

Effects of Orally Ingested Arsenic on Respiratory Epithelial Permeability to Bacteria and Small Molecules in Mice

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BACKGROUND: Arsenic exposure via drinking water impacts millions of people worldwide. Although arsenic has been associated epidemiologically with increased lung infections, the identity of the lung cell types targeted by peroral arsenic and the associated immune mechanisms remain poorly defined.

OBJECTIVES: We aimed to determine the impact of peroral arsenic on pulmonary antibacterial host defense.

METHODS: Female C57BL/6 mice were administered drinking water with 0, 250 ppb, or 25 ppm sodium arsenite for 5 wk and then challenged intratracheally with *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, or lipopolysaccharide. Bacterial clearance and immune responses were profiled.

RESULTS: Arsenic had no effect on bacterial clearance in the lung or on the intrapulmonary innate immune response to bacteria or lipopolysaccharide, as assessed by neutrophil recruitment to, and cytokine induction in, the airspace. Alveolar macrophage TNF α production was unaltered. By contrast, arsenic-exposed mice had significantly reduced plasma TNF α in response to systemic lipopolysaccharide challenge, together suggesting that the local airway innate immune response may be relatively preserved from arsenic intoxication. Despite intact intrapulmonary bacterial clearance during pneumonia, arsenic-exposed mice suffered dramatically increased bacterial dissemination to the bloodstream. Mechanistically, this was linked to increased respiratory epithelial permeability, as revealed by intratracheal FITC-dextran tracking, serum Club Cell protein 16 measurement, and other approaches. Consistent with barrier disruption at the alveolar level, arsenic-exposed mice had evidence for alveolar epithelial type 1 cell injury.

CONCLUSIONS: Peroral arsenic has little effect on local airway immune responses to bacteria but compromises respiratory epithelial barrier integrity, increasing systemic translocation of inhaled pathogens and small molecules. <https://doi.org/10.1289/EHP1878>

Introduction

Arsenic is a naturally occurring metalloid found in soil, water, and air that has been linked to the development of a wide range of human cancers (i.e., lung, bladder, liver, skin, kidney), as well as cardiovascular disease and other nonmalignant disorders (Hughes et al. 2011). Although occupational exposure to arsenic may occur via inhalation, it is thought that the major route of exposure, involving >140 million people worldwide, is via drinking water due to natural contamination of groundwater with inorganic arsenic from the earth's crust (Hughes et al. 2011; States et al. 2011). Although comparatively a much larger problem in countries such as Bangladesh, Chile, India, and Taiwan where arsenic concentrations >1 ppm have been documented in drinking water, arsenic was ranked first on the U.S. Agency for Toxic Substances and Disease Registry's 2015 "Priority List of Hazardous Substances" (<https://www.atsdr.cdc.gov/spl/>) due to elevated levels at several locations in the United States.

In recent years, there has been increasing recognition of the lung as a uniquely susceptible target organ for arsenic ingested via the oral route. In addition to lung cancer (Celik et al. 2008; D'Ippoliti et al. 2015; Smith et al. 2009), epidemiologic studies have associated arsenic exposure via drinking water with decreased lung function (Das et al. 2014; Dauphiné et al. 2011; Parvez et al. 2013; Recio-Vega et al. 2015; von Ehrenstein et al. 2005), respiratory symptoms and signs (Mazumder et al. 2000; Parvez et al. 2010; Steinmaus et al. 2016), pneumonia (George et al. 2015; Rahman et al. 2011), bronchiectasis (Mazumder et al. 2005; Smith et al. 2006), and chronic obstructive pulmonary disease (COPD) (D'Ippoliti et al. 2015). Although the underlying mechanisms have remained somewhat obscure, studies in rodent models have shown that the lung has substantial arsenic metabolizing capacity through arsenite methyltransferase (Healy et al. 1998), that some methylated arsenic species are preferentially distributed to the lung (Hughes et al. 2003; Kenyon et al. 2005), and that oral arsenic alters pulmonary gene expression (Andrew et al. 2007; Boellmann et al. 2010; Kozul et al. 2009b) and induces DNA single strand breaks selectively in the lung (Yamanaka et al. 1989). Toxic effects of arsenic on respiratory epithelial cells and macrophages have been identified *in vitro*. However, little work to date has been done *in vivo* to identify specific cell types in the lung targeted by arsenic and the associated effects on pulmonary immune responses to the environment.

Here, our objective was to define the effect of orally ingested arsenic upon antibacterial host defense in the lung. Mice were exposed to arsenic by drinking water for 5 wk and then challenged intratracheally with bacteria or lipopolysaccharide (LPS). The intrapulmonary innate immune response was quantified. Bacterial burden was quantified in the lung in order to assess local microbial clearance, and in blood and spleen in order to assess extrapulmonary bacterial dissemination. Permeability of the respiratory epithelium to endogenous airspace proteins was assessed through the measurement of plasma Club Cell protein 16 (CC16). Permeability of the respiratory epithelium to exogenous small molecules was evaluated through measuring translocation of FITC-dextran from airspace to plasma.

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Methods

Reagents

Sodium arsenite was from Sigma (St. Louis, MO). Dulbecco's Modified Eagle Medium (DMEM) was from Gibco™ (Gaithersburg, MD). Fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA) was used in cell culture studies.

Animals and Arsenic Dosing

Female, 8-wk-old C57BL/6 mice weighing 18–22 g 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 NIEHS Animal Care and Use Committee. Female mice were used in order to facilitate group housing and group exposure. Animals were treated humanely with due consideration to the alleviation of distress and discomfort. Four liter volumes of drinking water were freshly prepared with 250 µg/L (ppb) or 25 mg/L (ppm) sodium arsenite every 2 wk for *ad libitum* consumption. Mice consumed 2–5 mL of arsenic-containing water (or water control) daily for ~5 wk prior to experimentation. In most studies, mice were fed NIH-31 chow. For a subset of studies, mice were fed AIN-93M diet (D10012M; Research Diets, Inc.; New Brunswick, NJ) for 2 wk prior to and during the 5-wk arsenic drinking water exposure.

In Vivo Bacterial Exposures

Klebsiella pneumoniae [ATCC 43816; 2,000 CFU (colony-forming units)] or *Streptococcus pneumoniae* (ATCC 6303; $6.5 - 9 \times 10^5$ CFU) were delivered to the lung via oropharyngeal aspiration while the mice were under 4% isoflurane anesthesia, as previously reported (Madenspacher and Fessler 2016). In other experiments, *K. pneumoniae* (960, 6,200, or 92,000 CFU) was injected intravenously (i.v.), and tissues were collected 4 or 18 h later. Splenic homogenate and whole blood were serially diluted and plated on tryptic soy agar (TSA) plates for *K. pneumoniae* or TSA with 5% sheep's blood for *S. pneumoniae* and incubated overnight for bacterial quantification.

In Vivo LPS Exposures

Mice were exposed to aerosolized *Escherichia coli* 0111:B4 LPS (300 µg/mL, Sigma-Aldrich, St. Louis, MO) for 30 min, as previously reported (Draper et al. 2010; Smoak et al. 2008). Bronchoalveolar lavage fluid (BALF) was collected immediately after euthanasia. BALF was centrifuged to pellet cells, and the supernatant was collected and frozen at -80°C prior to analysis. Cell count and differential calculation was performed using Wright's stain. Alternatively, mice were injected i.p. with 0.5 mg/kg LPS for serum cytokine analysis.

Immunoblotting

BALF was collected as previously described (Draper et al. 2010). 400 µL of BALF was centrifuged (100,000 g, 2 h, 4°C), and the pellet was resuspended in 65 µL Laemmli sample buffer with 50 mM DTT and boiled for 8 min. Samples were run on a 10% sodium dodecyl sulfate–polyacrylamide gel and transferred to nitrocellulose using standard methods. The membrane was probed with anti-T1 α /podoplanin antibody (AF3244; R&D Systems, Minneapolis, MN) and detection performed with a secondary antibody labeled at 800 nm (LiCor, Lincoln, NE) and an Odyssey Fc (LiCor). Quantification was performed using ImageStudio 5.0 (LiCor).

Flow Cytometry

Lungs were digested with porcine elastase (4.5U; E7885, Roche Diagnostics, St. Louis, MO) and 100 U/mL DNase I (Catalog No. 79254; Qiagen, Valencia, CA). Cells in suspension were then

filtered and processed. Pacific Blue/V450 streptavidin-conjugated (Catalog No. 560797; BD Biosciences, San Jose CA) secondary antibody was used to label cells after staining with biotinylated antibodies against CD45 (Catalog No. 553078; BD Biosciences), CD31 (Catalog No. 558737; BD Biosciences), and CD34 (Catalog No. 119304; BioLegend, San Diego, CA). Cells were also stained with APC (allophycocyanin)-conjugated rat anti-mouse EpCAM (Catalog No. 17-5791-82; eBioscience, San Diego, CA) and 7-aminoactinomycin D (Catalog No. A1310; Invitrogen, Grand Island, NY). Cell isolation was performed using a FACS Aria II (Becton Dickinson, Franklin Lakes, NJ). Epithelial cells were defined as CD31 $^{-}$ CD34 $^{-}$ CD45 $^{-}$ (i.e., Lin $^{-}$), EpCAM $^{+}$.

FITC–Dextran Permeability Analysis

Small-molecule permeability of the respiratory epithelial barrier was assayed using a modification of a reported method (Chen et al. 2014). FITC–dextran (3,000 MW; D3305, Life Technologies, Grand Island, NY) was dissolved in sterile saline and introduced to the airspace via oropharyngeal aspiration (55 µL, 5 mg/kg). After 1 h, mice were euthanized via intraperitoneal (i.p.) sodium pentobarbital injection, and blood was collected by cardiac puncture into EDTA-containing plasma separation tubes (BD). Serial dilutions of plasma were loaded into 96-well plates and analyzed for fluorescence intensity using a BioTek Synergy (Winooski, VT) plate reader and 485/528 nm filter.

RNA Isolation and RT-qPCR

RNA was isolated by RNEasy kit (Qiagen). Complementary DNAs (cDNA) were generated from 500 ng of purified RNA using TaqMan reverse transcription reagents from Applied Biosystems (Foster City, CA). Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was performed in triplicate with Taqman PCR Mix (Applied Biosystems) in the HT7900 ABI sequence Detection System (Applied Biosystems). Predesigned primers for Claudin-3 (Mm00515499_s1) and Claudin-18 (Mm00517321_m1) were purchased from Life Technologies. Gene expression was normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Mm99999915_g1).

Protein Measurement

TNF α ELISA was from BioLegend (San Diego, CA). ELISAs for IgM (immunoglobulin M) and albumin were from Bethyl. CC16 ELISA was from Novatein Biosciences (Woburn, MA). All ELISAs were conducted per the manufacturer's instructions.

Peripheral Blood Leukocyte Typing and Enumeration

Blood samples were analyzed using the HEMAVET 1700 hematology analyzer (Drew Scientific, Dallas, TX). Manual WBC (white blood cell) differential counts were reported and smear estimates used for confirmation.

Histology

Tissues were fixed in 10% neutral buffered formalin, trimmed, processed for paraffin embedding, sectioned (5 µm), and stained with hematoxylin & eosin. The slides were scanned using an Aperio AT2 slide scanner (Leica Biosystems, Buffalo Grove, IL) and images were captured using Aperio ImageScope.

Statistical Analysis

Experiments were conducted using six mice per treatment condition and were repeated two to three times except where indicated in figure legends. Statistical analysis was performed using Prism 6.0 software (Graphpad Software, Inc., La Jolla, CA). Statistical significance was determined using Student's *t*-test, analysis of variance (ANOVA) for comparisons of more than two groups, or

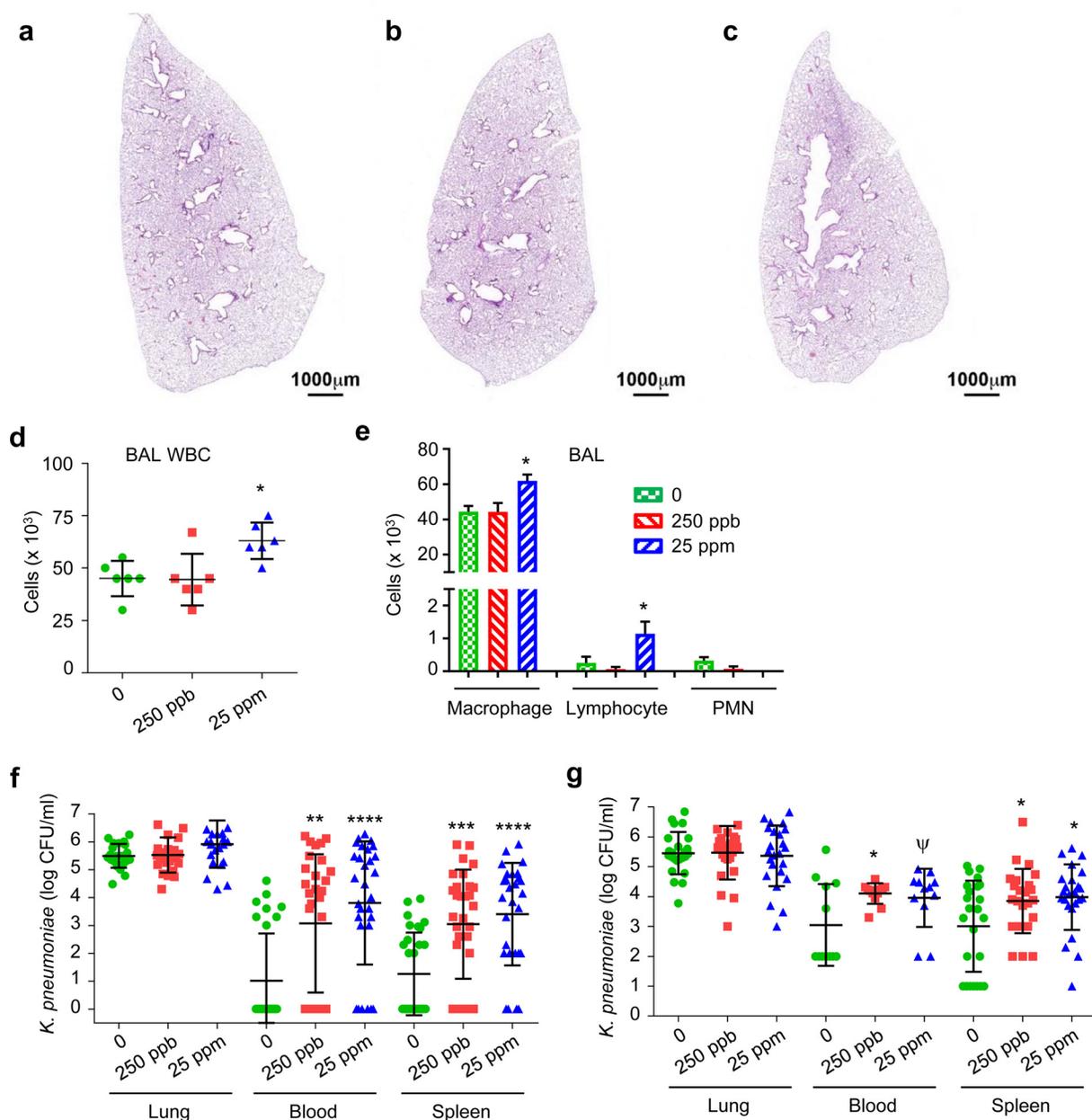


Figure 1. Intrapulmonary compartmentalization of bacteria following oral arsenic exposure. (a–c) Mice were exposed to 0 (a), 250 ppb (b), or 25 ppm (c) sodium arsenite via drinking water for 5 wk, and then lung histopathology was evaluated after hematoxylin and eosin staining. Results shown are representative of six mice per treatment. (d, e) BAL total WBC (d); and macrophages, lymphocytes, and neutrophils (PMN) (e) were counted in C57BL/6 mice after a 5-wk exposure to drinking water with 0, 250 ppb, or 25 ppm sodium arsenite ($n = 6/\text{treatment}$, representative of three independent experiments). (f) Mice exposed as in (d) were infected i.t. with 2,000 CFU *K. pneumoniae*. 24 h post-infection, bacterial burden was quantified in lung, blood, and splenic homogenate ($n = 23\text{--}30/\text{treatment}/\text{tissue}$). (g) The study design in f was repeated, except mice were fed AIN-93M low-arsenic diet instead of NIH-31 chow (mice in all panels other than this were fed NIH-31) ($n = 12\text{--}24/\text{treatment}/\text{tissue}$). (h) Mice exposed to water conditions shown were infected i.t. with $\sim 1 \times 10^6$ CFU *S. pneumoniae*, and then bacteria were quantified in the indicated tissues 24 h later ($n = 6/\text{treatment}$, representative of two independent experiments). (i) Survival was monitored following i.t. inoculation with 360 CFU *K. pneumoniae* in mice exposed to arsenic or tap water as shown ($n = 10/\text{treatment}$, repeated twice). Note: BAL, bronchoalveolar lavage; CFU, colony-forming units; i.t., intratracheal; PMN, neutrophil; WBC, white blood cell. Data shown are mean \pm SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$, $\psi p = 0.058$.

Mantel-Cox log-rank analysis (survival). Statistical significance was defined as $p < 0.05$.

Results

Effects of Oral Arsenic on Pulmonary and Extrapulmonary Bacterial Burden following Intratracheal Challenge

Arsenic has been associated with increased rates of pneumonia and with lung diseases such as bronchiectasis, tuberculosis, and

COPD that are characterized by chronic or recurrent bacterial lung infection (D'Ippoliti et al. 2015; George et al. 2015; Mazumder et al. 2005; Smith et al. 2011). Given this, we hypothesized that arsenic may compromise pulmonary host defense and reasoned that a murine model of bacterial pneumonia might provide insight into the underlying mechanisms. To our knowledge, the only studies to date that have tested the effect of peroral arsenic on lung infection *in vivo* have been in the specific setting of influenza A, where reduced viral clearance

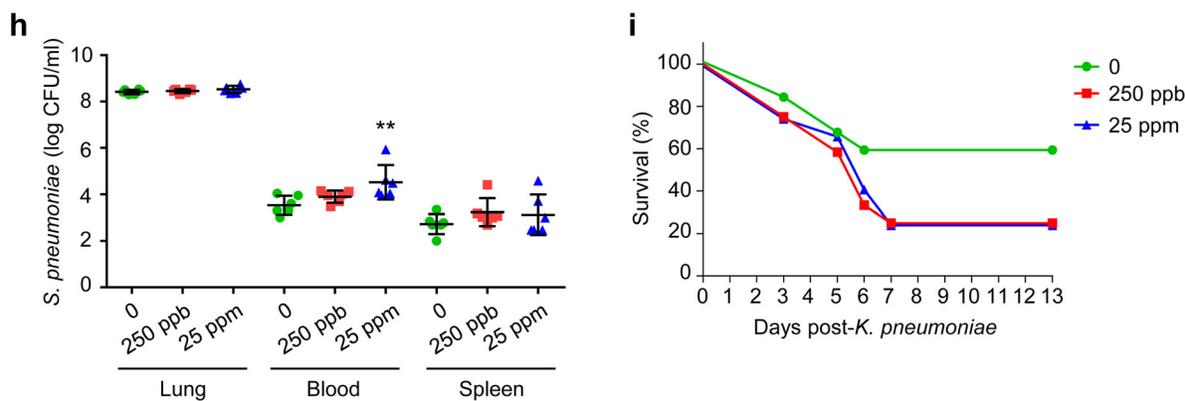


Figure 1. (Continued.)

from the lung has been noted (Kozul et al. 2009a; Ramsey et al. 2013).

In preliminary studies, we exposed C57BL/6 mice for 5 wk to drinking water with 0, 250 ppb, or 25 ppm sodium arsenite and then profiled immune cell populations in the airspace. No significant change was noted upon hematoxylin & eosin staining of the lungs of arsenic-exposed mice (Figure 1a–c). Mice exposed to 25 ppm but not 250 ppb of arsenic had a modest increase in airway total leukocyte count that was driven by an increase in alveolar macrophages and lymphocytes (Figure 1d, e).

We next challenged 5-wk arsenic-exposed mice intratracheally (i.t.) with the clinically relevant Gram-negative bacterium *Klebsiella pneumoniae* (Draper et al. 2010) and quantified bacteria in the lung and peripheral tissues. Arsenic had no effect on bacterial burden in the lung 24 h post-infection (Figure 1f), suggesting that it does not compromise pathogen killing in the airspace. Despite this, mice from both arsenic groups had markedly increased bacterial counts in blood and spleen (Figure 1f), indicating increased extrapulmonary bacterial dissemination. Consistent with our prior findings (Madenspacher and Fessler 2016), a substantial number of mice had no detectable extrapulmonary bacterial dissemination 24 h post-infection with *K. pneumoniae*, consistent with an as-yet unbreached alveolocapillary barrier. Consistent with the accelerated escape of bacteria from the arsenic-exposed lung, bacteremia was detected in 8/29 (0), 18/29 (250 ppb), and 23/29 (25 ppm) mice, and splenic bacteria were detected in 18/29 (0), 24/29 (0 ppb), and 25/27 (25 ppm) mice.

Given that our standard facility chow (NIH-31) was determined to have a total arsenic concentration of ~340 ppb, we

repeated the *K. pneumoniae* infection studies, substituting in the purified AIN-93M diet, which has <50 ppb arsenic (vendor data). As shown in Figure 1g, similar results, namely, increased extrapulmonary but unchanged pulmonary bacterial burden, were also found on the low-arsenic diet. Given this, all subsequent studies were conducted using NIH-31 chow.

In order to assess the generalizability of our findings to other bacterial pathogens, we next challenged arsenic-exposed and control mice i.t. with the Gram-positive bacterium *Streptococcus pneumoniae*. Although a much more modest effect and only statistically significant in the blood at 25 ppm, a similar finding of increased extrapulmonary bacterial burden was also noted (Figure 1h).

Given the enhanced extrapulmonary spread of bacteria we questioned whether arsenic exposure might reduce survival during pneumonia with the pathogen *K. pneumoniae*. Although a relative reduction in survival was noted in both arsenic groups starting after 5 d of pneumonia, this was not statistically significant (Figure 1i). Taken together, peroral arsenic exposure compromises compartmentalization of Gram-negative and Gram-positive bacteria to the infected airspace during pneumonia but does not significantly reduce survival in this setting.

Effects of Oral Arsenic on Innate Immune Responses in Lung and Systemic Circulation

To more directly evaluate whether peroral arsenic alters the local innate immune response within the lung, we next evaluated leukocyte recruitment to the airspace 24 h post-infection. Neutrophils (PMNs) are rapidly recruited from the circulation to

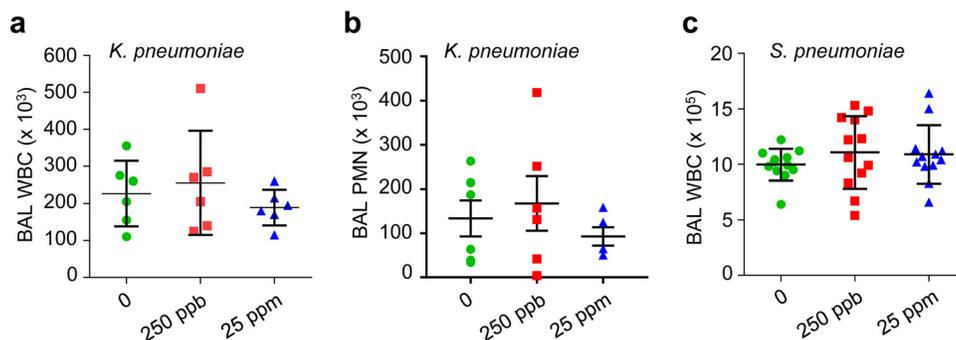


Figure 2. Leukocyte recruitment to the infected lung following oral arsenic exposure. Mice exposed for 5 wk to drinking water with the indicated arsenic content were infected i.t. with *K. pneumoniae* (a, b) or *S. pneumoniae* (c). BAL WBCs and PMNs were quantified 24 h post-infection as shown. Note: BAL, bronchoalveolar lavage; i.t., intratracheal; PMN, neutrophil; WBC, white blood cell. Data shown are mean ± SEM. Data in a, b derive from n = 6/treatment and are representative of two independent experiments. Data in c derive from n = 12/treatment.

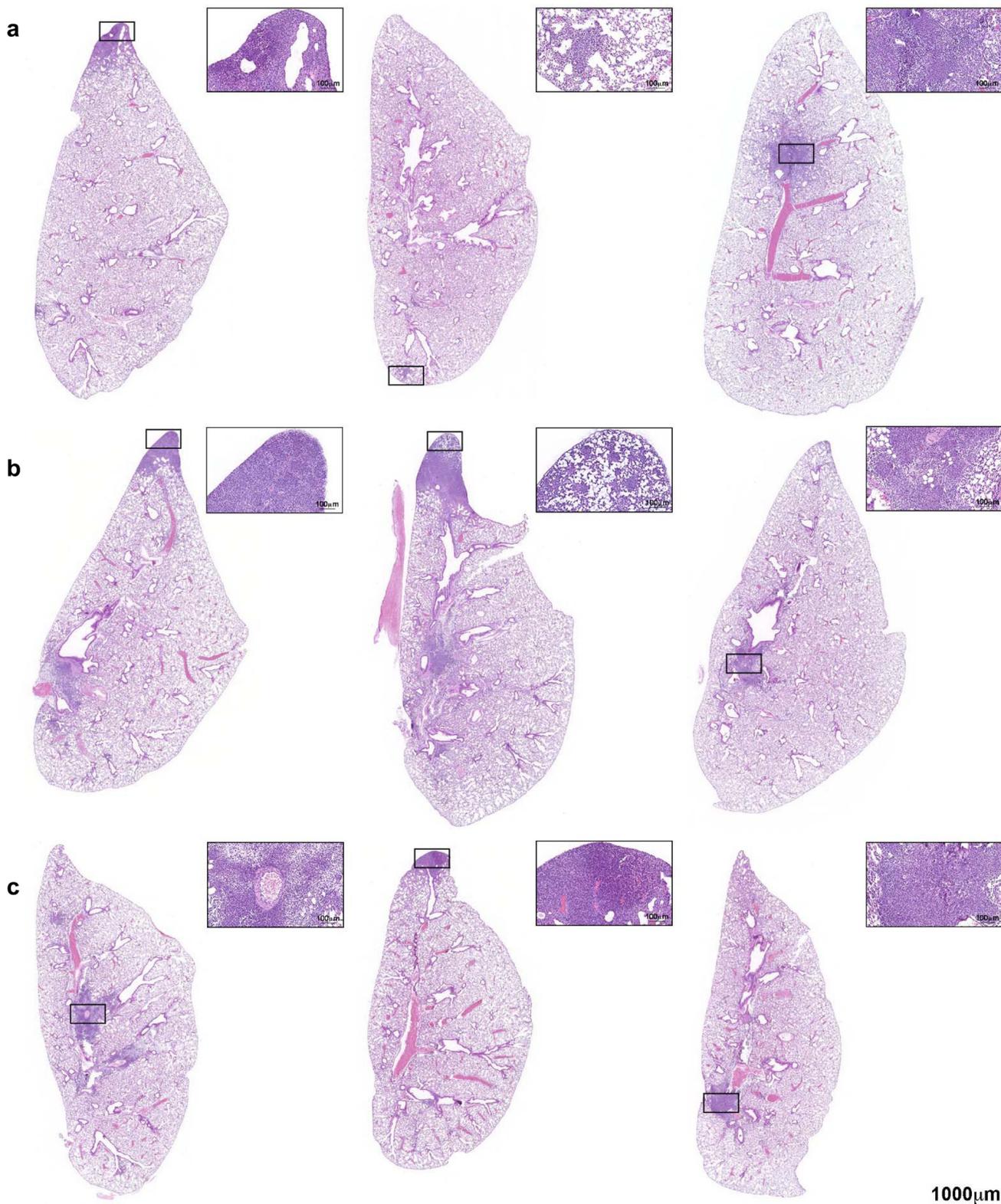


Figure 3. Lung histopathology during pneumonia following oral arsenic exposure. Mice were exposed to 0 (a), 250 ppb (b), or 25 ppm (c) sodium arsenite via drinking water for 5 wk and then infected in the lungs with 2,000 CFU *K. pneumoniae* via oropharyngeal aspiration. Twenty-four hours post-infection, lungs were fixed and stained with hematoxylin & eosin; lungs from three mice/condition are shown and are representative of $n=6$ /condition. Patchy, focal consolidative infiltration of neutrophils was observed in all mice. Note: CFU, colony-forming units. Scale bar applies to all lungs shown; insets are 20 \times original.

the infected airway by cytokines and chemokines produced by alveolar macrophages (Draper et al. 2010). In addition to playing a critical role in pathogen killing, PMNs cause bystander injury to

the respiratory epithelium and thereby increase permeability of the alveolocapillary barrier (Zemans et al. 2009). Arsenic exposure did not alter recruitment of total leukocytes (Figure 2a) or

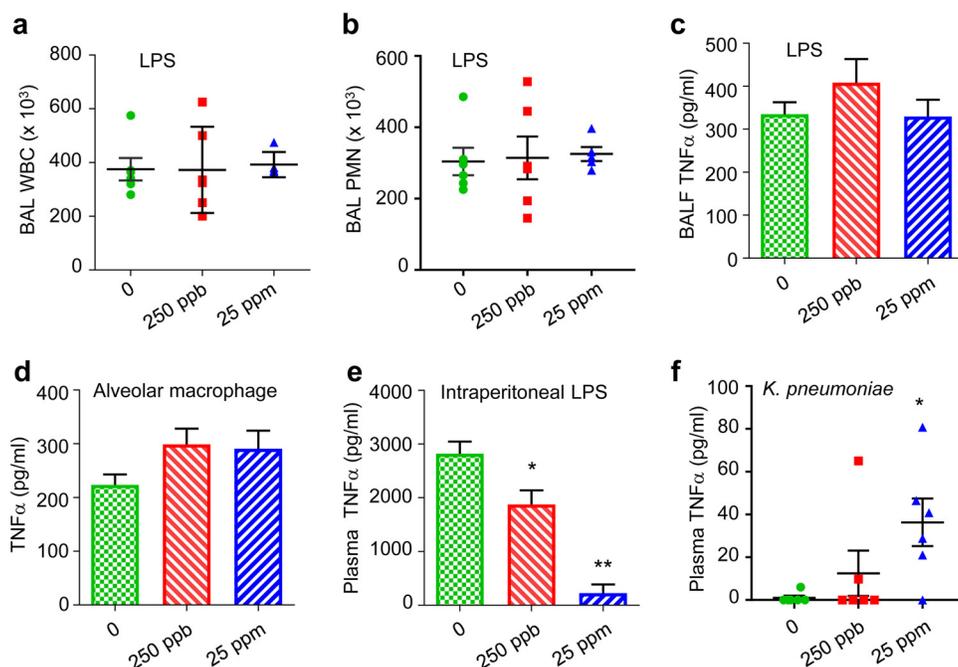


Figure 4. Intrapulmonary and systemic innate immune responses following oral arsenic exposure. (a–c) Mice maintained for 5 wk on drinking water with the indicated arsenic content were exposed to aerosolized LPS (300 $\mu\text{g}/\text{mL}$, 30 min). BAL total WBCs (a) and PMNs (b) were measured 24 h post-LPS, and BALF TNF α (c) was measured 2 h post-LPS. $n=6/\text{treatment}$; representative of two independent experiments. (d) Mice were treated with drinking water and inhaled LPS as in c, except that alveolar macrophages were lavaged 2 h post-LPS and then cultured overnight, after which time the culture media was assayed by ELISA for TNF α . $n=12/\text{treatment}$. (e) Mice were exposed to the indicated drinking water for 5 wk, injected i.p. with 0.5 mg/kg LPS, and then sacrificed for plasma TNF α measurement 90 min post-LPS. $n=6/\text{treatment}$. (f) Plasma TNF α was measured 24 h post-lung infection with *K. pneumoniae* in mice that had been exposed to the indicated drinking water conditions for 5 wk. $n=6/\text{treatment}$. Note: BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; i.p., intraperitoneal; LPS, lipopolysaccharide; PMN, neutrophil; WBC, white blood cell. Data shown are mean \pm SEM. * $p < 0.05$; ** $p < 0.0001$.

PMNs (Figure 2b) to the *K. pneumoniae*-infected airway. Similar findings were noted after lung infection with *S. pneumoniae* (Figure 2c). Histopathologically, *K. pneumoniae* delivered to the lung via oropharyngeal aspiration induced sparse, patchy neutrophilic infiltration, but there was no overt effect of arsenic on either the severity or nature of histological pneumonia (Figure 3). Together, this indicates that the acute cellular immune response in the airspace to Gram-negative and Gram-positive bacteria is unaltered by peroral arsenic, and also suggests that the excess bacterial dissemination in arsenic-exposed mice is unlikely to arise from increased cell-mediated inflammatory injury in the infected lung.

Lipopolysaccharide (LPS) is a glycolipid of the outer cell wall of Gram-negative bacteria that triggers the innate immune response by activating Toll-like Receptor 4 (TLR4) (Rosadini and Kagan 2017). LPS inhalation has been used widely to model the early kinetics of the innate immune response of the lung (Draper et al. 2010). We found that arsenic had no effect on recruitment of either total leukocytes (Figure 4a) or PMNs (Figure 4b) to the LPS-exposed lung. The concentration of TNF α in bronchoalveolar lavage fluid (BALF) of LPS-exposed mice was also unaltered by arsenic (Figure 4c). Further indicating that peroral arsenic has no overt effect on the local innate immune response of the airspace, alveolar macrophages harvested from arsenic-exposed and control mice after LPS inhalation generated equivalent TNF α *ex vivo* (Figure 4d). Collectively, peroral arsenic has no substantial effect on infection-induced leukocyte recruitment nor local TNF α induction within the inflamed airspace.

Next, to determine whether our findings were specific to the lung, we administered LPS via the i.p. route, a model of systemic sepsis. Intraperitoneal LPS triggers rapid induction of plasma cytokines that are thought largely to derive from splenic and

hepatic macrophages (Vollmar et al. 1996), cells that, unlike air-space macrophages, are directly exposed to portal venous drainage from the intestinal tract prior to first-pass metabolism. Of interest, we found a marked arsenic dose-dependent reduction in i.p. LPS-induced plasma TNF α (Figure 4e). Given the increased bacteremia in arsenic-exposed mice during lung infection with *K. pneumoniae* (Figure 1f), we also measured plasma TNF α in this setting as a readout of the systemic immune response to exacerbated sepsis. Mice exposed to 25 ppm arsenic exhibited a dramatic increase in plasma TNF α compared with controls (Figure 4f).

Effects of Oral Arsenic on Intravascular Clearance of Bacteria and Peripheral Blood Leukocyte Distributions

The increase in extrapulmonary bacteria in arsenic-exposed mice in the face of unchanged pulmonary bacterial burden suggested to us two mechanistic possibilities, that arsenic may a) impair killing of bacteria in the bloodstream after translocation from the airspace, and/or b) directly compromise the integrity (i.e., increase the permeability) of the pulmonary epithelial barrier to bacteria. Our finding of attenuated plasma cytokine induction after systemic LPS challenge, suggesting dysfunction of peripheral tissue macrophages and/or blood leukocytes, potentially supported the former possibility.

To formally test bloodstream killing of bacteria, we bypassed the lung, inoculating mice i.v. with a range of doses of *K. pneumoniae*, and then cultured the blood and spleen at different time points in order to profile bacterial clearance. Arsenic exposure did not alter bloodstream or splenic counts of bacteria 4 h after i.v. injection of 9.2×10^4 or 6.2×10^3 CFUs of *K. pneumoniae* (Figure 5a). Similarly, bloodstream bacterial burden was equivalent in arsenic-exposed and control mice 18 h after i.v. injection

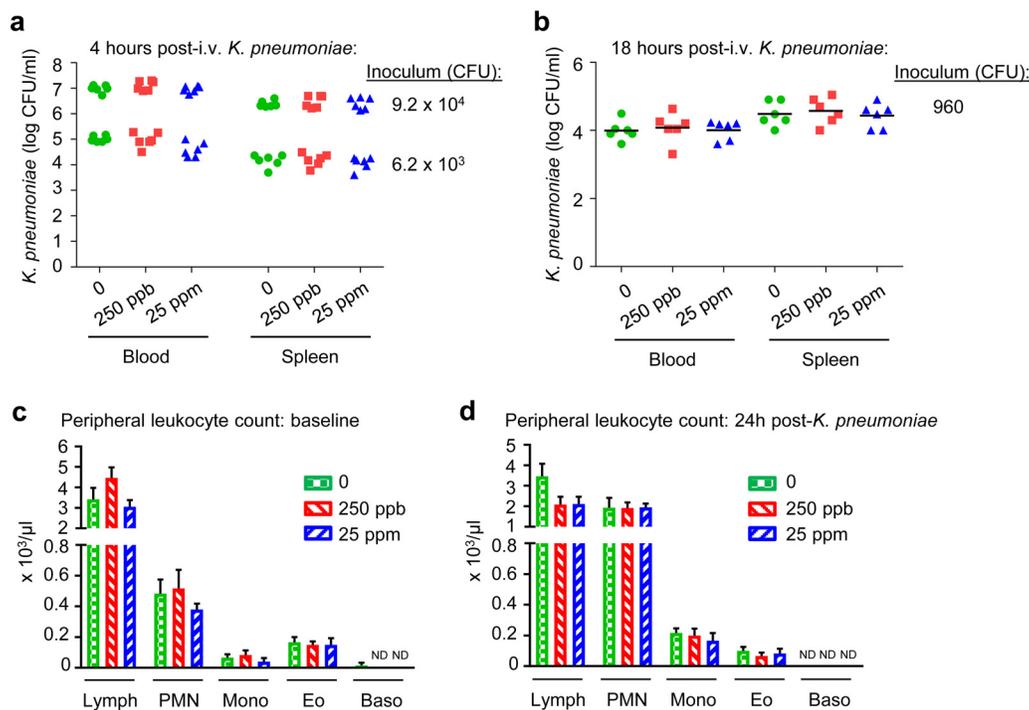


Figure 5. Intravascular *K. pneumoniae* clearance following oral arsenic exposure. (a, b) Mice maintained for 5 wk on drinking water with the indicated arsenic content were injected i.v. with 9.2×10^4 or 6.2×10^3 CFUs of *K. pneumoniae*. Bacteria were quantified in blood and spleen 4 h later (a). Alternatively, bacteria were quantified 18 h after i.v. injection of 960 CFUs (b). $n = 6$ /treatment/condition; representative of two independent experiments. (c, d) Circulating leukocytes were counted in arsenic water–exposed mice preceding (c) or 24 h following i.t. infection with 2,000 CFU *K. pneumoniae* (d). $n = 6$ /treatment; representative of two independent experiments. Data shown are mean \pm SEM. Note: baso, basophil; CFU, colony forming unit; eo, eosinophil; i.t., intratracheal; i.v., intravenous; lymph, lymphocyte; mono, monocyte; ND, not detected; PMN, neutrophil.

of 960 CFUs of *K. pneumoniae* (Figure 5b). Further, suggesting that arsenic does not compromise either basal or emergency granulopoiesis (i.e., release from the bone marrow of bactericidal PMNs into the bloodstream in response to bacteremia and cytokines), we found that the number of PMNs and other leukocyte subtypes in peripheral blood of mice in the steady state as well as 24 h post-i.t. *K. pneumoniae* was not altered in arsenic-exposed mice (Figure 5c, d).

Effects of Arsenic on Airspace-to-Plasma Translocation of Bacteria and Small Molecules

The finding of intact bacterial clearance in the bloodstream suggested to us that the increased peripheral bacterial burden in arsenic-exposed mice during pneumonia might instead arise from increased permeability of the airspace to pathogens. In support of this, we found evidence for arsenic dose-dependent acceleration of *K. pneumoniae* dissemination from the airspace during pneumonia. Specifically, at 12 h post-i.t. *K. pneumoniae*, a time point at which lung bacterial burden was equivalent across arsenic conditions and no bloodstream bacteria could yet be cultured in nonarsenic-exposed mice, 1 of 6 mice at 250 ppb and 4 of 6 mice at 25 ppm arsenic exposure ($p < 0.05$) had detectable bacteremia (Figure 6a).

During pneumonia, intra-alveolar bacteria must first transit the epithelial barrier and then the endothelium in order to access the bloodstream. Bacterial penetration may in principle either be paracellular, via penetration through intercellular tight junctions, or transcellular, following invasion and/or damage to cells. Measurement in BALF of the levels of two plasma proteins, albumin (MW, ~ 67 kDa) and IgM (MW, ~ 970 kDa in pentameric form), is commonly used as a metric of endothelial permeability in the lung to circulating medium-sized and large molecules,

respectively (Herrero and Matute-Bello 2015). BALF albumin and IgM were both increased after exposure of mice to inhaled LPS, as expected; however, levels were equivalent between control and arsenic-exposed mice in both the naïve and post-LPS condition (Figure 6b, c). This finding argues against overt compromise of the pulmonary endothelial barrier by peroral arsenic exposure.

We next evaluated the permeability of the respiratory epithelium using two independent approaches. CC16 is endogenously released into the airspace by epithelial Club cells; increased bloodstream levels of CC16 have been widely used as a biomarker indicating increased permeability of the respiratory epithelium (Lakind et al. 2007). Of interest, we found that peroral arsenic induced a dose-dependent increase in serum CC16 both in uninfected mice and in mice 24 h post-i.t. *K. pneumoniae* (Figure 6d). In a second approach designed to measure epithelial permeability to an exogenous marker under inflammatory conditions, we instilled FITC-labeled dextran i.t. 5 h post-LPS inhalation and then measured fluorescence intensity in the plasma 1 h later (Chen et al. 2014). Increased plasma FITC signal was detected in arsenic-exposed mice (Figure 6e), suggesting that arsenic also increases the permeability of the respiratory epithelium to inhaled foreign agents.

We questioned whether arsenic increases permeability by compromising epithelial cell viability. However, no increase in uptake of the nuclear dye 7-aminoactinomycin D (7-AAD) by total pulmonary epithelial ($CD31^- CD34^- CD45^- EpCAM^+$) cells was seen by flow cytometry in arsenic-exposed mice (Figure 7a). This suggests that either arsenic does not cause a lytic mode of respiratory epithelial cell death that is detectable in pooled total epithelial cells subjected to an enzymatic lung digest protocol, or that, if it does, the affected cells are homeostatically cleared *in vivo*.

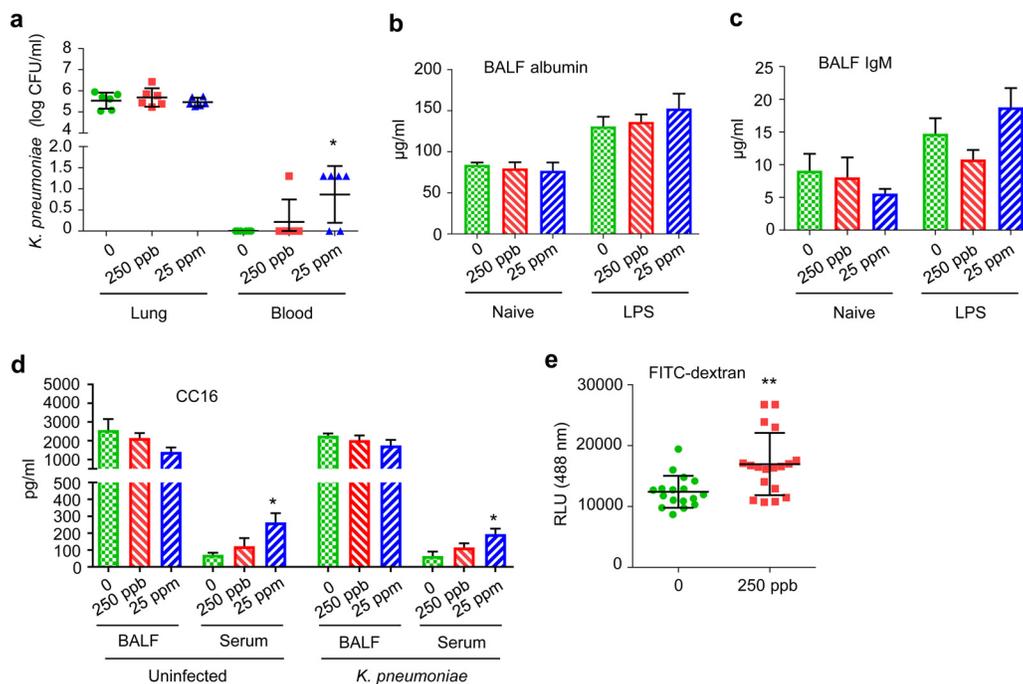


Figure 6. Effects of oral arsenic on markers of alveolocapillary barrier permeability. (a) Mice maintained for 5 wk on drinking water with the indicated arsenic content were infected i.t. with 2,000 CFU *K. pneumoniae*, and then sacrificed for lung and blood bacterial quantification 12 h later. $n=6$ /treatment/tissue. (b, c) Mice under the indicated drinking water conditions as in a were left untreated, or exposed to aerosolized LPS (300 µg/mL, 30 min) and sacrificed 24 h later. BALF albumin (b) and IgM (c) were then quantified by ELISA. $n=6-18$ /treatment. (d) Mice exposed to drinking water conditions shown were left uninfected or infected i.t. with 2,000 CFU *K. pneumoniae* for 24 h. BALF and serum CC16 were quantified. $n=6$ /condition; representative of three independent experiments. (e) Mice treated with drinking water conditions shown were exposed to aerosolized LPS. 5 h post-LPS, mice received i.t. FITC-dextran and then were sacrificed 1 h later for fluorescence detection in plasma. Note: BALF, bronchoalveolar lavage fluid; CFU, colony-forming unit; IgM, immunoglobulin M; i.t., intratracheal; LPS, lipopolysaccharide; RLU, relative light unit. $n=17-18$ /condition. Data shown are mean \pm SEM. * $p < 0.05$; ** $p < 0.01$.

The claudin family of tight junction proteins serve as key regulators of permeability in the lung (Koval 2013). Of particular importance in the terminal airspaces are claudin 18, an alveolar epithelial-specific protein that is the predominant claudin of alveolar epithelial type 1 (AT1) cells, and claudin 3, which is relatively enriched in alveolar epithelial type 2 (AT2) cells (Koval 2013; LaFemina et al. 2014). Permeability defects in a variety of lung diseases have been shown to arise from abnormal changes, upward and downward, in pulmonary claudin expression. Of interest, we found that arsenic exposure via drinking water induced a dose-dependent increase in claudin 18 mRNA in lung homogenates, whereas claudin 3 expression was unaltered (Figure 7b). This suggested that arsenic might be increasing epithelial permeability through intoxication of AT1 cells. Consistent with this, we found elevated BALF levels of podoplanin/T1 α protein, an established marker of AT1 injury (LaFemina et al. 2014), in arsenic-exposed mice (Figure 7c).

Discussion

In recent years, a growing number of epidemiologic reports have linked arsenic exposure via drinking water with a wide range of respiratory symptoms, signs, and diseases (D'Ippoliti et al. 2015; Mazumder et al. 2005; Steinmaus et al. 2016). In parallel, controlled exposure studies in rodents have suggested that the lung, perhaps due to its local arsenic metabolism and highly oxidizing microenvironment, may be uniquely susceptible to toxicity from orally ingested arsenic (Yamanaka et al. 1989). Indeed, arsenic has been shown to localize to the mouse lung within 1 h of a single oral administration and shows progressive accumulation upon repeated dosing (Hughes et al. 2003; Kenyon et al. 2005). To date, very few studies have been reported that address mechanisms by which oral arsenic modifies immune/inflammatory

responses in the lung *in vivo*. Direct exposure of cultured macrophages with *in vitro* arsenic, or of airway macrophages via inhaled arsenic, suppresses antimicrobial and inflammatory functions (Lantz et al. 1995). However, the susceptibility of lung cells to arsenic ingested orally—the route most relevant to global health—remains largely undefined.

Here, we provide evidence that *in vivo* arsenic exposure via the oral route elicits differential functional effects upon macrophages of the intrapulmonary versus extrapulmonary compartment. Thus, following LPS inhalation, alveolar macrophages from arsenic-exposed mice had intact cytokine induction associated with normal *in vivo* BALF concentrations of TNF α , whereas plasma TNF α following i.p. LPS, largely a product of hepatosplenic macrophages (Vollmar et al. 1996), was markedly attenuated in this setting. The latter finding is generally consistent with a prior report that i.p. challenge of mice with sodium arsenite reduces LPS-induced nitric oxide production by purified splenic macrophages (Sengupta and Bishayi 2002). Alveolar macrophages have a different ontogeny and immune phenotype than macrophages from other tissues (Guilliams et al. 2013). Future studies are thus warranted to better define whether pulmonary and extrapulmonary macrophages have different intrinsic susceptibility to arsenic. Alternatively, the microenvironments of the alveolus and peripheral tissues may expose local macrophages to different concentrations and profiles of arsenic metabolites and/or program differential susceptibility through other local factors.

Our finding that arsenic increases alveolar-to-plasma permeability of the lung to bacteria, endogenous proteins (CC16), and exogenous small molecules (FITC-dextran), and that this may arise from compromise to AT1 (and perhaps other epithelial) cells, suggests a novel mechanism by which orally ingested arsenic may interact with inhaled exposures to amplify lung injury and augment systemic penetration of airborne agents. Of interest,

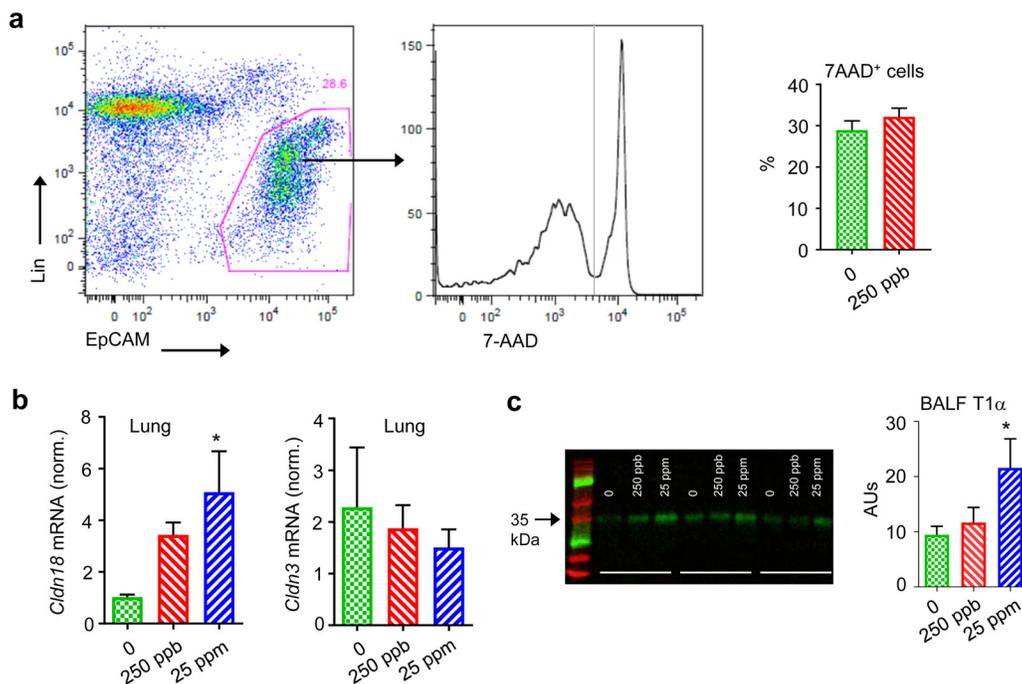


Figure 7. Effects of oral arsenic on pulmonary epithelial viability and injury. (a) Epithelial cells from lungs of arsenic-exposed and control mice were evaluated for viability by 7-AAD uptake. As shown by cytometry gating at left, epithelial cells were defined as Lin-negative (i.e., CD31⁻ CD34⁻ CD45⁻ EpCAM⁺). This cell population (circled in the cytometry gating plot) was then evaluated for 7-AAD uptake, as shown in the histogram at the middle of the panel. 7-AAD⁺ cells (rightmost peak in histogram) are quantified in the bar graph at right ($n=7$ /condition). (b) Claudin 18 and claudin 3 mRNA (normalized to GAPDH) was quantified in lung homogenates of mice following 5 wk of exposure to drinking water with the indicated arsenic content. $n=6$ /condition. (c) Ultracentrifuged BALF was assayed by immunoblot for T1 α , a marker of alveolar epithelial type 1 cell injury. Gel image shows BALF from nine independent mice, treated across the three conditions shown. T1 α signal densitometry is quantified in the bar graph at the right. Note: AAD, 7-aminoactinomycin D; AU, arbitrary unit; BALF, bronchoalveolar lavage fluid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Lin, lineage. Data shown are mean \pm SEM. * $p < 0.05$.

a few clinical studies have provided indications that oral arsenic may injure the alveolar epithelium in humans. Thus, sputum levels of receptor for advanced glycation end-products (RAGE), a protein with high expression in AT1 cells, are reduced in patients with elevated urinary inorganic arsenic and are negatively correlated with the concentration of methylarsenic metabolites in exposed humans (Olivas-Calderón et al. 2015).

CC16 (a protein of pulmonary epithelial origin that is released into the airway lumen) has a complex relationship with environmental exposures. Although elevated plasma CC16 has been interpreted as a biomarker of increased respiratory epithelial permeability after acute lung insults (Lakind et al. 2007), reduced plasma CC16 has been interpreted as a biomarker of Club cell (i.e., epithelial) toxicity, typically in the setting of chronic injurious exposures (Beamer et al. 2016). Of interest, reduced plasma and urinary CC16 have been found in humans with chronic arsenic exposure (Beamer et al. 2016; Parvez et al. 2008). We speculate that a more prolonged arsenic exposure than 5 wk (the protocol used in the present study), or perhaps *in utero* exposure, as is presumed to occur in human populations (Rahman et al. 2011), may have instead caused progressive Club cell injury and resultant reduction in BALF and serum CC16. Future studies are warranted to better define the kinetics and reversibility of this biomarker in experimental oral arsenic exposure. Serum CC16 could potentially serve as a useful indicator of acute versus chronic injury to the respiratory epithelium in human populations, both during exposure and environmental mitigation.

In vitro studies have begun to suggest mechanisms by which arsenic may compromise the respiratory epithelium. Arsenic treatment of primary mouse tracheal epithelial and immortalized human bronchial epithelial cells reduces transepithelial electrical resistance, a measure of barrier function, and also alters

expression of claudins (Sherwood et al. 2013b), similar to the findings in the present study. Arsenic also attenuates wound-induced purinergic calcium signaling and wound repair in respiratory epithelial cells *in vitro* (Sherwood et al. 2011, 2013a). It has been suggested that arsenic may either alter purinergic receptor function through direct binding to protein thiols (Hughes et al. 2011), or indirectly compromise wound repair (Olsen et al. 2008) and impair tight junction barrier function (Vermeer et al. 2009) through induction and activation of epithelial matrix metalloproteinase-9. Additional mechanisms revealed by *in vitro* studies by which arsenic may potentially compromise pulmonary epithelial cells include degradation of cystic fibrosis transmembrane conductance regulator (Bomberger et al. 2012), DNA damage (Xie et al. 2014), glycolytic reprogramming (Zhao et al. 2013), and proapoptotic effects on mitochondria (Larochette et al. 1999).

Because aquaporins promote arsenic uptake into cells (Roggenbeck et al. 2016) and are highly expressed by the alveolar epithelium (Kreda et al. 2001), it is intriguing to consider that these channel proteins may, at least in part, underlie the apparent susceptibility of respiratory epithelia to arsenic. Given that arsenic-induced cellular transformation has been associated with epithelial–mesenchymal transition (Li et al. 2011; Xu et al. 2012), it seems plausible that the capacity of arsenic to disorder the epithelial barrier and to induce neoplasms of epithelial origin may possibly reflect different stages on a spectrum of molecular pathogenesis in the lung.

Optimal dosing and administration of arsenic in rodent models of human disease have remained somewhat controversial (States et al. 2011). Although the environmental relevance of the high arsenic dose used in the present study (25 ppm) may be questioned, ppm-range arsenic levels have been documented in drinking water outside the United States (Das et al. 1995).

Moreover, mice have much higher capacity for arsenic metabolism than humans, and 50 ppm oral arsenic in rodents has been shown to achieve arsenic concentrations in liver tissue similar to those documented in human subjects from high arsenic-exposure regions (States et al. 2011). The major findings in our study, including increased bacterial dissemination and *in vivo* epithelial permeability, were observed with 250 ppb arsenic. The relevance of the findings observed only at the 25 ppm dose [i.e., increased steady-state airway leukocytes (Figure 1), *S. pneumoniae* bacteremia (Figure 1), plasma TNF α after *K. pneumoniae* infection (Figure 4), and BALF T1 α (Figure 7)] is uncertain, and caution is thus warranted in interpreting these results. Whether lower doses (e.g., <10 ppb, the current U.S. EPA standard [epa.gov]) would induce similar changes is an interesting question worthy of further study. Prior reports have in fact documented significant changes in gene expression and immune response in mouse lung at these very low levels (Kozul et al. 2009a, 2009b). Our studies also raise the important possibility that background arsenic in the widely used NIH-31 diet may be an underappreciated variable in rodent studies that involve the pulmonary epithelium. Because our studies were limited to female mice, future studies are warranted to determine whether arsenic has similar or distinct effects in male mice.

Conclusions

We report for the first time that orally ingested arsenic compromises the respiratory barrier to inhaled pathogens and exogenous small molecules and provide evidence that this effect may stem, at least in part, from compromise to the lung epithelium. Respiratory epithelial barrier integrity has been shown to be pivotal to the pulmonary innate and adaptive immune response (Georas and Rezaee 2014), and to play a role in regulating atopic (Schuijs et al. 2015), fibrotic (Barkauskas and Noble 2014), and reparative (Liang et al. 2016) events in the lung, as well as translocation of air pollutants into the systemic circulation (Thorley et al. 2014). Taken together, we speculate that orally ingested arsenic may possibly interact synergistically with a wide range of inhaled exposures to promote pulmonary and perhaps systemic diseases of great public health significance.

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