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Arsenic alters transcriptional responses to *Pseudomonas aeruginosa* infection and decreases antimicrobial defense of human airway epithelial cells

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Abstract

Arsenic contamination of drinking water and food threatens the health of hundreds of millions of people worldwide by increasing the risk of numerous diseases. Arsenic exposure has been associated with infectious lung disease in epidemiological studies, but it is not yet understood how ingestion of low levels of arsenic increases susceptibility to bacterial infection. Accordingly, the goal of this study was to examine the effect of arsenic on gene expression in primary human bronchial epithelial (HBE) cells and to determine if arsenic altered epithelial cell responses to *Pseudomonas aeruginosa*, an opportunistic pathogen. Bronchial epithelial cells line the airway surface, providing a physical barrier and serving critical roles in antimicrobial defense and signaling to professional immune cells. We used RNA-seq to define the transcriptional response of HBE cells to *Pseudomonas aeruginosa*, and investigated how arsenic affected HBE gene networks in the presence and absence of the bacterial challenge. Environmentally relevant levels of arsenic significantly changed the expression of genes involved in cellular redox homeostasis and host defense to bacterial infection, and decreased genes that code for secreted antimicrobial factors such as lysozyme. Using pathway analysis, we identified Sox4 and Nrf2-regulated gene networks that are predicted to mediate the arsenic-induced decrease in lysozyme secretion. In addition, we demonstrated that arsenic decreased lysozyme in the airway surface liquid, resulting in reduced lysis of *Micrococcus luteus*. Thus, arsenic alters the expression of genes and proteins in innate host defense pathways, thereby decreasing the ability of the lung epithelium to fight bacterial infection.

Graphical abstract

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Conflict of interest

The authors declare that we have no conflicts of interest to disclose

Keywords

arsenic; lung; RNA-seq; immunity; lysozyme

Introduction

Arsenic has been identified as a primary environmental contaminant of concern by the World Health Organization and the Agency for Toxic Substances and Disease Registry because of its widespread presence in drinking water and pleiotropic effects on human health (ATSDR, 2007). It is estimated that over 200 million people worldwide are exposed to drinking water containing arsenic above the WHO and U.S. EPA recommended maximum contaminant level (MCL) of 10 µg/L (Naujokas et al., 2013; World Health Organization, 2006). Exposure to high levels of arsenic in drinking water, which exceed 1000 µg/L as a result of naturally occurring sources in regions of Asia, South America and other parts of the world, is associated with increased risk of bronchiectasis, pneumonia, tuberculosis and other infectious lung diseases (Mandal and Suzuki, 2002; Parvez et al., 2013; Smith et al., 2006, 2011). However, until recently, association of lower levels (<100 µg/L) of arsenic exposure with lung disease had not been reported.

In the United States, approximately 3 million people drink well water containing arsenic levels above 10 µg/L, but concentrations up to 1000 µg/L have been detected in private wells, which are not subject to public water supply regulations (ATSDR, 2007). A growing body of epidemiological studies have identified an association between low to moderate arsenic exposure in drinking water and adverse effects on immune and respiratory function (Bailey et al., 2016; Carlin et al., 2016; Fry et al., 2007; Olivas-Calderón et al., 2015). In New Hampshire, increased respiratory infections were observed in infants exposed to arsenic *in utero* (Farzan et al., 2013, 2016). However, an adverse outcome pathway linking cellular mechanisms of arsenic with increased incidence of lung infection has not been established. Thus, the goal of this study was to identify gene expression changes caused by arsenic in lung epithelial cells that modulate susceptibility to bacterial infection.

Humans are exposed to arsenic in water and some foods, including rice and rice based products. In drinking water, arsenic is present in the inorganic forms of arsenite (iAs^{III}) and arsenate (iAs^V). In the human body, inorganic arsenic is metabolized by the liver through a sequential process involving reduction to trivalent intermediates and oxidative methylation to stable metabolites including monomethylarsonic acid (MMA^V) and dimethylarsinic acid (DMA^V) (Vahter, 2002). Foods such as rice and fruits can contain both inorganic and organic forms of arsenic (Jackson et al., 2012; Jara and Winter, 2014). This may be of

particular concern for infants, who are exposed via rice cereals and formula; action levels for arsenic in rice cereal and apple juice have only recently been established by the US FDA (Carignan et al., 2015; Nachman et al., 2017). Most people therefore ingest a mixture of arsenic species in food and water, which are metabolized via a series of intermediates before excretion in the urine. For individuals whose drinking water contains less than 10 µg/L arsenic, diet is the primary route of arsenic exposure, whereas when drinking water contains more than 10 µg/L arsenic, water becomes the dominant route of exposure (Kurzius-Spencer et al., 2013). Arsenic exposure in water and food can result in levels of total arsenic as high as ~75 µg/L in serum, in a ratio of iAs (7–52%), MMA (18–39%), and DMA (33–67%) (Hall et al., 2007). Thus, in this study we exposed human bronchial epithelial (HBE) cells to a biologically relevant mixture of arsenic species (5, 10 or 50 µg/L in a ratio of iAs (25%), MMA (25%), and DMA (50%)).

Little is known about the effects of organic arsenic species on immune function at concentrations relevant to human populations, but several studies have demonstrated that low concentrations of iAs^{III} in drinking water decrease the ability of animals to clear lung infections. Kozul et al. found that mice exposed to 100 µg/L iAs^{III} in drinking water were unable to clear influenza infection (Kozul et al., 2009a). In utero exposure to 100 µg/L iAs^{III} in drinking water disrupted expression of immune genes, altered lung development, and decreased mucociliary function in mice (Ramsey et al., 2013). In zebrafish, 10 µg/L iAs^{III} induced aberrant gene expression and significantly reduced clearance of bacterial and viral infections (Nayak et al., 2007). The mechanism by which arsenic increases susceptibility to bacterial and viral lung infections is not well understood. In human lung epithelial cells, arsenic induces degradation of the cystic fibrosis transmembrane regulator (CFTR) protein, which is important for maintaining fluid balance in the lung and mucociliary clearance of pathogens (Bomberger et al., 2012). Arsenic has additionally been shown to inhibit wound repair, and block autophagy in lung epithelial cells, leading to accumulation of p62 and activation of Nrf2, which regulates many antioxidant response genes (Lau et al., 2013b; Sherwood et al., 2013). While these pathways have been associated with development of lung cancers, it is not known how they affect the cellular responses to pathogens. In a previous study we showed that exposure to MMA^{III} alone, and a mixture of arsenic species (iAs (25%), MMA (25%), and DMA (50%)), altered the cytokine response of primary human bronchial epithelial cells to *Pseudomonas aeruginosa*, an important respiratory pathogen in individuals with chronic lung diseases (Notch et al., 2015).

In this study, we used a whole transcriptome approach to identify innate immune pathways in HBE cells exposed to a mixture of arsenic species at three concentrations relevant to human exposures. We examined how arsenic affects the response of HBE cells to *Pseudomonas aeruginosa*, a Gram negative, opportunistic pathogen that infects the lungs of immune-compromised individuals and is one of the most common bacterial species detected in patients with cystic fibrosis, pneumonia, bronchiectasis and chronic obstructive pulmonary disease (COPD) (Aksamit et al., 2017; Lavoie et al., 2011; Murphy et al., 2008; Tsai et al., 2017). In experiments with primary HBE cells from 6 donors, we show that arsenic induced a consistent increase in oxidative stress response genes, while decreasing expression of host defense response genes, including antimicrobial proteins. The gene expression changes were accompanied by a significant decrease in lysozyme secretion by

HBE cells. In addition, we demonstrated that the reduction in lysozyme decreased the bacteriolytic activity of the airway surface liquid. Lysozyme is one of the most abundant antimicrobial proteins in the lung, secreted by lung epithelial cells and macrophages, and provides important protection from bacterial infection (Dajani et al., 2005; Konstan et al., 1981). This is the first report of arsenic decreasing antimicrobial secretion by human cells, which is predicted to compromise a critical barrier function in the lung, thereby increasing chronic lung infections.

Methods

Chemicals

Sodium (meta)arsenite and dimethylarsinic acid were purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in distilled, ultrapure water to make 133 μM (10 mg/L) iAs^{III} and DMA^{V} stocks, respectively. Diiodomethylarsine (MMA^{III}) was synthesized by the Synthetic Chemistry Facility Core (Southwest Environmental Health Science Center, Tucson, AZ) according to previously published methods (Escudero-Lourdes et al., 2010; Millar et al., 1960) and dissolved in distilled, ultrapure water to make a 66.7 μM (5 mg/L) MMA^{III} stock. All stock solutions were stored at -20°C and concentrations verified by the Dartmouth Trace Elements Analysis Core by ICP-MS. Fresh dilutions were made in cell culture media for each experiment.

Cell culture and exposures

Primary human bronchial epithelial (HBE) cells from 6 non-diseased donors (2 male, ages 23–40, and 4 female, ages 23–75) were obtained from the UNC CF/Pulmonary Research and Treatment Center Tissue Procurement and Cell Culture Core (Chapel Hill, NC) and maintained at 37°C with 5% CO_2 as described previously (Fulcher and Randell, 2013). Passage 1 epithelial cells were expanded in Bronchiallife epithelial airway medium (Lifeline Cell Technology, Frederick, MD) supplemented with Penicillin/Streptomycin and seeded onto 12 or 24 mm clear Transwell 0.4 μm filters (Corning, New York, NY) coated with type IV human placental collagen (Sigma-Aldrich). Once a confluent monolayer was obtained, apical media was removed and cells were cultured at an air-liquid interface (ALI), and fed basolaterally every other day with ALI medium (LHC:Basal/DMEM-H, provided by the UNC TPC as described by Fulcher and Randell) for three to four weeks (Fulcher and Randell, 2013). Filters were rinsed with PBS weekly to remove excess mucus, and ciliary movement was visually confirmed before the beginning of exposure to arsenic. Polarized HBE cells were exposed to 0, 5, 10 or 50 $\mu\text{g/L}$ total arsenic in basolateral Bronchiallife media for 6 days, with media replaced every other day. Arsenic exposure concentrations were chosen to reflect concentrations measured in serum of US and Bangladeshi populations drinking water with 10–600 $\mu\text{g/L}$ arsenic (Hall et al., 2007; Sanders et al., 2012). Exposures were conducted with a mixture representative of arsenic speciation in human serum; 25% iAs^{III} , 25% MMA^{III} and 50% DMA^{V} as described previously (Hall et al., 2007; Notch et al., 2015).

Pseudomonas aeruginosa

A laboratory strain of *Pseudomonas aeruginosa* (PAO1) was cultured overnight at 37°C in Luria broth medium (Invitrogen, Grand Island, NY). *P. aeruginosa* cultures were washed three times in antibiotic-free cell culture media and the concentration determined using OD (600 nm). 24 hours prior to *P. aeruginosa* exposures, HBE cells were rinsed with PBS and the media was changed to antibiotic free ALI media. *P. aeruginosa* was resuspended in antibiotic free ALI media and 10⁷ colony forming units (MOI of 20) were added to the apical side of the monolayers and incubated for six hours as described previously (Moreau-Marquis et al., 2010). HBE cells were then rinsed with PBS, and cells homogenized in RNazol RT (Sigma-Aldrich) for total RNA isolation. Basolateral media was collected and stored at -80°C for analysis of lactate dehydrogenase (LDH) activity.

Intracellular arsenic detection

HBE cells polarized on 12-mm Transwell filters were exposed to 0, 5, 10 or 50 µg/L total arsenic in the basolateral media for 6 days as described above. After 6 days of exposure, the basolateral media was removed, filters were rinsed with PBS, and HBE cells were removed from filters by brief trypsinization. Cells were collected into soybean trypsin inhibitor and washed 3 times with PBS, with cells from two filters combined for each sample. Cells in each sample were counted with a BioRad TC20 automated cell counter, PBS was removed, cell pellets were weighed to obtain wet weight and stored at -80°C. Cell pellets were acid digested at the Dartmouth Trace Elements Analysis Core and total intracellular arsenic was detected by ICP-MS (Agilent 79000 and 8800) following U.S. EPA 200.8 but using He as a collision gas. The instrument was calibrated using National Institute of Standards and Technology (NIST) traceable standards.

LDH release

To measure cytotoxicity of HBE cells in response to arsenic and *P. aeruginosa*, lactate dehydrogenase (LDH) was detected in cellular media with the Cytotox 96 Non-radioactive cytotoxicity assay per manufacturer instructions (Promega, Madison, WI). Optical density (OD, 490 nm) was measured on a Biotek plate reader and is expressed as a percent of lysed HBE cell control (lysed with 0.1% Triton-X). To determine the effect of arsenic exposure on LDH release over time, aliquots of basolateral media (100 µl) were removed from HBE cells exposed to 50 µg/L total arsenic every 24 h. Aliquots were taken before feeding on days 2 and 4. To identify potential cytotoxicity resulting from combined exposure to arsenic and *P. aeruginosa*, basolateral media was collected from all arsenic treatment groups on day 6 following 6-hour exposure to *P. aeruginosa* or control.

RNA-Seq

RNA was isolated from HBE cells using RNazol RT per manufacturer instructions, and quantified using a Nanodrop spectrophotometer. The quality of each sample was analyzed on a Bioanalyzer with the RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA). RNA Integrity values for all samples were > 9. Libraries were prepared from 48 samples using Illumina Tru-seq stranded mRNA kits at the University of New Hampshire Hubbard Center for Genome Studies. 100 bp paired-end sequencing was conducted with an Illumina

Hi-Seq 2500 sequencer. We obtained 20–32 million reads per sample (Table S1). Reads were aligned to the human genome assembly GRCh38.82 downloaded from Ensembl in CLC Genomics Workbench Qiagen, Germantown, MD), and count tables of reads aligned to each gene were exported for further analysis with the R software environment for statistical computing and graphics (R Core Team, 2014). Data were submitted to the Gene Expression Omnibus database, Accession GSE97036.

RNA-Seq analysis in EdgeR

Count tables of reads aligned to each gene were analyzed in R using the EdgeR package (McCarthy et al., 2012; Robinson et al., 2010). Genes with very low counts across all libraries were removed from the dataset; genes expressed above a threshold of two counts per million (cpm) in at least 6 samples were included in further analysis. Libraries were normalized in EdgeR using the trimmed mean of M-values (TMM), and the Cox-Reid profile-adjusted likelihood (CR) method was used to estimate tagwise dispersion for generalized linear models (GLM). We tested for differentially expressed genes using the GLM likelihood ratio test with arsenic, *P. aeruginosa* and donor included as factors in the model. An interaction term was included to test for interactions between arsenic and *P. aeruginosa*. We modeled the data both with arsenic concentration as a continuous variable, and with arsenic as a discrete variable to identify significant genes that do not exhibit a linear dose response. Log2 fold change values for heatmaps were generated using the model with arsenic as a discrete variable, so that fold changes at individual arsenic concentrations could be visualized. Genes and sample groups were clustered for heatmap visualization using the Pearson correlation distance.

Gene Ontology and Pathway Analysis

Gene expression (fold change) and p values from the EdgeR analysis were analyzed using Goseq in R (Young et al., 2010) to identify biological processes of genes that exhibited similar expression patterns (enrichment $P < 0.05$). We used Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity) to predict biological networks, canonical pathways and upstream regulators. For the arsenic effect, we included genes that were significant with $P < 0.05$ and $\log_2 \text{FC} > .01$ per $\mu\text{g/L}$ arsenic. This resulted in 90 genes included in the analysis. The upstream analysis function was used to predict transcriptional regulators with an activation z score of 2 or greater and p value of overlap < 0.01 . For the *P. aeruginosa* effect, we included genes significant at $P < 0.05$ and a $\log_2 \text{FC} > 1$.

QPCR

RNA expression changes of a subset of significant genes identified in the RNA-seq study were confirmed in a separate set of experiments with HBE cells from three donors exposed to arsenic as described above. cDNA was generated from 1 μg of RNA using RetroscriptRT reverse transcriptase (Ambion, Austin TX). Taqman Gene Expression assay probes for *HMOX1*, *FTH1*, *LYZ*, *CRISP3*, *CAMP* and *GUSB* were purchased from Thermo Fisher Scientific. Triplicate quantitative RT-PCR (QPCR) reactions using Taqman universal master mix were amplified using an ABI StepOne thermocycler with the following cycling parameters: 95°C for 10 min, 40 cycles of 15 sec at 95°C and 1 min at 60°C. Gene expression changes were calculated using the delta Ct method with *GUSB* as a

housekeeping gene. We confirmed that neither treatment nor donor differences caused significant changes in *GUSB*. No amplification of product was observed in no-template and no-RT controls.

Lysozyme concentration and antimicrobial activity in airway surface liquid (ASL)

Polarized HBE cells were grown on Transwell filters and exposed to arsenic as described above for 6 days. 48 hours prior to ASL collection (day 4 of arsenic exposure), HBE cells were transferred to antibiotic-free ALI media, with arsenic exposure maintained as specified above. On day 5, the apical surfaces of filters were rinsed, basolateral media refreshed, and 300 μ L of ALI media was added to the apical surface. Filters were incubated for 24 hr. and apical and basolateral media were collected for analysis of secreted proteins. HBE cells were collected for RNA as described previously. ASL was immediately frozen at -80°C until analysis. Lysozyme concentrations in ASL were measured by ELISA (Abcam) according to the manufacturer's instructions. In order to determine the antibacterial activity of ASL collected from the HBE cells, lysis of *Micrococcus luteus* was measured with a turbidimetric assay based on published procedures with slight modifications (Lee and Yang, 2002; Shugar, 1952). Lyophilized *Micrococcus luteus* (*M. lysodeikticus*, Sigma Aldrich) were resuspended at 2.5 mg/mL in 66 mM phosphate buffer pH 6.25, vortexed and allowed to equilibrate for 30 min. 50 μ L of *M. luteus* suspension was then mixed with 50 μ L ASL, and optical density at 450 nm was measured once per minute for 1 hr with shaking on a Biotek plate reader. The OD_{450} was calculated by regression fit to the linear response in R. One unit of lysozyme activity was defined using the Shugar unit (decrease in OD_{450} of $.001 \text{ min}^{-1}$) (Shugar, 1952).

Statistics

Statistical analysis of LDH, QPCR, ELISA and lysozyme activity data was conducted in R with the nlme and base packages (R Core Team, 2014). Experiments were analyzed using mixed effect linear models with donor as a random effect. Data visualizations were created with the ggplot2 package (Wickham, 2009).

Results

Arsenic (0–50 $\mu\text{g/L}$) accumulates in HBE cells but is not cytotoxic

We first determined the extent to which the arsenic mixture in the basolateral media was taken up by polarized HBE cells, and measured LDH release to examine if the exposures were cytotoxic. We measured total arsenic in cell pellets and media following 6 days of exposure to 0, 5, 10 or 50 $\mu\text{g/L}$ total arsenic (as 25% iAs^{III} , 25% MMA^{III} and 50% DMA^{V}) via ICP-MS. Mean total arsenic detected in media was 0.1, 5.1, 10.9 and 52.5 $\mu\text{g/L}$ (Figure 1A). Arsenic accumulated in HBE cells in a concentration-dependent manner, where mean arsenic wet weight in cell pellets was 3.1, 27.4, 70.5 and 290.9 ng/g wet weight (Figure 1A).

We and others have previously shown that the range of exposures used in this are well below arsenic levels that induce cytotoxicity in HBE cells (Notch et al., 2015; Xie et al., 2014). To confirm that the effects of arsenic observed in this study were not due to cell death, we measured lactate dehydrogenase (LDH) release into basolateral media during the 6-day

arsenic exposure and following apical exposure to *P. aeruginosa*. Assessment of daily LDH release during the 6-day exposure to 50 µg/L total arsenic showed no significant difference between arsenic-exposed and control cells (Figure 1B). Cell counts determined on day 6 confirmed that arsenic exposure did not decrease cell number (Table S2). We measured LDH release following the 6 hour challenge with *P. aeruginosa* on day 6. None of the arsenic exposure concentrations significantly altered LDH release, alone or in combination with *Pseudomonas*. Exposure to *P. aeruginosa* induced a slight increase in LDH release, which was significant but less than 1% of the lysed control (Figure 1C). Together, these data show that while arsenic is readily accumulated by polarized HBE cells, the low concentrations used in this study do not induce cytotoxicity.

Arsenic effect on transcriptional responses of HBE cells to *Pseudomonas aeruginosa*

RNA-seq analysis of gene expression was used to identify the transcriptional response of HBE cells to *P. aeruginosa*, and to investigate how *P. aeruginosa*-induced pathways in HBE cells were affected by arsenic. *P. aeruginosa* or vehicle were added to the apical surface of polarized HBE cells exposed to 0, 5, 10 or 50 µg/L arsenic, and samples were collected for RNA-seq 6 hours later. In total, 20–32 million reads were obtained per sample. 92% (± 0.92 SD) of the sequences aligned to *Homo sapiens* genome version GCh38 (Table S1). At this sequencing depth, 13,846 genes had a minimum of two counts per million (cpm) coverage in at least six samples and were considered sufficiently expressed for further analysis.

P. aeruginosa alone significantly altered the expression of 560 genes (FDR adjusted $P < 0.1$, log₂ Fold Change (FC) > 1). Expression of 420 genes increased, while a smaller set of 140 genes decreased in expression (Figure 2A). Genes most induced by *P. aeruginosa* were *CSF3*, *IL17C*, *TNFAIP6* and *TNF* (Table S3). We created a bidirectional clustered heatmap of the log₂FC values of the 560 *P. aeruginosa* regulated genes in HBE cells compared to control HBE cells across all treatment groups (Figure 2B). The response genes (Figure 2B, rows) clustered into two distinct groups of genes that were either increased or decreased by *P. aeruginosa*. Biological processes associated with these clusters of genes were identified using GSeq analysis in R (Table S4 and summarized in Figure 2B). Genes increased by *P. aeruginosa* (Figure 2B, orange cluster) were enriched in the defense response, immune response, signal transduction and response to stimulus processes. Genes decreased by *P. aeruginosa* were involved in macromolecule biosynthesis and gene expression regulation (Figure 2B yellow cluster).

Clustering of treatment groups in the heatmap of genes significantly affected by *P. aeruginosa* (Figure 2B, columns) provided preliminary insight into the effect of arsenic on *P. aeruginosa*-induced HBE cell gene expression. As expected for this set of 560 genes, treatment groups with *P. aeruginosa* had much more similar patterns of expression to each other than to groups treated with arsenic alone. We noted, however, that the arsenic plus *P. aeruginosa* combination exposures clustered in order of arsenic concentration, suggesting a dose-dependent effect of arsenic on genes induced by *P. aeruginosa*. Decreased gene expression in arsenic plus *P. aeruginosa* exposures compared to *P. aeruginosa* alone is apparent in the heatmap (Figure 2B).

We explored the interaction between arsenic and *P. aeruginosa* by examining the interaction term in our statistical model (EdgeR), but did not identify significant interaction genes with FDR-corrected $P < 0.1$. This lack of statistically significant interaction, despite notable changes in mean expression values, is a result of differences in the extent to which the *P. aeruginosa* response was altered by arsenic in samples from different donors. While donor differences may be an important area for investigation in future studies, we focused additional analysis on the consistent main effects genes significantly affected by arsenic.

Arsenic alters expression of host defense response genes

To understand how arsenic regulates gene expression in HBE cells, we tested for an arsenic effect over a range of concentrations (0, 5, 10 or 50 $\mu\text{g/L}$). Because non-monotonic transcriptional responses have previously been observed with arsenic, we modeled the data both with arsenic concentration as a continuous variable, and with arsenic as a discrete variable to identify significant genes at individual concentrations. We did not identify genes uniquely altered at low concentrations (5 and 10 $\mu\text{g/L}$) that were not significant in the linear model. We therefore focused additional analysis on genes that exhibited a significant response in the more powerful model with arsenic as a continuous variable, and present fold change and significance values for the linear arsenic effect. A significant arsenic effect (FDR < 0.1 , $\log_2\text{FC} > 0.01$ per $\mu\text{g/L}$ arsenic, equivalent to an estimated 2-fold change at 100 $\mu\text{g/L}$) was identified for 16 genes. We considered these genes, which include *HMOX1*, *FTH1*, and *JMJD7*, high-confidence arsenic genes. While the stringency of controlling for FDR is useful for identifying potential biomarker genes with consistent responses across all donors, it filters out more variable genes that may contribute important information about the biological response. Filtering based on fold change and a less stringent P value cutoff has led to increased reproducibility between gene expression studies (Shi et al., 2006). For gene ontology enrichment and pathway analysis, we therefore included genes with $P < 0.05$, $\log_2\text{FC} > 0.01$ per $\mu\text{g/L}$ arsenic. This set of 102 genes is comprised of 44 genes that increased with arsenic exposure, and 58 genes that decreased (Figure 3A, Table S3). A heatmap of $\log_2\text{FC}$ values (compared to untreated control) for each arsenic concentration shows that the significant arsenic genes clustered into 4 groups (Figure 3B, rows). A large cluster of genes that increased with arsenic (Figure 3B, green cluster) was enriched in genes involved in cell proliferation, response to oxygen-containing compounds, transmembrane and ion transport. This cluster contains *HMOX1* (Heme oxygenase 1), *FTH1* (Ferroptin heavy chain 1) and other redox-responsive proteins regulated by NRF2 (Lau et al., 2013a). A smaller cluster of genes that decreased at the lower arsenic concentrations but increased at 50 $\mu\text{g/L}$ (Figure 3B, purple cluster) was not enriched in genes from any biological processes, but included *UGT1A8*, a UDP glycosyltransferase involved in xenobiotic excretion. Both clusters of genes that were decreased by arsenic (Figure 3B, orange and yellow) were enriched in genes involved in the cellular defense response. These genes included *LYZ* (lysozyme), *STATH* (statherin), *CRISP3* (cysteine rich secretory protein 3), *SCGB1A1* (Secretoglobulin), *PLA2G7*, *NOS2* (nitric oxide synthase 2) *IL19*, and *HLA-DQA1*, several of which are secreted proteins.

We used Ingenuity Pathway Analysis to predict upstream transcriptional regulators of genes affected by arsenic. NFE2L2 (also known as NRF2) and SOX4 are transcription factors that,

based on significant gene expression changes induced by arsenic, were predicted to be activated (activation z score > 2, p value of overlap < 0.01). The Ingenuity Knowledgebase contains curated findings in the literature, which have shown that NRF2 and SOX4 regulate many of the genes that were most affected by arsenic (Figure 3C, genes that significantly increased or decreased in red or green, respectively). Biological effects of these gene expression changes were also predicted using the Ingenuity knowledgebase. Increased *IL6*, *HMOX1*, *SLC7A11* and decreased *NOS2* were predicted to increase metal transport (Figure 3C, predicted effect in orange). Changes in these same genes, along with decreased *LYZ*, were predicted to decrease cell death of immune cells (Figure 3C, predicted effect in blue). The biological effects of these gene expression changes are particularly interesting in the context of the lung, where lysozyme (*LYZ*) is secreted and has important antibacterial activity in the airway epithelium.

Arsenic regulates antimicrobial gene expression

We conducted a separate set of additional experiments to validate arsenic effects on the gene expression network shown in Figure 3C, and to further investigate the effect on antimicrobial peptides secreted by HBE cells. Using QPCR, we confirmed that 50 µg/L arsenic, significantly increased *HMOX1* and *FTH1* gene expression compared to control, as was observed in the RNA-seq analysis (Figure 4 A, B, C and D). We investigated two secreted antimicrobial peptides, *LYZ* and *CRISP3*, which decreased in the RNA-seq analysis (Figure 4, E and G). In our QPCR experiments, we confirmed that both genes were significantly decreased by 50 µg/L arsenic (Figure 4, F and H). Differences in gene expression between donors were particularly notable with the antimicrobial genes, and we found that these differences were highly repeatable between experiments and across platforms. Despite the variability in baseline expression of these genes, arsenic exposure caused a consistent and significant decrease in both *LYZ* and *CRISP3* gene expression.

Arsenic decreases lysozyme secretion and the antimicrobial activity of ASL

We next investigated whether the decreased expression of *LYZ* mRNA we observed translated to a change in antimicrobial peptide secreted into the airway surface liquid (ASL) overlying HBE cells. Apical lysozyme secretion was measured in ASL from control and arsenic-exposed HBE cells by ELISA. Donor differences in lysozyme gene expression translated to corresponding differences in basal lysozyme release, which ranged from 5–43 ng/mL in apical supernatants (Figure 5A). We estimate that our reported ASL concentrations represent a ~ 100-fold dilution, as a result of the addition of 300 µl of media for collection. This is based on previous studies demonstrating a mean airway surface liquid height of 7 µM in primary bronchial epithelial cells in culture (Tarran, 2004). Pre-dilution concentrations on the filter surfaces are estimated to be 0.5–4.3 µg/mL, which is consistent with concentrations of lysozyme (0.52–10 µg/mL) reported in human airway lavage fluid (Thompson et al., 1990; Travis et al., 1999). Exposure to 50 µg/L total arsenic for 6 days caused a highly consistent decrease in lysozyme secreted into the apical surface liquid.

To gain insight into the biological effect of decreased lysozyme, we tested the bacteriolytic activity of ASL collected from arsenic-exposed and control HBE cells. Lysozyme hydrolyzes peptidoglycans in bacterial cell walls, causing bacterial lysis. While lysozyme

has been reported to have additional bactericidal activity via other mechanisms, gram-negative bacteria are generally less sensitive to lysozyme because of lower peptidoglycan content and the protective outer membrane (Nash et al., 2006). *Micrococcus luteus* are gram positive bacteria routinely used to assess lysozyme activity in biological fluids (Lee and Yang, 2002; Shugar, 1952). Thus, we measured lysis of *M. luteus* using a turbidimetric assay, and found that ASL from HBE cells exposed to 50 µg/L arsenic had significantly reduced bacteriolytic activity (Figure 5B). Differences in bactericidal activity between individual donors mirrored differences in lysozyme concentrations detected by ELISA, with arsenic causing a consistent and significant decrease in all donors tested.

Discussion

Recent epidemiological and animal-based studies provide strong evidence that exposure to low levels of arsenic can adversely affect immune function of the lung (Farzan et al., 2016; Kozul et al., 2009a; Lantz et al., 2009; Nadeau et al., 2014). The lung, which is an entry point to the body for bacterial and viral pathogens, has been identified as a target organ in several populations exposed to arsenic in drinking water (Liao et al., 2011; Smith et al., 2011). Early development may be a particularly sensitive window; individuals exposed to arsenic in utero have increased risk of developing lung infections later in life (Smith et al., 2006, 2013). We investigated how low-dose arsenic exposure affects the response of HBE cells to *P. aeruginosa*, an opportunistic bacterial pathogen. The goal of this study was to elucidate mechanisms by which arsenic increases the risk of bacterial lung infection observed in human populations.

We employed a broad discovery approach using RNA-seq to measure all genes expressed in primary HBE cells from 6 donors exposed to arsenic as a mixture representative of speciation and concentrations detected in serum of human populations (Hall et al., 2007). The mixture represents a starting point for discovery of arsenic effects; we note that metabolism differences result in wide differences in speciation between individuals. The extent to which individual species contribute to effects observed will be an interesting direction for future studies. By testing for significant signals across 6 donors, our study by design did not detect arsenic effects unique to an individual or cell line, but rather consistent effects that are more likely to be observed across a population.

The airway epithelium not only provides a physical barrier against pathogens that enter the lungs, but plays a critical role in the innate immune response by regulating airway surface liquid, clearing bacteria through the mucociliary elevator, directly killing bacteria via secreted antimicrobial peptides and reactive oxygen species, and producing cytokines and chemokines to recruit and activate professional immune cells (Ryu et al., 2010). As expected, HBE cells responded to *P. aeruginosa* with robust changes in expression of innate immune response genes, including cytokines *CSF3*, *TNF*, *IL1B* and *IL8*. When HBE cells were exposed to arsenic prior to bacterial challenge, we observed reduced mean fold changes in many *Pseudomonas*-induced genes, though the interaction was not significant, potentially due to variability in donor responses.

Examining the arsenic affect alone, regardless of *P. aeruginosa* exposure, we found that the biological processes most significantly affected were related to immune function and host defense response. This has also been observed in transcriptomic studies of mice exposed to low-dose arsenic in drinking water (Andrew et al., 2007; Kozul et al., 2009b; Ramsey et al., 2013). The gene most responsive to arsenic in our study is a well-known oxidative stress response gene, *HMOX1*, which has been proposed as a biomarker of arsenic exposure (Efremenko et al., 2015). Many of the genes that were decreased by arsenic in our study are involved in immune processes. *LYZ*, *STATH*, *CRISP3*, *SCGB1A1* and *IL19* all encode secreted proteins with immune functions, and were decreased by arsenic. Several Major Histocompatibility Complex I and II genes also decreased significantly. Andrew et al. also observed that arsenic decreased the expression of antigen presentation genes in mouse lung (Andrew et al., 2007).

Based on our pathway analysis, we predict that arsenic activates transcription factors Nrf2 and Sox4 to mediate many downstream gene expression changes. Nrf2 activates transcription of *HMOX1*, *FN1*, *FTH1* and *SLC7A11*, genes that were most induced by arsenic, via antioxidant response elements (AREs) in their promoters. Sox4 has not previously been shown to be activated by arsenic, but was strongly predicted in our analysis, and is associated with induction of epithelial-to-mesenchymal transition and metastasis in lung cancers (Vervoort et al., 2013).

While many of the differentially expressed genes have important functions in the lung, decreased expression of lysozyme is particularly interesting because of its role in direct bacterial killing. Lysozyme was first described in 1922 by Alexander Fleming, and is one of the most abundant antimicrobial factors secreted in the lung, as well as in saliva, tears, milk and egg whites (Fleming, 1922). A cationic enzyme, lysozyme catalyzes hydrolysis of_{1,4} β linkages in bacterial cell walls and is particularly effective at killing gram-positive bacteria, but studies have shown activity against a wide range of bacteria, including *P. aeruginosa*, especially in combination with other cofactors (Ganz, 2004; Iacono et al., 1980; Travis et al., 1999). Unlike other antimicrobial compounds that are secreted primarily by professional immune cells such as neutrophils and macrophages, the bulk of lysozyme in the lungs is secreted by epithelial cells and submucosal glands (Konstan et al., 1981). Lysozyme is abundant in airway lavage fluid (10 µg/mL) and human sputum (> 1 mg/mL). In mice, lysozyme deficiency decreases the ability to clear *P. aeruginosa* (Cole et al., 2005). This is the first time, to our knowledge, that arsenic has been shown to affect lysozyme secretion in human cells. A study of killifish exposed to arsenic reported that lysozyme expression was decreased by a low concentration of arsenic in water (Gonzalez et al., 2010). In addition, we reanalyzed gene expression data published by Kozul et al. and found that expression of both *LYZ* homologs, *Lyz1* and *Lyz2*, were significantly decreased in lungs of mice exposed to iAs^{III} (10 µg/L) in drinking water (Kozul et al., 2009b). Interestingly, significant effects on lysozyme gene expression were only observed at low concentrations of arsenic (< 100 µg/L) in both the fish and mouse studies.

While lysozyme has long been known to be important for host defense at mucosal surfaces, only a few transcriptional regulators of *LYZ* have been identified (Kai et al., 1999; Suico et al., 2004). Unlike many innate immune response genes, *LYZ* expression was not induced by

P. aeruginosa exposure in HBE cells. In chicken macrophages, *LYZ* is induced by LPS via toll-like receptor signaling (Lefevre et al., 2008). Our data suggest that *LYZ* expression in the airway epithelium is regulated by a different mechanism. *LYZ* synthesis has also been shown to be decreased by glucocorticoids in human mononuclear leukocytes, suggesting a role of the glucocorticoid receptor in *LYZ* regulation (Panarelli et al., 1994). We found that basal expression of *LYZ* varied between cells isolated from different donors, and the relative differences in expression of the *LYZ* gene correlated with lysozyme concentration and activity detected in ASL. Regardless of basal secretion, 50 µg/L arsenic decreased *LYZ* expression and bactericidal activity in all donors by 11–35%. This decrease is predicted to decrease bacterial clearance in the lung. Moreover, the differences in basal levels of antimicrobial factors between donors suggests that individual susceptibility may be an important area for further investigation. Future studies will investigate the role of Sox4 and Nrf2 in *LYZ* transcriptional regulation, how arsenic disrupts expression and the role donor differences may play in leading to individual susceptibilities.

In summary, environmentally relevant levels of arsenic altered the expression of genes involved in redox homeostasis and host defense to *P. aeruginosa*. Arsenic reduced the expression of multiple genes that code for secreted antimicrobial proteins, including lysozyme, that provide an important first defense against bacterial lung infections. These data, showing disruption of innate immune functions of the airway epithelia, contribute to an emerging mechanistic explanation for increased infectious lung disease observed in human populations exposed to arsenic.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Arsenic alters transcriptional responses of lung cells to *Pseudomonas aeruginosa*
- Environmentally relevant arsenic exposure decreases expression of host defense genes
- Arsenic decreases lysozyme release and bactericidal activity in airway surface liquid

Figure 1. Intracellular arsenic concentrations and *Pseudomonas aeruginosa* exposure do not cause cytotoxicity

Accumulation of arsenic (As) by HBE cells was assessed by measuring total arsenic in cell pellets and media following 6 days of exposure to 0, 5, 10 or 50 $\mu\text{g/L}$ total arsenic (as 25% iAs^{III} , 25% MMA^{III} and 50% DMA^{V}) via ICP-MS. Data represent experiments with 6 donors $\pm$ SEM. (A). Cytotoxicity induced by arsenic exposure was assessed by measuring LDH release in basolateral media using the Promega CytoTox 96 Non-Radioactive Cytotoxicity assay. Aliquots of media from HBE cells exposed to 50 $\mu\text{g/L}$ arsenic or control were analyzed for LDH release every 24 hr during the exposure period. Data are expressed as percent of lysed control cells and represent means of 3 donors $\pm$ SEM; no significant effect of arsenic on LDH release was observed using a mixed effect linear model with donor as a random effect (B). LDH was measured on day 6 of arsenic exposure following challenge with *P. aeruginosa* (Pa) or control. Data are expressed as percent of lysed control cells; no significant effect of arsenic was observed using a mixed effect linear model with donor as a random effect. *P. aeruginosa* alone caused a statistically significant but small ($<1\%$) increase in LDH release ($P < 0.05$) (C).

Figure 2. *Pseudomonas aeruginosa* induces innate immune gene expression in HBE cells
 RNA-seq data were analyzed in EdgeR to identify genes that were significantly differentially expressed in HBE cells exposed to *P. aeruginosa* (*Pa*). Significance (FDR adjusted $-\log_{10}$ p values) compared to $\log_2$ Fold Change (FC) of the *P. aeruginosa* effect for all genes detected in the analysis are shown in (A), with significant genes ($P < 0.05$, $\log_2\text{FC} > 1$) highlighted in blue. We compared expression of the 560 genes that were significantly changed by *P. aeruginosa* across all treatment groups in the experiment. $\log_2\text{FC}$ values for each treatment group (columns) compared to untreated control are shown in a bidirectional clustered heatmap (B). $\log_2\text{FC}$ values of each gene (rows) are scaled relative to the mean, with increased expression in yellow and decreased expression in blue. Clusters of genes with similar expression patterns are annotated with biological processes summarized from Gene Ontology enrichment analysis.

Figure 3. Arsenic alters expression of host defense response genes in HBE cells

Genes significantly affected by arsenic (As) in the RNA-seq analysis were identified in EdgeR. Significance (FDR adjusted $-\log_{10}$ P values) compared to $\log_2$ Fold Change (FC) of the arsenic effect for all genes detected in the analysis are shown in (A). High confidence arsenic genes are represented above the horizontal line delineating an FDR-adjusted P value of 0.1. Genes that met our significance criteria for pathway analysis (unadjusted $P < 0.05$, $\log_2\text{FC} > 0.01$ per $\mu\text{g/L}$ total arsenic) are highlighted in blue. We compared $\log_2\text{FC}$ values of these 102 genes across the three arsenic exposure concentrations (5, 10 or 50 $\mu\text{g/L}$) in a bidirectional clustered heatmap (B). Yellow indicates increased expression, while blue indicates a decrease compared to unexposed control. Clusters of genes with similar expression patterns are annotated with biological processes summarized from Gene Ontology enrichment analysis. Upstream regulators of significant arsenic-induced gene

expression changes were predicted using Ingenuity Pathway Analysis. A network of significant transcription factors Nrf2 and Sox4 (activation z score > 2, P value of overlap < 0.01) and predicted downstream targets is shown in (C) Genes significantly increased by arsenic are in red, significantly decreased genes are in green. Biological effects predicted to increase are shown in orange, effects predicted to decrease are in blue.

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Figure 4. QPCR validation of gene expression changes induced by arsenic

We conducted a separate set of experiments using HBE cells from the same six donors exposed to 50 µg/L total arsenic (As) or control, and compared expression changes detected in HMOX1, FTH1, LYZ, and CRISP3 by QPCR with data from our RNA-seq analysis. Log₂ counts per million detected by RNA-seq are shown in **A** (HMOX1), **C** (FTH1), **E** (LYZ) and **G** (CRISP3). FDR-adjusted P values for the arsenic effect were determined using the GLM method in EdgeR. Log₂ Fold change values detected by QPCR are shown in **B** (HMOX1), **D** (FTH1), **F** (LYZ) and **H** (CRISP3). Data are represented as log₂ fold change compared to

the mean of the unexposed control group, P values for the arsenic effect were determined using a mixed effect linear model with donor as a random effect.

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Figure 5. Arsenic decreases lysozyme concentration and bactericidal activity of airway surface liquid

We determined the effect of arsenic on lysozyme secretion by measuring lysozyme in the airway surface liquid (ASL) overlying HBE cells exposed to 50 $\mu\text{g/L}$ arsenic or control for 6 days. Arsenic exposure significantly decreased the amount of lysozyme accumulated in ASL over a 24 hr collection window. Data represent means of three replicate experiments conducted with each donor, and were analyzed with a mixed effect linear model with donor as a random effect (**A**). Bactericidal activity of ASL samples were determined with a *M. luteus* photometric turbidity assay, with 1 unit of lysozyme activity defined as a decrease in O.D.₄₅₀ of .001/min. Data represent the mean of two replicate experiments conducted with each donor, and were analyzed with a mixed effect linear model with donor as a random effect (**B**).