ARSENIC COMPROMISES THE ALVEOLOCAPILLARY BARRIER, FACILITATING BACTEREMIA IN A MOUSE PNEUMONIA MODEL

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ABSTRACT

Michael Wayne Henderson: Arsenic compromises the alveolocapillary barrier, facilitating bacteremia in a mouse pneumonia model
(Under the direction of: Mike B. Fessler)

The greatest threat to public health regarding arsenic exposure is consumption of contaminated drinking water. Chronic oral consumption of inorganic arsenic has been linked with respiratory dysfunction as well as immunotoxicity. Here we examined the effects of consuming either of two concentrations of sodium arsenite (250 ppb, 25 ppm) via drinking water in a mouse model of bacterial pneumonia. Arsenic exposure did not alter recruitment of leukocytes to the airway upon bacterial infection, nor pulmonary clearance of bacteria, but was associated with increased dissemination to extrapulmonary tissues. Mice exposed to arsenic had intact bloodstream killing of bacteria, suggesting that the increased extrapulmonary bacterial burden in lung-infected mice derived from increased escape from the lung. Analysis of early events in lung-infected mice revealed that arsenic led to accelerated escape of bacteria from the alveoli, providing new evidence that chronic exposure to arsenic-contaminated drinking water disrupts the architecture of the alveoli, permitting pathogen escape into the bloodstream.

ACKNOWLEDGEMENTS

All of my successes, past, present, and future, would not have been possible without the support of trusted friends and colleagues. First, thanks to Drs. Roland Tisch and Peggy Cotter for giving me the time to develop myself as a scientist, and supporting all my endeavors. I appreciate all of the discussion offered by Drs. Mirek Stýblo, David Thomas, Erik Tokar, and Alex Merrick and the sharing of their vast expertise in the field of arsenic toxicity. Laura Miller, Greg Whitehead, Deborah King, and Jennifer Madenspacher deserve significant praise for all of the experimental assistance that they offered to me. Thanks to my dear friends Drs. Robert Immormino, Peter Thompson, and Keith Miller for their boundless support. Lastly, my unending gratitude to Dr. Michael Fessler, who has supported me at the sacrifice of many hours of his own life, which is something I can only hope to repay.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANOVA	analysis of variance
APC	allophycocyanin
AsIII	arsenite
AsV	arsenate
BALF	bronchoalveolar lavage fluid
BMDM	bone marrow-derived macrophages
CBC	complete blood count
CC16	Club cell secretory protein
CFU	colony forming units
CXCL	chemokine (CXC-motif)
DMAIII	dimethylarsinous acid
DMAV	dimethylarsinic acid / cacodylic acid
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
DTT	dithioreitol
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
EPA	Environmental Protection Agency
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GMCSF	granulocyte macrophage colony-stimulating factor
IgM	immunoglobulin M

IHC	immunohistochemistry
IL	interleukin
iNOS	nitric oxide synthase (inducible)
IP	intraperitoneal
IT	intratracheal
IV	intravenous
LPS	lipopolysaccharide
MMAIII	monomethylarsonous acid
MMP9	matrix metallopeptidase 9
mRNA	messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NIEHS	National Institute of Environmental Health Sciences
NLRP3	NOD-like receptor family, pyrin domain containing 3
NO	nitric oxide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
TEM	transmission electron microscopy
TGFβ	transforming growth factor beta
TLR	toll-like receptor
TNFα	tumor necrosis factor alpha
TSA	tryptic soy agar
WHO	World Health Organization
γH2AX	H2A histone family, member X; phosphorylated serine 139

CHAPTER 1: INTRODUCTION

Arsenic is a naturally occurring metalloid, widely dispersed within the earth's crust and sediment. Many species of arsenicals exist in the environment and are classified as inorganic or organic depending on the combined elements. The main forms of arsenic relevant for human exposure are the inorganic pentavalent (arsenate AsV) and trivalent (arsenite AsIII) species. Humans are exposed to anthropogenic and natural sources through inhalation or through ingestion, likely the predominant route globally. Inhalation toxicity is usually caused by inhaling the manufactured species, arsenic trioxide, which is used in many industrial settings including coal combustion, glass manufacturing, smelters, and pesticide manufacturing facilities. Additionally, arsenic trioxide can be incidentally ingested by consuming runoff from wood-preserving facilities or from pesticide-treated crops. However, most ingestion exposures worldwide are from consumption of drinking water from areas with naturally high levels of arsenic present in nearby rocks or soil. Inorganic arsenic is readily absorbed from the gastrointestinal tract [1], and undergoes a series of reductive and oxidative changes as well as methylation before ultimately being metabolized to dimethlyarsenate (DMAV) [2-5]. The intermediate metabolites methylarsonous acid (MMAIII) and dimethylarsinous acid (DMAIII) have been demonstrated to be more toxic than their inorganic precursors [6].

In one of the most tragic ironies, the World Health Organization (WHO) and the World Bank installed groundwater wells in low income countries within the Indochina Peninsula as a measure of combatting death due to diarrheal diseases resulting from consumption of fetid surface water. These populations were inadvertently exposed to toxic levels of arsenic in their

drinking water due to contamination from the natural deposits within the earth [7-9]. Reports from 1995 [10, 11] enlightened the world that these populations were exposed to toxic levels of arsenic and described the disease state of the affected individuals, prompting several epidemiologic investigations that today serve as the predominant source for our understanding of patterns of arsenic-induced disease in humans. Prolonged consumption of arsenic-contaminated drinking water in humans has been demonstrated to cause arsenical dermatosis (hyperpigmentation and scaly keratosis), skin cancer, bladder cancer, urinary tract cancer, and kidney cancer [12-19]. Arsenic was recognized as a carcinogen in 1980, and then formally classified as a Group 1 carcinogen by IARC in 2004/05 [20, 21]. Epidemiological data has also demonstrated a link between consumption of arsenic contaminated drinking water and vascular fibrosis, endothelial damage and leak [22-24], immune suppression [25-32], and bone marrow depletion [33-36]. Recent literature has also identified important associations of arsenic exposure in humans with chronic structural lung disease, including pulmonary fibrosis, bronchiectasis, and reduced lung function [37-39]. This is of particular interest given that it has been demonstrated in rodent studies of chronic oral arsenic exposure that the lung is second only to liver as the tissue that retains the greatest levels of arsenic [5, 40, 41], in particular dimethylated species [5, 42].

In addition to its reported structural and functional effects on mucosal organs such as the lung, arsenic has been demonstrated to suppress immunity. Reports indicate reduced IgM and IgG producing plasma cells, inhibition of clonal T cell expansion and macrophage activity, and decreases in CD4+ splenic T cells and IL-2 [18, 43-46]. Suggesting that arsenic might compromise pulmonary immunity in particular, a recent report from Chile [47] provided epidemiologic evidence that individuals within a demarcated region who consumed arsenic-contaminated drinking water over a period of ~14 years were twice as likely to die from tuberculosis (TB). In addition to this, another recent report corroborated that oral arsenic

ingestion compromises pulmonary host defense against influenza A virus in mice [48]. Despite these preliminary reports, however, most of the scant literature to date that has investigated potential roles for arsenic in immunity has used in vitro cell line exposures of uncertain physiologic significance or inhalational exposures to arsenic trioxide [49], a route and arsenic species of uncertain relevance to predominant global arsenic exposure.

In this report, our objective was to test whether inorganic arsenic exposure via drinking water impacts pulmonary host defense against common bacterial pathogens that cause community-acquired pneumonia, using a mouse model and the predominant human exposure route. Our findings indicate that a 5 week exposure to arsenite does not substantially affect the pulmonary or circulating leukocyte repertoire, or the intrapulmonary innate immune response, including bacterial burden within the lung. However, infection in arsenic-exposed mice results in rapid bacterial dissemination, and our data suggest that host defense functions of extrapulmonary macrophages are sensitive to the effects of arsenic toxicity. We identified the likely cause of increased and accelerated bacteremia to be a deficiency in the alveolocapillary barrier that compromises pathogen compartmentalization.

CHAPTER 2: METHODS

Reagents

Sodium arsenite, (S7400) and cacodylic acid (DMAV) (C0125) were purchased from Sigma (St. Louis MO). Lipopolysaccharide (LPS)(#421) for *in vivo* use was purchased from List biologicals (Campbell CA), and *Escherichia coli* 0111:B4 LPS (L2630) (Sigma) was used for all *in vitro* experiments. Dulbecco's modified eagle medium (DMEM)(11320-033) and phosphate buffered saline (PBS)(100-10049) was purchased from Gibco (Gaithersburg MD). Fetal bovine serum (FBS) (S11150, Atlanta Biologicals, Flowery Branch GA) was used in all cell culture or *ex vivo* macrophage studies. Baxter saline (BHL2F7124) was used as the delivery vehicle for all bacterial inoculations.

Animals and arsenic dosing

Female 7-8 week old and weighing 18–22g C57Bl/6 mice were obtained from the Jackson Laboratory and used in accordance with the Animal Welfare Act and the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals after review by the Animal Care and Use Committee of the NIEHS. Four liter volumes of drinking water were prepared with 250ug/L (ppb) or 25mg/L (ppm) sodium arsenite freshly every two weeks for consumption *ad libitum*. Mice consumed 2-5mL of arsenic-containing water (or vehicle control) daily for 5-6 weeks prior to experimentation. Body weight, food consumption, and behavior were evaluated every 5 days. For the results indicated in Supplementary Figure 1, AIN 93 (D10012M) diet was purchased from Research Diets Inc. (New Brunswick NJ). These animals consumed the special diet for 2 weeks prior to, and throughout, the 5 week treatment.

In vivo bacterial exposures

Klebsiella pneumonia (2000 CFU) (ATCC 43816) or *Streptococcus pneumonia* (6.5-9 x10^5 CFU); (ATCC 6303) were delivered to the lung via oropharyngeal aspiration following anesthesia using 4% isoflurane at 1.5L/min for experiments evaluating pathogenesis and dissemination. For analysis of systemic bacterial clearance, 3 doses of *Klebsiella pneumoniae* (960, 6200, or 92000 CFU) were injected intravenously, and tissues were collected 6 or 18 hours later. Spleen homogenate and whole blood were serially diluted and plated onto tryptic soy agar (TSA) plates for *Klebsiella pneumoniae* or TSA with 5% sheep's blood for *Streptococcus pneumoniae* and incubated overnight for bacterial quantification.

Immunoblotting

To evaluate markers of epithelial injury, bronchoalveolar lavage fluid (BALF) was collected and prepared as described in McElroy Dobbs (cite), with minimal modifications. In brief, 400ul of BALF samples were centrifuged at 100,000g for 2 hours at 4°C and the pellet was resuspended in 65ul Laemmli Sample buffer with 50mM DTT as the reducing agent, and boiled for 8 minutes at 100°C. Samples were run on a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel, using prestained standards (1610374, BioRad, Hercules CA) for validation of molecular weight. Proteins were transferred to a nitrocellulose membrane using transfer buffer with 20% methanol. Antibody (AF3244, R&D Systems, Minneapolis MN) was used at .3ug/ml to detect mouse T1alpha /podoplanin. Detection was performed with a secondary antibody labeled at 800nm (925-32214, LiCor, Lincoln NE) and measured using an Odyssey Fc (LiCor). Quantification was performed using ImageStudio 5.0 (LiCor).

Primary macrophage harvest

Bone marrow-derived macrophages were prepared by culturing cells harvested from the femur and tibia of C57BL/6 mice in DMEM supplemented with 10% FBS. L929 cell supernatants

(in DMEM) were used as a source of growth factors and were added to the media at 25% of the total volume. Macrophages were cultured for 7 days at 37°C, humidified, with 5% CO_2, with fresh media being added every 3 days; adherent cells were retained and enumerated prior to seeding for experimentation. Peritoneal macrophages were collected via peritoneal lavage 96 hours after elicitation via injection of 2ml of 4% Brewer's thioglycollate. Macrophages were counted, plated at $7.5e^5$ cells per well in DMEM + 10% FBS, and allowed to settle overnight prior to experimentation. Bone marrow derived macrophages were exposed to arsenite or DMAV *ex vivo* for 8 hours prior to additional treatments, unless otherwise noted. MTT assay (CT02, Millipore, Billerica MA) was performed according to the manufacturer's recommendations following arsenic intoxication. For experiments analyzing gene expression, arsenic-treated macrophages were stimulated with 10ng/ml LPS (Sigma) for 2 hours in the presence of arsenic before mRNA was collected. A nitric oxide quantitation kit (40020, Active Motif, Carlsbad CA) was used to detect nitrate production in bone marrow derived macrophages following arsenic exposure and 24 hour stimulation using 1ng/ml LPS (Sigma).

LPS exposures

To assess early cytokine response, and neutrophil recruitment, mice were exposed to 300ug/ml of aerosolized *Escherichia coli* 0111:B4 LPS (List Biologicals, Campbell CA) for 30 minutes using a custom nebulizer chamber. BALF was collected immediately after euthanasia in volumes calibrated to overall body weight. BALF was centrifuged to pellet cells, and supernatant was collected and frozen at -80°C prior to analysis. Cell count and differential calculation was performed using Wright's stain as described in Draper et al (ref). Lavage supernatant was analyzed via ELISA immunoassay for IgM (E90-101) and albumin (E90-134) (Bethyl) as markers of alveolocapillary barrier integrity and cytokines were measured via multiplex assay (M600009RDPD, BioRad).

Fluorescent assisted cell sort and quantitative microarray of respiratory epithelium

Lungs are digested with porcine elastase (4.5U; E7885, Roche Diagnostics, St. Louis MO) and 100 U/ml DNase I (79254, Qiagen, Valencia CA). Cells in suspension are then filtered and sorted by FACS. The following antibodies were used: Pacific Blue/V450 streptavidin-conjugated (560797, BD Biosciences, San Jose CA) secondary antibody was used to label biotinylated CD45 (553078, BD), CD31 (558737, BD), and CD34 (Lin)(119304, BioLegend, San Diego CA) expressing cells; APC-conjugated rat anti-mouse EpCam (17-5791-82, eBioscience, San Diego CA) and 7-aminoactinomycin D (A1310, Invitrogen, Grand Island NY). Cell isolation was performed using a FACS Aria II (Becton Dickinson, Franklin Lakes NJ) cell sorter for epithelial cells (Lin-EpCam+). Collected cells are stored in TRIzol (15596-018 Invitrogen) for RNA isolation.

FITC –dextran permeability analysis

Disruption of alveolocapillary barrier was evaluated by measuring leakage of FITC-dextran (D3305, Life Technologies, Grand Island NY) from the airways into the bloodstream. FITC –dextran (3000MW) was dissolved into sterile saline and was introduced to the airspace through oropharyngeal aspiration (55ul, 5mg/kg) following completion of the arsenic-water exposure regime. Mice were allowed to recover for 1 hour prior to euthanasia via sodium pentobarbital. Blood (400ul) was collected by cardiac puncture into an ETDA-containing plasma separating column (365985, BD). Serial dilutions of plasma were loaded into a 96 well plate and analyzed for fluorescence intensity (FI) using a BioTek Synergy (Winooski VT) plate reader and 485/528nm filter.

Electron Microscopy

Lungs were fixed with 4% paraformaldehyde at 25cm H2O hydrostatic pressure and fixed for 24 hours as described in [50]. Left lung lobes were sectioned longitudinally at 1-2mm intervals and submitted in cassettes to the electron microscopy (EM) laboratory for transmission electron microscopy (TEM) processing and evaluation. Each lung sample was removed from the cassettes and further trimmed into 1mm3 cubes then processed in the automatic Leica® Tissue Processor using the routine TEM program. Following processing, the samples were embedded into six resin blocks. Each resin block was trimmed and sectioned at 700-800nm then stained with 1% Toluidine blue with subsequent examination by light microscopy. Based on the Toluidine blue-sections, two blocks for each animal were selected for thin sectioning (a section ~70 to 90 nm or "gold"). The thin sections were placed on a 100-mesh Formvar copper grid then stained with uranyl acetate and lead citrate. The grids were examined on a FEI Tecnai 120KV (Hillsboro OR) transmission electron microscope. Digital photomicrographs of each mouse were taken and evaluated.

RNA isolation and qPCR

RNA was isolated by RNEasy kit (74104, Qiagen). Complementary DNAs (cDNA) were generated from 500 ng of purified RNA using TaqMan reverse transcription reagents from Applied Biosystems (Foster City, CA). Real-time PCR was performed in triplicate with Taqman PCR Mix (Applied Biosystems) in the HT7900 ABI sequence Detection System (Applied Biosystems). Predesigned primers for Cxcl10 (Mm00445235_m1), IL-1β (Mm00434228_m1), TNFα (Mm00443258_m1), and iNOS (Mm00440502_m1) were purchased from Applied Biosystems. Gene expression was normalized to GAPDH (Mm99999915_g1) and expression levels in untreated control samples were set as a value of 1.0.

Inflammasome induction

Bone-marrow derived macrophages were exposed to arsenite at concentrations from 0-2μM for 4 hours prior to and during 4 hours of exposure to 10ng/ml LPS. Following this, cells were washed once with phosphate buffered saline and fresh media ± inflammasome trigger (ATP- 1191, Calbiochem, Billerica MA; Nigericin- N7143, Sigma) was given for 1 hour. Supernatants were collected for ELISA analysis; values were normalized to total cellular protein.

Statistical Analysis

Statistical analysis was performed using Prism 6.0 software from Graphpad Software, Inc. Statistical significance was determined using the unpaired student's *t*- test, analysis of variance (ANOVA), or Mantel-Cox log-rank analysis. Significance indicated as: *=*P* 0.01 - 0.05, **=*P* 0.01 - 0.001, ***=*P* 0.001 - 0.0001, ****=*P* <0.0001. Post- test for linear trend (Figure 3B) was used to evaluate dose-dependent effects of arsenic toxicity; significance in this study indicated as † *P*= <0.001.

Works in progress:

Cytokine analysis

TNFα, IL-6, IL-10 ELISAs were purchased from BioLegend; MMP9, IL-1β, TGF-β, and GM-CSF ELISAs were from R&D Systems. All were used according to manufacturer's instructions. Bio-Plex Pro mouse 23-plex assay (M600009RDPD, BioRad) was used to evaluate cytokine production in lavage fluid.

Immunohistochemistry

Formalin-fixed, paraffin-embedded mouse tissues were deparaffinized and rehydrated. Antigen retrieval was performed with heat and pressure in a decloaking chamber, using 10 mM

pH 6.0 1X Antigen Decloaker citrate buffer retrieval solution (Biocare Medical, Concord, CA). Endogenous peroxidase was blocked with 3% hydrogen peroxide. The sections were incubated with 10% normal donkey serum (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) for 20 minutes, followed by the avidin-biotin blocking kit (Vector Laboratories, Burlingame, CA). The sections were incubated with Caspase-3 (Catalog#CP229C, Lot#102312, Biocare Medical, Concord, CA) polyclonal primary antibody and an equivalent dilution of normal rabbit IgG (negative control; Jackson Immunoresearch Laboratories, Inc., West Grove, PA) for 60 minutes at 1:100 dilution. Sections were incubated with biotin-sp-conjugated donkey anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA for 30 minutes at 1:500 dilution and Vectastain Elite ABC reagent, RTU for 30 minutes. Antigen-antibody complex was visualized using DAB (Dako Corp., Carpinteria, CA) for 6 minutes. The sections were counterstained with hematoxylin, dehydrated, cleared and coverslipped.

Transepithelial electrical impedance assay

A549 lung epithelial cells were cultured to confluency onto proprietary gold-plated chamber slides (8W10E+; Applied Biophysics). Monolayer permeability was measured using an electrical substrate impedance sensing system (ECIS; Applied Biophysics, Troy NY) as described in (Imani Singh HSP 27). Once resistance stabilized between 1000-1500Ω, fresh media with 0, 0.625, or 1.25um sodium arsenite or the methylated arsenical cacodylic acid (DMAV). Application of 2000-3000Hz cycles was used to measure barrier function, and resistance was measured over 72 hours. These data report cell-cell junction, and not cell-matrix adhesion. All experiments were repeated 5 times; data are normalized to the average value of the resistance of media only cells.

CHAPTER 3: RESULTS

Minimal impact of oral arsenic on steady state lung and circulating immune cell populations

Both chronic and acute arsenic exposure through consumption of contaminated drinking water may cause bone marrow depression [51], leading to pancytopenia. Substantial *in vitro* information indicates that inorganic arsenic is immunotoxic [52-54], and *in vivo* when administered IT [55-57] or inhaled [58, 59]. However, there is limited information describing the immune composition within the lung following oral consumption of arsenic. To evaluate the resident immune cell populations in the lung, as well as hematopoietic effects in our model, we exposed 7 week old female mice to doses of 0, 250ppb, or 25ppm arsenic (sodium arsenite), for 5 weeks *ad libitum* in the drinking water for 5 weeks. Steady state alveolar leukocyte numbers and populations were defined by collecting bronchoalveolar lavage fluid (BALF) from arsenic-exposed animals (Figure 1). Animals receiving 25ppm arsenic had significantly more leukocytes in the BALF compared to animals receiving water alone (1A). However, differential analysis of leukocyte populations did not reveal significant changes among the treatment groups (1B). While previous studies have demonstrated increased rates of apoptosis in alveolar macrophages [60], these experiments analyzed cells exposed *ex vivo*. We further examined these animals using a complete blood count analysis, to determine if circulating leukocyte numbers or composition was significantly altered following arsenic exposure (1C). Animals receiving arsenic in their drinking water had an apparent dose-dependent effect on number of circulating monocytes, but these changes fell short of statistical significance (1D). These results

provide evidence for arsenic causing decreased numbers of circulating leukocytes, but suggest that leukocyte numbers within the alveolar compartment may be 'protected' from this effect.

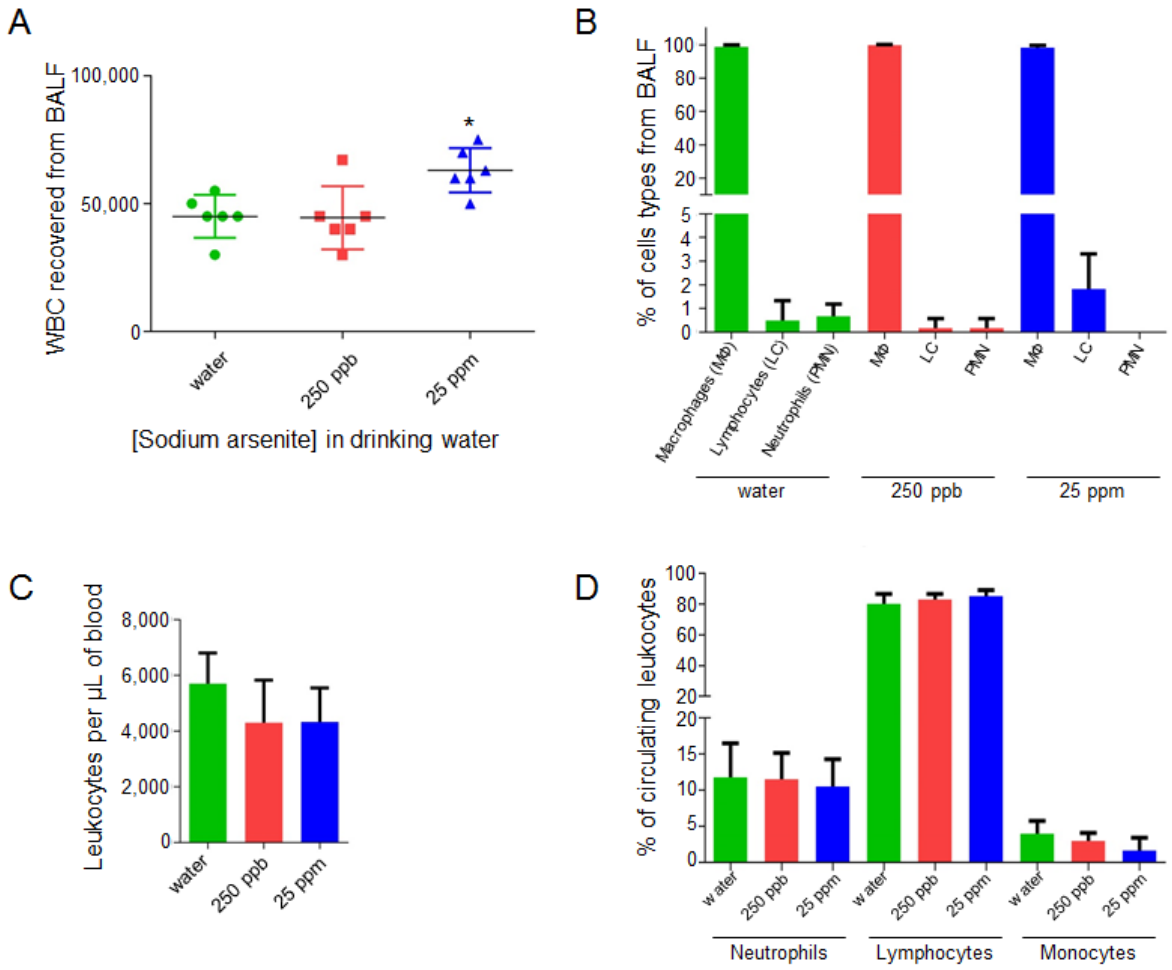

Figure 1: Consumption of arsenic drinking water has a modest effect on pulmonary, but not circulating leukocytes. Seven week old female C57Bl/6 mice were exposed *ad libitum* to drinking water containing sodium arsenite (0-green; 250ppb-red; 25ppm-blue) for 5 weeks prior to analysis. A) Total leukocyte numbers recovered from BALF. B) Leukocyte differential from BALF indicates percentages of macrophages, lymphocytes, or neutrophils across treatment groups. C) Total white blood cell count per microliter (µl). D) Complete blood count (CBC) analysis of exposure groups indicates percent differences in the major populations of circulating leukocytes. *=*P* 0.01 - 0.05.

Effect of oral arsenic on leukocyte recruitment to the microbially exposed airway

To determine if respiratory host defense functions remained intact following arsenic exposure, animals were exposed to 2000 CFU of the Gram-negative bacterium, *Klebsiella pneumoniae,* and then the host cellular immune response in the airway was quantified 24 hours later (Figure 2). Total white blood cell numbers in the airspace were not statistically different across the treatment groups (2A). Additionally, no significant quantitative differences were evident in leukocyte subpopulations within the BALF under any treatment (2B). Consistent with this, we did not detect any differences in complete blood count analysis across arsenic treatment groups (2C-D). To test the generalizability of these findings, we also infected arsenic-treated mice with the Gram-positive bacterium *Streptococcus pneumoniae* ($6.5e^5$ CFU). Similarly, we observed no differences in the total number of BALF leukocytes (2E). These data indicate that oral arsenic, under these exposure conditions, does not affect immune cell recruitment to the lung upon infection.

Accumulation of leukocytes in the airspace 24 hours post-infection is not a simple readout of the primary host response, but is rather a dynamic readout that is sensitive to secondary feedback from earlier time points such as early clearance/overgrowth rates of bacteria in the airspace in response to host defense in the first hours after infection. In order to isolate the effect of oral arsenic on the primary innate immune response in the airway more directly, we simplified our challenge model by using aerosolized E. coli LPS (300ug/ml for 30 min) instead of live bacteria. BALF was then collected 24 hours post-exposure for counting of airway leukocytes. Under these conditions, mice exposed to 25 ppm arsenic, but not those exposed to 250 ppb arsenic, had a modest, but statistically significant increase in alveolar leukocyte number (2F). These data suggest that arsenic consumption had no effect on recruitment of immune cells into the lung environment upon bacterial infections. However, a

moderate increase of leukocyte recruitment, seen only at the 25ppm dose, was observed in response to LPS.

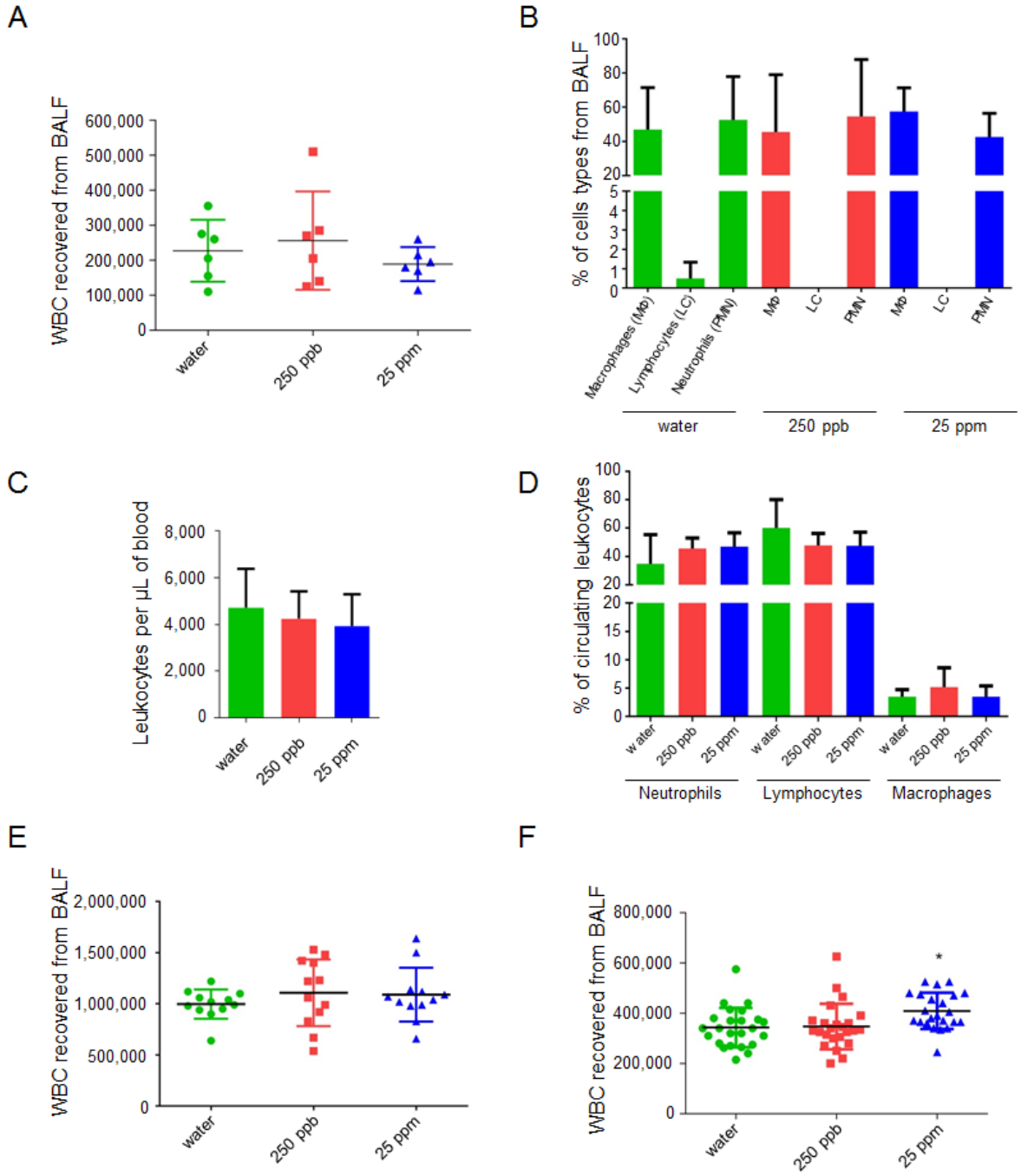

Figure 2: Responding immune cell composition is not significantly altered following respiratory challenge. A) Total leukocyte numbers recovered from BALF after 24 hours *K. pneumoniae* infection. B) Leukocyte differential from BALF indicates percentages of macrophages, lymphocytes, or neutrophils across treatment groups. C) Total white blood cell count per

microliter (µl). D) Complete blood count (CBC) analysis of exposure groups indicates percent differences in the major populations of circulating leukocytes. E) Total leukocyte numbers recovered from BALF after 24 hours *S. pneumoniae* infection. F) Total leukocytes recovered from lavage fluid 24 hours after LPS inhalation. *=*P* 0.01 - 0.05.

Effect of oral arsenic on bacterial clearance/compartmentalization during pneumonia

To determine if arsenic impacts bacterial clearance, arsenic (or control) -exposed mice were infected intratracheally (IT) with *Klebsiella pneumoniae* (2000 CFU). Lung, spleen, and blood were analyzed for bacterial counts 24 hours after infection (Figure 3). We observed no significant differences in lung bacterial burden across the treatment groups. However, quantification of bacterial load within the circulation and the spleen indicated a significant increase in these tissues, indicating greater extrapulmonary bacterial dissemination (3A).

Given that dissemination of *Klebsiella sp.* has been demonstrated to be influenced by many factors [61], we decided to infect animals with another human-relevant pathogen, *Streptococcus pneumoniae*. Following the 5 week arsenic exposure course, mice were infected IT with $6.5e^5$ - $1.87e^6$ *S. pneumoniae* for 24 hours. As before, lung, spleen, and blood were collected, processed, and plated for bacterial enumeration. Arsenic consumption did not affect the bacterial burden within the lungs. Similar to *K. pneumoniae*, infection with *S. pneumoniae* displayed a statistically significant dose-dependent trend (*P*= <0.001) increase in bacterial burden in peripheral tissues as arsenic concentrations increased (3B).

Analysis of the standard chow NIH-31 used in our studies revealed that this diet contained 300-400ppb of arsenic content (analysis did not determine speciation). Many dietary arsenicals, such as arsenobetaine and arsenocholine undergo metabolism different from arsenite or arsenate [21] and are considered non-toxic [62]. Nonetheless, we were concerned that this significant degree of background arsenic exposure might be confounding our results, so we repeated the study using an arsenic-free specialty research diet, maintaining dosing solely

through water consumption (not shown). This cohort was infected with *K. pneumoniae* as well. Exposure to arsenic via drinking water on this arsenic-free diet had a similar effect on host defense as observed on NIH-31 chow, with unchanged bacterial burden in lung, but increased burden in extrapulmonary tissues. These results suggest that the dietary arsenic exposure did not significantly affect bacterial burden.

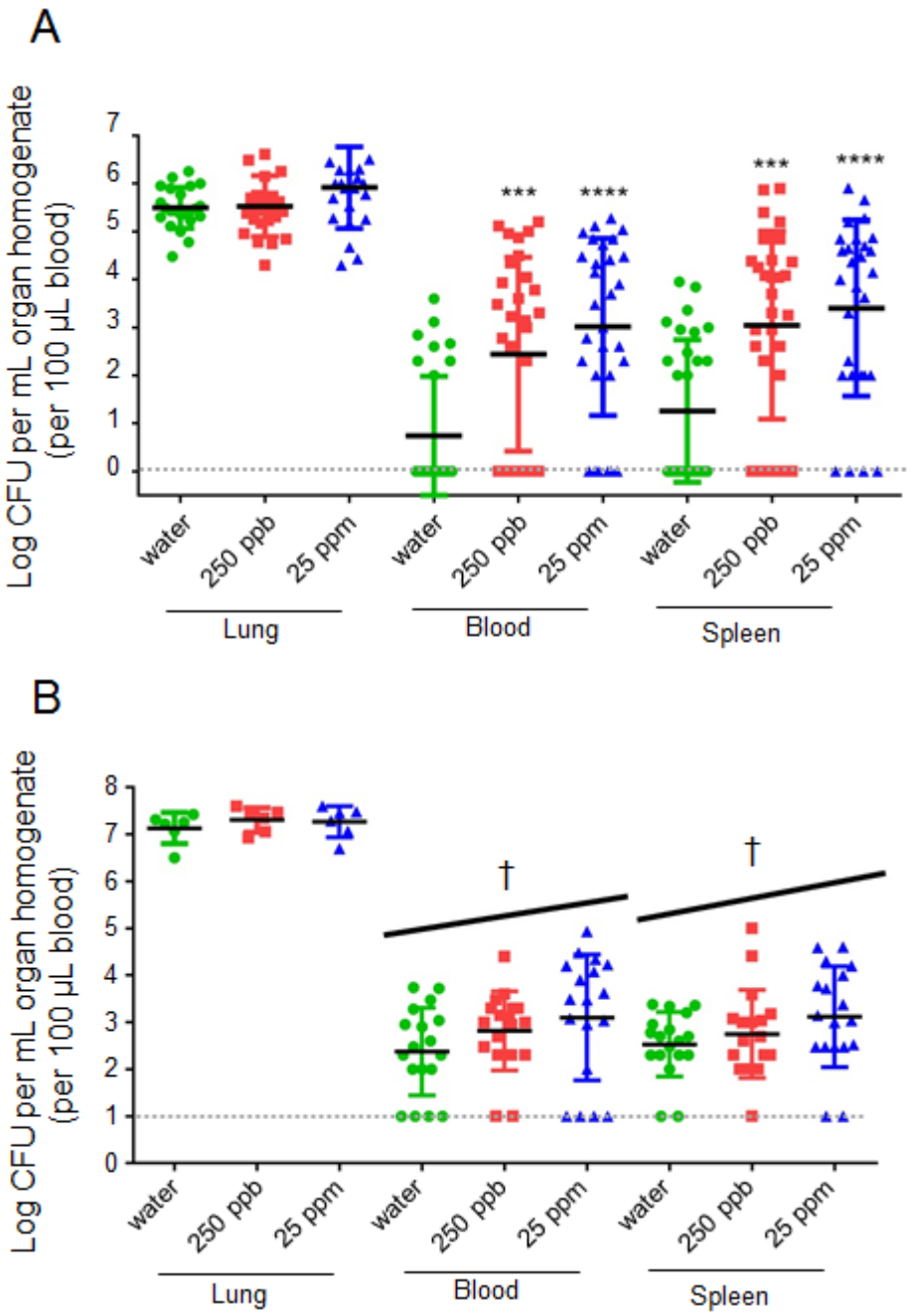

Figure 3: Enhanced extrapulmonary bacterial dissemination in arsenic-exposed mice. Seven week old female C57Bl/6 mice were exposed *ad libitum* to drinking water containing sodium arsenite (0-green; 250ppb-red; 25ppm-blue) for 5 weeks prior to analysis. Mice were infected IT, and lavage fluid was collected 24 hours later. A) Log CFU *Klebsiella pneumoniae* recovered from tissues. B) Log CFU *Streptococcus pneumoniae* recovered from tissues. ***=*P* 0.001 - 0.0001, ****=*P* <0.0001; †=*P* <0.001.

Taken together, our findings demonstrated increased bacteremia in animals following consumption of arsenic-contaminated drinking water at 24 hours post infection. In order to test the physiological significance of this impairment in host defense, we conducted a survival study by infecting animals with a lower dose of *K. pneumoniae* (1500 CFU) after a 5 week exposure to oral arsenic or control water. We monitored them post-infection for 2 weeks, with continuation of their assigned arsenic/water exposure. Under these conditions, there was a numerical, albeit not statistically significant reduction in survival in arsenic-exposed mice (Figure 4).

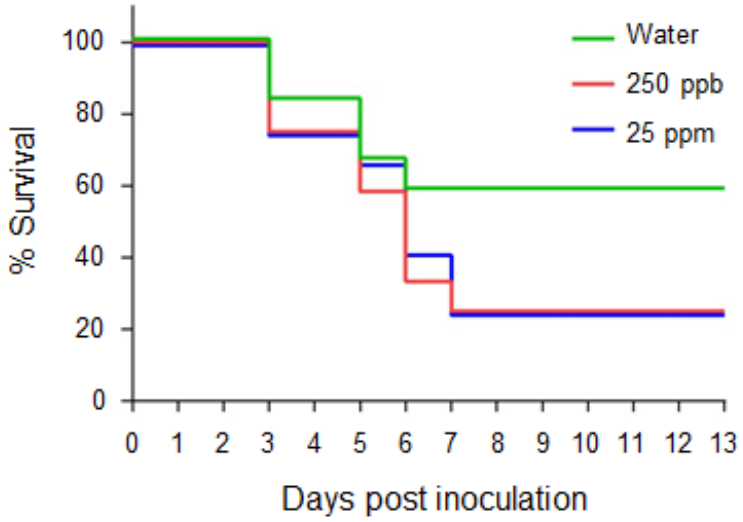

Figure 4: Effects of arsenic exposure on host survival following respiratory infection. Seven week old female C57Bl/6 mice were exposed *ad libitum* to drinking water containing sodium arsenite (0-green; 250ppb-red; 25ppm-blue) for 5 weeks prior to IT infection with 1500 CFU *Klebsiella pneumoniae.* Percent survival was calculated daily.

Differential effect of oral arsenic on LPS response of intrapulmonary vs. extrapulmonary macrophages

To evaluate for a possible difference in the effect of arsenic exposure on the innate immune response of lung-resident versus circulating macrophages, we dosed mice that had completed the 5 week arsenic exposure with aerosolized LPS (300ug/ml) or IP LPS (0.5mg/kg) and quantified the early cytokine response (Figure 5).Nuclear factor kappa B-dependent

cytokine response is one of the earliest quantifiable responses in response to LPS, including the production of tumor necrosis factor alpha (TNFα)[63]. TNFα promotes the inflammatory response, and is a key cytokine for activation of antimicrobial function. Measurement of cytokines in the airspace fluid serves as a readout of the local cellular response in the lung, while measurement in serum as a readout of the response in the extrapulmonary compartment. Production of TNFα in the LPS-exposed airspace is largely produced by alveolar macrophages; whereas, serum TNFα is thought to largely arise from production by liver macrophages (Kupffer cells) and splenic macrophages. Mice that were dosed with aerosolized LPS displayed no significant differences in production of TNFα within the BALF among arsenic treatment groups (5A). Consistent with this, alveolar macrophages harvested from mice that were exposed *in vivo* to inhaled LPS made equivalent levels of TNFα protein *ex vivo* across all water/arsenic treatment groups (5B). Conversely, mice injected IP with LPS showed a dose-dependent reduction in TNFα concentration within the bloodstream (5C). These data suggest that oral arsenic exposure has differential effects on intrapulmonary versus extrapulmonary macrophages, reducing the *in vivo* LPS responsiveness of the latter, but not the former.

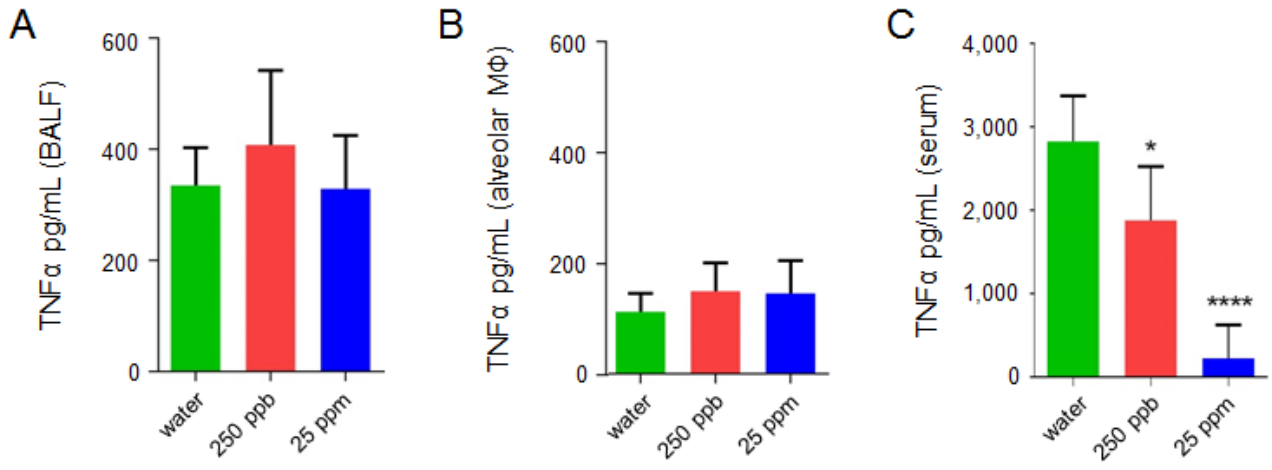

Figure 5: Arsenic-mediated suppression of cytokine response is compartment specific. Mice were dosed with LPS either IT (A,B) or IP (C). Concentrations of TNFα were measured in A) BALF; B) alveolar macrophages; C) serum. *=*P* 0.01 - 0.05, ****=*P* <0.0001

To test the effect of oral arsenic on a readily accessible peripheral macrophage, we harvested peritoneal exudate macrophages from mice that had completed a 3 week arsenic exposure (250 ppb only) and studied them *ex vivo* (Figure 6). Macrophages were exposed to LPS and assayed for production of TNFα. We observed no significant differences in production of TNFα in response to two different concentrations of LPS (6A). In addition, peritoneal macrophages from were tested for their phagocytic function. FITC-labeled *K. pneumoniae* was used at an MOI of 5 and macrophages were permitted time for engulfment. Flow cytometric analysis revealed that macrophages from arsenic-exposed were significantly less phagocytic than those from mice consuming water alone (6B). To study arsenic effect on macrophage LPS signaling in an even more direct and reductionist model, we harvested bone marrow-derived macrophages (BMDM) and exposed them *ex vivo* to arsenic or DMAV (Figure 7).

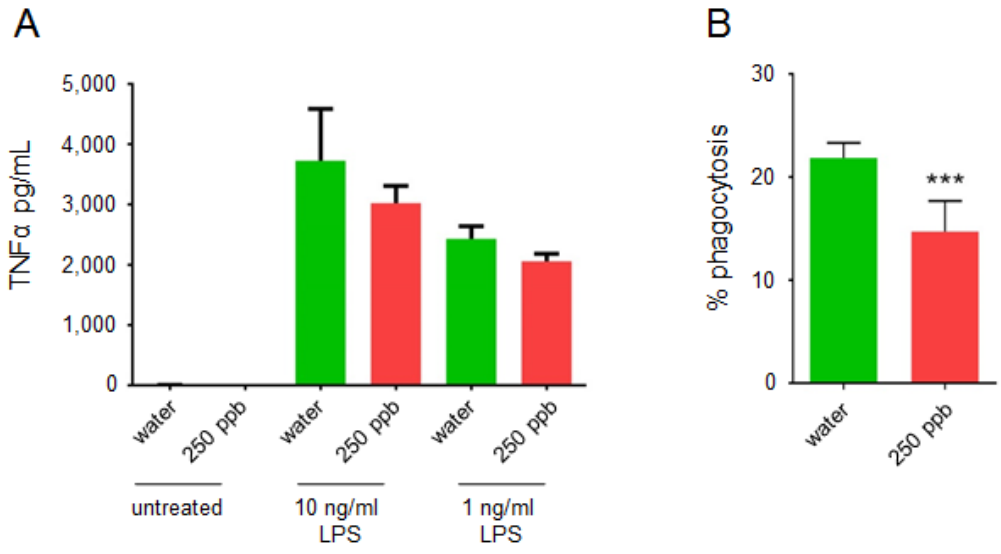

Figure 6: Immunotoxic effects on extrapulmonary macrophages following *in vivo* exposure. Seven week old female C57Bl/6 mice were exposed *ad libitum* to drinking water containing sodium arsenite (0-green; 250ppb-red) for 3 weeks prior to elicitation of macrophages using Brewer's thioglycollate and macrophages were collected 96 hours later. A) Macrophages were exposed to LPS (or vehicle) prior to quantification of TNFα in supernatants. B) Phagocytosis percentage of heat-killed *K. pneumoniae*. ***=*P* 0.001 - 0.0001.

The intended goal of arsenic methylation during metabolism is to produce a compound that facilitates excretion [64, 65]. However, some methylated metabolites of arsenic have been demonstrated to be cytotoxic and cell-signaling disruptors [66]. Additionally, the lung has been demonstrated to retain the methylated metabolite DMAV [5, 40]. BMDM were exposed to arsenite or the metabolite DMAV for 8 hours prior to analyzing viability using an MTT assay to determine if metabolism of the parent arsenic or exposure to a methylated metabolite was cytotoxic (7A). Doses of 10, 5, and 2.5um arsenite caused significantly reduced viability, whereas no tested dose of DMA V caused decreased viability in the macrophages. Macrophages were cultured in non-cytotoxic concentrations of arsenic before stimulation with LPS and processing of mRNA for quantitative analysis of cytokine transcripts (7B). Recognition of LPS drives transcription of both MyD88-dependent and -independent cytokines depending on signaling from different subcellular compartments (plasma membrane vs. endosome), which have distinct biological effects. These data indicate that transcript expression of both MyD88 – dependent and independent cytokines were suppressed at doses of 2uM; transcription of Cxcl10, was also decreased at the 0.5uM dose. We also analyzed transcription of nitric oxide synthase 2 (iNOS), an enzyme that generates nitric oxide (NO), a potent antimicrobial agent with a proven role against *Klebsiella pneumoniae* [67, 68]. Arsenic did not significantly affect LPS-mediated induction of iNOS in BMDM (7C). By contrast, total nitrate plus nitrite production, a surrogate measure for NO, was significantly reduced after exposure to non-cytotoxic doses of arsenic, suggesting a potential effect of arsenic either on protein-level expression or function of iNOS (7D). Tested concentrations of DMA V did not cause inhibition. Activation of these inflammatory and antibacterial processes has been demonstrated to contribute to host defense against *K. pneumoniae* [69].

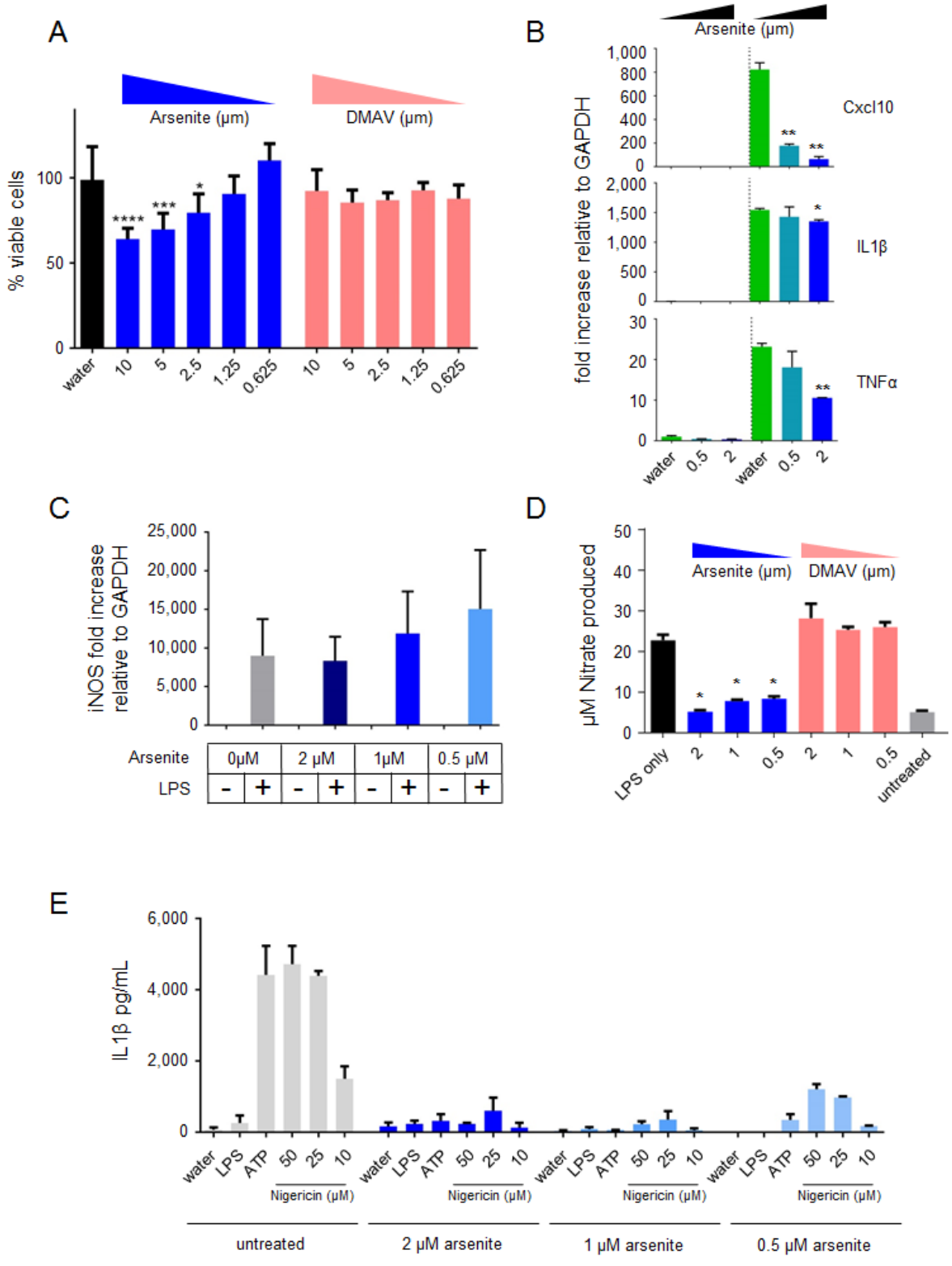

Figure 7: Arsenic-mediated cytotoxicity and inhibition of signal transduction and host defense functions. *In vitro* analysis of bone marrow-derived macrophaged (BMM) for viability and immune function following arsenic exposure. A) BMM were exposed to doses of arsenite or

dimethylarsinous acid (DMA V)for 8 hours prior to conducting a tetrazolium dye assay (MTT) to indicate viability. Doses pf 2.5 uM and higher caused significant decreases in the number of viable cells. B) Macrophages were exposed to 0, 0.5uM, or 2uM arsenite for 8 hours prior to the addition of 10ng/ml LPS or vehicle for 1 hour. Quantitative (ΔΔCT) real-time polymerase chain reaction (qRTPCR) was performed for analysis of transcription of the cytokines Cxcl10, pro IL1β, TNFα. C) BMM were analyzed for activation of the antimicrobial iNos pathway. Macrophages were exposed to doses of arsenitefor 8 hours prior to LPS (10ng/ml) for one hour. did not reveal a significant arsenic-specific difference in transcript. D) Griess assay was performed after exposing BMM to doses of arsenite (0, 0.5, 1, 2) or DMA V (0, 0.5, 1, 2) for 8 hours, then exposing cells to10ng/ml LPS for an additional 16 hours before quantifying nitrate production. E) BMM were exposed to doses of arsenic (0, 0.5, 1, 2) for 4 hours, and 10ng/ml LPS was added for a further 4 hours. Supernatants were removed media containined the inflammasome triggers ATP (2.5mM) or Nigericin (50, 25,10uM) for 30 minutes and mature IL1β was analyzed by ELISA. *=*P* 0.01 - 0.05, **=*P* 0.01 - 0.001, ***=*P* 0.001 - 0.0001, ****=*P* <0.0001.

More recently, activation of the inflammasome, a large multiprotein complex that promotes the maturation of the inflammatory cytokines IL-1β and IL-18, has been shown to be a critical component of immunity against *K. pneumoniae* infection [70]. Arsenic–treated BMDM were exposed to NLRP3 inflammasome priming and triggering and evaluated for production of mature IL-1β (7E). Non-cytotoxic doses of arsenic significantly inhibited IL-1β production by the NLRP3 inflammasome. Taken together, our *in vivo* experiments provide evidence that oral arsenic exposure reduces the innate immune response of extrapulmonary macrophages, a finding that is corroborated by direct *in vitro* exposure of cultured macrophages. By contrast, of interest, the TLR4 response of alveolar macrophages does not appear to be susceptible to oral arsenic exposure, perhaps suggesting that the alveolus may be a ‘protected’ immune compartment in the context of systemic arsenic intoxication.

Arsenic compromises integrity of pulmonary epithelial barrier

Our experiments with *K. pneumoniae* lung infection, revealing markedly increased bacteremia in the face of intact pulmonary bacterial clearance (Figure 3) suggested at least three possible mechanisms of arsenic effect: i) compromised bloodstream killing of bacteria; ii)

increased permeability of the alveolocapillary barrier to bacteria; and/or iii) impaired intrapulmonary host defense at an earlier time point than assayed (allowing for increased early extrapulmonary dissemination). To address the first possibility, we repeated *K. pneumoniae* infection, but bypassed the lung, directly injecting the bacteria intravenously (IV) (Figure 8). In one experiment, arsenic-exposed mice were injected with *K. pneumoniae* IV and allowed 4 hours of incubation time before collecting whole blood and spleen for bacterial quantification. Bacterial burden was not significantly altered in either tissue in arsenic-exposed mice (8A). A second experiment, designed to address the possibility that we had oversaturated the killing response with too high of an inoculum, was conducted using a lower dose and a longer response period (18h). Again, no difference in bacteremia or splenic bacteria was observed across arsenic treatment groups (8B). Taken together, these results indicate that, despite the depressed systemic TLR response observed in arsenic-exposed mice, arsenic does not appreciably compromise bloodstream killing of bacteria.

Next, in order to evaluate the effectiveness of pulmonary host defense at earlier time points, arsenic-exposed mice were dosed IT with *K. pneumoniae,* and then lung and peripheral organs assessed for bacteria 12h later (Figure 9). Consumption of arsenic drinking water did not affect bacterial numbers in the lung at this time point, suggesting that arsenic does not facilitate early bacterial overgrowth in the lung. No bacteria were detected in the bloodstream of control (water-exposed) mice, suggesting that this time point precedes dissemination of bacteria from the lung in the normal state. Of note, however, 1 of 6 mice exposed to 250 ppb arsenic and 4 of 6 mice exposed to 25 ppm arsenic ($P<0.05$) had detectable bacteremia. These data support the premise that the increased dissemination observed at 24 hours may derive from increased permeability of the alveolocapillary barrier to bacteria.

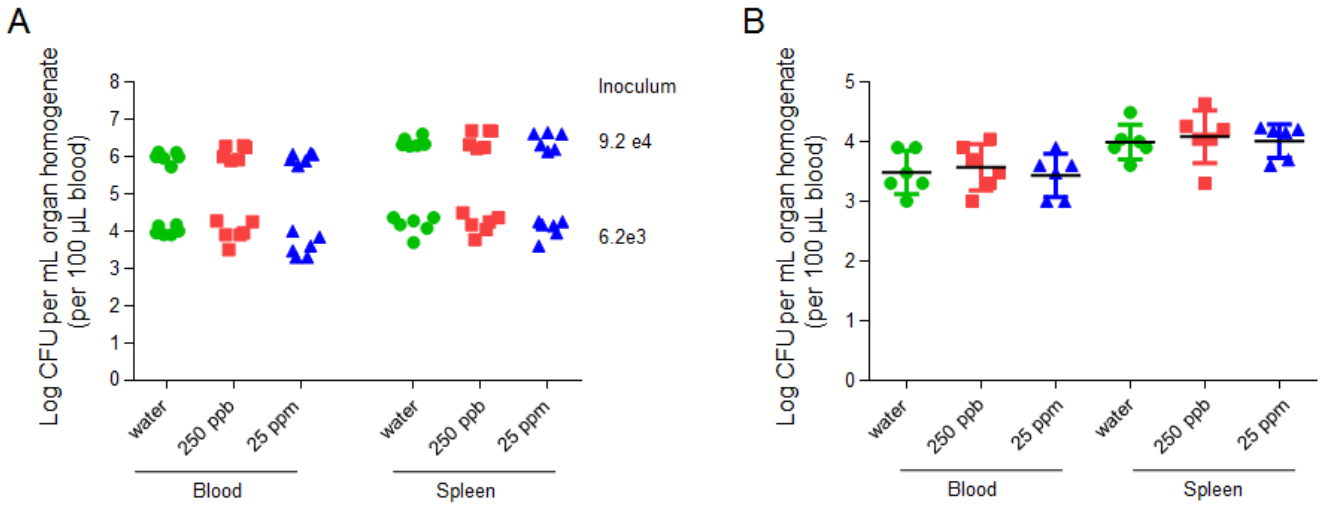

Figure 8: Peripheral clearance is not significantly affected by arsenic. After the normal arsenic exposure regime, mice were infected (IV) with *Klebsiella pneumoniae* for 4 (6.2e3, 9.2 e4 CFU) or 18 (960 CFU) hours prior to tissue collection. Bacterial burden was calculated from whole blood and spleen for each dose. There were no significant differences in recovered CFU at 18 (A) or 4 (B) hours after infection.

Measurement of plasma proteins in lung lavage fluid is an established indicator of disruption of microvascular (endothelial) integrity in the lung [71, 72]. To evaluate if arsenic caused disruption of the alveolocapillary barrier, BALF was collected from animals following the 5 week arsenic exposure regime, with or without subsequent exposure to aerosolized LPS, and analyzed for the presence of albumin or IgM, two established markers of pulmonary microvascular injury (Figure 10). Albumin (67 kilodalton, kDa) concentration within the BALF was not significantly changed by arsenic exposure, even when acute lung injury (ALI) was further promoted by LPS challenge (10A). Similarly, IgM (~970 kDa) levels were not affected by arsenic, and differences in LPS –induced injury were not evident across arsenic treatments (10B). These results argue against significant disruption of the pulmonary endothelial barrier, at least as indicated by plasma-to-alveolar translocation of protein markers 67 kDa or larger.

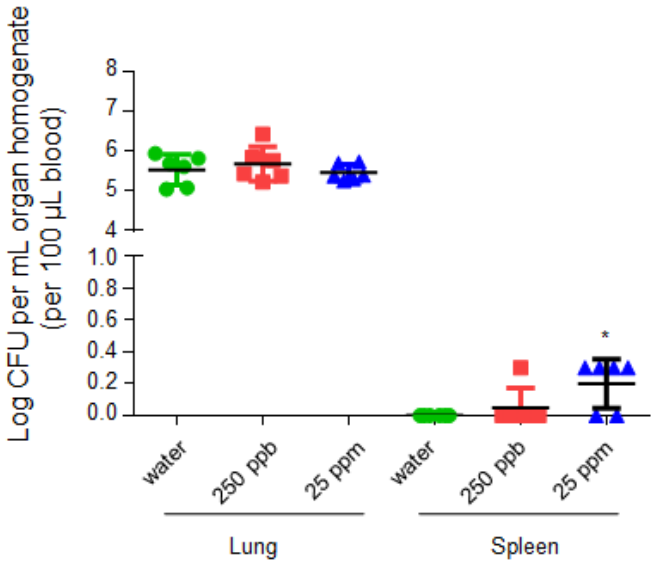

Figure 9: Early analysis respiratory infection reveals accelerated bacterial dissemination. To evaluate the early immune response, i.e. neutrophil effectiveness, mice were infected (IT) with *Klebsiella pneumoniae*. Lung tissue and whole blood were collected 12 hours after exposure and were plated for bacterial enumeration (calculated as log CFU per ml). Differences in peripheral burden (25ppm dose).
*=*P* 0.01 - 0.05

In order to evaluate for possible increased alveolar-to-plasma permeability in vivo, we measured plasma levels of CC16, an endogenous Club cell protein that is released into the airspace and whose levels in the plasma are an established indicator of pulmonary epithelial permeability [73] (Figure 11). BALF and serum were collected from mice completing 5 weeks of consumption of arsenic drinking water and analyzed via ELISA for CC16 concentration. Mice exposed to 25ppm arsenic had significantly greater amounts of CC16 in the serum, but not the airway (11A). These data suggest that arsenic facilitated translocation of a protein from the airway to the bloodstream, and indicating acute lung injury.

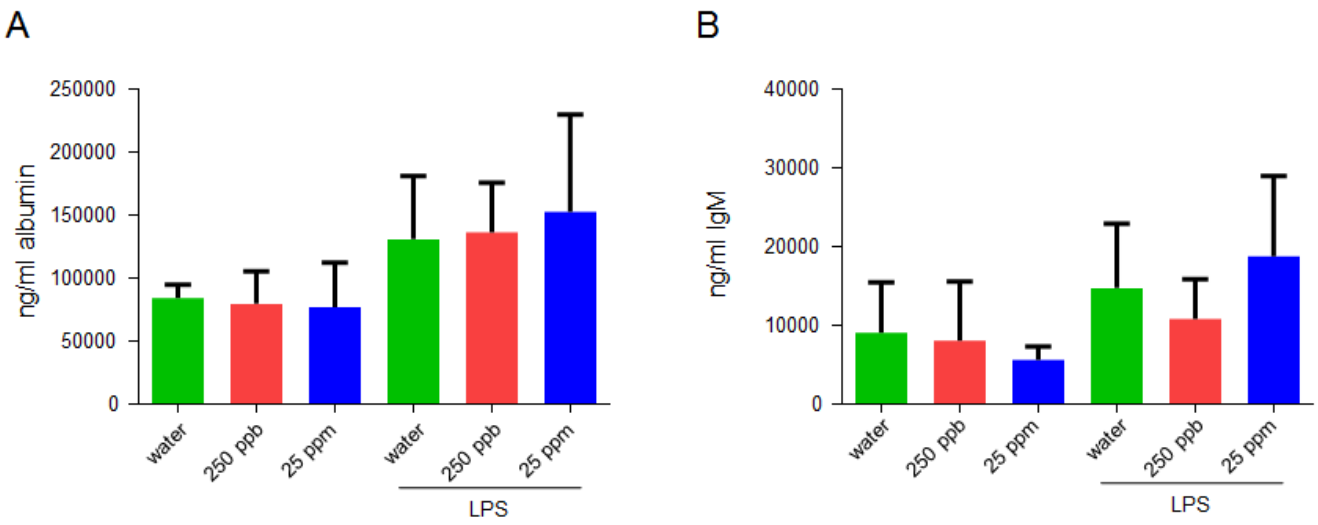

Figure 10: Effects of arsenic on pulmonary microvascular permeability. After the normal arsenic exposure regime, mice were infected (IV) with *Klebsiella pneumoniae* for 4 (6.2e3, 9.2 e4 CFU) or 18 (960 CFU) hours prior to tissue collection. Bacterial burden was calculated from whole blood and spleen for each dose. Bacterial CFU recovered at 18 (A) or 4 (B) hours after infection.

Alveolar-to-plasma transit has been measured using a variety of probes, radioactive and non-radioactive [74]. We used an established method for testing respiratory epithelial permeability by assaying for plasma presence of an airway-introduced fluorescent small molecule [75]. To test translocation from the alveoli to the bloodstream *in vivo*, FITC-dextran (3000MW) was instilled IT, and then plasma analyzed for fluorescence intensity (Figure 11B). Mice receiving 250ppb of arsenic in their drinking water demonstrated significantly greater fluorescence in the plasma, indicating that FITC-dextran translocation was enhanced by arsenic. Fluorescence was also numerically, but not statistically higher in the 25 ppm arsenic-exposed mice. To test more specifically for cytotoxic effects of arsenic on the alveolar epithelium *in vivo*, effects which might increase permeability, we performed western blot analysis of BALF for microvesicles containing T1α/podoplanin, an established marker of alveolar epithelial type I cell injury [76] (Figure 12A). Densitometry revealed that mice receiving 25ppm arsenic in their drinking water displayed significantly increased T1α signal within the BALF, (12B) suggesting injury to alveolar epithelial type I cells. To further investigate these findings,

we collected and fixed whole lung tissue from mice that had been exposed to water, 250ppb, or 25ppm arsenic for 5 weeks, prior to analysis via electron microscopy (EM)(Figure 13). Animals exposed to 25ppm demonstrated multiple apoptotic type I pneumocytes (13A), significant swelling from the basement membrane, and multiple platelets coating damaged endothelial lining. Examination of tissue collected from animals exposed to 250ppb revealed no examples of apoptotic pneumocytes, but revealed moderate swelling beneath the alveolar epithelial and endothelial basement membrane, with focal collagen dispersal, and fewer attached platelets on the endothelial lining. Animals consuming water alone displayed no dying type I cells, had very few observable platelets, and predominantly showed a thin, smooth endothelial lining (13B). Taken together, our data suggested that greater dissemination of a respiratory pathogen was likely not due to pulmonary or extrapulmonary immune compromise, but rather to facilitated escape from the lung compartment to the periphery due to a disruption of the alveolocapillary barrier, with some detectable effects on both alveolar and endothelial cells.

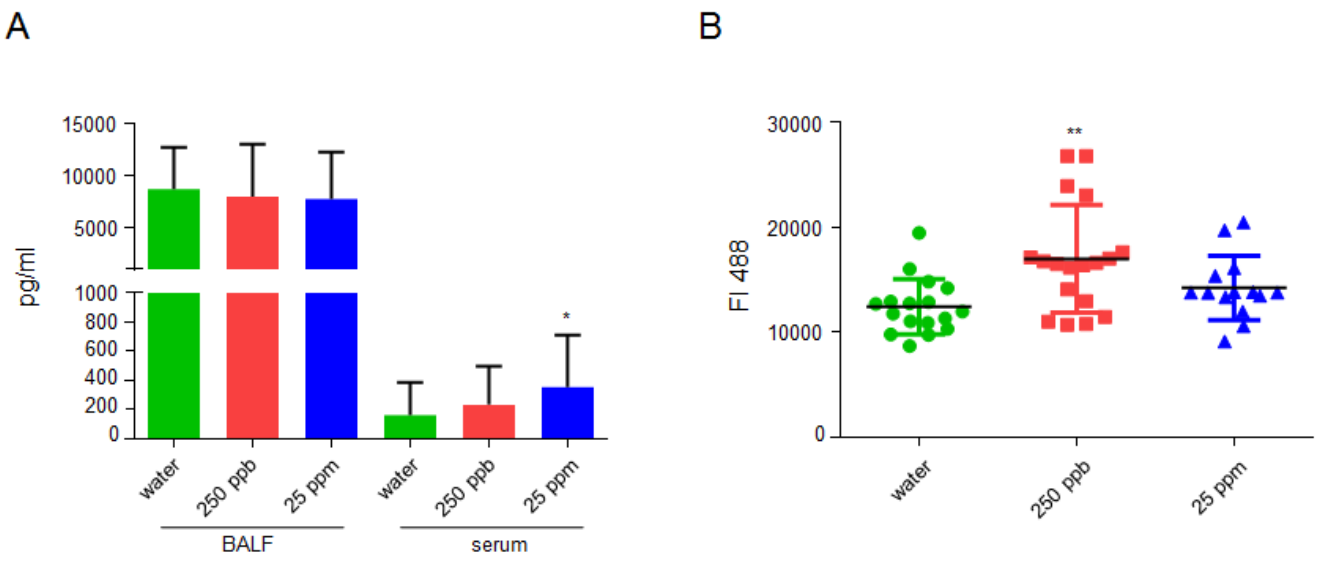

Figure 11: Indications of facilitated alveolar to circulatory transit after arsenic consumption. A) ELISA of Club cell protein 16 (CC16) from BALF and serum. B) Fluorescence intensity (FI) of plasma from arsenic-exposed animals after IT administration of 5mg/kg FITC-dextran. *=*P* 0.01 - 0.05, **=*P* 0.01 - 0.001.

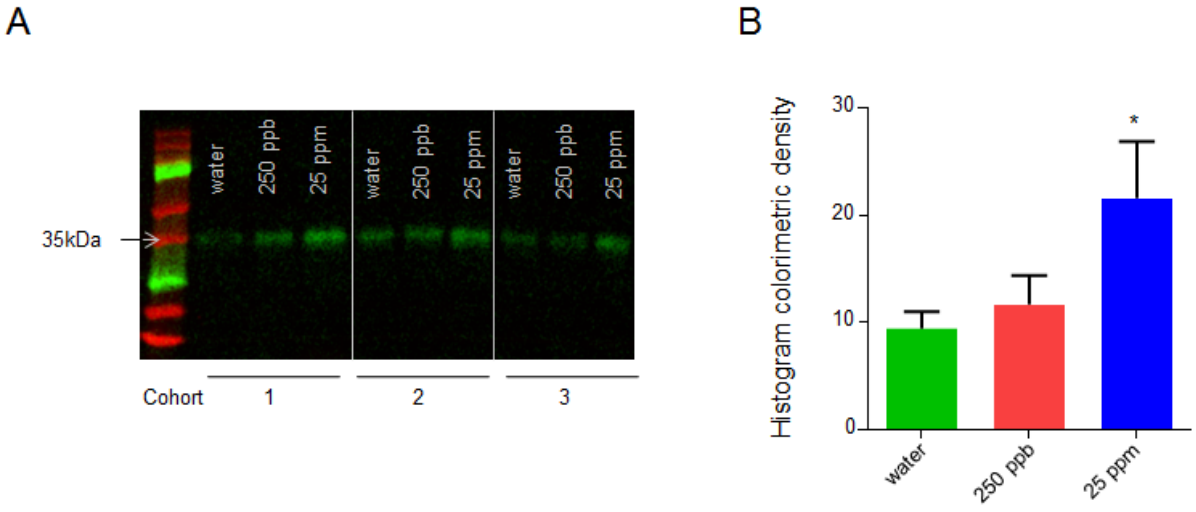

Figure 12: Consumption of arsenic drinking water causes damage to type I pneumocytes. A) Western blot of T1α (podoplanin) from vesicles present in lavage fluid. Results are representative samples from three independent experiments. B) Densitometric analysis of western blot image in A. *=*P* 0.01 - 0.05.

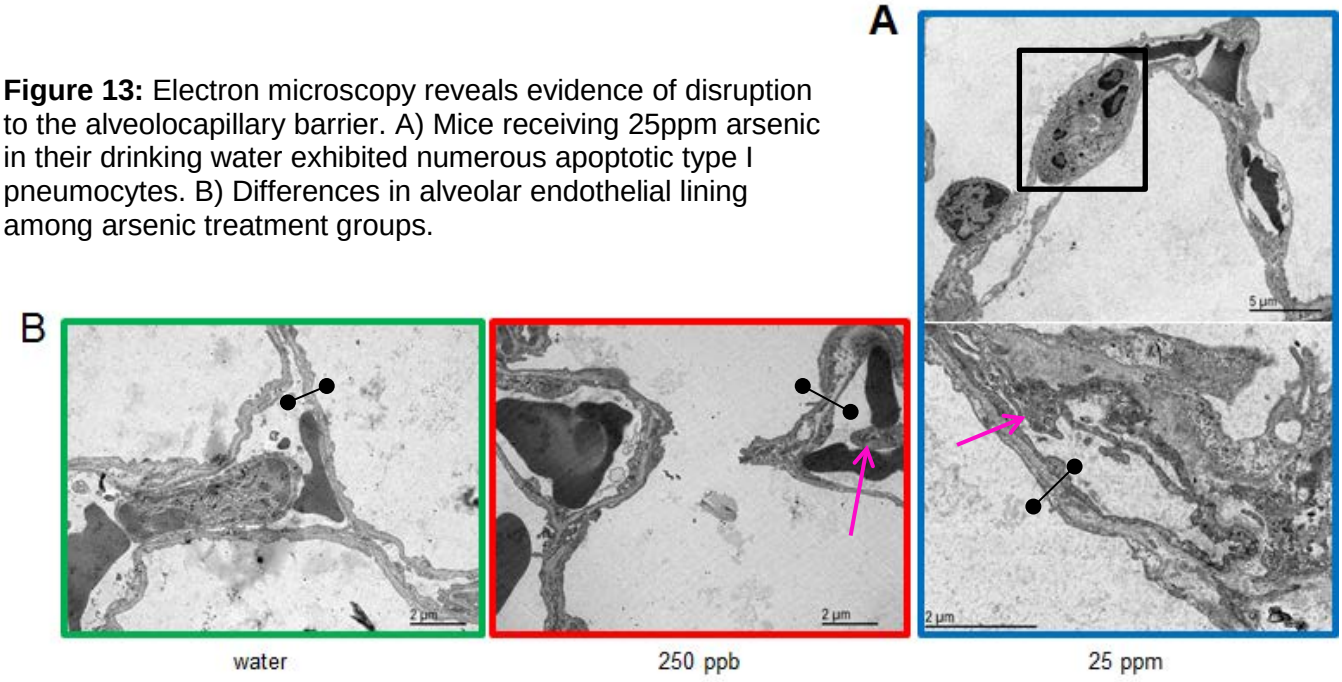

Figure 13: Electron microscopy reveals evidence of disruption to the alveolocapillary barrier. A) Mice receiving 25ppm arsenic in their drinking water exhibited numerous apoptotic type I pneumocytes. B) Differences in alveolar endothelial lining among arsenic treatment groups.

Additional mechanistic investigations of effect of arsenic on pulmonary epithelium underway

We will pursue possible molecular pathways that can impact alveolar cell fate using IHC, to potentially elucidate specific molecular mechanisms responsible for our cytotoxicity findings. Lung tissue sections are currently also being processed for immunohistochemistry. Sections will be stained for Ki-67, Caspase-3, γ-H2AXto evaluate for proliferation defects, cell death, and DNA damage, respectively. Additionally, dysregulation of MMP-9 has been identified as a biomarker for arsenic-mediated lung toxicity [77, 78], and tissues were evaluated for production.

Arsenic has been demonstrated *in vitro* to disrupt proteins that maintain junctional adhesions between epithelial cells [79]. Preliminary analysis of expression levels in the lung of arsenic-exposed mice of genes such as Zona occludens (ZO1), junctional adhesion molecules (JamA, JamC), occludin (OC), and claudins (Cldn3, Cldn18) suggests that arsenic disrupts normal expression (data not shown). We intend to conduct a much longer *in vitro* exposure time course using the mouse alveolar epithelial line E10, and the human alveolar line A549. Cells will be maintained within a range of arsenic concentrations for 24 hours, 1 week, 2 weeks, or 3 weeks. Fluorescent microscopy will be used to detect possible changes within the cell-cell adhesive proteins, and mRNA will be collected to determine differences in transcription. Electric cell-substrate impedance sensing (ECIS) will also be used to establish the permeability of these junctions to electrical current.

Pathway-specific transcriptome analysis will be conducted using Nanostring to search for and quantify the alteration of additional genes that are affected by consumption of arsenic-contaminated drinking water. Nanostring technology is a variation of the DNA microarray technology that is capable of quantitatively assaying transcript copy, without the need for signal amplification using PCR. We chose this technology due to its high sensitivity, necessary when

working with very low cell numbers. EpCam positive (CD31,CD34, CD45 negative) epithelial cells were collecting using flow cytometric sorting, and mRNA was isolated. The mouse PanCancer and Immunology (Nanostring) codeset was chosen to evaluate genes that are potentially responsible for the results found in our previous studies, as well as to elucidate early biomarkers of carcinogenesis due to inflammation, pneumocyte toxicity, and stressed repair systems.

CHAPTER 4: DISCUSSION

The guideline for arsenic concentration in drinking water was first established in 1942 by a standard provided by the U.S. Public Health Service. In 1974, Congress passed the Safe Drinking Water Act, urging the Environmental Protection Agency (EPA) to establish limits for inorganic chemicals within drinking water, and determining that the concentration of arsenic should be limited to 50 µg/L (ppb). This concentration was reevaluated based on new research, and was lowered in 2001 from 50 ppb to the current standard of 10 ppb [80]. These standards are upheld by law in the United States, yet globally, millions of individuals are exposed to doses exceeding 120 ppb [81-84]. Low income populations within the Indochina Peninsula are particularly susceptible to these exposure levels. Few published studies allow for direct comparison of dose-toxicity relationships between rodents and humans [4, 21, 40, 85-89]. Although our study used a 250 ppb dose that may be higher than encountered globally except for in rare circumstances, it is nonetheless a ~100-200-fold lower exposure than a large number of reports that have studied arsenic in rodent models, and certainly much shorter in duration than encountered in human populations. As the leading cause of death in low income countries with high arsenic exposure is lower respiratory infections, we aimed to use a tractable rodent pneumonia model system to determine whether consumption of arsenic-contaminated drinking water increases susceptibility to bacterial respiratory infections.

To determine whether our drinking water dosing scheme compromises the immune response to respiratory bacterial infection, we first addressed the toxic effects of arsenic

exposure alone. Our findings demonstrate that 250 ppb arsenic does not substantially affect pulmonary immune cell numbers, nor composition of the circulating leukocyte repertoire. This is in contrast to past studies that have used IT or inhalation delivery of arsenic in rodents, which have found reductions in the numbers of alveolar macrophages [55-57]. Of interest, we detected a slight, but statistically significant increase in total airspace leukocytes in mice exposed to 25 ppm arsenic. This effect was most likely due to an increase in lymphocytes, though the increase in lymphocyte number in our study did not quite achieve statistical significance. Consistent with our finding, lymphocytes were recently reported to be selectively increased in the lungs of mice exposed to low levels of arsenic in drinking water [48].

In order to test the innate immune response to microbial challenge, we infected mice with *Klebsiella pneumoniae* and *Streptococcus pneumoniae*, two clinically relevant bacterial pathogens. We observed no differences in the number or type immune cells recruited to the lungs, nor a significant reduction in any type of leukocyte in the bloodstream. Similar studies using viral challenge also demonstrated no significant differences in the number or type of immune cells 36 hours after infection [48]. In order to quantify host defense function, we next quantified bacterial burden in the lung and peripheral tissues. Unlike earlier inhalation studies [49] and the aforementioned viral challenge [48], we found no significant differences in microbial number within the lung compartment among arsenic treatment groups, suggesting that oral arsenic does not compromise pulmonary host defense within this early time frame. By contrast, mice exposed to either 250 ppb or 25 ppm oral arsenic had marked increases in bloodstream and splenic bacterial load, indicating greater extrapulmonary dissemination of bacteria.

Our experiments indicated that arsenic consumption increased the severity of the extrapulmonary dissemination, but had not determined the cause to be resultant from a failure in the lung compartment or the periphery. Other studies have demonstrated that *ex vivo* exposure

to inorganic arsenic to alveolar macrophages definitely compromised host defense functions [53, 56]. Yet we found no *in vivo* evidence for alveolar macrophage toxicity. These data provoked questioning the compartment-specific arsenic toxicity caused by oral exposure. We therefore conducted studies on peripheral macrophages using a dual exposure scenario: *in vivo* arsenic exposure and *ex vivo* stimulation; and *in vitro* exposure of primary cells. These studies demonstrated that the host defense functions peripheral macrophages directly exposed to arsenic, or its blood borne metabolites, were inhibited. To determine if this toxicity seen *in vitro*, would compromise peripheral bacterial clearance *in vivo* we bypassed the lungs by directly injecting bacteria into the bloodstream. Our results showed that arsenic exposure does not inhibit peripheral clearance of bacteria, and we began to investigate mechanisms of toxicity beyond immune suppression.

Given that consumption of arsenic has been linked with peripheral microvascular disease [90-93], we postulated that arsenic exposure might disrupt the architecture of the alveoli which would permit bacterial escape into the bloodstream. Our findings agree with previous studies demonstrating that oral arsenic exposure does not cause leak of serum albumin into the alveoli, even after respiratory challenge [48]. However, we observed biomarkers of airway epithelial damage in the bloodstream, and performed a functional study in which we observed the transit of a fluor-labeled probe from the airways to the serum, indicating that alveolocapillary barrier integrity was reduced after consumption of oral arsenic. Furthermore, we presented evidence of type I pneumocyte damage, and cytotoxicity, thus disrupting the alveolar epithelial barrier and increasing permeability [94]. Electron microscopy revealed significant changes to the alveolar endothelium, specifically disorganization of the endothelial lining and platelet accumulation in the vasculature. Taken together, these results support that greater bacterial dissemination was likely caused by disruption of the alveolocapillary barrier, rather than the

immunotoxic effects of arsenic in our model. Moreover, our data suggest the possibility that epithelial breach further compromises barrier integrity.

Type I pneumocyte toxicity was significantly detected at 25 ppm, but animals exposed to 250 ppb exhibited significantly greater translocation of the FITC-dextran tracer. We suspect that lower doses of arsenic (sub 1 ppm) disrupt the proteins responsible for cell-cell junctions, permitting paracellular translocation. *In vitro* experiments using intestinal epithelial cells have described a similar scenario with compromised barrier integrity following arsenic exposure, though their rationale was dependent on increased levels of local TNFα [95]. Exploratory studies were conducted analyzing whole lung mRNA following arsenic exposure, and revealed differences in gene expression of claudins, junctional adhesion molecules, and occludin (not shown). Knowing that chronic arsenic exposure causes obstructive pulmonary diseases in humans (COPD) [37-39], we suspect that TGFβ, which has been demonstrated to play a major role in airway remodeling and COPD [96, 97], levels are increased in the BALF. We demonstrated that our higher 25 ppm dose caused apoptosis of pneumocytes, and efferocytosis of that debris could potentiate TGFβ production [98]. Additionally arsenic has been demonstrated to directly upregulate expression of TGFβ in certain cell types [99]. Therefore, we presume that in higher dose exposures, the remodeling response is engendered and would limit the transit of our probe.

Compartmentalization of pathogens is a key function of the innate immune response. It can therefore be expected that compromised alveolocapillary barrier integrity would permit extrapulmonary dissemination, thus causing greater risk to the host. To our knowledge, this is the first report showing that consumption of arsenic in drinking water disrupts barrier function *in vivo*, and results in a worsened disease state upon infection. Many reports investigating the immunotoxicity of arsenic have suggested that exposed individuals would be at greater risk to

infection, and this work elaborates by describing significantly altered pathogenesis in a bacterial pneumonia model. Given the global health burden of arsenic drinking water contamination, we hypothesize that our results provide some rationale for the increased mortality due to respiratory infections in low-income populations of contaminated regions.

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