2012-01-01

Assessment of Carbon Nanoparticle Exposure on Murine Macrophage Function

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DEDICATION

I would like to thank my family and friends, especially my parents, Linda and Victor, my brother Ivan, my sister, Beatriz, and my aunt Mayra for their love, encouragement, and support, which made all of this possible. I would also like to thank Walker for his unconditional love, support, and patience. Your caring fun-loving attitude and belief in me has kept me going each step of the way - I love you with all my heart. Garza lab members, Amanda, Valerie, Marisol, Federico and Kristina, thank you for your enthusiasm, help, support, and fun times in lab. Dr. Jeff Sivils, thank you for all your knowledge, feedback, and advice – you are a great scientist and friend. To Jeff and Johanny, thank you for your friendship and encouragement, even from a distance.

I wish to express my most sincere gratitude to my mentor, Dr Kristine M. Garza, for all her support, effort, patience, constructive criticism, and mentorship throughout this process. Her knowledge, guidance, and commitment inspired and motivated me. I would also like to thank my committee members, Dr. Marc Cox, Dr. Igor Almeida, Dr. Kyle Johnson, and Dr. Lawrence Murr, for your advice, suggestions, and guidance. Finally, I would like to thank everyone who in one way or the other helped me throughout this journey - thank you for your support.

ASSESSMENT OF CARBON NANOPARTICLE EXPOSURE ON MURINE MACROPHAGE FUNCTION

by

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DISSERTATION

Presented to the Faculty of the Graduate School of

The University of Texas at El Paso

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

Department of Biological Sciences

THE UNIVERSITY OF TEXAS AT EL PASO

December 2012

ACKNOWLEDGEMENTS

Thanks to the Department of Biological Sciences at The University of Texas at El Paso, the Border Biomedical Research Center's Core Facilities - Cell Culture and Cytometry Core Facility, Biomolecule Analysis Core Facility, and the DNA Analysis Core Facility for the technology and equipment needed to conduct my experiments. I will also like to thank Dr. Lawrence Murr for providing the micron-sized and nano-sized particles used in our studies. Special thanks to Dr. Johanny Tonos for training me in Western Blot analysis. And I will also like to thank Kristina Barron, who collaborated in these studies. I would like to thank the Analytical Cytology Core Facility for the TEM images, Joanne T. Ellzey, Ph.D., Professor, and Laboratory Director, Marian Zappala M.S., Laboratory Manager, Ted J. Whitworth, Ph.D., and Peter Cooke, Ph.D., Laboratory Director, The Microscopic Imaging Core Suite at New Mexico State University, Department of Agricultural Sciences.

Funding Sources:

NIH RCMI Grant – (2G12RR008124)

NIH RISE Grant – (R25GM069621-02)

NIH SCORE Grant – (S06-GM08012)

Texas Higher Education Coordinating Board (THECB) - (14-8000-17)

UTEP Graduate School Dodson Dissertation Fellowship

UTEP Graduate School Research Support Award

ABSTRACT

There is growing concern about the potential cytotoxicity of nanoparticles. Exposure to respirable ultrafine particles (2.5μM) can adversely affect human health and have been implicated with episodes of increased respiratory diseases such as asthma and allergies. Nanoparticles are of particular interest because of their ability to penetrate into the lung and potentially elicit health effects triggering immune responses. Nanoparticles are structures and devises with length scales in the 1 to 100-nanometer range. Black carbon (BC) nanoparticles have been observed to be products of combustion, especially flame combustion and multi-walled carbon nanotubes (MWCNT) have been shown to be found in both indoor and outdoor air. Furthermore, asbestos, which have been known to cause mesothelioma as well as lung cancer, have been shown to be structurally identical to MWCNTs. The aims of these studies were to examine the effects of carbon nanoparticles on murine macrophage function and clearance mechanisms. Macrophages (MΦ) are immune cells that function as the first line of defense against invading pathogens and are likely to be amongst the first cells affected by nanoparticles. Our research focused on two manufactured nanoparticles, MWCNT and BC. The two were tested against murine-derived MΦ in a chronic contact model. We hypothesized that long-term chronic exposure to carbon nanoparticles would decrease MΦ ability to effectively respond to immunological challenge. Production of nitric oxide (NO), tumor necrosis factor alpha (TNFα), cell surface MΦ activation markers, reactive oxygen species formation (ROS), and antigen processing and presentation were examined in response to lipopolysaccharide (LPS) following a 144hr exposure to the particulates. Data demonstrated an increase in TNFα, and NO production; a decrease in phagocytosis and antigen processing and presentation; and a decrease in the expression levels of cell surface MΦ activation markers. The data suggests that carbon nanoparticle exposure alters MΦ responses to LPS marked by increased inflammation while potentially limiting clearance and ability to interact with effector T-cells. Thus, physiological exposure to carbon nanoparticles could potentially lead to ineffective pulmonary immunity.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1 NANOPARTICLES

Nanotechnology is defined as research and technology development at the atomic, molecular, and macromolecular scale, leading to the controlled manipulation and study of structures and devices with length scales in the 1 to 100-nanometer (nm) range (McNeil, 2005). Due to their small size and their particular physiochemical properties, research indicates that toxicological profiles of nanoparticles may be significantly different from those of larger particles composed of the same materials (Card *et al.* 2008). Indeed, research demonstrates that ultrafine or nanoparticles pose a much greater health risk than fine (2.5 μm) and course (10 μm) particulate matter (PM) (Donaldson *et al.* 2001; Donaldson *et al.* 2004; Machado *et al.* 2011; Murr *et al.* 2008; Nel *et al.* 2006; Oberdorster *et al.* 1994; Oberdorster, 2001; and Somasundaran *et al.* 2010).Moreover, nano-sized particles are able to disturb biological systems at the cellular and subcellular levels (Kang *et al.* 2008).

Individuals are exposed on a daily basis to large numbers of ambient nanoparticles in the form of air pollution (Donaldson *et al.* 2004) produced by industrial sources and naturally occurring combustion processes such as forest fires and volcanic eruptions (Oberdorster *et al.* 2005). Additionally, exposure to engineered nanomaterials commonly used in sporting goods, tires, stain-resistant clothing, sunscreens, cosmetics, and electronics occurs due to use normal and wear-and-tear of the materials (Nel *et al*. 2006). For carbon nanoparticles such as black carbon (BC) and multiwalled carbon nanotubes (MWCNT), the focus of our studies, they have been observed to be products of combustion, especially flame combustion, and have been identified in both indoor and outdoor air (Murr *et al.* 2005 and Murr, 2008). Additionally, BC is

commonly used in the production of tires as a strengthening agent making it abundant as tire wear debris along roadways (Murr, 2008). Furthermore, asbestos, known to cause mesothelioma as well as lung cancer, has been shown to be structurally identical to the engineered MWCNT (**Figure 1**) (Murr and Soto, 2004, Soto *et al.* 2004), while the commercial BC is identical in shape to anthropogenic diesel soot aggregates such as wood and candle particulate matter (**Figure 2**) (Garza *et al.* 2008).

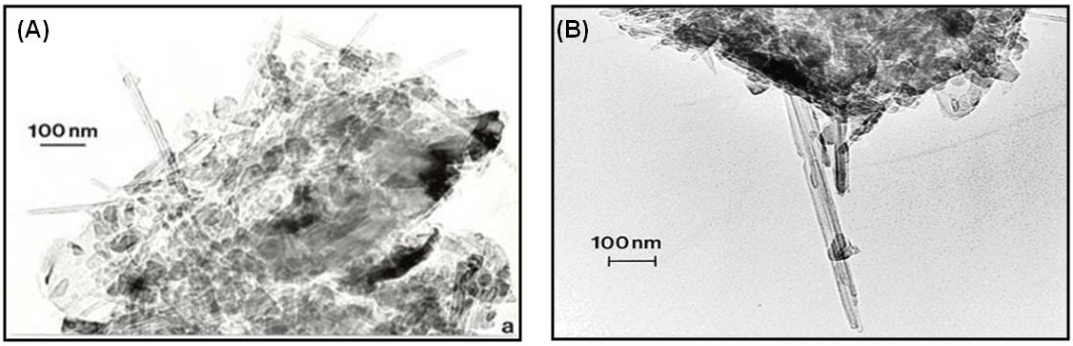

Figure 1. TEM image of commercially produced Multi-Walled Carbon Nanotubes (MWCNT) aggregate material (A) Soto *et al.* (2008) and (B) Garza *et al.* (2008)

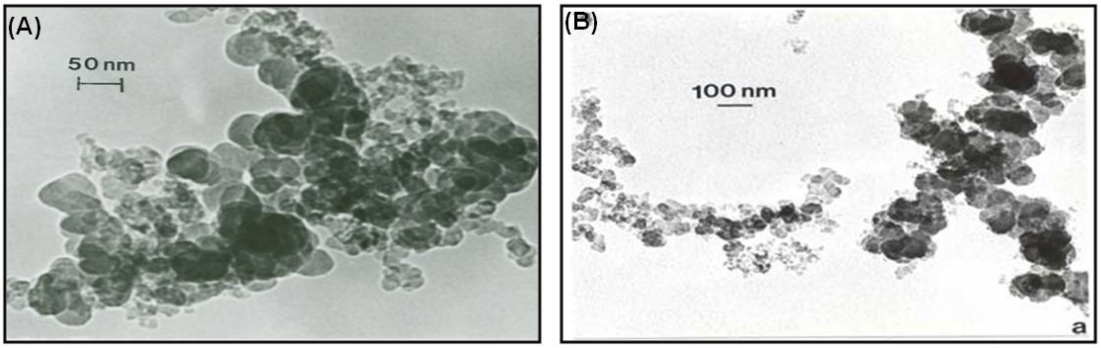

Figure 2. TEM image of commercially produced Black Carbon (BC) containing spherule and branched aggregates (A) Murr *et al.* 2005) and (B) Soto *et al.* (2008)

1.2 NANOPARTICLE PROPERTIES AND TOXICITY

Nanomaterials dimensions range from 1 to 100 nm (McNeil 2005), as a result of their small size and physical properties these can exhibit different characteristics when compared to the same material in bulk form. Their small particle size makes them more potent than larger

particles because of their higher surface area and reactivity (Brauner *et al.* 2007). Due to their size and shape, many nanoparticles can agglomerate and increase their net size, which can potentially cause adverse health effects and affect the effective dose on the cellular system (Oberdorster *et al.* 2005). Additionally, shape and agglomeration degree of particles in the culture medium are important determinants for cytotoxic effects of carbon nanoparticles (Hirano *et al.* 2010). Table 1 lists the physical properties of various nanoparticles, all made of carbon atoms but with distinct shape; these nanoparticles are hydrophobic materials because of their graphene structure and can easily agglomerate in aqueous solutions (Hirano *et al.* 2010).

Table 1. Physical properties of various carbon nanoparticles

Nanoparticles	Size	Aggregate size	Shape
Black Carbon (1) (fullerenes)	2 - 50 nm spherules; 20 nm (mean)	0.1 - 1 um; 0.5 um (mean)	Sphere/buckyball
MWCNT (1)	10 - 30 nm (diameter); 50 nm - 1 um length	0.1 - 3 um; approx. 2 um (mean)	Multiple sheets - Tube/cylinder/fibers
SWCNT (1)	10 - 200 nm (diameter)	2 - 20 um; 10 um (mean)	Single sheet - Tube/cylinder/fibers

(1) Soto *et al.* 2005

Black carbon (BC) is a common nanoparticle composed of fine particles of more than 97% elemental carbon, characterized by spherule graphite sheets of carbon (Figure 2) (Sellers *et al.* 2009). Its initial particle size is nano-size; however, van der Waals forces cause BC particles to aggregate and eventually adhere to form stable agglomerates that can reach 1 to over 100 μm in size. A different shaped carbon nanoparticle is carbon nanotubes (CNTs); these are known for their cylinder/fiber like shape and strong structural properties. CNTs are categorized as single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) (Figure 1). SWCNT consist of hollow cylinder like shape composed of one layer thin sheet of graphene, while MWCNTs are made of various layers. It is now well established that nanoparticles pose a much greater health risk than fine and course particulate matter

(Murr, 2008) and that these nano-sized particles are able to disturb biological systems (Kang *et al.* 2008).

The interaction of carbon nanoparticles with cellular system and toxicity is not well understood. However, many toxicity studies suggest that there are many variables involved such as nanoparticle shape, surface area, reactivity, and aggregation that can influence cell toxicity (Nel *et al.* 2006). These properties could be influencing cell and organismal toxicity through mechanisms such as oxidative stress and the generation of reactive oxygen species (ROS). ROS can be produced directly when cells come into contact with the surface of carbon nanoparticles, creating an oxidative stress reaction, which is a state of redox disequilibrium (Li *et al.* 2008). Oxidative stress reaction can also be triggered by carbon nanoparticles that enter the cells and are taken up by phagocytosis, which in turn induces oxidative stress by the generation of free radicals. Intracellular production of ROS is an important defense mechanism to mediate toxicity, however, over production of ROS can overwhelm the cellular antioxidant capacity. Thus, cells that have been stressed by exposure to foreign materials such as carbon nanoparticles can cause over production of ROS that cannot be neutralized by antioxidants and in turn leads to damage of DNA, proteins, and lipids by oxidation, as well as inflammation, increased calcium concentrations, and cell death (Xia *et al.* 2006). Oxidative stress caused by production of ROS can result in the release of various protein factors such as cytokines, chemokines, and cell growth factors that can initiate reactions that would involve immune and inflammatory cells and other reactive processes (Shatkin, 2008). The latter reaction is a result of inflammation, which is a protective response by the organism that is designed to eliminate it of the foreign material that is causing the injury. The overwhelming intracellular induction of ROS is mainly influenced by the surface and size of the carbon

particles, since nano-sized particles have a large surface area to mass ratio it allows for a greater induction of ROS than larger particles (Donaldson *et al.* 2003).

Carbon nanoparticles can also damage the mitochondria resulting in oxidative stress and cell death (Li *et al.* 2003, Donaldson *et al.* 2003, and Simko *et al.* 2011). Deposits of BC in the mitochondria can lead to the loss of mitochondrial membrane potential which releases cytochrome *c* to the cytosol leading to damage of the inner mitochondrial membrane, apoptosome formation, caspase 3 activation, *poly ADP-ribose polymerase* (PARP) cleavage, and DNA fragmentation, ultimately resulting in apoptotic cell death (Xia *et al*. 2004 and Hussain *et al.* 2010). Carbon nanoparticles can also interact with lysosomal membranes increasing lysosome membrane permeability, which releases lysosomal proteases that can promote toxicity and cell death. Since carbon nanoparticles are often internalized by phagocytosis, they interact with the phagolysosome membrane resulting in the release of lysosomal enzymes like cathepsin B into the cytosol to initiate apoptotic cell death. Studies suggest that loss in lysosomal permeability contributes to apoptosis by factors such as oxidative stress and inducing the release of tumor necrosis factor alpha (TNFα) (Thibodeau *et al.* 2004).

Surface area and reactivity of the carbon nanoparticles appears to influence cell morphology and cytotoxicity. For example SWCNTs and MWCNTs have a cylinder/fiber like shape (Xia *et al.* 2006). Cytotoxicity of SWCNTs and MWCNTs has been visualized by examining cell morphology after exposure; results indicated disruption of plasma membrane and presented with infiltration of the plasma membrane by MWCNT fibers (Hirano *et al.* 2010, 2008). These changes in cell morphology were attributed to hydrophobic contact between the carbon nanoparticles and the cell membrane, thus making it possible for particles to perforate the cell membrane, which is a unique property of insoluble particles (Geisser *et al.* 2005). The

cylinder/fiber-like shape could have increased the extent to which the particle associated with the plasma membrane ultimately causing damage to the cell membrane and possibly loss of cellular content and cell death.

1.3 *IN VITRO* AND *IN VIVO* CELL EFFECTS

There are various studies examining the effects of these materials both on *in vitro* and *in vivo* models to elucidate the potential adverse health effects of nanoparticle exposure. Nanoparticles have been shown to travel throughout the body, deposit in main organs, penetrate cell membranes, lodge in mitochondria, and induce toxic responses (Nel *et al.* 2006). Because of their nano-size these materials have the ability to redistribute from their site of deposition and travel to various locations in the organism (Donaldson *et al.* 2004).

SWCNT and MWCNT are structurally identical to asbestos fibers, which have raised concerns about the durability of these particles inside the organism after being inhaled. Consequently, CNTs could potentially cause asbestos-like injuries, such as pulmonary fibrosis and lung cancer. As a result, many examinations with these carbon nanoparticles have focused on toxicity in the pulmonary system. Mice inhalation studies with SWCNT demonstrated lung injury, inflammation, and granuloma formations (Warheit *et al.* 2004) likewise, results in mice exposed to MWCNT also indicated pulmonary fibrosis, granuloma formation, and inflammatory responses (Muller *et al.* 2005). The observed granulomas were associated with large CNT aggregates in the lung while pulmonary fibrosis was correlated with dispersed CNTs indicating a difference between aggregated and dispersed carbon nanoparticles. The data suggested that chronic inhalation of carbon nanoparticles is difficult to clear by phagocytic cells such as macrophages due to the constant overload in the animal's respiratory system. Long-term inhalation assessments of carbon nanoparticles have exhibited

an increase in systemic inflammation by production of pro-inflammatory cytokines and an increase in granulomatus inflammation in the lung and draining lymph nodes (Lam *et al*. 2004). Moreover, inhaled BC particles have been shown to produce inflammatory cytokines and cytotoxic cellular responses in response to oxidative stress causing lung injury as well as low clearance of these particles from the airways, therefore respiratory exposure to BC might influence the exacerbation and development of inflammatory conditions of the airways such as chronic obstructive pulmonary disease (COPD).

Animal studies have shown that particle size is critical to the ability of inhaled carbon nanoparticles translocation. Investigations have shown translocation of nano-size carbon particles from the alveolar region to the circulatory system followed by distribution throughout the body, including the vasculature, heart, liver, spleen, and bone marrow (Geiser and Kreyling, 2010, Kreyling *et al.* 2009, Nemmar *et al.* 2002, and Peters *et al.* 2006). However, only a low percentage of the carbon nanoparticles were able to translocate from the alveolar region to the circulatory system, indicating a low clearance mechanism for these nano-sized particles from their initial deposition site. Nevertheless, nanoparticle access to different tissues in the body could explain why exposure to ambient carbon nanoparticles has been associated with health conditions such as pulmonary and cardiovascular diseases (Li *et al.* 2007). Retention of carbon nanoparticles on organisms' tissues due to reduced clearance mechanisms can potentially interfere with effective immune responses. Lastly, large surface area of carbon nanoparticles appears to be the most important determinant of toxicity in cell and animal models mainly by uncontrolled ROS formation and oxidative stress.

1.4 MACROPHAGES

Nanoparticles are of particular interest because of their ability to penetrate deep into the lung and potentially elicit health effects triggering immune responses (Wegesseer *et al.* 2008), potentially through interactions with macrophages (MΦs). These phagocytes are specialized white blood cells that are considered sentinels and effectors of innate immunity (Reiner, 2009). Before differentiating into MΦs these cells are monocytes that originate from hematopoietic stem cell (HSC) derived progenitors with myeloid-restricted differentiation potential (Geissmann *et al*. 2010). Monocytes are produced in the bone marrow and released into the blood where they circulate or reside in the spleen before migrating into tissues during infection. For monocytes to migrate they use chemokine receptors CCR2 (also known as monocyte chemo-attractant protein 1 - MCP-1) and adhesion molecules such as cluster of differentiation 54 (CD54), which allow the monocytes to adhere to tissues where they differentiate to MΦs or DCs (Robbins and Swirski, 2010). For differentiation to occur, monocytes need specific growth or differentiation cytokines such as Macrophage Colony-Stimulating Factor (M-CSF), cytokine responsible for differentiation of monocytes into MΦs (Geissmann *et al.* 2010).

1.5 MACROPHAGE ACTIVATION

MΦs, the mature form of monocytes, are large (approximately 25 to 50 μm in diameter) antigen presenting cells (APCs) that are an important component of innate immune response to infection (Russell and Gordon, 2009). They are ubiquitous, located in many tissues such as the skin, lungs, spleen, lymph nodes, and liver, where they serve as filters for trapping foreign microbes. MΦ migrate into tissues in the steady-state or in response to inflammation where they are involved in tissue homeostasis by eliminating cellular debris generated during tissue repair. They are also involved in wound healing, removing cellular material from cells that

have gone through apoptosis and/or necrosis via the recognition of exposed intracellular membrane components.

MΦs respond to signals produced by innate immune cells and from APCs that arise after injury or infection (Mosser and Edwards, 2008). Upon recognition of a microbe or infected cell, MΦs engulf the foreign material to initiate MΦ-specific clearance mechanisms and to further process the ingested material as antigenic molecules for other immune cells to respond to (Palomaki *et al.* 2010). Thus, MΦs play a central role in host defense to pathogens by mediating important functions such as phagocytosis, removal by lysosomal degradation, production of cytokines and chemokines, and as APCs to primed T cells. The latter occurs through the major histocompatibility complex class II molecules (MHCII) by enzymatic degradation of antigens and binding of protein fragments (peptides) to MHC molecules to the cell surface for presentation (Russell and Gordon, 2009). Once peptide has been presented at the cell surface by MHCII binding to the T cell receptor (TCR) an additional signal is required such as stimulation by cytokines or through co-stimulatory molecules, such as CD80 and CD86 which in turn interact with CD28, the receptor protein on the surface of T cells, thus activating T cells to elicit a immune response to in turn activate innate immune cells to eliminate foreign pathogens.

For MΦs to perform most of their primary functions during an immune response, these cells must be activated (Xaus *et al.* 2000). MΦs are activated by toll-like receptor (TLR) ligands such lipopolysaccharide (LPS), which is a bacterial product of a complex glycolipid endotoxin that is a major component of gram-negative bacterial wall (Stout and Suttles, 1995) or by cytokines secreted by T cells such as interferon-γ (IFN-γ). When activated, MΦs can act as scavenger cells, participate in tissue repair, kill microbial pathogens including bacteria,

viruses, fungi, protozoa, and parasites; activated MΦs also secrete inflammatory cytokines, process antigens and present them to T cells. (Xaus *et al.* 2000). Furthermore, when MΦs become activated they up-regulate expression of cell surface molecules such as CD80, CD86, and major histocompatibility complex (MHC) class 2 antigen (MHCII), nitric oxide (NO) formation, produce pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα), interleukin molecules (IL) IL-6, and IL-12, and chemokines (CC and CXC) (He *et al.* 2008 and Javala *et al.* 2008). Additionally, cytokine secretion by MΦs can be triggered by microbial products, interaction with Type I Helper T cells (Th1), or by soluble factors including other cytokines. All of such activities aid MΦs in their innate and adaptive immune functions.

Phagocytosis of microbes by MΦs initiates the innate immune response that, in turn, finalizes and maintains the activation of the adaptive immune response at the target site of infection (Russell and Gordon, 2009). When foreign material is encountered by MΦs they become activated, in this state they increase phagocytosis a process that involves internalization of the pathogen by the plasma membrane that leads to fusion in a membrane bound vacuole, the phagosome. The phagosome that contains the pathogen then fuses with a lysosome (phagolysosome), followed by digestion of the pathogen by the lysosomal enzymes, resulting in the degradation of the ingested pathogen (Russell and Gordon, 2009). This process can lead to antigen presentation and removal of invading pathogens by presentation of an antigen from the pathogen on the cell surface. Mosser and Edwards (2008) describe that MΦs express low levels of MHC II, but the expression can be up-regulated by LPS as well as IFN- γ, allowing the MΦs to present antigens to primed T cells. Primed T cells enter the site of infection due to signals being provided by infected cells and by the MΦs. The MΦ – T cell peptide-specific interaction causes production of IL-12 by the MΦ to expand Th1 cell

population, which results in IFN- γ production by the T cells to activate MΦs and increase its activity to eliminate microbes, underscoring the critical role of MΦs in regulation of the adaptive immune system (Palomaki *et al.* 2010).

Studies suggest that MΦ phagocytic ability is reduced by the presence of fine and course particulate matter hence affecting the clearance of the particles from the alveolar region of the lung (Barlow *et al.* 2008). However, not much is known about the interaction of MΦ and carbon nanoparticles in the presence of a pulmonary infection and the ability of these APCs to produce a response to a bacterial infection.

1.6 DISSERTATION GOALS

The response of MΦs to carbon nanoparticulate materials is poorly understood. *This research is significant* because many biological aspects of specific constituents of nanoparticulate matter still remains unclear and requires further evaluation. Exposure to respirable ultrafine or nanoparticulate matter (PM) designated $PM_{0.1}$, indicative of particulates smaller than 0.1μm in diameter, can adversely affect human health and have been implicated with episodes of increased respiratory diseases such as asthma and allergies (Bush, 2000). Nanoparticles are of particular interest because of their ability to penetrate into the lung and potentially elicit health effects triggering immune responses (Wegesseer *et al.* 2008). *There is a lack of research* examining contemporary carbon nanoparticles and their potential ability to aggravate pulmonary immunity as well as affect our ability to fight future infections. Thus, physiological exposure to carbon nanoparticles could potentially lead to ineffective pulmonary immunity. Thus, the *goal of these studies is* to assess the chronic effects of carbon nanoparticles on murine MΦ function and clearance mechanisms. *The central hypothesis* is

that MΦ exposed to carbon nanoparticles would not suffer compromised immediate immune functions. However, long-term exposure to the nanomaterial would alter the MΦ's ability to respond appropriately to future microbial stimuli resulting in compromised immunity. Our *data* demonstrates that chronic *"pre-exposure"* to carbon nanoparticles, black carbon specifically, impedes MΦ response to a microbial stimulus. Thus, physiological exposure to carbon nanoparticles could potentially lead to ineffective pulmonary immunity. The *significance* for the studies described in this dissertation is that the identification of key cellular responses to engineered carbon nanoparticles can establish a strong framework in the study of how the environment and anthropogenic activities influence and alter MΦ function, which may ultimately interfere with effective pulmonary immune responses capable of increasing the development and progression of human diseases like chronic obstructive pulmonary disease (COPD).

CHAPTER 2

IN VITRO CYTOTOXIC EFFECTS OF CARBON NANOPARTICLES, MICRON-METALS, AND NANO-METALS

2.1 INTRODUCTION

Research demonstrates that ultrafine or nanoparticles of different compositions pose a much greater health risk than fine and course particulate matter (PM) (Buzea *et al.* 2007; Donaldson *et al.* 2001; Donaldson *et al.* 2004; Machado *et al.* 2011; Murr *et al.* 2008; Nel *et al.* 2006; Oberdorster *et al.* 1994; Oberdorster, 2001; and Somasundaran *et al.* 2010) and that these nano-sized particles are able to disturb biological systems at the cellular and subcellular levels (Kang *et al.* 2008). In the present study, we examined *in vitro* toxicity of two distinct engineered carbon nanoparticles on a murine macrophage (MΦ) cell line (RAW264.7). In addition, ballistic aerosols composed of ultrafine (nano-sized) and fine (micron-sized) metal particulates were tested for toxicity using a human lung epithelial cell line (A549). The studies detailed herein were conducted in order to evaluate in a controlled and simple experimental model, the potential cytotoxic effects of anthropogenic micron and nanoparticles on eukaryotic cells.

Nanoparticles are structures and devises with length scales in the 1 to 100-nanometer range and micron-sized particles describes fine particles that are less than or equal to 2.5-micrometer in diameter (McNeil, 2005). Black carbon (BC) nanoparticles are products of combustion, especially flame combustion; multi-walled carbon nanotubes (MWCNT) have been shown to be found in both indoor and outdoor air (Murr *et al.* 2005 and Murr, 2008). Of note is that asbestos, which has been known to cause mesothelioma as well as lung cancer, is

structurally identical to MWCNTs (Murr *et al.* 2005). Ballistic aerosols are generated by military gun firing at rod projectiles. These particles are released by kinetic energy penetrator rods of tungsten heavy alloys perforating steel target plates. Machado et al. (2010) collected ballistic aerosol debris after tungsten heavy alloy penetrators were fired into steel plates that were encapsulated in a steel containment vessel inside military armored vehicles. The study found that the generated ballistic aerosol debris was mostly composed of nanoparticulates with an average particle size of ~10 nm. Additionally, particle characterization by SEM and TEM revealed that they consist of various metal nanoparticles (iron [Fe], tungsten [W], nickel [Ni], and cobalt [Co]). Moreover, previous work in our laboratory in collaboration with Machado et al. (2010) performed cytotoxicity assays with filter collected ballistic particles on A549 cells using a direct contact cell culture method previously described by Garza *et al*. (2008) and Soto *et al*. (2008b). Results demonstrated that direct exposure of A549 cells to single stage filter samples was highly cytotoxic. In addition, previous experiments in our laboratory have shown that a wide range of anthropogenic carbon and metal particles are cytotoxic to eukaryotic cells (Garza, *et al*., 2008). Together, our work suggests that inhalation exposure to metal nanoparticles could be a potential hazard to human health. Thus, we decided to further examine this phenomena using engineered MWCNT's, BC carbon nanoparticles as well as various metal nano-sized and micron-sized particles as mimics for anthropogenic carbon and metal nanoparticles found in the environment (Murr, 2008). Cytotoxicity was measured as a function of cell viability and inflammatory cytokine production.

2.2 MATERIALS AND METHODS

Nano-sized and micron-sized metal particles. As described by Machado et al. (2011) various commercially available nano-sized and micron-sized metal particles (Fe, W, Ni, and Co) were purchased to simulate the elemental compositions of two tungsten heavy alloy rods. The tungsten alloy rods had been previously characterized (Machado *et al.* 2010) and found to contain 91% W, 5.6% Ni, 1.4% iron Fe, and 92% W, 6% Ni, and 2% Co. The commercially available nano-sized and micron-sized metal particles were further characterized by Machado et al. (2011) using a Hitachi S-4800 field-emission scanning electron microscope (FESEM) fitted with an EDAX energy- dispersive spectrometry system. Additionally, TEM equipped with a digital camera allowed direct magnifications of over 10,000,000X of the metal particles. These were placed onto a SiO/Formvarcoated Cu (200 mesh) TEM grid (3 mm diameter) and then sandwiched with another grid on top to contain the powder. Both nano-sized and micron-sized metal particles were suspended at 3 mg/mL in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) to create a stock solution. The solutions were further diluted in cell media for the assays and were re-suspended prior to administration to the cells using a vortex (1 min) to minimize metal particle agglomeration.

Carbon nanomaterials. Black carbon (BC) (Vulcan XC-72) from Cabot Corporation (Billerica, MA) and multi-walled carbon nanotubes (MWCNT) powder purchased from Rosseter Holdings, Inc (Limassol, Cyprus) were used for our experiments. Both carbon nanoparticles have been characterized by Soto et al. (2005) by transmission electron microscopy (TEM) using a Hitachi H-8000 analytical TEM operated at 200kV accelerating potential and fitted with a goniometer-tilt stage, and a noran energy-dispersive (X-Ray) spectrometer (EDS). Carbon nanoparticles were suspended at 5 mg/mL in DMSO (Sigma-Aldrich, St. Louis, MO) to create a stock solution. The solutions were further diluted in cell media for the experimental assays and

were re-suspended prior to administration to the cells using a vortex (1 min) to minimize carbon nanoparticle agglomeration.

Cell lines. Human epithelial lung cell line (A549) and murine MΦ RAW264.7 (TIB-71) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). A549 cells were cultured at 37°C and 5% CO_2 in F-12 Ham's media, supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (5%) from Fisher Scientific (Pittsburg, PA). RAW264.7 cells were cultured at 37°C and 5% CO_2 in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% fetal bovine serum (FBS), 5% penicillin-streptomycin, 5% sodium pyruvate, and 5% glutaMAX from Fisher Scientific (Pittsburg, PA).

Viability assays. Viability of A549 cells after exposure to nano-sized or micron-sized metals was assessed by a tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2- (4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay, which is a colorimetric method for determining the number of viable cells as a function of mitochondrial redox potential (CellTiter 96 Aqueous One Solution Reagent, Promega Corporation, Madison, WI). In brief, 1 x 10^5 cells per well were seeded in a 12-well plate (Fisher Scientific, Pittsburg, PA) and allowed to attach to the surface for 24 h prior to metal particle exposure. Subsequently, metal particles (nano-sized or micron-sized at 10 µg/mL, 5 µg/mL, and 2.5 µg/mL) and DMSO (vehicle control) were added for 48 hr. Following exposure, the cells were trypsinized, centrifuged, and re-suspended in 1 mL of complete DMEM; 100 µl of cell suspension was transferred to a 96-well plate (Fisher Scientific, Pittsburg, PA) in replicates of five and 20 µl of MTS solution was added to each well. The plate was then placed in the incubator at 37°C and 5% CO_2 for 2 hr. Relative viability was measured at 490 nm using a microplate spectrophotometer (VersaMax Microplate Reader, Molecular Devices).

Carbon nanoparticle dose response. We performed a carbon nanoparticle dose response assay, which allowed us to identify the concentration at which the materials exhibit a median lethal concentration (LC50). The viability of RAW264.7 cells after exposure to BC and MWCNT was assessed by CellTiter Glo luminescent assay as described by the manufacturer (Promega Corporation, Madison, WI). The CellTiter Glo assay is a luminescent method for distinguishing the number of viable cells in culture based on quantification of adenosine-5'-triphosphate (ATP), which signals the presence of metabolically active cells. Carbon nanoparticles (MWCNT and BC) were tested in a dose dependent concentration ranging from 10 μg/mL to 100 μg/mL. In brief, 3.5 X 10^5 cells per well were seeded in 6-well plates (Midwest Scientific, St. Louis, MO) and allowed to attach to the surface for 24 h prior to carbon nanoparticle exposure. Subsequently, carbon nanoparticles (MWCNT's and BC) and DMSO (vehicle control) were added for 48 hr. Following exposure, the cells were harvested, centrifuged, and re-suspended in 1 mL of complete DMEM; 100 μl of cell suspension was transferred to opaque-walled 96-well plate (VWR, Radnor, PA) in replicates of four. An equal volume of the Cell Titer Glo substrate was then added to each well. The plate was then placed on a shaker for two minutes to induce cell lysis. Next, viability was measured as a function of relative light units (RLU) using a microplate luminometer (Luminoskan Ascent, Thermo Scientific).

Lipopolysaccharide (LPS) dose response. LPS from *E.coli* was obtained from VWR (Radnor, PA) and used in a dose response assay to identify the optimal LPS concentration at which RAW264.7 MΦ cells become activated. We tested LPS-treated RAW264.7 cell viability by MTS assay and pro-inflammatory cytokine production (tumor necrosis factor alpha - TNFα) by enzyme-linked immunosorbent assay (ELISA). In brief, 3.5 x 10^5 cells per well were seeded in 6-well plates (Midwest Scientific, St. Louis, MO) and allowed to attach to the

surface. LPS stimulation was tested in a dose dependent concentration ranging from 0.1 µg/mL to 8 µg/mL. 24 hrs post-stimulation with LPS, culture supernatants were collected and stored -20□C for later analysis of cytokine detection by ELISA. To test for viability, the cells were harvested, centrifuged, and re-suspended in 1 mL of complete DMEM; 100 µl of cell suspension was transferred to a 96-well plate (Fisher Scientific, Pittsburg, PA) in replicates of five and 20 µl of MTS solution was added to each well. The plate was then placed in the incubator at 37°C and 5% CO_2 for 2 hr. Next, viability was measured at 490 nm using a microplate spectrophotometer (VersaMax Microplate Reader, Molecular Devices).

Cytokine assay. Pro-inflammatory cytokine production (tumor necrosis factor alpha - TNFα) by RAW267.4 cells was measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (BioLegend, San Diego, CA). The ELISA analysis was performed according to manufacturer's instructions. Briefly, microtiter ELISA plates were coated with anti-TNFα capture antibody overnight at 4°C. The plates were then blocked at room temperature with 3% bovine serum albumin in PBS. Cell culture supernatants from treated MΦ were added to the plates. Following binding of cytokines to the capture antibodies, the plates were incubated with biotin-conjugated anti-TNFα antibody, followed by streptavidin-HRP (Biosource/Life Technologies, Grand Island, NY). The chromogenic substrate 3,3'5,5'-tetramethylbenzidine (TMB) (Genscript, Piscataway, NJ) was utilized for color development and absorbance was measured by microplate spectrophotometer (VersaMax Microplate Reader, Molecular Devices). Cytokine concentrations were calculated against murine recombinant cytokines (BD Pharmingen, San Jose, CA).

Toxicity analysis in RAW264.7 cells. The viability of RAW264.7 cells after exposure to BC and MWCNT was assessed by CellTiter Glo luminescent assay as described by the

manufacturer (Promega Corporation, Madison, WI). The CellTiter Glo assay is a luminescent method for distinguishing the number of viable cells in culture based on quantification of adenosine-5'-triphosphate (ATP), which signals the presence of metabolically active cells. In brief, 4 x 10^5 cells per well were seeded in 6-well plates (Midwest Scientific, St. Louis, MO) and allowed to attach the surface for 24 h prior to carbon nanoparticle exposure. Subsequently, carbon nanoparticles (MWCNT's and BC at 30 µg/mL) and DMSO (vehicle control) were added for 48 hr. Following exposure, the cells were harvested, centrifuged, and re-suspended in 1 mL of complete DMEM; 100 µl of cell suspension was transferred to opaque-walled 96-well plate (VWR, Radnor, PA) in replicates of five. An equal volume of the Cell Titer Glo substrate was then added to each well. The plate was then placed on a shaker for two minutes to induce cell lysis. Next, viability was measured as a function of relative light units (RLU) using a microplate luminometer (Luminoskan Ascent, Thermo Scientific).

Toxicity analysis in RAW264.7 cells. Two methods were used to measure cell viability and cytotoxicity of RAW264.7 cells after chronic *"exposure"* and *"pre-exposure"* to BC and MWCNT. The first one to be used was a trypan blue viability assay; this method determines cell viability based on cell membrane integrity. The cells were divided into the following treatments: media (negative control), media stimulated (lipopolysaccharide, LPS 100 ng/mL – positive control), MWCNT treated (30 µg/mL), BC treated (30 µg/mL), and dimethylsulfoxide (DMSO) treated (vehicle control). On day 0, MΦ were plated into 6-well plates. The following day (day 1), carbon nanoparticles (BC and MWCNT) and vehicle control (DMSO) were added to the indicated wells. On day 4, the cells were washed with phosphate buffered saline (PBS) and treatments were refreshed. Subsequently, on day 6, the cells were washed with PBS and treatments were refreshed. Additionally on day 6, but only for the *"pre-exposure"* set of plates, lipopolysacharide (LPS -100 ng/mL) stimulus was added to all treatments except to the

negative control. The administration of LPS functioned as a mimic for a bacterial infection following a chronic exposure to the carbon nanoparticles. Alternatively, on day 6 for the *"exposure"* model, LPS (100 ng/mL) stimulus was only added to the positive control. This series of plates assessed the impact of chronic exposure, in the absence of infection, on the MΦ. Twenty-four hrs later, on day 7*,* the function of the MΦs were assessed for both "*pre-exposure*" and "*exposure*" models. Following carbon nanoparticle exposure, the cells were gently removed from the plate surface by scrapping, harvested, centrifuged, and re-suspended at 1 mL of complete DMEM. Next, 10 µl of cell suspension was mixed with 10 µl of 0.4% trypan blue followed by cell counting in the Cellometer Auto T4 Instrument (Nexcelom Bioscience, Lawrence, MA). The second method for testing cell viability and cytotoxicity was by the LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cell (Life Technologies, Grand Island, NY). The LIVE/DEAD assay is a two color fluorescent assay that identifies live versus dead cells on the basis of membrane integrity and esterase activity. Membrane-permeant calcein AM is cleaved by esterase activity in live cells to yield cytoplasmic green fluorescence, and membrane-impermeant ethidium homodimer-1 labels nucleic acids of membrane-compromised cells with red fluorescent (Life Technologies, Grand Island, NY). Following carbon nanoparticle chronic "*exposure*" and "*pre-exposure*", the cells were gently removed from the plate surface by scrapping, harvested, centrifuged, and re-suspended at 1 mL suspension of cells with 1 x 10^6 cells/mL in FACS buffer (1X phosphate buffered saline (PBS), 2% FCS, and 0.1% sodium azide). Next, we added 2 µl of 50 µM calcein AM working solution to 4 µl of the 2 mM ethidium homodimer-1 stock to each sample and incubate cells for 20 min at room temperature. Cells were then analyzed via flow cytometry (Beckman Coulter FC500 System).

Statistical Analysis. The data is presented as the mean ± SEM of replicate wells and is one of three representative experiments. The differences among treatment groups was evaluated using one-way ANOVA with a post-hoc Tukey test with a significance level of $p<0.05$. (GraphPad Prism software v. 5.00, San Diego, CA).

2.3 RESULTS

Nano-sized (nm) metals particles are more cytotoxic to A549 cells than micron-sized (μm) metals particles.

We investigated the effects of size-dependent particle toxicity on A549 cells using four different commercially available micron and nano-sized particles (Fe, W, Ni, and Co). The particles used were representative of the elemental compositions of two tungsten heavy alloy rods ballistic debris previously collected and characterized by Machado et al. (2010). A549 cells were exposed for 48 hrs to different concentrations (10 μg/mL, 5 μg/mL, and 2.5 μg/mL) of both micron and nano-sized metal particles and assessed for viability by the MTS assay. Results demonstrated that nano-sized metal particles at all concentrations **(Figure 3 A, C, and E)** were more toxic to A549 cells than the micron-size particles **(Figure 3 B, D, and F).** The results are consistent with studies that determined that smaller size particles, such as nanoparticles seem to be more toxic than fine and course particles (Buzea *et al.* 2007; Donaldson *et al.* 2001; Donaldson *et al.* 2004; Machado *et al.* 2011; Murr *et al.* 2008; Nel *et al.* 2006; Oberdorster *et al.* 1994; Oberdorster, 2001; and Somasundaran *et al.* 2010)

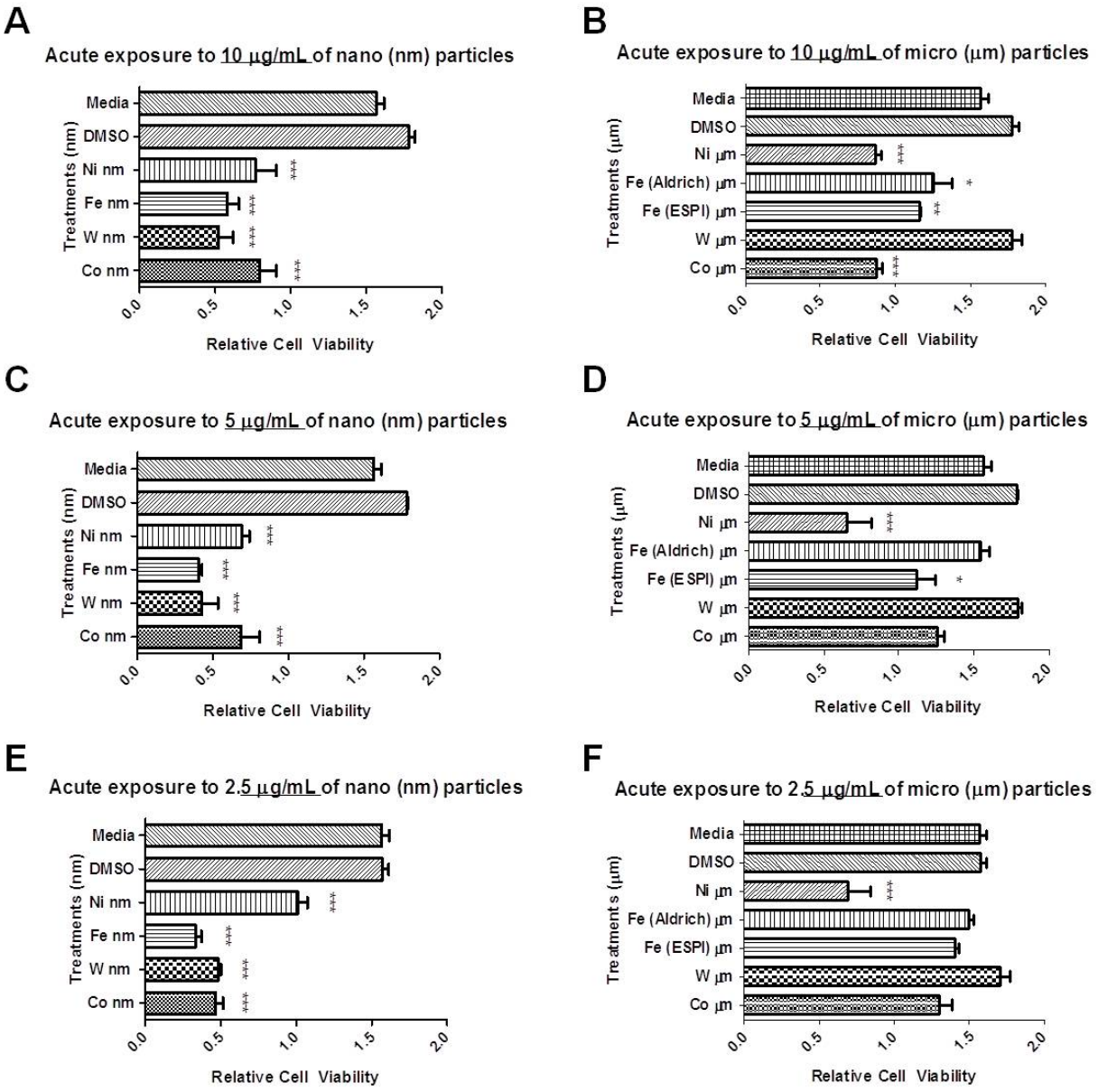

Figure 3. Nano-sized (nm) metals are more cytotoxic to A549 cells than micron-sized (µm) metals. A549 cells were treated with nano-sized (nm) or micron-sized (µm) metal particles at a concentration of 10 µg/mL (A, B), 5 µg/mL (C, D), and 2.5 µg/mL (E, F). The cells were tested for viability as a function of mitochondrial redox potential (MTS assay) 48 hrs post-treatment. Media was used as a negative control and DMSO (equal volume as to treatments) as a vehicle control. This data is presented as the mean ± SEM of duplicate wells and is one of three representative experiments. Notes: *P < 0.05; **P < 0.0015; ***P < 0.0001

BC is cytotoxic to RAW264.7 cells

To study the cytotoxicity effects of MWCNT and BC particles on MΦ and to determine the concentration of the particles to use in the chronic experiments, a dose response viability

assay to an acute exposure was first performed. MΦ were exposed for 48 hr to increasing concentrations of carbon nanoparticles, MWCNT and BC (10 µg/mL to 100 µg/mL) and assessed for viability by the CellTiter Glo assay. The luminescent-based assay was specifically selected to eliminate interference of the carbon materials with a colorimetric-based assay. Results demonstrated that BC and MWCNT carbon nanoparticles are cytotoxic to the MΦ in a dose dependent manner (**Figure 4**). Additionally, the cytotoxic assessment demonstrated that BC alters MΦ cell viability with an LC50 of 30 µg/mL. The results also demonstrated that BC is markedly more cytotoxic than MWCNT. Previous MWCNTs anthropogenic collection studies (Soto *et al.* 2005) indicate that this carbon nanoparticle is found in low concentrations in the environment, which makes it difficult to collect in sufficient quantities to perform adequate assays. Thus, rather than use MWCNT at its LC50, which would have been significantly high and likely not realistic to environmental exposure doses, we chose to keep MWCNT at the same concentration as the BC LC50.

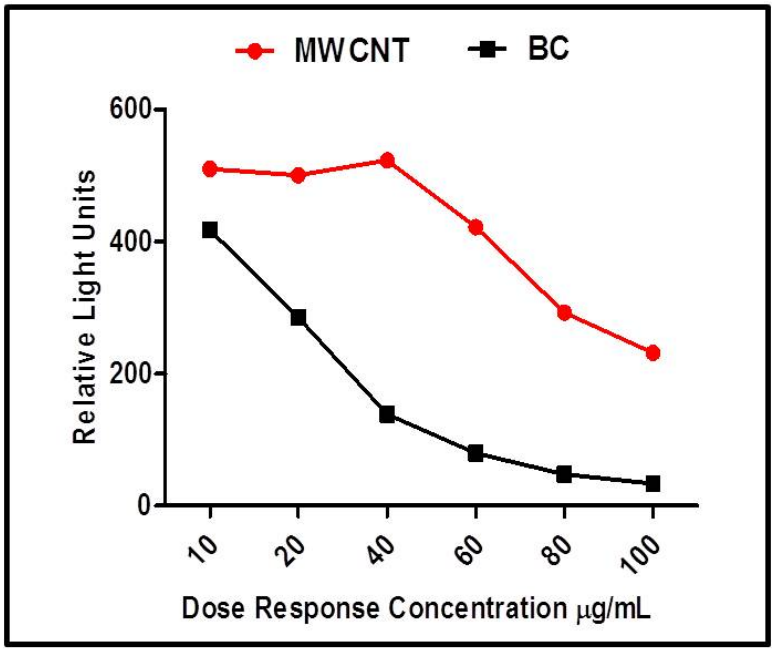

Figure 4. MWCNT and BC are cytotoxic to RAW264.7 MΦ. MΦ were treated with increasing concentrations of the indicated carbon nanoparticles and were assessed for viability using a luminescent assay 48hrs post-treatment. This data is presented as the mean ± SEM of triplicate wells and is one of three representative experiments.

Increasing concentrations of LPS do not affect RAW264.7 MΦ cell viability but reveals a dose dependent production of TNFα.

A dose response assay was performed to identify the optimal LPS concentration at which RAW264.7 MΦ cells become activated. This assay was necessary to identify the LPS concentration to be used as a positive control for MΦ activation in all *in vitro* experiments. MΦ were exposed for 24 hr to increasing concentrations of (0.1 μg/mL – 8 μg/mL) of LPS, and assessed for viability by MTS assay. Our results demonstrated no loss in viability (**Figure 5**). Additionally, the results for the cytokine detection ELISA revealed a dose dependent production of TNFα by the LPS treated RAW264.7 MΦ (**Figure 6**). Thus, we chose 0.1 μg/mL of LPS since it exerted no loss in viability and induced an exponential increase in pro-inflammatory cytokine production.

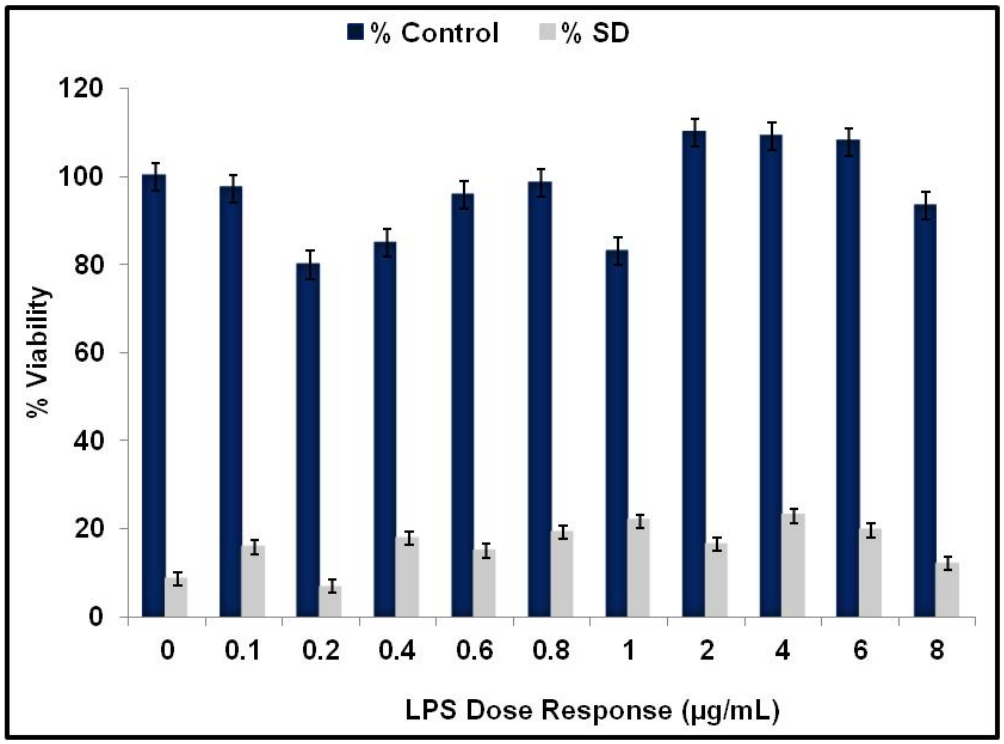

Figure 5. Increasing concentrations of LPS do not affect RAW264.7 MΦ cell viability. MΦ were treated with increasing concentrations of LPS and were assessed for viability by MTS assay 24hrs post-treatment. This data is presented as the mean ± SEM of duplicate wells and is one of three representative experiments.

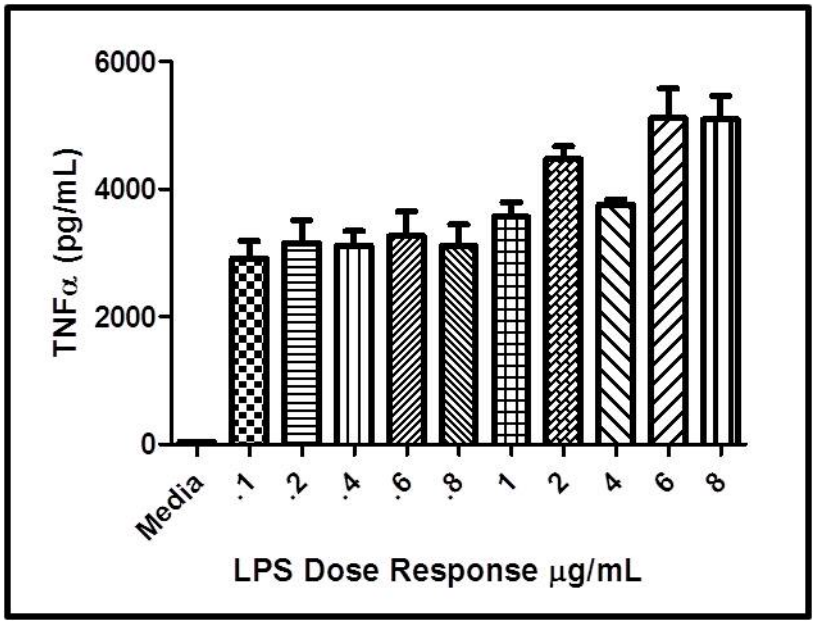

Figure 6. Increasing concentrations of LPS revealed a dose dependent production in TNFα by RAW264.7 MΦ. MΦ were treated with increasing concentrations of LPS and were assessed for TNFα production by ELISA. This data is presented as the mean ± SEM of duplicate wells and is one of three representative experiments.

Chronic "exposure" and "pre-exposure" to BC is cytotoxic to RAW264.7 cells.

Cytotoxicity assays were performed to determine the long term effects of carbon nanoparticles treatment on MΦ cells. After a 7 day chronic *"exposure"* and *"pre-exposure"* to MWCNT and BC, we assessed cellular cytotoxicity by the CellTiter Glo assay. The CellTiter Glo assay is a luminescent method for distinguishing the number of viable cells in culture based on quantification of adenosine-5'-triphosphate (ATP), which signals the presence of metabolically active cells. As shown in **Figure 7A** and **7B**, results from the CellTiter Glo assay demonstrate that both *"exposure"* and *"pre-exposure"* to BC decreases cell viability. However, no statistical difference was observed for any other measurement.

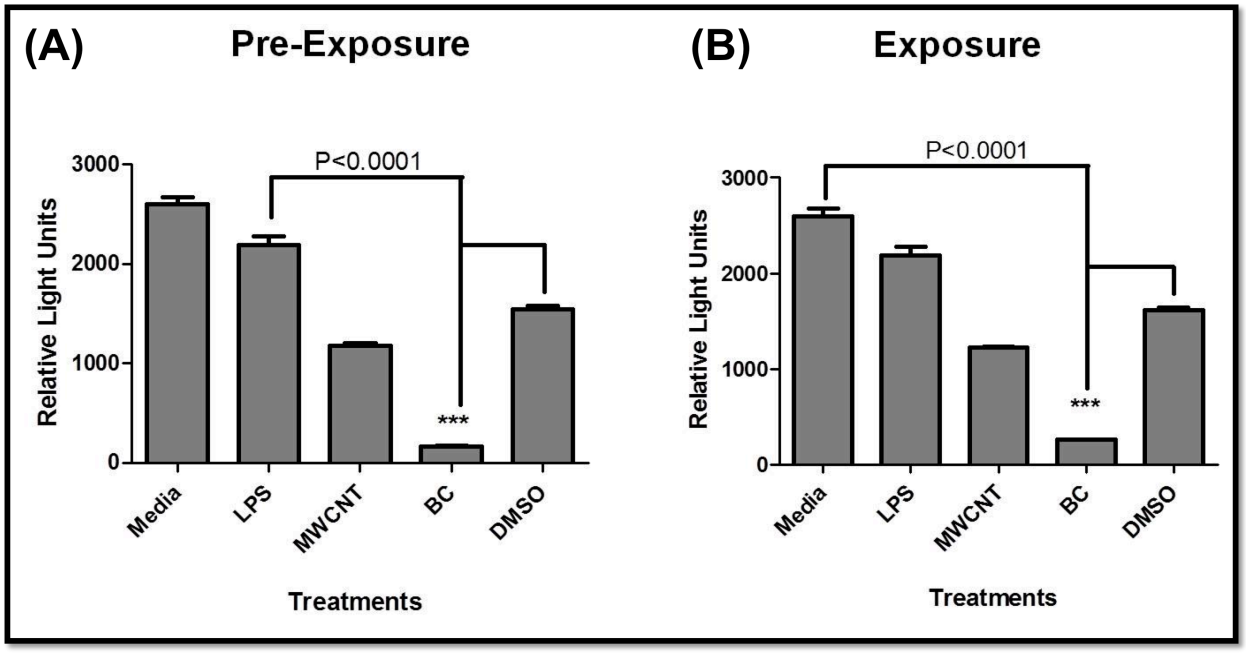

Figure 7. Chronic *"pre-exposure"* and *"exposure"* to BC is cytotoxic to RAW264.7 cells. MΦ were treated with MWCNT or BC and "Pre-Exposure" (A) samples were activated with 100ng/mL of LPS. Treatments were tested for cytotoxicity by CellTiter Glo assay 7 days post-treatment. Untreated-cells (media) and vehicle-treated cells (DMSO) were used as controls. This data is presented as the mean ± SEM of quadruplicate wells and is one of three representative experiments.

2.4 DISCUSSION

This study indicated that nanoparticles are markedly more cytotoxic to cells than micron-sized particles of the same composition. A549 cells showed decreased cell viability when exposed to nano-sized metal particles, whereas, micron-size particles were not as toxic. Similarly, other *in vitro* studies have observed increased cytotoxic response with nanomaterials when compared to micron-sized or larger particles (Buzea *et al.* 2007, Donaldson *et al.* 2004, Machado *et al.* 2011, Murr *et al.* 2008; Nel *et al.* 2006, Somasundaran *et al.* 2010, Soto *et al.* 2008a). Additionally, *in vivo* rat studies using TiO_2 or BC particles with two sizes show that the smaller particles (nanomaterial) exhibit greater inflammation and systemic effects when compared to larger size particles (Donaldson *et al.* 2001, Renwick *et al.* 2004, Oberdorster *et al.* 1994), and when inhaled can be found in lung tissues (Oberdorster, 2001). As described by Machado et al. (2010) there are several well characterized studies that examined the effects of Co, Ni, W, and Fe exposure and demonstrated that these metal particles are cytotoxic and exacerbate pulmonary fibrosis, asthma, pulmonary edemas, and pneumonia. This is likely due to their small particle size, which makes them more potent than larger particles because of their higher surface area and reactivity (Brauner *et al.* 2007). Due to their size and shape, many nanoparticles can agglomerate and increase their net size, which can potentially cause adverse health effects and affect the effective dose on the cellular system (Oberdorster *et al.* 2005). This study is of particular importance to human health because the nano-sized and micron-sized particles analyzed were from ballistic aerosol debris collected inside military armored vehicles (Machado *et al.* 2010).

The present study also examined two distinct carbon nanoparticles (BC and MWCNT) that are commonly found in our environment in the form of air pollutants. These particles are produced by combustion processes, especially flame combustion, and have been identified in both indoor and outdoor air (Oberdorster *et al.* 2005, Murr *et al.* 2005, Murr *et al.* 2006, and Murr, 2008). In order to investigate potential cell toxicity of carbon nanoparticles, cell viability was examined using commercially available particles as mimics for the anthropogenic materials. Cytotoxic assessments demonstrated that BC alters MΦ cell viability with an LC50 of 30 μg/mL and indicated that BC is more toxic than MWCNT. Our laboratory has previously shown that there is a decrease in cell viability in RAW264.7 and A549 cells treated for 48 hrs with BC or MWCNT (Murr *et al.* 2006, Soto *et al.* 2005). Additionally, two chronic six-day exposure models were also established, "*pre-exposure*" and "*exposure*". The *"pre-exposure"* model was formulated to investigate the potential ability of carbon nanoparticles to alter MΦ responses to future microbial insults; the *"exposure"* model was designed to test the ability of the carbon nanoparticles to directly activate the MΦ.

Cell cytotoxicity assessments demonstrated that both chronic *"exposure"* and *"pre-exposure"* to BC significantly decreases cell viability, in contrast, MWCNT showed no cytotoxic response, consistent with the treatment controls. Similarly, previous *in vitro* assays in our laboratory have demonstrated a cytotoxic response with long term (7 and 14 day) treatment of BC (Murr *et al.* 2006). The differences in cytotoxicty between the two test particles might be dependent on carbon nanoparticle structure and aggregation properties. BC is characterized by spherule graphite sheets and branched aggregates of carbon (Sellers *et al.* 2009), whereas, MWCNTs consist of hollow cylinder like shapes composed of various layers of thin sheets of graphene (Soto *et al.* 2005). Previously published studies (Murr *et al.* 2006, Murr *et al.* 2008, Soto *et al.* 2007) show that various aggregated nanoparticles are toxic to cellular systems.

These nanoparticles can overwhelm the biological system by penetrating deep into the lungs, compromising airway clearance capacity, resulting in injury to the lung tissue (Buzea *et al.* 2007). In conclusion, cytotoxic assessments demonstrate that nanomaterials exhibit higher toxicity to immune cells than larger particles and this activity might be a result of differences in structure, composition and aggregation of particles. Therefore, it is critical to further study the potential implications nanomaterials such as BC and MWCNT might have on adaptive immune responses.

CHAPTER 3

CHRONIC EXPOSURE TO BLACK CARBON COMPROMISES MACROPHAGE RESPONSES TO MICROBIAL STIMULUS

3.1 INTRODUCTION

Nanotechnology is defined as research and technology development at the atomic, molecular, and macromolecular scale, leading to the controlled manipulation and study of structures and devices with length scales in the 1 to 100-nanometer (nm) range (McNeil, 2005). Due to their small size and their particular physiochemical properties, research indicates that toxicological profiles of nanoparticles may be significantly different from those of larger particles composed of the same materials (Card *et al.* 2008). Research demonstrates that ultrafine or nanoparticles pose a much greater health risk than fine and course particulate matter (PM) (Donaldson *et al.* 2001; Donaldson *et al.* 2004; Machado *et al.* 2011; Murr *et al.* 2008; Nel *et al.* 2006; Oberdorster *et al.* 1994; Oberdorster, 2001; and Somasundaran *et al.* 2010) and that these nano-sized particles are able to disturb biological systems at the cellular and subcellular levels (Kang *et al.* 2008).

Disruption of biological activity is of increasing concern as exposure to nanomaterials is becoming more common. People are exposed to large numbers of ambient nanoparticles on a daily basis due to environmental air pollution (Donaldson *et al.* 2004). Such materials are generated by industrial sources, emissions from automobile combustion engines, and from natural sources resulting from combustion processes such as forest fires and volcano emissions (Oberdorster *et al.* 2005 and Card *et al.* 2008). Engineered nanomaterials also

serve as environmental contaminants. Such materials are commonly used in everyday devices such as sporting goods, tires, stain-resistant clothing, sunscreens, cosmetics, and electronics (Nel *et al*. 2006) and can be released into the environment during production or as a result of use.

Nanoparticles are available in a variety of compositions such as metal-based (titanium dioxide (TiO_2), silicon dioxide (SiO_2), tungsten (W), and nickel oxide (NiO)), or carbon-based (fullerenes like C_{60} or black carbon (BC) and single-walled (SW) or multi-walled (MW) carbon nanotubes (CNT)). The most abundant nanomaterial in both indoor and outdoor environments is the carbon-based nanoparticles (Murr and Garza 2009). BC nanoparticles predominantly form through anthropogenic activities such incomplete combustion. However, engineered BC is also released to the environment. It is commonly used in the production of tires as a strengthening agent and thus is released as tire wear debris (Murr, 2008). Carbon nanotubes, such as MWCNT, are commercially engineered for their electrical, thermal, and mechanical properties (Murr *et al*. 2005). Interestingly however, MWCNT has been found together with BC in both indoor and outdoor environments (Murr *et al.* 2005 and Murr 2008), as a consequence of combustion of natural gas, propane, wood, and candle (Murr and Garza 2009). Thus, carbon nanoparticles present a fairly common exposure risk.

Exposure to carbon nanoparticles has been reported to induce pulmonary toxicity (Lam *et al.* 2004; Warheit *et al.* 2004; Jia *et al.* 2005; Muller *et al.* 2005; Shevedova *et al.* 2005), as well as exacerbate respiratory and cardiovascular diseases (Li *et al.* 2007). Studies examining both anthropogenic and engineered carbon nanoparticles on human and murine cell lines have reported that these materials are cytotoxic, increase oxidative stress by generating reactive oxygen species (ROS), and promote inflammatory responses by production of pro-

inflammatory cytokines (Garza *et al.* 2008; Soto *et al.* 2005; Soto *et al.* 2006; Soto *et al.* 2007; Soto *et al.* 2008a; Soto *et al.* 2008b). These studies all suggest that environmental exposure to anthropogenic as well as engineered carbon nanoparticles can have detrimental effects on human health.

Animal experiments have supported the premise that exposure to airborne carbon nanomaterials is detrimental. Long-term *in vivo* inhalation assessments of carbon nanoparticles demonstrate an increase in systemic inflammation by production of pro-inflammatory cytokines and an increase in granulomatus inflammation in lungs and draining lymph nodes of treated animals (Lam *et al*. 2004). Data also suggests that respiratory exposure to BC might influence the exacerbation and development of inflammatory conditions of the airways such as chronic obstructive pulmonary disease (COPD). In addition to promoting inflammation, inhaled BC particles have been shown to induce oxidative stress causing cellular cytotoxicity and lung injury (Donaldson *et al.* 1998). Data also demonstrate that carbon nanoparticles acquired through chronic inhalation are difficult to clear by phagocytic cells leading to low clearance of these particles from the airways (Barlow *et al.* 2008). Interestingly, animal studies demonstrate translocation of inhaled carbon nanoparticle. Nano-size carbon particles have been shown to translocate from the alveolar region to the circulatory system followed by distribution throughout the body, including the vasculature, heart, liver, spleen, and bone marrow (Geiser and Kreyling, 2010; Kreyling *et al.* 2009; Nemmar *et al.* 2002; and Peters *et al.* 2006). However, only a low percentage of the carbon nanoparticles were able to translocate from the alveolar region to the circulatory system, indicating a low clearance mechanism for these nano-sized particles from their initial deposition site. Nevertheless, nanoparticle access to different tissues in the body could

explain why exposure to ambient carbon nanoparticles has been associated with health conditions such as cardiovascular diseases (Li *et al.* 2007).

Ambient carbon nanoparticles present a particularly unique health risk due to their ability to penetrate deep into the lung and to possibly circulate into other organs. Retention of carbon nanoparticles within tissues due to reduced clearance suggests interference with innate immunity, notably macrophages (MΦ). MΦ are phagocytes, responsible for recognizing, engulfing, and destroying potential pathogens. They are ubiquitously located in many tissues such as the skin, lungs, spleen, lymph nodes, and liver, where they serve as filters for trapping foreign material (Palomaki *et al.* 2010). When foreign material is encountered, MΦ can become activated; in this state they increase phagocytosis as well as up-regulate nitric oxide (NO) production and cytokine secretion, all of which are involved in the removal of invading pathogens (Javala *et al.* 2008 and Xaus *et al.* 2000). MΦ are typically activated by toll-like receptor (TLR) ligands, which are pathogen-associated molecules, (Stout and Suttles, 1995) or by cytokines secreted by T cells such as interferon-gamma (IFN- γ). MΦ also serve as antigen presenting cells (APC), processing and presenting peptide antigen of ingested material to T cells that have migrated into the site of infection. Activation of primed T cells completes elimination of the invading pathogen. Thus, activated MΦ are able to act as scavenger cells, kill microbial pathogens, secrete inflammatory cytokines for the recruitment of other immune cells, and to activate primed T cells (they also participate in tissue repair) (Xaus *et al.* 2000).

Previous studies demonstrating that carbon nanomaterial is predominantly retained *in situ* suggest that MΦ phagocytic ability is reduced by the presence of fine and course particulate matter hence affecting the clearance of the particles from the alveolar region of the lung (Barlow *et al.* 2008). Thus, considering that MΦ are critical for effective microbial

eradication, inhaled nanomaterials could severely inhibit anti-microbial immunity. Here, we assess the effect of carbon nanoparticles BC and MWCNT on MΦ function in an *in vitro* chronic exposure model. The data demonstrate that BC by itself, in a chronic "*exposure*" model, is capable of inducing an inflammatory response by murine MΦ as measured by NO formation and TNFα production. Additionally, in a "*pre-exposure*" model, BC suppresses MΦ activity in relation to adaptive immunity. The up-regulation of cell surface MΦ activation markers, required to interact with T cells, was prevented by *"pre-exposure"* to BC; moreover, BC *"pre-exposure"* suppressed phagocytosis (antigen acquisition), and prevented proteolytic processing of ingested material (antigen processing). In contrast, MWCNT causes little or no effect on murine MΦ. Thus, our data demonstrate that BC can promote innate immune activity and inhibit adaptive immune function of MΦ suggesting that carbon nanoparticles could potentially alter the effectiveness of alveolar MΦ to clear pulmonary infections. The data further suggests that this phenomenon might be dependent on carbon nanoparticle structure since MWCNTs demonstrated no impact on MΦ function. The findings underscore the potential detriment that carbon-based nanoscale air pollutants can impose on immunity.

3.2 METHODS

Carbon nanomaterials. Black carbon (BC) (Vulcan XC-72) from Cabot Corporation (Billerica, MA) and multi-walled carbon nanotubes (MWCNT) powder purchased from Rosseter Holdings, Inc (Limassol, Cyprus) were used for our experiments. Both carbon nanoparticles have been characterized by Soto *et al.* (2005) by transmission electron microscopy (TEM) using a Hitachi H-8000 analytical TEM operated at 200kV accelerating potential and fitted with a goniometer-tilt stage, and a noran energy-dispersive (X-Ray) spectrometer (EDS). Carbon

nanoparticles were suspended in a stock solution at 5 mg/mL in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO). The solutions were re-suspended, prior to administration to the cells, using a vortex (1 min) to minimize carbon nanoparticle agglomeration.

Cell line. Murine macrophage RAW264.7 (TIB-71) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured at 37□C and 5% CO_2 in Dulbecco's Modified Eagle Media (DMEM), supplemented with 10% fetal bovine serum (FBS), 5% penicillin-streptomycin, 5% sodium pyruvate, and 5% glutaMAX from Fisher Scientific (Pittsburg, PA).

Viability assay. The viability of RAW264.7 cells after exposure to BC and MWCNT was assessed by CellTiter Glo luminescent assay as described by the manufacturer (Promega Corporation, Madison, WI). The CellTiter Glo assay is a luminescent method for distinguishing the number of viable cells in culture based on quantification of adenosine-5'-triphosphate (ATP), which signals the presence of metabolically active cells. In brief, 4 x 10^5 cells per well were seeded in 6-well plates (Midwest Scientific, St. Louis, MO) and allowed to attach the surface for 24 h prior to carbon nanoparticle exposure. Subsequently, carbon nanoparticles (MWCNT's and BC at 30 μg/mL) and DMSO (vehicle control) were added for 48 hr. Following exposure, the cells were harvested, centrifuged, and re-suspended in 1 mL of complete DMEM; 100 μl of cell suspension was transferred to opaque-walled 96-well plate (VWR, Radnor, PA) in replicates of eight. An equal volume of the Cell Titer Glo substrate was then added to each well. The plate was then placed on a shaker for two minutes to induce cell lysis. Next, viability was measured as a function of relative light units (RLU) using a microplate luminometer (Luminoskan Ascent, Thermo Scientific).

Cytokine assay. Pro-inflammatory cytokine production (tumor necrosis factor alpha - TNFα) by RAW267.4 cells and interferon gamma (IFNγ) production by T cells was measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (BioLegend, San Diego, CA). The ELISA analysis was performed according to manufacturer's instructions. Briefly, microtiter ELISA plates were coated with capture antibody over night at 4□C. The plates were then blocked at room temperature with 3% bovine serum albumin in PBS. Cell culture supernatants from treated MΦ or from T cell and MΦ co-cultures were added to the plates. Following binding of cytokines to the capture antibodies, the plates were incubated with biotin-conjugated anti-cytokine antibody, followed by HRP-labeled avidin (Biosource/Life Technologies, Grand Island, NY). The chromogenic substrate 3,3'5,5'-tetramethylbenzidine (TMB) (Genscript, Piscataway, NJ) was utilized for color development and absorbance was measured by microplate spectrophotometer (VersaMax Microplate Reader, Molecular Devices). Cytokine concentrations were calculated against murine recombinant cytokines (BD Pharmingen, San Jose, CA).

Nitric oxide assay. Nitric oxide (NO) formation was measured by the Griess Reagent System (Promega Corporation, Madison, WI). This is a colorimetric assay that measures nitrite (NO_2^-), the stable metabolite, in culture supernatants. 144 hr post carbon nanoparticle treatment, culture supernatants were collected and placed into 96-well plate (Midwest Scientific, St. Louis, MO). According to the manufacturer's instructions, sulfanilamide and N-1-napthylethylenediamine dihydrochloride (NED) solution were added to all samples, which gave a purple/magenta color in the presence of (NO_2^-). Color change was detected by measuring the absorbance in a microplate spectrophotometer (VersaMax Microplate Reader, Molecular Devices) in the visible range at 520nm. Nitrite concentrations were calculated from a sodium nitrite standard curve using a linear curve fit (SOFTmax Pro software, Molecular Devices).

Measurement of cell surface activation molecules. Cell surface molecules CD40 and CD86 were analyzed by flow cytometry 144 hr after treatment with carbon nanoparticles. RAW264.7 cells were gently removed from the plate surface by scrapping. After 2 washes with ice cold FACS buffer (1X phosphate buffered saline (PBS), 2% FCS, and 0.1% sodium azide) cells were blocked with 50% normal mouse serum for 20 min. This was followed by an incubation with PE-conjugated anti-CD40 or anti-CD86 antibodies (BD Pharmingen, San Jose, CA). After a 45 min incubation, the samples were washed with FACS buffer and cells were fixed with 1% parafomaldehyde (PFA) (Sigma-Aldrich, St. Louis, MO) and analyzed via flow cytometry (Beckman Coulter FC500 System).

Phagocytosis assay. Phagocytosis activity was examined using the Vybrant Phagocytosis Assay Kit (Invitrogen, Carlsbad, CA), which determines phagocytosis by the internalization of fluorescently-labeled bio-particles. Briefly, cells were seeded at 1 x 10^5 cells per well in a 96-well flat bottom black-walled plate (VWR, Radnor, PA) and incubated with fluorescein-labeled *Escherichia coli* (*E. coli*) K-12 BioParticles. Following a 2 hr incubation at 37□C, the cells were treated with trypan blue to quench fluorescence of any *E. coli* bio-particles that were not uptaken by the RAW264.7 cells. Phagocytosis was analyzed with a fluorescence plate reader (excitation 480 nm, emission 520 nm) (Fluoroskan Ascent, Thermo Scientific).

Measurement of antigen processing. Antigen processing by treated RAW264.7 cells was examined by the addition of a self- quenched conjugate of ovalbumin (DQ-OVA), which exhibits bright green fluorescence upon proteolytic degradation due to the released dye molecules (Invitrogen, Carlsbad, CA). According to the manufactures instructions, following carbon nanoparticle treatment, cells were seeded at 1 x 10^5 cells per well in a 96-well flat

bottom black-walled plate in the presence of DQ-OVA (10 μg/mL). Fluorescence emission proportional to the extent of DQ-OVA processed was measured (excitation 485 nm, emission 518 nm) every 10 min for 320 min with a fluorescent plate reader (Fluoroskan Ascent, Thermo Scientific).

Measurement of reactive oxygen species (ROS) production. ROS generation by treated RAW264.7 cells was determined by flow cytometry using the CellROX™ Deep Red (Invitrogen, Carlsbad, CA). The reagent is a cell permeant indicator for ROS; the reagent is non-fluorescent while in a reduced state and upon intracellular oxidation it exhibits a fluorescent signal. After 144 hr carbon nanoparticle treatment, the cells were gently scraped and transferred to flow cytometry tubes. According to the manufacturer's instructions, CellROX™ Deep Red Reagent was added to each tube at a final concentration of 5 μM and incubated for 30 min at 37□C. The medium was then removed and cells were washed three times with PBS followed by fixation with 1% PFA. The samples were analyzed via flow cytometry (excitation 640 nm, emission 665 nm) (Beckman Coulter FC500 System).

Antigen presentation to antigen-specific T cells. To observe the ability of treated RAW264.7 cells to process whole protein and present processed peptides to T cells, carbon nanoparticle-treated MΦ cells were pulsed with soluble endotoxin-free ovalbumin protein (250 μg/mL) (BioVendor, Candler, NC) and were irradiated in a X-RAD 160 Biological X-Ray Irradiator (Precision X-Ray, North Branford, CT) at 3,000 cGy per min . Irradiated MΦs were then co-cultured with enriched ovalbumin-specific $CD4^+$ T cells isolated from lymph nodes of C.Cg-Tg(DO11.10)10Dlo/J mice (The Jackson Laboratories, Bar Harbor, ME) at a ratio of 1:10 MΦ to T cell for 72 hrs in a flat bottom 96-well plate (Midwest Scientific, St. Louis, MO). T cell activation was measured 72 hrs later as a function of IFN- γ production by ELISA.

Statistical Analysis. The data is presented as the mean ± SEM of replicate wells and is one of three representative experiments. The differences among treatment groups was evaluated using one-way ANOVA with a post-hoc Tukey test with a significance level of $p<0.05$. (GraphPad Prism software v. 5.00, San Diego, CA).

3.3 RESULTS

In this study we have examined two distinct engineered nanoparticle materials, Vulcan XC-72 black carbon (BC) (**Figure 8A**) and MWCNTs (**Figure 8B**). BC is a common nanoparticle composed of more than 97% elemental carbon, characterized by spherule graphite sheets of carbon (Sellers *et al.* 2009) (**Figure 8A**). Its initial particle size is nano-size; however, van der Waals forces cause BC particles to aggregate and eventually adhere to form stable agglomerates that can reach 1 to over 100 μm in size. A different shaped carbon nanoparticle is carbon nanotubes (CNTs) (**Figure 8B**); these are known for their cylinder/fiber like shape and strong structural properties. CNTs are categorized as single-walled carbon nanotubes (SWCNTs) or multiwalled (MWCNTs). SWCNT consist of hollow cylinder like shape composed of one layer thin sheet of graphene, while MWCNTs are made of various layers.

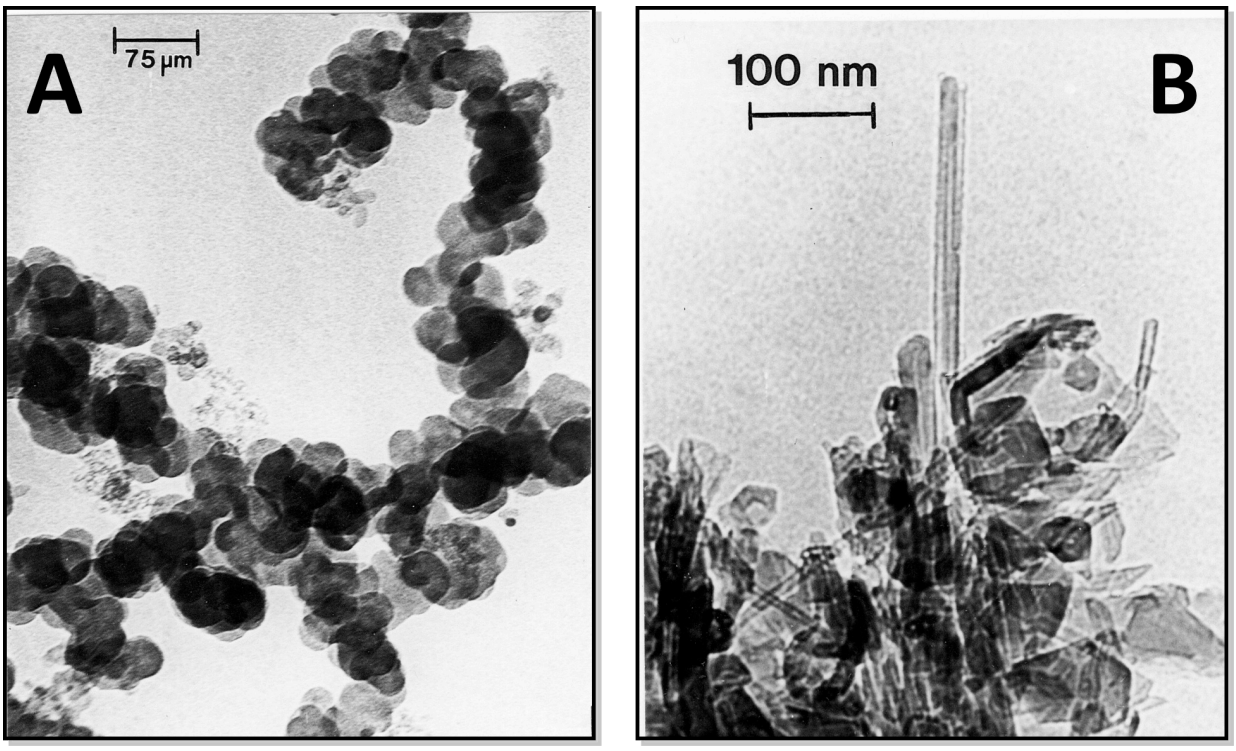

Figure 8. TEM image of commercially produced Carbon Nanoparticles. Black Carbon (BC) containing spherule and branched aggregates (A) and Multi-Walled Carbon Nanotubes (MWCNT) aggregate material (B).

The carbon nanoparticle materials used in this study were previously characterized by Soto et al. (2005) and (2006). Transmission electron microscopy (TEM) and ancillary techniques involving selected-area electron diffraction (SAED) to observe the particle crystallinity were performed for both carbon nanoparticles. As previously described, engineered BC nanoparticles are composed of branched aggregates of carbon spherules ranging in size from 2 to 50 nm; with a mean diameter of 20 nm; and SAED patterns show carbon spherules to be composed of graphene (graphite sheet nano-fragments). Engineered MWCNT nanoparticle aggregate sizes ranged from 10 nm to 30 nm and nanotube lengths ranged from 50 nm to >1 µm. SAED patterns show clumps or loose aggregates composed of multi-wall nanotubes and other carbon nanoforms (fullerene polyhedra). In addition to engineered nanomaterial, Soto et al. (2005) characterized anthropogenic carbon

nanoparticles. TEM and SAED demonstrated that anthropogenic BC and MWCNT nanoparticles are identical in morphology and composition to the engineered materials.

Garza et al. (2008) performed *in vitro* cytotoxicity assays with these nanomaterials using a human lung epithelial cell line (A549) as the indicator system. They observed significant cytotoxicity in response to the engineered BC and MWCNT and to various anthropogenic nanomaterials. Thus, the similarities in structure composition and cytotoxicity establish the grounds for using manufactured carbon nanoparticles for the current study. Moreover, these previous studies suggest that the similar cytotoxicity between the engineered and anthropogenic carbon nanomaterials deserve further investigation since the carbon nanoparticles could potentially lead to long term complications and exacerbations of respiratory diseases (Soto *et al.* 2005).

In this study, murine MΦs were chronically exposed *in vitro* to BC and MWCNT to assess the affects of these nanomaterials on MΦ function and clearance mechanisms. The *in vitro* model allowed us to evaluate, in a controlled and simple experimental model, the potential effect of engineered carbon nanoparticles on MΦ's and by inference, how the environment and anthropogenic activities may influence innate immunity. Two chronic six-day exposure models were established, "*pre-exposure*" and "*exposure*". The *"pre-exposure"* model was formulated to investigate the potential ability of carbon nanoparticles to alter MΦ responses to future microbial insults; the *"exposure"* model was designed to test the ability of the carbon nanoparticles to directly activate the MΦ.

Figure 9 outlines the experimental set-up for the chronic *in vitro* model. Murine macrophage-like cells, RAW264.7 were used as the model system. The cells were divided into the following treatments: media (negative control), media stimulated (lipopolysaccharide,

LPS 100 ng/mL – positive control), MWCNT treated (30 µg/mL), BC treated (30 µg/mL), and dimethylsulfoxide (DMSO) treated (vehicle control). On day 0, MΦ were plated into 6-well plates at 4 x 10^4 cells per well. The following day (day 1), carbon nanoparticles (BC and MWCNT) and vehicle control (DMSO) were added to the indicated wells. On day 4, the cells were washed with phosphate buffered saline (PBS) and treatments were refreshed. Subsequently, on day 6, the cells were washed with PBS and treatments were refreshed. Additionally on day 6, but only for the *"pre-exposure"* set of plates, lipopolysacharide (LPS -100 ng/mL) stimulus was added to all treatments except to the negative control. The administration of LPS functioned as a mimic for a bacterial infection following a chronic exposure to the carbon nanoparticles. Alternatively, on day 6 for the *"exposure"* model, LPS (100 ng/mL) stimulus was only added to the positive control. This series of plates assessed the impact of chronic exposure, in the absence of infection, on the MΦ. Twenty-four hrs later, on day 7, the function of the MΦs were assessed for both "*pre-exposure*" and "*exposure*" models.

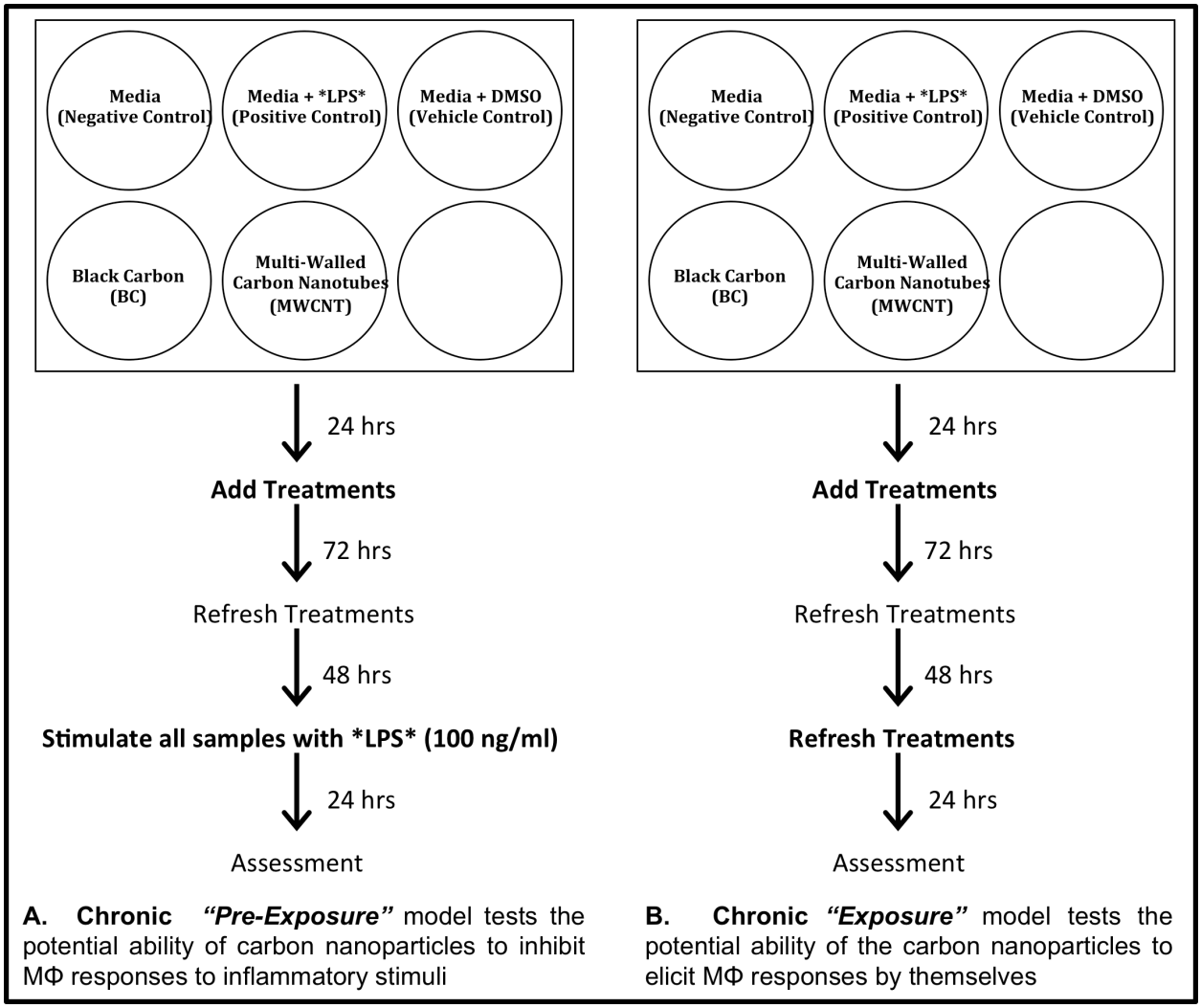

Figure 9. Chronic *in vitro* assay model. On day 0, MΦs are be plated in 6-well plates at 4 x 10^4 cells per well. The next day (d1), carbon nanoparticles (MWCNT's and BC) and vehicle control (DMSO) is added to the indicated wells and left in the incubator for 72 hrs. On d4, cells are washed with 1X PBS and treatments are refreshed; the cells are incubated for an additional 48 hrs. Subsequently, on d6, the cells are again washed with 1X PBS and treatments are refreshed. For the "pre-exposure" set of plates (A), LPS (100 ng/mL) stimulus is added to all treatments except the negative control. For the "exposure" model (B), the LPS (100 ng/mL) stimulus is added only to the positive control. Twenty four hours later (d7), the cells from both the "pre-exposure" and "exposure" plates are assessed for cell viability, TNFα production, NO formation, ROS production, phagocytosis ability, and up-regulation of MF cell surface markers.

To study the cytotoxicity effects of MWCNT and BC particles on MΦ and to determine the concentration of the particles to use in the chronic experiments, a dose response viability assay to an acute exposure was first performed. MΦ were exposed for 48 hr to increasing

concentrations of carbon nanoparticles, MWCNT and BC (10 µg/mL to 100 µg/mL), and assessed for viability by the CellTiter Glo assay. The luminescent-based assay was specifically selected to eliminate interference of the carbon materials with a colorimetric-based assay. Results demonstrated that BC and MWCNT carbon nanoparticles are cytotoxic to the MΦ in a dose dependent manner. Additionally, the cytotoxic assessment demonstrated that BC alters MΦ cell viability with an LC50 of 30 µg/mL. The results also demonstrated that BC is markedly more cytotoxic than MWCNT. Previous MWCNTs anthropogenic collection studies (Soto *et al.* 2005) indicate that this carbon nanoparticle is found in low concentrations in the environment, which makes it difficult to collect in sufficient quantities to perform adequate assays. Thus, rather than use MWCNT at its LC50, which would have been significantly high and likely not realistic to environmental exposure doses, we chose to keep MWCNT at the same concentration as the BC LC50.

MΦ also produce inflammatory mediators as a protective response or defense to eliminate foreign material or organisms that cause injury. Mediators include nitric oxide (NO), pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα), interleukin molecules (IL) IL-6, and IL-12, and chemokines (CC and CXC) (He *et al.* 2008 and Javala *et al.* 2008). Results from chronic "*exposure*" treatment in **Figure 10A and 10B** demonstrate an up-regulation in the formation of pro-inflammatory mediators NO and TNFα. However, no statistical difference was observed in the detection levels of NO and TNFα formation in chronic "*pre-exposure*" model (**Figure 10C and 10D**). The data indicates that BC by itself is capable of inducing an inflammatory response in murine MΦ by up-regulating NO formation and TNFα production, however MWCNT does not. In addition, neither BC nor MWCNT inhibit NO or TNFα production upon microbial stimulation.

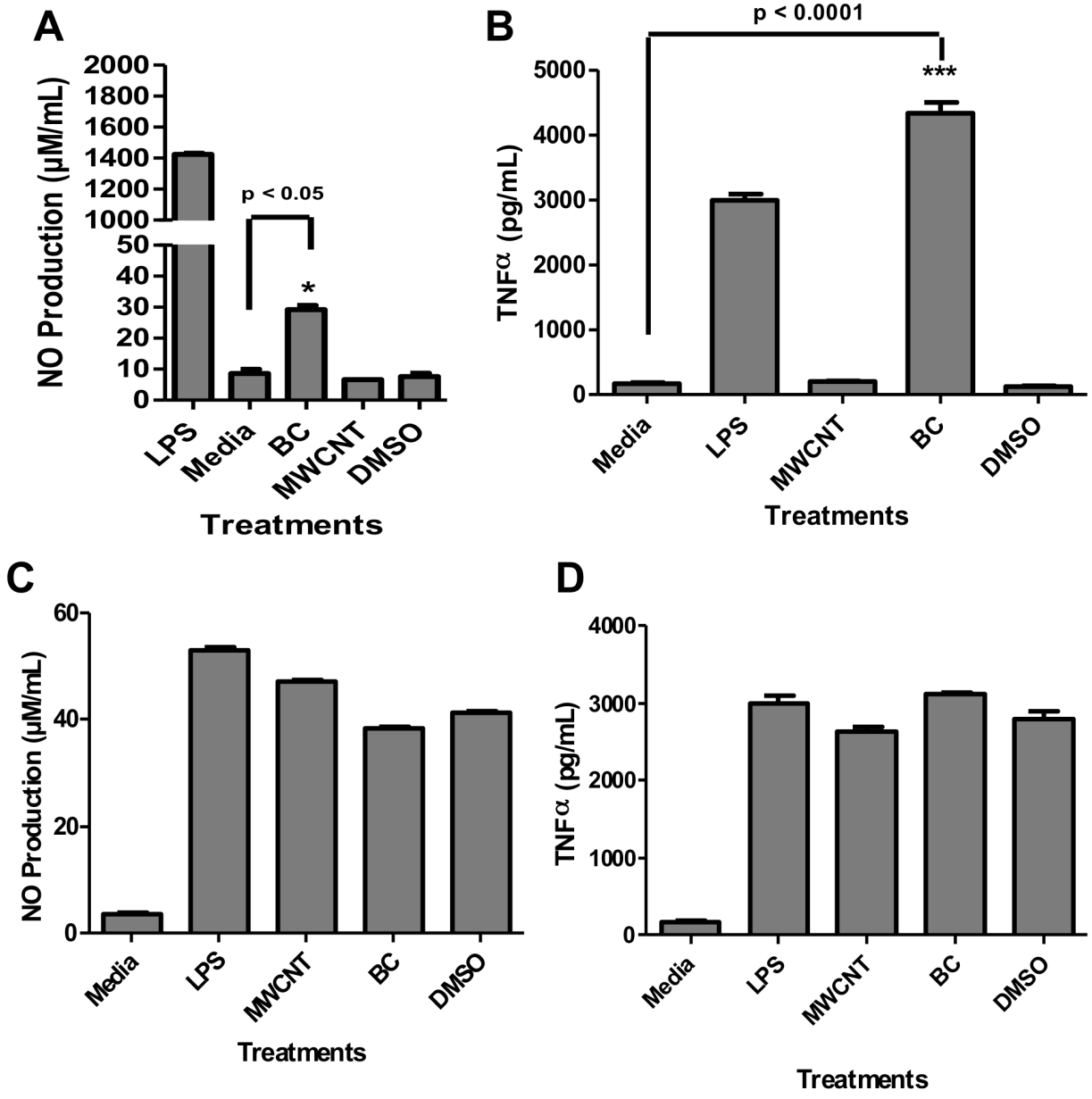

Figure 10. BC Chronic *"exposure"* up-regulates NO and TNFα formation in MΦ. MΦs were treated with MWCNT or BC and were tested for Nitric Oxide formation (A, B) and TNFα production (C, D) 7 days post-treatment. However, BC Chronic "*Pre-Exposure*" does not alter MΦ inflammation markers (C) and (D). This data is presented as the mean ± SEM of duplicate wells and is one of three representative experiments.

Intracellular production of reactive oxygen species (ROS) is also an important defense mechanism to mediate microbial clearance; however, over production of ROS can overwhelm

the cellular antioxidant capacity (Xia *et al.* 2006). Studies have implicated ROS formation as a cytotoxicity mechanism promoted when cells come into contact with the surface of carbon nanoparticles, creating an oxidative stress reaction, which is a state of redox disequilibrium (Li *et al.* 2008). In **Figure 11A**, we observed that BC "*pre-exposure*" significantly decreased ROS production in murine MΦ.

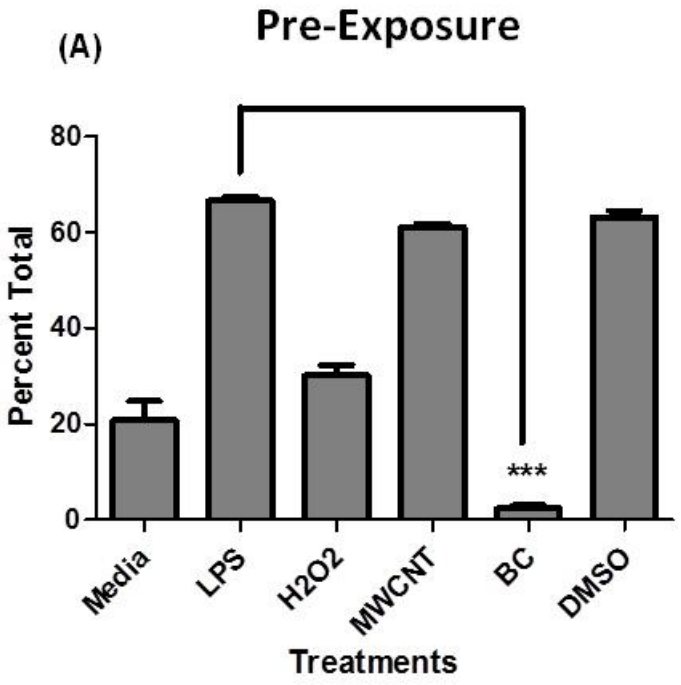

Figure 11. BC Chronic "pre-*exposure"* alters MΦ ROS production. MΦ were treated with MWCNT or BC and "Pre-Exposure" (A) samples were activated with 100ng/mL of LPS. Treatments were tested for ROS production by CellROX™ Deep Red fluorescent reagent 7 days post-treatment. This data is presented as the mean ± SEM of duplicate wells and is one of three representative experiments.

MΦ become activated and up-regulate expression of cell surface molecules when they engage toll-like receptors upon encountering and interacting with foreign materials or microbes. Activation molecules include CD80, CD86, and major histocompatibility complex (MHC) class II antigen (MHCII). These markers indicate MΦ activation and are used for optimal interaction with primed T cells. Results in **Figure 12A** and **12B** demonstrate that chronic "*pre-exposure*" to BC nanoparticles caused an inhibition of cell surface MΦ activation

markers CD40 and CD86; "*exposure"* alone did not induce up-regulation of these markers. Thus, it was observed that BC inhibits MΦ response to a microbial stimulus by preventing the increased expression of cell surface MΦ activation markers.

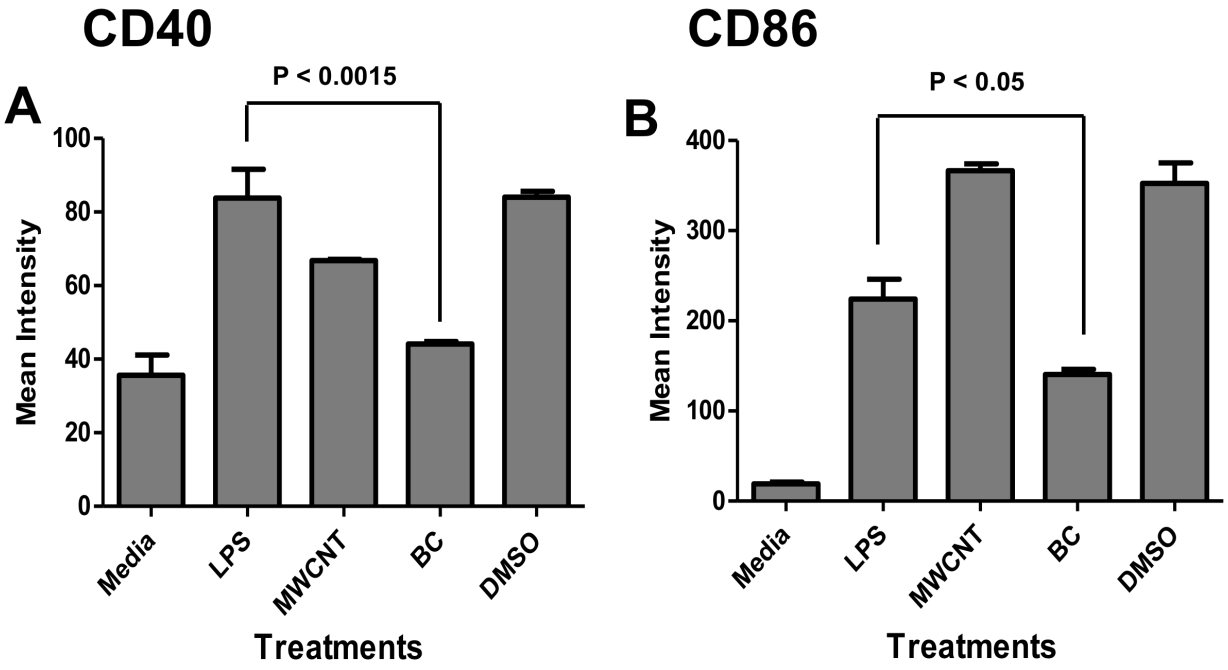

Figure 12. BC Chronic "p*re-exposure*" alters MΦ response to LPS. MΦ were treated with MWCNT or BC and activated with 100ng/mL of LPS. Post-treatment, the cells were harvested, washed, and then stained with PE-conjugated anti-CD40 (A) or anti-CD86 (B). This data is presented as the mean ± SEM of duplicate wells and is one of three representative experiments.

MΦs are highly effective in the uptake of pathogens by phagocytosis, which serves two functions; the removal of the invading microbe and maintenance of a T cell-mediated immune response. Protein material ingested by MΦs is ultimately processed by proteolytic cleavage into peptides for antigen presentation to primed T cells. Studies indicate that chronic inhalation of carbon nanoparticles are difficult to clear by phagocytic cells due to the constant overload in the animal's respiratory system (Barlow *et al.* 2008). We therefore also investigated antigen

acquisition/phagocytosis and antigen processing by the RAW267.4 cells exposed to the carbon nanomaterials. Phagocytosis was measured as uptake of fluorescently-labeled bioparticles; antigen processing was measured as intracellular exposure of fluorescently-labeled ovalbumin (OVA-DQ: exposure of fluorescence occurs following proteolytic removal of a protein cage). As shown in **Figure 13A and 13B**, after chronic "*pre-exposure*" to the carbon nanoparticles, we observed a significant decrease in phagocytosis and OVA-DQ processing by the BC treated MΦ. Results demonstrate that pre-exposure to BC severely compromises the ability of MΦs to ingest and degrade extracellular material.

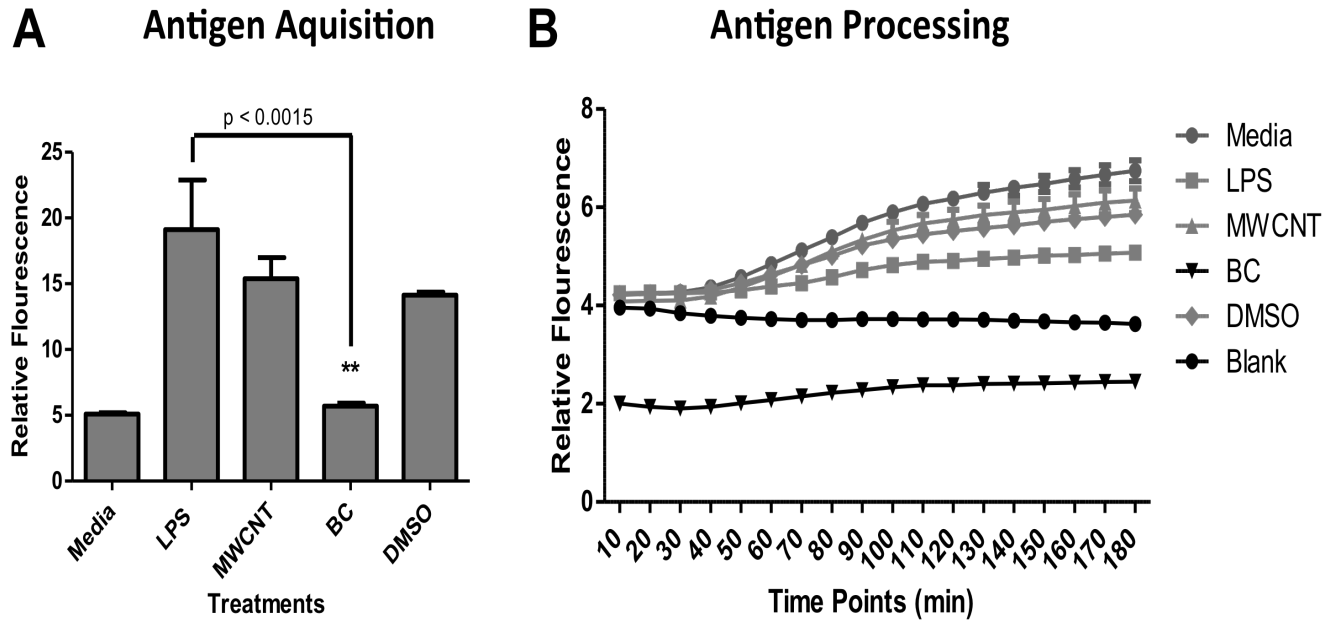

Figure 13. BC Chronic "*pre-exposure*" alters MΦ antigen acquisition and processing. MΦ were treated with MWCNT or BC and were additionally activated with 100 ng/mL of LPS. The cells were tested for Phagocytosis (A) and OVA-DQ processing (B) post-treatment. The data is presented as the mean ± SEM of duplicate wells and is one of three representative experiments.

MΦs are an important component of the innate immune system, as phagocytes these cells internalize and digest pathogens. This process ultimately leads to antigen presentation

and links MΦs to adaptive immunity. MΦs thus activate the adaptive immune system by breaking down antigens and binding of protein fragments (peptides) to MHC class II molecules that are then sent to the cell surface for presentation to primed T cells (Russell and Gordon 2009 and Underhill *et al.* 2009). In this way, MΦs engage primed T cells that have entered the site of infection, promoting pathogen clearance by adaptive immune cells (Russell and Gordon 2009 and Underhill *et al.* 2009). Figure 13 demonstrated that acquisition and processing of antigen is suppressed by pre-exposure to BC. We therefore investigated the ability of carbon nanoparticle treated RAW267.4 cells to process and present soluble endotoxin-free ovalbumin protein (250 µg/mL) (BioVendor, Candler, NC) to ovalbumin protein-specific $CD4^+$ T cells **(Figure 14A)**. The antigen-specific processing and presentation (APC – T cell interaction) results indicate a decrease (although not statistically significant) in T cell response (IFN-γ production) when MΦ are pre-treated with BC, indicating a suppressed ability to effectively acquire and/or process ovalbumin protein. Addition of ovalbumin protein requires the internalization by phagocytosis, proteolytic processing of protein and up-regulation of MHC class II peptides at the cell surface. As shown in **Figure 14A**, the positive controls pre-treated cells readily interact and activate T cells. Thus, the decreased induction of IFNγ by T cells when co-cultured with BC pre-treated MΦ is directly related to the MΦs inability to effectively phagocytose and/or process protein antigens.

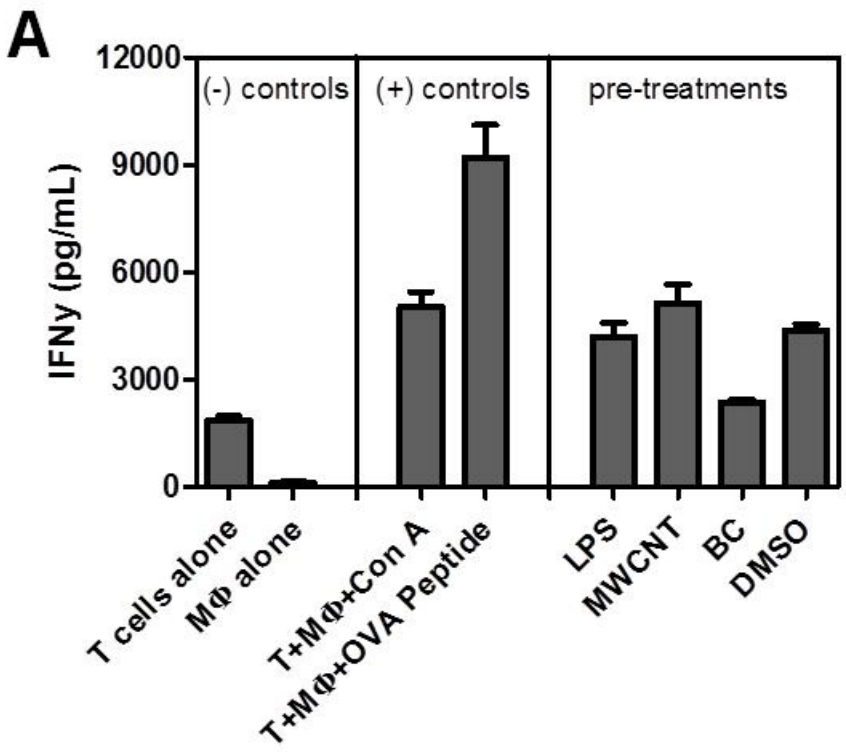

Figure 14. Chronic "*Pre-Exposure*" to BC suppresses MΦ antigen processing and presentation. MΦ were pre-treated with MWCNT or BC and were additionally activated with 100 ng/mL of LPS. 7 days post treatment the cells were irradiated and given endotoxin-free ovalbumin protein (250 µg/mL) (A). The cells were then used as antigen presenters to ovalbumin-specific enriched $CD4^+$ T cells harvested from C.Cg-Tg(DO11.10)10Dlo/J mice. T cell responses were measured as a function of IFNγ production 96 hrs post-co-culture. The data is presented as the mean ± SEM of five wells and is one of three representative experiments.

3.4 DISCUSSION

The present study demonstrates in *vitro* chronic exposure to BC nanoparticles alters MΦ function by promoting inflammation and compromising responses to microbial stimulation. Results indicated that BC by itself is capable of inducing an inflammatory response in murine MΦ by up-regulating TNFα alpha production and NO formation. Exposure to carbon nanoparticles such as BC has previously been shown in both *in vitro* and *in vivo* to increase the production of pro-inflammatory cytokine TNFα (Brown *et al.* 2002, Muller *et al.* 2005,

Donaldson *et al.* 2003, Pozzi *et al.* 2003, Monteiller *et al.* 2007). Similarly, Brown *et al.* (2002) reported that treatment with ultrafine BC (nanomaterial) resulted in TNFα increase in rat alveolar MΦ while other *in vitro* studies observed increases in TNFα production in the supernatants and mRNA levels of MΦ cultures (Muller *et al.* 2005). Additionally, Donaldson *et al.* (2003) treated human MΦ cells (MM6) and rats with BC nanomaterials and observed an increase in TNFα cytokine release and greater inflammation in the rat model. Furthermore, Pozzi *et al.* (2003) tested the induction of pro-inflammatory mediators by treating RAW264.7 MΦ cells with urban air particles and BC and observed an increase in TNFα production. Continuous secretion of TNFα has been linked to an increase inflammatory response with resulting pathological consequences (Brown *et al.* 2007). In addition, it has been reported that BC nanoparticles and TiO_2 exhibited pro-inflammatory effects on epithelial cells *in vitro* (Monteiller *et al.* 2007) and several studies have also indicated NO formation as a marker for inflammatory responses. Stoker *et al.* (2008) reported an increase in NO formation using human bronchial epithelial cells and human lung fibroblast exposed to carbon nanomaterials. Similarly, Park and Park (2009) investigated pro-inflammatory responses using *in vivo* and *in vitro* murine models treated with silica nanoparticles reporting an increase in NO formation, up-regulation of mRNA inflammatory related genes and cytokines in both models. A constant inflammatory response could potentially be detrimental to the immune system by causing systemic inflammation (Lam *et al*. 2004) as well as influencing health conditions such as pulmonary and cardiovascular diseases (Li *et al.* 2007).

Next, we compared the effects of carbon nanomaterial exposure on immune cells pre-treated with LPS to test the ability of a bacterial infection to suppress MΦ activity. Several studies have investigated the effects of BC nanomaterials and the mechanism by which they influence cell and animal toxicity by testing for oxidative stress by generation of intracellular

ROS (Donaldson *et al.* 2001 and 2003; Garza *et al.* 2008; Kagan *et al.* 2006; Koike and Kobayashi, 2006; Pulskamp *et al.* 2007; Shatkin, 2008; Shvedova *et al.* 2005; Soto *et al.* 2008a; Xia *et al.* 2006; Wilson *et al.* 2002). In contrast to several studies which indicate that BC nanomaterials can increase oxidative stress by the formation of ROS, our results indicate that BC *"pre-exposure"* to LPS inhibited ROS formation. Other studies also noticed the absence of intracellular ROS formation (Shvedova *et al.* 2005 and Crouzier *et al.* 2010) and suggest that it is due in part to the purity of the nanoparticles that were metal free structures (Cruzier *et al.* 2010). Furthermore, a recent study with engineered fullerenes (C_{60}) on human epidermal keratinocyte cells (HEK) reported an inhibition in total ROS levels (Gao *et al.* 2010). The authors showed that engineered fullerene (tris-C_{60}) induced cellular senescence, inhibited apoptosis and necrosis, cell proliferation, reproductive capacity, and cell cycle arrest. They suggest these effects could be the consequence of chemical modification of the engineered fullerene since the other two tested fullerenes did not exhibit cellular senescence. The discrepancy between ROS production studies could then be partly due to the functionalization and purity of the carbon nanoparticles being tested.

Co-stimulatory molecules CD40 and CD86 are involved in antigen presentation and play an important role in host defense when MΦ are activated (He *et al.* 2008 and Suttles and Stout, 2009). Activated MΦ cells treated with LPS up-regulate the expression of these cell surface receptors which allow for the interaction with ligands on T cells in order to present foreign antigen (He *et al.* 2008 and Becker *et al.* 2003). Few studies have compared the role and importance of adaptive immune responses to carbon nanoparticle exposure. These studies have reported that exposure to nanomaterial by itself can act as a stimulus resulting in increased expression of co-stimulatory molecules (CD80, CD86, and CD40) (Becker *et al.* 2003; de Haar *et al.* 2008; Koike *et al.* 2008; and Palomaki *et al.* 2010). In sharp contrast, our

findings indicate that chronic BC *"pre-exposure"* to LPS inhibited cell surface marker expression (CD40 and CD86). Recent *in vitro* studies on mouse MΦ and bone-marrow derived dendritic cells treated with carbon nanoparticles, exhibited an increase in the expression of co-stimulatory molecule CD86 (Palomaki *et al.* 2010). In addition, Koike *et al.* (2008) treated bone marrow derived dendritic cells with BC nanoparticles for 24 hrs and demonstrated an increase in the percentage of cells expressing CD86 and MHC class II and not CD80, suggesting that BC nanoparticles could potentially activate DCs. Furthermore, *in vitro* primary human alveolar MΦ studies observed that exposure to ultrafine particulate matter (nanomaterial) collected from the ambient air had no effect on co-stimulatory molecules (CD40, CD80 or CD86) (Becker *et al.* 2003). However, using human blood derived monocytes, the authors showed that ultrafine particulate matter (nanomaterial) increased the expression of these co-stimulatory molecules, suggesting that the phenomenon might be dependent on the cell type. Differently from our results, the latter studies were performed on DCs and not MΦ, nanomaterial exposure time was short-term (24 or 48 hrs), whereas our study exposed MΦ cells to carbon nanomaterials for 144 hrs followed by LPS treatment. These results suggest that chronic BC pre-exposure to LPS might affect MΦ cells antigen presenting capacity by inhibiting expression of these cell surface markers.

MΦ are phagocytes, responsible for recognizing, engulfing, and destroying potential pathogens through phagocytosis. In the present study, we demonstrate that BC pre-treatment suppressed phagocytosis (antigen acquisition) in murine MΦ cells. The results of this study are in accordance with others, for example, Lundborg *et al.* (1999) primed rat alveolar MΦ with IFNγ followed by exposure to carbon nanomaterial. IFNγ as well as LPS are a MΦ activator and can be used to mimic an infection (Hu *et al.* 2008). Their study demonstrated a marked

inhibition in phagocytosis in rat alveolar MΦ cells primed with IFNγ and exposed to ultrafine carbon particles (nanomaterial) for 6 hrs, however, no difference was observed in the cells that were treated with ultrafine carbon particles (nanomaterial) without IFNγ. The authors suggest that inhaled nanomaterial can gravely affect alveolar MΦ ability to properly remove ingested particles mainly after exposure to infection. Moreover, several *in vitro* murine MΦ studies (Donaldson *et al.* 2001, Donaldson *et al*. 2003, Renwick *et al.* 2001, Moller *et al.* 2002) and human alveolar MΦ studies have reported inhibition of phagocytosis after treatment with ultrafine carbon particles (nanomaterials) (Lundborg *et al.* 2001 and 2006). Donaldson *et al.* (2001 and 2003) reported that treatment of J774A.1 murine MΦ cell line with ultrafine BC (nanomaterials) inhibited phagocytosis. The authors suggest that after MΦ exposure to nanomaterials there is impairment in phagocytosis that leads to a release of inflammatory mediators and increase in oxidative stress caused by the large surface area of nanomaterials (Donaldson *et al.* 2001). Similarly, Renwick et al. (2001) indicated that treatment with ultrafine BC (nanomaterials) (.39 μg/mm^2) after 8 hr exposure impaired phagocytosis ability in J774.2 murine MΦ cells. The authors suggest that cell-cell contact is the mechanisms responsible for the inhibition in phagocytosis and further report that interaction between MΦ cells and ultrafine BC (nanomaterials) causes cells to transmit an inhibitory signal that prevents phagocytic activity of cells with which they make contact (Renwick *et al.* 2001). Additionally, they demonstrate that inhibition of phagocytosis is particle dependent because both ultrafine TiO_2 (nanomaterials) as well as ultrafine BC (nanomaterials) were tested, however ultrafine TiO_2 showed no difference. Moreover, *in vitro* studies on J774A.1 murine MΦ cells and primary alveolar MΦ from beagle dogs further demonstrate that exposure to ultrafine particles (nanomaterial) (100 μg/ml), specifically BC nanomaterial causes cytsokeletal dysfunction such as inhibition of phagocytosis abilities, however other nanomaterials such as TiO_2 does not

(Moller *et al.* 2002). Taken together, our findings suggest that uptake of BC nanoparticles by MΦ treated with a microbial product (LPS) interferes with their clearance functions and ultimate makes them incapable of adequately phagocytosing carbon nanoparticles that are coming into contact with the surface of the cells.

While phagocytosing engulfed material, MΦ also serve as APCs, processing and presenting peptide antigen of ingested material to T cells that have migrated into the site of infection, thus activating an adaptive immune response (Xaus *et al.* 2000). To our knowledge there are no published studies comparing the effect of carbon nanomaterial exposure on immune cells treated with LPS and their ability to acquire and process antigen. Therefore we investigated whether carbon pre-exposure to LPS would affect adaptive immune responses. Our study demonstrated that acquisition and processing of antigen is suppressed by *"pre-exposure"* to BC. Furthermore, we observed a decrease (although not statistically significant) in T cell response (IFN-γ production) when MΦ are pre-treated with BC. These results suggest that decreased induction of IFNγ by T cells when co-cultured with BC pre-treated MΦ is directly related to MΦs inability to effectively phagocytose and/or process protein antigens.

In contrast to BC nanoparticles, chronic *"exposure"* and *"pre-exposure"* to MWCNT caused no detectable changes in MΦ function in any of our functional assays. In sharp contrast, others have reported significant cytotoxicity, inflammatory effects, oxidative stress, ROS production (Bottini *et al.* 2006, Garza *et al.* 2008, Hirano *et al.* 2010, Sohaebuddin *et al.* 2010, Soto *et al.* 2008a), as well as suppression of systemic immunity in mice (Mitchell *et al.* 2009). It is likely that the variability in published results regarding MWCNT effects might be dependent on the cell type, concentration, purity, degree of agglomeration, and exposure time.

Taken together, our data demonstrates that chronic *"pre-exposure"* to carbon nanoparticles, black carbon specifically, impedes MΦ response to microbial stimuli, which could result in detrimental human health effects. If carbon nanoparticles are not cleared by macrophages and are retained within tissues these could potentially lead to ineffective pulmonary immunity. Understanding the key cellular responses to engineered carbon nanoparticles can establish a strong framework in the study of how the environment and anthropogenic activities influence and alter MΦ function, which may ultimately interfere with effective pulmonary immune responses capable of increasing the development and progression of human diseases like COPD.

In summary, we have demonstrated the BC by itself in a chronic *"exposure"* model is capable of inducing an inflammatory response in murine MΦ by up-regulating NO formation and TNFα production, however MWCNT does not. Moreover, our data also demonstrates that BC inhibits MΦ response to a microbial stimulus by down-regulating cell surface MΦ activation markers, phagocytosis, antigen processing, and ROS. Currently, the knowledge about the immune response of APCs to carbon nanoparticulate materials is poorly understood, additionally many biological aspects of specific constituents of nanoparticulate matter still remains unclear and requires further evaluation. However our study indicates that there is a dose dependent chronic exposure of RAW264.7 cells to BC inhibits MΦ response to a microbial stimulus, whereas MWCNT causes little or no effect on murine MΦ. Additionally, no changes in antigen specific presentation were observed with BC or MWCNT with or without microbial stimuli. This study suggests that some but not all anthropogenic carbon nanoparticles could alter the effectiveness of the immune systems innate response to future infections. Moreover, the data suggests this might be dependent on carbon nanoparticles

structure and concentration. This underscores the potential detriment that air pollutants can impose on immunity.

CHAPTER 4

EXTENDED DISCUSSION

4.1 DISCUSSION

In this dissertation we studied the chronic effects of carbon nanoparticle exposure on murine MΦ function and clearance mechanisms. Our hypothesis stated that MΦ exposed to carbon nanoparticles would not suffer immediate compromised function; however, long-term exposure to the nanomaterial would alter MΦ ability to respond appropriately to microbial stimuli resulting in compromised immunity. We demonstrated that chronic "*pre-exposure*" to BC nanoparticles specifically inhibited MΦ response to microbial stimuli (lipopolysaccharide - LPS). As a consequence, cell surface MΦ activation markers, ROS production, phagocytosis, antigen processing and presentation, all of which are needed for effective microbial clearance, were inhibited. Taken together, these data suggest that exposure to carbon nanoparticles could potentially lead to ineffective pulmonary immunity.

The first objective was to determine the cytotoxic effects of micron-sized and nano-sized metals. We determined that nanoparticles are markedly more cytotoxic to cells than micron-sized particles of the same composition. This is likely due to their small particle size, which makes them more potent than larger particles because of their higher surface area and reactivity (Brauner *et al.* 2007). Additionally, many nanoparticles can agglomerate and increase their net size, which can potentially cause adverse health effects and affect the effective dose on the cellular system (Oberdorster *et al.* 2005).

To further evaluate the cytotoxicity of nanoparticles, we turned our attention on two distinct nanoparticles, BC and MWCNT. Both are found in the environment as a result of

combustion processes and have been linked to adverse health effects. Our laboratory had previously shown that there is a decrease in cell viability in RAW264.7 and A549 cells treated for 48 hrs with BC or MWCNT (Murr *et al.* 2006, Soto *et al*. 2005). Short-term cytotoxic assessments with RAW264.7 cells demonstrated that BC alters MΦ cell viability with an LC50 of 30 μg/mL and indicated that BC is more toxic than MWCNT. For that reason we established two chronic six-day exposure models, "*pre-exposure*" and "*exposure*". The *"pre-exposure"* model was formulated to investigate the potential ability of carbon nanoparticles to alter MΦ responses to future microbial insults; the *"exposure"* model was designed to test the ability of the carbon nanoparticles to directly activate the MΦ. Cell cytotoxicity assessments demonstrated that both chronic *"exposure"* and *"pre-exposure"* to BC decreases cell viability, in contrast, MWCNT showed no cytotoxic response, consistent with the treatment controls. The differences in cytotoxicty between the two test particles might be dependent on carbon nanoparticle structure and aggregation properties, since BC nanoparticles aggregate and adhere to the surface of MΦ cells as demonstrated by light microscopy **(Figure 15)**. Our cytotoxic assessments demonstrated that the pulmonary-derived cells exhibit higher toxicity to nanomaterials than to the larger particles and this activity might be a result of differences in structure, composition and aggregation of particles.

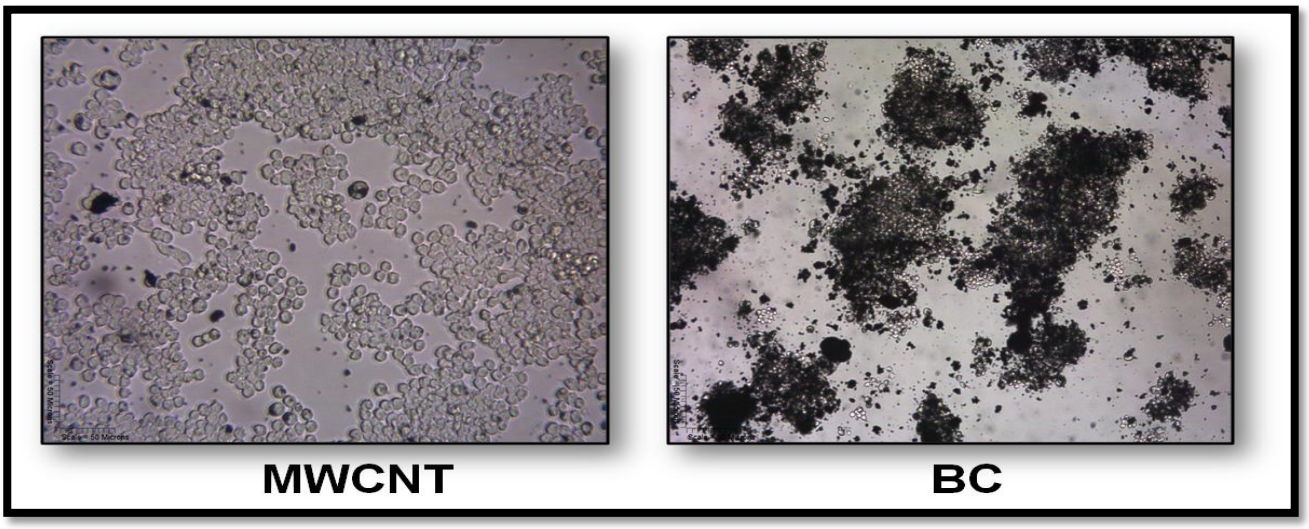

Figure 15. Chronic exposure to both MWCNT & BC causes blasting and aggregation of MΦs. MΦ were treated with MWCNT or BC, vehicle control (DMSO), and negative control (untreated) and were examined by light microscopy 7 days post treatment. (Magnification: 40x)

To further investigate the effects of carbon nanoparticles on murine MΦs we examined cellular responses of MΦ associated with the toxicity to the engineered carbon nanoparticles (MWCNT and BC). This was assessed following acute (48hr) and chronic (144hr) *"pre-exposure"* and *"exposure"* treatments. As previously stated, our *"pre-exposure"* model was formulated to investigate the potential ability of carbon nanoparticles to inhibit MΦ responses to microbial stimuli; our *"exposure"* model was designed to test the ability of the carbon nanoparticles to directly activate the MΦs. The results demonstrated that acute (48 hr) *"pre-exposure"* and "exposure" to BC and MWCNT does not alter RAW264.7 MΦ activity. Thus, we focused our studies on chronic (144hr) *"pre-exposure"* and *"exposure"* treatments. Results demonstrated that *in vitro* chronic "exposure" to BC nanoparticles alters MΦ function by promoting inflammation and compromising responses to microbial stimulation. The data also

indicated that BC by itself is capable of inducing an inflammatory response from murine MΦ by up-regulating TNFα production and NO formation.

We then compared the ability of MΦ chronically pre-treated with carbon nanomaterials to respond to bacterial stimuli. In contrast to several studies which indicate that BC nanomaterials can increase oxidative stress by the formation of ROS, our results indicate that BC *"pre-exposure"* inhibited ROS formation upon stimulation with LPS. Other studies also noticed the absence of intracellular ROS formation (Shvedova *et al.* 2005 and Crouzier *et al.* 2010) and suggest that it is due in part to the purity and functionalization of the nanoparticles. Our results also demonstrated that chronic BC *"pre-exposure"* inhibited cell surface marker expression (CD40 and CD86). Activated MΦ cells treated with LPS typically up-regulate the expression of these cell surface receptors that allow for enhanced interaction with T cells (He *et al.* 2008 and Becker *et al.* 2003). These results suggest that chronic BC *"pre-exposure"* prior to a microbial infection might negatively affect MΦ cells antigen presenting capacity by inhibiting expression of these cell surface markers. Indeed, studies have demonstrated that the absence or decreased expression of these key co-stimulatory molecules reduces the effectiveness of T cell-mediated immunity (Clarke, 2000).

To determine the effect of carbon nanoparticles on MΦ antigen presenting capacity, we examined phagocytosis by the internalization of fluorescein-labeled *E. coli* bio-particles. In addition, we investigated antigen acquisition and processing by the addition of a self-quenched conjugate of ovalbumin (DQ-OVA). We demonstrated that BC *"pre-exposure"* suppressed phagocytosis, antigen–acquisition and processing in activated murine MΦ cells. Furthermore, we assessed the ability of carbon nanoparticle treated RAW267.4 cells to process and present ovalbumin protein to ovalbumin protein-specific $CD4^+$ T cells. The antigen-specific presentation

results indicate a decrease (although not statistically significant) in T cell response (IFN-γ production) when MΦ are pre-treated with BC, indicating a suppressed ability to effectively acquire and/or process ovalbumin protein. Taken together, our findings demonstrate that uptake of BC nanoparticles by MΦ stimulated with a microbial product TLR 4 ligand (LPS) ultimately renders the macrophages incapable of adequately ingesting and degrading extracellular material thus, interfering with their microbial clearance functions and ability to properly present antigen to T cells. However, chronic *"exposure"* and *"pre-exposure"* to MWCNT caused no detectable changes in MΦ function in any of our functional assays. Similar findings were made when using TLR 2 ligand (zymosan) as the microbial stimulus. It is likely that the variability in results between BC and MWCNT might be dependent on carbon nanoparticle physical, chemical, and aggregation properties.

In addition, we investigated MΦ intracellular localization of ingested carbon nanoparticles after chronic treatment. Transmission electron microscopy (TEM) allowed us to identify carbon nanoparticles internalization location within MΦ. Ultrathin sections of carbon nanoparticle treated macrophage cells were cut, stained, and analyzed by TEM. Microscopic examinations revealed aggregated BC nanoparticles located and stored inside large cytoplasmic vacuoles **(Figure A1)**. We did not observe carbon nanoparticles in any organelle including the nucleus, mitochondria, or golgi apparatus. As such, our data is in contrast with observations by previous studies, who found ultrafine particles inside the mitochondria (Li *et al.* 2003) or inside the nucleus (Geiser *et al.* 2005). In addition, TEM analysis of MΦ cells treated with MWCNT did not indicate intracellular uptake or penetration of these nanoparticles **(Figure A2).**

Lastly, as we sought to determine a potential mechanism and examined the effect of carbon nanoparticles treated MΦ on mitochondrial membrane permeability, viability (Live/Dead and trypan blue exclusion), and stress responses (HSP70, iNOS, and Cox-2). Mitochondria membrane potential by treated RAW264.7 cells was determined by flow cytometry using the MitoProbe JC-1 assay kit (Molecular Probes, Eugene, OR). JC-1 dye measures accumulation in mitochondria, indicated by red fluorescence. Whereas, mitochondrial depolarization is indicated by a shift from red to green fluorescence intensity because JC-1 dye cannot aggregate in the mitochondria and stays in the cytoplasm where it fluoresces green (Molecular Probes, Eugene, OR). Results demonstrated that BC "pre-exposure" does not alter mitochondria membrane potential **(Figure A5)**. BC treated cells had polarized mitochondria as revealed by red fluorescence, whereas there was no indication of depolarized mitochondria when compared to the untreated and vehicle controls. Next we performed two additional viability assays and compared the results to the initial CellTiter Glo viability assay shown in Figure 7, which determines viability based on quantification of ATP. After a 7 day chronic *"exposure"* and *"pre-exposure"* to MWCNT and BC, we assessed cellular viability by LIVE/DEAD assay and trypan blue exclusion assay. The LIVE/DEAD assay is a two color fluorescent assay that identifies live versus dead cells on the basis of membrane integrity and esterase activity. As shown in **Figure A6**, results from the LIVE/DEAD assay demonstrate that both *"exposure"* and *"pre-exposure"* to BC is cytotoxic to RAW264.7 cells. However, no statistical difference was observed for any other measurement. These results are consistent with the trypan blue exclusion assay data **(Figure A7).** Interestingly, all viability assays demonstrate that both *"exposure"* and *"pre-exposure"* to BC is cytotoxic to MΦs, however, the CellTiter Glo assay shows a much more significant decrease in viability, while the other two assays demonstrate a decrease in viability but not as cytotoxic as previously indicated by the

CellTiter Glo. The LIVE/DEAD and trypan blue exclusion assay are in accordance with our microscopic observations and cell count numbers of all treatments the day of their analysis where there were a significant number of live cells in our BC treated samples. Next, we examined stress responses (HSP70, iNOS, and Cox-2) by western blot analysis. Results revealed no up-regulation of HSP70 or presence of iNOS and Cox-2. In overall our results demonstrate that BC treated MΦs have polarized mitochondria, meaning they are healthy, LIVE/DEAD assay revealed that BC is cytotoxic to MΦs but there is still a high percentage of live cells, and there was no up-regulation of stress responses. Taken together, these results might suggest that *"pre-exposure"* to BC is causing MΦs to undergo cellular senescence. This process limits the growth of cells but not necessarily the viability of the cell, meaning the cells become unresponsive. In addition, cellular senescence inhibits proliferation and apoptosis, activates cell cycle checkpoints, and prevents cell division by inducing cell cycle arrest (Gao *et al*. 2010). We need to perform further experiments to determine if cellular senescence is responsible for the inhibition of MΦs responses associated with chronic BC "pre-exposure" observed in this study.

In sum, we have demonstrated that exposure to anthropogenic nano-sized particles proves to be more cytotoxic than larger particles. Our study indicates that chronic exposure of RAW264.7 cells to BC inhibits MΦ response to a microbial stimulus, whereas MWCNT causes little or no effect on murine MΦ. Additionally, long-term accumulation chronic exposure, rather than short-term acute exposure to carbon nanoparticles interactions have deleterious effects on MΦ function. Exposure to BC nanoparticles by itself in a chronic *"exposure"* model is capable of inducing an inflammatory response in murine MΦ by up-regulating NO formation and TNFα production, however MWCNT does not. Inflammation brought upon by long term

chronic exposure to anthropogenic carbon nanoparticles requires careful observation since excessive inflammatory responses could further aggravate pre-existing diseases such as respiratory or cardiovascular diseases. Moreover, our data also demonstrates that BC inhibits MΦ response to a microbial stimulus by down-regulating cell surface MΦ activation markers, ROS, phagocytosis, antigen processing and presentation to T cells. Macrophage's main function is to engulf and process materials and thus create a link between innate and adaptive immune system. If carbon nanoparticles are not cleared by macrophages and are retained within tissues these could potentially lead to lung tissue injury and ineffective pulmonary immunity. Thus, understanding the key cellular responses to engineered carbon nanoparticles can establish a strong framework in the study of how the environment and anthropogenic activities influence and alter MΦ function, which may ultimately interfere with effective pulmonary immune responses capable of increasing the development and progression of human diseases like COPD.

4.2 FUTURE DIRECTIONS

Currently, the knowledge about the immune response of APCs to carbon nanoparticulate materials is poorly understood, additionally many biological aspects of specific constituents of nanoparticulate matter still remains unclear and requires further evaluation. This study suggests that some but not all anthropogenic carbon nanoparticles could alter the effectiveness of the immune system's innate response to future infections. Moreover, the data suggests this might be dependent on carbon nanoparticles structure and concentration. Although we do not know the exact mechanism of action by which carbon nanoparticles are inhibiting macrophage function, we can conclude that long-term chronic BC *"pre-exposure"*

impairs macrophage phagocytic and acquisition and processing abilities *in vitro*. This underscores the potential detriment that air pollutants can impose on immunity. Further studies, should include assessment of cellular senescence by examining the expression of **β**-galactosidase, activation of cell cycle checkpoints, and analysis of cell cycle phase distribution. In addition, future experiments should examine cellular immune responses to *Mycobacterium avium* (*M. avium)* infection. This model would be ideal since *M. avium* is an intracellular pathogen known to primarily infect and replicate in MΦ (Inderlied *et al.* 1993). Additionally, this bacterial infection is usually localized in the lungs and the primary route of infection is the respiratory tract (Horsburgh, 1991). Therefore, *M. avium* would provide an ideal basis to assess the effect of carbon nanoparticle exposure on murine pulmonary MΦ function and the ability of infected cells to clear a bacterial infection. Further understanding of the mechanisms involved between MΦs and immune responses to carbon nanoparticles would ideally be addressed in an *in vivo* mouse model study, where intranasal administration of the nanomaterials would precede an *M. avium* infection thus providing the opportunity to examine the ability of these mice to clear a lung infection in the presence of the carbon nanoparticles. Such studies would be quite beneficial to identify other factors that would increase understanding of nanoparticle effect in biological systems.

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GLOSSARY

OAPC – Antigen presenting cells

MΦ – Macrophages

DC - Dendritic cells

ROS – Reactive oxygen species

IFNγ – Interferon-γ

MHCII – major histocompatibility complex class 2 antigen

TNFα– tumor necrosis alpha

IL - interlukin molecules (e.g. IL-12, IL-4)

Th1 – Type 1 Helper T cells

ATP – Adenosine-5'-triphosphate

BC – Vulcan XC-72 black carbon

MWCNT – Multi-walled carbon nanotubes

CNT – Carbon nanotubes

PM – Particulate matter

TEM – Transmission electron microscopy

SAED – Selected area electron diffraction

TLR – Toll-like receptors ligands

NO – nitric oxide

CD – Cluster of differentiation (e.g. CD86, CD40)

LPS – Lipopolysaccharide

M. avium – mycobacterium avium

COPD – chronic obstructive pulmonary disease

RAW264.7 cells – murine macrophage cell line

A549 cells - Human epithelial lung cell line

OVA-DQ - Self- quenched conjugate of ovalbumin

LC-50 – lethal concentration

DMSO – dimethyl sulfoxide

RLU – relative light units

CO^2 – carbon dioxide

MEM – minimum essential medium

DMEM – Dulbecco's Modified Eagle's medium

FBS – fetal bovine serum

HBSS – Hanks buffered salt solution

PBS – phosphate buffered saline

MTS - 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt

SDS-HCL – sodium dodecyl sulfate in Hydrochloric Acids solution

MPER – mammalian protein extraction reagent

EDTA – ethylenediaminetetraacetic acid

Ab – antibody

SDS – sodium dodecyl sulfate

BSA – bovine serum albumin

[^{3}H] - Thymidine

ELISA – enzyme-linked immunosorbent assay

HSP – Heat shock proteins

APPENDIX

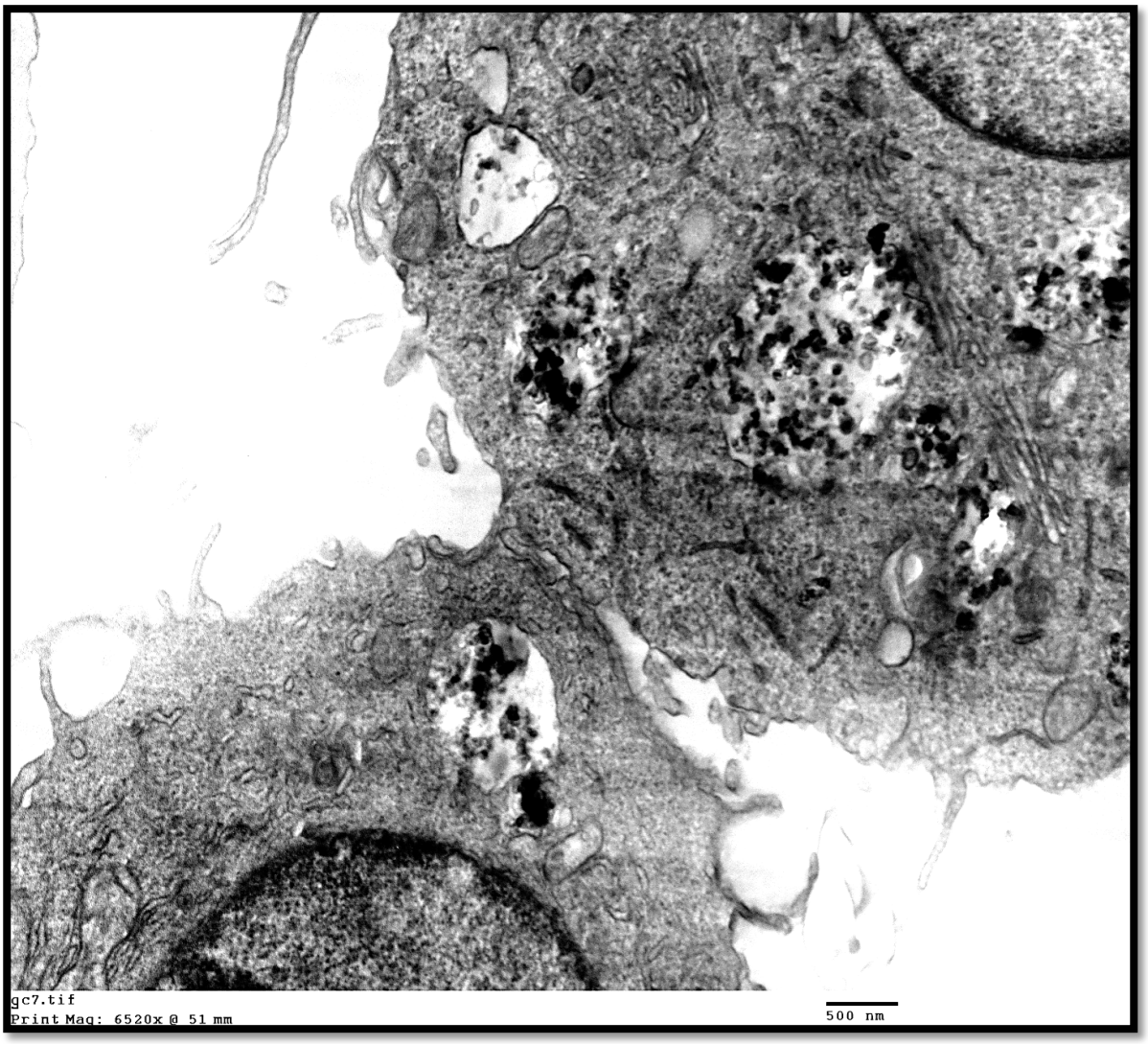

Figure A1. Transmission electron microscopy image of internalized BC aggregated nanoparticles inside cytoplasmic vacuoles. MΦ were treated with MWCNT or BC and were examined by TEM 7 days post treatment. Ultra-thin sections were cut, placed on copper grids, stained with uranyl acetate and lead citrate, and examined by TEM (80-kV)(Magnification: 20000x)

Figure A2. Transmission electron microscopy analysis of MΦ cells treated with MWCNT did not indicate intracellular uptake of these nanoparticles. MΦ were treated with MWCNT or BC and were examined by TEM 7 days post treatment. Ultra-thin sections were cut, placed on copper grids, stained with uranyl acetate and lead citrate, and examined by TEM (80-kV)(Magnification: 20000x)

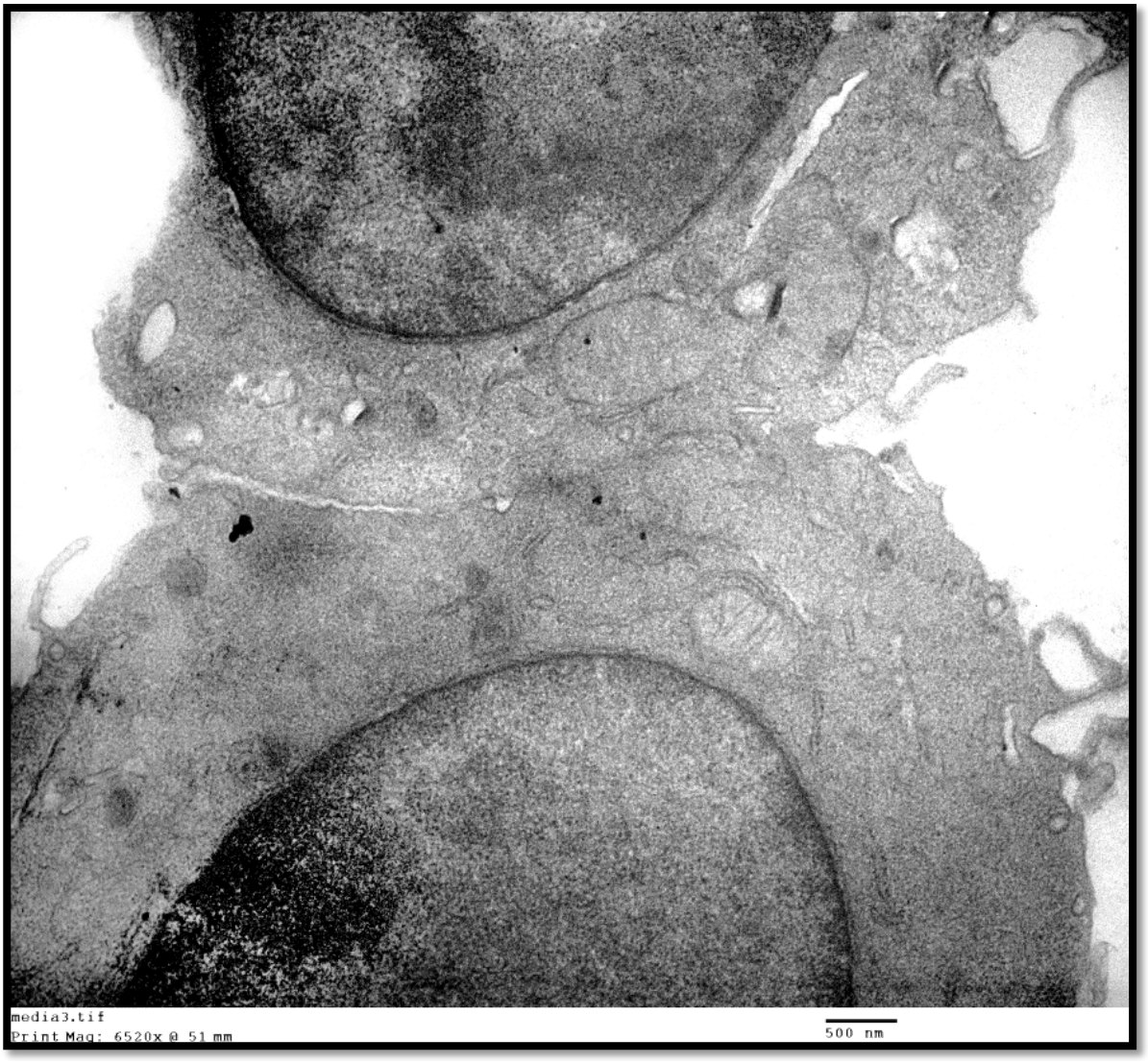

Figure A3. Transmission electron microscopy image of negative control (untreated) MΦs. MΦ were treated with MWCNT or BC, vehicle control (DMSO), and negative control (untreated) and were examined by TEM 7 days post treatment. Ultra-thin sections were cut, placed on copper grids, stained with uranyl acetate and lead citrate, and examined by TEM (80-kV)(Magnification: 20000x)

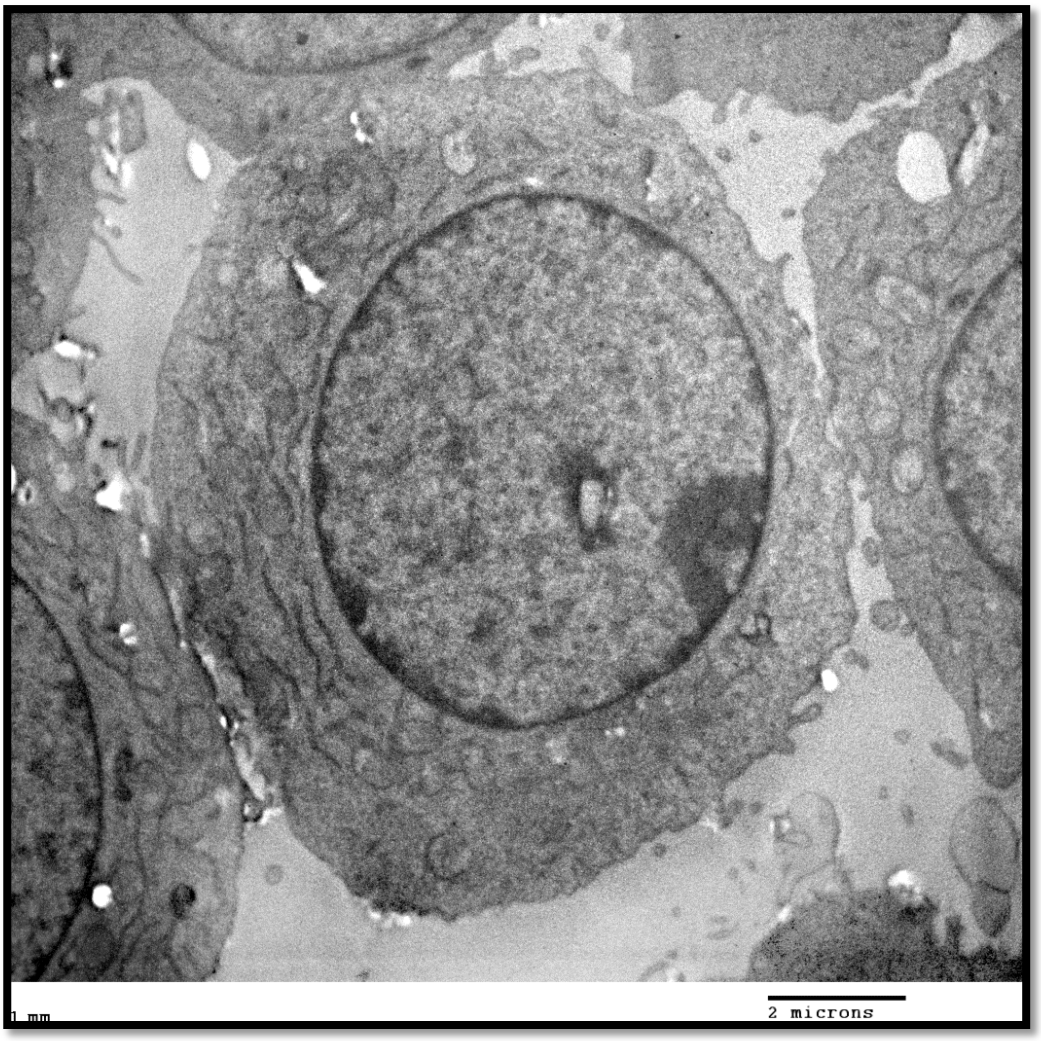

Figure A4. Transmission electron microscopy image of vehicle control (DMSO) treated MΦs. MΦ were treated with MWCNT or BC, vehicle control (DMSO), and negative control (untreated) and were examined by TEM 7 days post treatment. Ultra-thin sections were cut, placed on copper grids, stained with uranyl acetate and lead citrate, and examined by TEM (80-kV)(Magnification: 8000x)

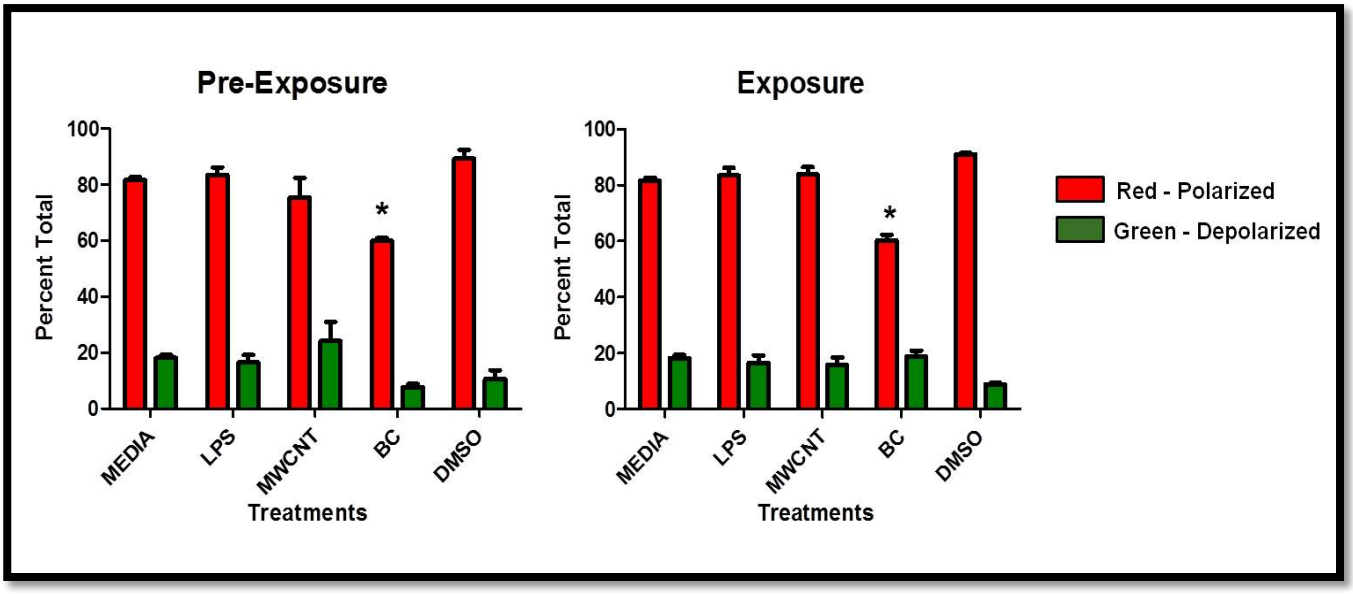

Figure A5. Chronic BC *"pre-exposure"* or *"exposure"* does not alter MΦ mitochondrial membrane potential. MΦ were treated with MWCNT or BC and "pre-exposure" (A) samples were activated with 100ng/mL of LPS. MΦs were tested for mitochondrial permeability using the MitoProbe™ JC-1 Assay Kit 7 days post-treatment and analyzed by flow-cytometry. Untreated-cells (media) and vehicle-treated cells (DMSO) were used as controls. This data is presented as the mean ± SEM of duplicate wells and is one of three representative experiments.

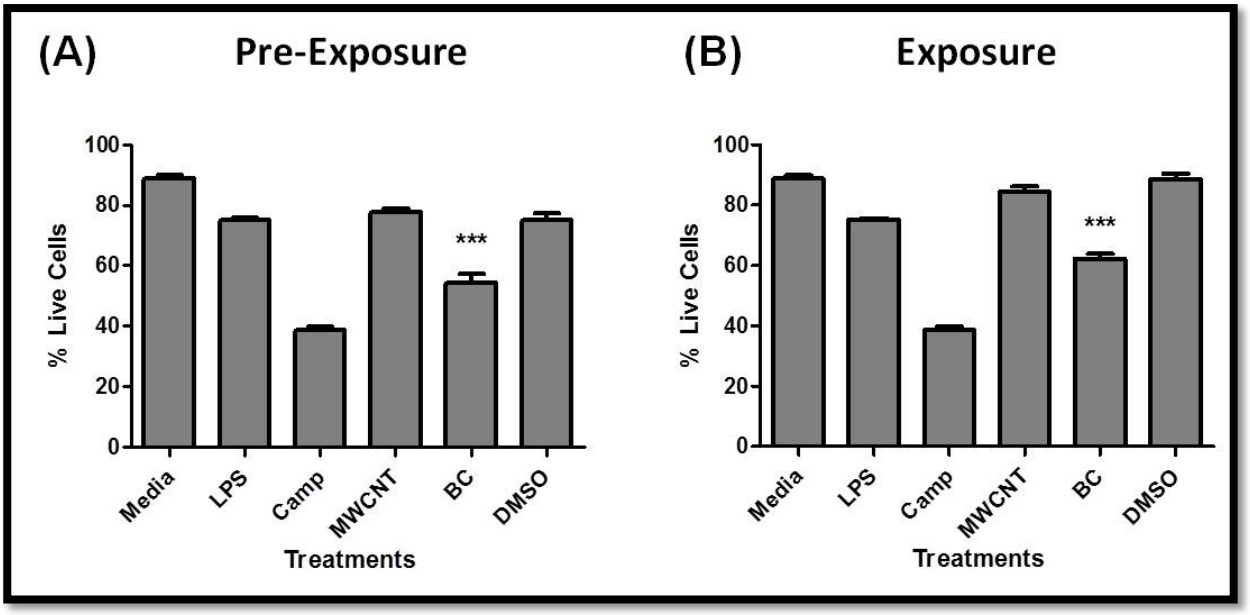

Figure A6. Chronic *"pre-exposure"* and *"exposure*" to BC is cytotoxic to RAW264.7 cells. MΦ were treated with MWCNT or BC and "pre-exposure" (A) samples were activated with 100ng/mL of LPS. Treatments were tested for cytotoxicity by LIVE/DEAD assay 7 days post-treatment. Untreated-cells (media), vehicle-treated cells (DMSO), and camptothecin-treated cells (Camp) were used as controls for the absence or presence of cell death. This data is presented as the mean ± SEM of quadruplicate wells and is one of three representative experiments.

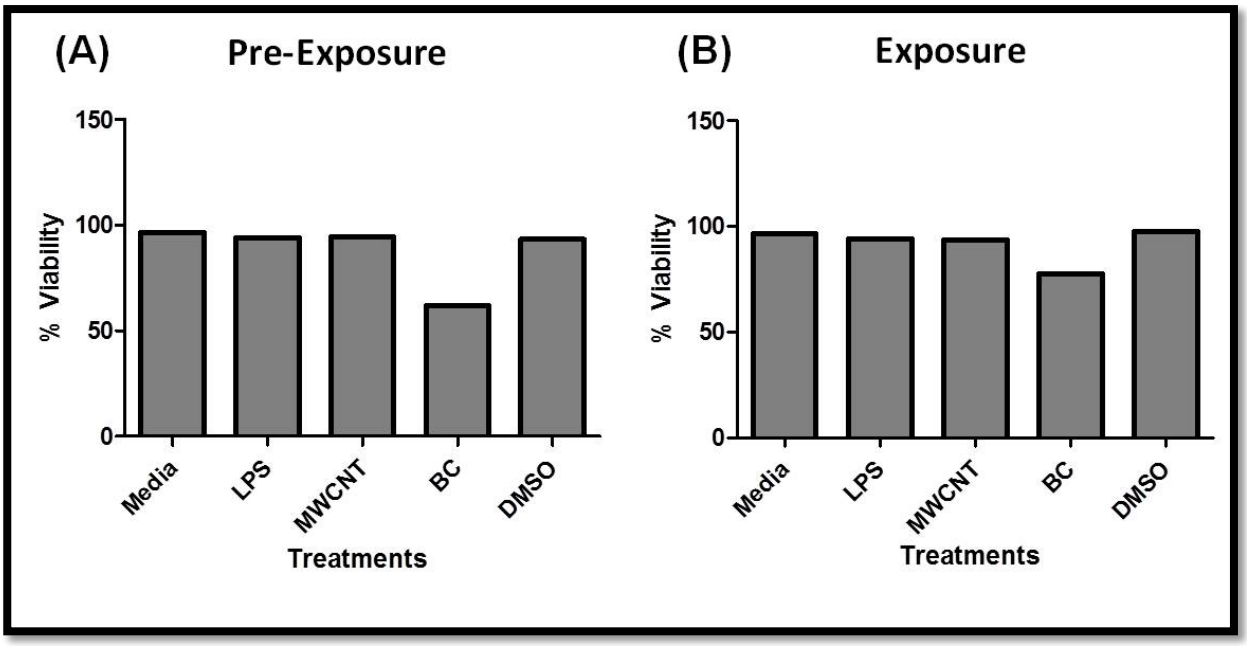

Figure A7. Chronic "*pre-exposure"* and "*exposure"* to BC is cytotoxic to RAW264.7 cells. MΦ were treated with MWCNT or BC and "Pre-Exposure" (A) samples were activated with 100ng/mL of LPS. Treatments were tested for cytotoxicity by trypan blue exclusion assay 7 days post-treatment. Untreated-cells (media) and vehicle-treated cells (DMSO) were used as controls for the absence of cell death. This data is presented as the mean ± SEM of duplicate wells and is one of three representative experiments.

CURRICULUM VITAE

Raquel M. Suro Maldonado earned her Bachelor of Arts degree in Geography from the University of Puerto Rico, Río Piedras campus in 2003. In the fall of 2003 she entered the Graduate Program at the Universidad Metropolitana (UMET) in San Juan, Puerto Rico. In 2004 she received the BRIDGES to the Doctorate Fellowship from the National Institute of General Medical Sciences (NIGMS – NIH). In the spring of 2006 she earned her Master's of Science degree in Environmental Science and Management of Natural Resources.

In the spring of 2007 she entered the Ph.D. Program in Pathobiology at the University of Texas at El Paso, where she received the Research Initiative for Scientific Enhancement (RISE) fellowship from NIGMS – NIH and worked as a research assistant under Dr. Kristine M. Garza. Her research focused on the effects of engineered carbon nanoparticles exposure on macrophage function.

As a graduate student at UTEP she received several scholarships, fellowships, and awards such as: RISE, HHMI, FASEB-MARC, BRIDGES-NIGMS, UTEP Graduate School Research Support Award, and the Dodson Dissertation Fellowship from UTEP Graduate School. While in the Doctoral program she worked as a Research Assistant for Dr. Kristine M. Garza and as an Adjunct Faculty at the El Paso Community College. She presented posters at the Society for the Advancement of Chicanos and Native Americans (SACNAS) annual meeting in 2008, 2009, and 2010, at the regional American Society for Microbiology meetings in 2009 and 2010, American Society for Cell Biology (ASCB) national conference in 2009, and the American Association of Immunologists (AAI) annual meeting in 2010. Additionally, she collaborated with Dr Lawrence E. Murr and the experiments resulted in a co-authored publication in 2010, *"Characterization and Cytotoxic Assessment of Ballistic Aerosol*

Particulates for Tungsten Alloy Penetrators into Steel Target Plates" in the International Journal of Environmental Research and Public Health and in 2011 *"Comparative Microstructures and Cytotoxicity Assays for Ballistics Aerosols Composed of Micronmetals and Nanometals: Respiratory Health Implications"* in the International Journal of Nanomedicine. In 2011 she also collaborated with Dr. Marc Cox and the research resulted in a co-authored publication *"Targeting the Regulation of Androgen Receptor Signaling by the Heat Shock Protein 90 Cochaperone FKBP52 in Prostate Cancer Cells"* in the Proceedings of the National Academy of Sciences (PNAS).

Raquel M. Suro Maldonado's dissertation entitled, *"Assessment of Carbon Nanoparticle Exposure on Murine Macrophage Function"*, was supervised by Dr. Kristine M. Garza.

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