and promoting fibroblast-to-myofibroblast transformation.* Chem Res Toxicol, 2011. **24**(12): p. 2237-48.

95. Mangum, J., et al., *Single-walled carbon nanotube (SWCNT)-induced interstitial fibrosis in the lungs of rats is associated with increased levels of PDGF mRNA and the formation of unique intercellular carbon structures that bridge alveolar macrophages in situ.* Part Fibre Toxicol, 2006. **3**: p. 15.

96. Manke, A., et al., *Effect of fiber length on carbon nanotube-induced fibrogenesis.* Int J Mol Sci, 2014. **15**(5): p. 7444-61.

97. Mercer, R.R., et al., *Pulmonary fibrotic response to aspiration of multi-walled carbon nanotubes.* Part Fibre Toxicol, 2011. **8**: p. 21.

98. Porter, D.W., et al., *Mouse pulmonary dose- and time course-responses induced by exposure to multi-walled carbon nanotubes.* Toxicology, 2010. **269**(2-3): p. 136-47.

99. Kisin, E.R., et al., *Single-walled carbon nanotubes: geno- and cytotoxic effects in lung fibroblast V79 cells.* J Toxicol Environ Health A, 2007. **70**(24): p. 2071-9.

100. Li, X., Y. Peng, and X. Qu, *Carbon nanotubes selective destabilization of duplex and triplex DNA and inducing B-A transition in solution.* Nucleic Acids Res, 2006. **34**(13): p. 3670-6.

101. Li, X., et al., *Carboxyl-modified single-walled carbon nanotubes selectively induce human telomeric i-motif formation.* Proc Natl Acad Sci U S A, 2006. **103**(52): p. 19658-63.

102. Ponti, J., et al., *Morphological transformation induced by multiwall carbon nanotubes on Balb/3T3 cell model as an in vitro end point of carcinogenic potential.* Nanotoxicology, 2013. **7**: p. 221 - 233.

103. Toyokuni, S., *Genotoxicity and carcinogenicity risk of carbon nanotubes.* Adv Drug Deliv Rev, 2013. **65**(15): p. 2098-110.

104. Sargent, L., et al., *Genotoxicity of multi-walled carbon nanotubes at occupationally relevant doses.* Proc Am Assoc Cancer Res, 2012. **53**: p. 1320.

105. Siegrist, K., et al., *Genotoxicity of multi-walled carbon nanotubes at occupationally relevant doses.* Particle and Fibre Toxicology, 2014. **11**(1): p. 6.

106. Sargent, L.M., et al., *Single-walled carbon nanotube-induced mitotic disruption.* Mutat Res, 2012. **745**(1-2): p. 28-37.

107. Sargent, L.M., S.H. Reynolds, and V. Castranova, *Potential pulmonary effects of engineered carbon nanotubes: in vitro genotoxic effects.* Nanotoxicology, 2010. **4**: p. 396-408.

108. Sargent, L.M., et al., *Induction of aneuploidy by single-walled carbon nanotubes.* Environ Mol Mutagen, 2009. **50**(8): p. 708-17.

109. Ryman-Rasmussen, J.P., et al., *Inhaled carbon nanotubes reach the subpleural tissue in mice.* Nat Nanotechnol, 2009. **4**(11): p. 747-51.

110. Tavares, A.M., et al., *Genotoxicity evaluation of nanosized titanium dioxide, synthetic amorphous silica and multi-walled carbon nanotubes in human lymphocytes.* Toxicol In Vitro, 2014. **28**(1): p. 60-9.

111. Srivastava, R.K., et al., *Multi-walled carbon nanotubes induce oxidative stress and apoptosis in human lung cancer cell line-A549.* Nanotoxicology, 2011. **5**(2): p. 195-207.

112. Ye, S.F., et al., *ROS and NF-kappaB are involved in upregulation of IL-8 in A549 cells exposed to multi-walled carbon nanotubes.* Biochem Biophys Res Commun, 2009. **379**(2): p. 643-8.

113. Hu, X., et al., *In vitro evaluation of cytotoxicity of engineered carbon nanotubes in selected human cell lines.* Sci Total Environ, 2010. **408**(8): p. 1812-7.

114. Garza, K.M., K.F. Soto, and L.E. Murr, *Cytotoxicity and reactive oxygen species generation from aggregated carbon and carbonaceous nanoparticulate materials.* Int J Nanomedicine, 2008. **3**(1): p. 83-94.

115. Herzog, E., et al., *Dispersion medium modulates oxidative stress response of human lung epithelial cells upon exposure to carbon nanomaterial samples.* Toxicol Appl Pharmacol, 2009. **236**(3): p. 276-81.

116. Muller, J., et al., *Absence of carcinogenic response to multiwall carbon nanotubes in a 2-year bioassay in the peritoneal cavity of the rat.* Toxicol Sci, 2009. **110**(2): p. 442-8.

117. Huizar, I., et al., *Novel murine model of chronic granulomatous lung inflammation elicited by carbon nanotubes.* Am J Respir Cell Mol Biol, 2011. **45**(4): p. 858-66.

118. Barna, B.P., et al., *Carbon nanotube-induced pulmonary granulomatous disease: Twist1 and alveolar macrophage M1 activation.* Int J Mol Sci, 2013. **14**(12): p. 23858-71.

119. Barna, B.P., M.A. Judson, and M.J. Thomassen, *Carbon Nanotubes and Chronic Granulomatous Disease.* Nanomaterials (Basel), 2014. **4**(2): p. 508-521.

120. Chen, R., et al., *Subchronic Toxicity and Cardiovascular Responses in Spontaneously Hypertensive Rats after Exposure to Multiwalled Carbon Nanotubes by Intratracheal Instillation.* Chemical Research in Toxicology, 2015. **28**(3): p. 440-450.

121. Erdely, A., et al., *Cross-talk between lung and systemic circulation during carbon nanotube respiratory exposure. Potential biomarkers.* Nano Lett, 2009. **9**: p. 36 - 43.

122. Erdely, A., et al., *Identification of systemic markers from a pulmonary carbon nanotube exposure.* J Occup Environ Med, 2011. **53**(6 Suppl): p. S80-6.

123. Mitchell, L., et al., *Mechanisms for how inhaled multiwalled carbon nanotubes suppress systemic immune function in mice.* Nat Nanotechnol, 2009. **4**: p. 451 - 456.

124. Mitchell, L.A., et al., *Pulmonary and systemic immune response to inhaled multiwalled carbon nanotubes.* Toxicol Sci, 2007. **100**(1): p. 203-14.

125. Oberdorster, G., *Safety assessment for nanotechnology and nanomedicine: concepts of nanotoxicology.* J Intern Med, 2010. **267**(1): p. 89-105.

126. Stapleton, P.A., et al., *Impairment of coronary arteriolar endothelium-dependent dilation after multi-walled carbon nanotube inhalation: a time-course study.* Int J Mol Sci, 2012. **13**(11): p. 13781-803.

127. Porter, D.W., et al., *Differential mouse pulmonary dose and time course responses to titanium dioxide nanospheres and nanobelts.* Toxicol Sci, 2013. **131**(1): p. 179-93.

128. Reddy, A.R., et al., *Translocation and extra pulmonary toxicities of multi wall carbon nanotubes in rats.* Toxicol Mech Methods, 2010. **20**(5): p. 267-72.

129. Monteiro-Riviere, N.A., et al., *Multi-walled carbon nanotube interactions with human epidermal keratinocytes.* Toxicol Lett, 2005. **155**(3): p. 377-84.

130. Wang, X., et al., *Multi-walled carbon nanotube instillation impairs pulmonary function in C57BL/6 mice.* Part Fibre Toxicol, 2011. **8**: p. 24.

131. Li, J.G., et al., *The pulmonary toxicity of multi-wall carbon nanotubes in mice 30 and 60 days after inhalation exposure.* J Nanosci Nanotechnol, 2009. **9**(2): p. 1384-7.

132. Bonner, J.C., et al., *Interlaboratory evaluation of rodent pulmonary responses to engineered nanomaterials: the NIEHS Nano GO Consortium.* Environ Health Perspect, 2013. **121**(6): p. 676-82.

133. Ding, L., et al., *Molecular characterization of the cytotoxic mechanism of multiwall carbon nanotubes and nano-onions on human skin fibroblast.* Nano Lett, 2005. **5**(12): p. 2448-64.

134. Warheit, D.B., et al., *Health effects related to nanoparticle exposures: environmental, health and safety considerations for assessing hazards and risks.* Pharmacol Ther, 2008. **120**(1): p. 35-42.

135. Wang, X., et al., *Pulmonary toxicity in mice exposed to low and medium doses of water-soluble multi-walled carbon nanotubes.* J Nanosci Nanotechnol, 2010. **10**(12): p. 8516-26.

136. Oberdorster, G., et al., *Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy.* Part Fibre Toxicol, 2005. **2**: p. 8.

137. Baron, P.A., et al., *Aerosolization of Single-Walled Carbon Nanotubes for an Inhalation Study.* Inhalation Toxicology, 2008. **20**(8): p. 751-760.

138. McKinney, W., B. Chen, and D. Frazer, *Computer controlled multi-walled carbon nanotube inhalation exposure system.* Inhal Toxicol, 2009. **21**(12): p. 1053-61.

139. Wong, B.A., *Inhalation Exposure Systems: Design, Methods and Operation.* Toxicologic Pathology, 2007. **35**(1): p. 3-14.

140. Walters, D.M., et al., *Genetic susceptibility to interstitial pulmonary fibrosis in mice induced by vanadium pentoxide (V2O5).* FASEB J, 2014. **28**(3): p. 1098-112.

141. Brody, A.R., et al., *IDentifying fibrosis susceptibility genes in two strains of inbred mice*.* Chest, 2002. **121**(3_suppl): p. 31S-31S.

142. Paun, A., et al., *Association Analysis Reveals Genetic Variation Altering Bleomycin-Induced Pulmonary Fibrosis in Mice.* American Journal of Respiratory Cell and Molecular Biology, 2013. **48**(3): p. 330-336.

143. Davis, G.S., K.O. Leslie, and D.R. Hemenway, *Silicosis in mice: effects of dose, time, and genetic strain.* J Environ Pathol Toxicol Oncol, 1998. **17**(2): p. 81-97.

144. Wynn, T.A., *Fibrotic disease and the T(H)1/T(H)2 paradigm.* Nature reviews. Immunology, 2004. **4**(8): p. 583-594.

145. Hardy, C.L., et al., *Differential uptake of nanoparticles and microparticles by pulmonary APC subsets induces discrete immunological imprints.* J Immunol, 2013. **191**(10): p. 5278-90.

146. Staal, Y.C., et al., *Inhaled multiwalled carbon nanotubes modulate the immune response of trimellitic anhydride-induced chemical respiratory allergy in brown Norway rats.* Toxicol Pathol, 2014. **42**(7): p. 1130-42.

147. Pauluhn, J., *The metrics of MWCNT-induced pulmonary inflammation are dependent on the selected testing regimen.* Regulatory Toxicology and Pharmacology, 2014. **68**(3): p. 343-352.

148. Teeguarden, J.G., et al., *Particokinetics In Vitro: Dosimetry Considerations for In Vitro Nanoparticle Toxicity Assessments.* Toxicological Sciences, 2007. **95**(2): p. 300-312.

149. Pauluhn, J., *Comparative pulmonary response to inhaled nanostructures: considerations on test design and endpoints.* Inhalation Toxicology, 2009. **21**(s1): p. 40-54.

150. Li, R., et al., *Surface charge and cellular processing of covalently functionalized multiwall carbon nanotubes determine pulmonary toxicity.* ACS Nano, 2013. **7**(3): p. 2352-68.

151. Sager, T.M., et al., *Effect of multi-walled carbon nanotube surface modification on bioactivity in the C57BL/6 mouse model.* Nanotoxicology, 2014. **8**(3): p. 317-27.

152. Hamilton, R.F., Jr., et al., *Purification and sidewall functionalization of multiwalled carbon nanotubes and resulting bioactivity in two macrophage models.* Inhal Toxicol, 2013. **25**(4): p. 199-210.

153. Aldieri, E., et al., *The role of iron impurities in the toxic effects exerted by short multiwalled carbon nanotubes (MWCNT) in murine alveolar macrophages.* J Toxicol Environ Health A, 2013. **76**(18): p. 1056-71.

154. Hamilton, R.F., Jr., et al., *NLRP3 inflammasome activation in murine alveolar macrophages and related lung pathology is associated with MWCNT nickel contamination.* Inhal Toxicol, 2012. **24**(14): p. 995-1008.

155. Ryman-Rasmussen, J., et al., *Inhaled multi-walled carbon Nanotubes potentiate airway fibrosis in murine allergic asthma.* Am J Respir Cell Mol Biol, 2009. **40**: p. 349 - 358.

156. Shvedova, A.A., et al., *Inhalation vs. aspiration of single-walled carbon nanotubes in C57BL/6 mice: inflammation, fibrosis, oxidative stress, and mutagenesis.* Am J Physiol Lung Cell Mol Physiol, 2008. **295**(4): p. L552-65.

157. Irwin, J. and S. Livnat, *Behavioral influences on the immune system: stress and conditioning.* Prog Neuropsychopharmacol Biol Psychiatry, 1987. **11**(2-3): p. 137-43.

158. Di Giorgio, M.L., et al., *Effects of single and multi walled carbon nanotubes on macrophages: cyto and genotoxicity and electron microscopy.* Mutat Res, 2011. **722**(1): p. 20-31.

159. Horie, M., et al., *Comparison of acute oxidative stress on rat lung induced by nano and fine-scale, soluble and insoluble metal oxide particles: NiO and TiO2.* Inhalation Toxicology, 2012. **24**(7): p. 391-400.

160. NIOSH. *NIOSH Recommends New Level of Exposure for Nanomaterials*. 2013 April 24, 2013 [cited 2015 July 15].

161. Nagai, H., et al., *Diameter and rigidity of multiwalled carbon nanotubes are critical factors in mesothelial injury and carcinogenesis.* Proceedings of the National Academy of Sciences, 2011. **108**(49): p. E1330–E1338.

162. Markowitz, S.B., et al., *Asbestos, asbestosis, smoking, and lung cancer. New findings from the North American insulator cohort.* Am J Respir Crit Care Med, 2013. **188**(1): p. 90-6.

163. Wraith, D. and K. Mengersen, *Assessing the combined effect of asbestos exposure and smoking on lung cancer: a Bayesian approach.* Stat Med, 2007. **26**(5): p. 1150-69.

164. Cesta, M.F., et al., *Bacterial lipopolysaccharide enhances PDGF signaling and pulmonary fibrosis in rats exposed to carbon nanotubes.* Am J Respir Cell Mol Biol, 2010. **43**(2): p. 142-51.

APPENDIX A: IACUC APPROVAL LETTERS FOR ANIMAL USE PROTOCOLS

East Carolina University

Animal Care and Use Commitee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

September 20, 2013

Dianne Walters, Ph.D.
Department of Physiology
Brody 6N-98
ECU Brody School of Medicine

Dear Dr. Walters:

Your Animal Use Protocol entitled, "Genetic Susceptibility to MWCNT" (AUP #Q322) was reviewed by this institution's Animal Care and Use Committee on 9/20/13. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

Enclosure