

LYMPHOCYTE SUBPOPULATIONS AND OXIDATIVE STRESS FOLLOWING
SUB-ACUTE EXPOSURE TO NATURAL DUST COLLECTED FROM
THE NELLIS DUNES RECREATIONAL AREA

by

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of

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DEDICATION

This thesis is dedicated to my wife, Sarah Merlyn Leetham. Without your patience, love, encouragement and unwavering support this thesis would have never been possible.

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ABSTRACT

Exposure to particulate matter containing heavy metals has been linked to adverse health effects when exposure occurs in industrial settings; however, little data exist on effects associated with natural exposure settings. In this study, markers of oxidative stress and lymphocyte subpopulations in mice were observed following sub-acute exposure to metals-containing dust collected from a natural setting used heavily for off-road vehicle (ORV) recreation. Adult female B6C3F1 mice were exposed to concentrations of dust collected from seven types of surfaces at the Nellis Dunes Recreation Area. Dust representing each of the seven map units was prepared with a median diameter of $\leq 4.5\mu\text{m}$ and suspended in PBS immediately prior to oropharyngeal aspiration at concentrations from 0.01 - 100 mg of dust/kg body weight. Four exposures were given a week apart over 28-days to mimic a month of weekend exposures. Thymi, spleens and blood for evaluation of oxidative stress markers and lymphocyte sub-populations were collected 24 hours after the final exposure. Blood markers of oxidative stress included levels of free radicals, superoxide dismutase, total antioxidant capacity, and total glutathione. CD4, CD8, FoxP3, CD25, IL-17, and B220 cell surface markers were used for T and B cell identification using flow cytometry. Overall, no single surface type was able to consistently induce markers of oxidative stress at a particular dose or in a dose-responsive manner. The two highest concentrations of dust from one surface type increased two markers of oxidative stress, but results of other surface types were inconsistent. No statistically significant changes were observed in the splenic B220+ cells following NDRA dust exposure. Three CBN units (1, 2, and 6) showed decreases in splenic CD4+/CD25+/FoxP3- cells. These observations were relatively consistent with TiO₂, where a significant change at the highest exposure level was observed in only one measure of oxidative stress. Additionally, the TiO₂ dosing groups showed no significant changes in lymphocyte subpopulations. These results indicate that exposure to these natural, mineral dusts, under the exposure scenario of our study, while are unlikely to considerably increase the risk of oxidative damage systemically, may induce a reduction in some cell populations in exposed individuals.

CHAPTER 1

INTRODUCTION AND BACKGROUND

Introduction

Recent research at Nellis Dunes Recreational Area (NDRA) in southern Nevada, USA has indicated that exposure to dust generated by both off-road vehicular (ORV) activities and natural winds may pose a public health hazard (Goossens and Buck, 2011a). Because there are currently no U.S. Environmental Protection Agency (USEPA) regulations in the United States for heavy metal inhalation exposures in recreational settings, there was a need for a site-specific human health risk assessment to estimate the potential risk of recreation at NDRA (Soukup et al., 2012). To investigate further, a human health exposure risk assessment was done to identify human health risks to dust obtained from different surface types at NDRA. Toxicology and human exposure data were collected for the study as a whole, with this thesis concentrating on the results from oxidative stress markers and lymphocyte sub-population analysis by flow cytometry.

Nellis Dunes Recreational Area (NDRA) is located approximately 8 km from the margin of the conurbation Las Vegas - North Las Vegas – Henderson and is the sole location in Clark County for off-road recreation. For more than 40 years, NDRA has been heavily used for ORV recreation. Off-road vehicle driving is one of the most prevalent and fastest growing recreational activities on public lands worldwide (Outdoor World Directory, 2010). The number of off-road drivers in southern Nevada has quadrupled in the last few years (Spivey, 2008). Research performed within NDRA has shown that

large amounts of dust are emitted throughout the year from this site and that some of this dust blows into the surrounding cities (Goossens and Buck, 2011b). This particulate matter (PM) carries naturally occurring heavy metals with known toxicity to humans.

This particulate matter, which is not only blown into Las Vegas, but also readily inhaled by those using ORVs, contains more than 20 trace metals with potential to negatively impact human health. NDRA was split into seven map units, referred to by their combination (CBN) number. Inhalation of particulate matter containing heavy metals has been linked to cancer, hypertension, cardiovascular disease, kidney damage, diminished intellectual capacity in children, and skeletal damage (Aschner et al., 2005; ATSDR, 2004, 2007, 2012; Klaassen et al., 2013; Costa et al., 2006; Jomova et al., 2010;). Arsenic (As) in particulate matter is of greatest concern due to the deleterious effects of the toxic metal of which will be discussed under background-metals. Extremely high concentrations (>300 ppm) of arsenic have been found in several soil and airborne dust samples from NDRA (Soukup et al., 2011). Other metals found in NDRA include aluminum, cesium, chromium, cobalt, copper, iron, lead, magnesium, manganese, strontium, uranium, vanadium, and zinc. Many of these metals are known to deleteriously affect human health, and characterization of the potential health risks as a result of recreation at NDRA is necessary.

Identification of splenic B and T lymphocytes using cell surface markers is considered a tier II procedure for detecting immune alterations following drug or chemical exposures in rodents, and is considered by the USEPA to be part of a testing battery for full immunotoxicological evaluation of potential risks associated with a toxic

substance (Luster et al., 1992; EPA, 1996). Moreover, the analysis of lymphocyte subpopulations has been shown to have a high association with predicting immunotoxicity with a concordance of 83% (Luster et al., 1992). In addition to the identification of standard splenic B and T lymphocyte, we also identified thymic T lymphocytes and splenic Regulatory T cells (Tregs). B-lymphocytes were identified using B220 as a surface marker, T lymphocytes were identified using CD4 and CD8 as surface markers, and CD4, FoxP3, CD25, and IL-17 surface markers were used to identify Tregs. Arsenic exposure in mice inhibits T cell proliferation and macrophage activity, decreases CD4 splenic cells number (Soto-Pena et al., 2006), suppresses proliferative lymphocytic response (Biswas et al., 2008), decreases IL-2 production (Biswas et al., 2008) and decreases Treg numbers specifically via oxidative stress (Thomas-Schoemann et al., 2012). In addition, chromium and cobalt are associated with CD8 lymphopenia (Hart et al., 2009).

Exposure to heavy metals at certain concentrations is known to alter the “redox balance” in an organism, or the maintenance of a normal ratio of reactive species that can induce cell damage and death and the antioxidant enzymes that break them down. Although nearly all cellular processes create reactive species, their ability to induce damage is typically reduced or eliminated by antioxidant enzymes. Damage, the result of oxidative stress, may occur when reactive species overwhelm the antioxidant enzymes or when the antioxidant enzymes themselves become damaged or depleted. One outcome of oxidative stress is inflammation, a response of the immune system to an injury. This thesis therefore presents results of a study to determine disruptions to the redox balance

via evaluation of redox markers in the plasma and red blood cells. Redox balance was evaluated by measuring the amount of reactive oxygen and nitrogen species, total glutathione, superoxide dismutase activity, and total antioxidant capacity in the blood of mice exposed to dust samples.

Background

Particulate Matter

Exposure to particulate matter of a size less than 10 μm (PM_{10}) above 0.15 mg/m^3 in 24 hours is associated with increased daily deaths and mortality from cardiovascular and respiratory illnesses (Samet et al., 2000, Katsouyanni et al., 2001), decreased pulmonary function in adults (Boezen et al., 1998) and bronchial hyper-responsiveness in children with an increased risk of lower respiratory symptoms (Boezen et al., 1998). More recently, particulate matter exposure has been linked with microglial activation, neurotoxicity, and triggering of the production of free radicals associated with oxidative stress (MohanKumar et al., 2008; Risom et al., 2005; Xia et al., 2006). With the amount of pollution seen in large cities today, particulate matter research is a popular topic. The focus of many of these studies has been on urban sources of particulate matter, particularly pollution. However, few studies have focused on particulate matter and dust exposure from non-urban sources and very little is known about the potential exposure levels and associated health effects of being exposed to natural dust while riding an ORV.

The dust from Nellis is a complex mixture of particulate matter and numerous metals found in their mineral metal form. These heavy metals are associated with human

toxicity. The behavior of particulate matter in the respiratory system is heavily dependent on the particle size. Particles of a $PM_{2.5}$ and below will deposit deep into the alveoli and bronchioles, whereas larger particles, PM_{10} , will distribute to the primary bronchi (Kelly et al., 2012). Much larger particles, $100\mu m$, will deposit in the nasopharynx and have a greater chance of entering the digestive tract (Kelly et al., 2012; Kish et al., 2013; Möller et al., 2004). The dust collected and used for exposure studies had an average particle size of $4.17\mu m$. Exposure to particulate matter of this small of a size is associated with increases in respiratory disease incidence such as asthma and acute bronchitis as well as increased mortality; also seen is an increase in cardiovascular disease and incidence of autoimmune diseases such as type 1 diabetes, juvenile onset arthritis, and rheumatoid arthritis (Samet et al., 2000; Katsouyanni et al., 2001; Farhat et al., 2011). Particulate matter exposure can create an immune response causing inflammation in the lung that has the potential to cause a systemic effect (Farhat et al., 2011).

Metals

For this study we collected topsoil from 17 different types of surfaces ("surface units") in the NDRA. These included vegetated and unvegetated sand dunes, areas with a thin (1-5 cm) layer of windblown sand, badland areas with aggregated and non-aggregated silts or mixtures of sand and silt, well-developed and degraded desert pavements, rocky, sandy and silty drainages, and bedrock. Special attention was paid to ensure that all surface types that occur within the NDRA were sampled, and that coarse-grained, medium-grained and fine-grained substrata were evaluated. This is important because grain size is one of the dominant parameters affecting the intensity of dust

emissions (Cowherd et al., 1990; MRI, 2001). The 17 surface units were later combined into seven combination (CBN) units as follows. This was based upon similar chemical compositions of the dust and a similar capacity to emit dust.

- a. CBN 1 (surface units 1.1 and 1.2): dune sands with and without vegetation.
- b. CBN 2 (surface unit 2.2): white and yellow silt/clay surfaces with gravel.
- c. CBN 3 (surface units 3.1, 3.2, 3.3, 3.4 and 3.5): desert pavements and other rock-covered areas on silt or sand.
- d. CBN 4 (surface units 4.1, 4.2, 4.3 and 2.1): active drainages and alluvial deposits adjacent to the drainage channel.
- e. CBN 5 (surface unit 1.5): badland areas with very fine yellow sand and coarse silt.
- f. CBN 6 (surface unit 1.4): patchy layers of sand over silty/rocky subsoil bordering dune field.
- g. CBN 7 (surface unit 2.3): badlands on brown, aggregated silt deposits.

Therefore, the geological samples collected from NDRA consisted of a complex mixture of both a variety of metals and particulate matter. Below 14 of the heavy metals found at NDRA are reviewed with special attention paid to immunotoxicity and oxidative stress effects.

Aluminum (Al)

Aluminum is the most common element in the earth's crust, and the third most abundant element on earth. Common routes of aluminum inhalation exposure include occupational, environmental, and cosmetics. Aluminum is minimally absorbed through

the lungs at approximately 1.5%, though inhalation is thought to be the most efficient route of exposure (ATSDR, 2008; Yokel and McNamara, 2001). Toxic effects of aluminum inhalation are thought to target the nervous system and impair lung function (ATSDR, 2008; Krewski et al., 2007). The nervous system toxicity, including dementia and possibly Alzheimer's Disease, is thought to result from direct olfactory transfer to the nervous tissue during inhalation (Bondy, 2010; Klaassen et al., 2013; Tjalve and Henriksson, 1998). Lung fibrosis from aluminum dust is well documented, but it is uncertain whether the fibrosis is a result of the dust or particulate matter (Sjogren et al., 2007).

Arsenic (As)

Arsenic is a known human carcinogen that is widespread in the environment with reported extensive health effects. The heavy metal occurs in three oxidation states, As^{III} , $\text{As}^{\text{III-}}$, and As^{V} , arsenite, arsenide, and arsenate respectively. Although arsenite is considered to be the most toxic to humans of the three, each form of arsenic is associated with human toxicity. Arsenic may enter the body via ingestion from contaminated water or inhalation and has been shown to increase oxidative stress, upregulate proinflammatory cytokines, and inactivates a form of an enzyme known as nitric oxide synthase, which is involved in vascular function (Singh et al., 2011). Arsenic exposure depletes antioxidant enzymes such as glutathione (Carter et al., 2003), leading to subsequent damage by oxidative stress. An increase in oxidative stress leads to DNA damage, chromosomal changes, interference with cellular signaling pathways, and ultimately, cancer (Singh et al., 2011). Inorganic arsenic is classified as a human

carcinogen causing tumors of the skin, lung, urinary bladder, and possibly liver (IARC, 2004).

Extremely high concentrations (> 300 ppm) of arsenic have been measured in airborne dust samples from NDRA (Soukup et al., 2011b). Exposure to arsenic has been linked to heart disease, hypertension, peripheral vascular disease, diabetes, immune suppression, acute respiratory infections, intellectual impairment in children, peripheral neuropathy, keratosis, cirrhosis, and skin, lung, bone, prostate, bladder, kidney and other cancers (ATSDR, 2007a; Chen, 1992; Jomova et al., 2010). In the form of arsenite, arsenic is thought to mimic estrogen, a sex hormone that plays roles in both females and males (Bridges et. al., 2005). Furthermore, arsenic exposure has been reported to damage lung tissue by inhibiting wound repair and modifying genes associated with immune function in lung tissue (Olsen et al., 2008; Kozul et al., 2009).

Arsenic affects both innate and adaptive immune responses (reviewed by Dangleben et al., 2013). Immunotoxic effects of arsenic have been shown in several animal models (Sikorski et al., 1989; Burns et al., 1993; Burchiel et al., 2009) as well as in humans (Soto-Peña et al., 2006). Mechanisms include alterations in key immune regulators, induction of apoptosis, oxidative stress and inflammation in circulating monocytes, impaired lymphocyte activation and macrophage function (Dangleben et al., 2013). Alterations in the humoral immune response are a sensitive target following arsenic inhalation toxicity, specifically T cell-dependent antibody production (Burchiel et al., 2009). A dose-dependent suppression of the secondary antibody-mediated response (IgG) to a T cell-dependent antigen, keyhole limpet hemocyanin (KLH), has also been

reported following subchronic exposure to arsenic, along with hepatotoxicity at doses above 4ppm (Nain et al., 2012).

Arsenic is also reported to target T cell function. Inorganic arsenic can alter the function of activated human T lymphocytes (Martin-Chouly et al., 2011) further impairing T cell-dependent antibody production and impairing the T-dependent humoral immune response (Burchiel et al., 2009). Arsenic (III) inhibits T cell proliferation and alters the T helper lymphocyte (Th) balance of cytokines secreted by co-stimulated T cells (Morzadec et al., 2012). Specifically, arsenic (III) reduces secretion of interferon-gamma without affecting IL-4 and IL-13 and this is reflected in mRNA levels. Arsenic (III) also increases expression of the redox-sensitive genes HMOX1, NQO1 and GCLM in activated T cells without altering the levels of reactive oxygen species (Morzadec et al., 2012). Arsenic exposure in mice inhibits T cell proliferation and macrophage activity, and decreases CD4 splenic cells number (Soto-Pena et al., 2006), suppress proliferative lymphocytic response (Biswas et al., 2008) and decrease IL-2 production (Biswas et al., 2008)

In the occupational environment, inhalation of arsenic may occur. Specifically, those that work in metal smelting, pesticide manufacturing or application, wood preservation, semiconductor manufacturing, welding, or glass production can be exposed to arsenic in air. Regulatory agencies such as the USEPA indicate that As is a hazardous air pollutant, while The National Institute for Occupational Safety and Health (NIOSH) and Occupation Safety and Health Administration (OSHA) have set guidelines that include 15 minutes permissible exposure limit of $2\mu\text{g}/\text{m}^3$ and 8 hour time weighted

average of $10 \mu\text{g}/\text{m}^3$ for inorganic arsenic compounds, respectively (ATSDR, 2007a). At this time, the USEPA does not report a reference concentration (RfC) for chronic inhalation exposure while the inhalation unit risk (IUR) is $0.0043 \mu\text{g}/\text{m}^3$ (USEPA IRIS, 1998; draft 2010).

Cesium (Cs)

Many cesium compounds are water-soluble and the metal is well absorbed through skin contact, inhalation, ingestion, and is rapidly distributed throughout the body (Klaassen et al., 2013). Limited data are available describing health effects associated with inhalation of cesium. It is known, however, that soluble cesium causes health effects via ingestion. Cardiac arrhythmia and long QT have been reported in patients exposed to stable cesium salts and gastrointestinal irritation has been reported after high-dose exposures (ATSDR, 2004c; Dalal et al., 2004; Neulieb, 1984). Isolated cases of neural stimulus following high dose oral cesium exposure are also seen in the literature (ATSDR, 2004c; Johnson et al., 1975).

Chromium (Cr)

Naturally occurring chromium is most commonly found in the trivalent state, whereas hexavalent chromium, a known carcinogen, is typically a by-product of industrial processes (Klaassen et al., 2013). Fully, chromium exists in oxidation states ranging from -2 to +6 (Cohen et al., 2006). Chromium is found readily in the environment, and is among one of the most common elements in the earth's crust. Trivalent chromium is an essential trace metal important to the metabolism of glucose

and is not highly toxic. An adequate intake of chromium is 20 – 45 µg Cr(III)/day for adults and children (IOM 2001).

Chromium is readily absorbed in the lungs and gastrointestinal tract, and when associated with strontium, the solubility is increased. Inhalation exposure of hexavalent chromium is strongly linked to health problems such as ulceration of the septum, epistaxis, dyspnea, increased risk of death due to noncancerous respiratory disease, lung fibrosis, abdominal pain, cirrhosis, and increased risk of respiratory, bone, prostate, hematopoietic, stomach, kidney, and bladder cancer (ATSDR, 2012; Klaassen et al., 2013; Costa and Klein, 2006). Risks of deleterious effects due to chromium exposure are exacerbated in those who exhibit chromium sensitivity.

Soluble chromium (VI) crosses cell membranes and then is reduced to chromium (III), which then binds serum proteins and eliminated via urine. The reduction of Cr(VI) to Cr(III) leads to the generation of free radicals, causing DNA strand breaks, crosslinking, adducts, chromosomal aberrations, modulation of p53, oxidative tissue damage, and oxidative stress (Bagchi et al., 2001; Shrivastava et al., 2002; Levina and Lay, 2005). More recently, high concentrations of chromium (III) in the cell have been shown to have carcinogenic properties in vitro to include DNA damage (Eastmond, 2008). Chromium (III) may form complexes with peptides, proteins, and DNA, resulting in DNA-protein crosslinks, DNA strand breaks, and alterations in cellular signaling pathways.

The immune system also is a target for inhaled and ingested chromium and its compounds as demonstrated in mouse models (ATSDR, 2012). Effects include

stimulation of the humoral immune system, increased phagocytic activity of macrophages, increased proliferative responses of splenic T and B cell mitogens and histopathological alteration of pancreatic lymph nodes (ATSDR, 2012). Occupational studies suggest that lymphocyte populations of CD4+ T cells, activated B cells and NK cells were decreased following hexavalent chromium exposure (Boscolo et al., 1997). Additionally, some animal models suggest that chromium exposure increases susceptibility to bacterial infections (Shrivastava et al., 2002).

Chromium measured in the blood and urine represents recent exposure. Elimination of chromium is multiphasic in that it is excreted in urine with three half-lives of 7 hours, 15-30 days, and 3-5 years (ATSDR, 2012). The USEPA reports chromium compounds as hazardous air pollutants and the IRIS data base indicates an inhalation RfC of 0.0001 mg/m³ for Cr(VI) particulates and 0.008 µg/m³ for chromic acid mists and dissolved Cr(VI) aerosols (US EPA IRIS, 1998). NIOSH has set a recommended exposure limit for an 8-hour time weighted period at 0.5 mg/m³ for Cr(III) compounds and a 10-hour time weighted exposure limit of 0.001 mg/m³ for Cr(VI) (ATSDR, 2012). OSHA guidelines are set for an 8-hour time weighted period at 0.5 mg/m³ for Cr(III) and 0.005 mg/m³ for Cr(VI) (ATSDR, 2012).

Cobalt (Co)

Cobalt (Co) is a component of cyanocobalmin, also known as vitamin B₁₂, and is characterized as an essential heavy metal with the potential for toxicity. Vitamin B₁₂ is acquired from the diet, particularly from seafood, pork, chicken and eggs and is found throughout the body, with the highest concentrations typically found in the liver, thyroid,

and kidneys. The most common disease state associated with vitamin B₁₂ and cobalt is pernicious anemia, which is seen in cases of B₁₂ vitamin deficiency. Although a Co deficiency is more prevalent, over-exposure to Co and inhalation exposure is associated with toxicities including asthma, decreased pulmonary function, cardiomyopathy, and pulmonary fibrosis (ATSDR, 2004, Klaassen et al., 2013).

The bioavailability of cobalt is largely dependent on the compound and the absorption varies from 5% - 45% for oral exposure and roughly 30% for inhalation (Lison et al., 1994). Once absorbed the majority of Co is rapidly excreted in the urine and then to a lesser extent the feces (Lison et al., 1994). Some of cobalt's toxicity can be attributed to its binding of sulfhydryl groups and its Co²⁺ form competing with divalent cations in normal biological processes. It also utilizes, interacts with, and blocks Ca²⁺ uptake mechanisms of the red blood cell, but is not excreted via Ca²⁺ pumps and can accumulate in the cytosol of RBCs (Simonsen et al., 2012; Klaassen et al., 2013). In addition, cobalt, along with manganese, competes for the iron transport system (Klaassen et al., 2013).

Inhalation exposure manifests primarily in the respiratory system, however thyroid effects and allergic dermatitis have been seen. Respiratory system effects include asthma, decreased pulmonary function, "hard metal" pneumoconiosis, pulmonary interstitial fibrosis, and more (Lauwerys et al., 1994; ATSDR, 2004; Chan, 2011; Klaassen et al., 2013). Many of the respiratory effects, general toxicity, and possible carcinogenicity of cobalt are thought to be due to the generation of free radicals causing oxidative stress. Thyroid hormone disruption has been observed in acute and chronic

exposures to cobalt. Patients receiving Co treatment for sickle cell anemia experienced varying thyroid disorders including goiter and hypothyroidism (Gross et al., 1955; Klinck, 1955; Kriss, 1955).

Cobalt is considered to be possibly carcinogenic to humans (group 2B) by the International Agency for Research on Cancer (IARC) and is reasonably anticipated to be carcinogenic in humans by the National Toxicology Program (NTP) (NTP, 2011; IARC, 1991); however, these findings are primarily based on animal studies. The immunotoxic potential of cobalt has not been fully assessed, although CD8 lymphopenia has been seen in patients receiving hip replacements containing chromium and cobalt (Hart et al., 2009), and Co is considered one of the most commonly implicated allergenic metals (Klaassen et al., 2013).

Copper (Cu)

Copper is an essential metal with the potential for toxicity. Most commonly, copper toxicity is seen in those with genetic disorders such as Menkes Disease and Wilson Disease, although toxicity from ingestion has been reported (ATSDR, 2004; Klaassen et al., 2013). Inhaled copper is a respiratory irritant that has been shown to induce lung inflammation, impair the immune response, and is thought to be the etiologic agent in Vineyard's sprayers lung (Drummond et al., 1986; Klaassen et al., 2013; Pettibone et al., 2008). Impaired immune response was observed in mice following inhalation exposure to copper sulfate at 0.13 mg/m³ 3 hours a day for 10 days (Drummond et al., 1986). Increased total white cell counts in blood alveolar lavage fluid

were observed in mice following an inhalation exposure of 4 hours per day, 5 days per week for 2 weeks of copper nanoparticles (Pettibone et al., 2008).

Iron (Fe)

Iron is an essential heavy metal, required for erythropoiesis and necessary for many enzymes. As such, iron deficiencies are a common side effect of blood loss (i.e. menstruation) and lack of iron in the diet, making iron deficiency the most common nutritional deficiency worldwide (Theil, 2011). Conversely, iron overload is seen in individuals with high iron content diets, hemochromatosis, and those needing frequent blood transfusions (Klaassen et al., 2013). Inhalation of iron at high levels for prolonged periods of time can lead to damage of the heart and liver as well as cause siderosis (Doherty et al., 2004; Sjogren et al., 2011). Historically, these conditions are encountered only in occupational settings such as steel manufacturing plants and hematic mines where the exposure values are now limited to 5 mg/m³ for iron oxide and 1 mg/m³ for soluble iron salts (Sjogren et al., 2011; Sentz et al., 1969). Systemic iron overload is a possible side effect of pulmonary siderosis, a disease that is common among welders (Doherty et al., 2004).

Lead (Pb)

Lead in the environment is generally as a result of anthropogenic activities and is considered to be a priority toxic substance by the EPA. Regardless of the path of absorption, the distribution of lead is largely the same, with approximately 95% of inhaled lead is absorbed through the lungs (ATSDR, 2007). In comparison, an

approximate ingested lead absorption rate for adults and children respectively is 15% and 42% (Klaassen et al., 2013). Thus, although inhalation exposure to lead is not a common problem, toxic effects can be achieved via inhalation with considerably lower amounts of the element. Submicron particles of inorganic lead can absorb into the blood stream via inhalation and within an hour after inhalation exposure, 50% of absorbed lead was detected in the liver (James et al., 1994).

Toxic effects of lead exposure are well characterized and due to the severe effects in humans at small doses, a minimal risk level (MRL) has not been identified. Blood levels determine lead toxicity, with 10µg/dL or higher being considered a high enough level to result in neurological damage (Jomova et al., 2011). It is hypothesized that lead enters cells through calcium channels, and the absorption of lead is inversely proportional to dietary calcium levels (Bridges et. al., 2005). The vast majority (99%) of lead in circulation is bound to intracellular erythrocyte proteins with the remainder bound and distributed by albumin and other plasma proteins, and is then stored mostly (93%) in the skeletal system (ATSDR, 2007b).

Lead is initially deposited into soft tissues where it is associated health effects such as encephalopathy, nephrotoxicity, renal failure, hypertension, immune system impairment, osteoporosis, abdominal pain, reduced fertility, and cancer of the lung, kidney, stomach, and others (ATSDR, 2007b; Klaassen et al., 2013). Lead is also known to affect the CNS, causing epilepsy, mental retardation, optic neuropathy, and learning disabilities in children with higher blood lead levels (ATSDR, 2007b; Bellinger, 2005; Goyer, 1990; Laraque and Trasande, 2005).

Similar to the other metal that have been reviewed, lead damages cells through free-radical induced oxidative damage. This damage is complex in that lead inhibits essential trace metal absorption, disrupts enzymes, effects membranes, and deactivates many of the cells antioxidant defense mechanisms (Ahamed and Siddiqui, 2007; Jomova et al., 2011). There are two ways lead goes about inducing oxidative stress, direct formation of ROS and depletion of cellular antioxidants (Ercal et al., 2001). A causal relationship has been identified in human and animal models between low-dose lead exposure and hypertension, linked to increased production of ROS (Jomova et al., 2011).

The immune system is a sensitive target for lead toxicity. The most commonly observed immunotoxic effect due to lead toxicity is a downgrade in Th1 responses from T helper cells with a simultaneous increase in the Th2 response (Klaassen et al., 2013). This has been concurrently seen with an increase in IgE levels, indicating immune dysfunction and an increased risk for allergies, especially in children (Luebke et al., 2006). Additionally, in adults exposed to lead in an occupational setting, a moderate decrease in B and T lymphocytes and T cell mitogen response has been observed with blood lead levels (BLLs) ranging from 30-70 µg/dL (Luebke et al., 2006; ATSDR, 2007b). Ewers et al. (1982) found that lead workers having a median BLL of 59 µg/dL exhibited significant suppression of IgM when compared to the control group (median BLL of 11.7µg/dL). Other occupational studies have found decreased populations of CD3+ and CD4+ cells, and decreased C3 and C4 complement levels in workers having a mean BLL of 74.8 µg/dL (Basaran and Ündeger, 2000; Fischbein et al., 1993; Ündeger et al., 1996).

Magnesium (Mg)

Magnesium is an essential heavy metal, required as a cofactor for many enzymes and is used as therapy for asthmatics to reduce bronchoconstriction (Rolla et al., 1986). Metal fume fever (MMF) is a rare side effect of inhalation of magnesium oxide, most commonly encountered in occupational settings (Klaassen et al., 2013). Conversely, at a median dose of 137 mg/m^3 , as reported by Kuschner et al. (1997), no MMF was seen nor indicated from human subjects. While overdose via ingestion can cause nausea, heart abnormalities, and CNS effects, the literature does not indicate that these effects are seen when the adsorption is through the lungs.

Manganese (Mn)

Manganese is an essential heavy metal, which is required for many metabolic functions with adequate intake at approximately 2.3 mg/day for adults (Aschner et al., 2005; Klaassen et al., 2013). Deficiency and excessive exposure of Mn have the potential to result in toxicity. Maintenance processes of immune, nervous, developmental, and reproductive functions depend on essential levels of Mn. Inhaled manganese particles $10\mu\text{m}$ and smaller are absorbed readily through the lungs into the blood stream or directly to the brain via nasal mucosa (ATSDR, 2013). Levels of manganese in the environment are typically naturally occurring; however some are a by-product of anthropogenic activities.

Manganese readily crosses the blood-brain barrier and accumulates in specific regions, thus the primary organ of concern for manganese toxicity is the brain (Aschner et al., 2005; Klaassen et al., 2013; Guilarte, 2010). Neurological manifestation of

manganese toxicity is referred to as manganism and produces a Parkinson's like disease (ATSDR, 2013; Bouchard et al., 2011). Other neurotoxicity demonstrated in occupational environments include decreased motor functions, mood alterations, decreased hand steadiness, insomnia, decreased eye-hand co-ordination, increased tremor, decreased response speed, limb paresthesia and decreased memory. The mechanism of action for Mn toxicity is not well understood and it is not clear which neurological endpoint is most sensitive for identifying subclinical Mn toxicity, although motor tasks may be considered (Mergler and Baldwin, 1997). It is understood, however, that when manganese enters the brain it is taken up into astrocytes and neurons, with astrocytes acting as the major storage site for Mn in the brain (Milatovic et al., 2009). Other health concerns related to manganese toxicity include, but are not limited to, inflammation of the lungs, pulmonary edema, pneumonia, hypotension, and reduced reproductive success (ATSDR, 2013).

Manganese is a common cofactor for the antioxidant superoxide dismutase (SOD), and essential levels of Mn are required to form Mn-SOD. Though its role in SOD, elevated levels of Mn may lead to and increase is oxidative stress and damage. Milatovic and colleagues (2009) report findings that suggest neurotoxicity may indeed be mediated by oxidative stress, mitochondrial dysfunction and neuroinflammation. The potential mechanisms for manganese-induced oxidative stress include the oxidation of catecholamines such as dopamine and excessive production of ROS (Erikson et al., 2004).

Because most studies demonstrate that the nervous system is the primary target, some reports indicate that Mn also affects cells of the immune system, however it is

unclear if Mn causes immune dysfunction (ATSDR, 2013). Suppression of T- and B-lymphocytes in male welders exposed to manganese by inhalation has been documented (Boshnakova et al., 1989) along with an increase in the amount of leukocytes and macrophages in the lung (Shiotsuka, 1984). Additionally, *in vitro* studies with human lung epithelial cells report that Mn (0.2-200uM concentrations) increase inflammatory cytokines IL-6 and IL-8, while levels of tumor necrosis factor alpha (TNF-alpha) were not altered (Pascal and Tessier, 2004).

Strontium (Sr)

In general, absorption of strontium both through the lungs and the gastrointestinal tract appears to be relatively efficient. Intratracheal doses of radioactive strontium in fly ash (90% less than 20 μm particle diameter) in rats demonstrated that the strontium entered plasma and other tissues and within one day, the tissue:plasma strontium concentration ratios were 0.3-0.5 in the liver, kidney, small intestine, and heart. Within a few days of the exposure, the tissue:plasma concentration ratios were >1 (1.5-2) in the liver, kidney, and <1 (0.7–0.9) in the spleen, heart, and brain (Srivastava et al., 1984). Following a nose-only exposure to aerosolized strontium (mean diameter of 1.4-2.7 μm) in dogs, 37% distributed to the bone within 12 hours while 84% was in the bone 4 days after the exposure (Fission Product Inhalation Project, 1967). Strontium has a half-life of 29 years residing primarily in bone.

Strontium may act as surrogate for calcium resulting in distribution throughout the body and in particular, the bone. Animal studies indicate that doses of strontium (≥ 500 mg/kg/day) caused a reduction in bone mineralization and altered organic bone

matrix. Aside from affecting bone mineralization, little is known about the health effects of excess strontium. *In vitro* studies have reported that strontium elicits degranulation of mast cells, causing histamine release (Alm and Bloom, 1981a, 1981b). Strontium is expected to be a carcinogen, particularly radioactive forms (ATSDR, 2004). In this case, a reduction in red blood cell counts can serve as a biomarker of exposure to the radioactive form of strontium.

Strontium is not known to cause any other deleterious effects in humans and no animal studies were found that indicated adverse effects following inhalation of stable strontium. However, strontium increases the solubility of hexavalent chromate (ATSDR, 2004).

Uranium (U)

Few studies have examined inhalation exposure to depleted uranium (DU) and even less for uranium. Depleted uranium is not an exposure in this NDRA study but due to the paucity of information, this section includes references to DU. Epidemiology studies indicate an increase in relative risk of lung cancer and fibrosis in uranium miners and workers in the nuclear industry (ATSDR, 1999). Moreover, an increase in chromosomal aberrations or genetic damage in blood samples was observed among Gulf War veterans exposed by embedded DU shrapnel fragments (McDiarmid et al., 2004) and in uranium-exposed miners (Meszaros et al., 2004; Zaire et al., 1996). Inhalation of uranium dust particles can lead to accumulation predominantly in the lungs and tracheobronchial lymph nodes (ATSDR, 1999). *In vivo* experimental animal results demonstrated that inhalation of DU could induce DNA damage in different rat cell types

and that DNA lesions were linked to the dose and independent of the solubility of uranium compounds (Monleau et al., 2006). Insoluble DU particles induced time-dependent increases in mRNA levels of cytokines and hydrogen peroxide production in rat lungs. In these same animals, only repeated exposure was able to induce DNA strand breaks in kidney cells (Monleau et al., 2006). *In vitro* studies with uranium-exposed macrophages have shown effects on cell viability (Kalinich et al, 2002; Tasat and De Rey, 1987) and an induction of TNF- α secretion and mitogen activated protein kinase (MAPK) activation important in gene expression, cell proliferation and programmed cell death (Gazin et al., 2004).

As compared to inhalation, more data are available regarding uranium toxicity via ingestion of contaminated drinking water sources. Human data indicate an average gastrointestinal absorption rate of 1-2% of the total ingested dose, with rapid clearance from the bloodstream and accumulation in the skeleton and kidneys. Nephrotoxicity (kidney toxicity) induced by U is the primary concern as this metal targets the proximal tubules, causing an increase in urinary b-2-microglobulin, alkaline phosphatase, and albumin. Correlative evidence also suggests an increase in urinary excretion of glucose, phosphate, and calcium with uranium exposure. The WHO established tolerable daily intake (TDI) is 0.6 mg/kg body weight/day based on a LOAEL of 60 mg/kg body weight/day from a sub-chronic 91-day study on rats using uranium in drinking water.

Uranium exposure also is associated with an increased risk of breast, liver, and bone cancer, diminished bone growth, DNA damage, and developmental and reproductive effects (Lemercier, 2003). Children, infants, pregnant and nursing women,

and women of childbearing age are at greater risk to radioactive forms of U (MTDEQ, 2009; Brugge et al., 2005; GDHR, 2011; Kurttio et al., 2002).

Vanadium (V)

Vanadium (V) is an element that is naturally occurring and widely distributed on the earth's crust, with exposure to humans typically occurring through polluted air, dust, and fumes (Valko et al., 2005). It has a number of valence states with V^{+4} and V^{+5} being of most toxicological relevance. Once vanadium enters the body it accumulates in the bone, kidney, liver, spleen, and to a small extent, in the lungs (Valko et al., 2005; Klaassen et al., 2013). While the absorption of vanadium is poor in the gastrointestinal tract, about 25% of inhaled soluble V is absorbed in the lungs. The excretion of vanadium observed by Oberg et al. (1978) was found primarily in the urine of rats and to a lesser extent, the feces. The primary targets of V toxicity resulting from oral exposure include the hematological system, the developing organism, and the gastrointestinal tract, and the respiratory system after inhalation exposure (ATSDR, 2012).

Industrial and occupational vanadium exposure has been shown to cause gastrointestinal distress, including abdominal pain, nausea, and vomiting, persistent coughing, respiratory distress, kidney damage, and heart palpitations (Zenz and Berg, 1967; Lagerkvist et al., 1986; Barceloux, 1999; ATSDR, 2012). Persistent coughing has been observed post acute V inhalation exposure at 0.6 mg V/m^3 (ATSDR, 2012). Inhalation studies in mice and rats found that both acute exposure (0.56 mg V/m^3) and chronic exposure (0.28 V/m^3) induced lung lesions, fibrosis and inflammation (ATSDR,

2012). Additional symptoms from exposures longer than two days included hyperplasia in nasal goblet cells and the larynx.

Once vanadium has entered into systemic circulation, it can reversibly bind transferrin and can also be taken up by erythrocytes. It is also important to note that brain levels of V post exposure are very low, indicating that the element does not effectively cross the blood brain barrier (Lagerkvist et al., 1986). While there have been no studies assessing V carcinogenicity in humans; there is evidence of lung carcinomas developing in chronically exposed rodents. Vanadium pentoxide has been classified as a 2B carcinogen by the IARC; however the US EPA and HHS have not given it any classification for carcinogenicity (ATSDR, 2012).

Immune system effects following vanadium exposure have not been well characterized in humans; however, in rodents, mild increases in spleen weight, decreases in spleen cellularity, and increases in blood leukocytes have been reported (ATSDR, 2012). Vanadium undergoes redox-cycling reactions (Valko et al., 2005). Vanadium and V compounds have insulin-enhancing effects in cell cultures and diabetic animals (Valko et al., 2005). It is hypothesized that the insulin-enhancing effects of V are due to antioxidant properties of the transition metal and changes in cellular GSH metabolism. V may participate in reactions resulting in ROS and further oxidative stress (Valko et al., 2006).

Zinc (Zn)

Zinc (Zn) is an essential heavy metal with a recommended dietary allowance of 11 mg Zn/day for men and 8 mg/day for women (ATSDR, 2005). In nature it occurs

primarily as zinc oxide, making up 20-200 ppm of the Earth's crust (ATSDR, 2005). Inhalation of zinc oxide is known to cause marked pulmonary inflammation. Metal fume fever (MFF) is a short-lived and self-limiting metal induced fever that is seen after large doses (77-600 mg Zn/m³) of inhaled zinc oxide; symptoms include fever, headache, fatigue, leukocytosis, chest pain, cough and shortness of breath (ATSDR, 2005; Cooper, 2008; Kaye et al., 2002). Long term effects of zinc inhalation and MFF are unknown at this time (ATSDR, 2005). Nausea, along with diarrhea, cramps and vomiting, has also been reported with exposure to high amounts of zinc oxide fumes and zinc has the ability to alter mitochondrial metabolism (ATSDR, 2005; Lemire, 2008). Inhalation of lower levels of zinc oxide (16.4 mg/m³) did not cause MFF, but did result in more polymorphonuclear leukocytes and lymphocytes in the BAL fluid, and higher levels of several cytokines (ATSDR, 2005). The allowable exposure to zinc oxide in the workplace is limited at 5.0 mg Zn/m³ (Beckett et al., 2005).

While excess dietary zinc is linked to pancreatic and neuronal toxicity, these effects have not been shown from inhalation exposures (Klaassen et al., 2013). Longer-term excess dietary zinc interferes with copper absorption, resulting in decreased levels or activity of copper-containing enzymes such as superoxide dismutase and monamine oxidase. More severe symptoms of copper deficiency include anemia and leucopenia (Summerfield et al., 1992). Loss of leukocytes and hence reduced leukocyte function can result from higher levels of chronic oral exposure to zinc (ATSDR, 2005). Regarding inhalation exposure, however, no RfC has been established for zinc and compounds.

Oxidative Stress

Many metals have been implicated in causing oxidative stress. These metals include arsenic, iron, copper, chromium, vanadium, cobalt, mercury, cadmium, and nickel. Of these, arsenic (As) is of greatest concern due to its ability to induce various adverse health outcomes in exposed humans, including several types of cancer. Many of the health problems associated with exposure to arsenic are thought to arise, in part, by a process known as “free radical-mediated oxidative damage”, which can be loosely termed “oxidative stress” (Jomova et al., 2010). Because of this concern, we used four markers to assess oxidative stress in mice exposed to dust from NDRA; total antioxidant capacity, reactive oxygen and nitrogen species quantification, total glutathione, and superoxide dismutase activity.

All aerobic organisms constantly produce reactive oxygen and nitrogen species (ROS/RNS) (Dalton et al., 1999; Guerin et al., 2001; Halliwell, 2001), but normal cellular process maintain a balance between ROS/RNS and the enzymes that break them down, often called “the redox balance”. An imbalance in the amount of ROS/RNS and antioxidants may result in oxidative stress and damage. Oxidative damage can take the form of DNA damage and/or lipid peroxidation, and perpetuate disease processes such as chronic inflammation and diabetes (Jomova et al., 2011). Examples of ROS include superoxide anion, singlet oxygen, peroxy radical, and hydrogen peroxide. ROS/RNS in excess can drain cellular energy reserves, disrupt the normal biological functions of cells, and contribute to carcinogenesis (Klaassen et al., 2013; Dalton et al., 1999). Glutathione (GSH) and antioxidant enzymes in general, are crucial to maintain the state of redox

balance within cells. In the cases where ROS/RNS overwhelm GSH, GSH depletion can lead to oxidative stress within a cell (Xia et al., 2006). Another important antioxidant is superoxide dismutase, which is capable of converting the free radical superoxide into hydrogen peroxide that can be further reduced by glutathione (Klaassen et al., 2013). Heavy metals that are redox active, such as chromium, possess the ability to produce reactive free radicals, and metals that are redox inactive, such as arsenic, are capable of depleting GSH and other thiols (Jomova et al., 2011; Valko et al., 2005).

An increase in oxidative stress leads to DNA damage, chromosomal changes, interference with cellular signaling pathways, and ultimately, cancer (Singh, et al., 2011). An imbalance in cellular redox is induced by oxidative stress and this has been found to be present in various cancer cells when compared with normal cells, thus this redox imbalance may be related to the stimulation of tumors and cancer (Valko et al., 2006). As reviewed above, many of the metals found in dust samples from NDRA are implicated in the production of ROS and oxidative stress, and it is likely that this plays a large role in the carcinogenicity of metals such as arsenic. The formation of DNA lesions, such as 8-OH-G, is seen in many cancers and is supportive of the role ROS play in the etiology of cancer (Valko et al., 2006).

There is strong evidence to suggest that mechanisms behind pulmonary and cardiovascular diseases caused by particulate matter exposure have partial etiology in oxidative stress. Damage due to particulate matter is highly dependent on the particle size. For instance, with particles of a diameter less than 100nm, the surface area of the particle is important, whereas for larger particles the chemical composition is more

critical (Risom et al., 2005). The model particulate pollutant for many of these studies is diesel exhaust particles (DEP), which have been shown to not only generate free radicals, but also cause inflammation, cytokine production, and increase myeloid cells (Xia et al., 2006). Within DEP it is likely that the pro-oxidative organic compounds and transition metals are the components responsible for the production of free radicals. Systemic effects of oxidative stress due to particulate matter exposure have been seen in the central nervous system, thus linking PM exposure and neurotoxic susceptibility (MohanKumar et al., 2008).

Immunophenotyping Lymphocytes

Few studies assessing particulate matter containing heavy metals have assessed the lymphocyte subpopulations post exposure. In this study, we immunophenotyped cells using flow cytometry from the spleen and thymus at each exposure level (0-100mg/kg), and statistically compared the lymphocyte quantities. Surface cell markers B220, CD4, CD8, CD25, FoxP3, and IL17 were used, each with respective controls. All surface markers were used on splenocytes, and thymocytes were analyzed using CD4 and CD8 surface markers only.

B lymphocytes (B cells) are a subset of cells critical for humoral immunity, and are responsible for the production of antibodies and some cytokines. B220 is a mouse isoform of CD45, a B cell marker that is present on all B cells, excluding those that are memory cells. T lymphocytes (T cells) will proliferate and differentiate into effector T cells after coming into contact with a foreign antigen. Effector T cells broadly function to kill, regulate, and activate cells. Regulatory T cells (Tregs) are a specialized subset of

effector T lymphocytes responsible for suppression of immune responses that are deleterious to the host organism. They are characterized by high surface expression of CD25 (the IL-2 receptor alpha chain) and intracellular expression of the master switch transcription factor forkhead box protein P3 (FoxP3) (Fontenot et al., 2003).

As noted above, many heavy metals found on dust samples from NDRA have been implicated in deleteriously affecting lymphocytes. Arsenic exposure in mice inhibits T cell proliferation and macrophage activity, decreases CD4 splenic cells and IL-2 production, and suppresses proliferative lymphocytic responses (Soto-Pena et al., 2006; Biswas et al., 2008). Chromium has been shown to significantly reduce CD4+ lymphocytes in workers exposed to contaminated dusts (Boscolo et al., 1997). *In vitro*, cobalt has been shown to reduce proliferation of T lymphocytes and IL-2 release (ATSDR, 2004). In addition, chromium and cobalt together, following exposure from items used for hip replacements, are associated with CD8 lymphopenia (Hart et al., 2009). Furthermore, multiple studies have described decreases in CD4+ cells following lead exposure (ATSDR, 2007).

T regulatory cell numbers have been shown to decrease, specifically via oxidative stress, following arsenic exposure (Thomas-Schoemann et al., 2012). Vanadium has been shown to decrease expression of CD11c surface marker on mouse thymic dendritic cells following a 4-week exposure (Ustarroz-Cano et al., 2012). This has relevance in that an immature dendritic cell subset that is tagged with CD11c+ is important for the development and function of CD4+ CD25+ regulatory Tregs (Tarbell et al., 2006).

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CHAPTER 2

OXIDATIVE STRESS AND LUNG HISTOPATHOLOGY FOLLOWING SUB-ACUTE
EXPOSURE TO NATURAL DUST COLLECTED FROM THE NELLIS DUNES
RECREATION AREA

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OXIDATIVE STRESS AND LUNG HISTOPATHOLOGY FOLLOWING SUB-ACUTE
EXPOSURE TO NATURAL DUST COLLECTED FROM THE NELLIS DUNES
RECREATION AREA

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Key Words

Oxidative Stress, Lung, Histopathology, Arsenic, Particulate Matter

Chapter Summary

Background: Exposure to particulate matter containing heavy metals has been linked to adverse human health effects when exposure occurs in industrial settings. However, little data exist on effects associated with exposure in natural settings. This study was designed to evaluate markers of oxidative stress and lung histopathology following sub-acute exposure to metals-containing dust collected from a natural setting used heavily for off-road vehicle (ORV) recreation.

Objectives: Determine whether exposure to natural dust containing heavy metals is associated with pathological changes in lungs and the balance of free radicals and antioxidants in plasma that have the potential to induce oxidative stress.

Methods: Adult female B6C3F1 mice were exposed to several concentrations of dust collected from seven different types of surfaces at the Nellis Dunes Recreation Area (NDRA), designated here as combined map units (CBN) 1-7. Dust representing each of the seven map unit locations was prepared with a median diameter of 4.5 microns or less and suspended in phosphate-buffered saline immediately prior to oropharyngeal aspiration in mice at concentrations from 0.01 to 100 mg of dust/kg of body weight. Exposures were given four times spaced a week apart over a 28-day period to mimic a month of weekend exposures. Lungs for histopathology and plasma for evaluation of markers of oxidative stress were collected 24 hours after the final exposure. Lung pathology was evaluated by an independent veterinary pathologist. Plasma markers of oxidative stress included levels of reactive oxygen and nitrogen species, superoxide dismutase, total antioxidant capacity, and total glutathione. These data were compared to

oxidative stress measures following *in vivo* exposure to titanium dioxide (TiO₂) devoid of a complex metal mixture.

Results: Exposure to 10 mg/kg of dust from CBN 4 increased superoxide dismutase and total glutathione and exposure to 100 mg/kg increased superoxide dismutase. Exposure to one or more concentrations of dust from CBN 1, CBN 6, or CBN 7 decreased total antioxidant capacity or total glutathione. While TiO₂ resulted in an increase in reactive oxygen and nitrogen species at the highest exposure level only, 100 mg/kg/d, no alteration in superoxide dismutase, total antioxidant capacity, and total glutathione was observed. Exposure to 100 mg/kg of dust from all map units but CBN 4 induced some level of lung inflammation. Based on the mean severity score, the potency of the CBNs to induce inflammation was CBN 5 > CBN 1 > CBN 3 > CBN 7 > CBN 6 > CBN 4 > CBN 2. Based on the number of lungs per dose exhibiting inflammation, the potency of the CBNs was CBN 1 > CBN 3 > CBN 5 > CBN 7 > CBN 6 CBN 4 > CBN 2. CBN 3 induced lung inflammation at all administered doses but 1 mg/kg. Exposure to 1 and 10 mg/kg from CBN 1, CBN 5, or CBN 7 induced inflammation. Exposure to 10 mg/kg from CBN 4 or CBN 6 also induced inflammation. No other pathological changes were noted.

Conclusions: Overall, results of assays to evaluate markers of oxidative stress indicate that no single CBN surface type was able to consistently induce markers of oxidative stress at a particular dose or in a dose-responsive manner. The two highest concentrations of dust from CBN 4 were able to increase two markers of oxidative stress, but results of other surface types were inconsistent. These observations are relatively

consistent with TiO₂ that contributed to a significant change at the high exposure level in only one measure of oxidative stress. All surface types were able to induce some level of lung inflammation, typically at the highest doses. These results indicate that exposure to these natural, metal-containing dusts, under the exposure scenario of our study, are unlikely to dramatically increase the risk of oxidative damage but it is possible that some level of exposure could induce a degree of inflammation in the lungs of exposed individuals.

Rationale

Studies of the effects of particulate matter on human health increasingly show a strong link to numerous diseases and mortality including asthma, heart disease, dementia, and cancer (Samet et al., 2000, Katsouyanni et al., 2001; Boezen et al., 1998). Studies of human populations have demonstrated that premature deaths may be from damage to the heart and lungs; however, the specific mechanisms by which this damage arises are not clear. A complicating factor is that particulate matter can have a wide range of characteristics, varying, for example, in chemical composition, grain size, and particle morphology. The particulate matter used for this study was natural soil dust collected from an area heavily used for off-road vehicle (ORV) recreation and prone to frequent wind erosion. The dust that is emitted from the topsoil contains a wide range of naturally occurring heavy metals including aluminum, iron, strontium, manganese, lead, zinc, vanadium, copper, arsenic, and chromium. Exposure to heavy metals at certain concentrations is known to alter the “redox balance” in an organism, or the maintenance of a normal ratio of reactive species that can induce cell damage and death and the antioxidant enzymes that break them down. While nearly all cellular processes create reactive species, their ability to induce damage is typically reduced or eliminated by antioxidant enzymes. Damage, the result of oxidative stress, may occur when reactive species overwhelm the antioxidant enzymes or when the antioxidant enzymes themselves become damaged or depleted. One outcome of oxidative stress is inflammation, a response of the immune system to an injury. This chapter therefore presents results of a study to determine disruptions to the redox balance via evaluation of redox markers in the

plasma, and signs of inflammatory injury or pathology in the lungs, the route of entry by which dust enters the systemic blood flow of an organism. Redox balance was evaluated by measuring the amount of reactive oxygen and nitrogen species, total glutathione, superoxide dismutase activity, and total antioxidant capacity in the blood of mice exposed to dust samples. Lungs were evaluated on the cellular level for signs of inflammation and other changes indicative of pathology.

Previous Studies

Nellis Dunes Recreation Area (NDRA) is a highly popular ORV area near Las Vegas, NV. Recent research at the NDRA has indicated that exposure to dust generated by both ORV activities and natural winds may pose a public health hazard (Goossens and Buck, 2011a). More than 20 trace metals that have the potential to affect human health have been identified in dust samples collected from the NDRA (Soukup et al., 2011a).

Metals of high concern, due to their well-known human health effects, include arsenic, cadmium, manganese, chromium, and strontium. Of these, arsenic (As) is of greatest concern due to its ability to induce various adverse health outcomes in exposed humans, including several types of cancer. Extremely high concentrations (> 300 ppm) of arsenic have been measured in airborne dust samples from NDRA (Soukup et al., 2011b). Exposure to arsenic has been linked to heart disease, hypertension, peripheral vascular disease, diabetes, immune suppression, acute respiratory infections, intellectual impairment in children, peripheral neuropathy, keratosis, cirrhosis, and skin, lung, bone, prostate, bladder, kidney and other cancers (ATSDR, 2007; Chen, 1992; Jomova et al.,

2010). In the form of arsenite, arsenic is thought to mimic estrogen, a sex hormone that plays roles in both females and males (Bridges et al., 2005). Furthermore, arsenic exposure has been reported to damage lung tissue by inhibiting wound repair and modifying genes associated with immune function in lung tissue (Olsen et al., 2008; Kozul et al., 2009). Many of the health problems associated with exposure to arsenic are thought to arise, in part, by a process known as “free radical-mediated oxidative damage”, which can be loosely termed “oxidative stress” (Jomova et al., 2010).

All aerobic organisms constantly produce reactive oxygen and nitrogen species (ROS/RNS) (Dalton et al., 1999; Guerin et al., 2001; Halliwell, 2001), but normal cellular process maintain a balance between ROS/RNS and the enzymes that break them down, often called “the redox balance”. An imbalance in the amount of ROS/RNS and antioxidants may result in oxidative stress and damage. Oxidative damage can take the form of DNA damage and/or lipid peroxidation, and perpetuate disease processes such as chronic inflammation and diabetes (Jomova et al., 2011). Examples of ROS include superoxide anion, singlet oxygen, peroxy radical, and hydrogen peroxide. ROS/RNS in excess can drain cellular energy reserves, disrupt the normal biological functions of cells, and contribute to carcinogenesis (Klaassen et al., 2013; Dalton et al., 1999). Glutathione (GSH) and antioxidant enzymes in general, are crucial to maintain the state of redox balance within cells. In the cases where ROS/RNS overwhelm GSH, GSH depletion can lead to oxidative stress within a cell (Xia et al., 2005). Another important antioxidant is superoxide dismutase, which is capable of converting the free radical superoxide into hydrogen peroxide that can be further reduced by glutathione (Klaassen et al., 2013).

Heavy metals that are redox active, such as chromium, possess the ability to produce reactive free radicals, and metals that are redox inactive, such as arsenic, are capable of depleting GSH and other thiols (Jomova et al., 2011; Valko et al., 2005).

Prior to the current study, we demonstrated that a three-day, *acute* exposure to dust samples collected from several surfaces within the NDRA resulted in local respiratory as well as systemic effects on immune function. Specifically, this included inflammatory changes in the lung, suppression of IgM antibody responses, and changes in splenic lymphocytic subpopulations (Proper et al., 2011). This initial study prompted us to evaluate additional surface types as well as biological endpoints that may be impacted by exposure to these complex mixtures of particulate matter and heavy metals.

For the current study we collected topsoil from 17 different types of surfaces ("surface units") in the NDRA. These included vegetated and unvegetated sand dunes, areas with a thin (1-5 cm) layer of windblown sand, badland areas with aggregated and non-aggregated silts or mixtures of sand and silt, well-developed and degraded desert pavements, rocky, sandy and silty drainages, and bedrock. Special attention was paid to ensure that all surface types that occur within the NDRA were sampled, and that coarse-grained, medium-grained and fine-grained substrata were evaluated. This is important because grain size is one of the dominant parameters affecting the intensity of dust emissions (Cowherd et al., 1990; MRI, 2001). Due to time constraints and budgetary reasons, the 17 surface units were later combined into seven (CBN) units. The CBN units were as follows:

- a. CBN 1 (surface units 1.1 and 1.2): dune sands with and without vegetation.

- b. CBN 2 (surface unit 2.2): white and yellow silt/clay surfaces with gravel.
- c. CBN 3 (surface units 3.1, 3.2, 3.3, 3.4 and 3.5): desert pavements and other rock-covered areas on silt or sand.
- d. CBN 4 (surface units 4.1, 4.2, 4.3 and 2.1): active drainages and alluvial deposits adjacent to the drainage channel.
- e. CBN 5 (surface unit 1.5): badland areas with very fine yellow sand and coarse silt.
- f. CBN 6 (surface unit 1.4): patchy layers of sand over silty/rocky subsoil bordering dune field.
- g. CBN 7 (surface unit 2.3): badlands on brown, aggregated silt deposits.

Therefore, the geological samples collected from NDRA consisted of a complex mixture of both a variety of metals and particulate matter.

Methods

Preparation and Verification of Dust Dosing Samples

Dust samples were carefully labeled, stored in sealed and dry containers, protected from light, and secured in a lock box in the laboratory. A stock dosing solution was prepared and administered to mice within 1-2 hours of preparation. Dust was prepared in sterile, endotoxin-free, phosphate buffered saline (ETF-PBS) at concentrations of 0.01, 0.1, 1, 10, or 100 mg of dust/kg of body weight. We considered that some elements have various degrees of solubility in water and addition of dust samples to saline for delivery into the mouse may change the distribution of insoluble

elements versus concentration of those elements in solution. To verify that adding the dust samples to PBS did not substantially alter the solubility of elements, a stability study was performed with the lowest concentration (0.01 mg/kg) and a higher concentration (10 mg/kg). Dust was added to ETF-PBS to ascertain stable time frames in which the solution could be used for animal exposures. Solutions were prepared and samples of the solutions were collected immediately after preparation, and then at 1, 2, 4, and 6 hours. Samples were immediately centrifuged, supernatants removed, and examined immediately using an ICP-MS to quantitate total soluble element concentrations. The analysis indicated that leaving the dust samples in an ETF-PBS solution for up to six hours did not substantially change the distribution of elements in solution. After six hours, soluble element concentrations in supernatant began to increase. That is, the amount of insoluble versus soluble remained constant for six hours in solution. We did not test for changes in speciation, but only total values. This additional quality control measure verified our dosing solution concentrations, potential for flux, and accounted for potential contamination from PBS or other steps in our preparation process. To control for contamination in this preparation process, no metal spatulas or any other metal items were used for weighing, storage, manipulation, or transport of dust samples. Samples were immediately centrifuged, supernatants removed, and examined immediately using an ICP-MS to quantitate total soluble element concentrations.

Animals and Animal Exposures

Mice were obtained from Charles River Laboratories and were acclimated to the conditions of the treatment room (12-h light/dark cycle, 22±2 °C, 60-65% relative

humidity) at the UNLV animal facilities, which are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All experiments were approved by the UNLV Institutional Animal Care and Use Committee. Mice were housed in ventilated polycarbonate shoebox cages with corncob bedding and were given unlimited access to food and water.

To simulate the potential health impacts of a month of weekend exposures to dust from the NDRA, adult female B6C3F1 mice were exposed to dust samples with a median diameter of 4.39 μm at 0, 0.01, 0.1, 1.0, 10, or 100 mg/kg of body weight once weekly for four weeks. Mice in the 0 mg/kg group received PBS only and served as a control group. To ensure availability of tissue for toxicology assays, each dose group contained 12 mice housed six per cage. In addition, three separate groups of mice were used for a total of three replicates for each dose. Samples for toxicity studies were collected from each group three days in a row.

Tissue and Plasma Collection

Whole blood was collected into sodium heparin microtainers following anesthetization of mice with CO_2 . Samples were kept cold, and centrifuged at approximately 7,000-10,000 g for 15 minutes 1-2 hours post collection in refrigerated conditions (6-10°C). Immediately after centrifugation, the plasma (supernatant) was removed and flash frozen in liquid nitrogen and stored to -80°C until testing. The remaining packed red blood cells (PRBCs) in the microtainer tubes were kept refrigerated (6-10°C) until processing.

Lungs were cannulated immediately after euthanasia, which involved the insertion

and fixation of a small tube into the trachea, transferred to PBS, and then perfused with formalin on a constant pressure inflation fixation apparatus. This apparatus allowed the formalin to flow into the lungs so that they were inflated, a requirement for optimal histological assessment.

Evaluation of Markers of Oxidative Stress

Plasma *in vitro* ROS/RNS

ROS and RNS were measured following directions provided with a kit purchased from Cell Biolabs Inc. Dichlorodihydrofluorescein DiOxyQ (DCFH-DiOxyQ), a green fluorogenic probe, was used to measure the total free radical population in the plasma of mice (**Figure 2.1**). Samples were fluorometrically analyzed and compared against a DCF standard.

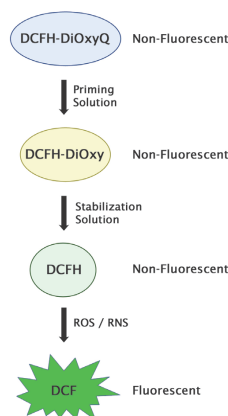


Figure 2.1. Mechanism of the ROS/RNS assay (Image from: Cell Biolabs, Inc., OxiSelect™ *In Vitro* ROS/RNS Assay Kit (Green Fluorescence), San Diego, CA). Plasma Superoxide Dismutase Activity

Superoxide dismutase (SOD) is a powerful antioxidant enzyme that is responsible for the dismutation of superoxide to hydrogen peroxide and oxygen. SOD activity was

measured in plasma using an inhibition activity assay purchased from Abcam (Cambridge, England). A water-soluble formazan dye is produced upon reduction with superoxide anion. The assay measures the ability of the superoxide ion to reduce xanthine oxidase activity through a colorimetric method. The principle of the assay is depicted in **Figure 2.2**.

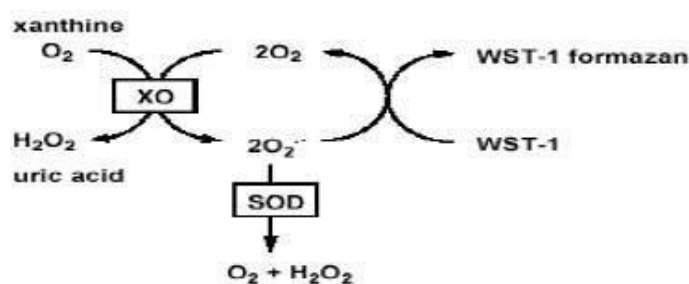


Figure 2.2. Superoxide dismutase activity assay principle. (Image from: Abcam[®], Superoxide Dismutase Activity Assay Kit (Colorimetric), USA).

Plasma Total Antioxidant Capacity

Total antioxidant capacity (TAC) of plasma samples was determined via a single electron transfer (SET) mechanism that measured the reduction of copper (II) to copper (I), following manufacturer's directions (Cell Biolabs Inc.; **Figure 2.3**). The absorbance values were proportional to the sample's total reductive capacity and were compared to a known uric acid standard curve.

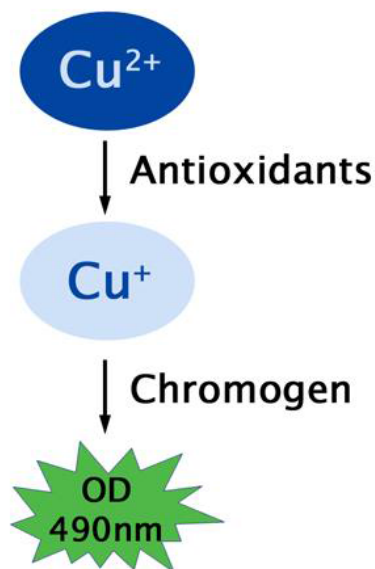


Figure 2.3. Total antioxidant capacity activity assay principle. ((Image from: Cell Biolabs, Inc., OxiSelect™ Total Antioxidant Capacity (TAC) Assay Kit, San Diego, CA).

Erythrocyte Lysate Total Glutathione (GSSG/GSH)

Total glutathione content in PRBC lysate was evaluated with a quantitative kinetic enzymatic assay purchased from Cell Biolabs Inc (San Diego, CA). One to six hours post blood collection, ice-cold 5% meta-phosphoric acid was added to PRBCs to remove interfering proteins and enzymes. Samples were held on ice for 10 minutes and then spun at 12,000 g for 10 minutes at 4°-6°C. The supernatant or PRBC lysate, was collected and stored at -80°C until testing. NADPH was used to reduce GSSG to GSH and the total amount of glutathione was determined by the rate of production over time, compared to a glutathione standard curve as depicted in **Figure 2.4**.

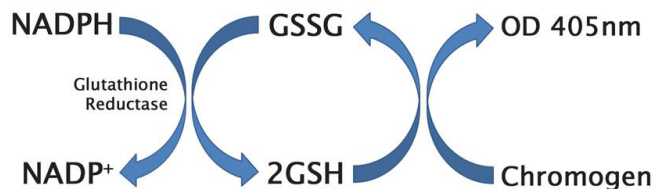


Figure 2.4. Total Glutathione assay principle. ((Image from: Cell Biolabs, Inc., OxiSelect™ Total Glutathione (GSSG/GSH) Assay Kit, San Diego, CA).

Lung Histopathology

After fixation in 10% neutral buffered formalin, whole lungs were shipped to the HistoPathology Laboratory in the Division of Human Pathology at Michigan State University. Once received, lungs were prepared for histopathological assessment. Briefly, this involved processing the lungs through several procedures that surround and infiltrate the lung tissues with paraffin (wax). This procedure does not damage the tissue and actually preserves it in a block of paraffin. Lungs were then cut into 10 micron sections, mounted onto glass slides, and stained with hemotoxylin and eosin (H&E), basic histological stains that highlight the basic cellular structures of biological tissues. Slides were shipped to Experimental Pathology Laboratories, Inc. (EPL), in North Carolina, a commercial laboratory that specializes in the pathological and histopathological examination and analysis of biological tissues. Pathologists from EPL evaluated and scored lung sections for basic signs of inflammation, including presence of macrophages, lymphocytes, or neutrophils in alveoli and alveolar ducts (cells involved in the inflammatory response) as well as the presence of foreign materials.

Statistics

Data were tested for normality and homogeneity and, if needed, appropriate transformations were made. A one-way ANOVA was used to determine differences among doses for each endpoint using JMP 9 (SAS Institute Inc., Cary, NC). When significant differences were detected ($p < 0.05$), Dunnett's t-test was used to compare treatment groups to the 0 mg/kg group. All oxidative stress assays were repeated twice, lung histopathology was done once per CBN unit. Data are presented as averages (means) $\pm$ the standard error of the mean (SEM; the standard deviation divided by the square root of the sample size). The SEM does not reflect variability; rather it reflects whether the mean of a dosed group is reflective of the mean of the 0 mg/kg group.

Results

Oxidative Stress

Plasma *in vitro* ROS/RNS

No statistically significant changes were observed when compared to control in the amount of ROS/RNS in any of the seven CBN units and among treatment groups within single CBN units (Data Table A). However, free radical production in the 100 mg/kg TiO₂ group was increased by 219.9% relative to the 0 mg/kg group (**Figure 2.5**).

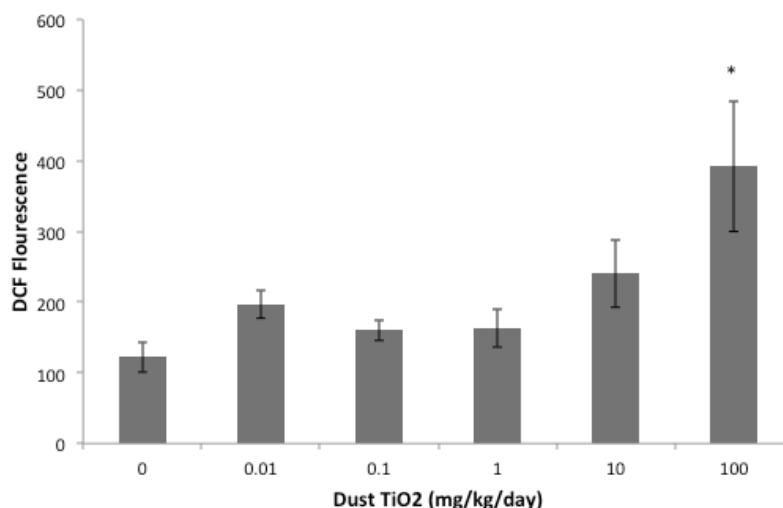


Figure 2.5. Plasma total free radicals in adult female B6C3F1 mice following oropharyngeal aspiration exposure to TiO₂ particles. Data are presented as mean $\pm$ SEM. Data presented are representative of two trial days. The (*) indicates data significantly different from the 0 mg/kg group ($p < 0.05$) and was determined from log transformed data. Sample size for each group was 2-3 plasma samples. 2', 7'-dichlorodihydrofluorescein (DCF) fluorescence intensity is proportional to the total ROS/RNS levels within the sample.

Plasma Superoxide Dismutase Activity

A statistically significant increase in SOD activity was observed in mice exposed to dust samples from CBN 4 (**Figure 2.6**). Activity in the 10 mg/kg group was increased by 14.3% and by 12.2% in the 100 mg/kg group relative to the 0 mg/kg group. No other statistical changes were observed for any other CBN unit or for the TiO₂ group (**Data Table B – 2.3**).

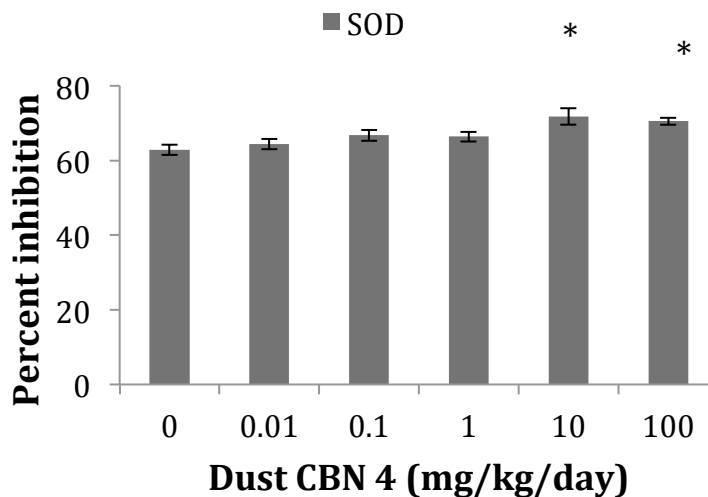


Figure 2.6. Plasma superoxide dismutase activity in adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust from CBN 4. Data are presented as mean $\pm$ SEM. Data presented are representative of two trial days. Sample size for each group was 5-6 plasma samples. The (*) indicates data significantly different from the 0 mg/kg group ($p < 0.05$) and was determined from ranked transformed data.

Plasma Total Antioxidant Capacity

A statistically significant decrease in total antioxidant capacity was detected in mice exposed to dust samples from CBN 6 (**Figure 2.7**). On average, capacity in the 0.1, 1, and 100 mg/kg groups was decreased by 26% relative to the 0 mg/kg group. No other statistical changes were observed for any other CBN unit or for the TiO₂ group (**Data Table C – 2.4**).

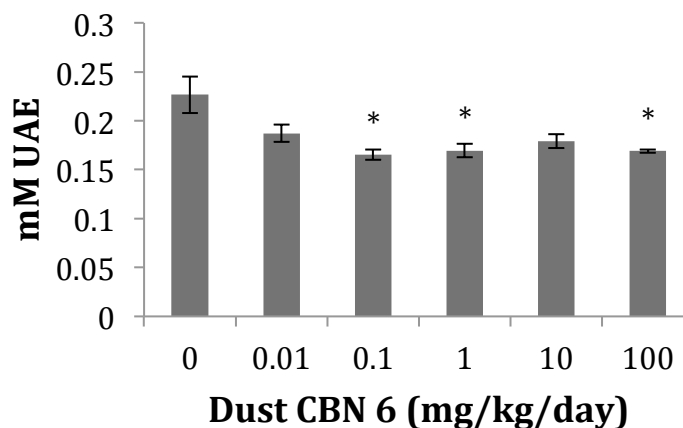


Figure 2.7. Plasma total antioxidant capacity in adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust from CBN 6. Data are presented as mean $\pm$ SEM. Data presented are representative of two trial days. Sample size for each group was 11-12 plasma samples. The (*) indicates data significantly different from the 0 mg/kg group ($p < 0.05$) and was determined from ranked transformed data. UAE-Uric Acid Equivalents. UAE sample values are equivalent to the sample's total antioxidant capacity.

PRBC Lysate Total Glutathione (GSSG/GSH)

No statistical differences were observed in GSSG/GSH from mice exposed to dust samples from CBN 2, 3, 5, or 6, or to TiO₂ (**Data Table D – 2.5**). In mice exposed to 10 mg/kg of dust sample from CBN 1, a 25.1% decrease in GSSG/GSH was observed relative to the 0 mg/kg group. In mice exposed to 0.01 or 10 mg/kg of dust sample from CBN 7, a 10% decrease in GSSG/GSH was observed relative to the 0 mg/kg group (**Figure 2.8A**). A 22.4% increase relative to the 0 mg/kg group was observed in mice exposed to 10 mg/kg of dust sample from CBN 4 (**Figure 2.8B**).

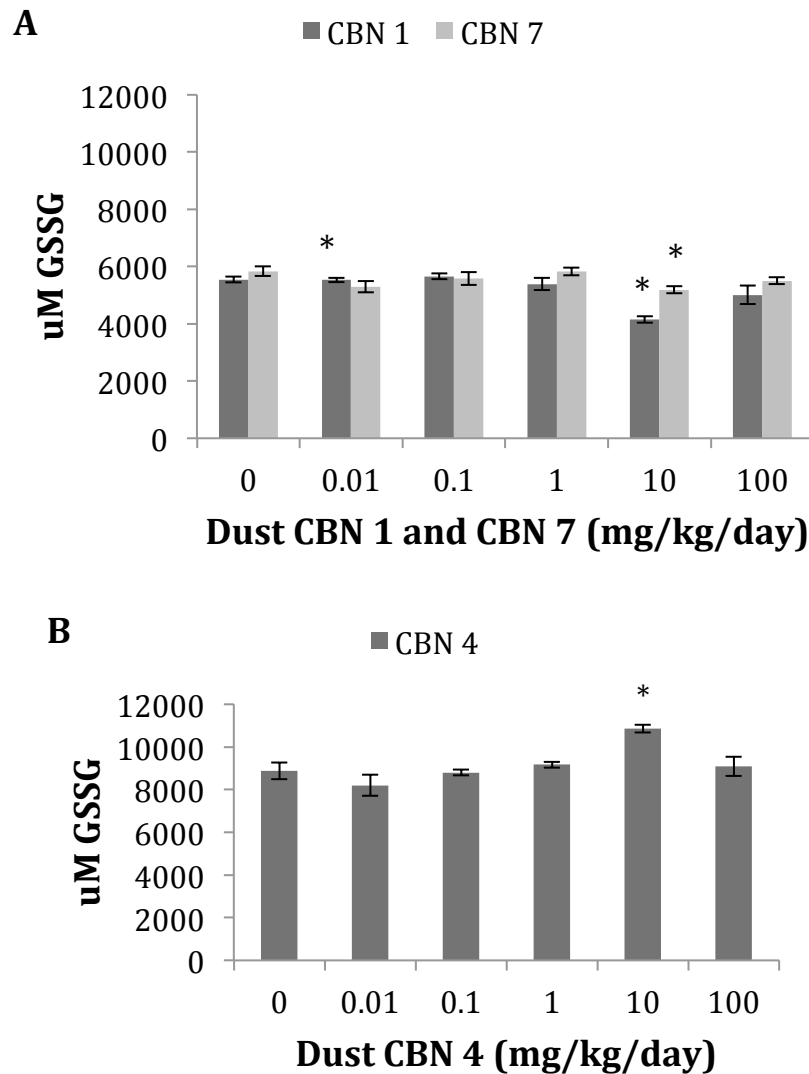


Figure 2.8. Total Glutathione in an packed red blood cell (PRBC) lysate from adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust from CBN 1 or CBN 7 (A) or from CBN 4 (B). Data are presented as mean $\pm$ SEM. Data presented are representative of two trial days. Sample size for each group was 11-12 (CBN 1 and CBN 7) or 6 (CBN 4) plasma samples. The (*) indicates data significantly different from the 0 mg/kg group ($p < 0.05$) and was determined from ranked transformed data.

Lung Histopathology

Lungs from mice exposed to dust samples from CBN units had signs of lung inflammation, generally at the highest doses administered (**Table 2.1**). The inflammation was characterized by infiltration of macrophages, lymphocytes, and/or neutrophils in alveoli and alveolar ducts. The inflammation most often was around the terminal bronchioles, alveolar ducts, and alveoli (termed “centriacinar inflammation”). Occasionally, the inflammation was characterized by perivascular or peribronchial cell infiltration (movement of inflammatory cells from outside of the lung into the lung); however, this was minimal to mild when observed. Lung foreign material was occasionally observed in macrophages and appeared to be mineral in origin. Based on the mean severity score, the potency of the CBNs to induce inflammation was CBN 5 > CBN 1 > CBN 3 > CBN 7 > CBN 6 > CBN 4 > CBN 2. Based on the number of lungs per dose exhibiting inflammation, the potency of the CBNs was CBN 1 > CBN 3 > CBN 5 > CBN 7 > CBN 6 CBN 4 > CBN 2.

Table 2.1. Summary of incidence and mean severity of inflammation and presence of foreign material in lungs from mice exposed to dust samples from NDRA.

<i>Inflammation (mean severity grade)*</i>						
<i>Combined map unit</i>	0 mg/kg	0.01 mg/kg	0.1 mg/kg	1 mg/kg	10 mg/kg	100 mg/kg
CBN1	-	-	-	0.2 (1/6)	0.7 (4/6)	1.0 (4/6)
CBN2	-	-	-	-	-	0.2 (1/6)
CBN3	0.2 (1/5)	0.3 (1/4)	0.2 (1/6)	-	0.3 (1/6)	0.7 (3/6)
CBN4	-	-	-	-	0.3 (2/6)	-
CBN5	-	-	-	0.3 (1/4)	0.7 (2/6)	1.0 (2/4)
CBN6	-	-	-	-	0.2 (1/6)	0.4 (1/5)
CBN7	-	-	-	0.2 (1/5)	0.3 (2/6)	0.2 (1/5)
<i>Foreign material**</i>						
<i>Combined map unit</i>	0 mg/kg	0.01 mg/kg	0.1 mg/kg	1 mg/kg	10 mg/kg	100 mg/kg
CBN1	-	-	-	-	3/6	3/6
CBN2	-	-	-	-	-	1/6
CBN3	-	-	1/6	-	1/6	3/6
CBN4	-	1/5	-	-	2/6	-
CBN5	-	-	-	1/4	2/6	2/4
CBN6	1/6	-	-	-	1/6	1/5
CBN7	-	-	-	-	-	1/5

*Inflammation was graded as mild (1), slight/mild (2), moderate (3), moderately severe (4), or severe (5). Values for each dose represent the total of the severity grade for each lung adjusted by the total number of lungs examined in that dose. Numbers in parentheses represent the number of lungs for that dose exhibiting inflammation.

**Foreign material is presented as the total number of lungs for that dose with visible foreign material out of the total number of lungs examined for that dose.

Discussion

Multiple heavy metals can be found in each of the CBN units and exposure to each of these metals has been associated with some degree of toxicity in humans. Inhalation of cadmium is known to cause pulmonary inflammation, cell damage, kidney damage, and alteration of systemic immune function (Blum et al., 2014; Ju et al., 2012). Manganese readily crosses the blood-brain barrier and accumulates in specific regions, thus the primary organ of concern for manganese toxicity is the brain (Aschner et al.,

2005; Klaassen et al., 2013; Guilarte, 2010). Neurological manifestation of manganese toxicity is referred to as manganism and produces a Parkinson's like disease. Arsenic is a known human carcinogen that is widespread in the environment with extensive health effects reported following exposure. Arsenic increases oxidative stress, upregulates proinflammatory cytokines and inflammatory mediators, and inactivates a form of an enzyme known as nitric oxide synthase, which is involved in vascular function. An increase in oxidative stress leads to DNA damage, chromosomal changes, interference with cellular signaling pathways, and ultimately, cancer (Singh, et al., 2011). Arsenic exposure depletes antioxidant enzymes such as glutathione (Carter et al., 2003), leading to subsequent damage by oxidative stress. Inorganic arsenic is classified as a human carcinogen causing tumors of the skin, lung, urinary bladder, and possibly liver (IARC, 2004). Chromium is readily absorbed in the lungs, and when associated with strontium, the solubility is increased (ATSDR, 2004). Inhalation exposure of hexavalent chromium is strongly linked to health problems such as ulceration of the septum, epistaxis, dyspnea, increased risk of death due to noncancerous respiratory disease, lung fibrosis, abdominal pain, cirrhosis, and increased risk of respiratory, bone, prostate, hematopoietic, stomach, kidney, and bladder cancer (ATSDR, 2012; Klaassen et al., 2013; Costa et al., 2006; Gatto et al., 2010). Risks of deleterious effects due to chromium exposure are exacerbated in those who exhibit chromium sensitivity. The International Agency for Research on Cancer (IARC) recognizes hexavalent chromium as a carcinogen via inhalation. Soluble strontium is readily absorbed in the lungs and accumulates in the

bones much like calcium. Bone growth problems can be seen in children with high levels of strontium, but only rarely (ATSDR, 2004).

Exposure to particulate matter of a size less than 10 μm (PM_{10}) is associated with increased daily deaths and mortality from cardiovascular and respiratory illnesses (Samet et al., 2000, Katsouyanni et al., 2001), decreased pulmonary function in adults (Boezen et al., 1998) and bronchial hyper-responsiveness in children with an increased risk of lower respiratory symptoms (Boezen et al., 1998). More recently, particulate matter exposure has been linked with microglial activation, neurotoxicity, and triggering of the production of free radicals associated with oxidative stress (MohanKumar et al., 2008; Risom et al., 2005; Xia et al., 2005). The focus of many of these studies has been on urban sources of particulate matter, particularly pollution. However, few studies have focused on particulate matter and dust exposure from non-urban sources and very little is known about the potential exposure levels and associated health effects of being exposed to natural dust while riding an ORV.

While there were no significant differences in the amount of free radicals present from the 0 mg/kg/day control group to the treatment groups (0.01-100 mg/kg/day) in each map unit, levels of free radicals were increased by more than 200% in the highest exposure group of TiO_2 . This indicates that exposure to 100 mg/kg of TiO_2 increased the amount of reactive oxygen or nitrogen species in the plasma. It has been demonstrated in the literature that zinc, as a redox inactive metal, has anti-oxidative properties (Powell, 2000; Prasad, 2009). Therefore, it is possible that the amount of Zn present on the dust

samples collected from NDRA was in great enough quantity to counter act any free radicals produced by the particulate matter and/or metals present on the dust.

The ability of inhalation exposure to TiO₂ to produce free radicals is inconsistent in the literature. Dick et al. (2003) reported that TiO₂ failed to produce significant free radical activity in primary rat alveolar macrophages after intratracheally-instilling 125µg of ultrafine TiO₂. However, LeBlanc et al. (2010) reported an increase in microvascular ROS production in rat coronary arterioles after exposure to ultrafine TiO₂ in an inhalation chamber with only 10µg being instilled into the lungs. Although these are only two studies, they are indicative of a literature base that indicates that in some tissues and systems, TiO₂ exposure may increase ROS whereas in others, it may not. In our study, exposure to TiO₂ increased only one marker of oxidative stress and only at the highest dose, so it is difficult to definitely assert that exposure to this “neutral” particle increases the risk of oxidative stress and damage. An increase in this plasma marker of oxidative stress suggests exposure to particles alone at high level (100 mg/kg/day) may induce oxidative stress, but additional work is required to determine if this stress is associated with damage to specific tissues or processes.

The response observed for TiO₂ was not reciprocated with dust samples collected from across the NDRA, suggesting that all particulate matter does not induce the same effects at equivalent doses. The increase in SOD activity and total glutathione for CBN 4 indicates an increase in plasma antioxidants for the groups dosed with 10 mg/kg/day; however when related back to the results of the total antioxidant capacity assay for CBN

4, it is apparent that while specific thiols are increased, the total number of total antioxidants in the plasma remains similar to the responses in the 0 mg/kg group.

Additionally, the time at which we measured oxidative stress, one day after the final exposure, may have been insufficient for capturing an active disturbance to the redox balance. Individual metals in the dust samples are known to increase oxidative stress, but perhaps one day after final exposure was too soon to measure those changes and too late after the earlier exposures. The redox balance is constantly shifting and the timing of measurements can impact detection of oxidative species and antioxidant enzymes. Also, we measured for systemic (blood) markers of oxidative stress. Alternatively, modeling oxidative stress in specific organs can be tested (Hays, 2006; Imai, 2008; Mittal and Flora, 2006; Resende, 2008; Shina, 2008). Lungs were not chosen as a tissue to measure oxidative stress because they were used for histopathology. While oxidative damage may have occurred at specific sites, such as the lungs, the blood may not reflect systemic change in antioxidants and free radicals. However, the lung histopathology results indicate inflammatory changes in the lungs (discussed below) and inflammation is a common effect of oxidative stress. So, although we did not detect changes in free radicals and antioxidant enzymes, inflammatory changes in the lungs can be indicative as a change to the redox balance. Systemic and organ specific effects related to immunotoxicological and neurotoxicological markers were seen in the mice as described in Chapters 10-16.

A previous study of dust samples from surface units 2.2, 3.1, and 3.2 (not combined) of the NDRA demonstrated that three consecutive days of exposure to each of

these map units resulted in histopathological changes in the lungs, but only at the highest administered doses of 100 and 1,000 mg/kg (Proper et al., 2011). The current study also found histopathological changes indicative of inflammation, with four exposures spaced a week apart and at lower doses. All CBNs, with the exception of CBN 4, induced inflammation in at least one animal exposed to 100 mg/kg of dust sample and with the exception of CBN 2, all CBNs induced inflammation in at least one animal exposed to 10 mg/kg of dust sample. In CBN 4, two animals showed signs of inflammation after exposure to 10 mg/kg of dust sample, but no other concentrations from CBN 4 induced inflammation. In terms of the average severity grade, which was averaged for all mice in a group, CBN 5 induced the most severe inflammation; however inflammation was never greater than slight/mild for any CBN. Based on the mean severity score, the potency of the CBNs to induce inflammation was CBN 5 > CBN 1 > CBN 3 > CBN 7 > CBN 6 > CBN 4 > CBN 2. Based on the number of lungs per dose exhibiting inflammation, the potency of the CBNs was CBN 1 > CBN 3 > CBN 5 > CBN 7 > CBN 6 CBN 4 > CBN 2. These inflammatory changes in the lungs indicate that geomorphology and/or the concentration of metals in a particular CBN unit may induce differential effects after an exposure similar to the one in this study.

Overall, results of assays to evaluate markers of oxidative stress indicate that no single surface type was able to consistently induce markers of oxidative stress at a particular dose or in a dose-responsive manner. The two highest concentrations of dust from CBN 4 were able to increase two markers of oxidative stress, but results of other surface types were inconsistent. All surface types were able to induce some level of lung

inflammation, typically at the highest doses. These results indicate that exposure to these natural dusts, under the exposure scenario of our study, are unlikely to dramatically increase the risk of oxidative damage systemically, but are likely to induce some level of localized inflammation in lungs of exposed individuals and that this level of inflammation is influenced by the geomorphology and/or the concentration of metals in a particular CBN unit.

SUPPLEMENT

2.2 Data Table A. Plasma <i>In Vitro</i> Reactive Oxygen and Nitrogen Species			
CBN Unit	TRT group (mg/kg/day)	Mean $\pm$ SEM	Significant (y/n) *
CBN 1	0	477.99 $\pm$ 90.00	na
	0.01	454.77 $\pm$ 78.42	n
	0.1	231.83 $\pm$ 49.03	n
	1	358.81 $\pm$ 48.11	n
	10	439.78 $\pm$ 82.19	n
	100	255.21 $\pm$ 52.88	n
CBN 2	0	85.63 $\pm$ 7.81	na
	0.01	105.78 $\pm$ 11.16	n
	0.1	87.90 $\pm$ 8.38	n
	1	88.09 $\pm$ 12.39	n
	10	94.63 $\pm$ 6.92	n
	100	83.65 $\pm$ 7.20	n
CBN 3	0	130.53 $\pm$ 14.87	na
	0.01	117.03 $\pm$ 15.92	n
	0.1	122.27 $\pm$ 12.68	n
	1	91.04 $\pm$ 7.08	n
	10	97.84 $\pm$ 9.83	n
	100	111.06 $\pm$ 12.58	n
CBN 4	0	147.58 $\pm$ 11.73	na
	0.01	143.28 $\pm$ 9.68	n
	0.1	134.03 $\pm$ 14.77	n
	1	143.82 $\pm$ 17.02	n
	10	144.82 $\pm$ 12.25	n
	100	144.61 $\pm$ 15.75	n
CBN 5	0	357.41 $\pm$ 77.76	na
	0.01	268.33 $\pm$ 42.89	n
	0.1	216.97 $\pm$ 35.01	n
	1	184.89 $\pm$ 28.84	n
	10	265.16 $\pm$ 59.73	n
	100	214.86 $\pm$ 23.31	n
CBN 6	0	470.48 $\pm$ 67.06	na
	0.01	660.81 $\pm$ 36.03	n
	0.1	408.55 $\pm$ 102.52	n
	1	339.89 $\pm$ 64.41	n
	10	454.91 $\pm$ 76.57	n
	100	464.38 $\pm$ 54.70	n
CBN 7	0	357.06 $\pm$ 25.65	na
	0.01	325.25 $\pm$ 31.84	n
	0.1	320.96 $\pm$ 25.93	n
	1	456.36 $\pm$ 85.12	n
	10	318.90 $\pm$ 33.19	n
	100	327.07 $\pm$ 29.26	n
* significance indicates a response statistically different from the control group (p< 0.05) y=yes, n=no.			

2.3 Data Table B. Plasma Superoxide Dismutase Activity			
CBN Unit	TRT group (mg/kg/day)	Mean $\pm$ SEM	Significant (y/n)*
CBN 1	0	58.38 $\pm$ 1.54	na
	0.01	62.30 $\pm$ 2.14	n
	0.1	65.18 $\pm$ 2.54	n
	1	61.78 $\pm$ 2.40	n
	10	57.05 $\pm$ 2.51	n
	100	63.97 $\pm$ 2.29	n
CBN 2	0	68.42 $\pm$ 2.41	na
	0.01	72.38 $\pm$ 0.73	n
	0.1	70.54 $\pm$ 1.84	n
	1	70.55 $\pm$ 1.54	n
	10	73.59 $\pm$ 1.64	n
	100	72.32 $\pm$ 1.28	n
CBN 3	0	74.86 $\pm$ 2.43	na
	0.01	75.69 $\pm$ 0.48	n
	0.1	74.30 $\pm$ 1.87	n
	1	75.35 $\pm$ 0.57	n
	10	75.08 $\pm$ 1.48	n
	100	78.04 $\pm$ 1.16	n
CBN 4	0	62.84 $\pm$ 1.36	na
	0.01	64.37 $\pm$ 1.45	n
	0.1	66.70 $\pm$ 1.44	n
	1	66.37 $\pm$ 1.30	n
	10	71.81 $\pm$ 2.24	y
	100	70.52 $\pm$ 0.97	y
CBN 5	0	71.88 $\pm$ 1.09	na
	0.01	77.24 $\pm$ 1.47	n
	0.1	72.33 $\pm$ 2.60	n
	1	73.29 $\pm$ 1.03	n
	10	74.24 $\pm$ 1.77	n
	100	76.22 $\pm$ 1.63	n
CBN 6	0	77.35 $\pm$ 2.06	na
	0.01	77.75 $\pm$ 1.54	n
	0.1	75.01 $\pm$ 1.22	n
	1	76.15 $\pm$ 1.09	n
	10	77.75 $\pm$ 1.46	n
	100	76.43 $\pm$ 1.10	n
CBN 7	0	65.75 $\pm$ 2.54	na
	0.01	69.56 $\pm$ 1.52	n
	0.1	70.83 $\pm$ 1.61	n
	1	69.63 $\pm$ 1.69	n
	10	68.28 $\pm$ 1.31	n
	100	71.54 $\pm$ 1.13	n
* significance indicates a response statistically different from the control group (p< 0.05) y=yes, n=no.			

2.4 Data Table C. Plasma Total Antioxidant Capacity				
CBN Unit	TRT group (mg/kg/day)	Mean $\pm$ SEM		Significant (y/n)*
CBN 1	0	0.178 $\pm$	0.007	na
	0.01	0.168 $\pm$	0.009	n
	0.1	0.167 $\pm$	0.012	n
	1	0.169 $\pm$	0.008	n
	10	0.178 $\pm$	0.009	n
	100	0.177 $\pm$	0.007	n
CBN 2	0	0.172 $\pm$	0.007	na
	0.01	0.152 $\pm$	0.010	n
	0.1	0.151 $\pm$	0.007	n
	1	0.155 $\pm$	0.010	n
	10	0.154 $\pm$	0.004	n
	100	0.159 $\pm$	0.004	n
CBN 3	0	0.190 $\pm$	0.006	na
	0.01	0.179 $\pm$	0.005	n
	0.1	0.192 $\pm$	0.007	n
	1	0.193 $\pm$	0.006	n
	10	0.185 $\pm$	0.005	n
	100	0.187 $\pm$	0.003	n
CBN 4	0	0.168 $\pm$	0.008	na
	0.01	0.151 $\pm$	0.008	n
	0.1	0.166 $\pm$	0.007	n
	1	0.169 $\pm$	0.006	n
	10	0.168 $\pm$	0.008	n
	100	0.164 $\pm$	0.003	n
CBN 5	0	0.214 $\pm$	0.008	na
	0.01	0.208 $\pm$	0.007	n
	0.1	0.221 $\pm$	0.013	n
	1	0.210 $\pm$	0.011	n
	10	0.203 $\pm$	0.010	n
	100	0.220 $\pm$	0.014	n
CBN 6	0	0.227 $\pm$	0.019	na
	0.01	0.187 $\pm$	0.009	n
	0.1	0.165 $\pm$	0.005	y
	1	0.170 $\pm$	0.007	y
	10	0.179 $\pm$	0.007	n
	100	0.169 $\pm$	0.002	y
CBN 7	0	0.185 $\pm$	0.010	na
	0.01	0.170 $\pm$	0.008	n
	0.1	0.183 $\pm$	0.005	n
	1	0.174 $\pm$	0.009	n
	10	0.163 $\pm$	0.007	n
	100	0.154 $\pm$	0.016	n
* significance indicates a response statistically different from the control group (p< 0.05) y=yes, n=no.				

2.5 Data Table D. Erythrocyte Total Glutathione			
CBN Unit	TRT group (mg/kg/day)	Mean $\pm$ SEM	Significant (y/n)*
CBN 1	0	5546.57 $\pm$ 108.25	na
	0.01	5527.63 $\pm$ 72.39	n
	0.1	5652.94 $\pm$ 102.46	n
	1	5385.64 $\pm$ 207.48	n
	10	4152.78 $\pm$ 117.61	y
	100	5008.68 $\pm$ 319.34	n
CBN 2	0	5476.86 $\pm$ 112.85	na
	0.01	5205.25 $\pm$ 129.44	n
	0.1	5362.32 $\pm$ 123.12	n
	1	5352.27 $\pm$ 147.66	n
	10	5345.66 $\pm$ 86.49	n
	100	5126.66 $\pm$ 127.04	n
CBN 3	0	5180.64 $\pm$ 94.03	na
	0.01	5010.04 $\pm$ 118.64	n
	0.1	4697.63 $\pm$ 220.29	n
	1	4506.35 $\pm$ 250.97	n
	10	4744.55 $\pm$ 117.63	n
	100	5052.85 $\pm$ 203.24	n
CBN 4	0	8873.19 $\pm$ 390.60	na
	0.01	8198.08 $\pm$ 486.70	n
	0.1	8798.96 $\pm$ 133.81	n
	1	9162.85 $\pm$ 138.50	n
	10	10860.15 $\pm$ 188.82	y
	100	9087.90 $\pm$ 447.21	n
CBN 5	0	7402.76 $\pm$ 306.17	na
	0.01	7357.76 $\pm$ 230.65	n
	0.1	7376.69 $\pm$ 236.83	n
	1	7419.38 $\pm$ 180.30	n
	10	6590.02 $\pm$ 217.18	n
	100	6958.38 $\pm$ 236.16	n
CBN 6	0	7701.21 $\pm$ 141.91	na
	0.01	7408.03 $\pm$ 225.52	n
	0.1	7315.39 $\pm$ 118.17	n
	1	7788.79 $\pm$ 96.86	n
	10	7893.70 $\pm$ 340.74	n
	100	8016.62 $\pm$ 101.05	n
CBN 7	0	5830.86 $\pm$ 166.01	na
	0.01	5297.23 $\pm$ 198.65	y
	0.1	5579.73 $\pm$ 224.62	n
	1	5824.67 $\pm$ 131.73	n
	10	5186.67 $\pm$ 130.78	y
	100	5501.25 $\pm$ 114.99	n
* significance indicates a response statistically different from the control group (p< 0.05) y=yes, n=no.			

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CHAPTER 3

LYMPHOCYTE SUBPOPULATIONS FOLLOWING SUB-ACUTE EXPOSURE TO
DUST COLLECTED FROM NELLIS DUNES RECREATION AREAIntroduction

As outlined in Chapter 1, the Nellis Dunes Recreational Area (NDRA) is a popular off-road-vehicle (ORV) riding area for both tourists and residents of southern Nevada, with annual visitors estimated at over 300,000 (Goossens and Buck, 2009). It is located in the northeastern corner of the Las Vegas valley in the Mojave Desert. Off-road vehicle driving is one of the most prevalent and fastest growing recreational activities on public lands worldwide (Outdoor World Directory, 2010), and the number of off-road drivers in southern Nevada has quadrupled in the last few years (Spivey, 2008). People have been driving at NDRA for over 40 years, and the area offers many different types of landscapes: sand dunes, badlands, dry drainages, and other typical desert terrain in its 36 km² area. The Bureau of Land Management (BLM) manages the NDRA. Research performed within the NDRA has shown large amounts of dust are emitted year round from this site and that some of this dust blows into the surrounding cities (Goossens and Buck, 2011).

This study as a whole was initiated due to concerns about riders and other visitors inhaling and ingesting dust at NDRA, as it is known to contain high concentrations of arsenic, with levels reaching over 300 ppm in areas (Soukup et al., 2012). At NDRA, both natural wind erosion and ORV activities cause dust emission (Goossens et al.,

2012). Dust in natural settings is often a complex mixture of organic and mineral compounds with highly variable chemical compositions and particle sizes ranging from nanometers to several tens of micrometers. Smaller particles, such as PM_{2.5}, are able to reach very deep within the lung and therefore are of greater concern for public health. The immune system is sensitive to heavy metal exposure, and with the dust found at NDRA carrying large amounts of heavy metals; we set to determine the effects of inhalation exposure on the immune system. While many assay endpoints were used to determine immunotoxicity for this study, including body weight, organ weight, immune organ cellularity, immunophenotyping, IgM plaque forming cells, and natural killer cell activity, only the results from immunophenotyping will be discussed within chapter 3 of this thesis.

There are two types of lymphocytes in the immune system, T lymphocytes (T cells) and B lymphocytes (B cells), each playing a critical role in the foundation of the immune system. Foreign antigens bind to the B cell receptor (BCR) on the surface of B cells and from there the lymphocyte will proliferate and differentiate into a plasma cell, which produces antibodies. Memory B cells are formed to target specific antigens to assist in adaptive immunity. T cells will proliferate and differentiate into effector T cells after coming into contact with a foreign antigen. Effector T cells broadly function to kill, regulate, and activate cells. These include T helper cells, which assist cells of the immune system with their functions, produce cytokines, and are CD4⁺; cytotoxic T cells, which are CD8⁺, kill virally infected and tumor cells; and regulatory T cells, which suppress other lymphocytes and are crucial for immunological tolerance. Like memory B cells,

memory T cells are long-lived antigen specific cells providing adaptive immunity to a reoccurring antigen.

The surface marker B220 is the mouse analog to the human CD45R. This protein functions as a signal transducer and is a pan B cell marker present from early pro-B cells onward. B220 is also present on activated T cells (Renno et al., 1998). The surface cell receptors CD4 and CD8 were used to identify T cells. T cells begin their maturation process in the thymus as double negative, or CD4-CD8-, cells and then mature to double positive, or CD4+CD8+. In healthy humans, 5% of the cells in the thymus are double negative, whereas 80% are double positive (Travers et al., 2012). After the double positive stage the T cells will mature into singly positive cells (CD8+ only or CD4+ only) that leave the thymus. CD4 is a co-receptor for T cell receptors and recognizes antigens bound to class II MHC molecules. As mentioned previously, CD4+ T cells differentiate into effector T cells that aid in B cell antibody production, secrete some cytokines, and activate macrophages. CD8 is also a co-receptor for T cell receptors and recognizes antigens bound to class I MHC molecules. CD8+ T cells differentiate into cytotoxic T cells that kill other cells, particularly those with intracellular pathogens.

Regulatory T cells (Tregs) are a specialized subset of T lymphocytes responsible for suppression of immune responses that are deleterious to the host organism. They are characterized by high surface expression of CD25 (the IL-2 receptor alpha chain) and intracellular expression of the master switch transcription factor forkhead box protein P3 (FoxP3) (Fontenot et al., 2003). By inhibiting activation, expansion, and function of other T cells within the host, Tregs maintain the homeostasis necessary to prevent the host

immune system from overreacting to self-antigens but still maintain a suitable defense against invasive pathogens and tumors. Disruption of this process can lead to autoimmune disease. Regulatory T cells that do not express FoxP3 have been shown to suppress auto-inflammatory disease in mice through an IL-10 independent mechanism (Travers et al., 2012).

Arsenic exposure in mice inhibits T cell proliferation and macrophage activity, decreases CD4 splenic cells (Soto-Pena et al., 2006), suppresses proliferative lymphocytic responses (Biswas et al., 2008), decreases IL-2 production (Biswas et al., 2008) and decreases Treg numbers specifically via oxidative stress (Thomas-Schoemann et al., 2012). In addition, chromium and cobalt are associated with CD8 lymphopenia (Hart et al., 2009).

Quantitation of splenic B and T lymphocytes using cell surface markers is considered a tier II procedure for detecting immune alterations following drug or chemical exposures in rodents, and is considered by the USEPA to be part of a testing battery for full immunotoxicological evaluation of potential risks associated with a toxic substance (Luster et al., 1992; EPA, 1996). Additionally, the analysis of lymphocyte subpopulations has been shown to have the highest association with predicting immunotoxicity, with an 83% concordance (Luster et al., 1992). In addition to the quantification of standard splenic B and T lymphocytes, we quantified thymic T lymphocytes and splenic Tregs. B lymphocytes were quantified using B220 as a surface marker, T-lymphocytes were quantified using CD4 and CD8 as surface markers, and

CD4, FoxP3, CD25, and IL-17 surface markers were used to quantify Tregs. All lymphocyte quantification was done using flow cytometry at the UNLV flow lab.

Methods

Preparation and Verification of Dust Dosing Samples

Dust samples were carefully labeled, stored in sealed and dry containers, protected from light, and secured in a lock box in the laboratory. A stock dosing solution was prepared and administered to mice within 1-2 hours of preparation. All CBN dust samples were prepared in sterile, endotoxin-free, phosphate buffered saline (ETF-PBS) at concentrations of 0.01, 0.1, 1, 10, or 100 mg of dust/kg of body weight. To verify that adding the dust samples to PBS did not substantially alter the solubility of elements, a stability study was performed with the lowest concentration (0.01 mg/kg) and a higher concentration (10 mg/kg). Dust samples were added to ETF-PBS to ascertain stable time frames in which the solution could be used for animal exposures. Solutions were prepared and samples of the solutions were collected immediately after preparation, and then at 1, 2, 4, and 6 hours. Samples were immediately centrifuged, supernatants removed, and examined immediately using an ICP-MS to quantitate total soluble element concentrations. The analysis indicated that leaving the dust samples in an ETF-PBS solution for up to six hours did not substantially change the distribution of elements in solution (data not shown). After six hours, soluble element concentrations in supernatant began to increase. That is, the amount of insoluble versus soluble remained constant for six hours in solution. We did not test for changes in speciation, but only total values. This

additional quality control measure verified our dosing solution concentrations, potential for flux, and accounted for potential contamination from PBS or other steps in our preparation process. To control for contamination in this preparation process, no metal spatulas or any other metal items were used for weighing, storage, manipulation, or transport of dust samples. Samples were immediately centrifuged, supernatants removed, and examined immediately using an ICP-MS to quantitate total soluble element concentrations.

Animals and Animal Exposures

Mice were obtained from Charles River Laboratories and were acclimated to the conditions of the treatment room (12-h light/dark cycle, 22 ± 2 °C, 60-65% relative humidity) at the UNLV animal facilities, which are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All experiments were approved by the UNLV Institutional Animal Care and Use Committee (IACUC). Mice were housed in ventilated polycarbonate shoebox cages with corncob bedding and were given unlimited access to food and water.

To simulate the potential health impacts of a month of weekend exposures to dust from the NDRA, adult female B6C3F1 mice were exposed to dust samples at 0, 0.01, 0.1, 1.0, 10, or 100 mg/kg of body weight once weekly for four weeks. Mice in the 0 mg/kg group received PBS only and served as a control. To ensure availability of tissue for toxicology assays, each dose group contained 12 mice housed six per cage. In addition, three separate groups of mice were used for a total of three replicates for each dose. Samples for toxicity studies were collected from each group three days in a row. **Table**

3.1 depicts the basic arrangement for replicates and sample collection.

12 mice per dose, three replicates Dose groups (mg/kg): 0, 0.01, 0.1, 1, 10, 100		
Replicate 1: <u>Set A: 6 mice/dose</u> Collect: blood, lungs and brains for histology, and organ weights (liver, spleen, brain, kidney) <u>Set B: 6 mice/dose</u> Collect: blood, lung weights, immune organs (spleen and thymus)	Replicate 2: <u>Set A: 6 mice/dose</u> Collect: blood, brains for histology, and organ weights (liver, lungs, spleen, brain, kidney) <u>Set B: 6 mice/dose</u> Collect: blood and immune organs (spleen and thymus)	Replicate 3: <u>Set A: 6 mice/dose</u> Collect: blood and organs for metal/metalloid analysis (liver, spleen, brain, kidney, thymus, lungs) <u>Set B: 6 mice/dose</u> Collect: blood and immune organs (spleen and thymus)

Table 3.1. Toxicology sample collection arrangement.

Mice were exposed by oropharyngeal aspiration, a procedure that allows the mice to “gasp” the dust-solution into their lungs. Briefly, mice were weighed, slightly anesthetized with isofluorane gas, and then suspended by their front incisors from a surgical thread strung across an intubation board. This permits the lower jaw to hang open and the tongue to slightly protrude. The appropriate amount of dosing solution, based on the body weight of the mouse being dosed, was drawn up into a pipette, the tongue was gently grasped and slightly pulled out of the mouth with a pair of straight forceps, and the dosing solution was dispensed into the back of the mouse’s throat. The tongue was let go and an audible gasp from the mouse verified that the dosing solution was inhaled. Mice were then placed into a recovery cage until they awakened from the anesthesia before being placed back into home cages. An average volume of 10 μ L was administered to each mouse; however, based on individual body weight changes, this

volume was adjusted to between 9-13 μ L per mouse.

Toxicity Studies

One day after the final dose was delivered, samples for toxicity studies were collected from animals euthanized by carbon dioxide asphyxiation. As depicted in **Table 3.1**, several groups of mice/dose were used to ensure adequate tissue and sera for all assays.

Body Weight, Organ Weights, and Immune Organ Cellularity

Body weight was monitored weekly during the study and terminal body weights were collected for all animals the day of euthanasia. Brain, kidney, liver, lung, spleen, and thymus were removed and weighed. Weights were adjusted for terminal body weights to determine absolute and relative organ weights. The total number of splenic and thymic lymphocytes (immune cells) was determined for each organ. Spleens and thymuses were suspended in complete medium (RPMI, 10% fetal calf serum, 50 IU penicillin and 50 μ g streptomycin) and were aseptically processed into single-cell suspensions by gentle grinding between two sterile, frosted microscope slides. An aliquot of each spleen or thymus suspension was manually counted on a hemocytometer to determine the number of live cells (viability of cells for each organ was generally greater than 95%). The total number of cells per spleen and thymus (cellularity), adjusted by the weight of each organ, was determined for each animal from Set B (see **Table 3.1**).

B Lymphocytes and CD4/CD8 Lymphocytes

The number of splenic B cells (B220) and splenic and thymic T cells (CD4+, CD8+, CD4+/CD8+, and CD4-/CD8-) were counted in single-cell suspensions of spleens and thymuses, diluted to a concentration of 1×10^7 cells/mL. Optimal concentrations of antibodies and reagents were determined in previous experiments. All experimental replicates included isotype controls (to estimate non-specific binding), unstained cells as negative controls, and single color controls as positive controls to determine color compensation. Flow cytometric analysis was performed using a BD FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA) and 10,000 events were collected from each sample. The total number of each cell type was determined from the spleen or thymus cellularity.

Regulatory T Lymphocytes (Tregs)

Splenic lymphocytes were added to 96-well round-bottomed plates (Nunc) at a concentration of 1×10^6 cells per well in RPMI medium containing 10% fetal bovine serum, 50 IU penicillin and 50 μ g streptomycin. Spleen cells were depleted of red blood cells via a 5-minute incubation in NH_4Cl lysis buffer at 37°C. Monoclonal antibodies coupled to fluorochromes specific for the following markers were used at a concentration of 1 μ g/ 10^6 cells: anti-mouse CD25-FITC, rat IgG1-PE isotype control, rat IgG2b-AF647 isotype control, and rat IgG2b-FITC isotype control (BD Pharmingen, San Diego, CA, USA). FoxP3, CD4, and IL-17 cells were stained using a commercial kit (BD Pharmingen, San Diego, CA, USA or eBiosciences, San Diego, CA, USA) according to manufacturer's instructions. Briefly, cells were fixed for 30 minutes in BD Pharmingen

Mouse FoxP3 Fixation Buffer at 4 °C. After a wash step, cells were permeabilized for 30 minutes at 37 °C in BD Pharmingen Mouse FoxP3 Permeabilization buffer. Following two wash steps, cells were stained with 20 µL Mouse Th17/Treg phenotyping cocktail. Anti-mouse CD25-FITC was added to wells following the Treg cocktail; appropriate positive, negative, and isotype controls were added to wells containing cells only. Cell suspensions were incubated for 30 minutes at room temperature in the dark. Following two wash steps, cells were resuspended in Analysis Buffer and stored at 4 °C in the dark prior to flow cytometry. Treg subsets were quantified using a BD FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA). 10,000 events were acquired for each sample. CD4⁺ lymphocytes in the lymphocyte fraction were gated, and the percentages of CD25⁺foxP3⁺ cells, CD25⁺foxP3⁻ cells, IL-17⁺, and IL-17⁻ cells were calculated.

Particle Positive Control

To help to separate “elemental effects” from “particle effects”, separate groups of mice were exposed to titanium dioxide (TiO₂; a “neutral” particle that contains no associated heavy metals and is less than 100 nm in size) at the same concentrations and via the same exposure paradigm as mice exposed to dust samples from NDRA combination units. Procedures described above were followed for these mice, with the exception of the Treg assay.

Statistical Analysis

Data were tested for normality and homogeneity and, if needed, appropriate transformations were made. A one-way analysis of variance (ANOVA) was used to

determine differences among doses for each endpoint using JMP 9 (SAS Institute Inc., Cary, NC) in which the standard error used a pooled estimate of error variance. When significant differences were detected by the F-test ($p < 0.05$), Dunnett's t-test was used to compare treatment groups to the control group.

Results

Dust Characterization

Total elemental concentration of dust samples from the combination units used in this study had a median diameter of 4.17 μm . Total digestion chemical compositions of the dust samples are shown in **Table 3.2**. Concentrations of aluminum, vanadium, manganese, iron, zinc, and strontium were particularly high for CBN 4 relative to most of the other CBN map units, and the highest concentration of arsenic was seen in CBN 5.

Table 3.2. Total elemental concentration ($\mu\text{g/g}$ in dry sample) of dusts collected from combination (CBN) map units (CBN) of the Nellis Dunes Recreation Area.

Sample Name	Median*	Al	V	Cr	Mn	Fe	Co	Cu	Zn
CBN 1	4.39	55090	70	33	511	21595	9.4	69	79
CBN 2	4.49	59377	74	39	387	26658	8.2	25	106
CBN 3	4.13	59993	62	44	552	28564	11	32	94
CBN 4	4.05	79651	100	54	752	33266	14	37	135
CBN 5	4.6	74530	99	43	357	33313	9.7	33	124
CBN 6	3.14	47692	56	35	376	21208	8.3	28	71
CBN 7	4.37	73239	103	50	294	36340	10	30	97
Sample Name	Median*	As	Sr	Cd	Sb	Cs	Tl	Pb	U
CBN 1	4.39	62	618	<0.47	<3.0	13	<8.3	25	4.7
CBN 2	4.49	137	424	<0.47	<3.0	26	<8.3	23	8.6
CBN 3	4.13	17	328	<0.47	<3.0	9.3	<8.3	23	2.2
CBN 4	4.05	71	666	<0.47	<3.0	15	<8.3	34	4.9
CBN 5	4.6	227	321	<0.47	<3.0	22	<8.3	44	13
CBN 6	3.14	24	445	<0.47	<3.0	9.4	<8.3	23	2.3
CBN 7	4.37	24	285	<0.47	<3.0	24	<8.3	26	5.1

* Median diameter (μm)

CBN 1 Immunophenotype

Neither the total number of splenic B cells nor the total number of splenic and thymic T cells was statistically changed by exposure to dust samples from CBN 1 (**Figures 3.1A-C**). The number of CD4+CD25+foxP3- Tregs was statistically reduced at all administered doses (**Figure 3.1D**). All dosed groups were decreased by 88.4%, on average, relative to numbers in the 0 mg/kg group.

CBN 2 Immunophenotype

No change was observed in splenic B and T lymphocytic subpopulations, or splenic B220 numbers following exposure to NDRA dust from CBN 2 (**Figure 3.2**). Significant decreases respective to the 0 mg/kg group in thymic CD4+/8+ subpopulations occurred following 0.01 (36.8%) and 100 (42.4%) mg/kg exposure to CBN 2 dust, but this was not dose-responsive. A 37.9% decrease as compared to the 0 mg/kg group was also observed in the 100 mg/kg exposure in the thymic CD4+ subpopulation.

The absolute number of splenic CD4+CD25-foxP3+ Tregs were statistically decreased by 37.1% as compared to the 0 mg/kg group in the 100 mg/kg exposure group (**Figure 3.2D**). Splenic CD4+CD25+foxP3- Tregs were statistically decreased by 74.2%, 63.4%, 67.2%, and 62.1% as compared to the 0 mg/kg group in the 0.01, 0.1, 10, and 100 mg/kg exposure groups, respectively.

CBN 3 Immunophenotype

The number of B cells and regulatory T cells (Tregs) from the spleen was not statistically altered by exposure to dust samples from CBN 3 (**Figures 3.3A and 3.3D**). The number of T cells from the spleen and the thymus was statistically altered by exposure to dust samples from CBN 3. In the spleen, CD4+, CD8+, and CD4+/CD8+ T cells were reduced by about 30% following exposure to 10 and 100 mg/kg relative to the 0 mg/kg group. In the thymus, all four subtypes of T cells (CD4+, CD8+, CD4+/CD8+, and CD4-/CD8-) were reduced by 37.4%, on average, following exposure to 100 mg/kg relative to the 0 mg/kg group. CD8+ and CD4+/CD8+ T cells also were reduced by about 30% following exposure to 0.01 and 10 mg/kg relative to the 0 mg/kg group.

Additionally, CD4+/CD8+ T cells were reduced by 21.6% after exposure to 0.1 mg/kg relative to the control group.

CBN 4 Immunophenotype

The number of B cells and regulatory T cells (Tregs) from the spleen and the number of T cells from the thymus was not statistically altered by exposure to dust samples from CBN 4 (**Figures 3.4A, 3.4C, and 3.4D**). The number of T cells from the spleen was statistically altered by exposure to dust samples from CBN 4 (**Figure 3.4B**). In the spleen, CD4+, CD8+, and CD4+/CD8+ T cells were reduced by about 30% following exposure to 10 and 100 mg/kg relative to the control group. CD8+ and CD4+/CD8+ T cells also were reduced by about 30% following exposure to 0.01 and 10 mg/kg relative to the control group. Additionally, CD4+/CD8+ T cells were reduced by 21.6% after exposure to 0.1 mg/kg relative to the control group.

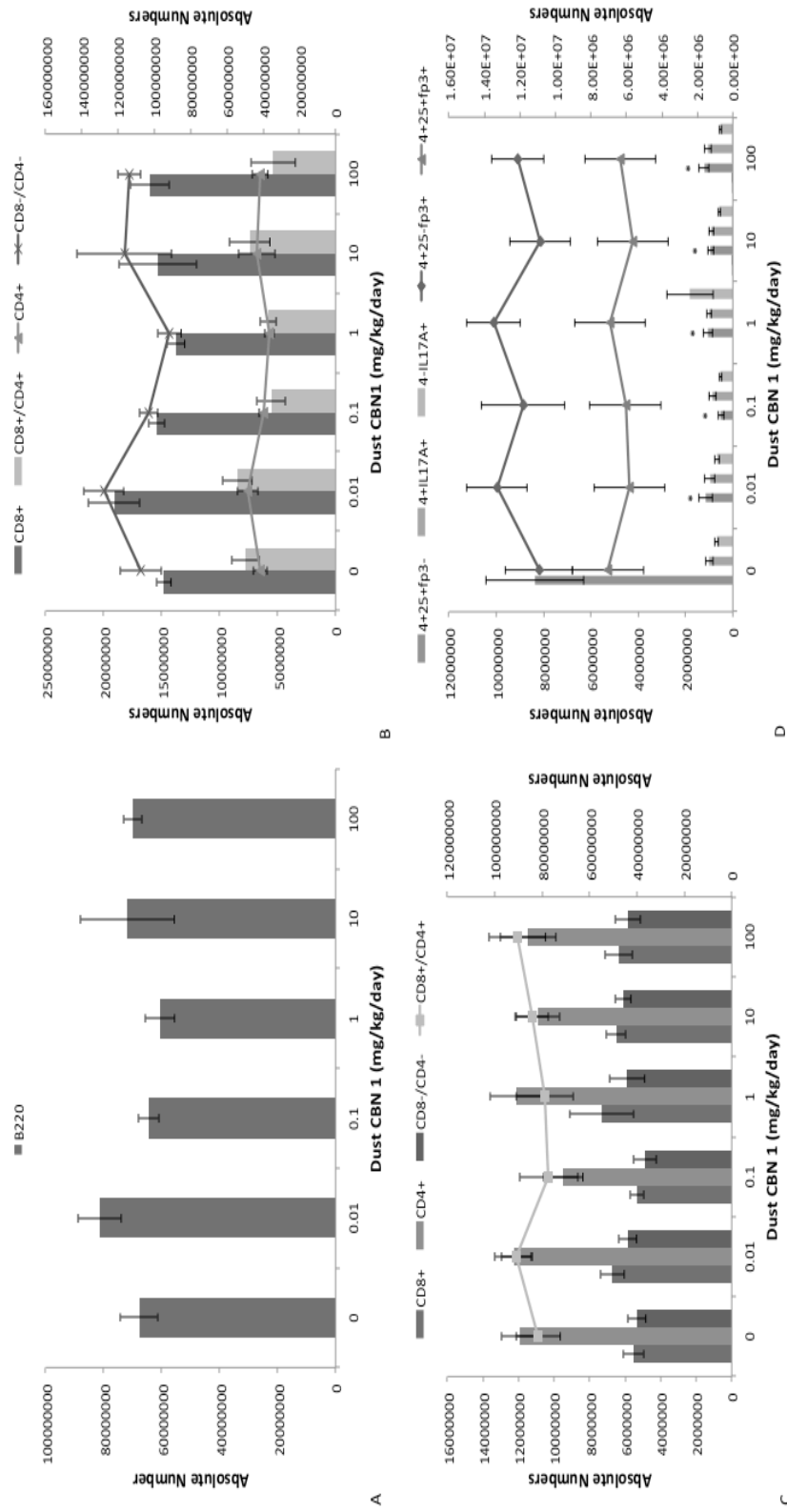


Figure 3.1A-D. Spleen and thymus B and T cell lymphocytes in adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust samples from NDRA combination unit CBN 1 (vegetated and non-vegetated sand dunes). Data are presented as mean $\pm$ standard error of the mean. Sample size for each group was 5-6 animals. Data presented are representative of three trial days. The (*) indicates a response statistically different from the control group ($p < 0.05$) and was determined from log transformed data. A) Splenic B cells. B) Splenic T cells (CD4+ and CD8-/CD4- on secondary axis). C) Thymic T cells (CD8+/CD4+ on secondary axis). D) Splenic regulatory T cells (4+/25-/fp3+ and 4+25+/fp3+ on the secondary axis).

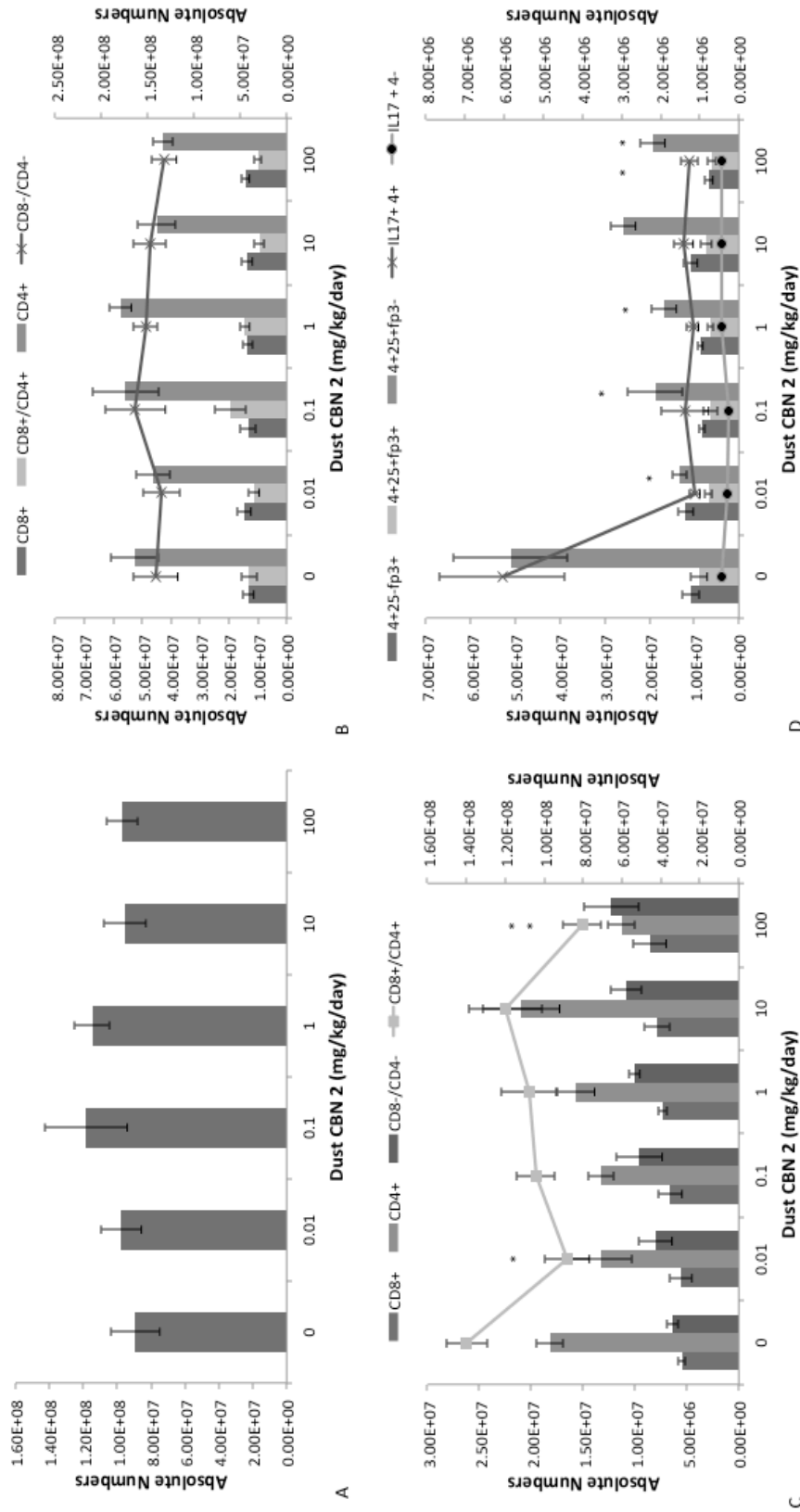


Figure 3.2A-D. Spleen and thymus B and T cell lymphocytes in adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust samples from NDRA combination unit CBN 2. Data are presented as mean $\pm$ standard error of the mean. Sample size for each group was 5-6 animals. Data presented are representative of three trial days. The (*) indicates a response statistically different from the control group ($p < 0.05$) and was determined from log transformed data. A) Splenic B cells. B) Splenic T cells (CD8-/CD4- on the secondary axis). C) Thymic T cells (CD8+/CD4+ on the secondary axis). D) Splenic regulatory T cells (IL17+4- on the secondary axis).

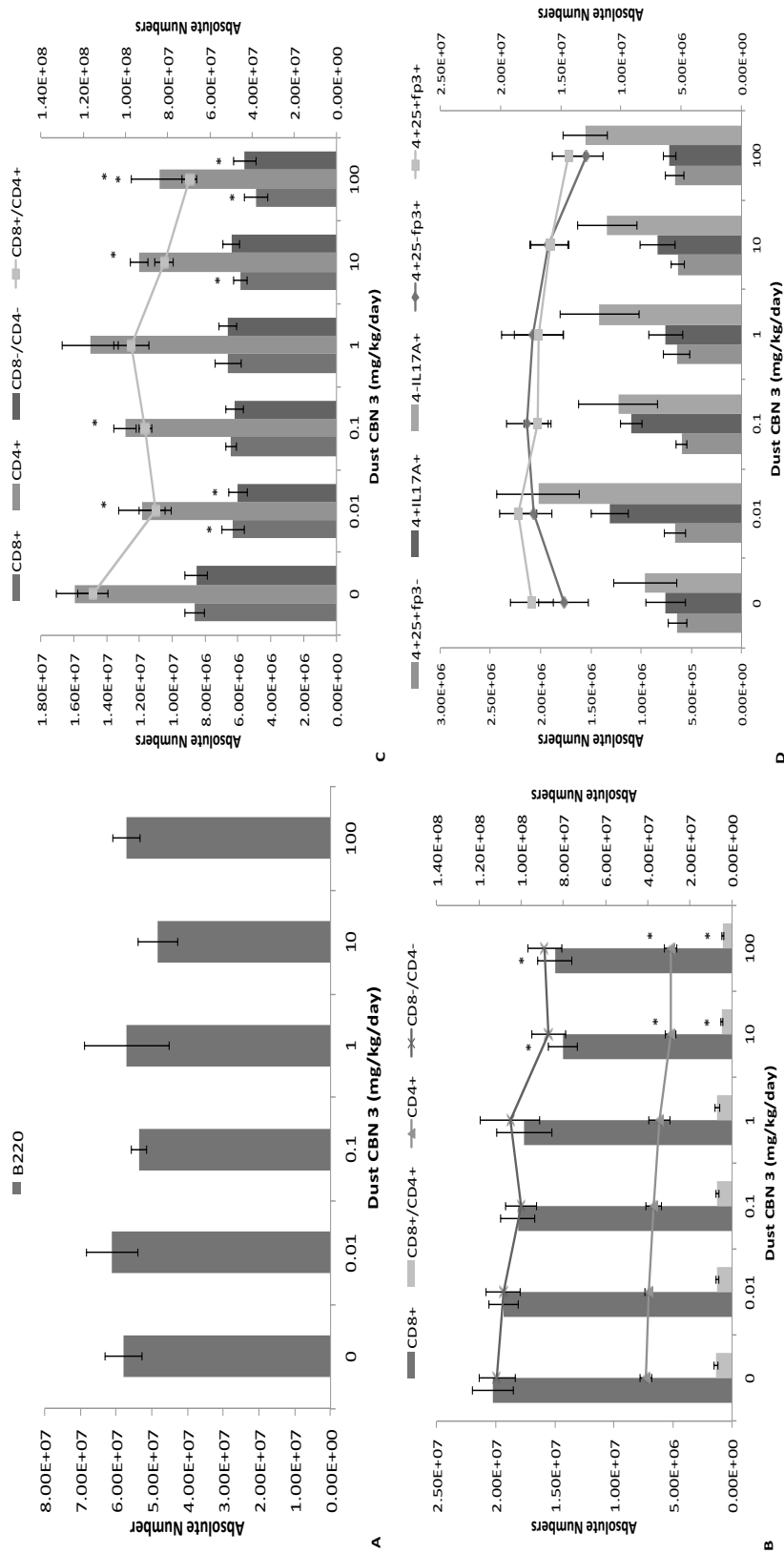


Figure 3.3A-D. Spleen and thymus B and T cell lymphocytes in adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust samples from NDRA surface units 3.1-3.5 combined ("CBN 3"). Data are presented as mean $\pm$ standard error of the mean. Sample size for each group was 5-6 animals. Data presented are representative of three trial days. The (*) indicates a response statistically different from the 0 mg/kg group ($p < 0.05$) and was determined from log transformed data. A) Splenic B cells. B) Splenic T cells (CD4+ and CD8-/CD4- on the secondary axis). C) Thymic T cells (CD8+/CD4+ on the secondary axis). D) Splenic regulatory T cells (4+23-fp3+ and 4+25-fp3+ on the secondary axis).

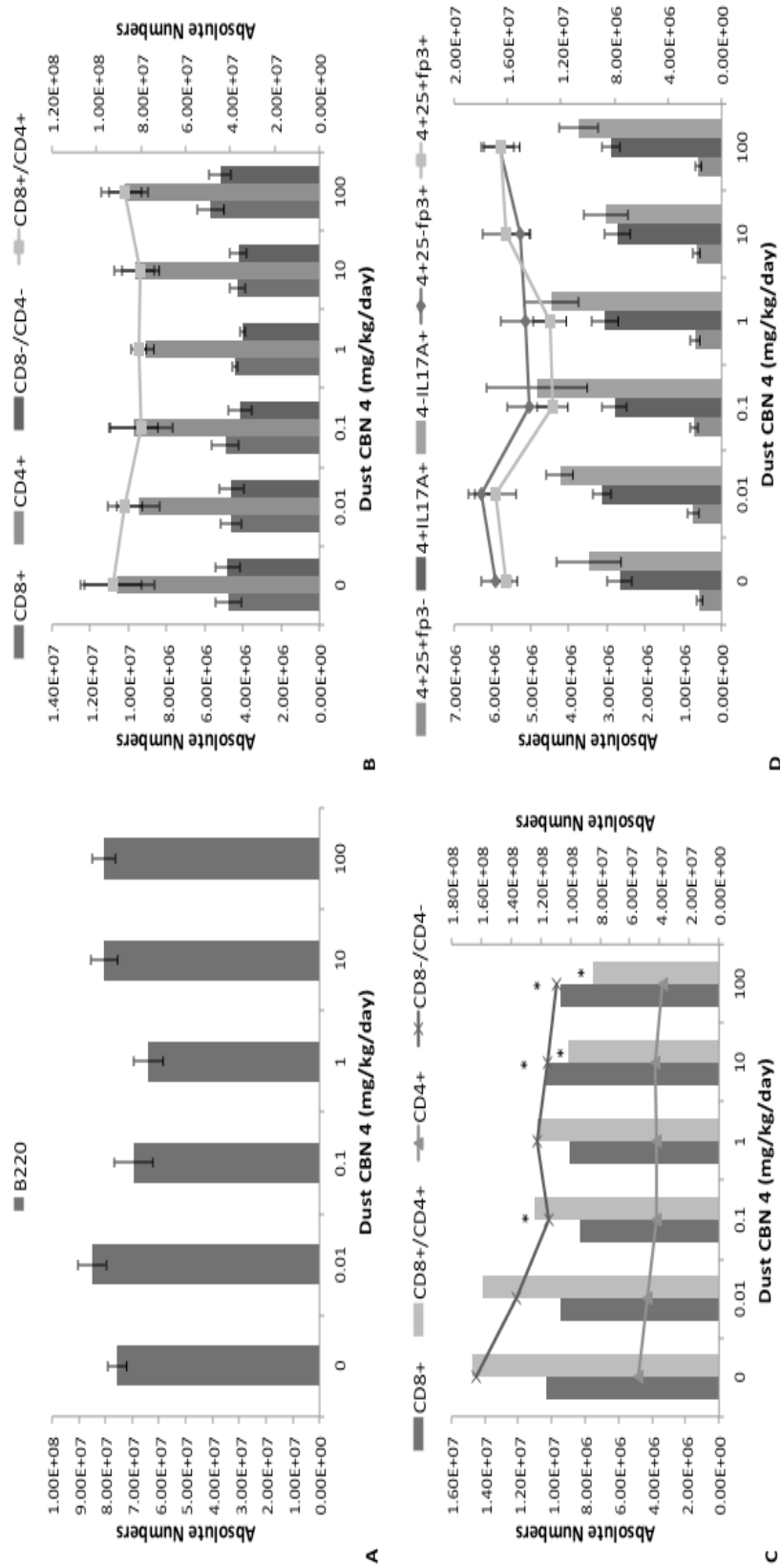


Figure 3.4A-D. Spleen and thymus B and T cell lymphocytes in adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust samples from NDRA surface units 4.1-4.3 and 2.1 combined ("CBN 4"). Data are presented as mean $\pm$ standard error of the mean. Sample size for each group was 5-6 animals. Data presented are representative of three trial days. The (*) indicates a response statistically different from the 0 mg/kg group ($p < 0.05$) and was determined from log transformed data. A) Splenic B cells. B) Splenic T cells (CD8+/CD4+ on the secondary axis). C) Thymic T cells (CD4+ and CD8-/CD4- on the secondary axis). D) Splenic regulatory T cells (4+25+fp3+ and 4+25-fp3+ on the secondary axis).

CBN 5 Immunophenotype

No change was observed in splenic and thymic B and T lymphocytic subpopulations or in splenic B220 numbers following exposure to NDRA dust from CBN 5. Additionally, no significant changes were observed in the spleen T-regulatory cell populations (**Figure 3.5**).

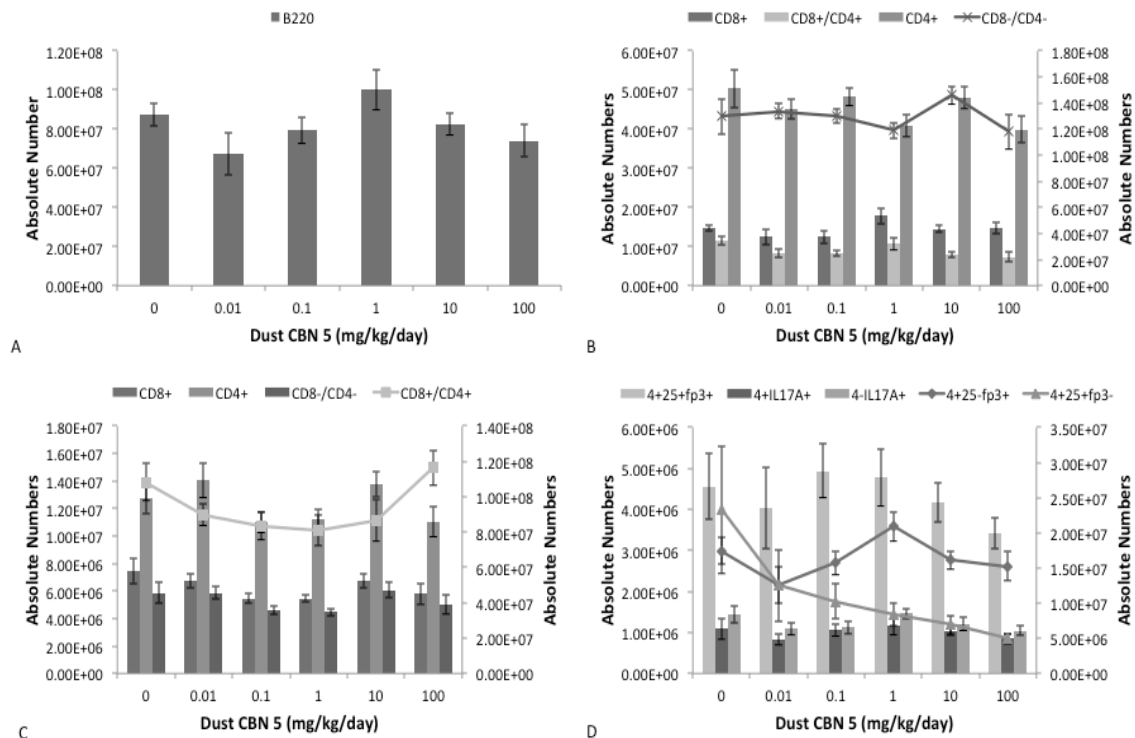


Figure 3.6. Splenic B and T cell lymphocytes in adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust samples from NDRA (“CBN5”). Graph A depicts absolute numbers of B220 lymphocytes in spleen. Graph B depicts splenic CD4/8 lymphocytic subpopulations with CD8-/CD4- on the secondary axis. Graph C depicts thymus CD4/8 lymphocytic subpopulations with CD8+/CD4+ on the secondary axis, while Graph D represents regulatory T cell populations in the spleen with 4+25-fp3+ and 4+25+fp3- on the secondary axis. Data are presented as mean $\pm$ standard error of the mean. Sample size for each group was 5-6 animals. Data presented are representative of three trial days.

CBN 6 Immunophenotype

No change was observed in splenic and thymic B and T lymphocytic subpopulations or in splenic B220 numbers following exposure to NDRA dust from CBN 6. The absolute number of splenic CD4+CD25+foxP3- Tregs was dose-responsively decreased beginning at the 0.1 mg/kg/day treatment group. As compared to control, the 0.1, 1, 10, and 100 mg/kg/day treatment groups were decreased by 76.2%, 82.4%, 88.9%, and 92.9%, respectively (**Figure 3.6**).

CBN 7 Immunophenotype

The number of B cells and regulatory T cells (Tregs) from the spleen and the number of T cells from the thymus was not statistically altered by exposure to dust samples from CBN 7 (**Figures 3.7A, C, D**). The number of T cells from the spleen was statistically altered by exposure to dust samples from CBN 7 (**Figure 3.7B**). In the spleen, CD4+, CD8+, and CD4+/CD8+ T cells were reduced by 35.6% to 56.8% following exposure to 10 and 100 mg/kg relative to the control group. In addition, CD4+ T cells were reduced by 35.3% and CD4+/CD8+ T cells were reduced by 62.1% following exposure to 1 mg/kg relative to the control group.

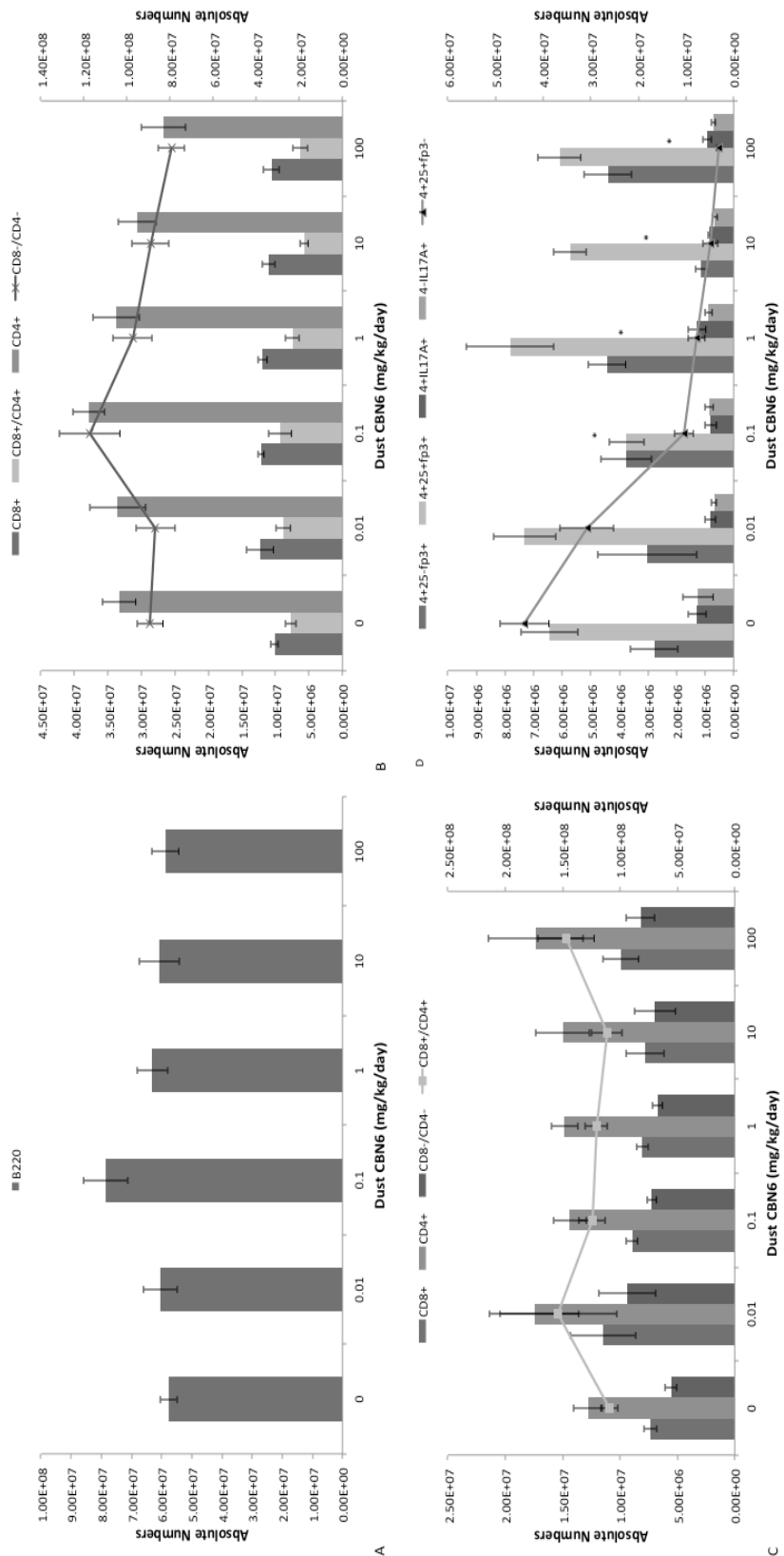


Figure 3.6. Splenic B and T cell lymphocytes in adult female B6C3F1 mice following OA exposure to dust samples from NDRA (“CBNG6”). (A) Absolute numbers of B220 lymphocytes in spleen. (B) Splenic CD4/8 lymphocytic subpopulations with CD8-/CD4- on the secondary axis. (C) Thymus CD4/8 lymphocytic subpopulations with CD8+/CD4+ on the secondary axis. (D) Treg cell populations in the spleen with 4+25+fp3- on the secondary axis. Data are presented as mean $\pm$ standard error of the mean. Sample size for each group was 5-6 animals. Data presented are representative of three trial days. The (*) indicates a response statistically different from the control group ($p < 0.05$).

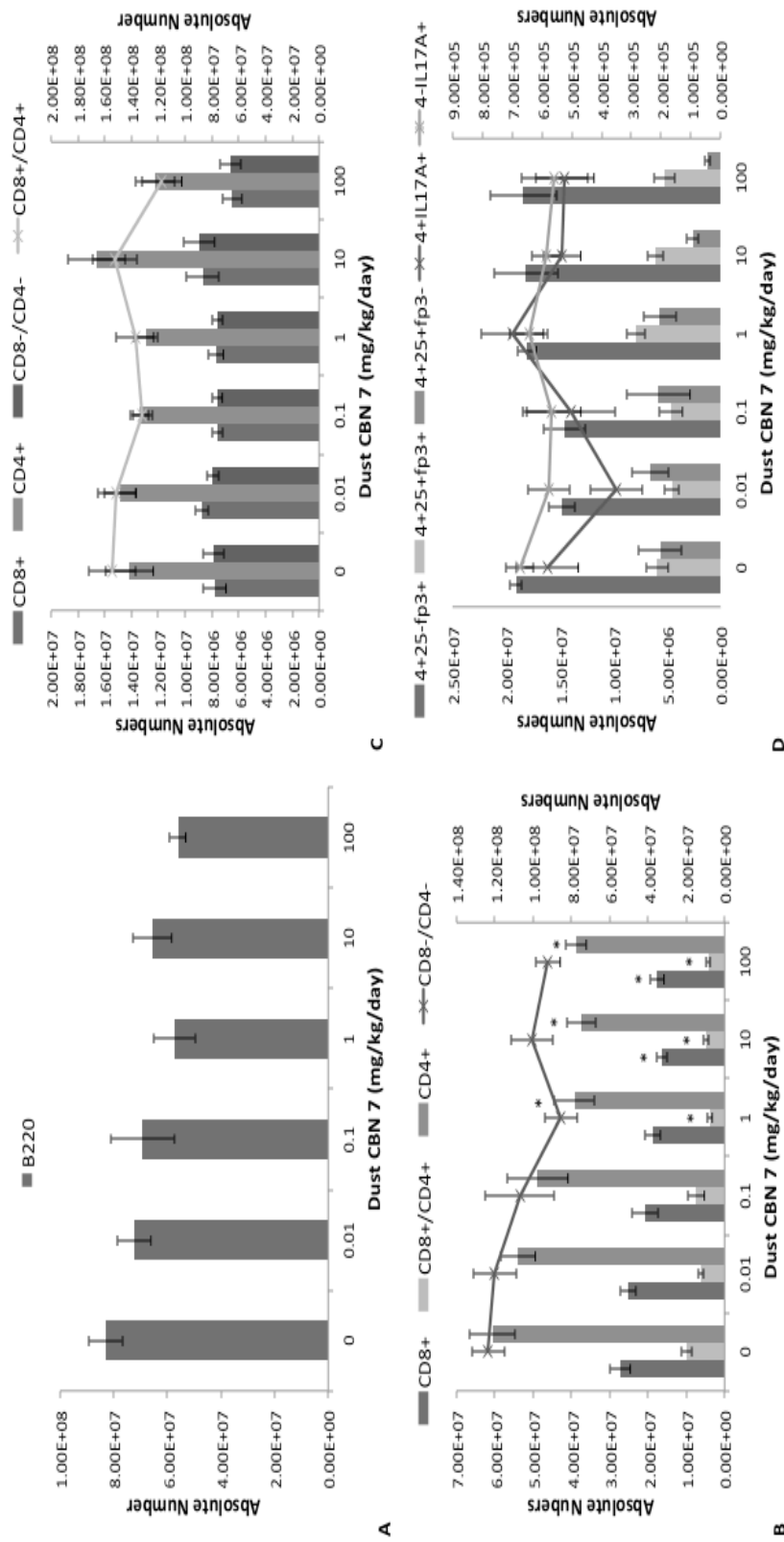


Figure 3.7. Spleen and thymus B and T cell lymphocytes in adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust samples from NDRA surface unit 2.3 (“CBN 7”). Data are presented as mean $\pm$ standard error of the mean. Sample size for each group was 5-6 animals. Data presented are representative of three trial days. The (*) indicates a response statistically different from the 0 mg/kg group ($p < 0.05$) and was determined from log transformed data. A) mean number of splenic B cells. B) mean number of splenic T cells (CD8-/CD4- on the secondary axis). C) mean number of thymic T cells (CD8+/CD4+ on the secondary axis). D) mean number of splenic regulatory T cells (4+IL17A+ and 4-IL17A+ on secondary axis).

TiO₂ Immunophenotype

No change was observed in splenic and thymic B and T lymphocytic subpopulations or in splenic B220 numbers following exposure to the particulate matter control, titanium dioxide (**Figure 3.8**).

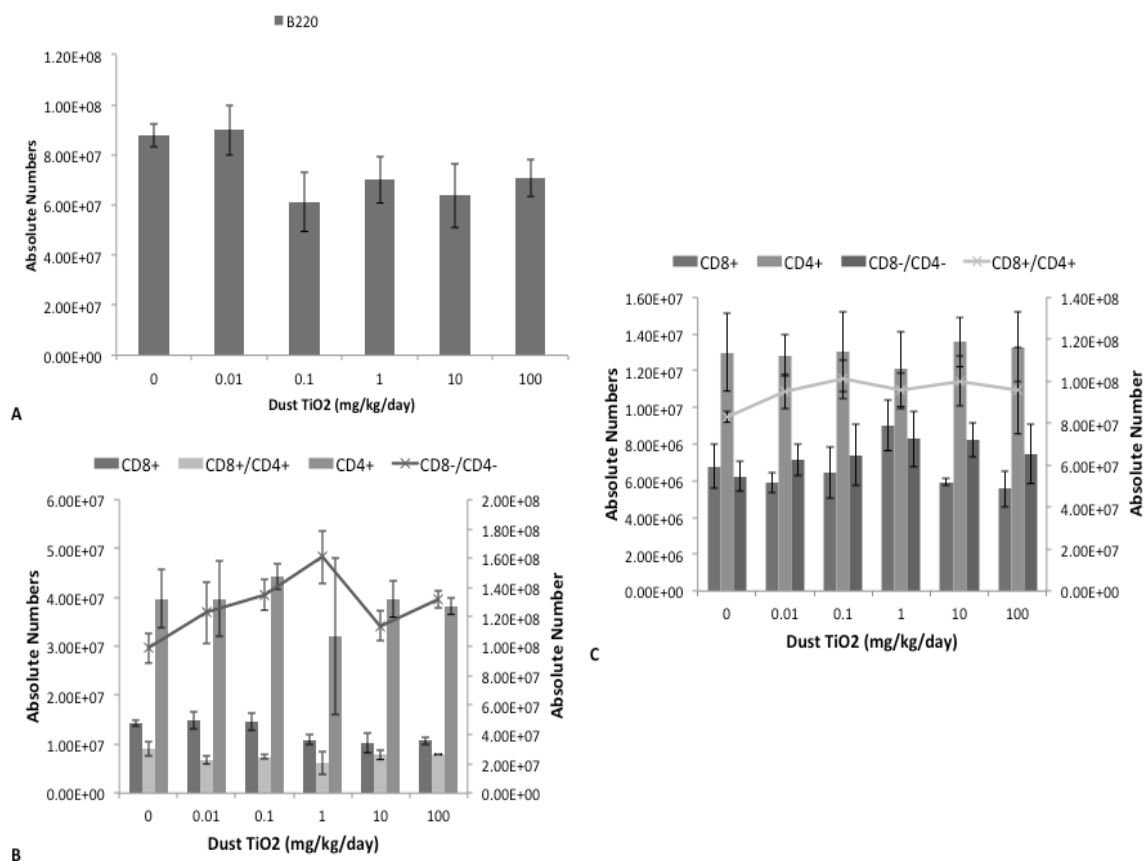


Figure 3.8. Spleen and thymus B and T cell lymphocytes in adult female B6C3F1 mice following oropharyngeal aspiration exposure to titanium dioxide. Data are presented as mean $\pm$ standard error of the mean. Sample size for each group was 3 animals. Data presented are representative of three trial days. A) mean number of splenic B cells. B) mean number of splenic T cells with CD8-/CD4- on the secondary axis. C) mean number of thymic T cells with CD8+/CD4+ on the secondary axis.

Effect Levels

Many assay endpoints were used to determine immunotoxicity for this study, including body weight, organ weight, immune organ cellularity, IgM plaque forming cells, and natural killer cell activity (Keil et al., 2014). As determined in Keil et al. (2014), exposure to natural dust collected from NDRA, given to mice in concentrations of 0.01 to 100 mg/kg had the potential to reduce the ability of the immune system to produce antibodies against a specific antigen. Several other assays also demonstrated effects of the dust exposure on responses in the immune and nervous systems as well as on lung histopathology, but only at higher concentrations. The lowest observed adverse effect level (LOAEL) and no observed adverse effect level (NOAEL) for each combination unit are outlined in **Table 3.3**. Based on the lack of statistical findings with a “neutral particle” (TiO₂), our results suggest that the concentrations of heavy metals or the combination of heavy metals with particulate matter in natural dust from the NDRA combination units are able to suppress antigen-specific antibody production. Exposure to natural dust from NDRA, at the concentrations and via a similar exposure paradigm as evaluated in this study and noted in **Table 3.3**, may alter the ability of organisms to produce an effective antibody response.

Table 3.3. Lowest observed adverse effect level (LOAEL) and no observed adverse effect level (NOAEL) for each combination unit following exposure to dusts collected from combination (CBN) map units of the Nellis Dunes Recreation Area as determined using the plaque forming cell assay as described in Keil et al. (2014) N/A – not applicable.

CBN unit	NOAEL (mg/kg)	LOAEL (mg/kg)
1	N/A	0.01
2	0.01	0.1
3	0.01	0.1
4	0.01	0.1
5	N/A	0.01
6	N/A	0.01
7	0.01	0.1

Discussion

Recently, arsenic has been shown to affect immune regulation specifically targeting Tregs. Hernandez-Castro and colleagues (2009) used a rat *in vivo* studies to demonstrate that Treg populations would increase in number with low doses of arsenic. However, the opposite was true when a threshold concentration of greater than 100 ug/L decreased Treg depletion in the spleen. A low dose of arsenic trioxide, which is used to treat acute promyelocytic leukemia, has been shown to decrease Treg numbers specifically via oxidative stress (Thomas-Schoemann et al., 2012). In addition to arsenic, vanadium has been shown to decrease expression of CD11c surface marker on mouse thymic dendritic cells following a 4-week exposure (Ustarroz-Cano et al., 2012). This has relevance in that an immature dendritic cell subset that is tagged with CD11c+ is important for the development and function of CD4+ CD25+ regulatory Tregs (Tarbell et al., 2006). Based on a vanadium exposure, it may be that maturation of Tregs is reduced

leading to fewer splenic 4+25+ cells as observed in CBN units 1, 2, and 6 of this experiment.

Dust exposures from three CBN units, 3, 4, and 7, produced significantly decreased populations of double positive splenocytes in the 10 and 100 mg/kg dosing groups, and including the 1 mg/kg dosing group in CBN 7. CBN 3 and 7 also showed a decrease in single positive splenocytes (CD8+ only and CD4+ only) in the 10 and 100 mg/kg dosing groups. Interestingly, CBN 3 double positive thymocytes were decreased in the 0.01, 0.1, 10, and 100 mg/kg dosing groups, a similar pattern also seen in the 0.01 and 100 mg/kg double positive thymocytes in CBN 2. All remaining significant results occurred sporadically or at the highest dosing group only.

Of the elements in the complex dust mixtures collected from NDRA, arsenic can reduce the CD4 lymphocyte count (Soto-Pena et al., 2006), suppress proliferative lymphocytic response, decrease IL-2 production (Biswas et al., 2008), and suppress lymphocytes (Arnold et al., 2006). Chromium has been shown to significantly reduce CD4+ lymphocytes in workers exposed to contaminated dusts (Boscolo et al., 1997). *In vitro*, cobalt has been shown to reduce proliferation of T lymphocytes and IL-2 release (ATSDR, 2004). And finally, multiple studies have described decreases in CD4+ cells following lead exposure (ATSDR, 2007).

Notably, the CBN unit with the highest amounts of arsenic, cobalt, and lead was CBN 5, which in turn produced no statistically significant aberrations in lymphocyte subpopulations. None of the aforementioned CBN units, that caused significant changes in lymphocyte populations, stood out among the units to have exceedingly large amounts

of metals previously identified to disrupt lymphocyte subpopulations. Based on the lack of statistical findings with a “neutral particle” (TiO₂), these results suggest that the concentrations of heavy metals, working individually or synergistically, or the combination of heavy metals with particulate matter in natural dust from NDRA are able to suppress some lymphocyte subpopulations. Suppression of lymphocyte subpopulations, particularly splenocytes, is a sensitive indicator of immunotoxicity in experimental animal systems and is suggestive of the risk of immunotoxicity in other exposed organisms, including humans (Luster et al., 1992; EPA, 1996). Consequently, exposure to natural dust from a wide range of sites within NDRA, at similar concentrations and via a similar exposure route as our animal studies, may decrease lymphocyte subpopulations in humans. However, from the abovementioned results, additional studies to identify the role of each metal in this mixture would be required to gain insight to how this would affect the immune system as a whole.

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CHAPTER 4

THESIS SYNTHESIS AND SUGGESTIONS FOR FUTURE WORK

This study was initiated due to concerns about exposure to dust at Nellis Dunes Recreation Area (NDRA) containing high concentrations of arsenic (up to 312 ppm; Soukup et al., 2012). The actual dust exposures found across NDRA are complex mixtures of (mostly) inorganic particulates, with varying amounts of heavy metals, some of which are known to be immunotoxic. Metals mixtures considered in this study include total amounts of arsenic, chromium, lead, strontium, manganese, iron, vanadium, nickel, zinc, cadmium, uranium, magnesium, and molybdenum. Within NDRA, the most important exposure routes for PM and heavy metals are inhalation and ingestion of airborne dust. Because there are currently no EPA regulations in the United States for heavy metal inhalation exposures in recreational settings, there was a need for a site-specific human health risk assessment to determine potential risk of recreation at NDRA (Soukup et al., 2012).

Research Implications

A human-health risk assessment has been performed and modeling results, using data not presented within this thesis, indicates lifetime cancer risks and non-cancer risks are below levels of concerns (Simon and Warren, 2014). However, lowest observed adverse effect levels (LOAEL) and no observed adverse effect levels (NOAEL) determined using a plaque forming cell assay indicate suppression of IgM antibody

responses at doses as low as 0.01 mg dust/kg body weight (Keil et al., 2014).

In the previous chapters we have explored the results following dose-responsive experiments of inhalation exposure to 7 types of dust from NDRA, specifically monitoring lymphocyte subpopulations and parameters indicative of oxidative damage. Oxidative stress assays suggest that no single CBN surface type was able to consistently induce markers of oxidative stress at a particular dose or in a dose-responsive manner. These observations are relatively consistent with TiO_2 , which contributed to a significant change at the high exposure level in only one measure of oxidative stress. These results indicate that oxidative damage due to dust exposure from NDRA is likely to be related to the particulate matter exposure in addition to any redox active metals present on the dust.

Two lymphocyte subpopulations stood out as being repeatedly sensitive to NDRA dust exposure. Splenic $\text{CD4}^+/\text{CD25}^+/\text{FoxP3}^-$ activated effector T cells and $\text{CD4}^+/\text{CD8}^+$ (double positive) splenocytes were decreased in at least the 10 and 100 mg/kg/d dosing groups in 3 out of 7 CBN units. Suppression of lymphocyte subpopulations, particularly splenocytes, is a sensitive indicator of immunotoxicity in experimental animal systems and is suggestive of the risk of immunotoxicity in other exposed organisms, including humans (Luster et al., 1992; EPA, 1996). Consequently, exposure to natural dust from a wide range of sites within NDRA, at similar concentrations and via a similar exposure route as our animal studies, may decrease lymphocyte subpopulations in humans and may cause oxidative stress at high concentrations.

Research Limitations

The aim of our exposure model was to mimic a consecutive 4 weeks of once a week inhalation exposures to dust at NDRA, exposing B6C3F1 female mice one combination unit at a time. Outdoor recreation at NDRA typically consists of three dust exposure scenarios, inhalation of airborne dust, and dermal contact and incidental ingestion with surface soils and airborne dust. It is unlikely for ORV riders to stay within one combination unit during their recreation and only be exposed to dust through inhalation of airborne dust particles. Thus, the more probable exposure scenario to dust at NDRA involves ORV drivers riding terrain across multiple combination units encountering multiple exposure scenarios. Mice, as a model species for humans, come with a large amount of limitations, mainly being that rodents are obligatory nose breathers with highly different nasal passageways when compared to humans (Hecht, 2005). However, B6C3F1 female mice are widely used in immunotoxicity evaluation and are considered by the National Toxicology Program to be the model animal when screening for effects on immune function (Yang et al., 2002).

Mice were exposed using oropharyngeal aspiration (OA), a method that allows for precise amounts of material to be instilled into the lungs. This exposure route differs from other inhalation exposure methods in that an OA exposure is an invasive, nonphysiological exposure that largely by-passes the upper respiratory tract. While an inhalation exposure model may have been more physiologically representative of human exposures, these scenarios are expensive and would require large amounts of dust, thus it was a non-viable option for our study design. Additionally, inhalation exposure models

create variability in that the overall amount of inhaled and ingested particles is unknown. A large amount of dust is deposited onto the pelt of the mouse and animal grooming creates for an additional and uncontrollable ingestion exposure (Hechet, 2005; De Vooght et al., 2009). Oropharyngeal aspiration provides for less variability and overall greater control of the exposure scenario when compared to inhalation and intratracheal instillation (De Vooght et al., 2009).

The time at which oxidative stress was measured, one day after the final exposure, may have been insufficient for capturing an active disturbance to the redox balance. While individual metals in the dust samples, such as arsenic and lead, are known to increase oxidative stress, one day after final exposure may have been too soon to measure changes and too late after the earlier exposures. The redox balance is constantly shifting and the timing of measurements can impact detection of oxidative species and antioxidant enzymes. Where our study measured for systemic (blood) markers of oxidative stress, oxidative stress modeling in specific organs can be done (Hays, 2006; Imai, 2008; Mittal and Flora, 2006; Resende, 2008; Shina, 2008). As the lungs were used for histopathology and saved for future metals analysis, they were not chosen as a tissue to measure oxidative stress. Oxidative damage may have occurred at specific sites, such as the lungs or spleen, however it is possible that the blood may not have reflected systemic change in antioxidants and free radicals.

As the CD4+CD25+FoxP3⁻ cell population was solely identified based on immunophenotyping, it is possible that these cells could have a number of identities. Travers et al. (2012) reports that CD4+CD25+FoxP3⁻ regulatory T cells have been

isolated, however, as we did not measure IL-10 production within this study, it is unknown if the identified suppressed CD4+CD25+FoxP3- cells fall into this category. It is most probable that this cell population is activated effector T cells. Only with further cytokine analysis on the quantified cell populations can we begin to identify their specific roles within the immune system and the consequence of this occasionally dose-responsive immune cell suppression.

Suggestions for Future Work

As the popularity of Nellis Dunes and off road vehicle recreation grow, further testing on the immunotoxic effects of NDRA dust exposure should be done. As has been previously stated, in addition to reduction of splenic CD4+/CD8+ and CD4+/CD25+/FoxP3- cells, NDRA dust also caused a dose-responsive suppression of IgM antibody responses (Keil et al., 2014). Further mechanistic analysis of why the immune system is targeted following dust exposure and the extent of immune system disturbances could affect the risk levels determined by Simon and Warren (2014). Further immune analysis, including bacterial challenge assays and tests addressing the functional ability for class switching, could elucidate the degree of immune distress. As the role of CD25 expression and its importance for the immune system may be species dependent (Triplett et al., 2012), further analysis of the CD4+CD25+FoxP3- cell population and its role in human and murine immune systems is necessary. Additionally, as heavy metal exposure is highly linked to oxidative stress and damage (Jomova et al., 2010), it is likely that using blood to measure oxidative stress from the oropharyngeal aspiration was

inadequate. Subsequent inhalation studies performed using dust from NDRA should analyze lung tissues for oxidative damage.

Many studies have found that exposure to airborne fine PM increases risks of respiratory and cardiovascular illnesses and mortality (Samet et al. 2000; Samoli et al. 2008; Beelen et al. 2008; Dockery et al. 1993). However, most research on PM has analyzed the health effects of anthropogenic (diesel exhaust, cigarette smoking, industry by-products etc.) air pollution in urban areas; airborne mineral dusts have been less studied, with geanthropogenic particulate matter exposure being a largely under-researched field. Furthermore, even less is understood about the potential exposure levels and associated health effects of being exposed to natural dust while riding an ORV.

Some studies have found that exposure to inorganic mineral dust events, such as dust storms, is associated with increases in respiratory disease incidence such as asthma and acute bronchitis as well as increased mortality, while others have documented increases in cardiovascular disease and related mortalities (Grineski et al. 2011; Chan et al. 2008; Mallone et al. 2011). Morman and Plumlee (2013) note, in a review of airborne dusts and human disease, that little research has been done concerning the local health effects of inorganic mineral dusts due to lack of monitoring. Additional research and human health risk assessments on geanthropogenic sources of PM exposure need to be carried out as off-road vehicle driving is one of the most prevalent and fastest growing recreational activities on public lands worldwide (Outdoor World Directory, 2010). Moreover, most research is focused on exposure to single compounds as this creates for less variability within the study design. However, this research style has created a lack of

information regarding the additive, interactive, and cumulative effects in real world exposure scenarios. Additional studies to identify the role of each metal in this mixture and their potential additive effects would be required to gain insight into the cumulative roles of metals and particulate matter toxicities.

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