Iraq Dust is Respirable, Sharp, Metal-Laden, and Induces Lung Inflammation with Fibrosis in Mice via IL-2 Upregulation and Depletion of Regulatory T Cells

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Abstract

Soldiers returning from Iraq have reported respiratory symptoms. Lung biopsies show constrictive bronchiolitis and vascular remodeling with polarizable crystals. We hypothesized that ground surface dust may be a contributing factor to Iraq Afghanistan War Lung Injury (IAW-LI) and analyzed soil grab samples from Camp Victory, Iraq to determine if particle sizes are respirable. Samples contain particles 2.5 micron in size and have sharp edges. Trace metals (including titanium), calcium and silicon are present. Mice with airway instillation of dust have polarizable crystals and septate inflammation. CD4+CD25+FOXP3+ regulatory T cells are decreased in spleen and thymus from mice exposed to dust. IL-2 is elevated from bronchoalveolar lavage taken

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from dust-exposed mice. Respirable Iraq dust leads to lung inflammation in mice similar to that seen in patients, particularly regarding polarizable crystals which, appear to be titanium.

Keywords

Iraq; Afghanistan; dust; burn pits; lung injury; inflammation; IL-2; regulatory T cells

INTRODUCTION

The U.S. Army Millennium Study determined that 14% of all soldiers in Iraq had new-onset respiratory symptoms. We found a similar finding that 14% of veterans deployed to Iraq and Afghanistan had new-onset respiratory symptoms. Recent reports of bronchiolitis and vascular remodeling among open lung biopsies of symptomatic military personnel supports the concept of inhalational lung injury.

Plausible exposures include inhalation of surface dust, particularly during sandstorms.

Iraq Afghanistan War Lung Injury (IAW-LI) has been described by our group to encompass new-onset respiratory symptoms among previously-healthy young soldiers after deployment to Iraq and Afghanistan.[1] The current study was designed to determine if Iraq dust has physico-chemical properties to yield particulate matter air pollution and if inhaled Iraq dust leads to immunosuppression via suppression of regulatory T cells (Treg), with upregulation of pro-inflammatory cytokines.

War-related lung injuries have emerged as previously-unrecognized health problems. Physicians did not recognize the nature of illness in the beginning of the Wars in Iraq and Afghanistan. During the last years of the Iraq War, as well as the ongoing Afghanistan War, new-onset lung problems have been increasingly recognized as being related to deployment in these two countries. Investigators, including our group, have identified asthmatic symptoms or pulmonary function test abnormalities. The Social Security Administration is now compensating for Iraq and Afghanistan War-related lung disease. The Millennium Cohort Study identified 14% of all U.S. soldiers in Iraq with new-onset respiratory symptoms.[2]

So far, we described a new entity called Iraq-Afghanistan War Lung Injury (IAW-LI), characterized by new, recent-onset respiratory symptoms necessitating spirometry, among 14% of 1,787 Long Island-based Veterans of the Wars in Iraq and Afghanistan.[1] Symptoms include dyspnea, exercise-induced shortness of breath, cough, wheezing, chest tightness. Six percent of 1,787 soldiers have spirometric evidence of new airway obstruction, of which half are reversible with bronchodilators; and, the other half has fixed airway obstruction.[3]
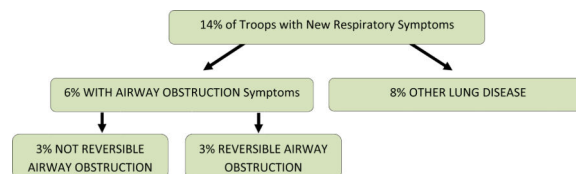
Among the 8% with other new lung diseases are those with: bronchiolitis and pulmonary vascular remodeling—similar to the pathology in lung transplant patients with rejection;[5] high levels of iron and titanium in lung tissue in some patients;[4] one of our patients has *Mycobacterium Avium Complex* in lung along with titanium; others have had patterns of granulomatous lung disease, suggestive of either sarcoidosis or hypersensitivity pneumonitis.

Plausible causes of IAW-LI include: Iraq dust, containing sharp particles (<5 μm in size), minerals, metals and sometimes microbes made airborne during sandstorms; "burn pit" fumes from open air burning of trash with jet fuel, JP-8, without incinerators, in large pits; outdoor and indoor aeroallergens;[8] improvised explosive devices aerosolizing metals; and blast overpressure, with shock waves related to explosions damaging lung tissue and rendering it vulnerable to further injury.

We seized the opportunity to test if a sample of dust from Camp Victory, Iraq, can induce lung injury with a specific aim to dissect the immunopathogenesis with respect to pro-inflammatory cytokine upregulation and suppression of immune-tolerant regulatory T cells.

MATERIALS AND METHODS

This study evaluated: 1) whether a dust sample from Iraq has physico-chemical properties as particulate matter air pollution; and 2) whether inhaled this Iraq dust in mice induces lung injury via immunosuppresion due to reduced regulatory T cell numbers and increased concentrations of pro-inflammatory cytokines. Iraq dust was analyzed by electron microscopy for particle size, shape, and metal constituents. Particle sizes were 5 microns or less and were sharp but not hollow. Titanium was present in trace amounts. Wild-type C57BL/6 mice were anesthetized and tracheotomized for instillation of 0.1 ml of 1 mg/ml g dust/ 1ml normal saline via tracheostomy tube. A dust sample was collected from Camp Victory, Iraq by the U.S. Army Corps of Engineers and compared to (National Institutes of Standards and Technology) NIST San Joaquin, California inert negative control soil samples and NIST Montana (positive control soil from a titanium mine), in comparison to untreated control mice. All dust-challenged mice had inflammation but the greatest histologic score was in Iraq dust-exposed mice (septate thickening, cellular inflammation, polarizable crystals). Bronchoalveolar lavage cytokine levels were highest for IL-2 in the Iraq dust group and suppression of splenic regulatory T cells CD4+CD25+ was the most in the Iraq dust group.

Dust

The mineral constituents of the dust sample were determined using a Scintag Pad X-ray diffractometer with a Cu Kα radiation ($\lambda = 1.54$ Å) from 4 to 90 degrees 2-theta in continuous scan mode at 2 degrees 2-theta/minute. X-ray fluorescence spectroscopy (Bruker S4 Pioneer) was used for elemental analysis. The specific surface area of the dust sample, determined with a QuantachromeNOVA5-point BET analyzer, is 26.429 m^2/g. The morphology and size of the dust particles was determined using a scanning electron microscope (SEM; LEO 1550 SFEG) with an energy dispersive X-ray spectrometer (EDS) operating at 15 kV and 30 μA.

Animal Model

Five 8-week-old male C57BL/6 mice served as untreated controls, without exposure to dust. We administered dust 0.1 ml of a 1 mg/ 1 ml sterile water solution intra-tracheally to eight additional age- and gender matched C57BL/6 mice. Mice were anesthetized with ketamine and xylazine prior to insertion of the tracheostomy tube. Mice were examined 1 month after dust exposure. The wild-type (WT) C57BL/6 mice used in this study are from Taconic Laboratories (Germantown, NY). All experiments and animal care procedures are approved by the Institutional Animal Care and Use Committee and conducted according to National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Method: Mice anesthetized with ketamine/xylazine were tracheotomized and then were exposed to 0.01g dust in 0.1 ml normal saline; then the tracheotomy tube was removed, the wound closed with Surgilock®, and mice were kept alive for 4 weeks. At 4 weeks, the mice again were anesthetized with ketamine, xylazine, a tracheotomy tube was inserted, and the lungs were inflation-fixed with formalin for Hematoxylin and Eosin staining. A pathologist blinded to the identities of the samples used the schemata above to score lung injury (crystal, septate thickening, inflammation in terms of lymphocytes per high power field.) To check for crystals, a polarizing light microscope was used.

For additional mice at 4 weeks, the mice again were anesthetized with ketamine, xylazine, a tracheotomy tube was inserted, and the thymus and spleen were removed as previously described (Szema, BMC Immunology) for flow cytometric analysis of CD4+CD25+FOXP3+ T regulatory cells as a percentage of total cells counted in the lymphocyte gate.

Another group of mice at 4 weeks were anesthetized with ketamine, xylazine, a tracheotomy tube was inserted, and bronchoalveolar lavage of 1.5 ml of a solution at a concentration of 1 tablet of Complete Mini antiprotease per 10 ml normal saline was used. Return lavage fluid was stored in an Eppendorf tube dipped in liquid nitrogen and frozen at −80C for analysis at Assaygate Corporation for concentrations in pg/ml of cytokines IL-2, IFN-g, IL-6, and IL-10.

Immunologic Evidence

Our flow cytometric analysis depended on the characteristic surface markers CD25 and CD4, identified by specific antibodies to identify Tregs.[7] We also used an additional antibody: FOXP3, an intracellular activation marker, which is critical for Treg survival.[9–14]

The spleen and thymus were removed and placed in a Falcon tube with RPMI media and stored on ice until labeling with antibodies for flow cytometric analysis. The spleen and thymus were teased apart and disaggregated on top of a cell strainer in a cell culture dish containing 5 ml of cold media (FACS wash). The cell suspension was transferred to a 50 ml conical tube, centrifuged 10 minutes, resuspended in 5 ml RBC lysis buffer, and incubated for 3 minutes at room temperature. The cells were then centrifuged and washed twice with 15 ml FACS wash buffer, $350 \times g$ 5 minutes and were resuspended at 5×10^6/ml.[7]

The cells were labeled with 5 microliter One step staining Mouse Treg FlowTM kit (FoxP3 Alexa Fluor® 488/CD25PE/CD4 Per CP) with 100 μ of cell suspension per 5 μl antibody for 20 minutes) followed by fixing with FoxP3 fix/perm buffer (Biolegend Cat. No. 421401) at room temperature for 20 minutes. The cells were then permeabilized in FoxP3 perm buffer (Biolegend Cat No. 421402,) for 20 minutes, centrifuged and washed twice with FoxP3 perm buffer. The cells were then labeled with anti-Helios (22f6) (Biolegend Cat. No. 137203 Alexa Fluor® 647) for 20 minutes at room temperature, centrifuged, washed twice, resuspended, and analyzed by flow cytometry. Lymphocyte and macrophage populations were defined by forward and sideward scatter, according to cell granularity and size. Mean control Treg counts in spleen of 435 was based on our earlier paper with N=10 mice.[7]

Histological evidence

To test for the presence of airway inflammation, a corollary of airway hyperresponsiveness in asthma, we subject the lungs from mice to tracheostomy under ketamine/xylazine anesthesia and histological examination after inflation fixation in formalin followed by hematoxylin and eosin staining by a pathologist who is blinded to the identities of the samples.[6] All abnormalities are graded 1, 2, 3, or 4, based on the intensity and extent of peribronchiolar and perivascular cellular infiltration by a pathologist blinded to the identities of the samples. The pathologist also graded the severity and location of crystal deposition and determined if the crystals, if present, were polarizable.

Experimental Design: Analyses (Collection of Data, Statistical Analysis, and Interpretation)

Results are expressed as mean±S.D. with n being the number of animals per group. For statistical analyses, responses are converted to their logarithms ($\log_{10}$) and differences among groups are analyzed using analyses of variance for repeated measurements (ANOVA) followed by Bonferroni or Tukey's multiple comparisons with $p < 0.05$ considered statistically significant.

We seal off the trachea with Vetglue or SurgiLock and examine in 1 month after dust administration.

At 1 month, we will use CO_2 euthanasia and then measure Treg from thymus and spleen using the methods described in the preliminary studies section, with flow cytometry for markers of Treg (CD4+CD25+FOXP3+).

Histologic sections of H&E-stained lung were graded by a blinded pathologist. We also conduct bronchoalveolar lavage and obtain lung tissue for genomics and proteomics analyses. ANOVA is used to compare Treg numbers among the four groups of dust-treated animals, compared to untreated controls.

Lung cytokines

As added evidence of possible airway inflammation, we perform bronchoalveolar lavage (BAL) in WT mice. The lungs of each mouse are lavaged three times with 1 ml of PBS, including an EDTA-free protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). The BAL fluid is centrifuged at 400 *g* for 5 min at 21°C, and the supernatant is analyzed by

a quantitative ELISA assay (Assaygate) for selected inflammatory cytokines, IL-2, IL-4, IFN-g, IL-10.

RESULTS

Dust

Particle sizes of 5 microns or less are present in the Iraq dust sample. The particles are angular in shape, with sharp edges, but are not hollow. Titanium, iron, silicon, calcium and other metals, are present in Iraq dust as shown by energy-dispersive x-ray analysis (Figure 1). Histologic airway inflammation and interstitial inflammation are not present in untreated C57BL/6 mice but are greatest in Iraq dust-exposed mice compared to San Joaquin or Montana dust experimental groups. The Iraq dust-treated group had the most crystals (which were polarizable), septate thickening, and inflammation. The San Joaquin dust generated less inflammation as a negative control compared to Montana dust (laden with titanium) as a positive control. The overall increase in the triad of crystals, septate thickening, and inflammation in the Iraq dust exposed mice suggests that titanium alone, while deleterious, does not account for the entire pathophysiologic process.

The immunopathogenesis of Iraq dust lung injury supports suppression of activated Treg (CD4+CD25+FOXP3+) cells which are immune-tolerant lymphocytes. These are anti-inflammatory. Suppression of total Treg (CD4+CD25+) is associated in these experiments with upregulation of pro-inflammatory cytokine IL-2. In contrast, IL-4, which skews B cells to produce the allergic antibody IgE, is not increased. The Montana dust, titanium-laden, strongly upregulated anti-inflammatory cytokine IL-10 uniquely among the groups.

Animal Model

Figure 2**: Histology Scores Among Lungs from Mice Exposed to dust from Iraq, California or Montana**

Iraq-exposed mice had the most lung injury, based on a scoring system utilizing: 1) presence of crystals, 2) septate thickening, and 3) inflammation.

A pathologist blinded to the identities of treated mice scored for the presence of crystals, septate thickening, or inflammation, using a grading scheme for 1=no crystal, 2= few crystals, and 3=numerous crystals; and 1=no septate thickening vs. 2=septate thickening present. A total score added the presence of all three characteristics. Crystals were identified by birefringence in polarizing light microscopy.

Three mice exposed to Iraq dust had the highest mean scores or 5.3, vs. 5.0 for San Juan, 4.5 for Montana, and 3.0 for no dust. The presence of titanium in Montana dust but lower score thresholds suggests that titanium alone is not sufficient to induce the combined presence of numerous crystals, septet thickening, and inflammation.

Immunologic evidence

Figure 3**: Flow Cytometric Scores from thymus and spleen of dust-exposed mice**

CD+CD25+ Treg cell percentages were lowest in spleen in Iraq-exposed mice compared to all other groups. Activated CD4+CD25+FOXP3+ Treg percentages in Iraq-exposed mice were the second lowest among all groups for both thymus and spleen.

Regulatory T cells (Treg) as defined by CD4+CD25+T cell percentages of total cells counted from thymus were lowest among the mice exposed to Iraq dust, suggesting the most immunosuppression. Treg are immune-tolerant cells; loss of Treg suggests lack of immune tolerance. In contrast Treg were highest in mice exposed to dust from Montana. Even though Treg in Iraq-exposed lungs were lowest among the 3 dust-treated groups, this number was similar to wild-type mice. Since the thymus is the central origin of Treg, a surrogate may be the spleen, since peripheral immune tolerance occurs in the latter organ.

Splenic Treg were lowest in the Iraq-exposed mice versus all other groups, including wild-type, Montana, and San Joaquin, California dust. These data indicate suppression of immune-tolerant CD4+CD25+ regulatory T cell numbers in the periphery.

Triple-staining CD4+CD25+FOXP3+ Treg indicate activation and survival. Percentages of these CD4+C25+FOXP3+ cells were all lower in dust-treated groups, compared to untreated mice. In descending order of percentages: Montana, followed by Iraq, then San Joaquin, California in thymus.

In the spleen, numbers of triple-staining, CD4+CD25+FOXP3+ Treg were lower in Iraq and San Joaquin-exposed mice, compared to wild-type and Montana experimental arms.

Lung cytokines

Figure 4**: Cytokine concentrations in bronchoalveolar lavage fluid from mice exposed to either of the 3 dusts**

Levels of cytokines were highest for IL-2 in the Iraq group, supporting a pro-inflammatory milieu with Iraq dust exposed, compared to San Joaquin, Montana, and untreated mice.

IL-4 was nearly undetectable, thus supporting the concept that this exposure does not induce an IgE-mediated allergic response. Interferon gamma levels were less pronounced than IL-2 but were highest in Iraq and untreated groups, possibly induced as a counter-regulatory response. Anti-inflammatory IL-10 was lowest in the Iraq group among the dust, with Montana and no dust having the highest response. IL-6 was higher in San Joaquin compared to the other groups, which were negligible.

DISCUSSION

Particulate matter air pollution induces vascular inflammation and is associated with premature death from cardiovascular and lung disease, including myocardial infarction and asthma exacerbations.[15, 16]

In the case of particulate matter air pollution from an Iraq dust sample, we now know that dust size is respirable, with 2.5 micron-sized particles present, and that these particles exhibit sharp edges. The physical properties are a concern, akin to asbestos fibers, as the

histological slides show incomplete phagocytosis by pulmonary alveolar macrophages. A plausible theory as to shape is that heavy trucks and tanks may have crushed surface dust to alter its rheology. These are solid particles. We did not see hollow particles discussed by Captain Mark Lyles at the 1st Scientific Symposium on Lung Health After Deployment to Iraq and Afghanistan, Stony Brook University, March, 2011(personal communication, Captain Mark Lyles, U.S. Naval War College, Newport, RI). So, these particles cannot transport substances as containers or "nano-carrier vehicles." Concentrations of trace metals and minerals in the dust are concerning, since some forms of titanium may be pro-fibrotic[17] and is not digestable, and calcium is an airway irritant.

Instillation of Iraq dust yields lymphocytic airway inflammation and interstitial changes which are patchy. Subtle changes in lungs of patients with dust inhalation may not necessarily be visualized with chest x-rays or observed with in-office spirometry, as seen in the cohort of patients studied by Matt King and Robert Miller's group.[18] These patients have lungs which reflect the concept that histologic bronchiolitis and vascular remodeling may be present in the absence of radiographic and pulmonary function physiologic abnormalities. The polarizable crystals we see in mice with dust inhalation are similar to that reported by Miller's group. Among our three patients we have examined by open lung biopsy, all have crystals, and titanium is the suspected culprit by micro x-ray fluorescence.

Interleukin 2 or IL-2 is a pro-inflammatory cytokine expressed by lymphocytes which enhances further lymphocyte proliferation. The striking finding of higher levels of IL-2 in Iraq dust-treated mice supports a pro-inflammatory milieu, especially since anti-inflammatory IL-10 levels are not upregulated. This is a specific inflammation profile, since IL-6, another pro-inflammatory cytokine, notably increased in Acute Respiratory Distress Syndrome from lung stretch,[19] is not increased. IFN-g is also not increased.

Regulatory T cells or Treg are immune-tolerant cells which are anti-inflammatory. They are centrally located in the thymus and also may be found in the periphery from a splenic origin. Finding suppressed Treg staining for CD4+CD25+ surface markers suggests that there is peripheral immunosuppression in dust-treated mice.

While a plethora of airborne hazards may have been experienced by military personnel during deployment to Iraq—detonation of mortars by Al-Qaeda, improvised explosive devices, vehicle improvised explosive devices, blast overpressure from shock waves resulting from explosions, dust and sandstorms called Shamal and Sharq, fumes from burning trash with jet fuel JP-8 in continuously incendiary burn pits, indoor and outdoor aeroallergens (depending on geographic location), infectious vectors from organic components in the dust and air—both bacterial and viral and fungal—our model allows the isolation of dust and the ability to examine its sundry inorganic components and their effect on lung health.

Since only half of patients with new-onset dyspnea after deployment to Iraq and Afghanistan in our cohort[1] respond to asthma medications, targeting the specific, characteristic immune response with respect to Treg and IL-2 may be a more focused approach.

While this research provides some insight into the role of dust encountered in Iraq and Afghanistan Wars on the production of respiratory disease observed in some of the troops deployed there, more work is needed to understand the role of other airborne contaminants in producing these effects. Additionally, the study results are based on only dust samples obtained at Camp Victory, Iraq. Furthermore, the number of animals tested was relatively small. Holding some animals beyond one month to evaluate long-term effects would be of interest.

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CLINICAL RELEVANCE

Surface dust from Iraq is respirable particulate matter air pollution, with physico-chemical properties leading to inflammation and immunosuppression in mice. This suggests inhaled dust may contribute to development of new-onset dyspnea among soldiers.
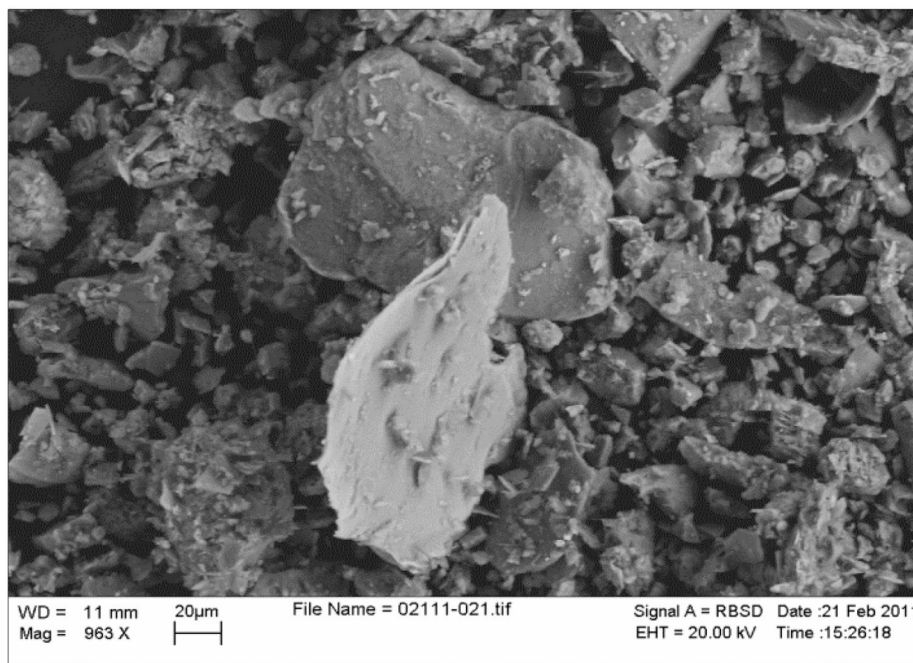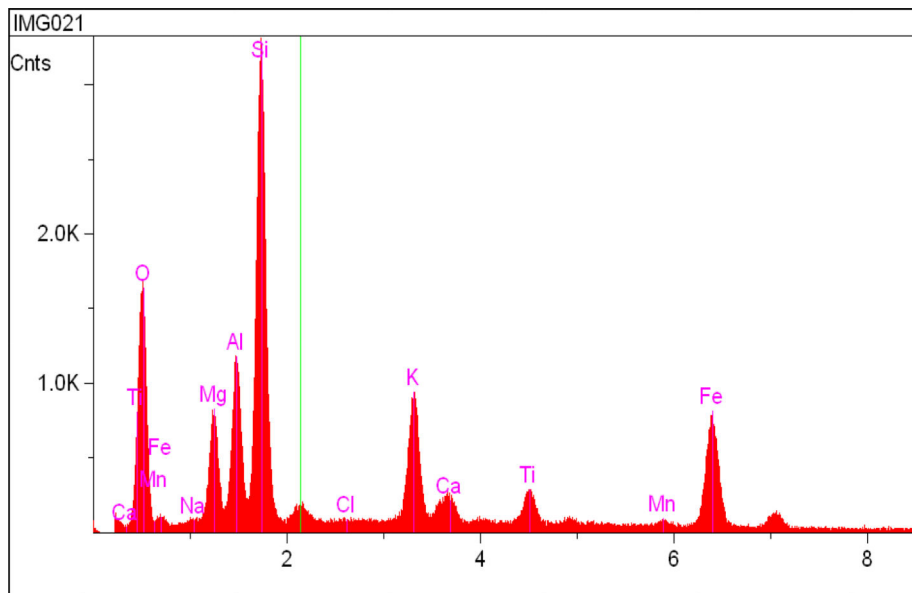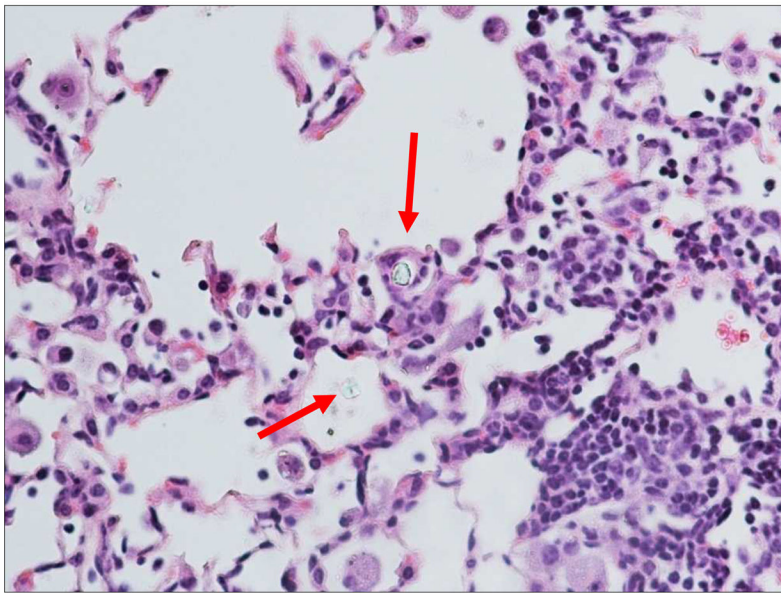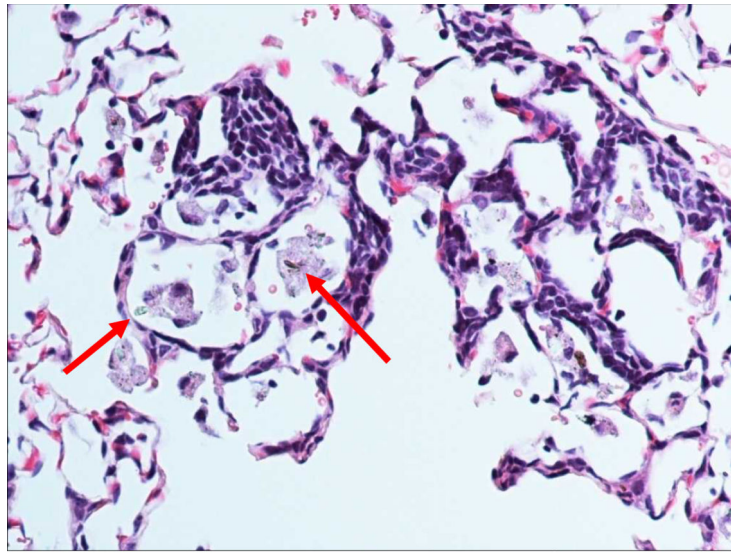
Figure 1). Dust
Energy-dispersive X-ray analysis of representative particle in Iraq dust sample, indicating elements detected (top). Electron micrograph of Camp Victory, Iraq, dust sample shows variable particle sizes, including fraction less than 5μm (respirable). Particles are angular with sharp edges (bottom).
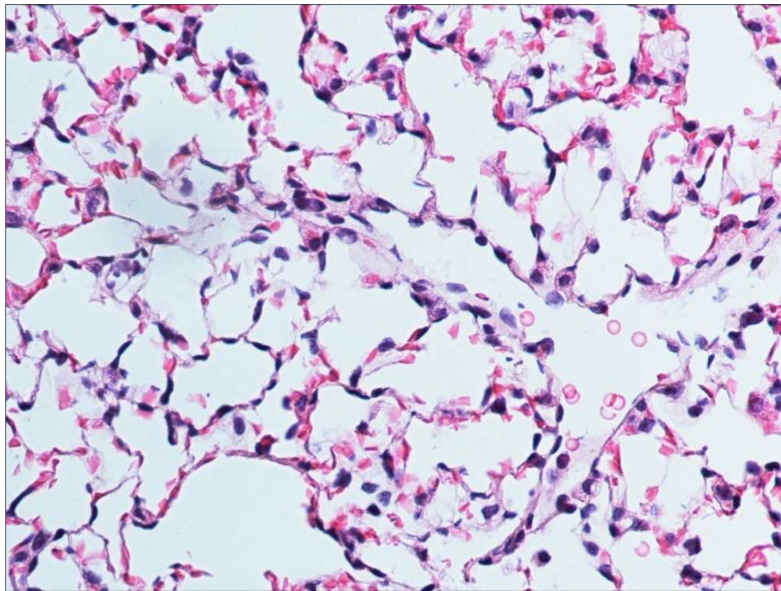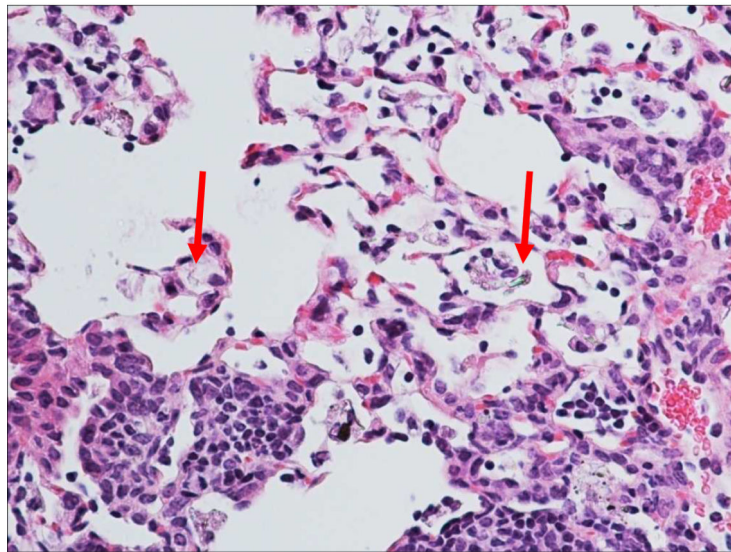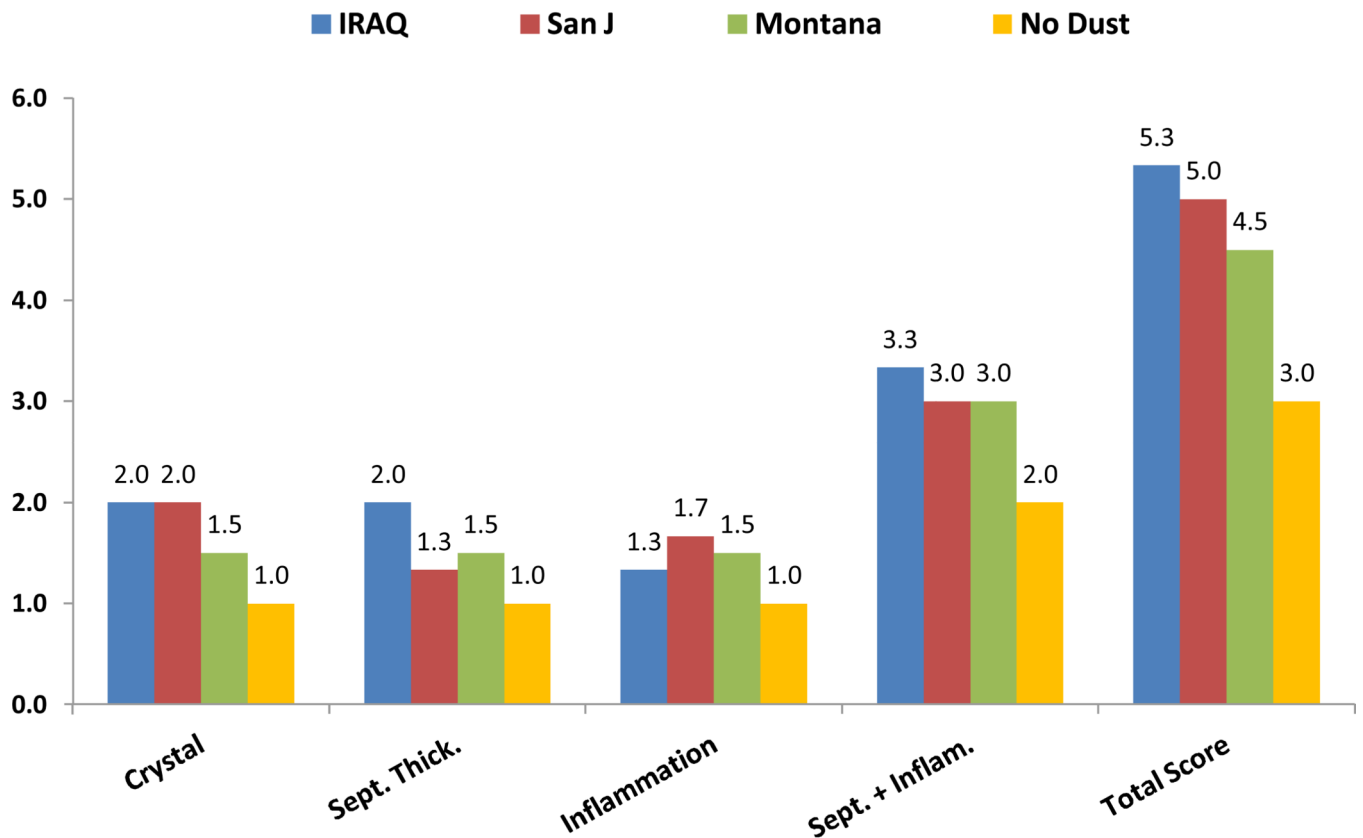
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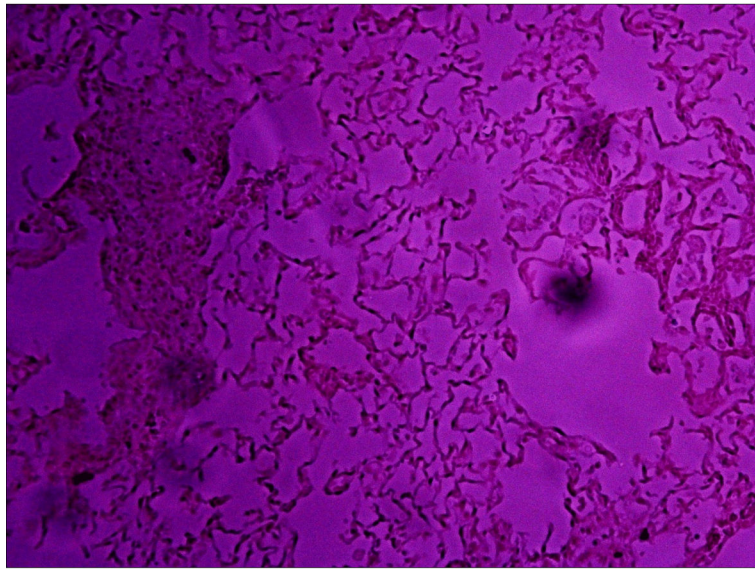

Montana Dust: 400X

Iraq Dust: 400X

San J. Dust: 400X

Histology_Statistical Analysis

No dust (n=5); Montana (n=2); Iraq (n=3); San J (n=3)

Polarizable crystals are bright white seen in Iraq dust-exposed lungs.

x200
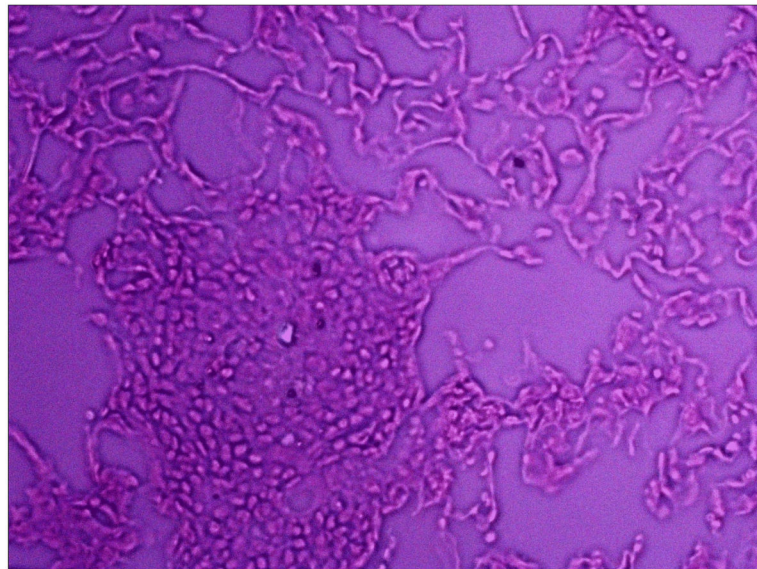
X 400

Figure 2). Histology
Histologic examples of lungs from each experimetnal arm shows: no dust at 400× with normal histology in C57BL/6 mouse lung; San Joaquin, California dust with a minimal amount of focal lymphocytic accumulation in the lower right hand corner of the field; Montana dust with significant inflammation, more widespread; and Iraq dust with septate thickening, interstitial inflammation, incompletely phagocytosed crystals.
No dust (n=5); Montana (n=2); Iraq (n=3); San J (n=3)
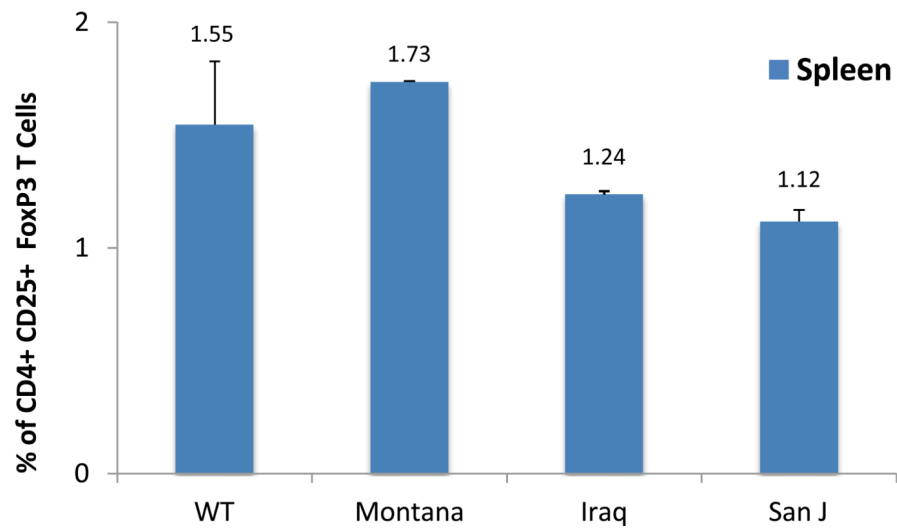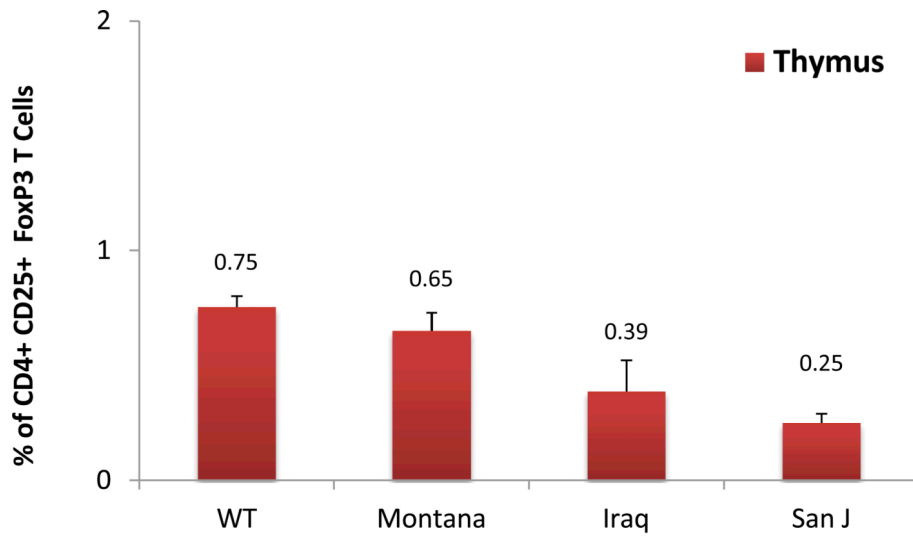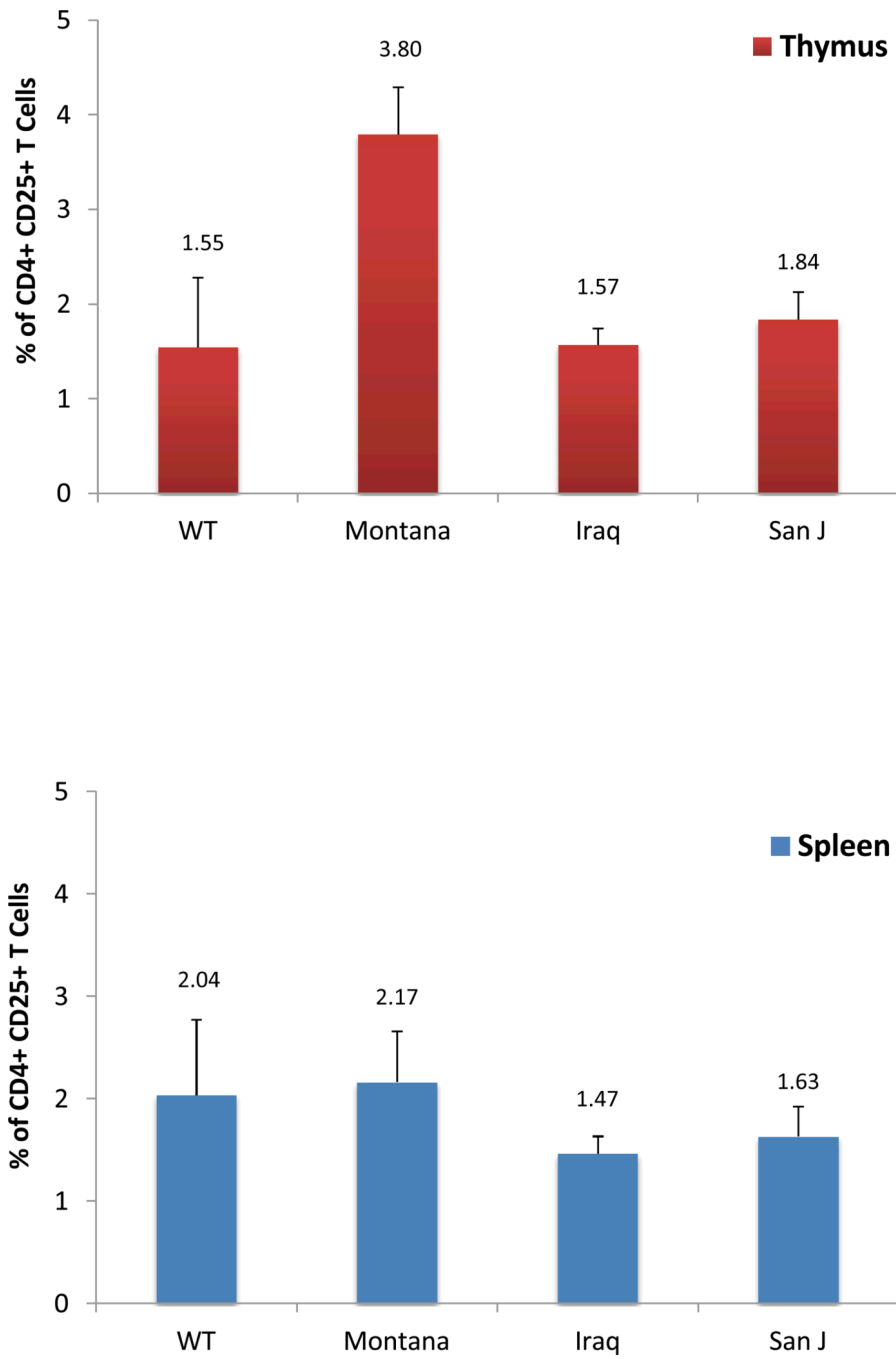
Figure 3). Flow cytometry
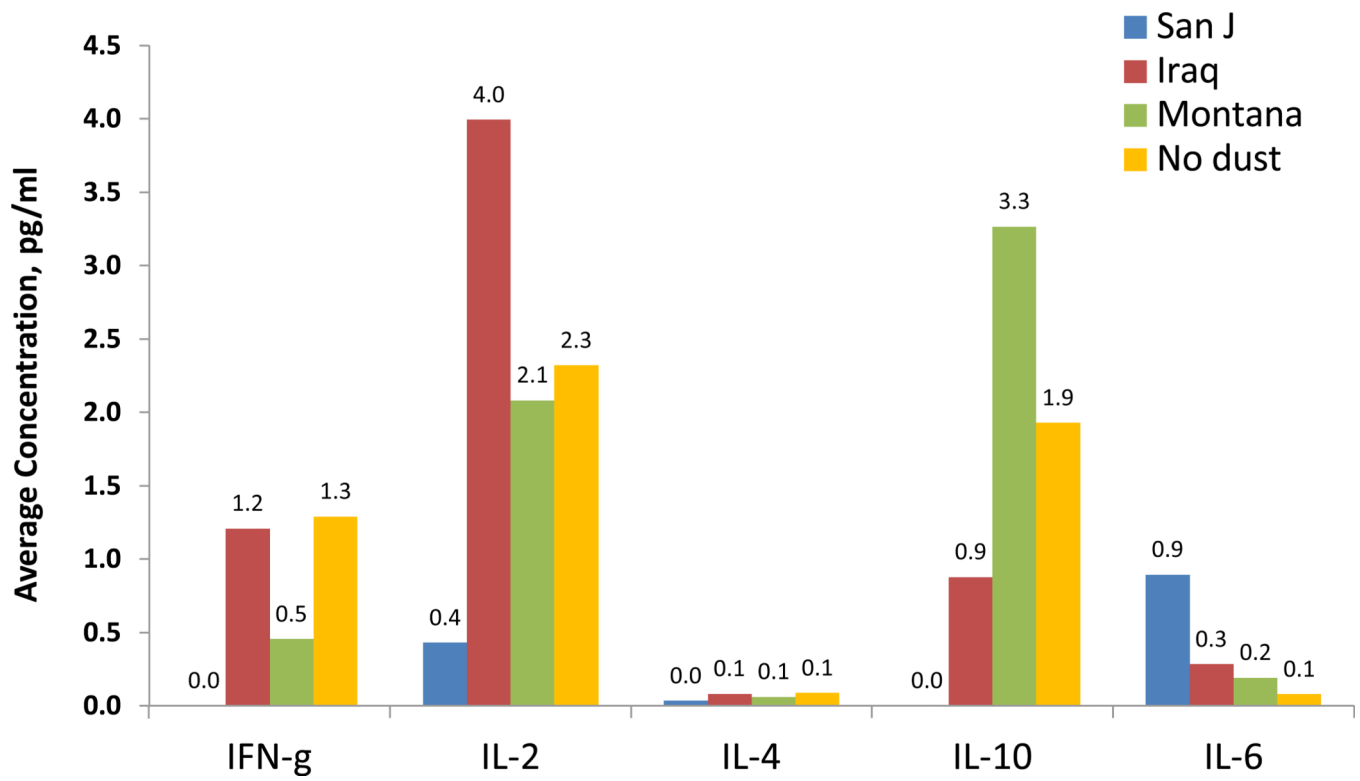
Figure 4). Cytokines analysis

TABLE 1

P values based on ttest

	Crystal	Septate Thickening	Inflammation	Septate + Inflammation	Total Score
WT/Mont	0.117	0.117	0.117	0.117	0.117
WT/Iraq	0.000	0.000	0.220	0.002	0.000
WT/Sanj	0.055	0.220	0.034	0.055	0.055
Mont/Iraq	0.272	0.272	0.789	0.724	0.537
Mont/Sanj	0.591	0.789	0.789	1.000	0.806
Iraq/Sanj	1.000	0.116	0.519	0.643	0.795

The highest total lung injury score was in the Iraq dust-exposed group, with more crystals, which were polarizable, consistent septate thickening, and inflammation, both airway and interstitial.

Flow cytometry_Statistical Analysis

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Thymus_CD4CD25	Between Groups	11.300	3	3.767	2.820	.099
	Within Groups	12.020	9	1.336		
	Total	23.320	12			
Spleen_CD4CD26	Between Groups	.957	3	.319	2.211	.156
	Within Groups	1.298	9	.144		
	Total	2.255	12			
Thymus_CD4CD25_FoxP3	Between Groups	.576	3	.192	9.452	.004
	Within Groups	.183	9	.020		
	Total	.759	12			
Spleen_CD4CD25_FoxP3	Between Groups	.652	3	.217	1.233	.353
	Within Groups	1.586	9	.176		
	Total	2.237	12			

1= No dust (n=5); 2= Montana (n=2); 3= Iraq (n=3); 4= San J (n=3)

P value-Based on Tukey Post Hoc Test

Thymus_CD4CD25_FoxP3	Tukey HSD		
1	2	0.819
	3	0.027
	4	0.004
2	1	0.819
	3	0.248
	4	0.054
3	1	0.027
	2	0.248
	4	0.656
4	1	0.004
	2	0.054
	3	0.656

P value based on unpaired ttest

Thymus CD4 CD25

	WT	Montana	Iraq	San J		
	4.45	3.3	1.91	1.51	WT/Mont	0.799
	1.38	4.29	1.44	2.42	WT/Iraq	0.106
	3.52		1.37	1.58	WT/SanJ	0.158
	2.38				Mont/Iraq	0.014
	5.55				Mont/Sanj	0.034
					Iraq/Sanj	0.479
Mean	1.55	3.80	1.57	1.84		
SEM	0.74	0.50	0.17	0.29		

Spleen CD4 CD25

	WT	Montana	Iraq	San J		
	2.24	2.33	1.49	1.54	WT/Mont	0.779
	1.16	2	1.4	1.67	WT/Iraq	0.134
	2.67		1.51	1.69	WT/SanJ	0.266
	1.99				Mont/Iraq	0.013
	2.14				Mont/Sanj	0.030
					Iraq/Sanj	0.045
Mean	2.04	2.17	1.47	1.63		
SEM	0.25	0.17	0.03	0.05		

Thymus CD4 CD25 +FoxP3

	WT	Montana	Iraq	San J		
	0.87	0.57	0.25	0.32	WT/Mont	0.311
	0.58	0.73	0.66	0.18	WT/Iraq	0.022
	0.81		0.25	0.25	WT/SanJ	0.000
	0.73				Mont/Iraq	0.252
	0.78				Mont/Sanj	0.015
					Iraq/Sanj	0.392
Mean	0.75	0.65	0.39	0.25		
SEM	0.05	0.08	0.14	0.04		

Spleen CD4 CD25 +FoxP3

	WT	Montana	Iraq	San J		
	1.83	1.73	1.24	1.02	WT/Mont	0.703
	0.53	1.74	1.26	1.13	WT/Iraq	0.439
	2.18		1.21	1.2	WT/SanJ	0.296
	1.44				Mont/Iraq	0.000
	1.75				Mont/Sanj	0.003
					Iraq/Sanj	0.092
Mean	1.55	1.74	1.24	1.12		
SEM	0.28	0.01	0.01	0.05		

TABLE 3

BAL Cytokines_Statistical Analysis ANOVA

		ANOVA				
		Sum of Squares	df	Mean Square	F	Sig.
IFNg	Between Groups	3.867	3	1.289	.832	.509
	Within Groups	13.945	9	1.549		
	Total	17.812	12			
IL2	Between Groups	19.174	3	6.391	3.203	.076
	Within Groups	17.959	9	1.995		
	Total	37.132	12			
IL4	Between Groups	.004	3	.001	.287	.834
	Within Groups	.046	9	.005		
	Total	.051	12			
IL10	Between Groups	14.911	3	4.970	1.697	.237
	Within Groups	26.360	9	2.929		
	Total	41.271	12			
IL6	Between Groups	1.323	3	.441	.780	.534
	Within Groups	5.088	9	.565		
	Total	6.411	12			

1= No dust (n=5); 2= Montana (n=2); 3= Iraq (n=3); 4= San J (n=3)

P value based on unpaired ttest	IFN-g	IL-2	IL-4	IL-10	IL-6
WT/Mont	0.569	0.708	0.738	0.515	0.527
WT/Iraq	0.942	0.236	0.924	0.487	0.106
WT/Sanj	0.275	0.006	0.401	0.221	0.263
Mont/Iraq	0.211	0.433	0.789	0.106	0.579
Mont/Sanj	0.272	0.011	0.696	0.047	0.588
Iraq/Sanj	0.009	0.093	0.421	0.017	0.533