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Measurement of Inflammatory Markers Resulting from Exposures to Southwest Asian Particulate Matter and Burn Pit Emissions in a Rat Model

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STATEMENTS

Disclaimer

The views expressed in this Technical Report are those of the author and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, nor the U.S. Government.

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Animal Care and Use statement

The study protocol was reviewed and approved by the Wright-Patterson Air Force Base, OH, Institutional Animal Care and Use Committee in compliance with all federal regulations governing the protection of animals and research.

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ABSTRACT

During recent conflicts in Iraq and Afghanistan, U.S. military personnel have been deployed to austere environments associated with exposure to inhalation hazards including sand particulate matter (PM) and emissions from burn pits (i.e. smoke released from the burning/combustion of waste). Upon returning from deployment, many veterans reported persistent respiratory symptoms that were associated with chronic cough, asthma and constrictive bronchiolitis. Defining the risk associated with exposure to PM (e.g. sand dust) and burn pit emissions and identifying markers of exposure is critical to the effort to quantify, understand, mitigate and, when necessary, treat the effects of these exposures to U.S. Service Members. This study is an *in vivo* rodent study conducted by the Naval Medical Research Unit-Dayton (NAMRU-D) to characterize the inflammatory responses induced by inhalational exposures to sand and/or burn pit emissions, and investigate the use of several immune molecules as potential biomarkers of lung exposure and/or effect. The study was accomplished by measuring both pulmonary and systemic levels of pro-inflammatory and pro-fibrotic cytokines, multiple antibody isotypes, neutrophil activation, and surfactant proteins in the bronchoalveolar lavage fluid (BALF) and serum samples that were collected for the previous study, "Respirable Southwest Asian Particulate Matter and Burn Pit Emission Exposures in Rats". BALF and serum samples from rats exposed to clean air (control), sand, sand and burn pit emissions, or burn pit emissions only, were collected at 4, 32, and 90 days post-exposure and frozen at -80 ℃ until analyzed. These samples were used to quantitate levels of the following immune molecules to assess pulmonary and systemic inflammation:

1. Inflammatory and pro-fibrotic cytokines: IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-17A, KC/GRO, TNF-α, and TGF-α.
2. Antibody isotypes: IgM, IgE, IgG, and IgA.
3. Acute inflammation markers: NE and MMP-9.
4. Surfactant proteins: SP-A and SP-D

This report documents measurements for the three exposure groups at three distinct time points following exposure, and the comparisons that were made across groups as well as against a control group. The primary finding was the persistent increase in TNF-α and IgA in BALF lasting ≥90 days following exposures to the combined sand and burn pit emissions.

INTRODUCTION

Many U.S. military personnel deployed during the recent conflicts in Southwest Asia (SWA) were exposed to high concentrations of particulate matter (PM) in the form of sand and dust, as well as other airborne pollutants such as emissions from open-pit burning of waste. Upon returning from deployment, many veterans have experienced respiratory issues that have included acute eosinophilic pneumonitis with unknown etiology (Shorr et al., 2004); exercise-related dyspnea (King et al., 2011); new onset asthma and other occupationally-related illnesses (Szema, 2013); respiratory fibrosis, and possibly constrictive bronchiolitis (King et al., 2011). One factor that has been pointed out as a potential cause of the issue is exposure to solid waste burn pit emissions (BPE) (Taylor et al., 2008; DeFraites, 2010; Smith et al., 2012; Liu et al., 2016). Additionally, exposure to high levels of ambient PM from the frequent dust storms in the area has been postulated to contribute to these diseases (Weese and Abraham, 2009; Abraham, 2014).

The exposure of a general population to air pollution has been a public health concern, particularly since the historic occurrence of major air pollution incidents with observable morbidity and mortality rate increases, such as in Donora, PA in 1948 (Helfand, et al., 2001) and London in 1952 (Bell and Davis, 2001). Recently, mortality or morbidity have been linked to the long-term inhalation of PM, specifically with fine-sized particles with diameters less than 2.5 micrometers (μm) (PM2.5). These particles have a greater probability of being inhaled and reaching the deep lung and potentially cause adverse health effects (Brook, et al., 2010). Major sources of PM in the United States and other industrialized regions come from fossil-fuel combustion and vehicle emissions while primary sources of PM in SWA may differ, with a significant fraction of airborne sand and dust particles having an aerodynamic diameter of less than 2.5 μm (Perdue et al., 1992). Previous NAMRU-D-sponsored inhalation studies of sand dust from Iraq with cigarette smoke, and sand from Iraq and Afghanistan found no adverse health effects in rats exposed to sand dust concentrations at 1 mg/m^3 for 20 hours/day, 5 days/week, for 2 weeks (Dorman et al., 2012).

Burn pits provided the primary means of municipal solid waste (MSW) disposal among deployed U.S. forces up through 2011. The combustion conditions in these pits and the nature of the MSW that was being burned likely resulted in the emission of products of incomplete combustion. Personnel in the vicinity or downwind of the combustion sites may have been exposed to these emissions of particulate matter and noxious chemicals, including dioxins and other toxic air pollutants. In 2011, the Department of Veterans Affairs commissioned the Institute of Medicine (IOM) to study the human health risks from PM exposure in Iraq and Afghanistan, with a particular focus on exposure to burn pits. The IOM report concluded that there was evidence suggestive of an association between exposure to combustion products and reduced pulmonary function (IOM, 2011).

Inhalational exposures to environmental hazards such as excess smoking have been shown to elicit an inflammatory response (Comandini et al., 2009). Increases in inflammatory cytokine levels are commonly found in asthma patients (Anderson & Morrison 1998, Chiappori et al., 2015), chronic obstructive pulmonary disorder (COPD) (Barnes 2008), and constrictive bronchiolitis (also referred to as bronchiolitis obliterans) (Rosewich et al., 2015). An increase in the pro-inflammatory cytokine TNF-α in bronchial

alveolar lavage fluid (BALF) following diesel exhaust exposure has been reported in mice (Kumar et al., 2017). In addition to cytokine production, inflammation can also trigger the production of antibodies. Depending on the degree of immune activation, it is possible that these antibodies, that were initially generated to protect, can become "auto-antibodies" and attack the individual's essential proteins. Antibodies are proteins produced by immune cells to identify and neutralize foreign antigens such as bacteria and viruses. Auto-antibodies are antibodies that neutralize one or more of the individual's own (self) proteins, and are the underlying factors of all autoimmune diseases. However, the cause for the spontaneous generation of auto-antibodies is still unknown. Some studies suggest environmental exposures can trigger production of auto-antibodies (Brent, 2010; Pfau et al., 2005). There are different classes of antibodies (IgM, IgG, IgE, IgA) with IgM antibodies being the first type of antibody produced in an immune response, making them ideal candidates for early biomarkers of exposure or effect. In fact, it has been shown that there is elevated IgM level at the early onset of non-small cell lung cancer (NSCLC) (Pedchenko et al., 2013). Furthermore, IgE has been associated with allergic reactions, such as asthma (Chiappori et al., 2015; Tarlo & Lemiere 2014), whereas elevated IgG has been correlated with occupational asthma, smoking, cystic fibrosis, COPD and interstitial lung disease (Brandsma et al., 2012; Daffa et al., 2014; Budding et al., 2015; Connors et al., 2010; Tarlo & Lemiere 2014). IgA is the primary immunoglobulin present in mucous membranes and plays a critical role in protection against pathogens and allergens (Woof and Kerr 2006). In addition, one study showed that IgA can be secreted in COPD (Ladjemi et al., 2015).

Neutrophil elastase (NE) expression has been established to be a reliable indicator of neutrophil activation. Neutrophils are recruited first to a site of trauma or injury and thus NE has been used as an indicator for acute inflammation (Comandini et al., 2009). Surfactant proteins ensure that lung compliance is sufficiently high for proper ventilation. Specifically, surfactant protein A and D (SP-A, SP-D) regulate inflammatory responses in the lung, and thus have been proposed as biomarkers of lung injury (Greene et al., 2002; Pan et al., 2002). To date, there is no published data characterizing the independent or combined effects of sand and burn pit emission inhalational exposures on the immune response. Previous studies have demonstrated that such responses can induce immunological activities that can be detrimental or disruptive to normal lung function.

Defining the risk of exposure to sand dust (e.g. SWA PM) and burn pit emissions, and identifying markers of exposure or effect is critical to the effort to quantify, understand, mitigate and, when necessary, treat the effects of these exposures to our personnel. A thorough characterization of inflammatory immune responses may lead to the identification of a set of biomarkers of effect due to sand and/or burn pit exposures. These biomarkers may be used clinically to assess the exposure to SWA PM and/or burn pit emissions or serve as early indicators of a biological/physical effect, as well as in understanding their potential contribution to respiratory health issues observed in returning personnel.

To identify biomarkers of exposure or effect in response to exposure to SWA PM and/or burn pit emissions, we evaluated the BALF and serum samples that were collected for the previously conducted

study, “Respirable Southwest Asian Particulate Matter and Burn Pit Emission Exposures in Rats.” (Wong et al., 2020). The study design and methods for that study are briefly described. Several immune molecules were quantitatively assessed in the BALF and serum samples from rats that have been exposed to SWA PM (sand dust) and burn pit emissions either alone or in combination.

STUDY DESIGN

Male Sprague-Dawley rats were divided into three cohorts, each consisting of a control and three experimental groups. In Cohort 1, rats were euthanized at 4 days following completion of exposures. In Cohort 2, rats were euthanized at 32 days following completion of exposures. In Cohort 3, rats were euthanized at 90 days following completion of exposures. In each of the cohorts, rats in Group 1 were exposed to clean air (control) (C); rats in Group 2 were exposed to sand only (So); rats in Group 3 were exposed to the combined sand and burn pit emissions (S+BPE); rats in Group 4 were exposed to the burn pit emissions only (BPEo). Rats in Group 2 and 3 rats were exposed by inhalation to the SWA PM (sand) at a concentration of 4.0 mg/m^3 for 20 hours/day, 5 days/week, for 4 weeks or 20 exposure days at the NAMRU-D laboratory located at Wright-Patterson Air Force Base (WPAFB), Ohio. Following the sand exposures, all groups were transported to a mobile laboratory located next to the ambient breeze tunnel (ABT) facility operated by Battelle at their West Jefferson facility near Columbus, OH, where burn pit emissions (BPE) exposures were conducted for rats in Groups 3 and 4. There rats were exposed to the combustion (burn pit) emissions of municipal solid waste at an average concentration of 0.8 mg/m^3 for 6 hours/day for 5 consecutive days. BALF and serum samples from rats were collected at 4 (Cohort 1), 32 (Cohort 2), and 90 (Cohort 3) days post-exposure. BALF and serum samples remained frozen at -80°C until analysis. Samples were collected and stored on May- August 2015. Assays were conducted on August - December 2017.

METHODS

Animal and Animal Exposures

A total of 136 Sprague-Dawley male rats (6 weeks of age) were purchased from Charles River Laboratories (Wilmington, MA). The rats were provided husbandry conditions consistent with the practices recommended by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), and in compliance with guidelines for laboratory animal care (NRC, 2011). After arrival at WPAFB, the rats were quarantined in the animal vivarium over a two-week period, which included acclimation to the exposure cage units. Following acclimation, the rats were placed in the cages for the duration of the four-week sand dust inhalation portion of the study at NAMRU-D, except when cages were changed (weekly) and for urine collection during weekend periods between weekly exposures. Following the four-week sand dust exposure period, the rats were transported approximately 45 miles to the surrogate “burn pit” in the ambient breeze tunnel (ABT) located on the Battelle West Jefferson, OH facility, using an approved animal transport vehicle. During the 5-day emissions exposure period the rats were housed in stainless steel cage units when being exposed (6 hours/day), and returned to polycarbonate metabolism cages between exposure periods for urine collection. The rats were domiciled inside

environmentally controlled facilities, provided food and water ad libitum, and kept on a 12 hour light/dark cycle throughout the study. Upon completion of the exposures, the rats were returned to the WPAFB animal vivarium for toxicological assessment.

Chemicals and Exposures

Camp Victory Particulate Matter: Surface sand (topsoil) obtained by the U.S. Army Corps of Engineers from Camp Victory in Iraq was autoclaved and aerosolized using a Wright Dust Feeder. The animals were exposed in stainless steel and glass whole body exposure chambers (H1000, Lab Products, Inc., Seaford, DE). Two chambers were used, one for exposure to the SWA PM, and the other to clean air.
Municipal solid waste (MSW): A list of materials that were representative of the waste stream that was historically generated in theatre between 2006 and 2009 was compiled by the US Amy (USALIA, 2013). Materials were combusted at the entrance to the ABT. Combustion emissions were extracted from the smoke plume and transferred to the adjacent mobile laboratory where the animals were exposed in whole body exposure chambers.

Bronchoalveolar Lavage Fluid (BALF) Analysis

After an animal had been euthanized, a bronchial alveolar lavage was conducted by opening the chest cavity, exposing the lungs and trachea. A 16 gauge blunted needle was inserted into a cut made between the cartilaginous rings in the anterior portion of the trachea. A syringe containing 3 ml of phosphate buffered saline (PBS) was used to wash the PBS in and out of the lung two times, and the withdrawn solution was transferred to a centrifuge tube (15 ml conical tube). This process was conducted three times with the wash solution being put into separate vials. The wash solutions were analyzed by combining the washes and counting the total number of cells in the wash (Cellometer, Nexcelom, Lawrence, MA). A cell differential was conducted by centrifuging cells from the wash onto a glass slide (cytospin), staining and enumerating the different cell types on the slide using microscopy. BALF samples were frozen at -80°C for biomarkers analysis.

Biochemical measurements

The levels of following cytokines: IFN-γ, IL-1β, IL-4, IL-5, IL-6, IL-10, IL-13, KC/GRO, and TNF-α were determined by the electrochemiluminescence-based measurements using our SECTOR Imager 2400A and the commercially available pro-inflammatory multi-spot plates (Meso Scale Discovery). IL-2, IL-8, TGF-β and IL-17A levels were determined using an ELISA protocol with a standard microplate reader. The levels of antibody isotypes IgA, IgE, IgG, IgG1, IgG2a, IgG2b, IgM were quantitated by using standard ELISA approach. Similarly, the levels of SP-A, SP-D, NE and MMP-9 were assessed using the ELISA approach. All ELISA and multiplex assays were conducted as duplicate determinations, with all results expressed as mean values ± standard error (SEM).

Data Analysis

Levels of these immune molecules were measured in both the BALF and serum from rats exposed to clean air (C, Group 1), sand only (So, Group 2), sand and burn pit emission (S+BPE, Group 3) and burn pit emission only (BPEo, Group 4). BALF and serum were collected at specified time points from animals euthanized on day 4, 32, and 90 is referred to as Cohort 1, 2, and 3 respectively on the figures. Data were

compared not only among the four experimental groups but also among the three time points. For statistical analysis, one way analysis of variance (ANOVA) was used along with the appropriate post-hoc comparison analysis such as the Holm-Sidak method.

RESULTS

Inflammatory and pro-fibrotic cytokines

TNF-α

There were statistically significant increases in the average levels of TNF-α in BALF of exposed animals exposed compared to control beginning at 4 days post-exposure (one way ANOVA P = 0.025) (Tables 1-3, Figure 1A). Post hoc multiple comparison testing using the Holm Sidak method yielded statistical significance between the C and So (P = 0.05) as well as between the C and S+BPE (P = 0.031) at 4 days post-exposure. ANOVA post hoc multiple comparison testing yielded statistical significance between the C and S+BPE at 32 days and 90 days post-exposure (P = 0.005, 0.028, respectively). TNF-α levels in serum of exposed animals appeared to be elevated above controls for the 4-day and 90-day cohorts, but the increase was not statistically significant (Tables 4-6, Figure 1B).

IL-1β

No significant change in IL-1β was observed in BALF of rats exposed to So, S+BPE or BPEo (Tables 1-3). There was a trending increase in BALF IL-1β level in rats exposed to the S+BPE at 32 days post-exposure but the increase was not statistically significant. However, there was a significant difference in serum IL-1β level observed across all experimental groups at 4 days post-exposure (one way ANOVA P = 0.009) (Table 4, Figure 2A). Post hoc multiple comparison analysis using Holm Sidak method yielded a statistically significant decrease between the So group and the S+BPE group (P=0.008) and between the So group and the BPEo group (P=0.048) (Figure 2A). The average IL-1β from the So group was also less than the average IL-1β from C animals but the decrease was not statistically significant according to the post hoc multiple comparison test (P = 0.157).

IL-5

No significant change in IL-5 was observed in BALF of rats exposed to So, S+BPE, or BPEo (Tables 1-3). However, there was a significant difference in serum IL-5 levels observed across all experimental groups at 4 days post-exposure (one way ANOVA P<0.001) (Table 4, Figure 2B). Post hoc multiple comparison analysis using Holm Sidak method yielded a statistically significant decrease in serum IL-5 from rats exposed to So compared to C (P=0.048) (Figure 2B). This decrease was not detected at day 32 or day 90 post-exposure.

IL-13

No significant change in IL-13 was observed in BALF of rats exposed to So, S+BPE, or BPEo (Tables 1-3). However, there was a significant difference in serum IL-13 levels observed across all experimental groups at 4 days post-exposure (one way ANOVA P=0.001) (Table 4, Figure 2C). Post hoc multiple comparison analysis using Holm Sidak method yielded a statistically significant decrease in serum IL-13

from rats exposed to So compared to C (P=0.023) (Figure 2C). This decrease was not detected at day 32 or day 90 post-exposure.

IL-10

No significant change in IL-10 was observed in BALF of rats exposed to So, S+BPE, or BPEo (Tables 1-3). However, there was a significant difference in serum IL-10 levels observed across all experimental groups at 4 days post-exposure (one way ANOVA P=0.015) (Table 4, Figure 2D). Post hoc multiple comparison analysis using Holm Sidak method yielded a statistically significant increase in serum IL-10 from rats exposed to BPEo compared to rats exposed to So (P=0.022) (Figure 2D). This increase was no longer statistically significant at day 32 nor day 90 post-exposure.

IL-6

There were no statistically significant differences in BALF or serum level of IL-6 across all 4 experimental groups (Tables 1-6). There were slight increases in the average of both BALF and serum IL-6 level without statistical significance at 4 and 32 days post-exposure that were not observed at 90 days post-exposure.

TGF-β

Levels of TGF-β was not detected in BALF of animals across all experimental groups (Tables 1-3). An increase in TGF-β was seen in the serum of animals exposed to BPEo compared to control animals at 4 days post-exposures that was almost statistically significant (ANOVA Holm Sidak comparison P = 0.051) (Tables 4). But this increase was no longer observed at 32 or 90 days post-exposure (Tables 5-6).

Other cytokines

Levels of other cytokines were measured in both the BALF and serum of control and exposed animals that did not result in statistical significance. A list of all cytokines measured is shown in Tables 1-6.

Table 1. Average levels of cytokines in BALF at 4 days post-exposure. Units ± standard error (SEM) are in pg/ml unless otherwise indicated. Values labeled * are statistically significant at $P < 0.05$ via one way ANOVA and post-hoc multiple comparison analysis using the Holm Sidak method.

4 days post-exposure	**CONTROL**	**SAND ONLY**	**SAND & BURN PIT**	**BURN PIT ONLY**
IFN-γ	2.81 ± 0.35	2.49 ± 0.16	2.47 ± 0.22	2.41 ± 0.19
IL-1β	18.94 ± 1.43	18.44 ± 0.85	18.41 ± 1.11	16.69 ± 0.71
IL-4	0.7203 ± 0.08	0.78 ± 0.03	0.80 ± 0.06	0.77 ± 0.05
IL-5	12.30 ± 1.69	11.98 ± 0.72	10.70 ± 0.78	11.09 ± 0.63
IL-6	56.55 ± 8.14	55.90 ± 3.31	63.65 ± 18.51	44.55 ± 5.29
KC/GRO	72.85 ± 7.99	87.75 ± 8.36	89.32 ± 11.20	67.33 ± 8.52
IL-10	6.25 ± 0.66	7.61 ± 0.51	6.84 ± 0.70	6.31 ± 0.44
IL-13	2.79 ± 0.49	2.59 ± 0.15	2.22 ± 0.20	2.21 ± 0.26
TNF-α	1.19 ± 0.14	2.25 ± 0.27*	2.43 ± 0.41*	1.29 ± 0.11
TGF-β (ng/ml)	*undetectable*	*undetectable*	*undetectable*	*undetectable*

Table 2. Average levels of cytokines in BALF at 32 days post-exposure. Units ± standard error (SEM) are in pg/ml unless otherwise indicated. Values labeled * are statistically significant at $P < 0.05$ via one way ANOVA and post-hoc multiple comparison analysis using the Holm Sidak method.

32 days post-exposure	**CONTROL**	**SAND ONLY**	**SAND & BURN PIT**	**BURN PIT ONLY**
IFN-γ	2.36 ± 0.14	2.27 ± 0.16	2.37 ± 0.16	2.59 ± 0.11
IL-1β	37.38 ± 1.63	36.58 ± 2.62	43.67 ± 2.47	37.07 ± 2.15
IL-4	1.37 ± 0.06	1.47 ± 0.08	1.27 ± 0.04	1.51 ± 0.16
IL-5	21.16 ± 0.80	22.29 ± 1.39	22.52 ± 0.85	23.83 ± 1.06
IL-6	26.38 ± 1.56	29.67 ± 2.38	30.41 ± 3.35	25.89 ± 2.44
KC/GRO	80.16 ± 6.56	85.47 ± 10.49	124.37 ± 28.6	71.05 ± 8.58
IL-10	7.65 ± 1.01	7.34 ± 0.81	6.61 ± 0.55	8.89 ± 1.42
IL-13	2.07 ± 0.11	2.28 ± 0.14	2.18 ± 0.11	2.35 ± 0.18
TNF-α	1.68 ± 0.06	2.061 ± 0.12	2.43 ± 0.22*	1.80 ± 0.14
TGF-β (ng/ml)	*undetectable*	*undetectable*	*undetectable*	*undetectable*

Table 3. Average levels of cytokines in BALF at 90 days post-exposure. Units ± standard error (SEM) are in pg/ml unless otherwise indicated. Values labeled * are statistically significant at $P < 0.05$ via one way ANOVA and post-hoc multiple comparison analysis using the Holm Sidak method.

90 days post-exposure	**CONTROL**	**SAND ONLY**	**SAND & BURN PIT**	**BURN PIT ONLY**
IFN-γ	1.94 ± 0.25	1.95 ± 0.30	2.35 ± 0.26	1.92 ± 0.25
IL-1β	35.33 ± 3.03	35.17 ± 2.21	34.17 ± 1.67	30.46 ± 1.67
IL-4	0.65 ± 0.09	0.82 ± 0.11	0.86 ± 0.11	0.77 ± 0.08
IL-5	15.87 ± 2.07	16.00 ± 1.67	19.22 ± 2.67	13.29 ± 1.27
IL-6	71.85 ± 39.16	49.86 ± 14.77	49.94 ± 12.58	40.21 ± 5.72
KC/GRO	102.92 ± 15.36	90.63 ± 12.63	108.14 ± 12.93	93.55 ± 11.44
IL-10	4.88 ± 0.83	5.61 ± 1.19	4.73 ± 0.71	4.40 ± 0.61
IL-13	2.64 ± 0.22	2.88 ± 0.35	3.29 ± 0.29	2.58 ± 0.23
TNF-α	1.42 ± 0.12	1.753 ± 0.17	1.96 ± 0.10*	1.37 ± 0.11
TGF-β (ng/ml)	*undetectable*	*undetectable*	*undetectable*	*undetectable*

Table 4. Average levels of cytokines in serum at 4 days post-exposure. Units ± standard error (SEM) are in pg/ml unless otherwise indicated. Values labeled * are statistically significant at $P < 0.05$ via one way ANOVA and post-hoc multiple comparison analysis using the Holm Sidak method.

4 days post-exposure	**CONTROL**	**SAND ONLY**	**SAND & BURN PIT**	**BURN PIT ONLY**
IFN-γ	0.24 ± 0.03	0.18 ± 0.02	0.22 ± 0.03	0.27 ± 0.04
IL-1β	8.85 ± 0.58	6.92 ± 0.48*	10.19 ± 1.00	9.43 ± 0.54
IL-4	0.89 ± 0.04	0.78 ± 0.03	0.91 ± 0.04	0.95 ± 0.04
IL-5	12.05 ± 0.34	10.50 ± 0.32*	12.70 ± 0.63	13.02 ± 0.34
IL-6	26.73 ± 3.19	23.33 ± 1.43	32.64 ± 3.35	31.90 ± 2.41
KC/GRO	79.73 ± 6.41	94.09 ± 7.75	110.92 ± 13.61	89.54 ± 5.99
IL-10	10.18 ± 0.55	9.49 ± 0.79	11.58 ± 0.36	12.06 ± 0.58*
IL-13	2.83 ± 0.16	2.36 ± 0.11*	2.91 ± 0.07	2.98 ± 0.11
TNF-α	2.04 ± 0.09	2.41 ± 0.22	2.24 ± 0.08	2.41 ± 0.07
TGF-β (ng/ml)	180.85 ± 13.44	179.62 ± 12.32	211.95 ± 21.35	232.21 ± 10.85 (*P=0.051*)

Table 5. Average levels of cytokines in serum at 32 days post-exposures. Units ± standard error (SEM) are in pg/ml unless otherwise indicated. Values labeled * are statistically significant at $P < 0.05$ via one way ANOVA and post-hoc multiple comparison analysis using the Holm Sidak method.

32 days post-exposure	**CONTROL**	**SAND ONLY**	**SAND & BURN PIT**	**BURN PIT ONLY**
IFN-γ	0.32 ± 0.11	0.23 ± 0.13	0.13 ± 0.03	0.22 ± 0.07
IL-1β	20.55 ± 2.00	19.64 ± 1.42	24.08 ± 1.70	22.03 ± 1.52
IL-4	0.58 ± 0.04	0.66 ± 0.06	0.59 ± 0.03	0.65 ± 0.05
IL-5	13.10 ± 0.45	13.22 ± 0.58	13.53 ± 0.27	14.22 ± 0.47
IL-6	21.66 ± 2.68	23.14 ± 2.25	24.70 ± 2.47	27.99 ± 2.37
KC/GRO	72.53 ± 9.78	52.41 ± 8.09	68.61 ± 7.21	67.73 ± 24.60
IL-10	8.02 ± 0.43	8.09 ± 0.68	7.97 ± 0.33	8.56 ± 0.54
IL-13	2.45 ± 0.13	2.46 ± 0.11	2.41 ± 0.08	2.79 ± 0.10
TNF-α	1.03 ± 0.05	0.99 ± 0.06	1.06 ± 0.04	1.19 ± 0.09
TGF-β (ng/ml)	177.34 ± 9.98	162.07 ± 12.74	183.97 ± 14.51	161.45 ± 9.09

Table 6. Average levels of cytokines in serum at 90 days post-exposures. Units ± standard error (SEM) are in pg/ml unless otherwise indicated. Values labeled * are statistically significant at $P < 0.05$ via one way ANOVA and post-hoc multiple comparison analysis using the Holm Sidak method.

90 days post-exposure	**CONTROL**	**SAND ONLY**	**SAND & BURN PIT**	**BURN PIT ONLY**
IFN-γ	0.81 ± 0.05	0.81 ± 0.05	0.81 ± 0.05	0.93 ± 0.05
IL-1β	26.78 ± 1.71	24.50 ± 1.48	22.80 ± 2.42	25.43 ± 1.81
IL-4	0.76 ± 0.04	0.72 ± 0.03	0.79 ± 0.03	0.83 ± 0.04
IL-5	15.74 ± 0.77	15.95 ± 0.61	15.82 ± 0.57	15.91 ± 1.18
IL-6	23.33 ± 1.53	22.73 ± 1.17	22.77 ± 1.22	26.73 ± 2.53
KC/GRO	115.32 ± 18.98	121.31 ± 24.15	155.83 ± 22.23	105.95 ± 19.37
IL-10	4.12 ± 0.27	4.00 ± 0.22	4.14 ± 0.18	4.56 ± 0.25
IL-13	3.34 ± 0.11	3.22 ± 0.08	3.24 ± 0.11	3.50 ± 0.09
TNF-α	1.48 ± 0.07	1.74 ± 0.15	1.61 ± 0.12	1.91 ± 0.15
TGF-β (ng/ml)	205.01 ± 18.82	201.84 ± 27.27	189.17 ± 10.81	165.03 ± 11.72

Antibody isotypes

IgA

There was a statistically significant elevation of the IgA levels in the BALF of exposed animals compared to control at 32 and 90 days post-exposure (one way ANOVA P = 0.01, <0.001, respectively) (Tables 8-9, Figure 3A). Post hoc comparison analysis using the Holm Sidak method yielded a statistically significant IgA increase in the BALF of animals exposed to S+BPE compared to C at 32 and 90 days post-exposure (P = 0.01, <0.001, respectively). Corresponding increases in IgA were not observed in the serum (Figure 3B, Tables 11-12). Statistical analysis of serum IgA levels indicated significant differences among experimental groups (one way ANOVA P = 0.046). In contrast to the BALF data, post hoc comparison analysis revealed a statistically significant decrease in the average IgA level in the serum of animals exposed to S+BPE at 4 days post-exposure (P = 0.049) (Table 10, Figure 3B). However, this decrease was no longer observed at 32 or 90 days post-exposure (Tables 11-12).

IgE

There was a statistically significant decrease in IgE levels in BALF of animals exposed to So at 90 days post-exposure (one way ANOVA P = 0.011, post hoc comparison test P = 0.009) (Table 9, Figure 4A). The average IgE level in BALF of animals exposed to the S+BPE and BPEo also decreased at 90 days post-exposure but statistical significance was not reached. Serum level of IgE significantly increased in the BPEo group at 32 days post-exposure (one way ANOVA P < 0.001, post hoc comparison test P < 0.001) (Table 11, Figure 4B). But at 90 days post-exposure, the averaged IgE level in the serum of animals exposed to burn pit alone slightly decreased without statistical significance (Table 12, Figure 4B).

IgG, IgG1

No statistically significant changes were detected in IgG levels I BALF or serum of exposed animals compared to control (Figure 5, Table 7-9). There was a reduction in the average of IgG1 levels in both BALF and serum of animals exposed So and the S+BPE at 90 days post-exposure (Table 9, 12, Figure 5). However, statistical significance was reached only in BALF (one way ANOVA P = 0.001) but not in serum (one way ANOVA P = 0.08). Post hoc multiple comparison testing in BALF IgG1 levels yielded significant decrease in So compared to C (P = 0.002) and in the S+BPE compared to C (P = 0.027). Although the average IgG1 level in the serum also decreased in the So and S+BPE compared to C, the reduction was not statistically significant in the serum.

IgG2a, IgG2b

Levels of BALF IgG2a level in BALF was significantly reduced in the So group compared to C at 90 days post-exposure (one way ANOVA P = 0.019, post hoc multiple comparison P = 0.037) (Table 9, Figure 5C). The reduction in IgG2a level was also observed in the serum of the So group at 90 days post-exposures but was not statistically different from C (P = 0.057) (Table 12, Figure 5D). Levels of BALF and serum IgG2b levels were not statistically affected although a trending increase in exposed animals was at 32 days post-exposure that was not statistically significant (P = 0.08) (Tables 7-12).

IgM

IgM levels in the BALF were undetectable using our assay methodology. IgM levels in the serum were detectable but did not statistically change resulting from exposures.

Table 7. Average levels of immunoglobulins in BALF at 4 days post-exposure. Units ± standard error (SEM) are in µg/ml or ng/ml as indicated. Values labeled * are statistically significant at $P < 0.05$ via one way ANOVA and post-hoc multiple comparison analysis using the Holm Sidak method.

4 days post-exposure	**CONTROL**	**SAND ONLY**	**SAND & BURN PIT**	**BURN PIT ONLY**
IgA (µg/ml)	2.58 ± 0.53	3.80 ± 0.76	3.36 ± 0.42	1.98 ± 0.35
IgE (ng/ml)	11.95 ± 1.84	10.44 ± 2.48	11.22 ± 1.77	9.40 ± 1.76
IgM	*undetectable*	*undetectable*	*undetectable*	*undetectable*
IgG (µg/ml)	4.79 ± 0.90	5.29 ± 0.91	5.48 ± 0.81	4.00 ± 0.61
IgG1 (ng /ml)	105.68 ± 26.62	145.97 ± 54.00	121.04 ± 30.24	153.97 ± 27.22
IgG2a (ng/ml)	409.25 ± 37.34	403.89 ± 43.66	437.15 ± 49.21	518.52 ± 49.27
IgG2b (ng/ml)	700.06 ± 68.88	613.02 ± 78.92	813.98 ± 86.38	793.07 ± 101.39

Table 8. Average levels of immunoglobulins in BALF at 32 days post-exposures. Units ± standard error (SEM) are in µg/ml or ng/ml as indicated. Values labeled * are statistically significant at $P < 0.05$ via one way ANOVA and post-hoc multiple comparison analysis using the Holm Sidak method.

32 days post-exposure	**CONTROL**	**SAND ONLY**	**SAND & BURN PIT**	**BURN PIT ONLY**
IgA (µg/ml)	2.01 ± 0.29	2.49 ± 0.34	3.41 ± 0.48*	1.77 ± 0.20
IgE (ng/ml)	8.81 ± 1.55	6.29 ± 0.50	9.04 ± 1.94	7.62 ± 1.05
IgM	*undetectable*	*undetectable*	*undetectable*	*undetectable*
IgG (µg/ml)	4.27 ± 1.04	4.39 ± 0.47	4.56 ± 0.47	4.65± 0.40
IgG1 (ng/ml)	114.61 ± 32.71	117.51 ± 38.40	78.94 ± 21.42	100.93 ± 18.83
IgG2a (µg/ml)	1.08 ± 0.15	1.28 ± 0.15	1.31 ± 0.13	1.55 ± 0.22
IgG2b (µg/ml)	0.69 ± 0.11	0.925 ± 0.15	1.10 ± 0.31	1.20 ± 0.31

Table 9. Average levels of immunoglobulins in BALF at 90 days post-exposure. Units ± standard error (SEM) are in µg/ml or ng/ml as indicated. Values labeled * are statistically significant at $P < 0.05$ via one way ANOVA and post-hoc multiple comparison analysis using the Holm Sidak method.

90 days post-exposure	**CONTROL**	**SAND ONLY**	**SAND & BURN PIT**	**BURN PIT ONLY**
IgA (µg/ml)	2.93 ± 0.26	3.07 ± 0.38	5.13 ± 0.45*	2.66 ± 0.32
IgE (ng/ml)	8.09 ± 2.06	4.34 ± 0.44*	5.20 ± 0.84	6.18 ± 1.12
IgM	*undetectable*	*undetectable*	*undetectable*	*undetectable*
IgG (µg/ml)	7.46 ± 1.09	5.06 ± 0.74	6.45 ± 0.76	4.64 ± 0.66
IgG1 (ng/ml)	147.43 ± 20.78	55.50 ± 8.60*	81.68 ± 12.74*	147.23 ± 26.80
IgG2a (µg/ml)	1.50 ± 0.11	1.05 ± 0.07*	1.34 ± 0.10	1.57 ± 0.18
IgG2b (µg/ml)	1.25 ± 0.23	1.05 ± 0.23	1.24 ± 0.22	1.22 ± 0.22

Table 10. Average levels of immunoglobulins in serum at 4 days post-exposure. Units ± standard error (SEM) are in µg/ml or mg/ml as indicated. Values labeled * are statistically significant at $P < 0.05$ via one way ANOVA and post-hoc multiple comparison analysis using the Holm Sidak method.

4 days post-exposure	**CONTROL**	**SAND ONLY**	**SAND & BURN PIT**	**BURN PIT ONLY**
IgA (µg/ml)	54.85 ± 1.96	53.82 ± 3.02	43.77 ± 2.60*	47.95 ± 3.73
IgE (µg/ml)	18.66 ± 1.50	16.11 ± 0.96	18.19 ± 1.39	19.37 ± 0.97
IgM (µg/ml)	142.47 ± 11.34	146.95 ± 14.90	148.70 ± 8.55	125.86 ± 5.95
IgG (mg/ml)	5.80 ± 0.72	6.08 ± 0.43	5.42 ± 0.57	6.07 ± 0.67
IgG1 (µg/ml)	138.85 ± 17.32	132.22 ± 22.02	147.32 ± 24.08	205.39 ± 39.11
IgG2a (mg/ml)	1.80 ± 0.34	1.73 ± 0.11	1.59 ± 0.17	2.07 ± 0.36
IgG2b (mg/ml)	1.47 ± 0.13	1.47 ± 0.14	1.52 ± 0.18	1.43 ± 0.19

Table 11. Average levels of immunoglobulins in serum at 32 days post-exposure. Units ± standard error (SEM) are in µg/ml or mg/ml as indicated. Values labeled * are statistically significant at $P < 0.05$ via one way ANOVA and post-hoc multiple comparison analysis using the Holm Sidak method.

32 days post-exposure	**CONTROL**	**SAND ONLY**	**SAND & BURN PIT**	**BURN PIT ONLY**
IgA (µg/ml)	52.04 ± 4.59	53.69 ± 2.77	56.83 ± 5.75	51.20 ± 2.26
IgE (µg/ml)	13.95 ± 0.79	11.77 ± 0.54	13.11 ± 0.94	19.77 ± 1.48*
IgM (µg/ml)	133.15 ± 13.36	146.89 ± 12.98	130.42 ± 17.38	138.89 ± 14.61
IgG (mg/ml)	6.37 ± 0.59	7.35 ± 0.53	7.54 ± 0.58	7.87 ± 0.49
IgG1 (µg/ml)	156.82 ± 26.63	157.73 ± 25.12	114.95 ± 19.80	225.36 ± 34.44
IgG2a (mg/ml)	1.54 ± 0.20	1.72 ± 0.12	1.92 ± 0.009	1.94 ± 0.19
IgG2b (mg/ml)	0.93 ± 0.16	1.28 ± 0.17	1.22 ± 0.10	1.57 ± 0.24

Table 12. Average levels of immunoglobulins in serum at 90 days post-exposure. Units ± standard error (SEM) are in µg/ml or mg/ml as indicated. Values labeled * are statistically significant at $P < 0.05$ via one way ANOVA and post-hoc multiple comparison analysis using the Holm Sidak method.

90 days post-exposure	**CONTROL**	**SAND ONLY**	**SAND & BURN PIT**	**BURN PIT ONLY**
IgA (µg/ml)	55.92 ± 4.53	56.36 ± 3.92	61.27 ± 5.80	63.41 ± 3.54
IgE (µg/ml)	15.30 ± 0.78	15.66 ± 0.95	15.48 ± 0.99	13.33 ± 0.85
IgM (µg/ml)	175.93 ± 18.78	184.83 ± 13.95	204.43 ±17.94	199.22 ± 16.58
IgG (mg/ml)	8.19 ± 0.68	7.00 ± 0.40	7.75 ± 0.85	7.55 ± 0.46
IgG1 (µg/ml)	213.22 ± 20.23	147.60 ± 18.85	155.45 ± 23.67	212.41 ± 36.55
IgG2a (mg/ml)	2.11 ± 0.13	1.56 ± 0.14	1.66 ± 0.24	1.95 ± 0.10
IgG2b (mg/ml)	1.15 ± 0.11	1.40 ± 0.20	1.38 ± 0.14	1.14 ± 0.98

Surfactant proteins

No statistically significant changes were observed in the levels of surfactant proteins (SP-A or SP-D) in BALF of exposed versus control animals (Table 13). However, there were statistically significant reductions in SP-A in serum of rats exposed to So and S+BPE at 4 and 32 days post-exposure (ANOVA $P = 0.036$, 0.02, respectively) (Table 14, Figure 6). Post hoc multiple comparison yielded significant reduction between C and S+BPE ($P = 0.031$) at 4 days post-exposure. At 32 days post-exposure, there were significant reductions in both the S+BPE ($P = 0.031$) and the So ($P = 0.04$) groups compared to C. No statistically significant change in serum SP-D was observed.

Table 13. Average levels of surfactant proteins SP-A and SP-D in BALF at 4, 32, and 90 days post-exposure. Units ± standard error (SEM) are in ng/ml or pg/ml as indicated. Values labeled * are statistically significant at $P < 0.05$ via one way ANOVA and post-hoc multiple comparison analysis using the Holm Sidak method.

4 days post-exposure	**CONTROL**	**SAND ONLY**	**SAND & BURN PIT**	**BURN PIT ONLY**
SP-A (pg/ml)	84.90 ± 10.71	94.76 ± 13.73	82.43 ± 10.57	76.29 ± 7.87
SP-D (ng/ml)	2.72 ± 0.67	2.72 ± 0.65	3.65 ± 0.73	2.56 ± 0.21
32 days post-exposure				
SP-A (pg/ml)	115.38 ± 14.33	111.00 ± 15.67	103.42 ± 23.48	91.24 ± 11.75
SP-D (ng/ml)	2.75 ± 0.43	2.41 ± 0.43	3.30 ± 0.34	2.12 ± 0.17
90 days post-exposure				
SP-A (pg/ml)	129.66 ± 11.31	107.19 ± 5.74	135.37 ± 12.40	133.85 ± 9.50
SP-D (ng/ml)	2.36 ± 0.34	2.25 ± 0.45	2.52 ± 0.34	2.37 ± 0.23

Table 14. Average levels of surfactant proteins SP-A and SP-D in serum at 4, 32, and 90 days post-exposure. Units ± standard error (SEM) are in ng/ml as indicated. Values labeled * are statistically significant at $P < 0.05$ via one way ANOVA and post-hoc multiple comparison analysis using the Holm Sidak method.

4 days post-exposure	**CONTROL**	**SAND ONLY**	**SAND & BURN PIT**	**BURN PIT ONLY**
SP-A (ng/ml)	25.20 ± 2.19	21.04 ± 0.80	19.45 ± 1.24*	22.34 ± 0.92
SP-D (ng/ml)	1.00 ± 0.08	0.98 ± 0.10	1.16 ± 0.14	1.08 ± 0.17
32 days post-exposure				
SP-A (ng/ml)	21.28 ± 1.44	16.84 ± 0.93*	16.56 ± 1.28*	17.91 ± 0.68
SP-D (ng/ml)	1.00 ± 0.06	0.88 ± 0.04	1.00 ± 0.05	0.99 ± 0.08
90 days post-exposure				
SP-A (ng/ml)	26.51 ± 2.01	27.34 ± 3.44	28.22 ± 1.69	30.08 ± 1.71
SP-D	*undetectable*	*undetectable*	*undetectable*	*undetectable*

Other biomarkers of acute inflammatory responses

We also measured levels of neutrophil elastase (NE) and matrix metalloproteinase 9 (MMP-9) in serum and BALF. NE was undetectable in serum and we saw no significant changes in BALF levels across experimental groups (not shown). MMP-9 was undetectable in BALF and we saw no significant changes in serum level across experimental groups (not shown).

DISCUSSION

In order to characterize the inflammatory responses induced by inhalational exposures to PM (i.e. sand) and/or burn pit emissions, and investigate the use of several immune molecules as biomarkers of lung exposure or effect, we quantitatively measured both pulmonary and systemic pro-inflammatory and pro-fibrotic cytokines, multiple antibody isotypes, neutrophil activation, and surfactant proteins in BALF and serum samples from exposed rodents. Rats were exposed for a prior study, "Respirable Southwest Asian Particulate Matter and Burn Pit Emission Exposures in Rats." (Wong et al., 2020). Briefly, in that study, male Sprague-Dawley rats were exposed by inhalation to the SWA PM at a concentration of 4.0 mg/m^3 for 20 hours/day, 5 days/week, for 4 weeks or 20 exposure days at the NAMRU-D facility at WPAFB in Ohio. Following the exposure to sand, all groups were transported to a mobile laboratory located next to the Battelle ambient breeze tunnel (ABT) facility in West Jefferson, OH. Two groups (sand+burn pit emissions and burn pit emissions only groups) were exposed to the combustion (burn pit) emissions of municipal solid waste at an average concentration of 0.8 mg/m^3 for 6 hours/day for 5 consecutive days, while the remaining two groups (clean air, sand only groups) were exposed to clean air. BALF and serum samples from rats were collected at 4, 32, and 90 days post-exposure.

Exposures induced lasting increases in TNF-α

Inhalational exposures to environmental hazards such as excess smoking have been shown to elicit an inflammatory response (Comandini et al., 2009). Increases in inflammatory cytokine levels are commonly found in patients suffering from asthma (Anderson & Morrison 1998, Chiappori et al., 2015), chronic obstructive pulmonary disorder (COPD) (Barnes 2008), and constrictive bronchiolitis (also known as bronchiolitis obliterans) (Rosewich et al., 2015). TNF-α is a cell signaling protein involved in systemic inflammation and is one of the cytokines that make up the acute phase reaction. TNF-α has been associated with the development of inflammatory response that leads to asthma (Thomas 2001) and an increase in TNF-α has been correlated with COPD (Aaron et al., 2001). In this study, we saw a statistically significant increase in BALF TNF-α levels in both So and the S+BPE groups starting from 4 days (~2-fold increase) post-exposure (Figure 1). The elevated BALF TNF-α persisted in the S+BPE group for at least 90 days post-exposure as a significant increase was still observed at 32 (1.4-fold increase) and 90 days (1.4-fold increase) post-exposure. TNF-α increase in COPD patients' sputum has been reported to be ~3-fold during an exacerbation that eventually returned to the baseline value in 30 days (Aaron et al., 2001). Further work is needed to determine whether the ~2-fold increase we initially observed in rats exposed to S+BPE at day 4 can be compared to the increase seen in COPD patients during an exacerbation. However, unlike a

COPD exacerbation, the increase we observed in rats did not return to baseline and remained elevated at approximately 1.4-fold even at 90 days post-exposure. Further investigation would be appropriate to determine whether this prevalent increase in TNF-α can induce or enhance susceptibility to pulmonary dysfunction or other inflammatory-mediated disorders.

Increases in TNF-α induced by an inflammatory event typically coincide with increases in other inflammatory markers. However, here we did not observe lasting increases in the other inflammatory cytokines tested. IL-5 and IL-6 have been implicated as playing key roles in pulmonary inflammation such as asthma and COPD (Hoshi et al., 1995; Greenfeder et al., 2001; Gorska et al., 2018). We did not see significant increases in serum or BALF levels of IL-6. The average IL-5 levels in the BALF increased slightly in the combined sand and burn pit group, but this increase was not statistically significant. The only other cytokine that increased with statistical significance was IL-10 in serum of rats exposed to sand on day 4 following exposures. But this increase was transient, and was no longer statistically significant on day 32 and day 90 post-exposure.

Exposures induced persistent increases in BALF IgA level

IgA plays a key role in immune protection against inhaled pathogens and allergens as it is the main class of immunoglobulin secreted in the mucosal surface of the respiratory tract (Woof and Kerr 2006). Here, we found that the levels of IgA were increased by approximately 1.7-fold in the BALF in rats exposed to S+BPE at 32 days and 90 days post-exposure (Figure 3). Increases in IgA have been associated with asthma as well as COPD. A previous study reported that patients suffering from asthma demonstrated ~2.7-fold increase in their IgA (van de Graaf et al., 1991). Further work is needed to determine whether the delayed 1.7-fold increase beginning at 32 days post-exposures that we observed in rats can be compared to that seen in asthma patients. Interestingly, we found significant decreases in BALF levels of IgE, IgG1 and IgG2 in the So group of rats (Figure 4, 5). It is unclear what the decrease in these immunoglobulins signify. One hypothesis is that this is a potential compensatory mechanism resulting from an initial immunologic response. Additional studies investigating how exposures to sand and/or burn pit emissions affect BALF IgA levels is worthwhile and may potentially lead to further hypotheses that can rationalize the observed reduction in BALF IgE as well as IgG1 and IgG2a.

Exposures reduced SP-A levels in serum

Surfactant proteins ensure that lung compliance is sufficiently high for proper ventilation. Specifically, surfactant protein A and D (SP-A, SP-D) regulate inflammatory responses in the lung, and thus have been proposed as biomarkers of lung injury (Greene et al., 2002; Pan et al., 2002). We found reduced serum SP-A levels in the S+BPE group beginning from day 4 and day 32 post-exposure (Figure 6). The reduction in SP-A was also seen in the So rats at day 32 post-exposures (Figure 6). However, this statistically significant reduction in SP-A was not seen at 90 days post-exposure. Previous studies tend to correlate pulmonary dysfunction with increased levels of serum SP-A (Kinder et al., 2009; Wang et al., 2017). It is unclear what a reduction in serum SP-A would signify. It would be appropriate to determine whether the observed transient reduction in serum SP-A (not observed at 90 days post-exposure) can confer

increased susceptibility to development of pulmonary dysfunction. A reduction in SP-A in BALF has been observed in patients suffering from cystic fibrosis correlating with increased neutrophil inflammation monitored over a 3 year period (Griese et al., 2005). However, in this study we only detected reduction in SP-A at the serum level and we did not detect a statistically significant reduction of SP-A in BALF of exposed animals.

In summary, exposures to sand with and without burn pit emissions led to persistent increases in TNF-α as well as IgA in the BALF of rats, lasting >90 days post-exposure. Serum SP-A level was transiently reduced in the sand and the combined sand and burn pit groups starting at day 32 and day 4 respectively but that ultimately reversed by day 90 days post-exposure. Furthermore, transient increases in serum IL-10 and IgE were observed at day 4 and 32, respectively. Small decreases in other inflammatory cytokines in the serum (IL-1β, IL-5, IL-13) and other immunoglobulins (IgG1, IgG2a) in the BALF were also detected. Additional studies should investigate these changes and the potential impact on long term pulmonary function.

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FIGURES

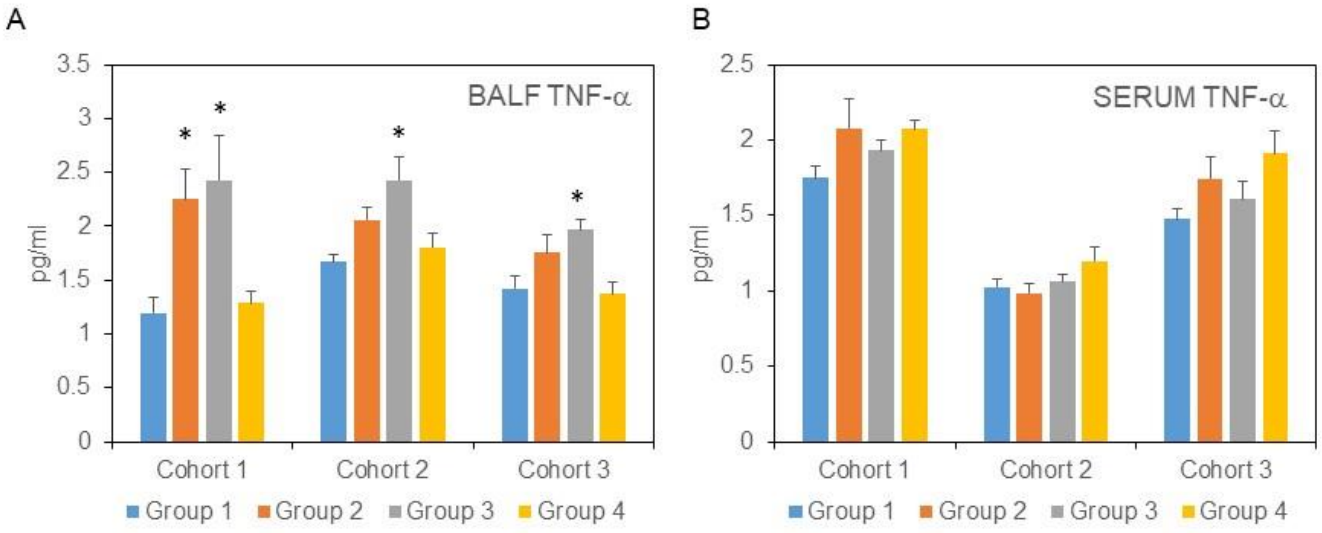

Figure 1. Effects of sand and/or burn pit exposures on levels of TNF-α. Groups 1, 2, 3, and 4 correspond to exposure groups C, So, S+BPE and BPEo, respectively. Cohorts 1, 2 and 3 were animals euthanized at day 4, 32, and 90 following completion of exposures, respectively. **(A)** Levels of TNF- α was measured in BALF. There was a statistically significant increase in TNF- α level in the So group (P=0.05) and the S+BPE group (P=0.025) groups compared to C on day 4. There was a statistically significant increase in TNF- α level in the S+BPE group (P = 0.005) at day 32. The increase in TNF- α in the S+BPE group remained significant (P=0.028) 90 days following exposures. P values were calculated using the post-hoc multiple comparison testing using the Holm-Sidak method following one way ANOVA testing in which $P<0.05$. **(B)** There were no statistically significant differences among experimental groups in serum level of TNF-α at day 4, 32, or 90 days following exposures.

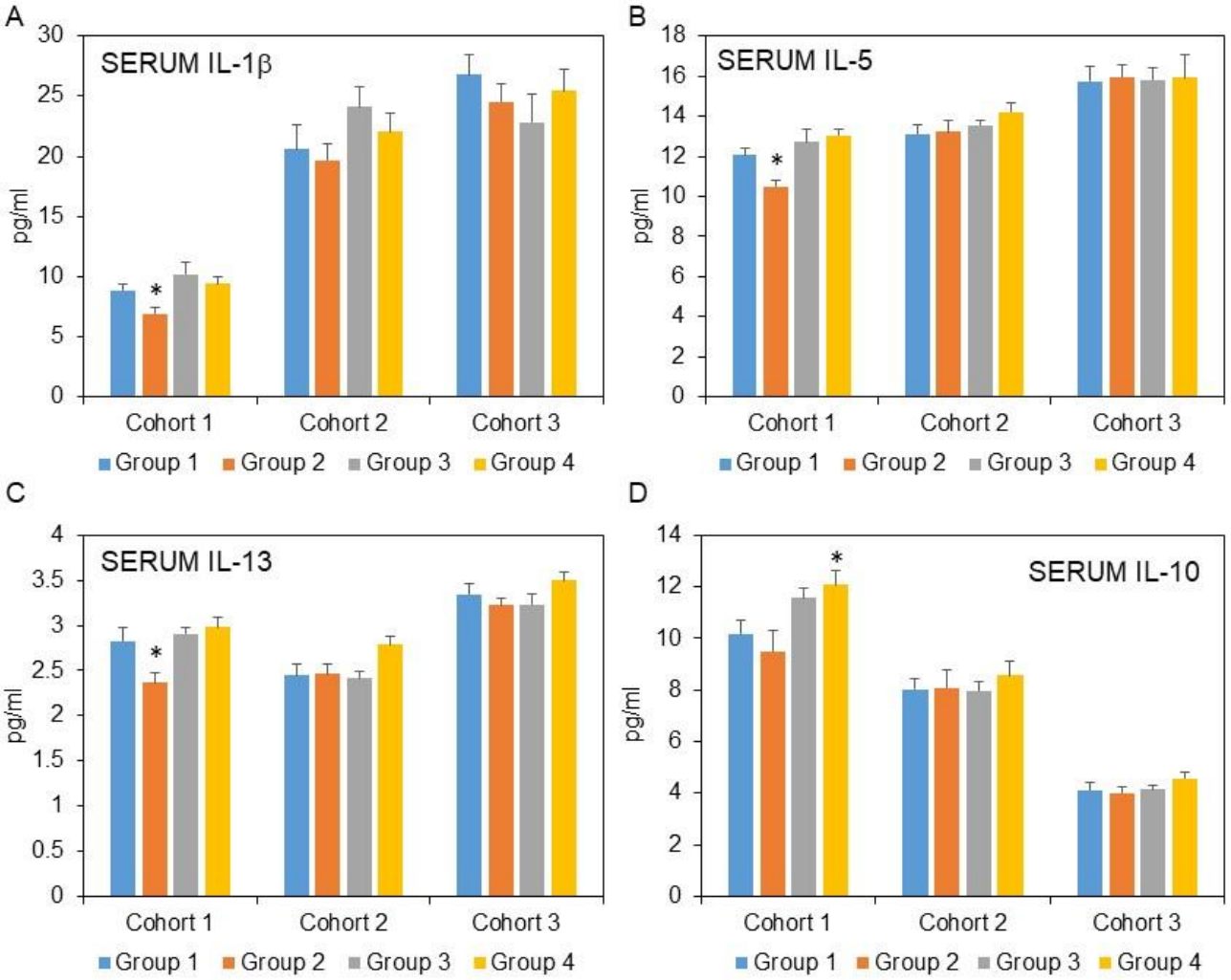

Figure 2. Effects of sand and/or burn pit exposures on serum levels of other cytokines. Groups 1, 2, 3, and 4 correspond to exposure groups C, So, S+BPE and BPEo, respectively. Cohorts 1, 2 and 3 were animals euthanized at day 4, 32, and 90 following completion of exposures, respectively. **(A)** There was a small but statistically significant decrease in the average levels of serum IL-1β in rats exposed to So compared to rats exposed to S+BPE (P=0.008) or BPEo (P=0.048). This decrease was not statistically significant compared to C (P=0.2). At day 32 and day 90, there were no statistically significant changes in serum level of IL-1β among experimental groups. **(B)** There was a small but statistically significant decrease in the average levels of serum IL-5 in rats exposed to So compared to C (P=0.048). This decrease was also statistically significant compared to the S+BPE group (P=0.004) and the BPEo group (P<0.001). At day 32 and day 90, there were no statistically significant changes in serum level of IL-5 among experimental groups. **(C)** There was a small but statistically significant decrease in the average levels of serum IL-13 in rats exposed to So compared to C (P=0.023). This decrease was also statistically significant compared to the S+BPE group (P=0.008) and the BPEo group (P<0.002). At day 32 and day 90, there were no statistically significant changes in serum level of IL-13 among experimental groups. **(D)** There was a small but statistically significant increase in the average levels of serum IL-10 in rats exposed to So compared to rats exposed to BPEo (P=0.022). This decrease was not statistically significant compared to C (P=0.8). At day 32 and day 90, there were no statistically significant changes in serum level of IL-10

among experimental groups. All P values were calculated using the post-hoc multiple comparison testing using the Holm-Sidak method following one way ANOVA testing in which P<0.05.

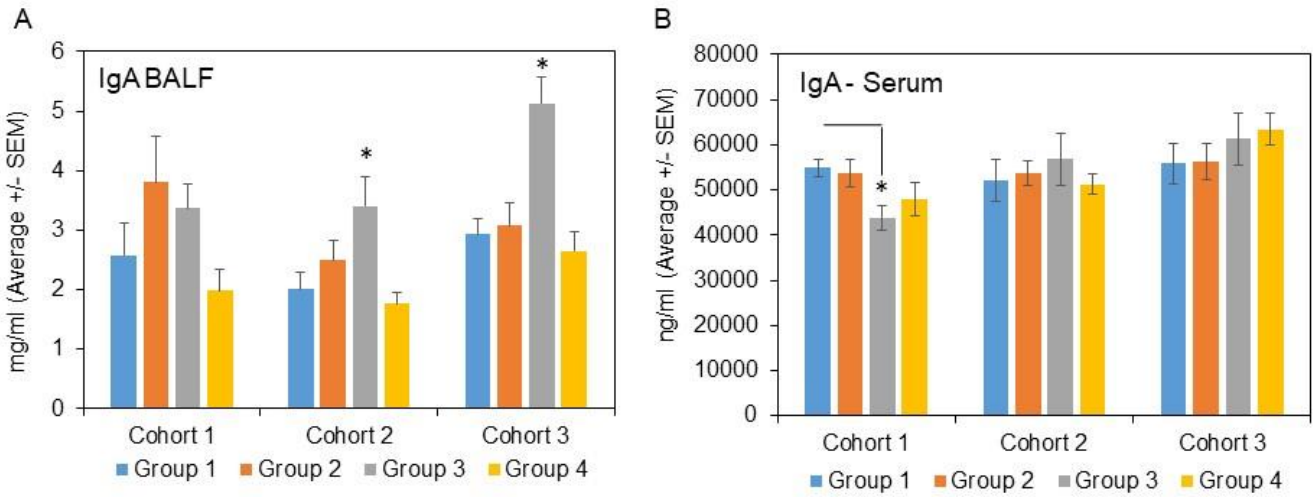

Figure 3. Effects of sand and/or burn pit exposures on levels of IgA. Groups 1, 2, 3, and 4 correspond to exposure groups C, So, S+BPE and BPEo, respectively. Cohorts 1, 2 and 3 were animals euthanized at day 4, 32, and 90 following completion of exposures, respectively. **(A)** Levels of IgA was measured in BALF. There was a statistically significant increase in IgA level in the S+BPE group (P=0.039) compared to C on day 32. The increase in IgA in the S+BPE group remained significant (P=0.004) on day 90 following completion of exposures. **(B)** Levels of IgA was measured in serum. There was a statistically significant reduction in serum IgA in the S+BPE group on day 4 post-exposure (P=0.049). Average values for serum IgA level in the S+BPE group increased over C on day 32 and day 90 post-exposure but the slight increases were not statistically significant. All P values were calculated using the post-hoc multiple comparison testing using the Holm-Sidak method following one way ANOVA testing in which P<0.05.

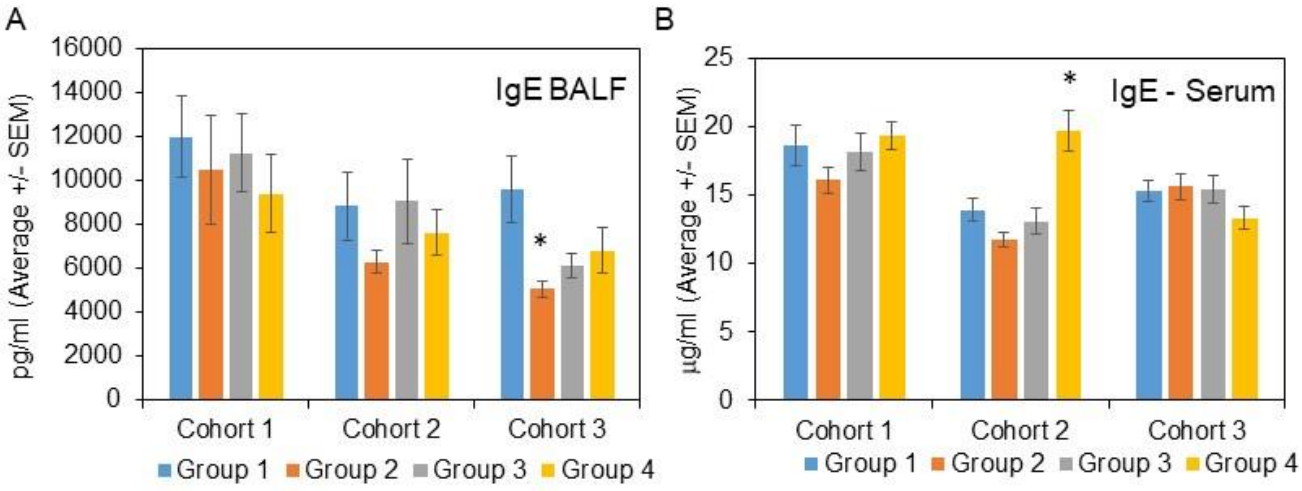

Figure 4. Effects of sand and/or burn pit exposures on levels of IgE. Groups 1, 2, 3, and 4 correspond to exposure groups C, So, S+BPE and BPEo, respectively. Cohorts 1, 2 and 3 were animals euthanized at day 4, 32, and 90 following completion of exposures, respectively. **(A)** Levels of IgE was measured in BALF. There was a statistically significant decrease in BALF IgE level in rats exposed to So compared to C on day 90 ($P=0.009$). **(B)** Levels of IgE was measured in serum. There was a statistically significant increase in serum IgE in the BPEo group on day 32 post-exposures compared to C ($P<0.001$). All P values were calculated using the post-hoc multiple comparison testing using the Holm-Sidak method following one way ANOVA testing in which $P<0.05$.

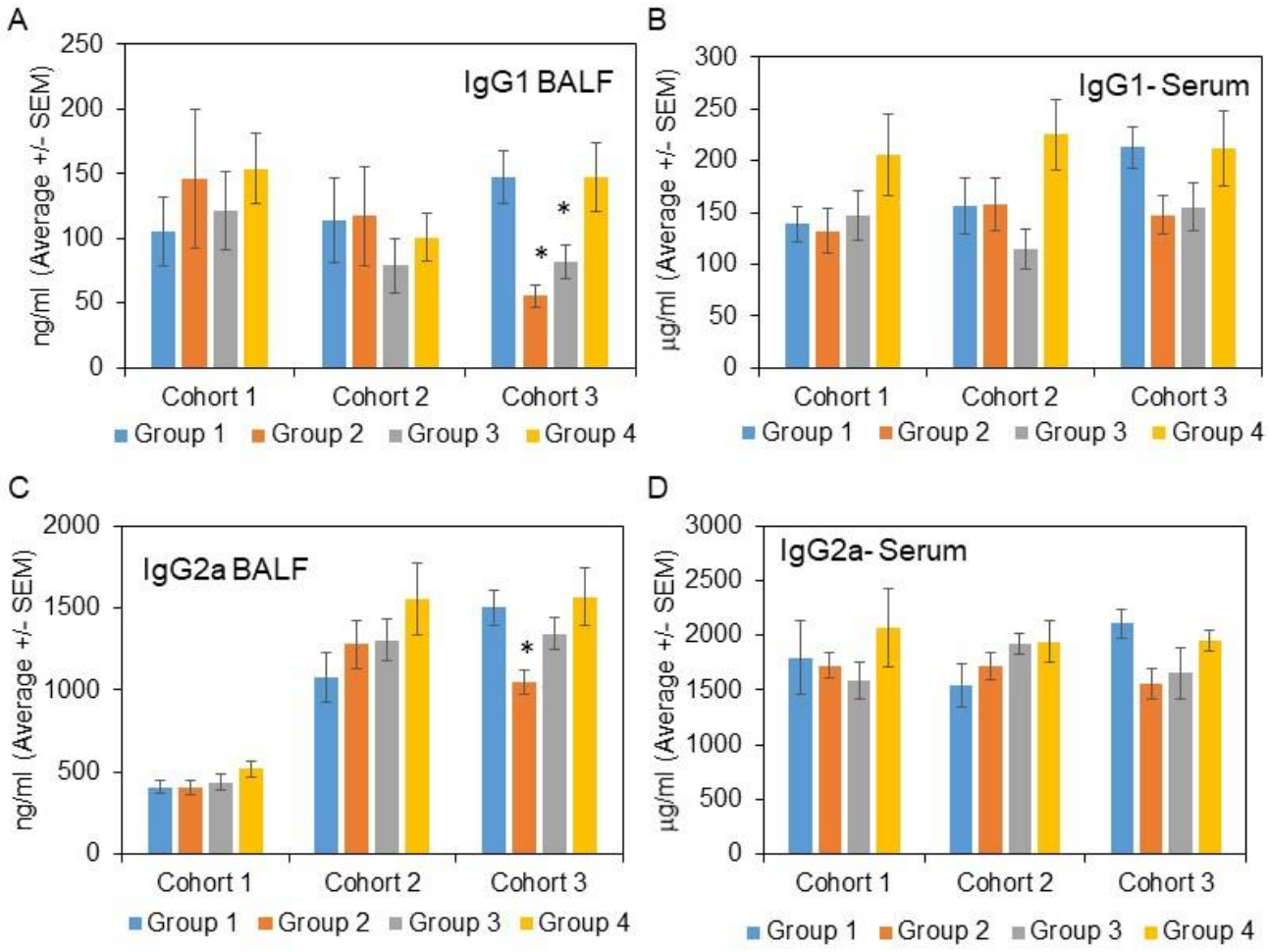

Figure 5. Effects of sand and/or burn pit exposures on levels of IgG1 and IgG2a. Groups 1, 2, 3, and 4 correspond to exposure groups C, So, S+BPE and BPEo, respectively. Cohorts 1, 2 and 3 were animals euthanized at day 4, 32, and 90 following completion of exposures, respectively. **(A)** Levels of IgG1 was measured in BALF. There was a statistically significant decrease in BALF IgG1 level in rats exposed to So (P=0.002) and S+BPE (P=0.027) compared to C on day 90. P values were calculated using the post-hoc multiple comparison testing using the Holm-Sidak method following one way ANOVA testing at P<0.05. **(B)** Levels of IgG1 was measured in serum. There was a reduction in the average serum level of IgG1 in rats exposed to So and S+BPE compared to C on day 90, but the reduction was not statistically significant (P=0.08, ANOVA). **(C)** Levels of IgG2a was measured in BALF. There was a statistically significant decrease in BALF IgG2a level in rats exposed to So compared to C on day 90 (P=0.037). P values were calculated using the post-hoc multiple comparison testing using the Holm-Sidak method following one way ANOVA testing at P<0.05. **(D)** Levels of IgG2a was measured in serum. There was a reduction in the average serum level of IgG2a in rats exposed to So compared to C on day 90, but the reduction was not statistically significant (P=0.057, ANOVA).

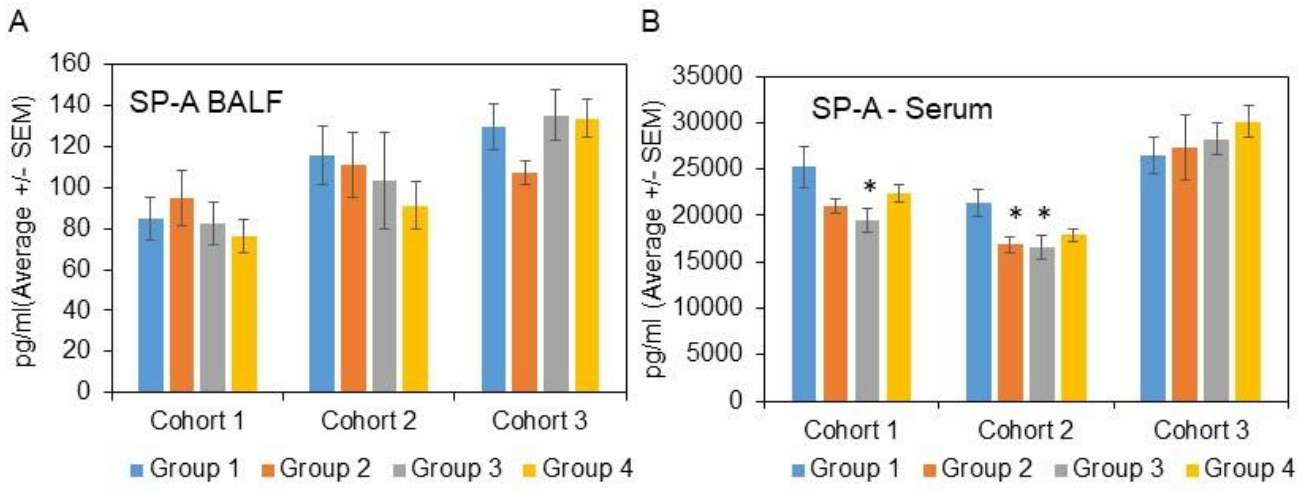

Figure 6. Effects of sand and/or burn pit exposures on levels of SP-A. Groups 1, 2, 3, and 4 correspond to exposure groups C, So, S+BPE and BPEo, respectively. Cohorts 1, 2 and 3 were animals euthanized at day 4, 32, and 90 following completion of exposures, respectively. **(A)** Levels of SP-A was measured in BALF. There were no statistically significant differences among experimental groups in BALF levels of SP-A in day 4, day 32, or day 90 following exposures. **(B)** Levels of SP-A was measured in serum. There was a statistically significant decrease in serum SP-A level in the S+BPE group ($P=0.031$) compared to C on day 4. On day 32, there was a statistically significant decrease in serum SP-A level in the So group ($P=0.04$) and the S+BPE group ($P=0.031$) compared to C. P values were calculated using the post-hoc multiple comparison testing using the Holm-Sidak method following one way ANOVA testing at $P<0.05$.