



# Health effects following subacute exposure to geogenic dust collected from active drainage surfaces (Nellis Dunes Recreation Area, Las Vegas, NV)



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## ABSTRACT

The specific health effects of direct inhalation of fine minerogenic dusts generated by natural soil surfaces remain poorly known and relatively little researched. To learn more about this exposure and its contribution to human health effects, we surveyed surface sediment and characterized dust from the Nellis Dunes Recreation Area (NDRA) in Clark County, Nevada, a popular off-road vehicle (ORV) recreational site. Dry drainage systems at NDRA are commonly used as natural trail systems for ORV recreation; these surfaces also are characterized by high concentrations of heavy metals. Geogenic dust with a median diameter of 4.05  $\mu\text{m}$ , collected from drainage surfaces at NDRA contained a total elemental concentration of aluminum (79,651  $\mu\text{g/g}$ ), vanadium (100  $\mu\text{g/g}$ ), chromium (54  $\mu\text{g/g}$ ), manganese (753  $\mu\text{g/g}$ ), iron (33,266  $\mu\text{g/g}$ ), cobalt (14  $\mu\text{g/g}$ ), copper (37  $\mu\text{g/g}$ ), zinc (135  $\mu\text{g/g}$ ), arsenic (71  $\mu\text{g/g}$ ), strontium (666  $\mu\text{g/g}$ ), cesium (15  $\mu\text{g/g}$ ), lead (34  $\mu\text{g/g}$ ), and uranium (54.9  $\mu\text{g/g}$ ). Adult female B6C3F1 mice exposed via oropharyngeal aspiration to 0.01–100 mg dust/kg body weight, four times, a week apart, for 28-days, were evaluated for immuno- and neurotoxicological outcomes 24 h after the last exposure. Antigen-specific IgM responses were dose-responsively suppressed at 0.1, 1.0, 10 and 100 mg/kg. Splenic lymphocytic subpopulations, hematological and clinical chemistry parameters were affected. In brain tissue, antibodies against NF-68, and GFAP were not affected, whereas IgM antibodies against MBP were reduced by 26.6% only in the highest dose group. A lowest observed adverse effect level (LOAEL) of 0.1 mg/kg/day and a no observed adverse effect level (NOAEL) of 0.01 mg/kg/day were derived based on the antigen primary IgM responses after subacute exposure to this geogenic dust.

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## 1. Introduction

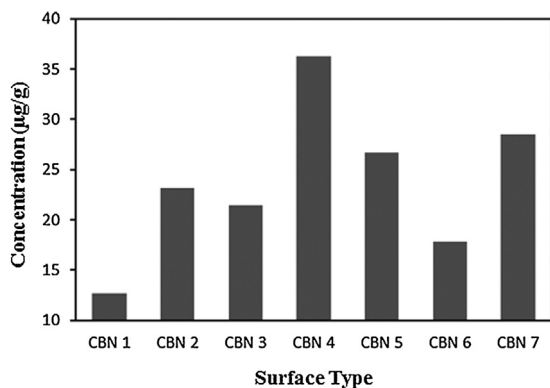
Desertification threatens dry land areas that comprise over a third of the earth's total land area and have the potential to affect populations of 2 billion or more [22]. Furthermore, there is an increasing concern that the frequency and volume of dusts produced will increase with climate change [22]. Desert land surfaces are more susceptible to wind erosion, particularly following anthropogenic disturbances such as off road vehicle activities,

construction, and other activities that disturb surfaces. Following disruption of a desert surface, for example, dust emissions may increase long after the initial disturbance [13].

Most research on particulate matter (PM) has analyzed the health effects of anthropogenic air pollution in urban areas; what is less well understood are health effects from naturally-derived mineral particles or "geogenic dust" [9]. In a comprehensive review of the health effects of exposure to geogenic dusts, Morman and Plumlee [28] noted that there is little research about the local health effects of inorganic mineral dusts, because there is very little PM monitoring in rural arid and agricultural areas where people are most often exposed to these dusts.

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**Fig. 1.** Total concentration of heavy metals in each of the seven mapped regions of the NDRA. Data are for the topsoil fraction (<2 mm from surface). Concentration represents total µg/g of the following metals: Ag, As, B, Cd, Co, Cr, Cs, Cu, Hg, Mo, Ni, Pb, Sb, Sr, Tl, U, and V (a broader assessment of metals than for the current study) and represents one sample for 3–5 sites/mapped region. The drainage sediments (CBN 4) have the highest concentration of metals in the NDRA (from [35]).

Drainage channels distribute and collect sediment from the whole hydrologic basin they drain. The sediment is supplied by runoff and concentrates in the main flow channel. In dry areas, drainages are often intermittent and remain dry during a large part of the year. As a result, much of the mineral and metal content transported by water is deposited in the channel sediment when the drainage dries out. The consecutive succession of sediment supply and subsequent accumulation results in the progressive accumulation of heavy minerals and metals in the drainage sediment. Drainage channels in dry areas are, therefore, often characterized by high concentrations of such pollutants.

This manuscript reports on one of seven desert regions (designated as CBN 1 to CBN 7) at the Nellis Dunes Recreation Area (NDRA). These regions differ based on surface sediment types and therefore the type of geogenic dust that they generate. The NDRA is located only minutes from a major metropolitan population and has provided the only publicly accessible area in southern Nevada for legal off-road vehicle (ORV) driving, with annual visitors estimated at over 300,000 [15]. Many of the ORV trails follow or frequently cross the drainage channels of the area. These drainage areas contain sediments that have the highest concentrations of heavy metals of all seven of the CBN regions in the NDRA (Fig. 1).

In this study, we focused on all of the major active drainages at NDRA and inset Holocene geomorphic surfaces directly adjacent to the drainages (designated as CBN 4). As previously mentioned, due to the arid climate, these drainages only flow with water during intense storm events (e.g. flash floods). The drainage channels are unvegetated and characterized by unconsolidated sediments of varying grain sizes. McLaurin et al. [27] mapped these drainages into three specific units based on their potential to emit dust: course-grained drainages almost entirely free of fine-grained sediment, drainages with a mixture of sand and gravel, and drainages with silt and clay as well as the gravel. In addition to these three units, which only occur in the active channel itself, drainage sediment also occurs in areas of silty alluvium in middle to latest Holocene inset fans adjacent to the active channels [27]. This sediment commonly has well-developed biological crust, rock cover is sparse, and shrubs are isolated. The ensemble of the four types of drainage sediments in the NDRA have been designated in this study as CBN 4 and comprise approximately 252 acres. Although this acreage equates to only 2.8% of the surface area at NDRA, the trails in it are heavily used by ORV riders.

As would be expected, the mineralogy of CBN 4 reflects all of the surfaces that drain into it. It contains quartz, calcite, gypsum, feldspar, smectite, illite, palygorskite, kaolinite, chlorite and amphi-

bole minerals. These minerals have within their crystal structure or adsorbed on their surfaces, many different heavy metals and metalloids. We analyzed the dust 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), chromium (Cr), and silicon (Si).

The primary pathway of geogenic dust exposure is inhalation, yet there is insufficient evidence to identify differences in the health effects of dust particles with different chemical compositions [36]. This report presents a profile of toxicological effects with specific focus on select immune and nervous system endpoints. While we modeled our study after U.S. Environmental Protection Agency harmonized test guidelines for immunotoxicity, we included additional generalized markers of lung pathology and neurotoxicity. Lung pathology was evaluated in an accompanying manuscript [24] and we demonstrated that exposure to this dust failed to produce overt signs of lung inflammation or lung pathology. Therefore, the endpoints described in this study likely reflect systemic effects rather than secondary effects of lung inflammation or pathology. As the potential health consequences of exposure to these dusts is unknown, our study was a necessary descriptor of the potential health effects of these geogenic dusts before any studies designed to address potential mechanisms could be addressed. Adult female B6C3F1 mice were exposed via oropharyngeal aspiration to CBN 4 geogenic dust with a median grain size of 4.05 µm at concentrations of 0.01–100 mg of dust per kg of body weight for four exposures spaced a week apart over a 28-day period to model a month of weekend exposures. Additional measures of hematology, clinical chemistry, and descriptive and functional assays to assess toxicity to the immune and nervous systems were performed 24 h after the final exposure. Characterization included the establishment of no observed adverse effect levels (NOAEL) and/or lowest observed adverse effect levels (LOAEL).

## 2. Methods

### 2.1. Collection of geogenic dust

Composite samples were collected from the surface sediment (upper 0–4 cm) 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 area was carefully recorded. After drying, the samples were mixed together to form a single sample, which was then treated in a Soil Fine Particle Extractor (see [14]) to extract a sample with a median diameter of approximately 4 µm. This size particle was chosen to ensure deep penetration into the rodent lung and to ensure that we were mimicking the very small particle sizes that have the potential for greater health risk. This size was chosen based on work by Brown et al. [4] that for typical activity levels and breathing habits, a 50% cut-size for the thoracic fraction at an aerodynamic diameter of around 3 µm was estimated for adults whereas current ambient and occupational criteria suggest a 50% cut-size of 10 µm. The exact particle size distribution of the extract (Fig. 3) was determined in water with laser diffraction using a Malvern Mastersizer S laser particle size analyzer (Malvern Instruments Ltd., Malvern, UK).

### 2.2. ICP-MS analyses of geogenic dust

All samples were digested in accordance with the USGS Four-Acids Method [3] 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

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

**Fig. 2.** Toxicology sample collection arrangement.

US EPA Method 6020A [37] were followed. In addition, NIST SRM 8704 (Buffalo River Sediment) and NIST SRM 2711a (Montana II Soil) were used as standard reference materials (SRMs). The method quantitation limit (MQL) was three times the detection limit.

### 2.3. Arsenic speciation

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 [21].

### 2.4. Quantitation of silicon in geogenic dust

Analysis for the element silicon utilized a Thermo Scientific Niton XL3 t GOLDD+ portable XRF (X-ray fluorescence) instrument. For calibration, the NIST Standard Reference Material 2711A was run and the XRF results were in agreement with the certified values for silicon and within the margins of uncertainty for the soil standard. Six samples from the <60  $\mu\text{m}$  fraction were analyzed from CBN 4, 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 4 samples were carefully labeled, stored in sealed and dry containers, protected from light, and secured in a lock box in the laboratory. We included a fairly wide dose range to account for the complexity of the human exposure situation. Actual human exposures at the NDRA and in other arid regions will depend on the soil characteristics at a particular site (soil texture, vegetation, soil moisture, rock cover, etc.), meteorological conditions (rain, humidity), and size, type, and speed of ORV as well as whether the erosion type is wind or ORV-derived. Samples were prepared in sterile, endotoxin-free, phosphate buffered saline (ETF-PBS) at concentrations adjusted to a delivery volume of 10  $\mu\text{l}$  that would represent 0.01, 0.1, 1, 10, or 100 mg of dust per kg of body weight. Mice were exposed via the lungs within 1–2 h of dust solution preparation. Addition of dust samples to ETF-PBS 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 in ETF-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 4 dust was 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

Adult female B6C3F1 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,  $22 \pm 2^\circ\text{C}$ , 60–65% relative humidity) at the University of Nevada Las Vegas (UNLV) animal facilities, which are 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 corn-cob bedding and were given unlimited access to food and water. Female mice were used for these studies as all preliminary studies were conducted in female mice [19] and the mass of geogenic dust available for animal studies was low due to the complexity of the dry fractional procedure, thus necessitating a reduction in animal numbers overall.

### 2.7. Animal exposures

To simulate the potential health impacts of a month of week-end exposures to CBN 4 dust samples from the NDRA, mice were exposed to CBN 4 extracts with a median diameter of 4.05  $\mu\text{m}$  (Table 1) 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. Therefore, based on a 20 g mouse, 20  $\mu\text{g}$  was adminis-

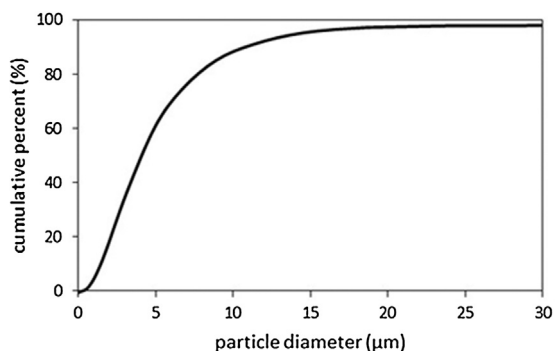
**Table 1**  
Total elemental concentration ( $\mu\text{g/g}$ ) in dry geogenic dust from CBN 4.

Median <sup>a</sup>	Al	V	Cr	Mn	Fe	Co	Cu	Zn	As	Sr	Cd	Sb	Cs	Tl	Pb	U	Si <sup>b</sup>
4.05	79,651	100	54	752	33,266	14	37	135	71	666	<0.47	<3.0	15	<8.3	34	4.9	199,740

<Indicates value is below method quantitation limit (MQL) and value shown is the MQL. Data are reported with a maximum of three significant figures.

<sup>a</sup> Median diameter ( $\mu\text{m}$ ) of geogenic dust delivered to animals.

<sup>b</sup> Si measured in the <60  $\mu\text{m}$  fraction and may not be representative to Si in geogenic dust delivered to animals.



**Fig. 3.** Particle size distribution of the CBN 4 geogenic dust used in this study.

tered to the lung via oropharyngeal aspiration. 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. Samples for toxicity studies were collected from each group three days in a row. Fig. 2 depicts the basic arrangement for replicates and sample collection.

Mice were exposed by oropharyngeal aspiration using isoflurane as an anesthetic agent. The dose of CBN 4 solution was based on current body weight of the mouse. An average volume of 10  $\mu\text{l}$  was administered to each mouse; however, based on individual body weight changes, this volume was adjusted to between 9–13  $\mu\text{l}$  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 4.

### 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 suspended in complete medium (RPMI, 10% fetal calf serum, 50 IU penicillin and 50  $\mu\text{g}$  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 Fig. 2).

### 2.9. Endpoints collected for toxicological assays

Briefly, as described in [18], sera, spleens, thymuses, and brain were prepared and evaluated in the following assays.

#### 2.9.1. 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 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 (serum). Hematologies were 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  $-80^\circ\text{C}$  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 volumes, whole blood values were determined only.

#### 2.9.2. 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  $1 \times 10^7$  cells/mL. Optimal concentrations of flow 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.

#### 2.9.3. Immunophenotyping of regulatory T lymphocytes (Tregs)

Splenic lymphocytes were adjusted to a concentration of  $1 \times 10^6$  cells per well and depleted of red blood cells via a 5-min incubation in  $\text{NH}_4\text{Cl}$  lysis buffer at  $37^\circ\text{C}$ . Monoclonal antibodies coupled to fluorochromes specific for the following markers were used at a concentration of 1  $\mu\text{g}/10^6$  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. 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 lym-

phocyte fraction were gated, and the percentages of CD25 + foxP3+ cells, CD25 + foxP3- cells, IL-17<sup>+</sup>, and IL-17<sup>-</sup> cells were calculated.

#### 2.9.4. 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  $\mu$ l 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  $1.0 \times 10^6$  cells/mL. A 10  $\mu$ l aliquot of the single-cell suspension was added to a tube containing 100  $\mu$ l of 25% SRBC in PBS, 40  $\mu$ l of RPMI medium (without additives), and 50  $\mu$ l 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<sub>2</sub> for 1–2 h. PFCs were counted microscopically and were reported as PFCs/million splenocytes.

#### 2.9.5. Natural killer cell activity

Natural killer (NK) cell activity was assessed via an in vitro cytotoxicity assay using <sup>51</sup>Cr-labeled Yac-1 cells as described previously [11,16]. 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  $2 \times 10^7$  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 <sup>51</sup>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<sub>2</sub>, the plates were centrifuged (1200 rpm, 3 min), and 25  $\mu$ l 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<sup>7</sup> splenocytes using 10% lysis as the reference point as described by [5]; equation 10. Essentially, this measure considers the target tumor cell activity in the context of both maximum and spontaneous release.

#### 2.9.6. 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 [12]. 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. Values that fell below the limits of detection were assigned a value of zero. Values that fell above the limits of detection were diluted upon the second evaluation. Values that still remained above the limits of detection after dilution were eliminated from the overall calculations due to insufficient amounts of sample.

#### 2.9.7. Brain histology

Two sections of cerebellum from each mouse brain, each 10  $\mu$ m 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

**Table 2**

Arsenic speciation results of dust collected from CBN 4.

AS(III)	AS(V)	MMAs	DMAs	Units
ND (<0.29)	15.7	ND (<0.22)	ND (<0.19)	$\mu$ g/g

ND = Not detected at the applied dilution.

MMAs = Monomethylarsonic acid.

DMAs = Dimethylarsinic acid.

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.

#### 2.10. Particle positive control

As described in Keil et al. [18], separate groups of mice (N=6/dose) were exposed to titanium dioxide (TiO<sub>2</sub>), a particle with no associated metals (21 nm in size), to help us to discern potential particle-only effects from the effects of metals and particles together. Mice were given matching concentrations (0.01–100 mg of TiO<sub>2</sub>/kg of body weight) and were exposed via the same paradigm, as the geogenic dusts. Toxicity testing was evaluated in the TiO<sub>2</sub>-exposed mice in parallel with CBN 2-exposed mice. Data, although relevant to this study too, have been reported in a previous publication with a different geogenic dust [18].

#### 2.11. 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 dose group to the dose-matched TiO<sub>2</sub> group.

#### 2.12. 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. CBN 4 geogenic dust characterization

Dust from CBN 4 used in this study had a median diameter of 4.05  $\mu$ m (Fig. 3 and Table 1). Total digestion chemical composition of the dust are shown in Table 1. All of the arsenic was As(V) (Table 2). Of the metals in this sample, aluminum and iron concentrations were the found to have the highest concentrations 79,651  $\mu$ g/g and 33,266  $\mu$ g/g respectively (Table 1). When com-

paring the different concentrations of metals among all seven CBN units in the NDRA, CBN 4 reflects the highest proportional amounts in Al, V, Cr, Zn, Sr and Pb (Fig. 1).

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

Body weight assessed at the beginning of the study compared to body weight assessed at the study terminus did not differ by dose for two of the three replicates. In one replicate, the average body weight change of the 1 mg/kg group was 125% greater relative to the 0 mg/kg group. This appears to be associated with several mice in the 1 mg/kg group that gained more than 3 g over the course of the study. This is unlikely to be treatment-related as the final body weights were still well within the normal weight range for adult female B6C3F1 mice and no other replicates had similar changes. No significant changes in organ weights or in spleen or thymus cellularity were observed for any replicate. No significant changes were observed in mice exposed to TiO<sub>2</sub> [18].

### 3.3. Hematology, clinical chemistry, and blood metals

Of the hematological endpoints measured in mice dosed with dust samples from CBN 4, hemoglobin (HGB) and mean corpuscular volume (MCV) were endpoints with statistical differences between exposed and unexposed animals. HGB concentration was elevated by about 5%, on average, in mice following exposure to 1, 10, and 100 mg/kg relative to mice from the 0 mg/kg group (Fig. 4A). In these same dose groups, MCV was decreased by about 20% in each group relative to concentrations in the 0 mg/kg group (Fig. 4B). Of the clinical chemistry endpoints, alanine aminotransferase (ALT) and plasma creatinine were altered. Following exposure to all doses, ALT was decreased by 45.6%–62.6% relative to the 0 mg/kg group (Fig. 5A). Creatinine levels were increased by 10% in both the 10 and 100 mg/kg dose groups relative to the 0 mg/kg dose group (Fig. 5B).

Blood from animals exposed to dust from CBN 4 had detectable concentrations of the metals/metalloids known to be associated with this dust (Table 3). Mean concentration did not differ by dose for all metals/metalloids but arsenic. Blood concentrations of arsenic in animals exposed to 1 mg/kg of dust from CBN 4 had levels that were 51% higher than animals in the 0 mg/kg group.

### 3.4. Immunophenotype

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 by exposure to dust samples from CBN 4 (Table 4). However, the number of T cells from the spleen was statistically altered by exposure (Table 4). 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. No significant changes were observed in mice exposed to TiO<sub>2</sub> [15].

### 3.5. Plaque forming cell (PFC) assay

Exposure to dust samples from CBN 4 reduced the number of plaque forming cells secreting IgM antibody to SRBC at all administered doses of 0.1 mg/kg and higher (Fig. 6). PFC/million of spleen cells was reduced by 39.9–62.4% in these dosed groups relative to the 0 mg/kg group. From these data, 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 mice exposed to TiO<sub>2</sub> reported in Keil et al., 2016.

### 3.6. Natural killer cell activity

Exposure to dust samples from CBN 4 had no significant effect on the ability of natural killer cells to lyse target cells (data not shown). No significant changes were observed in mice exposed to TiO<sub>2</sub>.

### 3.7. Neuronal autoantibody formation

Exposure to dust samples from CBN 4 did not alter IgM or IgG antibody production against NF-68 or GFAP or IgG antibody production against MBP, relative to antibody production in the 0 mg/kg group. Exposure to dust samples from CBN4 at a concentration of 100 mg/kg did, however, reduce IgM antibodies against MBP by 26.6% relative to the 0 mg/kg group (Fig. 7).

### 3.8. Brain histology

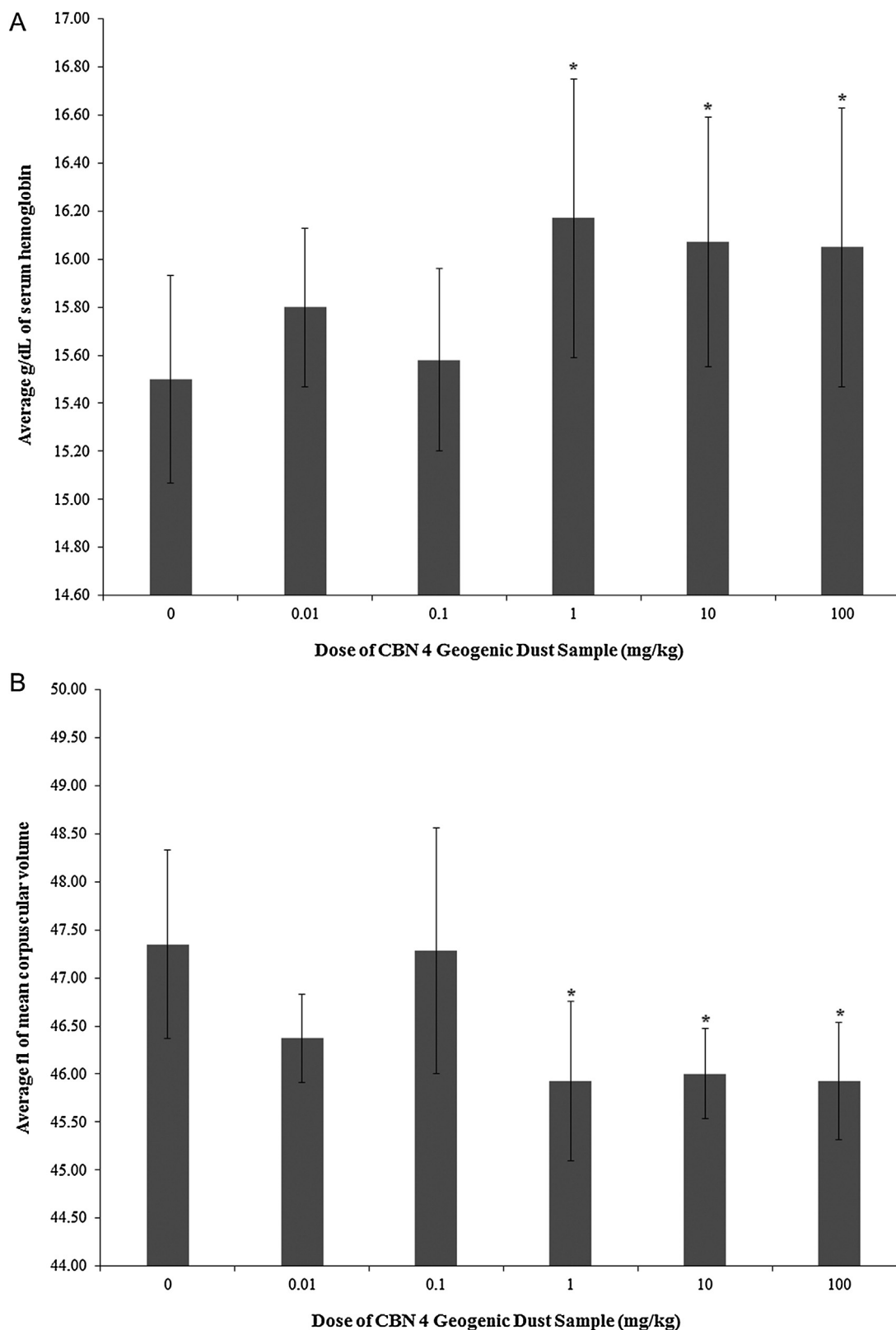
No CD3+ T cells were observed in any of the brains of animals exposed to dust samples from CBN 4. Relative to staining observed in the 0 mg/kg group, MBP staining in brain sections from animals exposed to 1 mg/kg was 18.8% less intense and was 29.5% less intense in brain sections from animals exposed to 100 mg/kg (Fig. 8).

## 4. Discussion

The National Academy of Sciences [31] emphasizes the need for a multipollutant approach. That is, an expansive, multidisciplinary strategy with the coordination of toxicology, exposure, geology, and atmospheric research has been designated a priority for understanding airborne dust exposures. Our research approach represents such an effort in defining potential health effects specifically focused on a natural, desert area in Clark County, Nevada.

Understanding health impacts of heavy-metal dust exposure is a global concern [1,8,23,38]. Our study examined a complex dust exposure representative of dusts generated from active drainages at the NDRA. In this study, the median diameter of dust was the fine fraction and a respirable size for both mice and humans, 4.05 μm, and aluminum and iron were the predominant metals in the dust samples. However, in general, the chemical composition of this geogenic dust is similar to that measured from other areas in the southwestern USA [32]. Mineral dusts are commonly high in silica and aluminum, reflecting that they are primarily composed of common crustal aluminum silicate minerals. The concentration of metals and metalloids will vary based on geologic processes that can concentrate specific elements in rocks. Other studies of geogenic dust typically analyze much coarser dust (~50 μm), which commonly contains lower concentrations of metals and metalloids and makes accurate comparisons with this study impossible. However, when the focus is on the fraction of airborne dust that penetrates deeply into the lungs, such as in our study, we have to work with the finest particles (~4 μm in this study). Because the data in other studies of geogenic dust are for coarser fractions, we cannot directly compare the metal/metalloid concentrations in dust from CBN 4 from NDRA to other desert environments in the southwestern USA or beyond. However, because the surfaces in the NDRA are very typical for the desert environments in southern Nevada and elsewhere in the world, we expect that when the fine fraction from other areas is analyzed, concentrations will at least be comparable to ours.

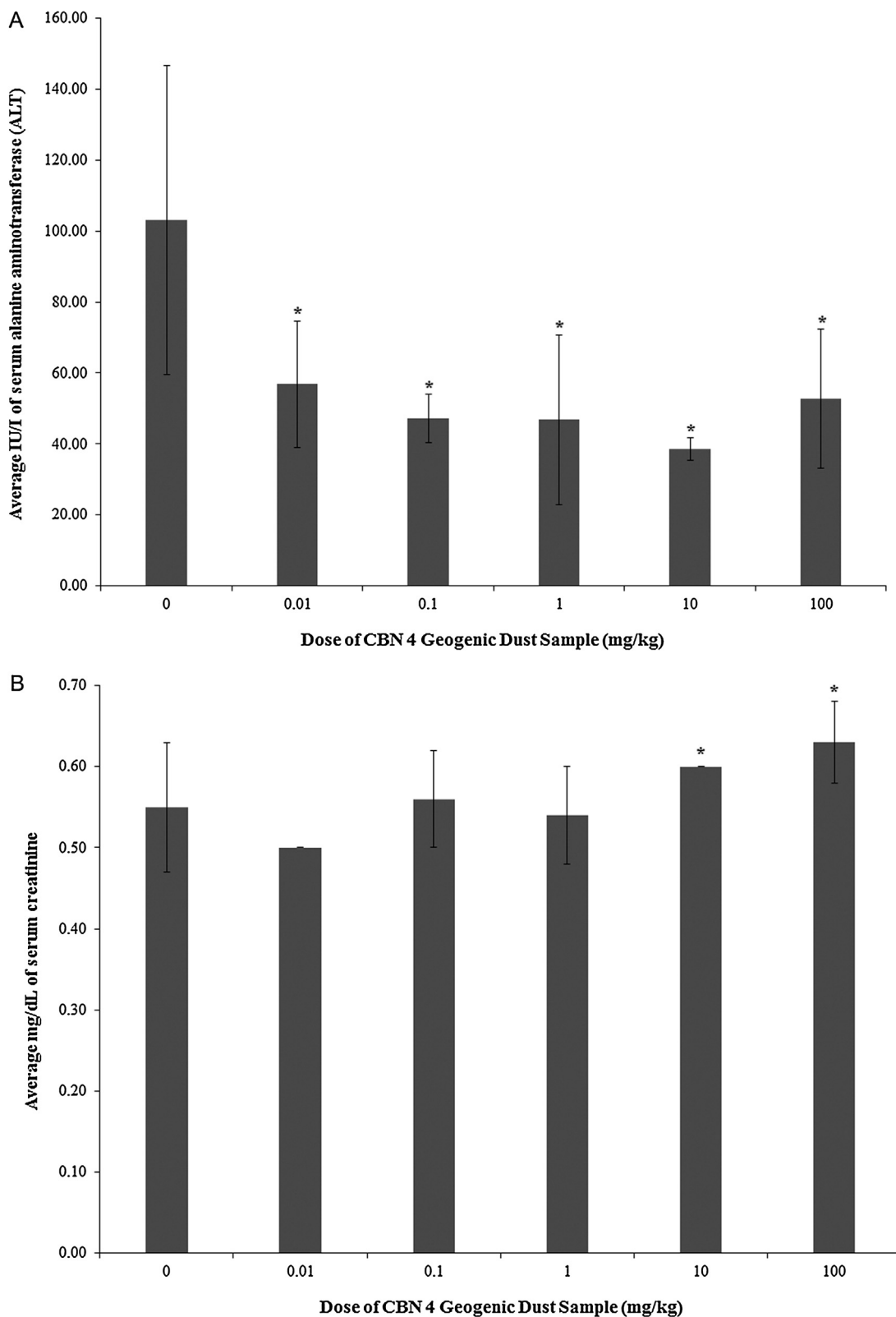
A large dose-response range was utilized to provide an opportunity to define a NOAEL and LOAEL. The plaque forming cell (PFC)



**Fig. 4.** Hematological endpoints measured in adult female B6C3F1 mice following oropharyngeal aspiration exposure to CBN 4 geogenic dust samples from NDRA each week for 28 days. Data are presented as mean percentage  $\pm$  standard deviation. Sample size for each group was 5–6 animals. Data presented are representative of one trial day. The (\*) indicates a response statistically different from the 0 mg/kg group ( $p < 0.05$ ). (A) Hemoglobin; (B) Mean corpuscular volume.

assay, NK cell assay, and flow cytometric evaluation of lymphocytic subpopulations were assessed in this study. These three assays are recommended by the US EPA when assessing the potential risk

of immunotoxicity to humans exposed to a particular agent. The PFC and NK cell assays are known to be predictive of alterations in immune function [25,26]. Suppression of the antigen-specific IgM



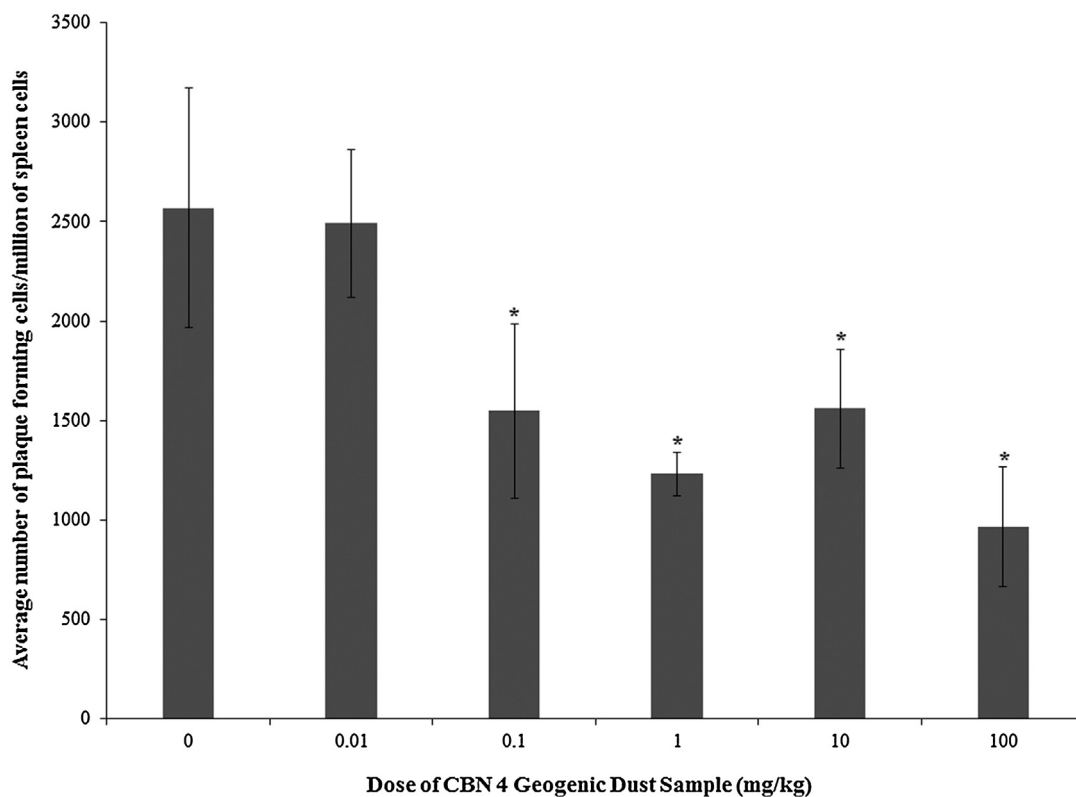
**Fig. 5.** Clinical chemistry endpoints measured in adult female B6C3F1 mice following oropharyngeal aspiration exposure to CBN 4 dust samples. Data are presented as mean  $\pm$  standard error of the mean. Sample size for each group was 5–6 animals. The (\*) indicates a response statistically different from the 0 mg/kg group ( $p < 0.05$ ). (A) Alanine aminotransferase (ALT); (B) Creatinine.

response suggests that the dust samples from CBN 4 impacted antibody production and/or secretion. The dose-responsive decrease of the PFC response indicates that this response is sensitive to this

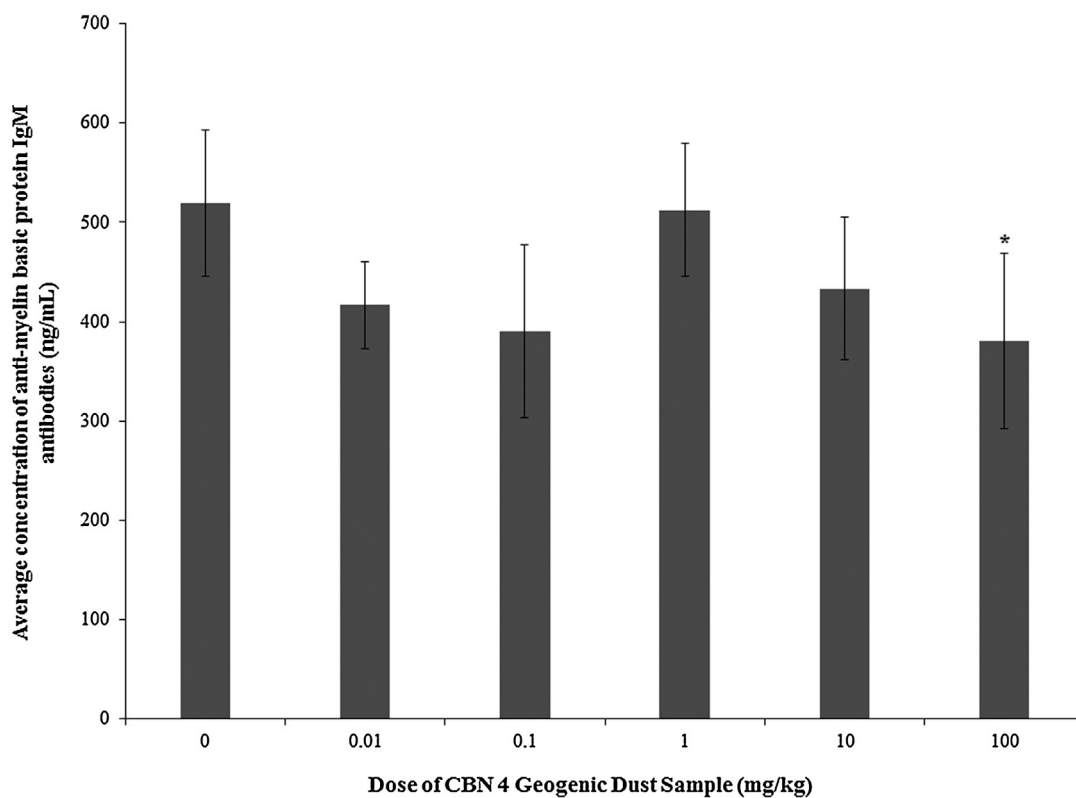
mixture of metals and PM and that similarly exposed humans may be at risk for alterations in this response.

The NOAEL identified in this study was 0.01 mg/kg and the LOAEL was 0.1 mg/kg. These values were based on reductions in





**Fig. 6.** Sheep red blood cell-specific-IgM antibody production in adult female B6C3F1 mice following oropharyngeal aspiration exposure to CBN 4 dust samples. Data are presented as mean  $\pm$  standard deviation. 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 ( $p < 0.05$ ).



**Fig. 7.** Antibody production against neuronal proteins in adult female B6C3F1 mice following oropharyngeal aspiration exposure to CBN 4 dust samples. Data are presented as mean  $\pm$  standard deviation. Sample size for each group was 5–6 animals. The (\*) indicates a response statistically different from the 0 mg/kg group ( $p < 0.05$ ).

**Table 3**  
Total elemental concentration ( $\mu\text{g/g}$  in wet sample) in whole blood of animals dosed with CBN 4 geogenic dust each week for 28 days.

	0 mg/kg	0.01 mg/kg	0.1 mg/kg	1 mg/kg	10 mg/kg	100 mg/kg
As	0.0037	0.0043	0.0041	0.0056*	0.0043	0.0038
Cd	a	a	a	a	a	a
Cr	a	a	a	a	a	a
Pb	a	0.0045	0.0021	0.0175	a	a
Mg	34.7	36.3	35.1	34.4	36.2	35.6
Mn	0.0211	0.0294	0.0234	0.0255	0.0226	0.0210
Mo	0.0151	0.0149	0.0169	0.0143	0.0140	0.0138
Ni	a	a	0.0686	0.0162	a	a
Sr	0.0080	0.0086	0.0083	0.0066	0.0076	0.0078
V	0.0117	0.0109	0.0115	0.0108	0.0108	0.0106
Zn	4.24	4.49	4.26	4.38	4.32	4.24

<sup>a</sup> Values not on the standard curve were not used.

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

**Table 4**  
Spleen and thymus B and T cell lymphocytes in adult female B6C3F1 mice following oropharyngeal aspiration exposure to CBN 4 geogenic dust each week for 28 days.

Geogenic dust (mg/kg)	Spleen				
	CD4+ (cells $\times 10^7$ )	CD8+ (cells $\times 10^6$ )	CD4+/CD8+ (cells $\times 10^7$ )	CD4-/CD8- (cells $\times 10^8$ )	B220 (cells $\times 10^7$ )
0	5.44 $\pm$ 1.30	10.37 $\pm$ 1.32	1.48 $\pm$ 0.65	1.64 $\pm$ 0.30	7.56 $\pm$ 0.873
0.01	4.86 $\pm$ 0.78	9.47 $\pm$ 1.46	1.14 $\pm$ 0.21	1.36 $\pm$ 0.33	8.49 $\pm$ 1.36
0.1	4.24 $\pm$ 0.41	8.35 $\pm$ 1.46	1.10 $\pm$ 0.19	1.14 $\pm$ 0.19*	6.95 $\pm$ 1.77
1	4.22 $\pm$ 0.99	8.96 $\pm$ 2.14	1.09 $\pm$ 0.39	1.22 $\pm$ 0.38	6.39 $\pm$ 1.22
10	4.25 $\pm$ 0.72	10.34 $\pm$ 0.78	0.90 $\pm$ 0.25	1.16 $\pm$ 0.17	8.05 $\pm$ 1.25
100	3.81 $\pm$ 0.36	9.45 $\pm$ 1.05	0.76 $\pm$ 0.17	1.09 $\pm$ 0.15*	8.07 $\pm$ 1.09
Geogenic dust (mg/kg)	Thymus				
	CD4+ (cells $\times 10^6$ )	CD8+ (cells $\times 10^6$ )	CD4+/CD8+ (cells $\times 10^7$ )	CD4-/CD8- (cells $\times 10^6$ )	
0	10.57 $\pm$ 4.78	4.76 $\pm$ 1.67	9.24 $\pm$ 3.12	4.80 $\pm$ 1.61	
0.01	9.46 $\pm$ 2.73	4.64 $\pm$ 1.38	8.73 $\pm$ 1.93	4.60 $\pm$ 1.64	
0.1	9.71 $\pm$ 3.08	4.93 $\pm$ 1.71	8.00 $\pm$ 3.52	4.14 $\pm$ 1.47	
1	9.09 $\pm$ 0.97	4.43 $\pm$ 3.20	8.11 $\pm$ 7.75	4.00 $\pm$ 0.26	
10	9.68 $\pm$ 2.54	4.27 $\pm$ 1.02	8.03 $\pm$ 2.07	4.24 $\pm$ 1.08	
100	10.19 $\pm$ 2.99	5.69 $\pm$ 1.70	8.72 $\pm$ 1.81	5.18 $\pm$ 1.41	
Geogenic dust (mg/kg)	Spleen				
	4+/25+/fp3+ (cells $\times 10^7$ )	4+/25-fp3+ (cells $\times 10^7$ )	4+/25+/fp3- (cells $\times 10^5$ )	4+/IL17A+ (cells $\times 10^6$ )	4-/IL17A+ (cells $\times 10^6$ )
0	1.61 $\pm$ 0.21	1.69 $\pm$ 0.27	5.71 $\pm$ 1.89	2.67 $\pm$ 0.80	3.47 $\pm$ 2.06
0.01	1.69 $\pm$ 0.38	1.79 $\pm$ 0.25	7.43 $\pm$ 3.75	3.13 $\pm$ 0.60	4.23 $\pm$ 0.84
0.1	1.26 $\pm$ 0.28	1.43 $\pm$ 0.40	7.06 $\pm$ 2.26	2.80 $\pm$ 0.76	4.83 $\pm$ 3.01
1	1.29 $\pm$ 0.28	1.46 $\pm$ 0.41	6.87 $\pm$ 2.91	3.05 $\pm$ 0.81	4.45 $\pm$ 1.66
10	1.61 $\pm$ 0.43	1.50 $\pm$ 0.19	6.53 $\pm$ 2.21	2.72 $\pm$ 0.82	3.03 $\pm$ 1.43
100	1.65 $\pm$ 0.36	1.66 $\pm$ 0.28	6.09 $\pm$ 2.01	2.90 $\pm$ 0.59	3.74 $\pm$ 1.24

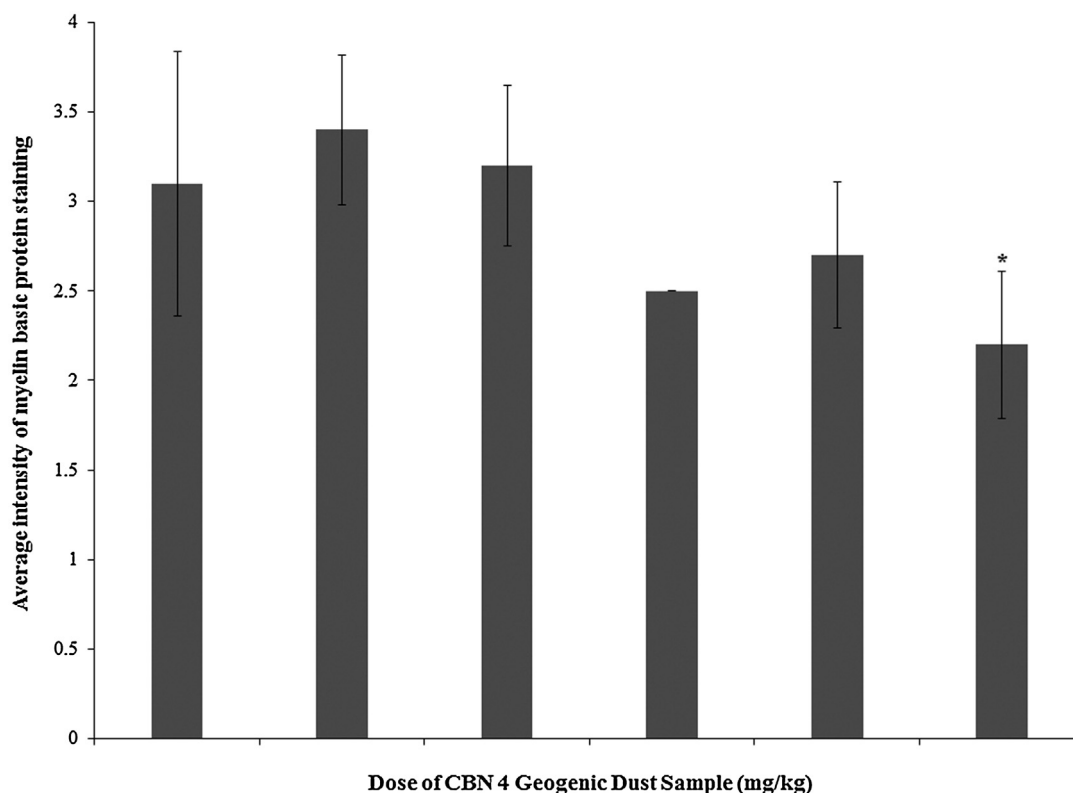
Data are presented as mean cell number  $\pm$  standard deviation. Sample size for each group was 5–6 animals. Data presented are representative of three trial days.

\* Response statistically different from the 0 mg/kg group ( $p < 0.05$ ) as determined from log transformed data.

antigen-specific antibody production and were supported by a serum biomarker of organ health (ALT). Dose-responsive suppression of IgM antibody production was the most sensitive parameter altered by exposure to dust samples from CBN 4, but reductions in ALT also were observed at these concentrations, including at 0.01 mg/kg. Suppression of IgM antibody production occurred at dust concentrations where no overt toxicity was indicated by a change in body weight from the 0 mg/kg group, which is often an indicator of overt or systemic toxicity. Additionally, the number of CD4-/CD8- and CD4+/CD8+ T cells from the spleen was reduced by exposure to 0.1 (CD4-/CD8- T cells only), 10, and 100 mg/kg of dust, which provides observations support of the functional deficit in IgM. Therefore, the NOAEL and LOAEL identified by the PFC assay likely are predictive of immunotoxicity posed by exposure to the geogenic dust from CBN 4. The LOAEL of 0.1 mg/kg was associated with a 39.8% reduction in IgM antibody production relative to the responses measured in the 0 mg/kg group. Additionally, the results of experiments with TiO<sub>2</sub>, a particle with no associated heavy metals, suggest that the metals in the mixture or the combination of metals and particles, rather than particles alone, were

likely contributing to the observed changes in immune function. We assert that this is a critical result associated with the geogenic dusts from CBN 4 and other geogenic dusts that contain associated heavy metals. Particles alone, as assessed by the response to TiO<sub>2</sub>, were insufficient to impact measured endpoints when given at the same mg/kg doses as the dust. While the blood levels of the associated metals were low (discussed below), we emphasize that it is likely that the combination of metals and particles was responsible for producing the toxicological outcomes that we observed in this study.

Hemoglobin was elevated and mean corpuscular volume (MCV) was reduced relative to the 0 mg/kg group after exposure to 1, 10, and 100 mg/kg. Hemoglobin is a protein associated with red blood cells that binds oxygen. In general, elevated serum hemoglobin is suggestive of anemia [30]. MCV is a measure of the size of red blood cells; a reduction in MCV indicates that the red blood cells are small and also can be used as an indicator of anemia [33]. The elevated serum hemoglobin and decreased MCV associated with exposure to higher concentrations of dust from CBN 4 indicates that its combination of dust and heavy metals can impact red blood cells at



**Fig. 8.** Intensity of myelin basic protein (MBP) staining in brain sections of adult female B6C3F1 mice following oropharyngeal aspiration exposure to CBN 4 dust samples. Data are presented as mean  $\pm$  standard deviation. Sample size for each group was 5–6 animals. The (\*) indicates a response statistically different from the 0 mg/kg group ( $p < 0.05$ ).

the higher exposure concentrations and for the duration of this study. Additional studies are warranted to determine the potential mechanisms by which dust from CBN 4 affects red blood cells.

ALT was reduced relative to the 0 mg/kg group after exposure to 0.01–100 mg/kg. ALT is a liver enzyme that may increase in detectable concentrations in the serum when the liver is damaged or diseased [29]; however, reductions in ALT are more challenging to interpret as decreases are not generally a marker of a specific disease. Increases in serum creatinine were observed after exposure to 10 and 100 mg/kg. Serum creatinine is a marker of kidney function and increasing levels suggest nephrotoxicity. Glomerular filtration rate (GFR) is typically used to assess renal function and is often determined by creatinine clearance. Creatinine is a byproduct of muscle metabolism and is transported in plasma before excretion in the urine. This production happens at a fairly constant rate and is relative to muscle mass. The reliability of creatinine production makes it a reliable analyte for assessing GFR in humans. However, estimating GFR based on creatinine clearance in the kidneys requires knowledge of gender, age, and weight as these parameters typically relate to muscle mass. We could not directly translate the calculation for human estimated GFR to our murine model; however, increased serum creatinine concentrations are generally associated with a decreased GFR [34].

The concentration of metals/metalloids in the blood of animals exposed to dust from CBN 4 did not differ by dose except for arsenic in animals exposed to 1 mg/kg. Concentrations of most of the other metals/metalloids occurred at very low levels, i.e., below the limits of quantitation. The metals with the highest average concentration were metals that are required for endogenous functions (magnesium and zinc) and that are a necessary part of a healthy rodent diet, so these higher levels relative to the other metals likely reflect dietary influences. The increased arsenic concentration in the blood

of animals exposed to 1 mg/kg of dust from CBN 4 was not associated with any toxicological changes specific to that dose group, including lung pathology [24]. It is possible that the observed effects of exposure to geogenic dust from CBN 4 on the immune system arose from systemic effects following local signals in the lungs induced by exposure to the combination of metals and particles. For example, although it is a different particle type and has a different array of associated toxicants, diesel exhaust particles are known to impact humoral immunity through mediation of macrophage signals in the lung [34]. 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, and cytokine receptor expression as well as genes involved in cell adhesion and migration [2,20]. Even though these reports examined a different route of exposure, it is possible that low levels of arsenic in the blood via a lung exposure may also lead to alterations of immune response as previously reported. Further, it is possible that all of the observed effects arose from the last administered exposure alone and that our results do not represent cumulative effects. We recommend that future studies evaluate the severity of the immune response with temporal models to determine if the robustness of the immune responses diminishes or changes over time, i.e., with subsequent exposures.

The reduction in serum anti-MBP IgM antibodies likely reflects an overall reduction in the immune response (as indicated by the PFC assay) rather than a direct effect of geogenic dust from CBN 4 on MBP-specific IgM antibody production. Additionally, no infiltration of T cells was observed in the brains of any mice, which suggests that dust from CBN 4 was not given at a concentration or for a duration sufficient to induce a strong enough inflammatory response to attract T cells from the periphery. The observed decrease in MBP in dosed groups (1 and 100 mg/kg) relative to

the 0 mg/kg group, however, can indicate inflammation-induced demyelination, which was detectable with immunohistochemistry, but not sufficient to attract immune cells from outside of the central nervous system. While neurotoxicity cannot be ruled out as a concern, under the conditions of this study, neurotoxicity was not evident.

As mentioned previously, the drainage areas of CBN 4 contain sediments that have the highest concentrations of heavy metals of all seven of the CBN regions in the NDRA and are heavily used for ORV activities. Our previous publications evaluating other types of sediments, including sand dunes with and without vegetation [18], arsenic-rich silty sediment [10], and desert pavements [17], however, suggest that geogenic dusts from CBN 4 do not suppress the humoral immunity as severely as dusts from these other three sites. Sediments from sand dunes produced the lowest LOAEL, at 0.01 mg/kg [18]. Sediments from desert pavements induced the largest impact on the humoral immune response; 100 mg/kg of geogenic dust from these sediments suppressed IgM antibody production by 92.8% relative to the 0 mg/kg group [17]. The impact of exposure to geogenic dusts from CBN 4 was the mildest relative to these other sites, which indicates that toxicity cannot be linked solely to sediment type or the concentration of metals, but very likely the combination of metals with the sediment type.

## 5. Conclusions

In a 2008 study, it was estimated that 44 million adults within the U.S. engage in ORV recreation and that the region of the U.S. with highest percentage of the population participating in ORV recreation is in the western, more arid states [6]. In addition, exposure to dust from dust storms is increasing worldwide due to climate change and land use that is encroaching into previously unoccupied or little-used arid lands [7]. In summary, exposure to geogenic dust collected from CBN 4 and given to mice in concentrations of 0.1–100 mg/kg, reduced the ability of the immune system to produce specific antibodies, which when affected by exogenous agents, is a robust predictor of immunotoxicity and therefore increased disease susceptibility [25,26]. This suppression of antibody production also was accompanied by a reduction in serum ALT, a measure of liver health. The LOAEL identified from both the reduction in antibody production and reduction in ALT was 0.1 mg/kg. Based on the lack of statistical findings with TiO<sub>2</sub>, our results suggest that the concentrations of heavy metals or the combination of heavy metals with particulate matter in natural dust from CBN 4 are able to suppress adaptive immunity. While the majority of our toxicological assessments were negative, the clear dose-response of exposure to geogenic dust from CBN 4 on antibody production are biologically significant. Therefore, exposure to natural dust from CBN 4, at the concentrations and via a similar exposure paradigm as evaluated in this study, may alter the ability of organisms to mount a fully effective immune response.

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