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RuX Largely Restores Lungs in Iraq PM-Exposed Mice, Up-Regulating Regulatory T-Cells (Tregs)

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Abstract

(Background)—Military personnel post-deployment to Iraq and Afghanistan have noted new-onset respiratory illness. This study's primary objective was to further develop an animal model of Iraq Afghanistan War Lung Injury (IAW-LI) and to test a novel class of anti-injury drug called RuX.

(Methods)—Particulate Matter (PM) samples were obtained in Iraq then characterized by spectromicroscopy. C57BL/6 mice underwent orotracheal instillation with PM, followed by drinkable treatment with RuX. Lung histology, inspiratory capacity (FlexiVent), thymic/splenic regulatory T cell (Treg) number, and whole-lung genomics were analyzed.

(Results)—Tracheal instillation of Iraq PM led to lung septate thickening and lymphocytic inflammation. PM-exposed mice had suppression of thymic/splenic regulatory T-cells (Tregs). Drinking RuX after PM exposure attenuated the histologic lung injury response, improved lung inspiratory capacity, and increased Tregs. Pooled whole lung genomics suggest differences among gene expression of IL-15 among control, PM, and PM+RuX groups.

(Conclusions)—RuX, a ruthenium and alpha-lipoic acid complex, attenuates lung injury by improving histology and inspiratory capacity via upregulation of Tregs in Iraq PM-exposed C57BL/6. Plausible genomic effects may involve IL=15 whole lung gene expression.

INTRODUCTION/BACKGROUND:

Military personnel deployed to regions with harsh environmental conditions often incur physiologic tolls beyond the repercussions of combat itself. Numerous studies have reported that servicemen and women, both during and after deployment to Iraq and Afghanistan (Operations Iraqi Freedom/Enduring Freedom/New Dawn), have new and distinct respiratory illnesses, which were not diagnosed prior to deployment. A screening process rules out potential servicemen and women diagnosed with major respiratory illness prior to deployment (1–6). Therefore, most studies have associated these illnesses to widespread exposure to hazardous airborne particulate matter (PM) in these severe, desert environments.

Respiratory Illness:

In an initial 2004 survey, over 69% of troops deployed to Southwest Asia reported respiratory distress (7), with 24% reporting at least one acute respiratory illness in a follow-up, mid-deployment survey (8). In a more recent study involving over 40,000 participants, 14% of returning deployed troops reported novel, persistent respiratory symptoms (9). Additionally, a retrospective study of 6,000 Veteran Administration (VA) medical records determined significantly higher rates of asthma in soldiers deployed to Iraq, compared to those stationed in the U.S. (6.6% vs. 4.3%, $P = 0.003$; odds ratio, 1.58; 95% confidence interval, 1.18 to 2.11) (3). The name 'Iraq Afghanistan War Lung Injury' has been coined to

describe these new respiratory illnesses. A more recent study of a cohort of soldiers deployed to Iraq/Afghanistan suggests higher rates of obstructive pulmonary diseases and respiratory symptoms post-deployment, relative to pre-deployment rates (5).

Perhaps most intriguing is the report of histologically-confirmed constrictive bronchiolitis (CB) in 38 of 49 previously-healthy soldiers--who returned from Iraq and Afghanistan--with unexplained dyspnea and inability to achieve the fitness standard for the 2-mile run that all had met prior to deployment (10). All subjects had normal chest x-rays, spirometry, diffusing capacity and lung volumes. While these data were within normal population ranges, they were, however, inferior compared to exercised soldiers from previously published data. Black pigment was seen on the visceral pleura in 37 of 38 at biopsy, and black peri-bronchiolar pigment containing polarizable (crystalline) material was observed contiguous to membranous bronchioles containing hypertrophic mural smooth muscle or fibrous thickening with luminal narrowing in the vast majority of lung biopsies (Figure 1) In cardiopulmonary exercise testing, these patients with constrictive bronchiolitis had mean levels of maximal oxygen consumption (MOC) and anaerobic thresholds (AT), which were at the lower limits of normal (MOC, 80% predicted; AT, 40% predicted MOC), but mean MOC ($85.1 \pm 15.2\%$ vs $105.4 \pm 14.3\%$ predicted value, $P < 0.001$) and AT ($45.0 \pm 9.5\%$ vs $78.2 \pm 15.3\%$ predicted value, $P < 0.001$) were significantly lower in these evaluated soldiers compared to military controls. Of possible pathophysiologic importance is the finding of marked intimal fibrosis and medial hypertrophy in pulmonary arteries adjacent to constricted bronchioles, despite no subjects being reported as hypoxemic. This raises the possibility that early attainment of AT, exertional dyspnea and limitation of exercise in these subjects were the result of pulmonary vascular remodeling, with elevated pulmonary pressure flow curves and limitation of cardiac output during exercise due to exertional pulmonary hypertension and adverse right ventricular effects on left ventricular compliance and performance (11).

Particulate Matter in Harsh Environmental Conditions in Iraq and Afghanistan:

The combat and support theatres in Iraq and Afghanistan posed some of the harshest environmental conditions endured by servicemen and women in decades. The distinguishing environmental factor of these hot, dry settings was the widespread exposure of personnel to airborne particulate matter (PM) (Figure 4). PM air pollution can be characterized by size as PM_{2.5} and PM₁₀. PM_{2.5} refers to particulate matter with a diameter smaller than 2.5 microns. PM_{2.5} is produced by combustion, including vehicle exhaust, and by chemical reactions between gases such as sulfur dioxide, nitrogen oxides, and volatile organic compounds (12). PM₁₀ is the abbreviation for fine particulate matter with a diameter smaller than 10 microns but larger than 2.5 microns (12). The prevalence of loose, unconsolidated sand, silt, and fine surface material, combined with frequent high winds, vehicular traffic, and a lack of extensive vegetative cover, resulted in airborne PM concentrations, which routinely exceeded US EPA and Military Exposure Guidelines (MEGs) (12,13). A study of PM at 15 locations in southwest Asia, including six locations in Iraq and two in Afghanistan, showed that long-term average PM_{2.5} concentrations exceeded the EPA and MEG values at all locations (14). The EPA's 150 $\mu\text{g}/\text{m}^3$ limit for PM₁₀ was exceeded at five of the six locations in Iraq, and the long-term average exceeded the 24-hr exposure MEG limit at three locations. These average PM conditions were greatly eclipsed

during severe PM and sandstorm events, which may have a frequency of 20–50 days/year in Iraq (15). Total suspended particle concentrations as high as 10,000 $\mu\text{g}/\text{m}^3$ have been measured for such events (16).

These ambient PM levels associated largely with re-suspension of surface material were compounded by other sources related to combat and support activities, including combustion, most notably at ‘burn pits’, and explosives. Much attention has been focused at the open-air burn pits that were widely used for waste disposal in the vicinity of personnel bases (17,18). In addition to dense smoke, emissions detected at burn pits included polycyclic aromatic hydrocarbons (PAH), volatile organic compounds (VOC), metals, and dioxins (18).

As cited above (*supra vide*), the role of PM inhalation in respiratory illness was underscored in the study by King *et al.*, showing crystalline particles in lung biopsy sections of returning Veterans (10). In a case report of a lung tissue biopsy sample from a soldier deployed in Iraq and diagnosed with nonspecific interstitial pneumonitis with peribronchiolar inflammation, synchrotron X-ray spectro-microscopy and diffraction (Figure 3) confirmed the presence of metal-bearing, crystalline particles in damaged tissue (6). This spectromicroscopy demonstrates that transition metals, including iron, titanium, zinc, and copper, are consistently associated with the particles observed in the alveoli. Some of this inhaled PM also likely contains associated microbial organisms that may contribute to lung injury, including viruses, bacteria, and allergens.

RuX:

We have developed a novel, anti-oxidant, ruthenium complex drug called RuX, which is a small peroxidase molecule with an active alpha-lipoic acid ingredient. Ruthenium complexes can be any suitable ruthenium compounds having the general formula:

(Me=metal made of organified palladium and ruthenium; Organified denotes the state of being bonded to an organyl group, in this case a charged arginine, by a coordination covalent bond.)

RuX is created when a bond between palladium and ruthenium is formed using zinc, alpha-lipoic acid, linoleic acid (fatty acid), formyl methionine (amino acid 1), and arginine (amino acid 2). The structure of RuX is as follows:

Me = Metal Made of Organified Palladium and Ruthenium

Zn = Zinc

C₈H₁₄O₂S₂ = alpha-Lipoic Acid

C₁₈H₃₂O₂ = Linoleic Acid

C₆H₁₁NO₃S = Formyl Methionine

C₆H₁₄N₄O₂ = Arginine

Ruthenium complexes have been published and identified in recent literature as anti-inflammatory and anti-cancer agents (19–25). RuX as an anti-inflammatory agent may be due to the higher coordination numbers of transition metals (ruthenium and palladium), allowing transition metal complexes to have more diverse molecular geometry, unknown to organic molecules created by carbon (coordination number 4) (26). RuX's geometric versatility can allow transition metal complexes to operate in chemical spaces unreachable by organic molecules and undergo unique reactions and mechanisms, potentially serving as therapeutic alternative mediator (26). An alternative explanation for RuX as an anti-inflammatory agent can be due to α -lipoic acid as an active ingredient (26,27). α -lipoic acid with its antioxidant property allows it to interact with and terminate free radicals, potentially serving as another therapeutic alternative mediator (26,27).

Hypothesis:

Based on the structure and pharmacology of specific compounds in RuX, we hypothesized that a remedial effective quantity can attenuate the pathology of mouse lungs injured from exposure to Camp Victory, Iraq PM. In this paper, we 1) confirm lung injury from exposure to Camp Victory, Iraq PM, 2) examine differences in histologic, physiologic, immunologic and genomic data from lungs of mice in different experimental groups, and 3) discuss possible anti-oxidant mechanisms of RuX.

METHODS:

Particulate Matter (PM) Characterization:

PM morphology was determined via a scanning electron microscope (SEM; LEO 1550 SFEG) with an EDAX energy dispersive X-ray spectrometer (EDS) using an accelerating voltage of 15 kV and a 30 μ m aperture. The composition and stability of surface grab samples from Camp Victory, Iraq, taken prior to 2011, was determined and includes minerals—chromium, magnesium, iron. The PM was largely insoluble. Surface grab samples were also taken from Fort Irwin, CA. Distinct respiratory illnesses have yet to be reported from servicemen deployed from this military base, which utilizes similar military vehicles to those in Camp Victory, Iraq, but have less harsh environmental conditions. So the PM entailed sample from this military base was used as a control.

PM analysis:

The contaminants used in all experiments originated near burn pits at Camp Victory, Iraq. The collection took place prior to 2013 and upon arrival to the United States the PM was subjected to UV radiation to rid the material of bacterial pathogens. The mineralogy of the PM was determined using a Scintag Pad X Diffractometer with a copper source ($\lambda = 1.54$

A) from 4 to 90 degrees 2 theta in continuous scan mode at 2 degrees 2theta/minute. X-ray fluorescence spectroscopy (Bruker S4 Pioneer) was used for elemental analysis. The specific surface area of the PM, determined with a QuantachromeNOVA5-point BET analyzer, was 26.429 m²/g. The PM morphology was determined on a scanning electron microscope (SEM; LEO 1550 SFEG) with an EDAX energy dispersive X-ray spectrometer (EDS) using an accelerating voltage of 15 kV and a 30 µm aperture (Figure 5).

Mice Experiments:

Two separate experiments have been conducted to elucidate the effects of RuX on mice lung injury due to Camp Victory, Iraq dust: experiment 1 (E1) and experiment 2 (E2). E1 spanned the course of four weeks, with a test group consisting of 24 weight-matched, two-month-old-male C57BL/6 mice (purchased from Taconic Laboratories, Germantown, NY). E2 spanned across three weeks, with a test group consisting of 13 weight-matched, two-month-old-female C57BL/6 mice (purchased from Taconic Laboratories, Germantown, NY). All experiments and animal care procedures are approved by the Institutional Animal Care and Use Committee (IACUC #239444) and conducted according to National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. (40).

In experiment 1 (E1), five, eight-week old age matched male, adult C57BL/6 mice were selected at random to serve as the control group, which only received water (treated animals received 5 µl of 1 g Camp Victory Iraq dust / 10 ml water) and no Camp Victory, Iraq PM. Ten male C57BL/6 were selected at random to serve as the experimental population group 1, which received the Camp Victory, Iraq PM and water (5 µl of 1 g / 10 ml water). Nine mice received Camp Victory, Iraq PM and RuX: four mice in low dosage (10-drop dose: 56.5 mL RuX dilution in 1443.5 mL of dH₂O), and five in high dosage (30 drop dose: 56.5 mL RuX dilution in 1443.5 mL of dH₂O).

In experiment 2 (E2), five, eight-week old age matched female C57BL/6 mice were selected at random to serve as the control group, which only received water (1 g / 10 ml water) and no Camp Victory, Iraq PM. Four female C57BL/6 were selected at random to serve as the experimental population group 1, which received the Camp Victory, Iraq PM and water (5 µl of dust at a concentration of 1 g / 10 ml water). Four other female C57BL/6 mice, received Camp Victory, Iraq PM and RuX (1:100 dilution on dH₂O).

All other variables remained constant for the duration of both experiments.

Orotracheal PM Instillation:

The mice were anesthetized using 0.1 mL of a 0.2mL ketamine/0.48 mL xylazine solution intraperitoneal using a 25 Ga needle and a 1 mL tuberculin syringe. Adequate anesthetization was established and confirmed by unresponsiveness to a foot-pinch pain stimuli test. Orotracheal instillation of 5 µl Camp Victory, Iraq PM (U.S. Army Corps of Engineers sample 2013) was administered after suspending the mice, vertically, by their teeth on a custom tray, with their nose pointed upwards, and using forceps to position the tongue away from the airway. A shutter-"gulp" type motion determined successful PM inhalation down the airway.

FlexiVent:

FlexiVent is a device used to measure respiratory and lung capacities using forced oscillation techniques after anesthetization. This system utilizes a piston pump that punctuates automated ventilation, controlled in volume and pressure, to ensure reliability and reproducibility. Flexivent is considered the standard for *in vivo* pulmonary measurements.

The mice were first anesthetized using 0.1 mL of a 0.2mL ketamine/0.48 mL xylazine solution intraperitoneal using a 25 Ga needle and a 1 mL tuberculin syringe. Once adequate anesthetization was established and confirmed by unresponsiveness to a foot-pinch pain stimuli test, we performed tracheostomy with a 22 Ga catheter inserted two tracheal rings below the larynx with a depth of approximately 5 tracheal rings. The catheter was secured using a 4'0 black silk suture and attached to the FlexiVent. Physiologic total lung capacity (TLC) is estimated using Deep Inflation perturbation, whereby lungs are slowly inflated from positive end-expiratory pressure to 30 cm H₂O.

Histology:

Hematoxylin and eosin staining was conducted on lungs that were inflated with formalin and embedded with paraffin. A pathologist, blinded to the experiment and the identities of the samples, scored the histological grading. The scale used to score ranged from 0, meaning no septate thickening, to 4, meaning prominent septate thickening.

Regulatory T-Cells (Tregs):

Spleens and thymuses were extracted for flow cytometric analysis.—For flow cytometry of thymus and spleen, we used cell surface markers CD25 and CD4 and FOXP3 (an intracellular activation marker critical for Treg survival) to identify T regulatory cells. We also used Helios antibody to determine which T cells originated from the central thymus, as opposed to the spleen, suggesting peripheral development, without Helios. FOXP3 intracellular antibody was used to determine viability of Treg, as it is critical for Treg survival.

Thymocytes and splenocytes from C57BL/6 mouse were stained with One Step Staining Mouse Treg Flow™ Kit (FOXP3 Alexa Fluor® 488/CD25 PE/CD4 PerCP) kit from Biolegend. This kit is composed of fluorochrome conjugated anti-mouse CD4, CD25 and FOXP3 antibodies and the critical buffers. It is a one-step staining kit for labeling both surface and intracellular antigens of Treg cells. (41–43)

Genomics Analysis:

Whole lungs were removed from each specimen one week after exposure to PM and immediately frozen in liquid nitrogen. These samples were next-day shipped overnight on 10 lbs. dry ice to QIAGEN for genetic analysis.

RNA was isolated using QIAGEN miRNEasy Mini Kit (Cat No. 217004). RNA concentration was determined by NanoDrop 2000. RNA quality check was performed using BioAnalyzer 2100; Reverse Transcription reaction was performed using RT2 First Strand Kit (Cat. No. 330401) using 750 ng RNA input per sample. Total RNA yield, 260/280, and

260/230 ratios were measured using a NanoDrop spectrophotometer (Thermo). RT-PCR was performed using the ABI 7900 in 384-well format using Mouse T-cell Anergy & Immune Tolerance PCR Array (Cat. No. PAMM-074Z) in combination with RT² SYBR® Green qPCR Mastermix (Cat. No. 330529). Ct values were normalized using the average of HKGs. Data were analyzed through the use of QIAGEN's Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity), where controls and test samples were designated as per the customer's description.

Statistics:

ANOVA with Dunnett's was calculated for $P < 0.05$ as statistically significant for three groups measured for inspiratory capacity: 1) control; 2) Iraq particulate exposed matter; 3) Iraq particulate exposed matter+RuX. ANOVA with Dunnett's was also calculated for histology scores among these groups.

Histological examination of lungs after inflation fixation in formalin, followed by hematoxylin and eosin (H&E) staining by a pathologist who was blinded to the identities of the samples was conducted. All abnormalities were graded 0, 1, 2, 3, or 4, based on the intensity and extent of peribronchiolar and perivascular cellular infiltration as well as septate thickening.

RESULTS:

Some of the following results will be denoted as E1 or E2 specific because the two experiments occurred under distinct time periods.

Particulate Matter (PM) Characterization:

PM morphology was determined on a scanning electron microscope (SEM; LEO 1550 SFEG) with an EDAX energy dispersive X-ray spectrometer (EDS) using an accelerating voltage of 15 kV and a 30 μ m aperture. The composition and stability of grab sample of surface material from Camp Victory, Iraq, taken prior to 2011, was determined and PM contains metals—including chromium, magnesium, iron. The PM was largely insoluble.

Histology:

E1 specific, according to Figure 12, airway inflammation was not present in the untreated control mice, which was seen in PM-treated mice. PM-treated plus RuX-exposed mice, using low and high doses of RuX, did not display any airway inflammation. Only high RuX dosage, PM-treated mice reached statistical significance. Figure 13 displays attenuation of histologic airway inflammation using RuX, low and high dosages. Figure 13 depicts the physiological effects of RuX, low and high dosage, on lung inflammation, a month after dust instillation. In either groups, lung histology depicts reduction of airway inflammation.

E2 specific, Table 3 and Figure 4 show septate thickening is highest in the untreated PM-exposed group. As seen in the center of figure 4, lung histology shows loss of airspace due to fibrosis. According to Table 3, RuX-treated groups have the lowest scores for septate thickening, despite PM exposure. In the control group ($n=5$), 4 of 5 mice did not have

septate thickening and showed normal lung histology, lacking inflammation. In the untreated PM-exposed group, not shown in the Figures, 2 of the 3 mice demonstrated septate thickening. 0 of 4 mice in the RuX-treated PM-exposed group presented with septate thickening. Table 3 shows the average scores of each group.

FlexiVent:

Table 1 depicts the pooled FlexiVent data from E1 and E2. Control mice showed normal lung volumes of 0.867 ± 0.181 mL. Both experimental group 1 receiving PM, untreated mice, and experimental group 2, RuX-treated, showed diminished lung volumes. However, RuX-treated PM-exposed mice had improved lung volumes 0.482 ± 0.010 mL compared to untreated PM exposed mice 0.312 ± 0.083 mL.

Tregs:

E1 specific, Treg were highest in spleens of control mice compared to PM-exposed mice at 2h and were the lowest in PM-exposed mice at 2 weeks. Both low dose RuX and high dose RuX increase Treg numbers in spleen, with the latter having a more pronounced effect (Figure 6). In the thymus, T reg increased at 2 h and 1 week in PM-exposed mice compared to control mice, with enhanced Treg numbers associated with RuX therapy, though the low dose appeared to have a more prominent effect than high dose (Figure 7).

E2 specific, overall PM-treated mice displayed lower Treg count at 1,826 as opposed to the non-PM-treated, control mice at 5,645 (Figures 10 and 11).

Genomics Analysis:

Fold-Change ($2^{(-C_t - C_t)}$) is the normalized gene expression ($2^{(-C_t)}$) in the Test Sample divided the normalized gene expression ($2^{(-C_t)}$) in the Control Sample. Fold-Regulation depicts fold-change results in a biologically meaningful way. Fold-change values greater than one (>1) indicate a positive, or 'up-regulation,' and the fold-regulation is equal to the fold-change. Fold-change values less than one (<1) indicates a negative, or 'down-regulation,' and the fold-regulation is the negative inverse of the fold-change. Fold-change and fold-regulation values greater than two (>2) are indicated in red; and fold-change values less than one-half (<0.5) and negative two (<-2) are indicated in blue.

E2 specific, Table 2 depicts the variations in gene expression among the three lung groups: control, experimental group 1 (PM-exposed) and experimental group 2 (PM-exposed + RuX-treated). As shown, PM-exposed mice had significantly up-regulated gene expression for CDK4. PM-exposed and RuX-treated mice also had significantly up-regulated gene expression of CDK4, but less than untreated mice. IL-6, Lep and PDCD1 are down regulated in PM-exposed mice. Lep and PDCD1 are less significantly in RuX-treated mice. IL-6 is down-regulated even further in RuX-treated mice than untreated. IL-10, EGR2 and IL-15 expression is not appreciably altered in PM-exposed mice in comparison to the control group however they are down-regulated 2, 5 and 9 fold in RuX-treated mice.

Statistics (Figures 8 & 9):

The histological score for the blind testing, both spleen (figure 8) and thymus (figure 9) were subjected to Kruskal-Wallis and Dunn's Multiple Comparison tests were used to determine if there were differences among the three groups: 1) control; 2) Iraq particulate exposed matter; 3) Iraq particulate exposed matter+RuX. An alpha-level of $<.05$ was set as the bar to determine statistical significance of the histology scores. According to the figures, Kruskal-Wallis reached significance both times and the ANOVA determined the group of significance (indicated in red).

DISCUSSION:

Our Iraq dust mouse model is similar to histopathology seen in soldiers exposed to Iraq dust since both sets of lung tissue demonstrate polarizable crystals in the airways and alveoli and metals such as titanium have been found in mice and man.

STATA output showed significant ANOVA results for one variable (alveolar wall thickening) and a significant trend for dust in bronchi. We were able to detect not only in the respiratory bronchioles but also distally in the alveolar walls. Carbonaceous particles were detectable and these particulates are polarizable, supporting the fact that surface grab samples from Camp Victory Iraq in 2003 contained burned particles.

In our earlier work in the **JOEM**, we reported a plausible mechanism of inhalational Iraq dust via upregulation of the lymphocyte chemoattractant and growth factor IL-2, which is consistent with the localization of lymphocytes in areas of dust injury as well as resulting fibrosis.

This current paper shows a mechanism of suppression of specific dose-dependent protective regulatory T cells (Tregs) by dust and a mechanism of action associated with increased Tregs with administration of the candidate drug RuX.

Limitations of the studies include the sample which was taken from one base, Camp Victory, Iraq, as a grab sample in 2013 at one time point. The intratracheal route is different from aerosolization. A plausible mechanism related to genomics is CDK4 differential expression comparing control vs. dust exposed and IL-15 gene expression with RuX exposure.

RuX is a novel, peroxidase, anti-oxidant drug, with ruthenium and α -lipoic acid as active ingredients. Compounds derived from these ingredients may have a remedial anti-inflammatory effect through the termination of oxygen free radicals. The results of this study strongly suggests that RuX, when administered orally, as a concentration of 0.113 mg/kg by mouse water bottle after Iraq PM instillation, greatly reduces histologic development of lung injury and attenuates the abnormal respiratory physiology (Figure 4).

Due to ruthenium's geometric versatility, it could behave as potential substitutes for organic molecules with anti-inflammatory properties, depending on the attached ligand (26). Ruthenium complexes with ligands have been published to induce anti-nociceptive and anti-inflammatory activity. Free ligands themselves, such as isonicotinic and nicotinic acids, can be inactive. Ruthenium ligand complexes- $[\text{Ru}-(\text{NH}_3)_5(\text{ina})](\text{BF}_4)_2$, trans- $[\text{Ru}(\text{NO})_+]$

(NH₃)₄(ina)](BF₄)₃, [Ru- (NH₃)₅(Hnic)](BF₄)₂], and trans-[Ru(NO⁺)(NH₃)₄(Hnic)](BF₄)₃- all display anti-inflammatory activity, not seen in noncoordinated, free ligands. Ligand coordination with transition metals forms transition metal complexes, in this case ruthenium, activating pathways to release NO, enhancing analgesic and anti-inflammatory activity. Though the structures of these ruthenium complexes differ from RuX, the non-activity of ligands without the coordination of ruthenium suggests its association to anti-inflammatory properties.

One potential free ligand and commonly employed dietary supplement, α-lipoic acid (LA), is well characterized chemically and demonstrates a number of antioxidant activities, both direct and indirect. Among these are: direct scavenging of hydroxyl radicals and hypochlorous acid and complexation of redox-active metals. (29) LA activates Nrf-2-dependent transcriptional activity, thereby upregulating expression of a plethora of antioxidant defense mechanisms through activation of the antioxidant response element (ARE). (29,30) LA also activates the anti-aging histone deacetylase sirtuin 1 (SIRT1). (31,32) LA has been shown to prevent asthmatic airways remodeling in mice (33,34) and bleomycin-induced lung fibrosis in rats. (35) Most importantly for translational application of our findings, LA is already available for use in human diseases, and in doses of 600 to 1800 mg per day has been shown to significantly improve symptoms in human patients with diabetic neuropathy (29,36,37–39). Thus, if effective in mouse models, LA might be proposed as a readily available therapy for veterans with CB.

The present study includes several limitations. Because RuX is a novel substance (patent is pending), the full extent of its mechanism of action and biological pathways has yet to be determined. The side effects of this compound are unknown, though no adverse effects have been reported to the FDA with its use as a supplement for over five years.

RuX may have broad scientific applicability to treat other etiologic mechanisms of lung fibrosis as well as other forms of organ scarring and wound healing. Since there are currently no available therapies for soldiers affected with new-onset respiratory illnesses post-deployment to the Middle East, RuX may be an excellent drug candidate.

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Figure 1.

Constrictive bronchiolitis, arteriopathy and peribronchial pigment deposition in an Iraq veteran. This H&E section from a patient in ref 6 shows airway smooth muscle hypertrophy (black arrow) with marked intimal fibrosis and medial hypertrophy of adjacent pulmonary artery (white arrow) and peribronchilar pigment depositon (arrow-head). From King et al., 2011.(King et al., 2011)

Figure 2. Seabees assigned to Naval Mobile Construction Battalion (NMCB) 5 work through a sand storm at the Barley Company Pre-Fabrication Yard at Camp Natasha, Helmand Province, Afghanistan, May 15, 2009.

Photo by Mass Communication Specialist 1st Class E. Rosario. This image was released by the United States Navy.http://commons.wikime-dia.org/wiki/File:US_Navy_090518-N-9410R018_Seabees_as-signed_to_Naval_Mobile_Construction_Battalion_%28NMCB%29_5_work_through_a_sand_storm_at_the_Barley_Company_PreFabrication_Yard_at_Camp_Natasha.jpg

Figure 3. Lung tissue section (left) and corresponding synchrotron X-ray map (right), showing elemental associations. 'Hot spots' correspond to metal-containing crystalline particles embedded in alveolar tissue. From Szema et al. (ref. 16).

Figure 4.

(Left) Lung from female C57BL/6 2-month-old untreated control mouse (40X magnification) with normal histology (H&E stain). (Center) Lung from 2-month-old female C57BL/6 mouse 3 weeks after 50 microliters orotracheal instillation of Camp Victory Iraq PM 0.00 mg/ml. (40X magnification, H&E stain). There is prominent septal thickening, inflammation and loss of airspaces. (Right) Lung from 2-month-old female C57BL/6 mouse 3 weeks after 50 microliters orotracheal instillation of Camp Victory Iraq PM 0.001 mg/ml. RuX was dosed by adding it in the water bottle immediately after orotracheal instillation of PM (40X magnification, H&E stain). The lung is histologically near normal despite exposure to PM.

Figure 5a.

PM morphology was determined on a scanning electron microscope (SEM; LEO 1550 SFEG) with an EDAX energy dispersive X-ray spectrometer (EDS) using an accelerating voltage of 15 kV and a 30 µm aperture. The composition and stability of surface grab samples from Camp Victory, Iraq, taken prior to 2011, was determined and includes metals—chromium, magnesium, iron. The PM was found to be insoluble. (Szema *et al.* JOEM 2014)

Figure 5b.
x ray diffraction scans

Figures 6:

Mean counts of CD4+CD25+ regulatory T cells in spleen and thymus increased with both low and high dose RuX treatment, compared to 2 h PM. For spleen 2h PM averaged 1529, whereas low RuX was 3754 and high RuX was 4110. For thymus, 2h PM was 862, while, low-dose RuX was 2663 and high dose was 1482.

Figures 7:

Mean counts of CD4+CD25+ regulatory T cells in spleen and thymus increased with both low and high dose RuX treatment, compared to 2 h PM. For spleen 2h PM averaged 1529, whereas low RuX was 3754 and high RuX was 4110. For thymus, 2h PM was 862, while, low-dose RuX was 2663 and high dose was 1482.

Kruskal-Wallis test

P value: 0.0129

Dunn's Multiple Comparison**P value Summary**

Control vs 2 hr dust

P > 0.05ns

Control vs 1 week dust

P > 0.05ns

Control vs Low Rux

P > 0.05ns

Control vs High Rux

P > 0.05ns

2 hr dust vs 1 week dust

P > 0.05ns

2 hr dust vs Low Rux

P > 0.05ns

2 hr dust vs High Rux

P > 0.05ns

1 week dust vs Low Rux

P > 0.05ns

1 week dust vs High Rux

P < 0.05*

Low Rux vs High Rux

P > 0.05ns

Figures 8:

Kruskal-Wallis and Dunn's Multiple Comparison tests to determine significance.

Kruskal-Wallis test

P value 0.0429

Dunn's Multiple Comparison Test

P value Summary

Control vs 2 hr dust

P > 0.05ns

Control vs 1 week dust

P > 0.05ns

Control vs Low Rux

P < 0.05*

Control vs High Rux

P > 0.05ns

2 hr dust vs 1 week dust

P > 0.05ns

2 hr dust vs Low Rux

P > 0.05ns

2 hr dust vs High Rux

P > 0.05ns

1 week dust vs Low Rux

P > 0.05ns

1 week dust vs High Rux

P > 0.05ns

Low Rux vs High Rux

P > 0.05ns

Figures 9:

Kruskal-Wallis and Dunn's Multiple Comparison tests to determine significance.

Figures 10: Tregs = 5645 in control mice.

Example of Tregs are at baseline high levels of 5645 in the control mice above, whereas another mouse treated with PM (below) after 2 hours has suppression of Tregs to 1826.

Figure 11: Tregs = 1826 in PM-treated mice.

Example of Tregs are at baseline high levels of 5645 in the control mice above, whereas another mouse treated with PM (below) after 2 hours has suppression of Tregs to 1826.

Control					
Mouse#	Sex	Lung			Comments Figure
		Peribronchiolar Inflammation	Perivascular Inflammation	Vascular Thickening	
1	M	0	0	0	
2	M	0	0	0	(2+) Focal Central inflammation, not localized
3	M	0	0	0	
4	M	0	0	0	
5	M	0	0	0	
Mean ± SEM		0 ± 0.0	0 ± 0.0	0 ± 0.0	
Dust					
1	M	1	0	0	Intra-alveolar and intra-bronchiolar dust
2	M	2	0	0	Intra-alveolar and intra-bronchiolar dust
3	M	3	0	0	Intra-alveolar and intra-bronchiolar dust
4	M	1	0	0	
5	M	0	0	0	
6	M	1	0	0	Intra-bronchiolar dust
7	M	0	0	0	
8	M	2	0	0	Intra-bronchiolar dust
9	M	0	0	0	
10	M	1	0	0	
Mean ± SEM		1.10 ± 0.31	0 ± 0.0	0 ± 0.0	
Dust + Low Rux					
Mouse#	Sex	Lung			Comments
		Peribronchiolar Inflammation	Perivascular Inflammation	Vascular Thickening	
1	M	0			Dust (intra-bronchiolar)
2	M	0			Dust (intra-bronchiolar)
3	M	0			Dust (intra-bronchiolar)
4	M	0			Dust (intra-bronchiolar)
Mean ± SEM		0 ± 0.0	0 ± 0.0	0 ± 0.0	
Dust + High Rux					
1	M	0			Dust (intra-bronchiolar)
2	M	0			Dust (intra-bronchiolar)
3	M	0			Dust (intra-bronchiolar)
4	M	0			Dust (intra-bronchiolar)
5	M	0			Dust (intra-bronchiolar)
Mean ± SEM		0 ± 0.0	0 ± 0.0	0 ± 0.0	

Figure 12:

Airway inflammation was not present in untreated C57BL/6 control mice (N=5), scored as 1.100.31 in PM-treated mice (N=10), some with intra-alveolar and intra-bronchiolar PM, vs. scores of zero for low-dose and high-dose RuX, N=4 and N=5, respectively. Due to sample size, only the high-dose RuX achieved statistical significance with both non-parametric and parametric testing.

Figure 13:
Pre-treatment of drinkable RuX in mice water bottles daily for 1 month prior to PM instillation attenuates histologic airway inflammation from Iraq burn pit PM at low and high doses.

Figure 14:
Differential expression of gene CDK4 comparing control vs. dust exposed mouse lungs.
Reduction in IL-15 gene expression with RuX.

Table 1:**FlexiVent**

Inspiratory Capacity (mL) in Unexposed, Iraq-Particulate Exposed and Exposed, Peroxidase-Treated Mice

Control	0.86 ± 0.18
Iraq Particulate Exposed Mice	$0.31 \pm 0.08^*$
Iraq-Particulate Exposed Mice + RUX	0.48 ± 0.01

*
P < .05 vs control mice not exposed to particulates (ANOVA with Dunnett's)

Table 2:

Gene Fold Regulation among Control vs. PM, Control vs. PM & RuX, PM vs. PM & RuX

	Up-Down Regulation (comparing to Untreated)		Fold Change (comparing to PM)
	PM	PM & RuX	PM & RuX
CDK4	11.1	9.9	-1.1
EGR2	-1.1	-5.0	-4.50
IL-10	-1.1	-2.3	-2.04
IL-15	1.0	-9.0	-9.7
IL-6	-2.4	-3.0	-1.3
Lep	-5.5	-2.6	2.1
PDCD1	-2.5	-1.4	1.7

Table 3:

Group Averages for Blinded Histology Score

	Control vs. PM	Control vs. PM+RUX	PM vs. PM+RUX
CDk4	11.1	9.9	-1.1
Egr2	-1.1	-5.0	-4.5
IL-10	-1.1	-2.3	-2.04
IL-15	1.0	-9.0	-9.7
IL-6	-2.4	-3.0	-1.3
Lep	-5.5	-2.6	2.1
Pdcd1	-2.5	-1.4	1.7