The Effect of Arsenic Exposure in an Adult Mouse Tuberculosis Model

by
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Abstract

Arsenic is a well-known human carcinogen, naturally forming in the earth's crust and leaching into drinking water sources. However, there is growing evidence that arsenic may also affect the immune system, thereby manipulating the host's response to infection. Specific to this study, there is epidemiological evidence of increased tuberculosis mortality rates following arsenic exposure in a unique study area in the North of Chile. The effect of arsenic on tuberculosis mortality risk appeared stronger in men compared to women. Here, we aim to verify this increase of tuberculosis burden post-arsenic exposure in an adult mouse model. Adult male C57Bl/6 and A/J mice (age 5 weeks) were chronically exposed to 0ppb, 100ppb or 1,000ppb inorganic arsenic, in the form of sodium (meta-)arsenite, in their drinking water. Three weeks after exposure started, mice were infected at 7-8 weeks of age to 100 or 10,000 colony forming units (CFU) of H37Rv strain of *Mycobacterium tuberculosis* (*Mtb*). CFU in lungs were assessed at 1, 7, and 28 days following infection to assess arsenic-induced differences in *Mtb* growth. Serum was also analyzed for select cytokine concentrations at these same time points and pre-infection. Our preliminary data indicates that arsenic may affect the innate and adaptive immune responses to *Mtb* infections, leading to higher *Mtb* burden in the lung at day 7 and 28 post-infection. In addition, we aim to investigate how arsenic specifically changes the function of macrophages, critical immune cells for controlling an *Mtb* infection. To this end, we analyzed nitric oxide, phagocytosis, and cytokine production of *in vivo* As-exposed macrophages. Our findings suggest a change in

macrophage function that may alter overall host immune response to *Mtb* infection. Future studies will focus on further investigating the causal mechanisms involved in the increased tuberculosis risk after arsenic exposure.

Dedication

I would like to dedicate this thesis to my mom and dad, who gave me everything to become an academic. I would also like to dedicate this work to Sara, the love of my life, for her everlasting support and the belief that I could do anything.

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Background

Arsenic

Arsenic is a metalloid that naturally forms in the Earth's crust in an inorganic, trivalent, or pentavalent form (Morton et al. 1994). Although naturally occurring, this chemical element has been associated with increases in skin, lung, bladder, and several other cancers, leading to its classification of a known human carcinogen in 2004 (IARC 2004). Arsenic has been found to leach into groundwater, a major source of drinking water for many communities, leading to wide scale human exposure (Ravenscroft et al. 2009). Although the World Health Organization (WHO) has set the permissible limit on the concentration of arsenic in drinking water to 10 μg/L (WHO 1996), countries such as India and Chile show concentrations several orders of magnitudes higher (Banerjee, Banerjee et al. 2009, Smith, Marshall et al. 2011). Cancer risks in these areas have been observed to be much greater than nearby regions that have drinking water arsenic concentrations near the WHO standard.

In drinking water, arsenic is most commonly in its inorganic form with a valency of 3 (As^{III}; arsenite) or 5 (As^{V}; arsenate) (Naujokas, Anderson et al. 2013). It is not until after ingestion that arsenic is methylated into monomethylarsonic acid (MMA^{V}), reduced to monomethylarsonous acid (MMA^{III}), methylated once more to dimethylarsonic acid (DMA^{V}), and to reduced dimethylarsinous acid (DMA^{III}) (Gamble, Liu et al. 2006). The reduction step is carried out by thiols, especially glutathione (GSH) (Buchet and

Lauwerys 1988). During methylation, a methyl group is mainly donated from S-adenosyl methionine (SAM) by arsenic-3-methyltransferase (AS3MT) (Marafante and Vahter 1984, Buchet and Lauwerys 1985, Marafante, Vahter et al. 1985, Buchet and Lauwerys 1987). Methyl group donations from SAM also have a role DNA methylation; indeed, arsenic exposure has been observed to interfere with proper methylation of genes across the genome (Nohara, Baba et al. 2011). This pathway has been suggested as a mechanism of arsenic-induced carcinogenesis (Mass and Wang 1997).

In mammals, when arsenic is consumed by drinking water it is immediately absorbed into the bloodstream. Arsenate is reduced to As^{III} mainly in the blood (Nemeti and Gregus 2004, Gregus and Nemeti 2005) and liver (Buchet and Lauwerys 1985, Marafante, Vahter et al. 1985), but also possibly in the stomach and GI tract (Herbel, Switzer Blum et al. 2002). Although the liver is the primary site of arsenic metabolism, there is also high methylating activity in the testes, kidney, and lung tissues (Healy, Casarez et al. 1998). As^{III} is then taken up by cells more readily than As^{V} (Lerman, Clarkson et al. 1983) via aquaglyceroporins 7 and 9 (Liu, Shen et al. 2002) and methylated to MMA^{V} and DMA^{V}, the main excreted forms of human urine (Crecelius 1977). These forms can further be reduced to MMA^{III} and DMA^{III} (Gamble, Liu et al. 2006).

There are dosimetric differences of arsenic concentration and species between mice and humans. For example, the final form of methylated arsenic in mice is trimethylarsine oxide (TMAO) (Vahter et al. 1984), but this form is not observed in human excretions (Le, Ma et al. 2000). Instead, human urine contains 10-15% inorganic arsenic, 20-25%

MMA^V, and 45-50% DMA^V 2-4 days after ingesting As^{III} (Vahter 1994). Mice have also been observed to metabolize and excrete arsenic more effectively than humans, processing 75-95% of inorganic arsenic in 48 hours while humans only processes 40-70% in the same amount of time (Cohen, Arnold et al. 2006). This difference can be observed at the blood and plasma level. A study by Hall et al. observed 10 ppb total arsenic in blood levels after human injection of <50 ppb in drinking water (Hall, Chen et al. 2006). In the case of mice, Chen et al. observed only 20 ppb after 1,000 ppb in drinking water, suggesting that mice are nearly 5 times more efficient at methylating and excreting arsenic than humans (Chen, Arnold et al. 2011).

Sex differences have also consistently been observed in human arsenic exposure studies, with males being at higher risk of developing skin lesions (Rahman, Vahter et al. 2006) and decrements in lung function (von Ehrenstein, Mazumder et al. 2005) than females. These differences may be due to the sex hormone-driven ability of females to methylate arsenic better than males, increasingly so during pregnancy (Lindberg, Ekstrom et al. 2008). As estrogen levels are heightened in pregnancy, it is suggested that estrogen has a role in arsenic methylation efficiency. To address sex differences in the effect of arsenic exposure on APCs, Ferrario et al. exposed both human and mouse granulocyte-macrophage progenitor cells to several species of arsenic. Although they did not observe any significant differences in cytotoxicity, there was increased proliferation at very low concentrations of As^{III} in only female human and mouse granulocyte-macrophage progenitor cells (Ferrario, Croera et al. 2008). This may serve as a protective mechanism

for females, but future studies addressing the effect of arsenic exposure on both APC progenitor cells and mature APCs are required to elucidate the mechanisms involved.

Exposure to arsenic is considered a world-wide issue, as the WHO estimates that over 200 million persons are chronically exposed at levels greater than the WHO standard (WHO 2008). Indeed, high arsenic levels have been observed in the United States as well. Regions such as the Midwest, New England, and Rocky Mountain System have measured drinking water concentrations exceeding the regulatory standard (Welch, Westjohn et al. 2000). As of 2001, an estimated 13 million people are exposed to arsenic levels in drinking water in the United States exceeding the WHO standard (U.S. EPA 2001). The human carcinogenicity of this chemical is well established, but recent studies have suggested other adverse health outcomes such as cardiovascular disease (Moon, Guallar et al. 2013) and diabetes (Kuo, Howard et al. 2015) Important here is evidence of immune system impairment by arsenic exposure (Selgrade 2007, Vahter 2008). Most recently, animal studies of arsenic in drinking water has been suggested a negative influence on immune cells involved in antigen presentation (Choudhury, Gupta et al. 2016, Xu, McClain et al. 2016, Soria, Perez et al. 2017).

Immune Response Changes in APCs

It has long been known that antigen presenting cells (APCs), particularly macrophages and dendritic cells, bridge the innate and adaptive immune responses (Joffre, Segura et al. 2012, Blum, Wearsch et al. 2013). This connection not only assists an early infection defense, but also the opportunity to fend off pathogens more effectively upon sequential infections. Fisher et al. were some of the first to observe negative effects of arsenic on APCs when they determined that sodium arsenite decreased the phagocytic ability of bovine alveolar macrophages (Fisher, McNeill et al. 1986). Without proper structure and function, APCs and their role in the immune response are compromised.

Phagocytosis is a key strategy APCs use to contain an infection (Li, Petrofsky et al. 2002, Leemans, Thepen et al. 2005). Compromising this ability disrupts the process of pathogen destruction and antigen presentation. Several groups over the last twenty years have recorded similar findings to Fisher et al.'s dose-dependent decline in phagocytosis upon arsenic exposure, in both macrophages and dendritic cells (Sengupta and Bishayi 2002, Banerjee, Banerjee et al. 2009, Mehrzad, Mahmudy Gharaie et al. 2017). Arsenic may have negative effects on the cytoskeleton, a structure required for efficient phagocytosis (Goodridge, Underhill et al. 2012). Banerjee et al. found a decrease in F-actin expression in human monocyte-derived macrophages from an Indian population exposed to arsenic in drinking water (Banerjee, Banerjee et al. 2009). Although the authors drew the conclusion of decreased cell adhesion, F-actin is also necessary for phagocytosis (May and Machesky 2001). Indeed, the decrease in F-actin may be a driver

in a possible inhibition of filament lengthening required to produce pseudopods in this protective mechanism.

Reactive oxygen species (ROS), nitric oxide (NO), and superoxide (SO) are additional mechanisms by which APCs use to combat pathogens. Released as free radicals, these chemical compounds are toxic to intracellular bacteria and parasites. Interference of these defenses would leave APCs unable to break down engulfed pathogens. Arkusz et al. observed a dose-dependent decrease in ROS production, as well as an inhibition of NO production in peritoneal macrophages after exposing mice to sodium arsenate in drinking water (Arkusz, Stanczyk et al. 2005). Interestingly, a previous report by Lantz et al. found SO production differences from alveolar macrophages between exposure methods and arsenic species. *In vivo* exposure of rats exhibited a greater increase of SO with As^{III} than As^{V}. However, *in vitro* exposure decreased SO in a dose-dependent manner, again greater with As^{III} than As^{V} (Lantz, Parliman et al. 1994). The increased sensitivity to As^{III} than As^{V} is to be expected, as intracellular transport of the trivalent form is more efficient than the pentavalent form (Lerman, Clarkson et al. 1983). However, the differences in SO production between *in vivo* and *in vitro* arsenic exposure is most likely due to the vast difference in cellular-level dose. Although the *in vitro* concentrations of 0.1-100 µg/L (0.1-100 ppb) are environmentally relevant, an intratracheal installation of 1 mg/kg (0.2 mg, or 200,000 µg, for a 200 g rat) is not. Epidemiological studies published since have shown conflicting results when extracting peripheral blood mononuclear cells (PBMCs) (Banerjee, Banerjee et al. 2009, Luna, Acosta-Saavedra et al. 2010). Banerjee et al. observed a decrease in NO production, but Luna et al. found a positive association

between arsenic exposure and NO, as well as SO, production. The discrepancy of NO production may be due to Banerjee et al. culturing monocytes until maturation into macrophages before experimentation, while Luna et al. performed their methods on undifferentiated monocytes. Although the increase of SO production with increased arsenic exposure observed by Luna et al. coincides with the *in vivo* exposure by Lantz et al., again, the studies tested two difference cell types. To elucidate changes in these free radicals, future studies should test macrophages from several areas of the body and match dose levels at the cellular level for *in vitro* and *in vivo* exposures.

Changes in expression of genes with roles in APC immune response have also been reported (Kozul, Hampton et al. 2009). Kozul et al. observed differences in expression of immune response genes involved in cell migration, adhesion, differentiation, and proliferation after arsenic exposure in mice at the WHO standard level (Kozul, Hampton et al. 2009). The same group also determined 100 ppb of arsenic exposure in drinking water suppresses the mouse immune response to influenza A virus (Kozul, Ely et al. 2009). A prenatal study by Rager et al. demonstrated an association between decreased expression levels of immune response-related miRNAs in newborn cord blood and maternal urinary arsenic levels (Rager, Bailey et al. 2014). A follow-up study showed that expression of genes encoding glucocorticoid receptors, receptors that play a regulatory role during responses to infectious diseases, was perturbed with prenatal exposure to inorganic arsenic (Rager, Yosim et al. 2014). Heideveld et al. demonstrated that glucocorticoid receptor activation differentiates monocytes to anti-inflammatory tissue macrophages, therefore suggesting changes in glucocorticoid receptor gene

expression impact the phenotypes of differentiating macrophages (Heideveld, Hampton-O'Neil et al. 2018). These studies suggest that arsenic may not only affect immune signaling gene expression, but may also directly impair the body's ability to combat and clear microbial infections.

Another animal model used to observe the effect of arsenic exposure on the immune system is the zebrafish. In a study by Hermann and Kim, an exposure to 50 µM or 100 µM of sodium arsenite to zebrafish larvae decreased Tumor Necrosis Factor (TNF) expression, which was associated with a decrease in respiratory burst from phagocytic cells (Hermann and Kim 2005). Navak et al. also observed a decline in TNF expression and respiratory burst activity, as well as several-fold increase in viral and bacterial loads in zebrafish embryos (Nayak, Lage et al. 2007). Both studies align with human studies of arsenic exposure in Bangladesh and Mexico, where down-regulation of *TNF* was observed (Argos, Kibriya et al. 2006, Salgado-Bustamante, Ortiz-Perez et al. 2010). Interestingly, arsenic exposure suppresses the release of TNF from macrophages of both humans (Banerjee, Banerjee et al. 2009) and animals (Lantz, Parliman et al. 1994), although one study did not observe any change (Arkusz, Stanczyk et al. 2005). These observations suggest a compromise of the innate response, perhaps through changes in morphology or cytokine production of antigen presenting cells (APCs) such as macrophages and dendritic cells.

Morphological Changes in APCs

The maturation of monocytes to macrophages is dependent on colony stimulating factors produced by T cells, natural killer cells, and other macrophages (Hamilton, Stanley et al. 1980). Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) differentiates monocytes to the proinflammatory M1 phenotype, while Macrophage Colony Stimulating Factor (M-CSF) drives differentiation to the anti-inflammatory M2 phenotype (Verreck, de Boer et al. 2004, Verreck, de Boer et al. 2006). Therefore, any upregulation of these growth factors will not only increase monocyte differentiation, but polarize the inflammatory microenvironment they reside in. Indeed, macrophages have demonstrated the ability to change between phenotypes *in vivo* and *in vitro* (Guiducci, Vicari et al. 2005, Saccani, Schioppa et al. 2006). An epidemiological study of arsenic exposure in children in Hidalgo, Mexico, observed an association between increased secretion of GM-CSF by macrophages with increasing total arsenic in urine (Soto-Pena, Luna et al. 2006). This may suggest an increase in differentiation to the M1 phenotype. Opposing this view is an *in vitro* study of human lung epithelial cells by Cui et al., demonstrating exposure to sodium arsenite polarizes macrophages to the M2 phenotype (Cui, Xu et al. 2017). Interestingly, an *in vitro* study by Sakurai et al. found sodium arsenite to be cytotoxic to macrophages stimulated with M-CSF and produced macrophages with low adhesion, high T cell inducing capabilities, higher expression of antigen presentation receptors, and decreased phagocytic capabilities after GM-CSF stimulation (Sakurai, Ohta et al. 2005). These studies suggest arsenic exposure may not specifically polarize macrophages to specifically the known M1 or M2 phenotypes, but a complex hybrid

phenotype that ultimately is incapable of adhering to a necessary location and phagocytosing pathogens. Additionally, M-CSF+arsenic-induced cytotoxicity of macrophages is a clear mechanism for immunosuppression.

Sakurai et al. reported that *in vitro* exposure to inorganic and organic arsenic to induced cytolethality in mature, extracted peritoneal and alveolar macrophages (Sakurai, Ohta et al. 2004). Indeed, prior to their 2004 study they had knowledge of patterns of macrophage cell death due to both inorganic and organic (i.e. methylated) arsenic species. The inorganic species mainly induced necrosis, while methylated arsenic induced mostly apoptotic cell death (Sakurai, Kaise et al. 1998). While it is unclear though what mechanisms these endpoints occur by, this study supports the idea of different forms of arsenic affecting the immune system through different pathways. It may be suggested that inflammatory conditions are induced by inorganic forms and less so by methylated forms. Since pathogens such as *Listeria monocytogenes* (Rogers, Callery et al. 1996), *Mycobacterium tuberculosis* (*Mtb*) (Watson, Hill et al. 2000), and *Yersninia pseudotuberculosis* (Monack, Mecsas et al. 1998) induce apoptosis rather than necrosis of immune cells to avoid a heightened immune response, it may be speculated that methylated forms of arsenic at the cellular level may lead to enhanced susceptibility to infection.

The following year, Sengupta and Bishayi also published findings of abnormal shaping of murine splenic macrophages after the same exposure treatment, describing the shape as "deviating from spherical outline" (Bishayi and Sengupta 2003). An epidemiological

study by Banerjee et al. of an adult human population chronically exposed to arsenic in West Bengal, India, observed data that both supports and opposes this abnormal shaping. Although they found decreased expression of F-actin, an important protein for cytoskeletal structure and function (Tang, Janmey et al. 1999), they observed "cell rounding" instead of deviations from spherical outline, upon arsenic exposure (Banerjee, Banerjee et al. 2009). They instead look to the "fried-egg"-like appearance of blood monocyte-derived macrophages as the normal morphology (Young, Lowe et al. 1990). Although there is disagreement on healthy cell structure, both groups found a loss of adhesion capabilities in the arsenic-exposed macrophages. Loss of migration capabilities upon exposure to sodium arsenite have also been observed in dendritic cells (Patterson, Vega et al. 2004, Kozul, Ely et al. 2009). These adhesion capabilities are important for the ability of macrophages to collect around sites of inflammation and infection, where they migrate to and are needed most (Pixley and Stanley 2004). Therefore, loss of these capabilities may negatively impact an immune response to infection.

Cytokine Changes and Possible Mechanisms

Proinflammatory cytokines are generally beneficial for combating infections. In inflammatory conditions, proinflammatory cytokines such as TNF are produced mainly by APCs (Kozul, Hampton et al. 2009). Kozul et al. found significant increases in TNF mRNA expression and protein, but decreases in interleukin (IL)-1β mRNA expression and protein, following *in vivo* exposure to 10 and 100 ppb sodium arsenite (Kozul, Hampton et al. 2009). It is unclear why some inflammatory cytokines increase while others do not, especially since LPS can induce TNF and IL-1β genes through the same pathway (Reimann, Buscher et al. 1994). Mehrzad et al., opposing Kozul et al., found *in vitro* sodium arsenite exposure to increase dendritic cell production of IL-6 and IL-1β (Mehrzad, Mahmudy Gharaie et al. 2017). These studies suggest that exposure to arsenic may drive macrophages and dendritic cells to inflammatory cytokine production, but through different mechanisms not yet distinguished.

Sakurai et al. found that organic arsenicals added *in vitro* also increased production of TNF in peritoneal macrophages from male CDF_1, ICR, C3H/HeN, and C3H/HeJ mice (Sakurai, Kaise et al. 1998). Importantly, Sakurai et al. also observed a decrease in TNF production from macrophages exposed to methylated arsenic species (Sakurai, Kaise et al. 1998). This finding suggests that the methylation of arsenic as a potentially protective mechanism to prevent APCs from creating inflammatory microenvironments, but perhaps also creating an environment susceptible to infection. Interestingly, Lantz et al. observed a decrease in lipopolysaccharide (LPS)-induced production of TNF by male Sprague-

Dawley rat alveolar macrophages after exposure to both As(III) and As(V) *in vivo*, but not *in vitro* (Lantz, Parliman et al. 1994). As^{III} had a greater suppression of TNF production than As^{V}. These findings oppose the findings from Sakurai et al. (increased TNF) (Sakurai, Kaise et al. 1998). However, a caveat may be that Lantz et al. observed LPS-induced inflammatory cytokine production as opposed to basal levels by Sakurai et al. Additionally, the studies used two different animal models. Taken together, there is not yet conclusive evidence of an increased production of inflammatory cytokines by APCs upon exposure to arsenic.

Vulnerability to Infectious Diseases

In 2000, Bishayi injected heat-killed *S. aureus* into mice after two weeks of daily intraperitoneal injections of 0.5 mg/kg body weight of sodium arsenite. Bishayi found that infecting mice with *S. aureus* after 2 weeks sodium arsenite exposure resulted in a lower titer of agglutinating antibodies when compared to the antibody titers produced without sodium arsenite exposure (Bishayi 2000). This is evidence that sodium arsenite impacted the magnitude of immunoglobulin production by B cells in response *S. aureus*. When infected with viable bacteria, peritoneal macrophages exposed to sodium arsenite showed a decreased capacity for intracellular killing capacity and ability to chemotax to bacteria (Bishayi 2000). Sengupta and Bishayi investigated the immune response further by studying splenic macrophages, cells that are vital to defense against a broad spectrum of pathogens. They reported that arsenite treatment resulted in decreased NO release, reduced phagocytic capacity, and increased DNA fragmentation in splenic macrophages, suggesting increased apoptosis (Sengupta and Bishayi 2002). To test the hypothesis that a reduction in the functional capacity of splenic macrophages leads to increased vulnerability to infection, Bishayi and Sengupta injected live *S. aureus* and found a reduction in the chemotactic index, an increased bacterial load and delayed bacterial clearance (Bishayi and Sengupta 2003).

When taking into consideration the findings of Bishayi and Sengupta, they suggest that exposure to arsenic increases vulnerability to challenge withpathogens that require NO production and phagocytosis for control and clearance. NO release has been shown to be

an important component in the immune response against intracellular protozoan parasites such as *Leishmania* and in control of malaria.(Mellouk, Green et al. 1991, Seguin, Klotz et al. 1994, Klotz, Scheller et al. 1995). The role of phagocytosis in infection control is perhaps most evident in tuberculosis (TB), and thus arsenic exposure is likely to have a detrimental effect on the outcome of infection with *Mtb* (Li, Petrofsky et al. 2002, Leemans, Thepen et al. 2005). In support of this idea, Smith et al. found exposure to arsenic in drinking water to increase TB mortality rates in an early-life arsenic exposed human population in Chile (Smith, Marshall et al. 2011).

Indeed, the incidence of several respiratory diseases and infections have been associated with arsenic exposure. A study by George et al. observed an association between pediatric arsenic urine levels and pediatric pneumonia in Bangladesh (George, Brooks et al. 2015). In the mouse model, lower respiratory tract infections (Rahman, Vahter et al. 2011) and worsened influenza morbidity (Kozul, Ely et al. 2009, Rahman, Vahter et al. 2011, Ramsey, Foong et al. 2013) were found in arsenic exposed groups. Lung diseases that may lead to respiratory infections have also been found to be associated with arsenic exposure, such as bronchiectasis (Smith, Marshall et al. 2006) and chronic obstructive pulmonary disease (D'Ippoliti, Santelli et al. 2015). Importantly, studies by both Smith et al. and D'Ippoliti et al. both found COPD outcomes from arsenic exposed patients to be more frequent in males than females, suggesting sex differences are maintained in the face of arsenic exposure (Smith, Goycolea et al. 1998, D'Ippoliti, Santelli et al. 2015).

Sex differences observed in infectious disease outcomes associated with arsenic exposure include the study by Smith et al., where males had significantly more excess pulmonary TB deaths than females (Smith, Marshall et al. 2011). Increased acute respiratory tract infections by arsenic exposure were only observed in male infants, adding more evidence to males experiencing more immunotoxicity (Raqib, Ahmed et al. 2009). Sex-dependent increases in risk of infectious respiratory diseases, and perhaps infections in general, may be partly explained by females methylating arsenic more efficiently than males (Lindberg, Ekstrom et al. 2008). These mechanisms may be addressed in a mouse model exposed to arsenic and infected with *Mtb,* leading to a disease where APCs are vital in defense.

Tuberculosis

Approximately 2 billion persons have latent or active tuberculosis (TB) infections (Rhines 2013). To date, TB continues to be a top cause of death due to a single infectious agent (Lozano, Naghavi et al. 2012). In 2013, nearly 1.5 million persons died due to TB-related deaths worldwide (Centers for Disease Control and Prevention, 2014). The pathology of this disease centers around the aerobic bacterium *Mtb* entering the alveolar passages in aerosol droplets, where mainly dendritic cells and macrophages engulf these bacilli (Guirado, Schlesinger et al. 2013). After migrating throughout the lung tissue, these infected cells may also induce dissemination of the disease to other organs via the circulatory and lymphatic systems (Frieden, Sterling et al. 2003).

Under ideal conditions, APCs can efficiently degrade bacteria and present bacterial antigens on their surface by the major histocompatibility complex (MHC) class II pathway. In this way, the antigen can be detected by T cells to initiate the adaptive immune response (Amigorena, Drake et al. 1994). However, some strains of *Mtb* can prevent the fusion of the phagosome and lysosome to escape acidic conditions and degradation (Armstrong and Hart 1975). Under these conditions, an accumulation of infected macrophages and a surrounding border of lymphocytes form a granule to prevent spread to the rest of the body (Cosma, Sherman et al. 2003).

Sex differences have been noted in epidemiological studies of TB. The disease is more prevalent in men than women, observed in 2004 with 1.4 million smear-positive male

cases compared to 775,000 female (Dye 2006). Interestingly, there is evidence of smoking being a significant effect modifier of the sex differences in TB notifications (Watkins and Plant 2006). Furthermore, underreporting of cases in women may be likely due to the social stigma of diagnosis and less availability of healthcare to women (Thorson and Diwan 2001, Thorson, Hoa et al. 2004). Ultimately, these studies suggest that behavior and environmental factors may account for much of observed sex differences in TB. However, sex hormones such as estradiol have been shown to play a role in IFNγ production by iNKT cells (Gourdy, Araujo et al. 2005). Although there is a general dearth of studies providing concrete evidence of biological factors that determine sex differences in TB outcomes, investigations pairing arsenic exposure, having well known biological sex differences, and TB infections may elucidate the mechanisms of sex-determined immune response.

Macrophages and TB

Macrophages are vital to the immunological response against *Mtb* and TB pathology, as they are the main cell infected and play a key role in the formation of the granulomas that contain the spread of infection (Li, Petrofsky et al. 2002, Leemans, Thepen et al. 2005). Decreased phagocytic capabilities observed after arsenic exposure (Sengupta and Bishayi 2002, Banerjee, Banerjee et al. 2009) may inhibit the ability of macrophages to contain the *Mtb* infection (Dannenberg 1989). Upon Toll-like receptor 2 (TLR2) recognition of *Mtb*, macrophages produce proinflammatory cytokines such as TNF and IL-6 that, along with IFNγ, generate a Th1 response for controlling and clearing the infection (Law, Weiden et al. 1996). When activated by IFNγ from activated CD4+ T cells, the macrophage generates nitric oxide (NO) radicals from L-arginine via inducible Nitric Oxide Synthase (iNOS) to kill the engulfed bacterium (Morris and Billiar 1994, Nathan and Xie 1994). Importantly, NO species have been shown to be vital in combating TB and NO-resistant strains correlating with high virulence (Chan, Xing et al. 1992). Proinflammatory cytokine production and high production of NO are part of the classical (M1) phenotype, opposite to the alternative (M2) phenotype with low production in both (Cui, Xu et al. 2017). To efficiently clear an *Mtb* infection, macrophages are polarized to the M1 phenotype (Sahu, Kumar et al. 2017). Although more consistent evidence is necessary, studies suggest that TNF production may decrease with arsenic production (Lantz, Parliman et al. 1994, Sakurai, Kaise et al. 1998). After phagocytosis, F-actin-dependent endocytic trafficking and antigen presentation are also vital to an effective immune response against *Mtb* infection (Tang, Janmey et al. 1999). Influences of arsenic

on macrophage adherence, cytokine production, phagocytosis, and antigen presentation may explain the doubling of TB mortality upon arsenic exposure observed in Chile (Smith, Marshall et al. 2006).

Study Design

No published data to date has investigated the immunological effect of arsenic in an *Mtb* infection model. Sillé et al., in unpublished data, observed a significant increase in colony forming units (CFU) between 100 ppb and 1,000 ppb in arsenic-exposed mice upon infection with 400 CFU Erdman *Mtb* (unpublished, Sillé et al. 2016, Supplemental Figure 3). In our current study, our group also exposed mice to inorganic arsenic, in the form of sodium arsenite, in drinking water 3 weeks before infection to replicate the effects seen by Bishayi and Sengupta and Kozul et al. suggesting vulnerability to infectious disease outcomes (Sengupta and Bishayi 2002, Bishayi and Sengupta 2003, Kozul, Ely et al. 2009).

In the work presented in this thesis we will test the hypothesis that ingesting environmentally relevant levels of sodium arsenite in drinking water during adulthood will affect macrophage function in a way that will lead to worsened health outcomes during *Mtb* infection. **Aim 1** of this study is to develop an *in vivo* TB-arsenic mouse model. Under this aim, our subhypotheses are 1) the effect of arsenic on *Mtb* burden is dose dependent and 2) arsenic exposure induces an anti-inflammatory microenvironment to inhibit a proper immune response to an *Mtb* infection. **Aim 2** of this study is to observe *ex vivo* the effect of *in vivo* arsenic exposure on macrophages. Subhypotheses under this aim are 1) upon arsenic exposure, macrophages are less effective at destroying *Mtb* and 2) arsenic-exposed macrophages create a microenvironment more susceptible to damage by *Mtb* via changes in phenotype and function.

To address Aim 1, we tested our adult mouse model of arsenic exposure and *Mtb* infection at different arsenic concentrations and with different mouse strains. Increasing arsenic doses allowed us to determine the presence of a dose-response relationship. Dose was determined by calculating environmentally relevant doses at the animal, tissue, and cellular level. For every part per billion (ppb) of sodium arsenite in drinking water, an immune cell in the mouse should be exposed to intracellular levels of 1 nM of total arsenic (Xu, Lauer et al. 2016). Therefore, we expect a 10-fold loss as 1 nM = 0.1ppb. Concentrations of 100 ppb and 1,000 ppb have been reported as environment exposures in , India (Banerjee, Banerjee et al. 2009) and Chile (Smith, Marshall et al. 2011). Additionally, higher concentrations are necessary to properly extrapolate findings to humans as mice are 5 times more efficient at methylating arsenic than humans (Hall, Chen et al. 2006, Chen, Arnold et al. 2011)

C57BL/6 was the main strain used here due to the extensive studies on macrophage function (Dietert 2009), arsenic immunotoxicity (Waalkes, Liu et al. 2004, States, Barchowsky et al. 2011), and *Mtb* studies (Chackerian and Behar 2003). Testing both the most resistant (C57BL/6) (Medina and North 1998) and semi-resistant (A/J) (Jagannath, Hoffmann et al. 2000) strains of mice to infection with the H37Rv strain of *Mtb* created an opportunity to assess immune response changes in multiple *Mtb* susceptibility models. Body weight was assessed to monitor disease progression and for mortality studies, and CFU counts were performed as a quantitative assessment of bacterial load in select organs after infection. These assessments were taken at Day 1 for baseline, and Day 7 and 28 to investigate the innate and adaptive immune responses against *Mtb* infections,

respectively. To qualitatively assess *Mtb* burden, histology was performed to visualize bacterial load, granuloma formation, and immune cell infiltration in the lung. The microenvironment was assessed by testing concentrations in serum before and after *Mtb* infection.

To address Aim 2, we performed functional and molecular assays on stimulated and unstimulated peritoneal macrophages *ex vivo* after *in vivo* arsenic exposure. A phagocytosis assay was performed to determine changes in ability of exposed macrophages to engulf bacteria. A nitric oxide (NO) assay was performed to determine intracellular killing ability of exposed macrophages. To determine differences in cytokine output, a Luminex assay was performed on the supernatant of exposed peritoneal macrophages.

Methods

Animal Care

C57BL/6 and A/J mice were obtained from Charles River Laboratories and delivered at 5 weeks of age. Mice were fed a low arsenic diet (Product #D10001 [a.k.a. AIN-76A]); protein 20.8% kcal, carbohydrate 67.7% kcal, fat 11.5% kcal) *ad libitum*). Groups of mice were exposed to 0, 100, or 1,000 ppb sodium-(meta) arsenite (Sigma; St. Louis, MO; Catalog #S7400-100G) in drinking water that was tested to have arsenic levels of <10 ppb (Crystal Geyser Spring Water). Mouse weights were measured and drinking solutions were freshly prepared every 2-3 days. Food was replenished weekly. Drinking water and food intake were calculated per cage and averaged per mouse. All experiments using mice and murine macrophages were conducted with the approval by the Institutional Animal Care and Use Committee, Johns Hopkins University, Baltimore.

Mycobacterial Strains

Mtb strain H37Rv was used for *in vivo* mouse infections. Middlebrook 7H9 broth with 0.2% glycerol, 10% oleic acid-albumin-dextrose-catalase (OADC) (Fisher Scientific; Hampton, NH), and 0.05% Tween 80 (Sigma-Aldrich; St. Louis, MO) was used for *in vitro* cultivation of H37Rv (Xu, Tasneen et al. 2018).

Aerosol Infection Model

Male C57BL/6 and A/J mice (8 to 9 weeks of age) were infected with H37Rv using the inhalation exposure system (Glas-Col; Terre Haute, IN) and a fresh log-phase broth

culture with an optical density at 600 nm of approximately 0.014 or 0.5 for exposure to 100 and 10,000 CFU, respectively (Xu, Tasneen et al. 2018). Day of infection was 3-4 weeks following the initiation of sodium-(meta) arsenite exposure. Weight loss threshold for euthanasia was 15% of the pre-infection weight (Franco, Correia-Neves et al. 2012).

Colony Forming Unit (CFU) Analysis

One to three mice from each exposure group were sacrificed 1 day after infection to evaluate inoculation rate. Five mice from each exposure group were sacrificed 7 and 28 days after infection. The lungs from each day 7 and day 28 animals and the spleens and livers from Day 28 animals were homogenized in 2.5 mL of PBS and serial diluted before plating. 500 μL of homogenate was plated on each 7H11 agar plate containing polymyxin B (200,000 units/L); Sigma-Aldrich; St. Louis, MO), carbenicillin (50 mg/L); Sigma-Aldridge; St. Louis, MO), trimethoprim lactate (20 mg/L) Sigma-Aldrich; St. Louis, MO), and amphotericin B (5 mg/L); Sigma-Aldrich; St. Louis, MO). Mycobacteria were incubated at 37°C for 3 weeks before counting. Counting was performed by two researchers, one blind to the exposure groups.

Isolation of Peritoneal Macrophages

Mice were injected intraperitoneally with 1.0 mL of sterile 3% thioglycollate medium (Sigma-Aldrich; St. Louis, MO); lavage fluid was harvested 4 days after injection as previously described (Zhang, Goncalves et al. 2008). Briefly, 8.0 mL PBS with 10% FBS was injected in the peritoneal cavity. The mouse was then gently shaken before withdrawing the solution. Cells were centrifuged at 1400 rpm for 10 minutes, red blood

cells were lysed with ACK lysis buffer, then centrifuged again before suspension in Dulbecco's Modified Eagle's Medium without phenol red (Sigma-Aldrich; St. Louis, MO) plus 10% fetal bovine serum (FBS; Sigma-Aldrich; St. Louis, MO), L-glutamine (2 mM; Gibco; Montgomery County, MD), and murine M-CSF (20 μg/mL; BioVision; San Francisco, CA).

TNF ELISA

To measure the TNF concentration in serum, a mouse TNF ELISA was purchased from BioLegend (San Diego, CA) and performed according to manufacturer's instructions. Serum samples were diluted 1 to 1 in 10% Fetal Bovine Serum. The lower limit of sensitivity of the assay was 7.8125 ng/mL.

Luminex Assay

A 32-plex mouse kit (Millipore; Burlington, MA) was used to detect eotaxin, G-CSF, GM-CSF, IFN- γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12, IL-17, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1a, MIP-1b, MIP-2, RANTES, TNF, and VEGF concentrations in serum and cultured supernatant. The Luminex assay was performed according to the manufacturer's protocol using DropArray™ 96-well coated plates (Curiox; San Carlos, CA).

Phagocytosis Assay

pHrodo™ Red and Green Bioparticles® Conjugates for phagocytosis were purchased (Thermo Fisher Scientific; Waltham, MA) and performed according to manufacturer's

instructions. Briefly, 1 mg/mL pHrodo™ *E. coli* was suspended by 2 mL Uptake Buffer and vortexed. Suspension was then transferred to a clean glass tube and vortexed. Culture medium was removed from plate of adhered cells and replaced with 100 µL pHrodo™ Bioparticles® suspension. Plate was then covered and transferred to incubator set at 37ºC for 3 hours. Measurements of 494 nm absorbance were taken once every hour after the fluorogenic dye-conjugated *E. coli* suspension was added to cells. Net phagocytosis is represented as Percent Effect, calculated by dividing the next experimental absorbance by the net positive control absorbance.

Nitric Oxide Assay

Nitric oxide production by macrophages was measured by modifications of what has been previously described (Arkusz, Stanczyk et al. 2005). Briefly, isolated cells (2 x 10^5/well) were stimulated for 24 hours with LPS (Sigma-Aldrich; St. Louis, MO; 1mg/mL) plus murine IFNγ (Invitrogen; Carlsbad, CA; 6.25 ng/mL) and PAM(3)CSK(4) (Invitrogen; Carlsbad, CA; 1 mg/mL) plus murine IFNγ (Gibco; Montgomery County, MD; 6.25 ng/mL). After stimulation, 50 µL of the supernatant was mixed with a freshly prepared Griess reagent (1% sulfanilamide, 0.1% napthylethylenediamine dihydrochloride, 2% phosphoric acid). The standard curve for nitrite was prepared using sodium nitrite (Sigma-Aldrich; St. Louis, MO) at concentrations of 0–200 µM. After 10 minutes of incubation at room temperature, the absorbance of the samples and standards was measured at 546 nm. Crystal violet solution (Fisher Scientific; Hampton, NH) was added to the cells and read at 540 nm absorbance to control for cell count per well.

Statistical Analysis

Lung CFU counts (x) were log transformed (as $x + 1$) prior to analysis and mean CFU counts were compared by 2-way ANOVA with Bonferroni's correction for multiple comparisons tests (Xu, Tasneen et al. 2018). Mouse body weight changes over time were analyzed by linear regression. Analyses of TNF production, phagocytic activity, and NO production were also done by 2-way ANOVA with Bonferroni's correction for multiple comparisons tests. Analyses of cytokines in Figures 5 and 8 were done by Students *t*-test with Welch's correction. All analyses were performed using GraphPad Prism version 7 (GraphPad; San Diego, CA).

Results

Mycobacterial Load

Colony forming units (CFU) were used to quantitatively assess the mycobacterial load of the collected organs. CFU increased with time post-infection, however, with no significant differences between 100 and 1,000 ppb arsenic exposure levels (Fig. 1). Although there were no significant differences between exposure groups, trends of increased CFU were observed at Day 7 and 28 post-infection. When observed as individual points, these trends more obviously show an increase in the arsenic-exposed groups at both 100 and 1,000ppb (Fig. 2). Mortality, assessed by weight, was not significantly different between exposure groups of C57BL/6 or A/J strains (Supp. Fig. 1). No significant differences were observed in food or water intake.

In alignment with the weight threshold set by Franco *et al.* (Franco, Correia-Neves et al. 2012), C57BL/6 and A/J mice were sacrificed at Day 20 and 19, respectively, once >15% of pre-infection body weight was lost. No significant differences or trends were observed in lung CFU counts between mouse strains or between arsenic-treated or untreated within strains. In the liver, CFU counts were significantly higher in 1,000 ppb C57BL/6 than in the 0 ppb and 1,000 ppb A/J. No significant differences were observed in spleen CFUs. Trends between exposures within the same strains were also observed in liver and spleen CFU.

Cytokines

Tumor Necrosis Factor (TNF) levels were measured in serum of mice exposed to 0 ppb or 1,000 ppb arsenic starting 3 weeks prior to infection until sacrifice (Fig. 3). Serum was collected prior to infection, and at days 1, 7, and 28 post-infection. At pre-infection, the 1,000 ppb arsenic-exposed group concentrations were greater than 0 ppb. Day 1 and 7 post-infection, there was a trend for the TNF levels in the serum to be lower in arsenic-exposed group than unexposed. These post-infection levels in arsenic-exposed group increased production of this cytokine at Day 28. None of the differences were statistically significant.

Overall, 32 cytokines were measured by the Luminex assay. However, in serum, only 16 of the assays had readings in the detectable range (eotaxin, G-CSF, IFNγ, IL-1α, IL-1β, IL-5, IL-6, IL-17, IL-9, IP-10, KC, MCP-1, M-CSF, MIG, MIP-2, and RANTES). Of this group, cytokines demonstrating differences in concentration between exposure groups in pre-infection serum were eotaxin, G-CSF, IP-10, and IL-17 (Fig. 4). IP-10 concentrations were significantly reduced in arsenic-exposed groups, and eotaxin and G-CSF demonstrated trends in lower concentrations than the unexposed groups. IL-17 was higher in the arsenic-exposed group but the difference was not statistically significant.

Of the same 32-plex cytokine panel, only IFNγ, IL-6, KC (CXCL1), and MIP-2 (CXCL2) had readings at detectable levels. Of these, IFNγ and IL-6 had observable differences between groups (Fig 8). A trend of decreased IFNγ and increased IL-6 concentrations

were found in the 1,000ppb compared to 0ppb arsenic group. There were no statistically significant differences between exposure groups.

Phagocytosis

Phagocytosis was expressed as 'percent effect' in peritoneal macrophages isolated from mice exposed to 0, 100 or 1,000 ppb of arsenic for 3 weeks (Fig. 6). After 1 hour, there appears to be a trend for a negative relationship between phagocytosis and arsenic levels in unstimulated cells. However, in the LPS/IFNγ -stimulated cells there were equal levels of the percent effects for all treatment and control groups. None of the differences were statistically significant.

After 2 hours, the 1,000 ppb-exposed group remained lower than percent effect of the unexposed group in the unstimulated cells. Interestingly, the 100 ppb-exposed group appeared to not only increase in phagocytic activity, but also have greater percent effect than the unexposed group. In the stimulated groups at this time point, there appears to be a trend of increased phagocytosis with increasing arsenic levels. Overall, the average phagocytic activity levels are still comparable to the 1 hour time point. Again, none of the differences were statistically significant.

After 3 hours, there is a noticeable decrease in the phagocytic percent effect overall due to degradation of the *E. coli* present by the cells. In the unstimulated cells, similar the 2-hour time point, the 1,000 ppb-exposed group remains lower than water and 100 ppb-exposed groups. Again, the 100 ppb-exposed cells have the highest percent effect of the

exposure groups. In the stimulated cells, the trend of increasing phagocytosis with increasing arsenic levels becomes more evident. None of the mean values were statistically significantly different from each other.

NO Production

Nitric oxide (NO) production was measured *ex vivo* in peritoneal macrophages from mice exposed to 100 or 1,000 ppb arsenic or water for 3 weeks (Fig. 7). In cells stimulated with LPS and IFNγ, the 100 ppb-exposed and 1,000 ppb-exposed groups produced more NO than unexposed. This was emulated in groups stimulated by PAM(3)CSK(4) and murine IFNγ, (Fig. 7) but with higher levels in each exposure group. In both groups, 100 ppb-exposed cells had the highest NO concentrations of the exposure groups. NO was not detected in unstimulated cells of any of the exposure groups. The differences in the NO levels were not statistically significant.

Discussion

Several areas of the world with a high prevalence of TB, such as Chile and Bangladesh, have also been exposed to high levels of arsenic. For this reason, it is important to understand the potential synergistic effects on health outcomes of these two agents. In this study, we aim to illuminate the mechanisms that may explain epidemiological findings that appear to document an increased mortality from TB in populations chronically exposed to high levels of arsenic (Smith, Marshall et al. 2011). In our animal model, we aimed to assess mortality, morbidity, and mycobacterial load in mice exposed to increasing concentrations of arsenic. We did not observe significant differences between weight gain or loss due to infection between any of the exposure groups (Supp. Fig. 1). However, CFU counts revealed a trend of increasing bacterial load from increasing arsenic concentration, analogous to the findings by Sillé et al. (unpublished, Sillé et al. 2016). Although not statistically significant, differences of +/- 0.5 log in CFU are defined as biologically significant. Therefore, the +0.25 changes in CFU for arsenic-exposed mice may be considered a biologically relevant trend (Tasneen, Williams et al. 2015). This data is particularly convincing as neither weight nor food and water intakes by any exposure groups were significantly different. Unlike Sillé et al., we did not observe differences in lung bacterial load between arsenic exposure doses.

To assess lung bacterial load and dissemination differences between C57BL/6 and A/J mouse strains, we collected the lung, liver, and spleen of both strains with respective exposure groups. Jagannath et al. observed significantly greater CFU in the lungs, liver,

and spleen of A/J compared to C57BL/6 mice when exposed to a 1,000 CFU *Mtb* infection (Jagannath, Hoffmann et al. 2000). The authors describe the difference observed as due to a genetic defect in the A/J strain that results in a deficiency of the fifth complement component (C5) (Cinader, Dubiski et al. 1964, Wetsel, Fleischer et al. 1990), an important component for opsonizing *Mtb* for phagocytosis (Schorey, Carroll et al. 1997, Schlesinger 1998). Here, we observed either no significant difference in the number of CFU between A/J than C57BL/6 mice at 19/20 days post-infection. One difference to note is our administration of a 10,000 CFU infection. This inoculation resulted in a shorter pathological timeline than the study by Jagannath et al., perhaps preventing necessary development of disease to develop a difference in bacterial loads. Another dissimilarity was the choice of vendor; our group purchased both strains from Charles River Laboratories, whereas Jagannath et al. purchased from Jackson Laboratories (Jagannath, Hoffmann et al. 2000). Recent studies have determined immunological endpoints to be dependent on mouse vendor source, including severity of infection and microbiota composition (Ericsson, Davis et al. 2015, Villarino, LeCleir et al. 2016). Of note, there was a trend for increased liver and spleen CFU in 1,000 ppb-treated groups for both C57BL/6 and A/J strains. This suggests differences in the ability of the immune system to contain *Mtb* infection and prevent dissemination to the rest of the body due to the migration of infected macrophages and dendritic cells (Chackerian, Alt et al. 2002).

After 3 weeks of arsenic exposure, we tested serum for differences in cytokines relevant to combating TB before and after aerosol infection. A cytokine well known for this role is

TNF, a cytokine released by macrophages upon infection that is associated with increased NO production (Flynn, Goldstein et al. 1995). Here, we sampled serum from pre-infection, as well as Day 1, 7, and 28 post-infection. At pre-infection, our observations reflect the *in vitro* findings by Sakurai et al. of increased TNF with arsenic exposure (Sakurai, Kaise et al. 1998). However, as we exposed mice to arsenic *in vivo,* the arsenic species in our mouse serum are likely to be a metabolized form due to methylation by the blood and liver (Buchet and Lauwerys 1985, Marafante, Vahter et al. 1985, Nemeti and Gregus 2004, Gregus and Nemeti 2005). Kozul et al. observed an increase of TNF expression after 5-6 weeks of sodium arsenite exposure in drinking water, suggesting an increase in its production as well (Kozul, Hampton et al. 2009). On Day 1 and Day 7 post-infection, production of this cytokine is less in arsenic-exposed groups than unexposed. The difference is most striking in Day 1, suggesting the arsenic-exposed group is more susceptible to the infection shortly after *Mtb* aerosol. This finding is analogous to the zebrafish infection/arsenic-exposure models by Hermann et al. and Nayak et al. where decreased concentrations of TNF were observed (Nayak, Lage et al. 2007, Kozul, Hampton et al. 2009). It isn't until Day 28 post-infection that TNF levels in arsenic-exposed animals are once again greater than those of unexposed groups. This U-curve of arsenic-exposed TNF production during infection may suggest an interplay between arsenic and infection. Though TNF levels in unexposed mice gradually increased overtime with infection, as expected, there seems to be a shunting effect in the arsenic-exposed group early on in infection that later recovers around the time of adaptive immunity. Therefore, it may be speculated that arsenic exposure is most damaging to the immune response against *Mtb* early on in infection by negatively

effecting the innate immune response. Perhaps cells with roles in innate immunity, such as the macrophage, may experience functional changes due to arsenic exposure.

Also sampled in this study were 32 different cytokines from the blood at pre-infection after 3 weeks arsenic exposure. Here, we observed a significant decrease in IP-10 in the serum of arsenic-exposed mice. IP-10, also referred to as CXCL10, is an interferon gamma-induced chemokine that is expressed by APCs that binds to the receptor CXCR3 on monocytes, NK cells and T cells and is considered a downstream marker of cell mediated immunity against *Mtb* (Ruhwald, Aabye et al. 2012). Indeed, numerous clinical studies have shown that IP-10 is on par with and, in the case of children and persons with low CD4 T cell counts, a better biomarker for TB infection diagnosis than IFN_{γ} (Ruhwald, Aabye et al. 2012). Therefore, an arsenic-mediated decrease in IP-10 may compromise the immune response to *Mtb* infection. IP-10 has even been suggested as a biomarker to differentiate between stages of tuberculosis infection (Wergeland, Pullar et al. 2015). In future studies of TB mouse models, tracking IP-10 levels may serve as an effective way to track tuberculosis progression in the context of arsenic exposure.

Two other cytokines evaluated here that showed differences in expression were eotaxin (CCL11) and Granulocyte Colony-Stimulating Factor (G-CSF/CSF1). Both cytokines demonstrated a trend for decreased levels in the serum in the arsenic-exposed groups. Eotaxin has a role in eosinophil recruitment to inflammatory tissues and has also been used as a tuberculosis biomarker, specifically for detecting pulmonary tuberculosis (Choi, Kim et al. 2016). Good responders to anti-tuberculosis treatments demonstrated increased

levels of eotaxin (Choi, Kim et al. 2016), and eotaxin is lower in active tuberculosis patients compared to those with latent infections (Djoba Siawaya, Beyers et al. 2009). Therefore, it is possible that lower eotaxin levels is indicative of an immune system that is less apt to control an *Mtb* infection. Just as IP-10, eotaxin too can be used as a biomarker in future TB mouse models.

G-CSF, known for its essential role in the induction of the Th1 response (Gonzalez-Juarrero, Hattle et al. 2005, Rothchild, Stowell et al. 2017), frequently increases with *Mtb* infection to produce macrophages with the phenotype appropriate to combat this pathogen (Higgins, Sanchez-Campillo et al. 2008, Cho, Park et al. 2013). Additionally, Szeliga et al. determined that GM-CSF (CSF2), another member of the CFS family, has an essential role of protecting alveolar structure and regulating macrophages and dendritic cells to contain *Mtb* in granulomas (Szeliga, Daniel et al. 2008). Therefore, it may be suggested that a decrease in GM-CSF may leave the body more vulnerable to the damaging effects of TB. The levels of GM-CSF in our model can only be speculated, as it was not at detectable levels in the serum from naïve (uninfected) mice (data not shown).

IL-17, as opposed to the other select cytokines, had a trend of increased concentration in serum from arsenic exposed groups. This increase in IL-17 could reflect a direct response to *Mtb* or could represent a response to a break in barrier integrity and exposure to components of the microbiome. Vested with the ability to recruit neutrophils to mucosal sites (Miyamoto, Prause et al. 2003) and modulate granulopoiesis (Schwarzenberger, La Russa et al. 1998), IL-17 appears as only beneficial for fighting an *Mtb* infection. However, a shift from Th1 to Th17 response may produce excessive IL-17

to, in turn, extensively recruit neutrophils and cause tissue damage (Torrado and Cooper 2010). It may not be so beneficial to respiratory health in TB patients if arsenic induces an increase in IL-17.

To assess immunogenic changes at the cellular level, we tested a functional attribute of peritoneal macrophages *ex vivo* after 3 weeks of *in vivo* exposure to 0, 100, or 1,000 ppb arsenic. The phagocytic activity of the macrophages was assessed by fluorogenic reagents every hour for 3 hours. For unstimulated cells, there is a trend for decreased phagocytosis with increasing arsenic concentrations. This pattern was also observed by Fisher et al. in an *in vitro* bovine model, and Banerjee et al. in an *in vitro* human model (Fisher, McNeill et al. 1986, Banerjee, Banerjee et al. 2009). However, neither of these studies stimulated their macrophages before their respective phagocytosis assay. In our study, when we stimulated these macrophages with LPS and IFNγ a trend of a positive relationship between arsenic and phagocytosis was observed. This has not been published before, and it suggests deeper complexity to the relationship between arsenic and phagocytic ability. Future studies should focus on the differences in the interaction with arsenic with the cytoskeleton of unstimulated and stimulated macrophages, as changes in this network are known to affect phagocytosis (May and Machesky 2001, Banerjee, Banerjee et al. 2009). Unlike the 1st hour, the 2nd and 3rd hour of the assay showed that the 100 ppb arsenic-exposed macrophages had the greatest phagocytic ability. Readings were made out to these time points to look for potential delay in phagocytosis. High phagocytosis readings at 100 ppb compared to the 1,000 ppb and 0 ppb treatments suggests a non-linear dose

response that numerous studies have found in arsenic exposure models (Arkusz, Stanczyk et al. 2005, Burchiel, Lauer et al. 2014, Xu, McClain et al. 2016).

Once phagocytosis is complete, the role of the macrophage is to kill the pathogen in the phagolysosome. The cell produces nitric oxide to degrade the bacteria and remove it. By assessing NO production, we evaluated the peritoneal macrophages' potential to intracellularly kill *Mtb*. Here again observed that cells exposed to 100 ppb of arsenic had the highest NO production of the exposure groups. Overall, there is a trend for increasing NO production with increasing levels of *in vivo* arsenic exposure. This pattern disagrees with the *in vitro* exposure murine cell study by Arkusz et al. and the *ex vivo* human cell study by Banerjee et al. which both indicate that arsenic suppresses NO production in macrophages (Arkusz, Stanczyk et al. 2005, Banerjee, Banerjee et al. 2009). However, Luna et al. observed a positive relationship with NO production and arsenic levels in the urine of environmentally exposed children (Luna, Acosta-Saavedra et al. 2010). As their group suggests, this finding may be due to increasing oxidative stress. Arsenic has been shown to induce oxidative stress through reactive nitrogen species (RNS) and reactive oxygen species (ROS) (Wu, Chiou et al. 2001, Ding, Hudson et al. 2005). Indeed, the relationship between SO and arsenic exposure has also been controversial (Lantz, Parliman et al. 1994, Luna, Acosta-Saavedra et al. 2010). Future studies of RNS and ROS with arsenic exposure must account for the actual arsenic species the macrophage is interacting with. Accounting for this should tease out discrepancies between studies of RNS/ROS association with arsenic association.

To assess the microenvironment created by macrophages after *in vivo* arsenic exposure, we analyzed supernatant from stimulated macrophages. Although not statistically significant, the trend of decreased IFNγ was particularly noteworthy due to its vital role in *Mtb* resistance (Flynn, Chan et al. 1993). Although primarily produced by lymphocytes, IFNγ by neighboring macrophages may also be necessary for proper stimulation of macrophages (Nathan, Murray et al. 1983). Hermann et al. observed increased IFNγ expression with increased sodium arsenite doses, but it should be noted that this was in a zebrafish fish pathogen model and that concentrations of IFNγ were not measured at any level (Hermann and Kim 2005). A trend of increased macrophage IL-6 production was observed in our model, which to our knowledge has not been reported before in an arsenic model. However, Mehrzad et al. reported increased IL-6 production by porcine dendritic cells, another APC (Mehrzad, Mahmudy Gharaie et al. 2017). There have also been reports of increased IL-6 levels in the lung in arsenic-exposed mice, suggesting an imbalance in redox status and disturbance of the Th1/Th2 balance (Li, Zhao et al. 2017).

Our study suggests a compromised immunogenic microenvironment induced by arsenic influencing the function of macrophages vital for the immune response against *Mtb* infection. Yet, our results also demonstrate the complexity of how arsenic interacts with and influences cells of the innate immune response. Additionally, there is vast literature on the effect of arsenic on T cells, known to produce IFNγ to create the M1 phenotype macrophages and the Th1 response (Soto-Pena and Vega 2008, Cho, Ahn et al. 2012). Future studies of the TB mouse model exposed to arsenic should aim to discriminate

between the effects arsenic has on the function of macrophages, dendritic cells, and T cells and their influence back on the regulation of the immune system responding to pathogens such as *Mtb*. In conclusion, we found relevant levels of arsenic in drinking water may compromise the immune response against *Mtb* infection. To this end, immunotoxicity at low levels of arsenic should be accounted for in public policy dictating environmental arsenic concentration limits, especially in areas of the world where tuberculosis is prevalent.

Figures

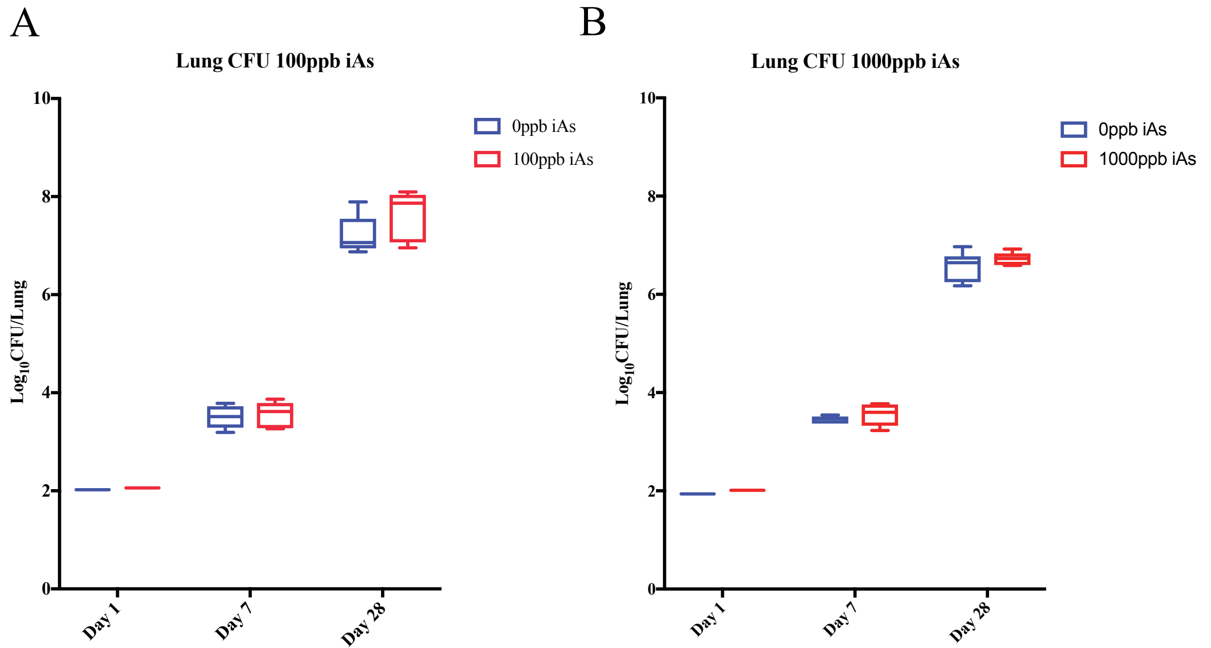

Figure 1 Lung CFU of varied iAs exposures with *Mtb* infection. Colony formation units (CFU) over post-*M. tuberculosis* (*Mtb*) infection timepoints. C57BL/6 mice were exposed to 0 or A) 100 or B) 1,000 parts per billion (ppb) of arsenic (iAs) 3 weeks prior to infection until sacrifice. Mice were infected with A) 100 or B/C) 10,000 colony formation units (CFU) of *Mtb*. Mouse strain was C57BL/6. No statistically significant differences in CFU were observed between treatment groups in either study (*p-value* > 0.05). Trends of increased CFU in iAs-exposed mice were observed.

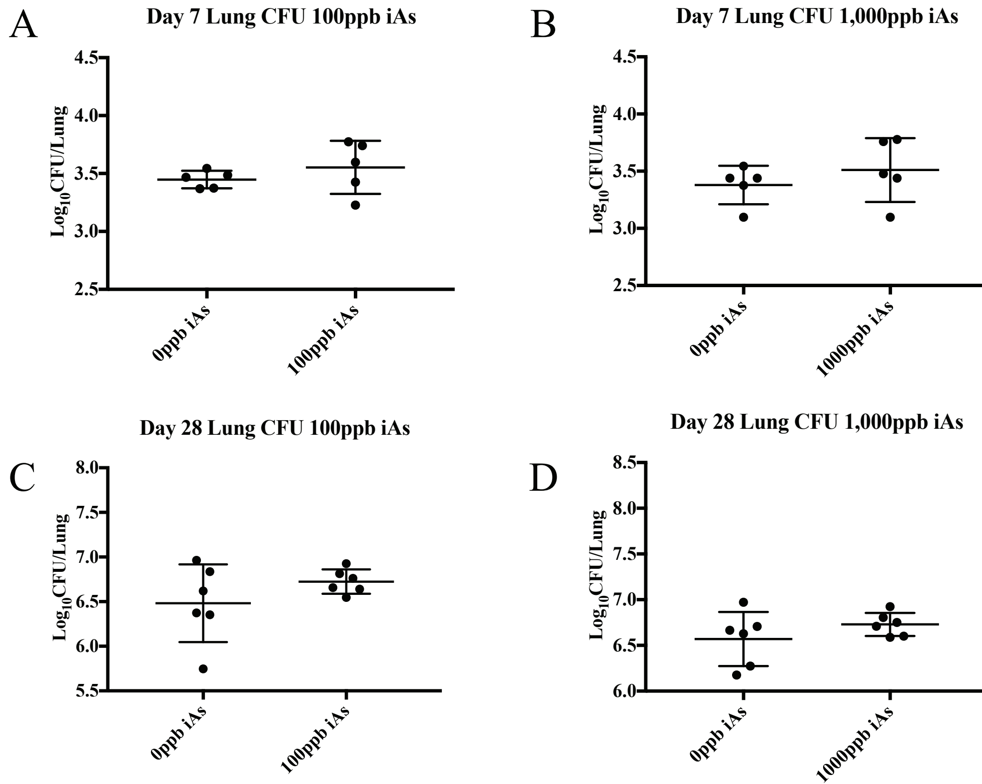

Figure 2 Lung CFU of 100ppb and 1,000ppb iAs exposure with *Mtb* infection. Colony forming units (CFU) for A/B) Day 7 and C/D) Day 28 post-*M. tuberculosis* (*Mtb*) infection from the studies represented in Figure 1. Dilutions of A/C) 10^{-1} and B/D) 10^{-5} are representative of the bacterial loads in the lung. Mice were exposed to A/C) 100 or B/D) 1000 parts per billion (ppb) of inorganic arsenic (iAs) or water starting 3 weeks prior to infection and until sacrifice. Mice were infected with 100 colony formation units *M. tb*. Mouse strain was C57BL/6. No statistically significant differences in CFU were observed between treatment groups in either study (*p-value* > 0.05). Trends of increased CFU within treatment groups were observed.

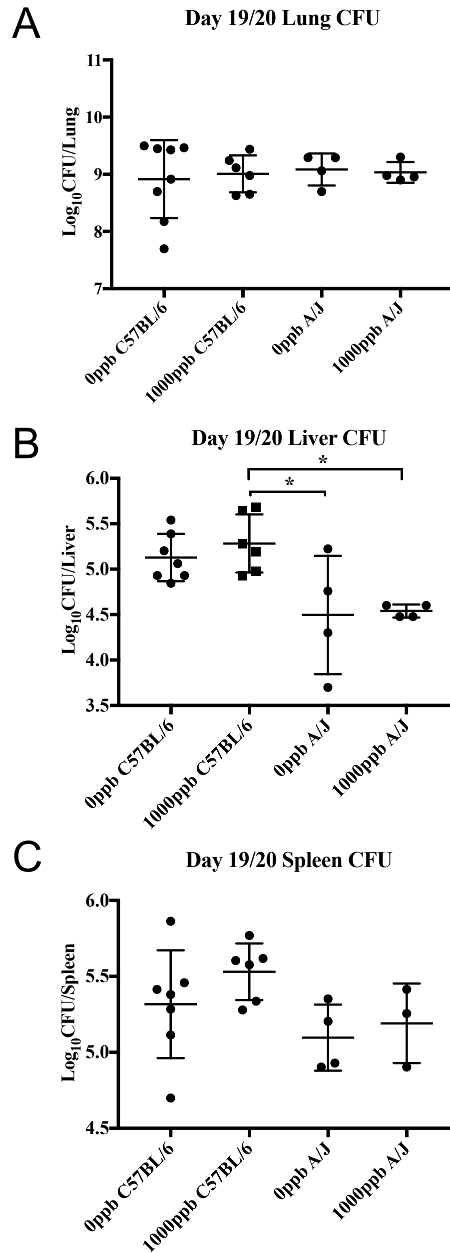

Figure 3 C57BL/6 and A/J CFU of iAs exposure and *Mtb* infection. Colony forming units (CFU) for A/J and C57BL/6 strains 19/20 days post-10,000 CFU *Mtb* infection, respectively. Dilutions of A) 10^{-7} and B/C) 10^{-3} are representative of the mycobacterial loads in the A) lungs, B) livers, and C) spleens.

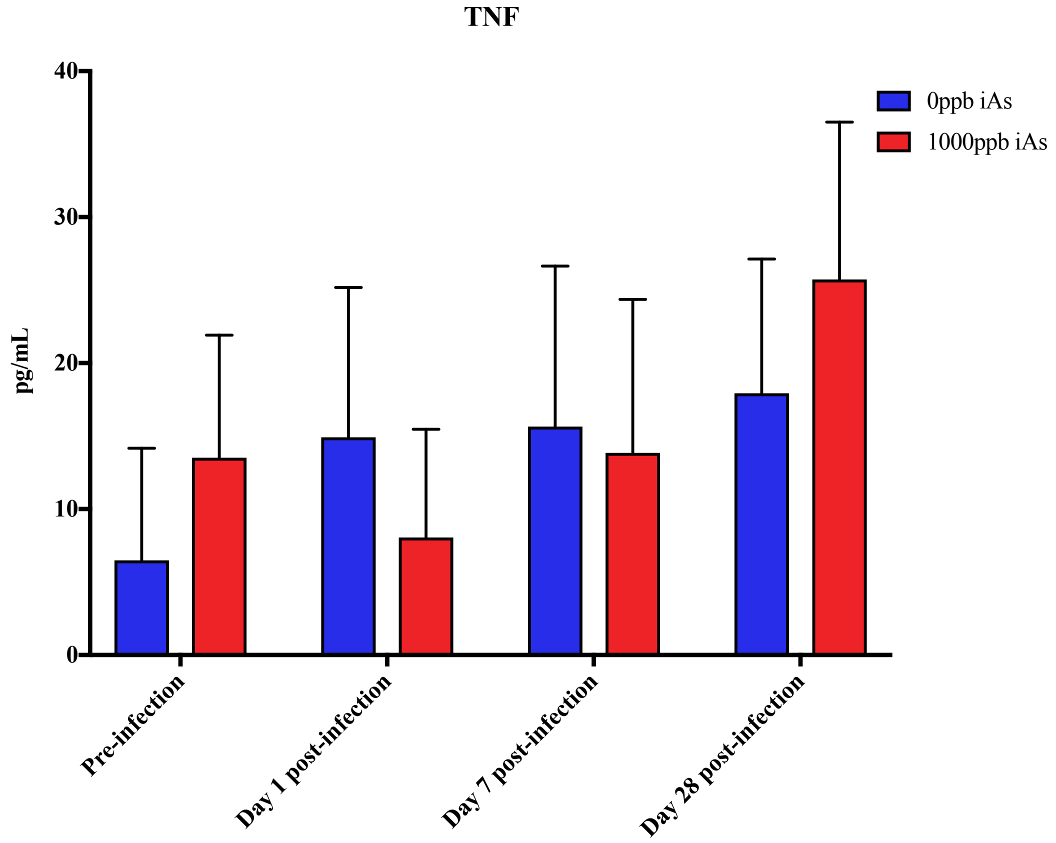

Figure 4 TNF serum levels with iAs exposure and *Mtb* infection. Tumor Necrosis Factor (TNF) serum concentrations pre- and post-100 CFU *M. tuberculosis* (*Mtb*) infection timepoints in the study represented by Figure 1B. Mice were treated with 0 or 1,000 parts per billion (ppb) of inorganic arsenic (iAs) starting 3 weeks prior to infection until sacrifice. Mouse strain was C57BL/6. No statistically significant differences in CFU were observed between treatment groups (*p-value* > 0.05).

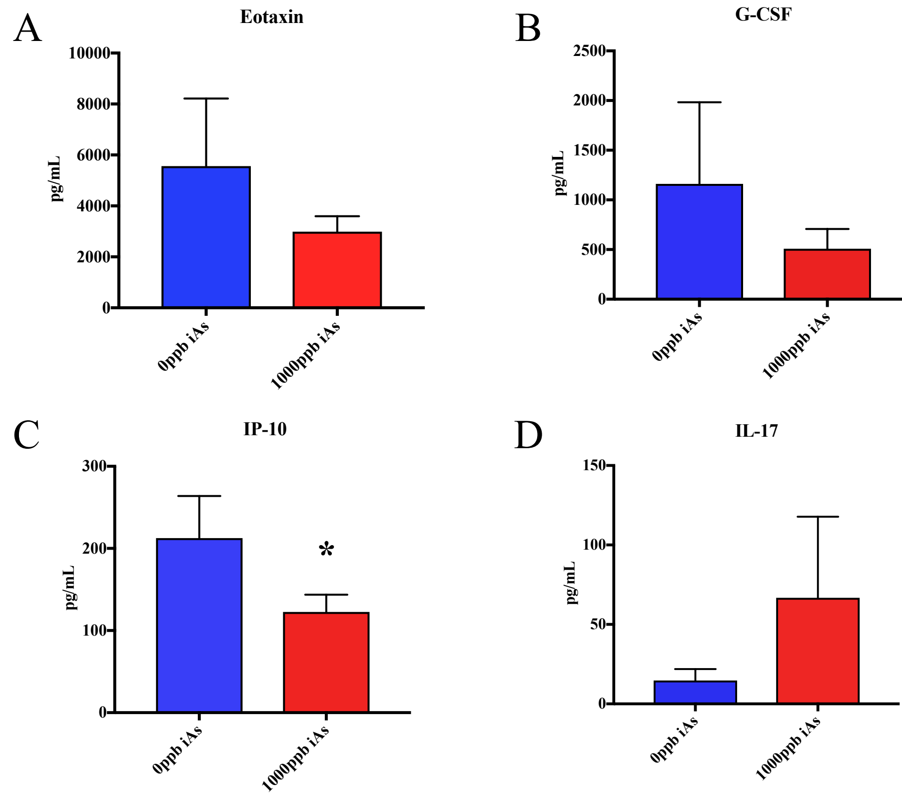

Figure 5 Serum cytokines post-iAs exposure. Select cytokine concentrations measured in serum before *M. tuberculosis* (*Mtb)* infection in the study represented in Figure 1B. Mice were exposed to 1000 parts per billion (ppb) of inorganic arsenic (iAs) or water for 3 weeks (**p-value* <0.05).

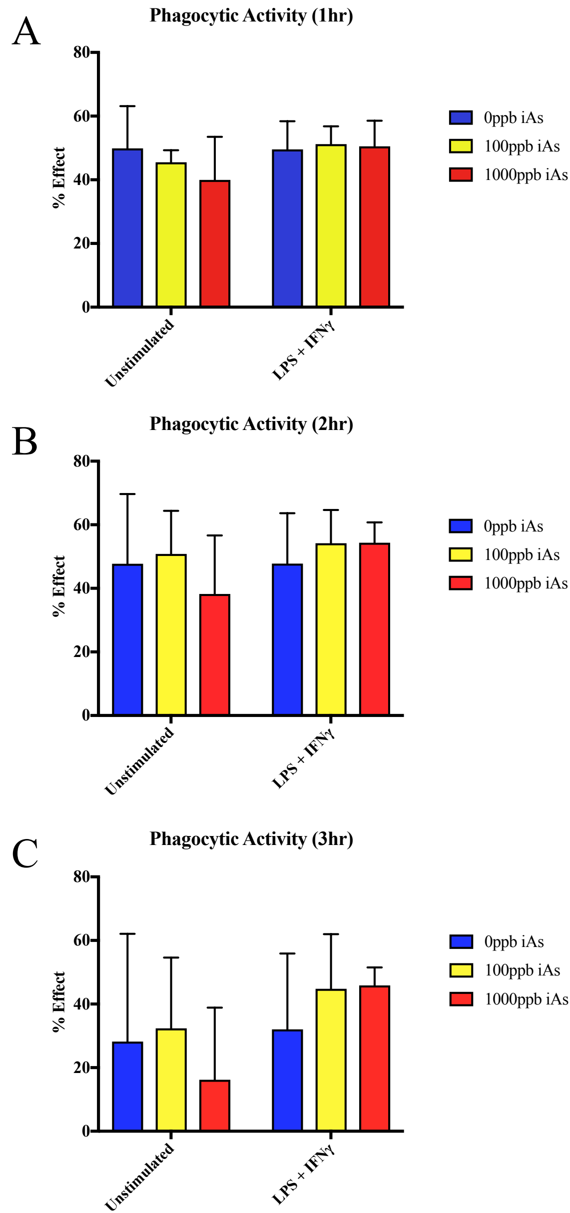

Figure 6 Phagocytic activity post-iAs exposure. Phagocytic activity of C57BL/6 mice peritoneal macrophages after A) 1 hour B) 2 hours and C) 3 hours after phagocytosis effector treatment. Cells were tested *ex vivo* after 3 weeks *in vivo* exposure to 0, 100 or 1000 parts per billion (ppb) inorganic arsenic (iAs). No statistically significant differences in CFU were observed between treatment groups (*p-value* > 0.05).

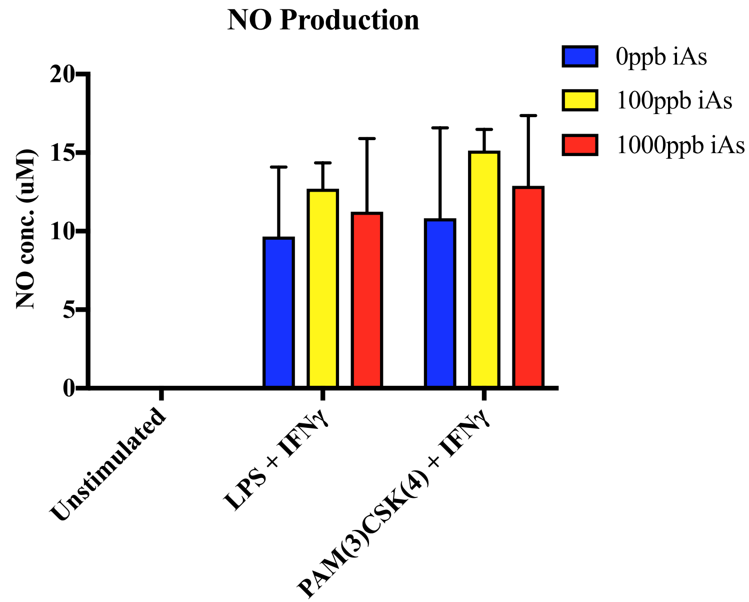

Figure 7 NO porduction post-iAs exposure. Nitric Oxide (NO) production by C57BL/6 mice peritoneal macrophages *ex vivo* after 48 hours stimulation. Cells were tested *ex vivo* after 3 weeks *in vivo* exposure of 0, 100 or 1,000 parts per billion (ppb) inorganic arsenic (iAs). Mouse strain was C57BL/6. No statistically significant differences in CFU were observed between treatment groups (*p-value* > 0.05).

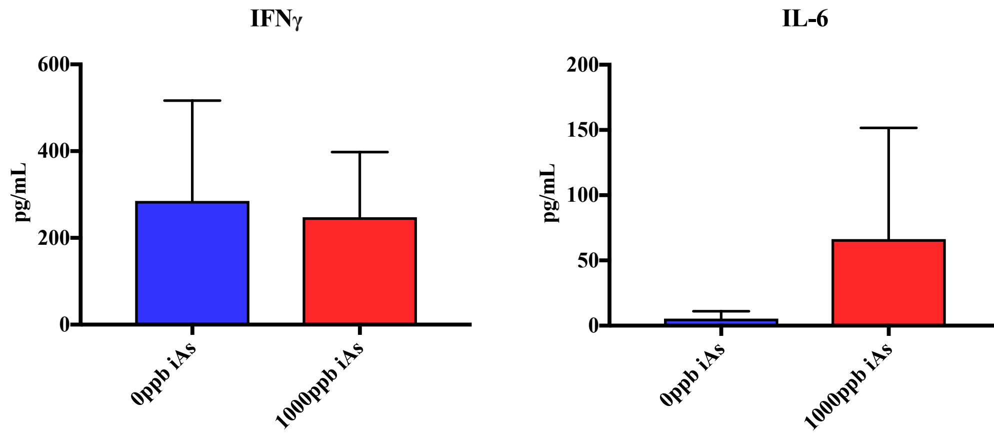

Figure 8 Macrophage cytokine production post-iAs exposure. Select cytokine concentrations measured in peritoneal macrophage supernatant after LPS + IFNγ stimulation. Mice were exposed to 0 or 1000 parts per billion (ppb) of inorganic arsenic (iAs) for 3 weeks. No statistically significant differences were observed between exposure groups (*p-value* > 0.05).

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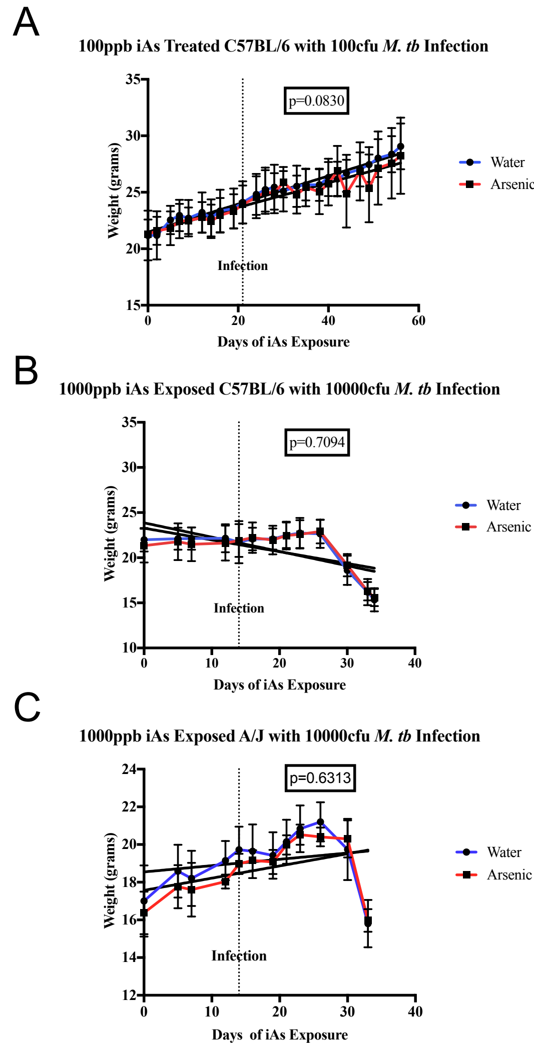
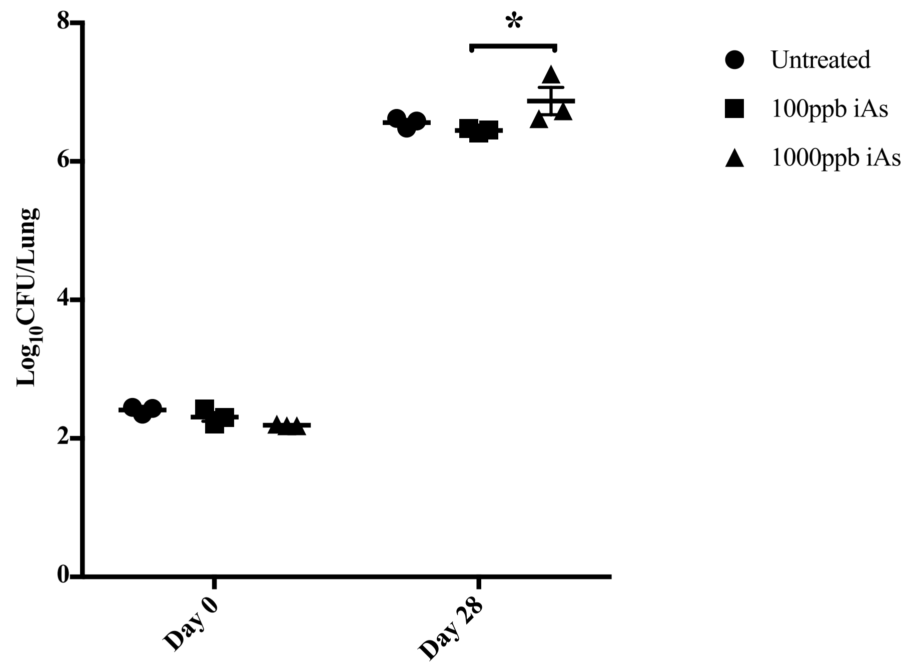
Supplemental Figures



Supplemental Figure 1 Body weight changes with iAs exposure and *Mtb* infection. Mouse weights (grams) by treatment group in study A (N=12), B (N=14), and C (N=8). Mice were exposed to A) 100 or B) 1000 parts per billion (ppb) of arsenic (iAs) or water starting 3 weeks before infection and until sacrifice. Mice were infected with A) 100 or B/C) 10000 colony formation units (cfu) of *M. tuberculosis* (*M. tb*). Mice strains were A/B) C57BL/6 or C) A/J. Infection occured on Day 21 and Day 14 in study A and B/C, respectively. No statistically significant differences in weight increase or decrease were observed between treatment groups in either study (*p-value* > 0.05).



Supplemental Figure 2 iAs exposure and *Mtb* infection lung CFU preliminary data. Unpublished data of C57BL/6 mice exposed to 0, 100, and 1000ppb arsenic (iAs) throughout *M. tuberculosis* (*Mtb*) infection and post-infeciton days. (*$P<0.05$)

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As part of the LeClair lab, my undergraduate research focused on epithelial and immune cell migration regenerative tissue of a zebrafish model by confocal microscopy.

2014-2015 — **Research Intern, Molecular Neurobiology**
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As part of the Obrietan lab, my rotation research focused on the effect of forebrain circadian clock timing on memory stem cell growth by behavioral assays and tissue immunolabeling.

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ABSTRACTS:

I. Sanchez, K.A. Rychlik, H. Zhang, C.M. Steinmaus, M.T. Smith, F.C.M. Sillé. The effect of arsenic exposure in an adult mouse tuberculosis model. Society of Toxioclogy Meeting, San Antonio, TX, March 11-15, 2018.

I. Sanchez, M. Vieira, A. Nelson, K. Fricke, M. Shin, V. Polotsky, and W. Mitzner. Diet-induced obesity attenuates airway hyperresponsiveness in mice. American Thoracic Society Conference, Washington, D.C., May 19-24, 2017.

I. Sanchez, K. Hansen, K. Snider, S. Impey, K. Obrietan. Transcriptional profiling of circadian clock-controlled gene expression in the hippocampus. Ohio State University Wexner Medical Center Trainee Research Day, Columbus, OH, April 15-16, 2015.

F. Figueroa, **I. Sanchez**, and E. E. LeClair. Wnt signaling, wound healing, and the immune system response in zebrafish maxillary barbell development and regeneration.

51st Annual Midwest Regional Society of Developmental Biology Meeting, St. Louis, MO, September 26-28, 2013.

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March 2018	Society of Toxicology Meeting, San Antonio, TX. "The effect of arsenic exposure in an adult mouse tuberculosis model." (Poster)
May 2017	American Thoracic Society Conference, Washington, D.C. "Diet-induced obesity attenuates airway hyperresponsiveness in mice." (Poster)
April 2015	Ohio State University Wexner Medical Center Trainee Research Day, Columbus, OH. "Transcriptional profiling of circadian clock-controlled gene expression in the hippocampus." (Poster)
Feb 2014	Louis Stokes Alliances for Minority Participation Conference, Chicago, IL. "Wound healing and the immune response in zebrafish maxillary barbell development and repair." (Oral presentation)
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