



DEPARTMENT OF THE AIR FORCE
59TH MEDICAL WING (AETC)
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5 DEC 2016

MEMORANDUM FOR ST

ATTN: HUI XIA

FROM: 59 MDW/SGVU

SUBJECT: Professional Presentation Approval

1. Your paper, entitled **A Multiplex Quantitative Analysis of Secreted Proteins in Bronchoalveolar Lavage Samples from War Veterans with Chronic Respiratory Symptoms** presented at/published to **Poster: A Multiplex Quantitative Analysis of Secreted Proteins in Bronchoalveolar Lavage Samples from War Veterans with Chronic Respiratory Symptoms** in accordance with MDWI 41-108, has been approved and assigned local file #**16395**.
2. Pertinent biographic information (name of author(s), title, etc.) has been entered into our computer file. Please advise us (by phone or mail) that your presentation was given. At that time, we will need the date (month, day and year) along with the location of your presentation. It is important to update this information so that we can provide quality support for you, your department, and the Medical Center commander. This information is used to document the scholarly activities of our professional staff and students, which is an essential component of Wilford Hall Ambulatory Surgical Center (WHASC) internship and residency programs.
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4. Congratulations, and thank you for your efforts and time. Your contributions are vital to the medical mission. We look forward to assisting you in your future publication/presentation efforts.

PAUL T. BARNICOTT, GS-15-DAF
Deputy Director, Clinical Research Division

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"The voluntary, fully informed consent of the subjects used in this research was obtained as required by 32 CFR 219 and DoDI 3216.02_AFI 40-402, Protection of Human Subjects in Biomedical and Behavioral Research."

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PROTOCOL TITLE - [NOTE: For each new release of medical research or technical information as a publication/presentation, a new 59 MDW Form 3039 must be submitted for review and approval.] Study of Active Military Personnel for Pulmonary Disease Related to Environmental Dust Exposure – 13 Comprehensive Dyspnea Evaluation (STAMPEDE-CDE)			
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APPROVING AUTHORITY'S PRINTED NAME, RANK, TITLE Valtier, Sandra, PhD, GS13, CAMD Director	APPROVING AUTHORITY'S SIGNATURE VALTIER.SANDRA.1230996403 <small>Digitally signed by VALTIER.SANDRA.1230996403 DN: cn=VALTIER.SANDRA.1230996403, o=USAF, ou=AFMILCONTRACTOR, email=VALTIER.SANDRA.1230996403 Date: 2016.11.16 14:44:31 -0800</small>		DATE Nov 16, 2016

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A multiplex quantitative analysis of secreted proteins in bronchoalveolar lavage samples from war veterans with chronic respiratory symptoms

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ABSTRACT

A significant number of veterans who participated in Iraq and Afghanistan wars are infected with chronic lung problems. Many such active duty personnel and veterans have been the subjects of a study termed STAMPEDE – Study of Active Duty Military for Pulmonary Disease related to Environmental Deployment Exposure. However, so far no definitive causal relationships between the characteristic clinical and pathological lung conditions and extrinsic factors has been established. The current study aimed to analyze 37 cytokines and other secreted proteins in the bronchoalveolar lavage (BAL) samples from healthy as well as the patients. The lavage samples were centrifuged to pellet cells and other debris, and supernatants were saved for further analysis. The analysis employed the Lumex bead-based high-throughput approach to simultaneously analyze the level of each of the 37 secreted cytokines and other proteins. Total protein concentration in each sample was determined by a fluorescence method using the Qubit. The individual protein determinations in each sample were then normalized to the total protein in the same sample. The results show that for many of the proteins analyzed there was little or no difference in levels between the healthy and lung disease patients. However, for many others there were marked differences in levels. The proteins whose levels were about 1.5 to nearly 4 times higher in the lung disease samples than the healthy ones were sIL-6 receptor, BAFF, and IL-8. The proteins that showed higher levels in the control samples than the disease samples were APRIL (1.5 fold) and INF-beta (2 fold). Many others appeared to be at the same level in healthy and disease samples. These results suggest the possibility that secreted protein profile in these patients in comparison to the healthy individuals may help distinguish the lung conditions due to exposure in the war theater. However, more extensive studies would be needed.

INTRODUCTION

Military personnel who served in Iraq and Afghanistan are at an increased risk of developing respiratory symptoms, compared with non-deployed troops. According to a study involving more than 760,000 veterans, 6 percent of veterans have one or more chronic respiratory symptoms. Several studies have attributed the chronic respiratory disease to harsh environmental exposures, such as dust, burn pits and other chemical hazards. Many chronic respiratory disease, like asthma and chronic obstructive pulmonary disease, are inflammatory disease of the airways. Multiple cytokines play a key role in the inflammatory airway diseases. In chronic inflammation, the level of cytokines become unbalanced. For example, the level of cytokines, such as TNF-alpha, IL-8, IFN-gamma, is increased in asthma. In this study, we compared the level of inflammation cytokines in the respiratory disease from the patients who deployed to Iraq and Afghanistan to that seen in healthy subjects. The discovery of anti-inflammatory targets will be helpful for developing new therapeutic strategies in the chronic respiratory disease.

Table 1. Bio-Plex Pro Human Inflammation Panel 1, 37-Plex

APRIL/TNFSF13	IL-11	LIGHT/TNFSF14
BAFF/TNFSF13B	IL-12 (p40)	MMP-1
sCD30/TNFSF8	IL-12 (p70)	MMP-2
sCD163	IL-19	MMP-3
Chitinase 3-like 1	IL-20	Osteocalcin
gp130/sIL-6R beta	IL-22	Osteopontin
IFN-alpha 2	IL-26	Pentraxin-3
IFN-beta	IL-27 (p28)	sTNF-R1
IFN-gamma	IL-28A/IFN-lambda 2	sTNF-R2
IL-2	IL-29/IFN-lambda 1	TSIP
sIL-6R alpha	IL-32	TWEAK/TNFSF12
IL-8	IL-34	
IL-10	IL-35	

METHODS

SAMPLES

BAL (bronchoalveolar lavage) fluid samples from 38 healthy control subjects and 193 patient subjects were collected according to a standardized method.

PROTEIN QUANTIFICATION

Total protein in BAL fluid was measured using Qubit protein assay kits and the Qubit 3.0 fluorometer (ThermoFisher Scientific).

ASSAY

Cytokines in BAL fluid were quantified using the Bio-Plex Pro Human Inflammation Panel 1, 37-Plex Assay kit (Bio-Rad Laboratories, Inc.) following the manufacturer's recommendations. 50µl of each sample was assayed in duplicate on a 96 well plate, and then read on the Lumex MAGPIX instrument (Luminex Corporation). Each 96 well plate included the following controls, which were assayed in duplicate: an 8 point standard curve, a negative blank control, and a positive control (provided by the manufacturer, Bio-Rad Laboratories, Inc.).

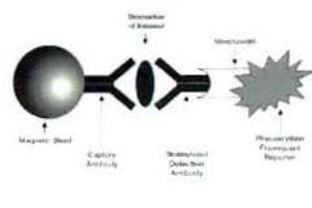
STATISTICAL ANALYSIS

For each of the 37 cytokines, the observed concentration of the cytokine (pg/mL) in each sample was divided by the total amount of protein (µg/mL) to calculate the final pg/µg value. The results of study were presented as means ± SEM. Group comparisons were done using an unpaired Student's t-test. Statistical significance defined as having a P value of less than 0.05.

Figure 1. A schematic depiction of the bead-based multiplex immunoassays for BAL fluid.



Figure 2. A depiction of the Bio-Plex sandwich immunoassay (Bio-Rad Laboratories, Inc.).

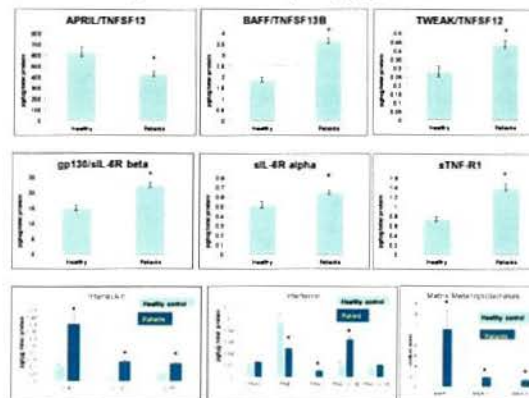


RESULTS

Table 2. Human inflammation biomarkers in BAL fluid between subjects with chronic respiratory symptoms and healthy control.

Cytokines and Cellular component	Healthy BAL (n=38)	Chronic Pulmonary Symptoms BAL (n=193)
Mean (range) pg/ml		
APRIL/TNFSF13	20.00 (0.00-100.00)	40.00 (0.00-100.00)
BAFF/TNFSF13B	10.00 (0.00-100.00)	20.00 (0.00-100.00)
sCD30/TNFSF8	1.00 (0.00-1.00)	1.00 (0.00-1.00)
sCD163	1.00 (0.00-1.00)	1.00 (0.00-1.00)
Chitinase 3-like 1	1.00 (0.00-1.00)	1.00 (0.00-1.00)
gp130/sIL-6R beta	1.00 (0.00-1.00)	1.00 (0.00-1.00)
IFN-alpha 2	1.00 (0.00-1.00)	1.00 (0.00-1.00)
IFN-beta	1.00 (0.00-1.00)	1.00 (0.00-1.00)
IFN-gamma	1.00 (0.00-1.00)	1.00 (0.00-1.00)
IL-2	1.00 (0.00-1.00)	1.00 (0.00-1.00)
IL-8	1.00 (0.00-1.00)	1.00 (0.00-1.00)
IL-10	1.00 (0.00-1.00)	1.00 (0.00-1.00)
IL-11	1.00 (0.00-1.00)	1.00 (0.00-1.00)
IL-12 (p40)	1.00 (0.00-1.00)	1.00 (0.00-1.00)
IL-12 (p70)	1.00 (0.00-1.00)	1.00 (0.00-1.00)
IL-19	1.00 (0.00-1.00)	1.00 (0.00-1.00)
IL-20	1.00 (0.00-1.00)	1.00 (0.00-1.00)
IL-22	1.00 (0.00-1.00)	1.00 (0.00-1.00)
IL-26	1.00 (0.00-1.00)	1.00 (0.00-1.00)
IL-27 (p28)	1.00 (0.00-1.00)	1.00 (0.00-1.00)
IL-28A/IFN-lambda 2	1.00 (0.00-1.00)	1.00 (0.00-1.00)
IL-29/IFN-lambda 1	1.00 (0.00-1.00)	1.00 (0.00-1.00)
IL-32	1.00 (0.00-1.00)	1.00 (0.00-1.00)
IL-34	1.00 (0.00-1.00)	1.00 (0.00-1.00)
IL-35	1.00 (0.00-1.00)	1.00 (0.00-1.00)
MMP-1	1.00 (0.00-1.00)	1.00 (0.00-1.00)
MMP-2	1.00 (0.00-1.00)	1.00 (0.00-1.00)
MMP-3	1.00 (0.00-1.00)	1.00 (0.00-1.00)
Osteocalcin	1.00 (0.00-1.00)	1.00 (0.00-1.00)
Osteopontin	1.00 (0.00-1.00)	1.00 (0.00-1.00)
Pentraxin-3	1.00 (0.00-1.00)	1.00 (0.00-1.00)
sTNF-R1	1.00 (0.00-1.00)	1.00 (0.00-1.00)
sTNF-R2	1.00 (0.00-1.00)	1.00 (0.00-1.00)
TSIP	1.00 (0.00-1.00)	1.00 (0.00-1.00)
TWEAK/TNFSF12	1.00 (0.00-1.00)	1.00 (0.00-1.00)

Figure 3. The comparison of Cytokine and Cellular component in BAL fluid between Healthy control and chronic respiratory symptoms.



Notes: All samples were run in duplicate. The healthy sample group includes 38 samples. The patient group includes 193 samples. The error bars represent the Standard Error of the Mean. * P<0.05.

DISCUSSION

1. Increased concentration of IFN-gamma in the patients, which is associated with T cell response from Th1 and Th2.
2. Increased concentration of Metalloproteinases (MMP1,2 and 3) in the patients, which can cause morphological changes in the lung.
3. Increased concentration of IL-8 and tumor necrosis factor superfamily (BAFF and TWEAK) in the patients.
4. Increased concentration of IL-19 in the patients, which was reported an increased level in the lungs of mice exposed to allergens.
5. Further assay for more cytokines will be finished.

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The voluntary, fully informed consent of the subjects used in this research was obtained as required by 32 CFR 219 and DoD 3216.02, AFM 40-402, Protection of Human Subjects in Biomedical and Behavioral Research