Toxicology Reports 5 (2018) 258–269

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

Nevada desert dust with heavy metals suppresses IgM antibody production

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ARTICLE INFO

Keywords:
Geogenic dust
Heavy metals
Particulate matter
Immunotoxicity
Neurotoxicity

ABSTRACT

Systemic health effects from exposure to a complex natural dust containing heavy metals from the Nellis Dunes Recreation Area (NDRA) near Las Vegas, NV, were evaluated. Several toxicological parameters were examined following lung exposure to emissive dust from three geologic sediment types heavily used for recreational off-road activities: yellow sand very rich in arsenic (termed CBN 5); a shallow cover of loose dune sand overlying a gravelly subsoil bordering dune fields (termed CBN 6); and brown claystone and siltstone (termed CBN 7). Adult female B6C3F1 mice were exposed by oropharyngeal administration to these three types of geogenic dusts at 0.01–100 mg of dust/kg of body weight, once per week for four weeks. The median grain sizes were 4.6, 3.1, and 4.4 μm, for CBN 5, 6, and 7, respectively. Each type of dust contained quantifiable amounts of aluminum, vanadium, chromium, manganese, iron, cobalt, copper, zinc, arsenic, strontium, cesium, lead, uranium, and others. Descriptive markers of immunotoxicity, neurotoxicity, hematology, and clinical chemistry parameters were assessed. Notable among all three CBN units was a systemic, dose-responsive decrease in antigen-specific IgM antibody responses. Geogenic dust from CBN 5 produced more than a 70% suppression in IgM responses, establishing a lowest adverse effect level (LOAEL) of 0.01 mg/kg. A suppression in IgM responses and a corresponding increase in serum creatinine determined a LOAEL of 0.01 mg/kg for CBN 6. The LOAEL for CBN 7 was 0.1 mg/kg and also was identified from suppression in IgM responses. These results are of concern given the frequent off-road vehicle traffic and high visitor rates at the NDRA, estimated at 300,000 each year.

1. Introduction

According to the World Health Organization (WHO), particulate matter from natural or anthropogenic sources affects more people than any other pollutant [1]. Specifically, exposure to desert dusts released from anthropogenic activities and dust storms signifies a global health issue [2–4] that has been understudied. In the more arid portions of the southwestern United States, weather factors and anthropogenic activities have significantly increased emissions of mineral dust [5–8]. These processes disturb soils and release the mineral dust into the air, leading to human exposures [4,8,9] at the local site and far beyond. Dust in natural settings is a complex mixture of mineral, metal and organic components in varying sizes ranging from nanometers to several tens of micrometers. The smaller dust particles with a median size of 10 μm or less, distribute deep within the lung and are reported to lead to severe health consequences [10,11]. Consequently, epidemiological studies have linked inhalation of desert dusts to risks of increased mortality [12], cardiopulmonary emergencies [13], hospitalizations for asthma [14], and occurrence of strokes [15].

The Nellis Dunes Recreation Area (NDRA) is a popular off-road vehicle (ORV) driving destination managed by the Bureau of Land Management (BLM) and is located just 6 km northeast of Las Vegas, NV, USA. For the past 40 years, the NDRA has provided the only publicly accessible area in southern Nevada for legal off-road vehicle driving with > 300,000 annual visitors [5,6]. Due to the high visitor rate and known health risks associated with particulate matter exposure, toxicological effects of these naturally derived geogenic dusts were examined in a mouse model. This study presents three of seven defined geomorphic units in the NDRA established on common surface features; the other units have been previously characterized in Keil et al. [16,17],

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https://doi.org/10.1016/j.toxrep.2018.01.006
Received 27 June 2017; Received in revised form 19 January 2018; Accepted 22 January 2018

DeWitt et al. [18,19], and Leetham et al. [20].

The National Research Council identified exposure to airborne particulate matter as a research priority with needs for characterization of emission sources and hazardous particulate matter components [21]. Accordingly, sources of geogenic dust presented in this study were characterized for size and metal composition. The until termed CBN 5 is primarily found in the northwestern portion of the NDRA and is characterized by badlands topography, where little to no vegetation grows and the soils are formed by weathering of underlying poorly consolidated, yellow sandstone deposits very rich in arsenic (up to 128 μg/g). These deposits are part of the Muddy Creek Formation. The unit termed CBN 6 is characterized by a thin layer of loose dune sand overlying calcareous subsoils primarily formed in Quaternary gravel deposits. These areas border a huge dune field in the center of NDRA. Finally, the unit termed CBN 7 consists of surfaces where brown claystone and siltstone of the Muddy Creek Formation is exposed. These surfaces are characterized by silt and clay aggregates up to 5 mm in diameter and are entirely devoid of vegetation. They occur primarily in badlands topography in the northwestern portion of NDRA.

Soils of these three units contain quartz, calcite, feldspar, and many clay minerals such as smectite, palygorskite, kaolinite, minor chlorite, and trace illite [22]. These minerals have within their crystal structure or adsorbed on their surfaces, many different heavy metals and metalloids. This study characterized the emissive dust of inhalable size for the following 16 elements: aluminum (Al), iron (Fe), strontium (Sr), manganese (Mn), zinc (Zn), vanadium (V), copper (Cu), arsenic (As), uranium (U), cobalt (Co), cadmium (Cd) antimony (Sb), cesium (Cs), thallium (Tl), lead (Pb) and chromium (Cr). The human lung is not designed to accomodate particulate matter and health effects have been reported with exposure to PM2.5 or PM10 (particulate matter < 2.5 μm or < 10 μm in diameter, respectively) containing metals such as lead, zinc, nickel, iron, manganese, chromium, and copper [23–26].

For some time, health effects attributed to a single toxicant have guided regulatory decisions with less emphasis on mixtures due to challenges in experimental design. In an effort to further describe the nature of immunotoxicological and related toxicological deficits produced by a complex mixture of geogenic dust and to facilitate future mechanistic studies, a mouse model was utilized. Adult female B6C3F1 mice were exposed to several concentrations of geogenic dust collected from these three NDRA units via oropharyngeal aspiration. Mice were given four exposures spaced a week apart over a 28-day period to mimic a month of weekend exposures. This study presents the descriptive and functional toxicological parameters that were performed 24 h after the final exposure.

2. Materials and methods

2.1. Collection of geogenic dust

Composite samples were collected from surface sediment (upper 0–4 cm) of CBN 5, 6 and 7, using a plastic scoop, and placed into a clean plastic bag, which was hermetically closed after collection. GPS position of the center of the collection areas was carefully recorded. After drying, the samples were treated in a Soil Fine Particle Extractor (see [5]) to extract a sample with a median diameter of approximately 4 μm. The particle size distribution of the samples was determined with laser diffraction using a Malvern Mastersizer S laser particle size analyzer (Malvern Instruments Ltd., Malvern, UK). Because the samples were given to the mice in a solution, the wet analysis option was chosen.

2.2. ICP-MS analyses of geogenic dust

The CBN 5, 6, and 7 samples were digested in accordance with the USGS Four-Acids Method [27] and subsequently analyzed using an Agilent 7700 inductively coupled plasma/mass spectrometry (ICP-MS) device (Agilent Technologies, Santa Clara, USA). To ensure quality control for the ICP-MS analyses, all quality control procedures set forth by US EPA Method 6020A (2007) were followed. In addition, NIST SRM 8704 (Buffalo River Sediment) and NIST SRM 2711A (Montana II Soil) were used as standard reference materials (SRMs).

2.3. Arsenic speciation in geogenic dust

Arsenite, arsenate, monomethylarsonic acid, and dimethylarsinic acid quantification speciation was performed using IC-ICP-CRC-MS at Brooks Rand Labs, LLC (formerly Applied Speciation and Consulting, LLC) according to an in-house developed method based on et al. [28].

2.4. Quantitation of silicon in geogenic dust

Analysis for the element silicon utilized a Thermo Scientific Niton XL3t GOLDD+ portable XRF (X-ray fluorescence) instrument. For calibration, the NIST Standard Reference Material 2711A was run and the XRF results agreed with the certified values for silicon and within the margins of uncertainty for the soil standard. Twelve samples were analyzed from each map unit (CBN 5, 6, and 7) and each sample was run twice for a total of 120 s for each analysis.

2.5. Preparation, stability and verification of geogenic dust for animal exposures

CBN 5, 6, and 7 extracts were carefully labeled, stored in sealed and dry containers, protected from light, and secured in a lock box in the laboratory. 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 per kg of body weight and were administered to mice within 1–2 h of preparation. Some elements have various degrees of solubility in water, and addition of dust samples to saline for delivery into the mouse may have changed 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). CBN 5, 6, or 7 dust samples were added to ETF-PBS to ascertain stable time frames in which the solution could be used for mouse exposures. Solutions were prepared, and samples of the solutions were collected immediately after preparation, and then at 1, 2, 4, and 6 h. Samples were immediately centrifuged, supernatants removed, and examined 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. At six hours, soluble element concentrations in supernatant began to increase, indicating that insoluble:soluble portions remained constant for six hours in solution. We did not test for changes in speciation, but only total values of elemental metals. 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.

2.6. Animals

Mice were obtained from Charles River Laboratories (headquartered in Wilmington, MA) and were acclimated for 7 days to the conditions of the treatment room (12-h light/dark cycle, 22 ± 2 °C, 60–65% relative humidity) at the University of Nevada Las Vegas (UNLV) animal facilities, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The UNLV Institutional Animal Care and Use Committee approved all experiments. Mice were housed in ventilated polycarbonate shoebox cages with

Table 1
Total elemental concentration (μg/g in dry sample) of dusts collected from CBN map units.

Sample Name	Median[a]	Al	V	Cr	Mn	Fe	Co	Cu	Zn	As	Sr	Cd	Sb	Cs	Tl	Pb	U	Si[b]
CBN 5	4.60	74530	99	43	357	33313	9.7	33	124	227	321	< 0.47	< 3.0	22	< 8.3	44	13	192,027
CBN 6	3.14	47692	56	35	376	21208	8.3	28	71	24	445	< 0.47	< 3.0	9.4	< 8.3	23	2.3	197,473
CBN 7	4.37	73239	103	50	294	36340	10	30	97	24	285	< 0.47	< 3.0	24	< 8.3	26	5.1	214,978

< Indicates value is below method quantitation limit (MQL). Value presented is MQL.
[a] Median diameter (μm).
[b] Indicates amount of silica in 60 nm fraction for each CBN unit.

corncob bedding and were given unlimited access to food and water.

2.7. Animal exposures

To simulate the potential health impacts of a month of weekend exposures to CBN 5, 6, or 7 dust from the NDRA (Tables 1 and 2), adult female B6C3F1 mice were exposed to each at 0, 0.01, 0.1, 1.0, 10, or 100 mg/kg of body weight once weekly for four weeks. Each dose administered was adjusted to body weight at the time of exposure. Therefore, based on a 20-g mouse, 20 μg was administered to the lung via oropharyngeal aspiration to achieve the appropriate mg/kg dose of dust. Mice in the 0 mg/kg group received PBS only and served as a vehicle control group. To ensure availability of tissue for toxicology assays, each dose group was comprised of 12 mice, housed six per cage. In addition, three separate groups of mice were used for a total of three replicates for each exposure group/dust type. Samples for toxicity studies were collected from each group and replicate as depicted in Fig. 1.

Mice were exposed by oropharyngeal aspiration using isofluorane as an anesthetic agent. An average volume of 10 μl was administered to each mouse; however, based on individual body weight changes, this volume was adjusted to between 9–13 μl per mouse. One day after the final dose was delivered, samples for toxicity studies were collected from animals euthanized by carbon dioxide asphyxiation. The following methods describe the assays used to determine a profile of toxicological effects specific to NDRA geogenic dust from CBN 5, 6, or 7.

2.8. 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. Spleens and thymuses were immediately suspended in complete medium (RPMI, 10% fetal calf serum, 50 IU penicillin and 50 μg streptomycin) and 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 (Fig. 1).

2.9. Blood and sera

For hematology and clinical chemistry endpoints, blood from anesthetized animals was collected into a microtainer tube containing EDTA, which kept the blood from coagulating, or in a microtainer tube with no anticoagulant for serum collection. Once collected, samples were sent overnight to the Montana Veterinary Diagnostic Laboratory (MVDL) in Bozeman, MT, for hematology (whole EDTA blood) and clinical chemistry analysis (sera). Hematology was run on all samples; however clinical chemistries performed were dependent on the volume of sample provided and not all samples were of sufficient volume. For this reason, hematology and clinical chemistry analyses were not performed in duplicate or triplicate as were other assays. For determination of blood metal and metalloid concentrations, blood from anesthetized mice was collected into a microtainer tube containing heparin. Using an analytical balance, each blood collection tube was weighed before and after collection of blood to determine the weight of each blood sample. Once collected, samples were frozen at −80 °C and then shipped to the Laboratory Services Bureau of the Montana Department of Public Health and Human Services for analysis of total levels of metal and metalloid concentrations. Blood metal/metalloid concentrations were not performed in duplicate or triplicate as were other assays due to limited blood volume collected from individual animals. Only whole blood values were measured.

2.10. Immunophenotyping of 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 diluted to a concentration of 1×10^7 cells/mL. Optimal concentrations of flow antibodies and reagents were determined in previous experiments [16]. All experimental replicates included isotype controls (to estimate non-specific binding), unstained

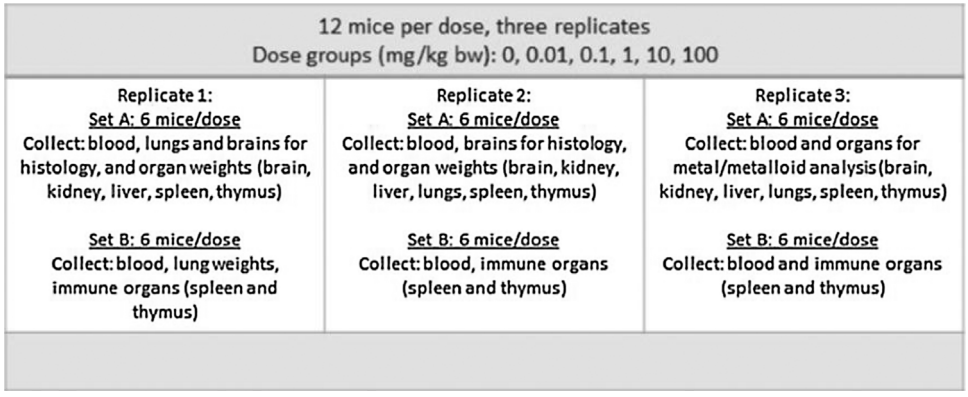

Fig. 1. Toxicology sample collection arrangement.

cells as negative controls, and single color controls as positive controls to determine color compensation. Monoclonal antibodies coupled to fluorochromes specific for the following markers were used: anti-mouse B220-PE, anti-mouse CD4-FITC, and anti-mouse CD8-PE. 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.

2.11. Immunophenotyping of regulatory t lymphocytes (Tregs)

Splenic lymphocytes were adjusted to a concentration of 1×10^6 cells per well and depleted of red blood cells via a 5-min 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 μg/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. Appropriate positive, negative, and isotype controls were added to wells containing cells only. 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.

2.12. Plaque forming cell (PFC) assay

The primary IgM response to sheep red blood cells (SRBC; Rockland, Gilbertsville, PA) was determined using a hemolytic plaque assay. Five days before euthanasia, mice were given an intraperitoneal injection of 100 μl of 25% SRBC in PBS. Single-cell suspensions (as previously described) were prepared from spleens of mice injected with SRBC and diluted to a concentration of 1.0×10^6 cells/mL. A 10-μl aliquot of the single-cell suspension was added to a tube containing 100 μl of 25% SRBC in PBS, 40 μl of RPMI medium (without additives), and 50 μl of guinea pig complement. Aliquots of the solution were placed into Cunningham chamber slides. The slides were sealed with paraffin and were incubated at 37 °C and 5% CO_2 for 1–2 h. PFCs were counted microscopically and were reported as PFCs/million splenocytes.

2.13. Natural killer cell activity

Natural killer (NK) cell activity was assessed via an in vitro cytotoxicity assay using ^{51}Cr-labeled Yac-1 cells as described previously [29]. To minimize radioactive waste, the procedure was adapted to 96-well plates that were read on a Packard Top Count scintillation counter. Spleen single-cell suspensions were adjusted to 2×10^7 cells/mL in complete medium and then the spleen cells and Yac-1 cells were added, in triplicate wells, in ratios of 100:1, 50:1, 25:1, and 12.5:1 spleen cells:labeled Yac-1 cells, and in a final volume of 0.2 ml per well. Maximum release was determined by lysing ^{51}Cr-labeled Yac-1 cells with 0.1% Triton X-100 in complete medium. Spontaneous release was determined by incubating Yac-1 cells only in complete medium. After a four-hour incubation at 37 °C and 5% CO_2, the plates were centrifuged (1200 rpm, 3 min), and 25 μl of supernatant was then transferred to a 96-well plate containing solid scintillant (LumaPlate). Plates were air dried overnight and within 24 h, were counted for 5 min, after a 10-min dark delay, using a Packard Top Count-NXT. The results were expressed in lytic units per 10^7 splenocytes using 10% lysis as the reference point as described previously [29].

2.14. Neuronal autoantibody formation

Blood was collected, held at room temperature for at least 30 min, and then centrifuged at 4 °C to separate serum, which was frozen at −80 °C until analysis of IgM and IgG antibody concentrations. IgM and IgG antibodies against glial fibrillary acidic protein (GFAP; American Research Products, Waltham, MA), myelin basic protein (MBP; Sigma-Aldrich, St. Louis, MO), or neurofilament 68 (NF-68; American Research Products, Waltham, MA) were determined with an ELISA assay developed by El-Fawal et al. [30]. Plates were read at 405 nm on a BioTek Synergy HT plate reader. Optical density values were converted to ng/mL concentrations using values obtained from a standard curve. All sera were assayed twice to verify results. Serum values that fell below the limits of detection were assigned a value of zero. Serum values that fell above the limits of detection were diluted upon the second evaluation. Serum values that remained above the limits of detection after dilution were eliminated from the overall calculations due to insufficient amounts of sample.

2.15. Brain histology

Two sections of cerebellum from each mouse brain, each 10 μm thick, were stained with either anti-CD3+ antibody (Abcam, Cambridge, MA) or anti-myelin basic protein (MBP) antibody (Abcam, Cambridge, MA). In sections stained with anti-CD3+, the number of T cells present throughout both sections was counted at 20X magnification. In sections stained with anti-MBP, the relative intensity of the stain was gauged relative to the intensity of the staining of the sections from the control brains. Control brains were scored as weak (1), mild (2), moderate (3), or strong (4). The intensity of the stain in brains from exposed animals was assigned a numerical value according to the following scale: 0 = no change in staining intensity relative to controls; 1 = very weak staining intensity relative to controls; 2 = mild intensity in staining relative to controls; 3 = moderate intensity in staining relative to controls; 4 = strong intensity in staining relative to controls; 5 = very strong intensity in staining relative to controls.

2.16. Metal-free particle

As described in Keil et al. [16], six separate groups of mice (N = 6/group) were exposed to titanium dioxide (TiO_2), a particle with none of associated metals found on geogenic dusts from the NDRA, to help us discern particle-only effects from the effects of metals and particles together. Mice were given matching concentrations of TiO_2 (0.01–100 mg/kg of body weight), and were exposed via the same paradigm as the geogenic dust. Toxicity testing was evaluated in the TiO_2-exposed mice in parallel with CBN exposed mice. Data, although relevant to this study too, have been reported previously in comparison to other geogenic dusts collected from the NDRA [16].

2.17. 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 0 mg/kg group. A Dunnett's *t*-test also was used to compare results of each geogenic dust dose group to the appropriate mg/kg TiO_2 group.

2.18. Quality assurance

This study was conducted as under the conceptual guidance of Good Laboratory Practices (GLP). Within this guidance, periodic audits of all

aspects of the project were conducted as well as extensive independent review of all documentation and data. In addition, each of the participating university sites conducting experiments (UNLV, MSU and ECU) were audited by an internal but independent Quality Assurance team. All final notebooks were reviewed and initialed by the Quality Assurance Team.

3. Results

3.1. Geogenic dust characterization

Dust isolated from the CBN 5, 6, and 7 map units and used in this murine exposure study had a median diameter of 4.6, 3.1, and 4.4 μm, respectively (Table 1). Metal composition following total digestion and ICP-MS analyses of the dust contained measurable amounts of aluminum, vanadium, chromium, manganese, iron, cobalt, copper, zinc, arsenic, strontium, cesium, lead, and uranium (Table 1). Of the metals in this sample, aluminum and iron concentrations were found in the highest concentrations in all CBN samples, while CBN 5 had approximately 9-fold higher levels of total arsenic as compared to CBN 6 or 7 (Table 1). Speciation was performed only on arsenic. In all samples, pentavalent arsenic [As(V)] was the primary species (Table 2).

3.2. Body weight, organ weights, and immune organ cellularity

Following a one-month exposure duration, no mice in any of the treatment groups demonstrated a significant change in body weight as compared to controls in any of the three experimental replicates for each geogenic dust sample. No significant changes in immune organ weights, cellularities, kidney, or brain weights were observed in any of the treatments. Notable were decreases in liver weight for CBN 5 and 6, but not 7. Following exposure to CBN 5, the adjusted liver weight as a percent of the whole body weight (LSI, liver somatic index) was decreased by 8.5%, 14.6%, 18%, and 17.2% in the 0.1, 1, 10, and 100 mg/kg exposure groups, respectively (Fig. 2); exposure to CBN 6 was associated with a significantly decreased liver weight only at 100 mg/kg (Fig. 2).

3.3. Hematology and clinical chemistry

Hematology included a complete blood count with a differential. Of the hematological endpoints measured in mice dosed with dust samples, only exposure to CBN 5 significantly affected red blood cells (RBC). Mean corpuscular volume in red blood cells (MCV) was statistically decreased in the 0.1 mg/kg–100 mg/kg groups, whereas the mean corpuscular hemoglobin concentration (MCHC) was increased in the 0.1 mg/kg through 100 mg/kg groups (Table 3). The MCHC is a calculated value derived from the measurement of hemoglobin and the hematocrit. The hemoglobin value is the amount of hemoglobin in a volume of blood while the hematocrit is the ratio of the volume of red cells to the volume of whole blood. Exposure to geogenic dusts from CBN 6 increased both neutrophil (PMN) percentage and absolute numbers in peripheral blood at 100 mg/kg (Table 3). Following exposure to 10 and 100 mg/kg of CBN 7 dust, total white blood cell (WBC) counts were increased by 93.8% and 109.4%, respectively, and lymphocyte concentrations were increased by 92.3% and 111.6%, respectively. Monocyte concentrations were increased by 247% in the 100 mg/kg group relative to the 0 mg/kg group (Table 3).

Clinical chemistry parameters were comprised of electrolytes and a basic metabolic panel. Of this panel, only creatinine, blood urea nitrogen (BUN) and bilirubin were affected following geogenic dust exposures. Plasma creatinine was significantly increased by all geogenic CBN dust exposures, but not by all exposure levels. Exposure to 10 and 100 mg/kg of CBN 5 (Table 3) increased creatinine levels relative to the 0 mg/kg group. All administered concentrations of CBN 6 dose-responsively increased plasma creatinine beginning at 0.1 mg/kg (Table 3). For CBN 7, BUN was decreased by 21.1%–30.9% after exposure to 1 mg/kg–100 mg/kg and plasma creatinine was significantly increased by 4.3% in the 100 mg/kg group (Table 3). Only CBN 7 was associated with a dose responsive increase (from 60% to 120%) in total serum bilirubin levels from animals dosed with 0.1 mg/kg through 100 mg/kg.

Table 2
Arsenic speciation results of dusts collected from CBN map units.

Sample Name/ID	As(III)	As(V)	MMAs	DMAs	Units
CBN 5/PM4/061112	ND (< 0.30)	73.1	ND (< 0.22)	ND (< 0.20)	μg/g
CBN 6/PM4/100812	ND (< 0.23)	6.93	ND (< 0.17)	ND (< 0.15)	μg/g
CBN 7/PM4/061112	ND (< 0.27)	6.16	ND (< 0.20)	ND (< 0.18)	μg/g

All results reflect the applied dilution and are reported as received (wet weight).
ND = Not detected at the applied dilution.
MMAs = Monomethylarsonic acid.
DMAs = Dimethylarsinic acid.

3.4. Blood metals

Blood was collected 24 h after the last exposure from animals exposed to CBN 5, 6, and 7. There were detectable concentrations of the metals/metalloids associated with these dusts (Table 4). Mean concentrations did not differ by dose in animals exposed to CBN 5. In blood from animals exposed to dust from CBN 6, mean concentrations of molybdenum (Mo) were 13.5–35.0% lower in the 0.01–100 mg/kg groups relative to the 0 mg/kg group and vanadium (V) was 26.8–42.7% higher in groups exposed to 1–100 mg/kg relative to the 0 mg/kg group (Table 4). Exposure to CBN 7 resulted in 13.3–20.0% lower levels of arsenic in the 1–100 mg/kg groups relative to the 0 mg/kg groups. Similarly, animals exposed to all concentrations of dust from CBN 7 had 22.2–37.7% lower levels of Mo relative to animals exposed to 0 mg/kg (Table 4).

3.5. Immunophenotype

Following exposure to CBN 5, no change was observed in splenic and thymic B and T lymphocytic subpopulations, splenic B220 numbers, and spleen T-regulatory cell populations (data not shown). For CBN 6, while no changes were observed in splenic and thymic B and T lymphocyte subpopulations or in splenic B220 numbers, the absolute number of splenic CD4 + CD25 + foxP3- activated T cells were dose-responsively decreased beginning at the 0.1 mg/kg. As compared to control, the 0.1–100 mg/kg/day treatment groups were decreased by 76.2%, 82.4%, 88.9%, and 92.9%, respectively (Table 5). For CBN 7 the number of B cells and regulatory T cells (Tregs) from the spleen and the number of T cells from the thymus were not statistically altered (data not shown) whereas 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 (Table 5). 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. No significant changes were observed in mice exposed to TiO_2, as previously reported in Keil et al. [16].

3.6. Plaque forming cell (PFC) assay

Exposure to dust samples from CBN 5, 6, and 7 dose-responsively reduced the number of SRBC-specific plaque forming cells. PFC/million of spleen cells was reduced by more than 70% in dosed groups relative to the control group in CBN 5 (Fig. 3A). A LOAEL of 0.01 mg/kg/day was identified and a NOAEL could not be established. Similarly, in

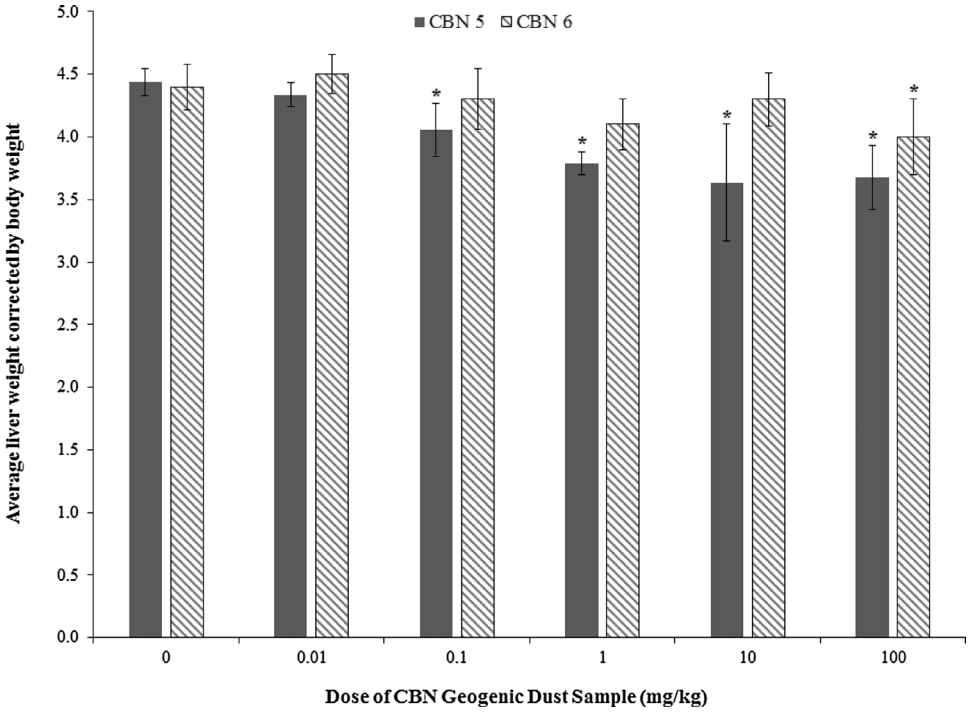

Fig. 2. Liver somatic index (liver weight corrected by body weight) of adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust samples from NDRA CBN 5 or CBN 6. Data are presented as mean ± standard deviation. Sample size for each group was 5–6 animals. The (*) indicates a response statistically different from the 0 mg/kg group ($p < 0.05$) for that CBN unit.

animals exposed to CBN 6, PFC/million of spleen cells was reduced by 20% beginning at 0.01 mg/kg (Fig. 3B). A LOAEL of 0.01 mg/kg/day was identified and like CBN 5, a NOAEL could not be determined. In contrast, CBN 7 reduced PFC/million of spleen cells at administered doses of 0.1 and above (Fig. 3C). PFC/million of spleen cells was reduced by 43.4-76.5% in these dosed groups relative to the 0 mg/kg group. For CBN 7, a NOAEL of 0.01 mg/kg and a LOAEL 0.1 mg/kg were identified for this response. No significant changes were observed in antigen-specific IgM antibody production in mice exposed to TiO_2 as previously reported in Keil et al. [16].

3.7. Natural killer (NK) cell activity

Exposure to CBN 5 was associated with significant decreases in NK cell activity at 10 and 100 mg/kg (Fig. 4). Exposure to CBN 6 induced significant decreases in NK cell activity only at 0.01, 0.1 and 1.0 mg/kg (Fig. 4). No useable data were available from the natural killer cell activity assay for CBN 7 due to the loss of target cells during this experiment.

3.8. Neuronal autoantibody formation

Exposure to dust samples from CBN 5, 6, and 7 altered production of IgM and IgG antibodies specific to neuronal proteins, but not in a dose-responsive way across each dust type. In fact, exposure was associated with decreases in the levels of these antibodies. Only exposure to CBN 6 was dose-responsive at levels of 1–100 mg/kg reducing both IgM (by about 20%) and IgG (by about 80–90%) antibody production against MBP (Fig. 5A), and IgM (by about 20%) antibody production against GFAP relative to 0 mg/kg (Fig. 5B).

3.9. Brain histology

Histological analyses of brains from animals exposed to geogenic dust from CBN 5 and CBN 6 did not indicate T cell infiltration or loss of MBP staining intensity (data not shown). Exposure to CBN 7 at 0.1, 1,

Table 3
Mean hematological and clinical chemistry changes measured in B6C3F1 female mice following oropharyngeal aspiration exposure to geogenic dusts from CBN map units.

Parameter	0 mg/kg	0.01 mg/kg	0.1 mg/kg	1 mg/kg	10 mg/kg	100 mg/kg
CBN 5						
Mean corpuscular volume (fl)	47.6 ± 0.46	46.8 ± 0.67	43.1 ± 0.56*	45.5 ± 0.47*	45.8 ± 0.88*	46.2 ± 0.53*
Mean corpuscular hemoglobin concentration (%)	33.0 ± 0.46	33.6 ± 0.46	34.2 ± 0.44*	34.6 ± 0.55*	34.4 ± 0.57*	34.2 ± 0.34*
Creatinine (mg/dL)	0.54 ± 0.06	0.53 ± 0.05	0.50 ± 0.0	0.57 ± 0.14	0.62 ± 0.05*	0.62 ± 0.05*
CBN 6						
Neutrophils (%)	14.5 ± 4.5	12.0 ± 4.0	16.8 ± 4.0	12.8 ± 3.3	15.4 ± 4.5	23.5 ± 4.8*
Neurtrophils (10^9/L)	0.64 ± 0.19	0.65 ± 0.27	0.67 ± 0.20	0.51 ± 0.16	0.82 ± 0.18	1.04 ± 0.40*
Creatinine (mg/dL)	0.40 ± 0.0	0.40 ± 0.0	0.48 ± 0.04*	0.48 ± 0.04*	0.60 ± 0.0*	0.70 ± 0.0*
CBN 7						
White blood cell count (10^9/L)	3.2 ± 0.95	3.0 ± 0.58	3.4 ± 0.90	3.6 ± 1.27	6.2 ± 0.148	6.7 ± 0.21*
Lymphocyte concentration (10^9/L)	2.6 ± 0.85	2.6 ± 0.51	2.7 ± 0.66	2.9 ± 1.09	5.0 ± 0.95*	5.5 ± 1.31*
Monocyte concentration (10^9/L)	0.08 ± 0.09	0.08 ± 0.07	0.16 ± 0.08	0.10 ± 0.07	0.25 ± 0.22*	0.29 ± 0.13*
Blood urea nitrogen (mg/dL)	30.7 ± 4.0	29.7 ± 3.1	27.3 ± 2.8	24.2 ± 2.2*	22.8 ± 3.1*	21.2 ± 3.3*
Creatinine (mg/dL)	0.40 ± 0.06	0.33 ± 0.05	0.33 ± 0.05	0.36 ± 0.06	0.38 ± 0.04*	0.42 ± 0.04*
Total bilirubin (mg/dL)	0.10 ± 0.06	0.12 ± 0.05	0.16 ± 0.06*	0.16 ± 0.06*	0.20 ± 0.06*	0.22 ± 0.04*

Data are presented as mean ± standard deviation. Sample size for each group was 5–6 animals. Data presented are representative of one trial day. This symbol (*) indicates a response statistically different from the control group ($p < 0.05$) for that CBN unit.

Table 4
Total elemental concentration (μg/g in wet sample) in blood of B6C3F1 female mice following oropharyngeal aspiration exposure to geogenic dusts from CBN map units.

Metal	0 mg/kg	0.01 mg/kg	0.1 mg/kg	1 mg/kg	10 mg/kg	100 mg/kg
CBN 5						
As	0.0038	0.0040	0.0038	0.0034	0.0032	0.0037
Cd	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Cr	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Pb	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Mg	36.7	35.9	36.6	36.0	35.9	36.9
Mn	0.0215	0.0201	0.0208	0.0231	0.0305	0.0208
Mo	0.0152	0.0135	0.0138	0.0104	0.0127	0.0162
Ni	< LOQ	0.0155*	< LOQ	< LOQ	< LOQ	< LOQ
Sr	0.0070	0.0076	0.0079	0.0068	0.0077	0.0076
V	0.0098	0.0087	0.0092	0.0080	0.0081	0.0085
Zn	4.41	4.34	4.37	4.33	4.46	4.51
CBN 6						
As	0.0040	0.0042	0.0039	0.0039	0.0039	0.0037
Cd	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Cr	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Pb	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Mg	36.6	35.8	36.1	34.6	34.7	35.0
Mn	0.0216	0.0200	0.0213	0.0199	0.0216	0.0202
Mo	0.0140	0.0117	0.0121	0.0100	0.0104	0.0091
Ni	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Sr	0.0081	0.0070	0.0073	0.0073	0.0084	0.0078
V	0.0082	0.0106	0.0093	0.0104	0.0117	0.0113
Zn	4.30	4.35	4.60	4.29	4.27	4.24
CBN 7						
As	0.0045	0.0046	0.0042	0.0039**	0.0039**	0.0036**
Cd	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Cr	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0.0989*
Pb	0.0021	0.0025	0.0170	0.0027	< LOQ	< LOQ
Mg	34.7	35.5	34.9	35.1	35.8	35.3
Mn	0.0224	0.0217	0.0216	0.0239	0.0215	0.0207
Mo	0.0180	0.0140**	0.0134**	0.0114**	0.0135**	0.0112**
Ni	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Sr	0.0088	0.0079	0.0074	0.0076	0.0124	0.0088
V	0.0109	0.0115	0.0113	0.0114	0.0116	0.0110
Zn	4.26	4.42	4.23	4.25	4.41	4.31

< LOQ indicates value is below the limits of quantitation.
*This was based on one animal with a concentration above the LOQ. All other animals were below the LOQ.
**Indicates a concentration statistically different from the 0 mg/kg group ($p < 0.05$).

and 10 mg/kg led to 200–300% more T cells in the cerebellum relative to the 0 mg/kg group (Fig. 6) and there was no loss of MBP staining intensity was (data not shown).

4. Discussion

Regulatory decisions to determine exposure safety and health are typically gauged on the toxicity of single pollutant exposures and not mixtures. More recently, effort has been applied to learn more about humans exposed to air pollutant mixtures and the associated broader range of health impacts. There is no ideal method to characterize complex mixtures. Despite experimental limitations, it is important to begin this dialogue by identifying collective toxicological outcomes in the scope of metal composition trends in the geogenic dust samples.

In general, the chemical composition of NDRA dust is comparable to that measured from other areas in the southwestern USA [31]. Mineral dusts are typically high in silica and aluminum and the concentration of metals and metalloids will vary based on geologic processes that can concentrate specific elements in rocks. For example, in this study, CBN 5 had very high concentrations of arsenic. Regional values of arsenic in dust in the southwestern USA usually range between approximately 5–25 μg/g [32] whereas concentrations up to 128 μg/g occurred in CBN 5. To learn about human health effects attributed to these mixture exposures, a respirable, representative dust sample with median diameter around 4 μm was isolated from each NDRA unit for these toxicology studies.

The three studied units at the NDRA offer a collective comparison of the metal mixtures of select respirable, geogenic dust samples, within the scope of toxicological outcomes. Yet, there were specific differences in metal mixes that were expected to influence toxicity. Most notable was that CBN 5 has approximately 9-fold more total arsenic and 2-fold more lead as compared to CBN 6 or 7. CBN 6 differed somewhat in that the concentrations of aluminum and iron were two-thirds the concentrations present in CBN 5 and 7 (Table 1). For the most part, the other metals measured were in similar ranges and arsenic was pentavalent across all units (Tables 1 and 2).

Characterizing complex mixtures poses challenges in toxicology. Metals are regulated based on health effects based on exposure to the single metal and not within the complexity of multiple metals. It was anticipated that defining the internal blood levels of select metals in our animal model would lead to an improved understanding of which

Table 5
Selected populations of splenic B and T lymphocytes from B6C3F1 female mice following oropharyngeal aspiration exposure to geogenic dusts from CBN map units.

Cell Type	0 mg/kg	0.01 mg/kg	0.1 mg/kg	1 mg/kg	10 mg/kg	100 mg/kg
CBN 5						
B220 (cells × 10^7)	8.75 ± 1.43	6.74 ± 2.43	7.93 ± 1.62	9.99 ± 2.48	8.26 ± 1.37	7.41 ± 2.02
Splenic $CD4^+$ (cells × 10^7)	5.03 ± 1.20	4.51 ± 0.65	4.82 ± 0.55	4.08 ± 0.68	4.81 ± 0.69	3.99 ± 0.83
Splenic $CD8^+$ (cells × 10^7)	1.47 ± 0.19	1.25 ± 0.44	1.25 ± 0.43	1.78 ± 0.49	1.45 ± 0.21	1.47 ± 0.37
Splenic $CD4^+/CD8^+$ (cells × 10^6)	11.58 ± 2.89	8.25 ± 2.59	8.26 ± 1.93	10.74 ± 3.95	7.95 ± 2.05	7.39 ± 3.41
Splenic $CD4^-/CD8^-$ (cells × 10^8)	1.30 ± 0.33	1.34 ± 0.14	1.30 ± 0.13	1.19 ± 0.15	1.46 ± 0.17	1.18 ± 0.33
Splenic $CD4^+/CD25^+/fp3^-$ (cells × 10^7)	2.33 ± 2.21	1.25 ± 1.76	1.03 ± 0.61	0.84 ± 0.39	0.69 ± 0.29	0.49 ± 0.18
CBN 6						
B220 (cells × 10^7)	5.76 ± 0.71	6.03 ± 1.26	7.85 ± 1.80	6.30 ± 1.26	6.06 ± 1.48	5.88 ± 1.11
Splenic $CD4^+$ (cells × 10^7)	3.33 ± 0.60	3.36 ± 0.94	3.79 ± 0.57	3.38 ± 0.86	3.06 ± 0.65	2.27 ± 0.80
Splenic $CD8^+$ (cells × 10^7)	1.01 ± 0.14	1.22 ± 0.44	1.21 ± 0.10	1.20 ± 0.17	1.10 ± 0.22	1.06 ± 0.29
Splenic $CD4^+/CD8^+$ (cells × 10^6)	7.70 ± 2.02	8.87 ± 2.50	9.31 ± 4.17	7.46 ± 2.51	5.74 ± 1.41	6.32 ± 2.77
Splenic $CD4^-/CD8^-$ (cells × 10^8)	0.89 ± 0.15	0.87 ± 0.20	1.18 ± 0.35	0.97 ± 0.22	0.89 ± 0.19	0.79 ± 0.15
Splenic $CD4^+/CD25^+/fp3^-$ (cells × 10^7)	4.39 ± 1.26	3.08 ± 1.25	1.05 ± 0.40*	0.77 ± 0.42*	0.49 ± 0.34*	0.31 ± 0.87*
CBN 7						
B220 (cells × 10^7)	8.31 ± 1.58	7.24 ± 1.55	6.92 ± 2.91	5.72 ± 1.88	6.55 ± 1.75	5.61 ± 0.72
Splenic $CD4^+$ (cells × 10^7)	6.06 ± 1.43	5.36 ± 1.11	4.89 ± 1.96	3.92 ± 1.28*	3.73 ± 0.94*	3.87 ± 0.65*
Splenic $CD8^+$ (cells × 10^7)	2.73 ± 0.66	2.52 ± 0.50	2.07 ± 0.85	1.88 ± 0.51	1.62 ± 0.33*	1.76 ± 0.44*
Splenic $CD4^+/CD8^+$ (cells × 10^6)	9.92 ± 3.65	6.02 ± 1.67	7.45 ± 5.31	3.76 ± 1.36*	4.84 ± 1.67*	4.28 ± 1.26*
Splenic $CD4^-/CD8^-$ (cells × 10^8)	1.23 ± 0.21	1.20 ± 0.28	1.67 ± 0.45	0.85 ± 0.91	1.00 ± 0.27	0.92 ± 0.16
Splenic $CD4^+/CD25^+/fp3^-$ (cells × 10^7)	0.92 ± 0.60	0.78 ± 0.52	0.58 ± 0.54	0.56 ± 0.48	0.49 ± 0.32	5.17 ± 11.2

* Indicates a concentration statistically different from the 0 mg/kg group ($p < 0.05$).

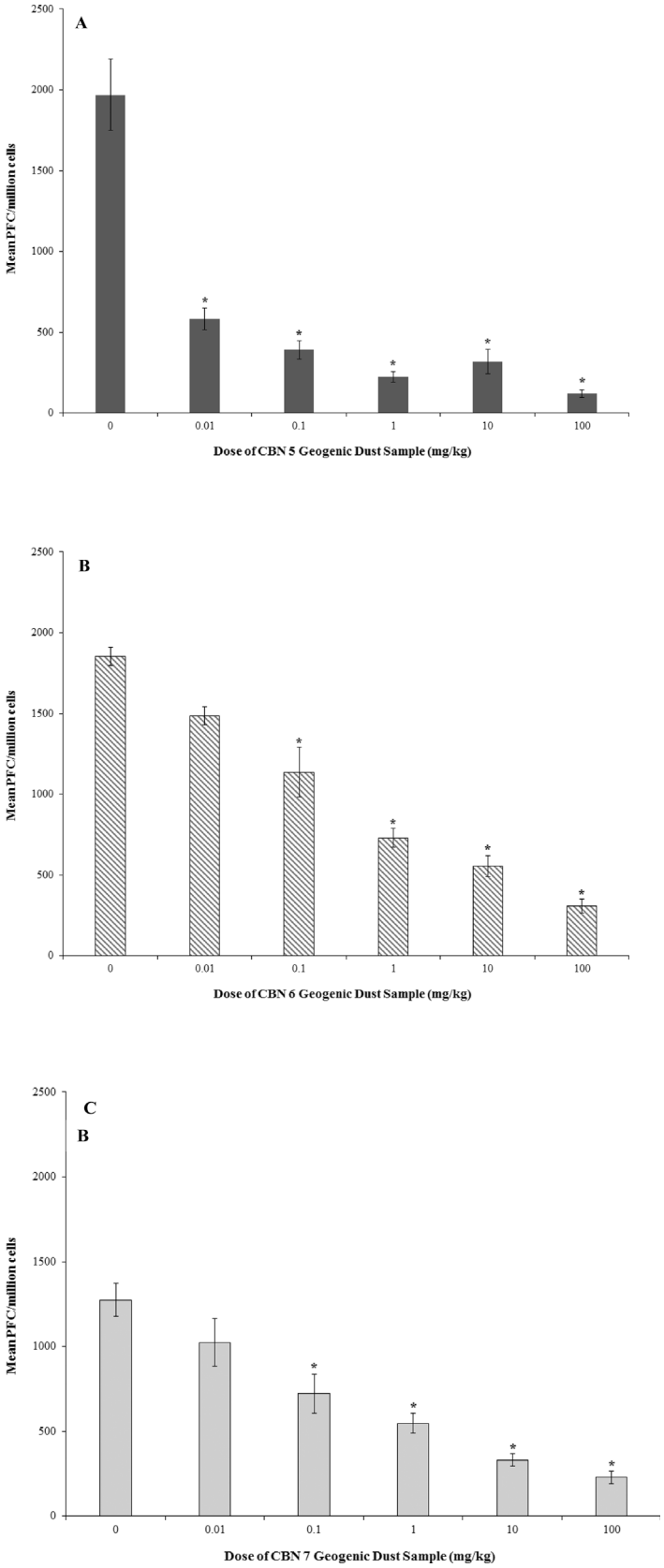

Fig. 3. Sheep red blood cell-specific-IgM antibody production in adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust samples from NDRA CBN 5 (A), 6 (B), and 7 (B). Data are presented as mean ± standard error of the mean. Sample size for each group was 4–6 animals. Data presented are representative of three trial days. The (*) indicates a response statistically different from the 0 mg/kg group ($p < 0.05$) for that CBN unit and was determined from log transformed data.

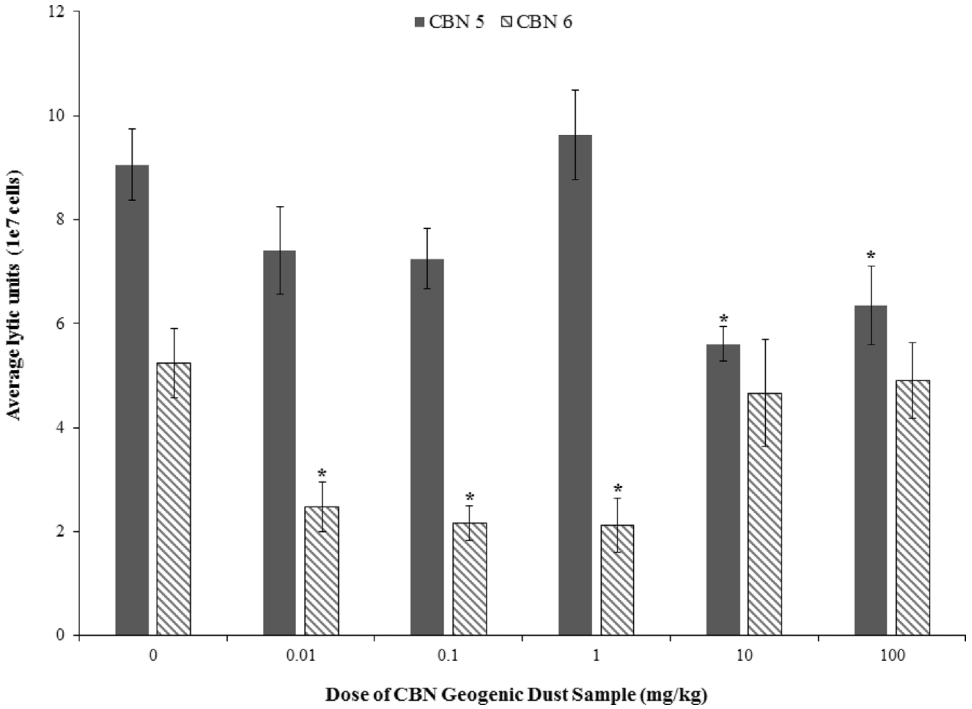

Fig. 4. Splenic natural killer cell activity in adult female B6C3F1 mice following oropharyngeal aspiration exposure to exposure to dust samples from NDRA CBN 5 or 6. Data are presented as mean lytic unit ± standard error of the mean. Sample size for each group was 5–6 animals. Data presented are representative of three trial days. The (*) indicates data significantly different from the 0 mg/kg group ($p < 0.05$) for that CBN unit.

metals may have a greater role in toxicity. It was also considered that this information might serve as a point of comparison with human blood levels in a volunteer study conducted in the NDRA [33]. Regarding bioavailability, blood was collected 24 h after the last dose from the 28-day exposure period. 'No-fold' differences in the blood metal levels were observed that reflected differences in the metals composition of CBN 5, 6 or 7 dust. For instance, we did not observe a 9-fold increase in arsenic in the blood in CBN 5 exposed animals as compared to CBN 6 and 7. Based on bioavailability studies performed for this study, it was learned that exposure to metals via oropharyngeal or intravenous exposure were typically baseline levels within 24 h (data not shown). The levels of metals in the blood of exposed animals did not allow us to clearly identify one or more metals that we could classify as readily bioavailable or a likely candidate for eliciting the toxicities that we observed with dust from each CBN unit.

Alterations in red blood cell size and hemoglobin capacity were affected following exposure to CBN 5, but not CBN 6 or 7. Of the metals associated with the dust, lead is known to cause microcytic, hypochromic anemia, which would account for the decreased MCV. Lead is understood to have direct effects on three enzymes in the hemoglobin synthesis pathway: delta-aminolevulicin acid dehydratase (ALAD), aminolevulinic acid synthetase (ALAS), and ferrochelatase [34,35]. Despite the effect of lead on the heme synthase pathway, the microcytic cells did not have proportionately lower levels of hemoglobin as demonstrated by the MCHC. The MCHC measures the proportion of hemoglobin within each red blood cell. It was noted that iron in the geogenic dust samples was > 21 ppm. It might be considered that this excess iron from the exposure presented a possible compensatory factor to drive hemoglobin synthesis by increased ferritin cellular stores or increased saturation of transferrin. In the NDRA, CBN 5 and dust from drainage sediments that were analyzed in a previous study [19] were the only two sediment types that showed any effect on red blood cell physiology and it should be noted that they contained the highest levels of lead compared to all other NDRA geogenic dust samples.

Increased levels of serum creatinine, a marker for kidney function, were evident in all three geogenic dust samples. Creatinine is a by-product of muscle metabolism and is carried in plasma before excreted in the urine. This production happens at a fairly constant rate and is relative to muscle mass. The reliability of creatinine production makes it one of the more ideal analytes for assessing glomerular filtration rate in humans. Each of the three dust samples contained comparable levels of uranium and manganese, while CBN 5 had higher levels of lead and arsenic. All four of these metals are known to lead to kidney toxicity. However, the lowest adverse effect level for creatinine was represented by CBN 6 at 0.01 mg/kg. Based on deduction, it is not clear if there was any one element driving this change. However, it was noted that zinc levels in CBN 6 geogenic dust was lowest for all three dust samples. This is a relevant observation as zinc has been reported to reduce the toxicity of arsenic [36].

An increased number of peripheral WBCs was observed for both the CBN 6 (neutrophils) and 7 (lymphocytes and monocytes) exposure, but not CBN 5. Arsenic and lead were higher in CBN 5 as compared to CBN 6 and 7. It is not clear how to account for these observations, but it is known that arsenic is not likely to permit a compensational increase in white blood cells as it decreases migration of white blood cells and impairs maturation of cells in the bone marrow [37,38].

Assessment of neuropathology is a prerequisite for evaluating neurotoxicity [39]. Thus, autoantibodies against nervous system proteins were measured. When there is cellular damage within the nervous system, liberated proteins may induce an autoimmune response measurable as serum autoantibodies [30]. For instance, [40] reported increases in IgM and IgG autoantibodies against neural and glial proteins in rats exposed to only one dose of trimethyltin. Lead also induced changes in these neural proteins leading to lead-induced neurotoxicity [41]. CBN 5, with elevated levels of lead and arsenic, did not dose-responsively alter neurotoxicity markers in this study. While statistically significant changes in autoantibodies against neural proteins were observed following exposure to CBN 6 and CBN 7, generally these levels were reduced in animals from dosed groups, rather than increased. This is, however, consistent with the overwhelming immune suppression observed in the PFC assay. The reduction in serum IgG antibodies against MBP, NF-68 and GFAP likely reflects an overall reduction in the immune response (as indicated by the PFC assay) rather than a direct effect of dust samples on neuronal protein-specific antibody production. While neurotoxicity cannot be ruled out as a concern, under the conditions of this study, neurotoxicity was not evident.

The standardized PFC assay measures the ability of an organism's immune system to mount a primary IgM immune response to a T-cell

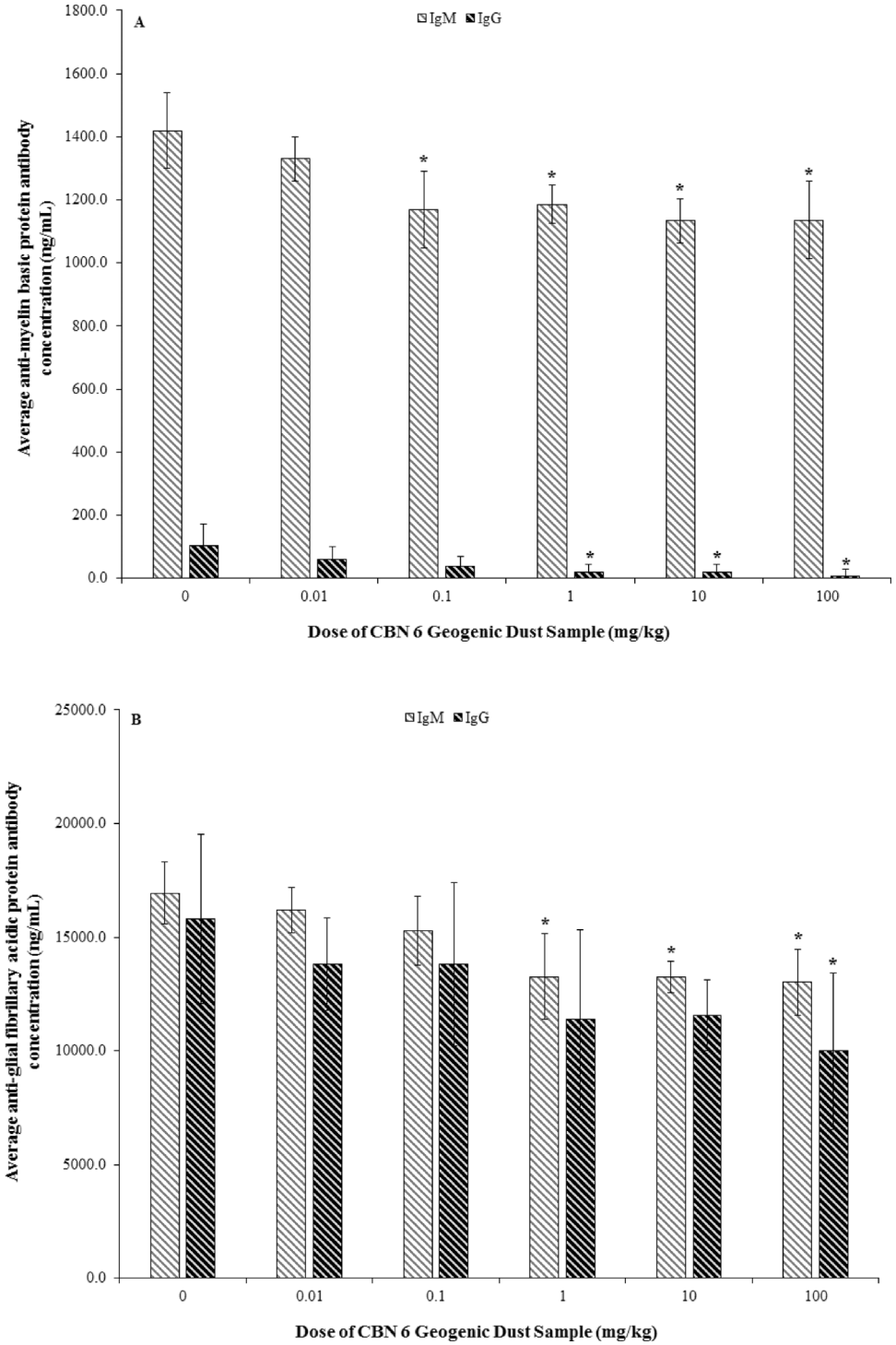

Fig. 5. Autoantibody production against (A) myelin basic protein (IgM and IgG) or (B) glial fibrillary acidic protein (IgM and IgG) in adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust samples from NDRA CBN 6. Data are presented as mean ± standard deviation. Sample size for each group was 5–6 animals. The (*) indicates a response statistically different from the 0 mg/kg group ($p < 0.05$).

dependent antigen, in this case, sheep red blood cells (SRBC). Such antigens require an appropriate response from both B cells, which secrete the antibody, and T cells, which 'help' the B cells. IgM levels were consistently and dose-responsively suppressed after exposure to each of the NDRA units in this study as well as previously published units [16–19]. These immune effects demonstrated from the complex mixture of metals are consistent with 'arsenic only' exposure studies that demonstrate a decrease in IgM humoral immune responses [37,38,42–44]. Additionally, low level exposure (< 50 ppb) to arsenic in drinking water has been reported to target systemic changes to include transcripts for humoral immune responses, antigen binding, cytokines, cytokine receptor expression as well as genes involved in cell adhesion and migration [45,46].

T-cell numbers and function can affect the outcome in the PFC assay or primary IgM response to a T-cell dependent antigen. That is, T helper cells provide a key intermediate step to achieving adequate levels of B-cell IgM production. Flow cytometric analysis demonstrated that exposure to CBN 6 and 7, but not CBN 5, affected T cell helper and

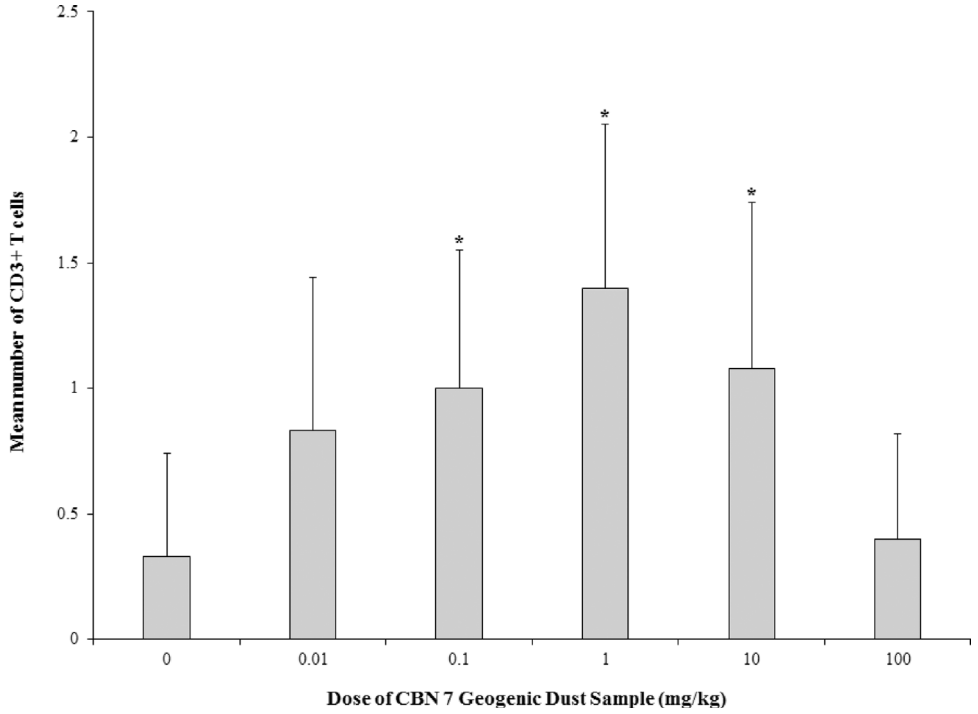

Fig. 6. Average number of CD3+ T cells observed in brains of adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust samples from NDRA CBN 7. Data are presented as mean ± standard deviation. Sample size for each group was 5–6 animals. The (*) indicates a response statistically different from the 0 mg/kg group ($p < 0.05$).

cytotoxic populations. Due to the proportionately higher levels of arsenic in CBN 5, it was anticipated that T cell populations would be decreased in this treatment, but this was not the case. This was unexpected as arsenic has been shown to affect T regulatory cell function, affect T cell migration, and reduce numbers mature and immature T cell populations [37,42,47]. These observations suggest that phenotypic T cell alterations may not be entirely driven by the highest level of metal in the mix, but some combination of the other metal components. Clearly, additional studies to identify the role of each metal in this mixture would be required offering more insight to this effect.

A strength of our study was that the design included an expanded dose response, an increased number of mice per treatment group, and the inclusion of many toxicological assays to maximize information gained. This approach was chosen as isolating dust at the median 3–4 μm level with enough sample to dose three replicate groups of mice required almost 1 ml of dust for the exposures in addition to another 1 ml required for analytical chemistry. Partitioning this geogenic dust from emissive surface types was no simple task. Although this study provided a comprehensive view of specific metal mixtures, it did not provide a clarity regarding the contribution of each metal to affected parameters.

An additional strength of our study design was the inclusion of the plaque forming cell (PFC) assay, NK cell assay, and flow cytometric evaluation of lymphocytic subpopulations. These three assays are recommended by the USEPA when assessing the potential risk of immunotoxicity to humans exposed to an exogenous agent. The PFC and NK cell assays are known to be predictive of alterations in immune function [48].

In summary, a clear dose-responsive suppression was observed in the IgM PFC humoral immune response from each unit and this observation established the NOAEL and LOAEL for each unit. The LOAEL established for CBN 5 and 6 was 0.01 mg/kg, while a NOAEL could not be established. For CBN 7, the NOAEL identified in this study was 0.01 mg/kg and the LOAEL established in this study was 0.1 mg/kg.

References

[1] World Health Organization (WHO). 2016. [Accessed 25 October 2016]. http://www.who.int/mediacentre/factsheets/fs313/en/.
[2] D.W. Griffin, C.A. Kellogg, E.A. Shinn, Dust in the wind: long range transport of dust in the atmosphere and its implications for global public and ecosystem health, Glob. Change Hum. Health 2 (2001) 20–33.
[3] E. Ganor, I. Osetinsky, A. Stupp, P. Alpert, Increasing trend of African dust, over 49 years, in the eastern Mediterranean, J. Geophys. Res. 115 (2010) D07201.
[4] M. Stafoggia, S. Zauli-Sajani, J. Pey, E. Samoli, E. Alessandrini, X. Basagaña, A. Cernigliaro, M. Chiusolo, M. Demaria, J. Díaz, A. Faustini, Desert dust outbreaks in Southern Europe: contribution to daily PM10 concentrations and short-term associations with mortality and hospital admissions, Environ. Health Perspect. 124 (2016) 413–419.
[5] D. Goossens, B. Buck, Dust emission by off-road driving: experiments on 17 arid soil types, Nevada, USA, Geomorphology 107 (2009) 118–138.
[6] D. Goossens, B. Buck, Dust dynamics in off-road vehicle trails: measurements on 16 arid soil types, Nevada, USA, J. Environ. Manage. 90 (2009) 3458–3469.
[7] D. Goossens, B. Buck, Effects of wind erosion, off-road vehicular activity, atmospheric conditions and the proximity of a metropolitan area on PM10 characteristics in a recreational site, Atmosp. Environ. 45 (2011) 94–107.
[8] D. Goossens, B. Buck, B. McLaurin, Contributions to atmospheric dust production of natural and anthropogenic emissions in a recreational area designated for off-road vehicular activity (Nellis Dunes, Nevada, USA), J. Arid Environ. 78 (2012) 80–99.
[9] J.L. Crooks, W.E. Cascio, M.S. Percy, J. Reyes, L.M.E.D. Neas Hilborn, The association between dust storms and daily non-accidental mortality in the United States, 1993–2005, Environ. Health Perspect. 124 (2016) 1735–1743.
[10] F.J. Kelly, J.C. Fussell, Health effects of airborne particles in relation to composition, size and source, in: R.M. Harrison, R.E.X. Hester Zuerol (Eds.), Airborne Particulate Matter: Sources, Atmospheric Processes and Health, Royal Society of Chemistry, London, 2016, pp. 344–382.
[11] C. Samara, D. Voutsa, Size distribution of airborne particulate matter and associated heavy metals in the roadside environment, Chemosphere 59 (2005) 1197–1206.
[12] L. Perez, A. Tobias, X. Querol, N. Kunzli, J. Pey, A. Alastuey, M. Viana, N. Valero, M. Gonzales-Cabre, J. Sunyer, Coarse particles from Saharan dust and daily mortality, Epidemiology 19 (2008) 800–807.
[13] C.C. Chan, K.J. Chuang, W.J. Chen, W.T. Chang, C.T. Lee, C.M. Peng, Increasing cardiopulmonary emergency room visits by long range transported Asian dust storms in Taiwan, Environ. Res. 106 (2008) 393–400.
[14] K.T. Kanatani, I. Ito, W.K. Al-Delaimy, Y. Adachi, W.C. Mathews, J.W. Ramsdell, Desert dust exposure is associated with increased risk of asthma hospitalization in children, Am. J. Respir. Crit. Care Med. 182 (2010) 1475–1481.
[15] J.H. Kang, T.C. Liu, J. Keller, H.C. Lin, Asian dust storm events are associated with an acute increase in stroke hospitalization, J. Epidemiol. Community Health 67 (2013) 125–131.
[16] D. Keil, B. Buck, D. Goossens, Y. Teng, M. Leetham, L. Murphy, J. Pollard, M. Eggers, B. McLaurin, R. Gerads, J. DeWitt, Immunotoxicological and

neurotoxicological profile of health effects following subacute exposure to geogenic dust from sand dunes at the Nellis Dunes Recreation Area, Las Vegas, NV, Toxicol. Appl. Pharmacol. 291 (2016) 1–12.
[17] D.E. Keil, B. Buck, D. Goossens, Y. Teng, J. Pollard, B. McLaurin, R. Gerads, J.C. DeWitt, Health effects from exposure to atmospheric mineral dust near Las Vegas NV, USA, Toxicol. Rep. 3 (2016) 785–795.
[18] J. DeWitt, B. Buck, D. Goossens, Q. Hu, R. Chow, W. David, S. Young, Y. Teng, M. Leetham-Spencer, L. Murphy, J. Pollard, D. Keil, Health effects following subacute exposure to geogenic dusts from arsenic-rich sediment at the Nellis Dunes Recreation Area, Las Vegas, NV, Toxicol. Appl. Pharmacol. 304 (2016) 79–89.
[19] J.C. DeWitt, B.J. Buck, D. Goossens, Y. Teng, J. Pollard, B.T. McLaurin, R. Gerads, D.E. Keil, Health effects following subacute exposure to geogenic dust collected from active drainage surfaces (Nellis Dunes Recreation Area, Las Vegas, NV), Toxicol. Rep. 4 (2016) 19–31.
[20] M. Leetham, J. DeWitt, B. Buck, D. Goossens, Y. Teng, J. Pollard, B. McLaurin, R. Gerads, D.E. Keil, Oxidative stress and lung pathology following geogenic dust exposure, J. Appl. Toxicol. 36 (2016) 1276–1283.
[21] National Research Council (US), Committee on research priorities for airborne particulate matter, Research Priorities for Airborne Particulate Matter: Continuing Research Progress IV National Academies Press, 119, 2004.
[22] D. Soukup, B.J. Buck, D. Goossens, Y. Teng, D. Baron, Mineralogical composition of soil samples in the Nellis Dunes Recreation Area, in: D. Goossens, B.J. Buck (Eds.), Assessment of Dust Emissions, Chemistry, and Mineralogy for Management of Natural and Disturbed Surfaces at Nellis Dunes Recreation Area, Nevada, Final Report to Bureau of Land Management for Task Agreement Number FAA010017, 2011, pp. 171–187.
[23] A. Kulshrestha, P.G. Satsangi, J. Masih, A. Taneja, Metal concentration of PM2.5 and PM10 particles and seasonal variations in urban and rural environment of Agra, India, Sci. Total Environ. 407 (2009) 6196–6204.
[24] J. Aldabe, D. Elustondo, C. Santamaría, E. Lasheras, M. Pandolfi, A. Alastuey, X. Querol, J.M. Santamaría, Chemical characterisation and source apportionment of PM2.5 and PM10 at rural: urban and traffic sites in Navarra (North of Spain), Atmos. Res. 102 (2011) 191–205.
[25] R.M. de Miranda, M. Fatima Andrade, A. Fornaro, R. Astolfo, P.A. de Andre, P. Saldiva, Urban air pollution: a representative survey of PM(2.5) mass concentrations in six Brazilian cities, Air Qual. Atmosp. Health 5 (2012) 63–77.
[26] J. Wang, Z. Hu, Y. Chen, Z. Chen, S. Xu, Contamination characteristics and possible sources of PM10 and PM2.5 in different functional areas of Shanghai, China, Atmos. Environ. 68 (2013) 221–229.
[27] P.H. Briggs, A.L. Meier, The determination of forty-two elements in geological materials by inductively coupled plasma-mass spectrometry, in: J.E. Taggart (Ed.), Analytical Methods for Chemical Analysis of Geologic and Other Materials: U.S. Geological Survey Open-File Report 02-0223, 1999, pp. I1–I14 (Also available online at http://pubs.usgs.gov/of/2002/ofr-02-0223/).
[28] K.M. Kubachka, N.V. Shockey, T.A. Hanley, S.D.D.T. Conklin Heitkemper, US FDA Method 4.11 Version 1.1: Arsenic Speciation in Rice and Rice Products Using High Performance Liquid Chromatography-Inductively Coupled Plasma-Mass Spectrometric Determination [Internet], (2012) Available from: http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm328363. htm.
[29] D. Keil, A. Dudley, J. EuDaly, C. Allen, G. Gilkeson, M. Peden-Adams, *N,N*-diethyl-*m*-toluamide (DEET) alters immune function in B6C3F1 mice, Toxicol. Sci. 108 (2009) 110–123.
[30] H.A.N. El-Fawal, S.J. Waterman, A. DeFeo, M.Y. Shamy, Neuroimmunotoxicology: humoral assessment of neurotoxicity and autoimmune mechanisms, Environ. Health Perspect. 107 (1999) 767–775.
[31] M.C. Reheis, J.R. Budahn, P.J. Lamothe, R.L. Reynolds, Compositions of modern dust and surface sediments in the Desert Southwest: United States, J. Geophys. Res. 114 (2009) 1–20.
[32] D. Soukup, B. Buck, D. Goossens, A. Ulery, B. McLaurin, D. Baron, Y. Teng, Arsenic concentrations in dust emissions from wind erosion and off-road vehicles in the Nellis Dunes Recreational Area Nevada, USA, Aeolian Res. 5 (2012) 77–89.
[33] B.J. Buck, D. Keil, D. Goossens, J. DeWitt, B. McLaurin, Nellis dunes recreation area: dust exposure and human health risk assessment, Final Report to Bureau of Land Management for Task Agreement Number L11AC20058, (2014).
[34] G. Flora, D. Gupta, A. Tiwari, Toxicity of lead: a review with recent updates, Interdiscip. Toxicol. 5 (2012) 47–58.
[35] T.L. Trombini, E. Oliveira, D.B. Berlese, R. Minozzo, T. De Deus, C.D. Muller, R. Linden, V.C. Figueiredo, C.M. Wannmacher, G.M. Conterato, L.R. Feksa, Inhibition of thiol-containing enzymes in erythrocytes of workers exposed to lead, Interciencia 40 (2015) 84–90.
[36] X. Zhou, X. Sun, C. Mobarak, A.J. Gandolfi, S.W. Burchiel, L.G. Hudson, K.J. Liu, Differential binding of monomethylarsonous acid compared to arsenite and arsenic trioxide with zinc finger peptides and proteins, Chem. Res. Toxicol. 27 (2014) 690–698.
[37] P.C. Ezeh, F.T. Lauer, D. MacKenzie, S. McClain, K.J. Liu, L.G. Hudson, A.J. Gandolfi, S.W. Burchiel, Arsenite selectively inhibits mouse bone marrow lymphoid progenitor cell development in vivo and in vitro and suppresses humoral immunity in vivo, PLoS One 9 (2014) e93920.
[38] S.W. Burchiel, L.A. Mitchell, F.T. Lauer, X. Sun, J.D. McDonald, L.G. Hudson, K.J. Liu, Immunotoxicity and biodistribution analysis of arsenic trioxide in C57Bl/6 mice following a 2-week inhalation exposure, Toxicol. Appl. Pharmacol. 241 (2009) 253–259.
[39] USEPA (U.S. Environmental Protection Agency), Method 6020A. Inductively Coupled Plasma-Mass Spectrometry. 6000 Series Methods, (2007) www.epa.gov/osw/hazard/testmethods/sw846/pdfs/6020a.pdf.
[40] H.A.N. El-Fawal, J.P. O'Callaghan, Autoantibodies to neurotypic and gliotypic proteins as biomarkers of neurotoxicity: assessment of trimethyltin (TMT), Neurotoxicology 29 (2008) 109–115.
[41] S.J. Waterman, H.A. El-Fawal, C.A. Snyder, Lead alters the immunogenicity of two neural proteins: a potential mechanism for the progression of lead-induced neurotoxicity, Environ. Health Perspect. 102 (1994) 1052.
[42] A.S. Andrew, D.A. Jewell, R.A. Mason, M.L. Whitfield, J.H. Moore, M.R. Karagas, Drinking-water arsenic exposure modulates gene expression in human lymphocytes from a U.S. population, Environ. Health Perspect. 116 (2008) 524–531.
[43] Q. Li, F.T. Lauer, K.J. Liu, L.G. Hudson, S.W. Burchiel, Low-dose synergistic immunosuppression of T-dependent antibody responses by polycyclic aromatic hydrocarbons and arsenic in C57BL/6J murine spleen cells, Toxicol. Appl. Pharmacol. 245 (2010) 344–351.
[44] M.K. Singh, S.S. Yadav, R.S. Yadav, A. Chauhan, D. Katiyar, S. Khattri, Protective effect of emblica-officinalis in arsenic induced biochemical alteration and inflammation in mice, Springer Plus 4 (2015) 1.
[45] A. Andrew, V. Bernardo, L. Warnke, J. Davey, T. Hampton, R. Mason, J. Thorpe, M. Ihnat, J. Hamilton, Exposure to arsenic at levels found in U.S. drinking water modifies expression in the mouse lung, Toxicol. Sci. 100 (2007) 75–87.
[46] C. Kozul, T. Hampton, J. Davey, J. Gosse, A. Nomikos, P. Eisenhauer, D. Weiss, J. Thorpe, M. Ihnat, J. Hamilton, Chronic exposure to arsenic in the drinking water alters the expression of immune response genes in mouse lung, Environ. Health Perspect. 117 (2009) 11805–11815.
[47] B. Hernández-Castro, L.M. Doníz-Padilla, M. Salgado-Bustamante, D. Rocha, M.D. Ortiz-Pérez, M.E. Jimenez-Capdeville, D.P. Portales-Pérez, A. Quintanar-Stephano, R. González-Amaro, Effect of arsenic on regulatory T cells, J. Clin. Immunol. 29 (2009) 461–469.
[48] M.I. Luster, C. Portier, D.G. Pait, G.J. Rosenthal, D.R. Germolec, E. Corsini, B.L. Blaylock, P. Pollock, Y. Kouchi, W. Craig, K.L. White, A.E. Munson, C.E. Comment, Risk assessment in immunotoxicology. II. Relationships between immune and host resistance tests, Fundam. Appl. Toxicol. 21 (1993) 71–82.