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**Kathryn A. Ramsey, Rachel E. Foong, Peter D. Sly,
Alexander N. Larcombe and Graeme R. Zosky**

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Early Life Arsenic Exposure and Acute and Long-term Responses to Influenza A Infection in Mice

Kathryn A. Ramsey^{1,2}, Rachel E. Foong^{1,2}, Peter D. Sly³, Alexander N. Larcombe^{1,2}, and Graeme R. Zosky^{1,2}

1. Division of Clinical Sciences, Telethon Institute for Child Health Research, Subiaco Western Australia, Australia.

2. Centre for Child Health Research, University of Western Australia, Perth, Western Australia, Australia.

3. Queensland Children's Medical Research Institute, University of Queensland, Herston, Queensland, Australia.

Corresponding author:

Kathryn A. Ramsey

Division of Clinical Sciences

Telethon Institute for Child Health Research

100 Roberts Road, Subiaco, Western Australia, 6008

Ph: +61 8 9489 7822

Fax: +61 8 9489 7700

Email: kramsey@ichr.uwa.edu.au

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Abstract

Background: Arsenic is a significant global environmental health problem. Exposure to arsenic in early life has been shown to increase the rate of respiratory infections during infancy, reduce childhood lung function and increase the rates of bronchiectasis in early adulthood.

Objectives: We aimed to determine if early life exposure to arsenic exacerbates the response to early life influenza infection.

Methods: C57BL/6 mice were exposed to arsenic *in utero* and throughout post-natal life. At 1 week of age a subgroup of mice were infected with influenza A. The acute and long term effects of arsenic exposure on viral clearance, inflammation, lung structure and lung function were assessed.

Results: Early life arsenic exposure reduced the clearance of and exacerbated the inflammatory response to influenza A, and resulted in acute and long term changes in lung mechanics and airway structure.

Conclusions: Increased susceptibility to respiratory infections combined with exaggerated inflammatory responses throughout early life may contribute to the development of bronchiectasis in arsenic exposed populations.

Introduction

Hundreds of millions of people throughout the world are exposed to arsenic through their drinking water at doses above the World Health Organization maximum contaminant level of 10µg/L (Mandal and Suzuki 2002). Chronic exposure to arsenic via drinking water has been shown to increase the risk of developing lung, liver, prostate, bladder, kidney and skin cancers (Ferreccio et al. 2000; Haynes 1983; Smith et al. 1992; Smith et al. 1998). Arsenic exposure has also been linked to the development of non-malignant lung diseases, including bronchiectasis, chronic bronchitis and chronic obstructive pulmonary disease (Guha Mazumder et al. 2005; Milton et al. 2001; Smith *et al.* 1998). An exposure event in northern Chile, whereby the residents of Antofagasta consumed high levels of arsenic (90 – 860µg/L) for two decades revealed the significance of early life arsenic exposure in the development of respiratory disease. In the years following the peak exposure event, children from Antofagasta presented with cough, dyspnea, bronchopulmonary disease and bronchiectasis (Borgono et al. 1977; Borgono and Greiber 1971; Rosenberg 1974; Zaldivar 1980). Long term follow up of these children revealed that the standardized mortality ratio for bronchiectasis was 46.2 (95% CI, 21.1–87.7; $p < 0.001$) in those born during the peak exposure event who were exposed to arsenic *in utero* and early childhood (Smith et al. 2006). Bronchiectasis is a progressive respiratory disease characterized by repeated lower respiratory tract infections and an intense inflammatory response leading to tissue damage, alterations to lung structure and premature death in adulthood (Barker 2002). Understanding whether arsenic exposure is able to increase the susceptibility to or exacerbate responses to respiratory infections in early life may help to explain the link between arsenic exposure and the development of bronchiectasis.

There is evidence that prenatal arsenic exposure can increase the susceptibility to respiratory infections in early life. Infants in Bangladesh exposed to arsenic at concentrations above 250 $\mu\text{g/L}$ *in utero* had a significantly increased risk of developing lower respiratory tract infections (RR = 1.69, 95% CI 1.36, 2.09) compared with those exposed to arsenic at concentrations below 39 $\mu\text{g/L}$ (Rahman et al. 2011). This increase may be linked to the known immunosuppressant activity of arsenic and/or the known effects of arsenic on impairing growth and development. Exposure during pregnancy can increase inflammatory cytokines and reduce T-cell numbers in the placenta (Ahmed et al. 2011) and impair thymic development in infants (Raqib et al. 2009). It is also associated with infants being born small for gestational age (Huyck et al. 2007; Rahman et al. 2009). Low birth weight is an important risk factor for the development of respiratory infections and worse lung function in childhood (Barker et al. 1991; Chan et al. 1989; Rona et al. 1993; Shaheen and Barker 1994; Vik et al. 1996) and mortality from chronic respiratory diseases in early adulthood (Stein et al. 1997; Stick 2000). We have previously shown in mice that *in utero* exposure to arsenic impairs lung growth and development, induces mucous cell metaplasia in the airways and alters the expression of genes that regulate lung morphogenesis and mucociliary clearance in the lung, all of which could contribute to the increased risk of respiratory infections in early life (Ramsey et al. 2013a). A combination of arsenic-induced impairments to lung growth and immune development are likely mechanisms for the increased rate of respiratory infections in early childhood.

We know little, however, about how arsenic may alter the physiological response to respiratory infections. Environmental exposure to cigarette smoke and particulate matter both not only increase the susceptibility to respiratory infections but also exaggerate the inflammatory responses to respiratory infections (Arcavi and Benowitz 2004; Ciencewicki and Jaspers 2007). A previous experimental study has shown that mice exposed to arsenic in

adulthood had a diminished ability to clear an influenza A (H1N1) infection, impaired CD8+ T cell responses, prolonged viral carriage and greater mortality compared with mice given influenza alone (Kozul et al. 2009). Exposure to arsenic in early life may increase the susceptibility to, and exacerbate the response to respiratory infections during a period of high susceptibility in infancy. A compromised response to influenza infection in early life may have a significant effect on infant morbidity and mortality, and play an important role in the development of bronchiectasis and other chronic respiratory diseases in arsenic exposed areas.

The present study investigated how exposure to arsenic *in utero* and throughout postnatal life may alter the response to an infection with influenza in infancy. We examined how arsenic modified both the acute inflammatory response to influenza infection, as well as the long term implications of this response for lung structure and function.

Methods

Animals and exposures

C57BL/6 mice were sourced from the Animal Resources Centre (Murdoch, Western Australia). Animals were treated humanely and with regard for alleviation of suffering. All studies were conducted according to the guidelines of the National Health and Medical Research Council Australia and approved by the institutional Animal Ethics Committee. We used a previously established model of *in utero* arsenic exposure whereby pregnant mice were given drinking water containing 0 (control) or 100 µg/L of arsenic from gestational day 8 until birth of their offspring (approx. E19) in the form of sodium arsenite (Ramsey *et al.* 2013a; Ramsey et al. 2013b). After their offspring were born, maternal exposure to either arsenic or control drinking water continued.

At one week postnatal age, offspring were inoculated intranasally with $10^{4.2}$ pfu Influenza A/Mem71 (H3N1) diluted in 10 μ L of virus production serum free medium (VP-SFM; Life Technologies, Mulgrave VIC) or the same volume of VP-SFM containing a preparation of mock-infected cells. Offspring were weaned at four weeks of age and continued to receive arsenic or control drinking water as per the prior exposure protocol. Outcomes were measured in the offspring at 3 and 7 days post-infection (during the acute stage of influenza infection), 21 days post-infection (after recovery from influenza infection) and 7 weeks post-infection (adulthood) (Table 1).

To investigate the effects of timing of arsenic exposure on long term lung structure and function outcomes, a separate group of female adult (8 week) C57BL/6 mice were exposed to either 100 μ g/L arsenic ($n = 7$) or control drinking water ($n = 7$) for the same period of time as the mice exposed from e8 gestation to 8 weeks post-natal age (10 weeks).

Inflammatory cells, viral titer and cytokines

Cellular inflammation was measured via total and differential cell counts in bronchoalveolar lavage fluid (BALf) taken from all offspring. Whole lungs were removed from infected offspring 3 and 7 days post-infection for quantification of viral titer. Inflammatory cytokines (IFN- γ , IL-6, TNF- α , MCP-1) were measured in BALf supernatants using a mouse inflammation Cytometric Bead Array (BD Biosciences, San Diego, CA, USA) as per the manufacturer's instructions. Measurement of total protein content of the BALf was carried out using the Bradford technique employing the Bio-rad Protein Assay kit according to manufacturer instructions (BIO-RAD, NSW, Australia). Further details are provided in Supplemental Material, p2.

Thoracic gas volume and lung mechanics

Lung volume and lung mechanics were measured in offspring at 7 days, 21 days and 7 weeks post-infection, and in mice exposed to arsenic in adulthood only. To measure lung mechanics *in vivo*, mice were anaesthetized, tracheotomized and mechanically ventilated. Plethysmography was used to measure thoracic gas volume (TGV) as described previously (Janosi et al. 2006). Lung mechanics were measured using the forced-oscillation technique as described previously (Sly et al. 2003). The forced-oscillation technique generates measures of airway resistance (R_{aw}), tissue damping (resistance) (G) and elastance (stiffness) (H). Further details are provided in Supplemental Material, p3.

Responsiveness to Methacholine

Hyper-responsiveness of the respiratory system to bronchoconstricting agents, such as methacholine, can reflect the presence of pulmonary inflammation or altered lung structure such as excess mucous production or increased airway smooth muscle (Lundblad et al. 2007, 2008). The responsiveness to methacholine (MCh) was measured in offspring at 7 weeks post-infection and mice exposed to arsenic as adults only. Mice received a saline aerosol followed by increasing doses of aerosolized MCh from 0.1 to 30 mg/mL for 90 seconds. Lung function was measured every minute for 5 minutes after the conclusion of each aerosol. Further details are provided in Supplemental Material, p4.

Airway remodeling

Following euthanasia, the lungs of offspring at 7 weeks post-infection were fixed through intratracheal instillation of 2.5% glutaraldehyde at 10 cmH₂O. The left lung was embedded in paraffin wax and 5 µm sections were cut at proximal, middle and distal parts of the lung for airway histology. Airway sections were stained and scored blind for airway smooth muscle and airway mucous cells. Further details are provided in Supplemental Material, p5.

Statistics

Statistical analyses were conducted using SigmaPlot software (v12.3 SPSS Science, Chicago, IL, USA). Group means were compared using two-way ANOVA with arsenic and influenza exposure as independent variables and Holm-Sidak post hoc analysis. If the interaction term was significant ($p < 0.05$) the interaction p value was reported and additional analysis was performed to determine if the effects were super-additive. We defined super-additive as an interaction between arsenic and influenza which produces an effect significantly greater than the sum of the individual effects. We determined if the interaction was super-additive by dividing the effect of combined arsenic and influenza by the sum of the effects of arsenic and influenza (Bates et al. 2008). A ratio significantly greater than 1 (one sample, two tailed t -test) indicates that the interaction is super-additive. Where necessary, data were log transformed to satisfy the assumptions of normality and homoscedasticity. A p value less than 0.05 was considered to be significant. Data are shown as mean (SD).

Results

Maternal outcomes

There were no significant effects of arsenic exposure on maternal body weight ($p = 0.51$), litter size ($p = 0.89$) or gestation period ($p = 0.11$). There were no effects of arsenic exposure or influenza infection on maternal water consumption (arsenic $p = 0.59$; influenza $p = 0.81$) (data not shown). At day 7 post-infection (2 weeks of age) and day 21 post-infection (4 weeks of age) there were no significant differences in any outcome between male and female offspring, so data were pooled.

Inflammatory cells

A significant inflammatory response to influenza infection was present at days 3 and 7 post-infection which was resolved 21 days post-infection (Figure 1). At day 3 post-infection, there were effects of both arsenic and influenza on the number of total cells (arsenic $p = 0.002$; influenza $p < 0.001$) and number of macrophages (arsenic $p = 0.01$; influenza $p < 0.01$) in the BALf, which were additive such that the mice exposed to both arsenic and influenza had greatest inflammatory response. At three days post-infection there was a significant effect of influenza ($p < 0.01$) on neutrophil number. At day 7 post-infection, there was a significant interaction between arsenic and influenza on the numbers of total cells ($p = 0.01$) and neutrophils ($p = 0.04$) in the BALf, such that the number of cells present in response to influenza was significantly higher if the mice had also been exposed to arsenic. There was a significant super-additive relationship between arsenic and influenza in determining the total number of cells (ratio 2.04 ± 2.49 , $p = 0.01$) and neutrophils (ratio 2.47 ± 4.54 , $p = 0.06$) in the BALf at day 7 post-infection. There was also a significant effect of influenza infection in increasing lymphocyte number ($p = 0.047$) at day 7 post-infection. By day 21 and 7 weeks post-infection the number of inflammatory cells had returned to control levels and there was no effect of arsenic ($p > 0.17$ in all cases) or influenza ($p > 0.15$ in all cases) on the number of inflammatory cells in the BALf.

Viral titer

The TCID₅₀ was significantly higher in the lungs of mice exposed to arsenic and influenza compared with those exposed to influenza alone at day 7 post-infection ($p = 0.04$) (Figure 2). There was some evidence of increased viral titer in the arsenic and influenza group compared with the influenza group at day 3 post-infection, however this was not statistically significant ($p = 0.06$).

Cytokines

There was an increase in cytokine levels in the BALf in response to influenza at day 3 and day 7 post-infection which recovered to baseline levels by day 21 (Figure 2). On day 3 post-infection there was an increase in IFN- γ ($p = 0.049$) and IL-6 ($p = 0.03$) in the BALf from influenza infected mice. Arsenic exposure alone had no effect on any cytokine at day 3 post-infection ($p > 0.22$ in all comparisons). At day 7 post-infection there was an increase in TNF- α ($p < 0.001$), IFN- γ ($p < 0.001$), MCP-1 ($p < 0.001$) and IL-6 ($p < 0.001$) and protein ($p = 0.003$) levels with influenza, but no effect of arsenic ($p > 0.087$ in all comparisons). There were no significant interactions between arsenic exposure and influenza infection on cytokine levels at any time-point ($p > 0.19$ in all comparisons).

Thoracic gas volume and lung mechanics

At day 7 post-infection (2 weeks of age) offspring infected with influenza were significantly smaller in weight ($p < 0.001$) and had larger TGV ($p = 0.01$) at day 7 post-infection compared with uninfected offspring (Supplemental Material, Table S1). There was no effect of arsenic on body weight ($p = 0.06$) or TGV ($p = 0.14$) at day 7 post-infection. To account for differences in TGV between groups, specific lung mechanics were calculated by multiplying the lung mechanics (airway resistance, tissue damping and tissue elastance) by the thoracic gas volume. Arsenic exposure ($p = 0.04$) and influenza infection ($p < 0.001$) alone significantly increased tissue damping. There was also an additive effect of the combination of arsenic and influenza on tissue damping (Supplemental Material, Table S1). Offspring infected with influenza had significantly higher tissue elastance ($p = 0.002$) compared with uninfected offspring, but there was no effect of arsenic on tissue elastance ($p = 0.17$). There was no effect of arsenic ($p = 0.48$) or influenza ($p = 0.56$) on airway resistance at day 7 post-infection.

At day 21 post-infection (4 weeks of age) offspring infected with influenza were significantly smaller in weight ($p = 0.002$) than uninfected offspring (Supplemental Material, Table S1). There was no effect of arsenic ($p = 0.75$) on body weight at day 21 post-infection. Arsenic exposure ($p = 0.004$) and influenza infection ($p = 0.049$) alone significantly increased TGV. There was also an additive effect of the combination of arsenic and influenza on TGV (Supplemental Material, Table S1). To account for differences in TGV between groups, specific lung mechanics were calculated. Both arsenic exposure ($p = 0.009$) and influenza infection ($p = 0.038$) increased tissue damping, resulting in additive effects in the group exposed to both arsenic and influenza. Offspring exposed to arsenic had significantly higher tissue elastance ($p = 0.007$) compared with unexposed offspring, but there was no effect of influenza infection on tissue elastance ($p = 0.20$). There were no effects of arsenic ($p = 0.44$) or influenza infection ($p = 0.86$) on airway resistance.

At 7 weeks post-infection (8 weeks of age), there was a significant difference in body weight between male and female offspring ($p < 0.001$), therefore, lung mechanics were analyzed separately in males and females (Supplemental Material, Table S2). There were additive effects of arsenic exposure (males, $p < 0.001$; females $p = 0.04$) and influenza infection (males, $p = 0.048$; females $p = 0.04$) on reducing body weight in both sexes, such that the mice exposed to both arsenic and influenza were the smallest. There were no differences in TGV between the groups in both males (arsenic, $p = 0.09$; influenza, $p = 0.48$) and females (arsenic, $p = 0.40$; influenza, $p = 0.10$). Offspring exposed to arsenic had significantly higher airway resistance compared with offspring exposed to control water in both males ($p = 0.007$) and females ($p = 0.01$). There was no effect of influenza on airway resistance in either males ($p = 0.085$) or females ($p = 0.344$). Male offspring infected with influenza had significantly higher tissue damping ($p < 0.001$) and tissue elastance ($p = 0.001$) values compared with uninfected males. There was no effect of arsenic on tissue mechanics (tissue damping, $p =$

0.07; tissue elastance, $p = 0.086$) in males and no effect of either arsenic (tissue damping, $p = 0.32$; tissue elastance, $p = 0.60$) or influenza (tissue damping, $p = 0.30$; tissue elastance, $p = 0.70$) on tissue mechanics in female offspring.

Responsiveness to methacholine

Responsiveness to methacholine was examined in offspring 7 weeks post-infection (Figure 3). In female offspring, exposure to arsenic ($p = 0.04$) and influenza ($p = 0.005$) increased airway resistance at the highest dose of MCh which was additive. In male offspring the maximum airway resistance to MCh was higher in arsenic exposed offspring ($p < 0.001$), but was not influenced by influenza infection ($p = 0.34$). In male and female offspring, maximum tissue damping to MCh was higher in offspring infected with influenza (male, $p = 0.03$; female, $p = 0.02$) compared with uninfected offspring, but was not influenced by arsenic exposure (males, $p = 0.37$; females, $p = 0.64$). In male offspring, maximum tissue elastance to MCh was significantly higher in influenza infected offspring ($p = 0.02$) compared with uninfected male controls. There was no effect of influenza infection on maximum tissue elastance in female offspring ($p = 0.91$), and no effect of arsenic exposure on maximum tissue elastance in males ($p = 0.85$) or females ($p = 0.48$).

Airway remodeling

Mice exposed to arsenic throughout life had a greater area of airway smooth muscle in the large ($p = 0.002$) but not medium ($p = 0.62$) or small ($p = 0.88$) airways at 8 weeks of age compared with control mice (Figure 4). There was no effect of influenza infection on airway smooth muscle area at 8 weeks ($p > 0.11$). Mice exposed to influenza in early life had greater number of mucous positive cells in the large ($p = 0.02$) and medium ($p = 0.04$), but not the small ($p > 0.05$) airways at 8 weeks of age compared with uninfected mice (Figure 4). There

was no effect of exposure to arsenic on the number of mucous producing cells in any sized airway ($p > 0.35$) compared with mice exposed to control water.

Effects of exposure to arsenic in adulthood only

Adult female mice exposed to arsenic for 10 weeks during adulthood were assessed for baseline lung mechanics and responsiveness to MCh. Exposure to arsenic in adulthood had no effect on baseline airway resistance ($p = 0.63$) or parenchymal mechanics ($p > 0.56$). Mice exposed to arsenic during adulthood only were not distinguishable from controls in terms of maximum airway resistance ($p = 0.28$) or parenchymal mechanics ($p > 0.59$) in response to MCh (Supplemental Material, Figure S1).

Discussion

In this study we investigated the role that arsenic plays in the acute and long term responses to early life influenza infection. Exposure to arsenic in drinking water prior to influenza infection increased the number of total cells and macrophages at day 3 post-infection. At day 7 post-infection, arsenic exposure prior to influenza infection increased viral titer, and there was a significant super-additive interaction between arsenic exposure and influenza infection on the number of total cells and neutrophils. Despite arsenic exposure altering the inflammatory cell response to influenza in BALf, exposure to arsenic had no effect on the level of cytokines measured in BALf at the time-points we measured. Exposure to arsenic and infection with influenza independently impaired lung mechanics in infant mice, and those mice exposed to both arsenic and influenza had the greatest deficits in lung mechanics (tissue damping). At 8 weeks of age, exposure to arsenic increased baseline airway resistance, airway responsiveness to methacholine and increased airway smooth muscle mass compared with control mice. Infection with influenza in early life increased tissue damping and tissue

elastance at baseline and the maximum dose of methacholine, and increased the number of mucous producing cells in the airways compared with control mice. Combined exposure to arsenic and infection with influenza resulted in additive increases in airway responsiveness in adult females. These data demonstrate how exposure to arsenic in early life can alter the response to influenza infection resulting in both acute and long term effects on respiratory health.

Early life exposure to arsenic increased viral titer during acute influenza infection (at day 7 post-infection) suggesting that arsenic may alter the innate immune response to influenza. Impaired clearance of viral and bacterial infections has been shown before in Zebrafish exposed to arsenic through swimming water (Nayak et al. 2007) and adult mice exposed to arsenic via drinking water (Kozul et al. 2009). Arsenic has been shown to be an immunosuppressant in humans. Exposure to arsenic can alter the expression of genes and cytokines involved in immune function, T-cell receptor signaling and inflammation in human lymphocytes (Andrew et al. 2008; Wu et al. 2003) and alter T cell proliferation and function (Gonsebatt et al. 1994; Hernandez-Castro et al. 2009). Urinary arsenic levels in children exposed to arsenic in drinking water were associated with reduced lymphocyte proliferation and interleukin-2 secretion (Soto-Pena et al. 2006). Arsenic exposure during pregnancy can increase oxidative stress and inflammation in the placenta, reduce placental T cells and alter the expression of cord blood cytokines (IL-1 β , IL-8, IFN γ , TNF α) (Ahmed et al. 2011) and infants exposed to arsenic *in utero* have impaired thymic development and higher levels of fever, diarrhea and acute respiratory infections in early life (Rahman et al. 2011; Raqib et al. 2009). In experimental studies, mice exposed to arsenic had suppressed antibody formation, inhibited T cell proliferation and macrophage activity, and altered cytokine expression (Burns et al. 1991; Corsini et al. 1999; Lantz et al. 1994; Sikorski et al. 1989; Soto-Pena and Vega 2008; Vega et al. 2001). Arsenic has also been shown to increase ubiquitinylation and

degradation of cystic fibrosis transmembrane conductance regulator (CFTR) chloride channels in the gills of killifish (Shaw et al. 2010; Shaw et al. 2007) and human airway epithelial cells (Bomberger et al. 2012). Arsenic exposure during pregnancy in mice can also increase the number of mucous producing cells and calcium activated chloride channel (CLCA3) protein, known to regulate mucous production and secretion (Ramsey *et al.* 2013a). Altered CFTR and CLCA3 expression in the airways has the potential to alter the properties of mucous and airway surface liquid in the lung resulting in impaired mucociliary clearance of respiratory pathogens and increased risk of bacterial and viral colonization. We have shown that arsenic exposure in early life can impair the clearance of respiratory pathogens in infancy during a period of high susceptibility. Recurrent respiratory infections in early life can result in morbidity and mortality from respiratory disease in early childhood and may increase the risk of developing chronic respiratory disease later in life.

Arsenic exposure also exacerbated the inflammatory response to influenza in the early stages of infection. While arsenic exposure in early life did not alter cytokine expression at the time-points we measured, exposure to arsenic increased the numbers of macrophages (day 3 post-infection) and neutrophils (day 7 post-infection) in the lung in response to influenza compared with mice infected with influenza alone. Excess neutrophilia in the lungs of mice exposed to both arsenic and influenza at 7 days post-infection corresponded with impaired tissue mechanics (tissue damping), indicating increased resistance or closure of the peripheral airways of the lung (Lutchen and Gillis 1997; Lutchen et al. 1996). The results of the present study show that arsenic exposure *in utero* and early postnatal life results in an exacerbated (super-additive) inflammatory response to influenza infection in early life. However, experiments in mice exposed to arsenic in adulthood resulted in a delayed and attenuated inflammatory response to influenza infection (Kozul *et al.* 2009). There are two major differences between these studies that may explain the alternate responses. Firstly, the mice

in the present study were infected with influenza at one week of age, therefore the peak inflammatory period occurred when the mice were young (10 – 14 days) during a period of immature immune development, compared with the adult infection study where the mice were 12 weeks of age during the acute inflammatory response to influenza. We have shown previously that mice infected with influenza at 3 weeks of age have higher viral titre, higher inflammation and worse lung function compared with mice infected with influenza in adulthood (Larcombe et al. 2011). Secondly, the mice in the present study were exposed to arsenic *in utero* and early postnatal life, which has been shown to impair immune function (Ahmed *et al.* 2011; Raqib *et al.* 2009). The differences in response to arsenic in early life compared with adulthood are highlighted by the alternate results between the two studies.

Exposure to arsenic and influenza in early life also resulted in long term alterations lung structure, lung mechanics and the responsiveness to methacholine. Seven weeks following infection with influenza, infected mice had an increased expression of mucous producing cells in the airways, increased resistance and stiffness of the lung parenchyma (tissue damping and elastance) and increased responsiveness to methacholine compared with controls. Mice exposed to arsenic *in utero* and postnatally until adulthood (8 weeks of age) had increased airway smooth muscle mass, increased airway resistance and airway hyper-responsiveness. Combined exposure to arsenic and infection with influenza resulted in additive increases in airway responsiveness in adult female mice. Airway hyper-responsiveness to methacholine can reflect a reduced airway caliber, increased airway wall thickness, increased airway smooth muscle and/or excess airway mucous production (Bates et al. 2008; Cockcroft and Davis 2006). These changes in airway structure result from chronic inflammation and airway remodeling (Woolcock and Permutt 2011). Airway hyper-responsiveness is a characteristic feature of chronic obstructive lung disease and often correlates with the severity of lung disease in humans (Berend et al. 2008; Cazzola et al.

1991; Ward et al. 2002). We have identified alternate mechanisms by which exposure to arsenic and infection with influenza are able to independently modify airway structure, lung mechanics and responsiveness to methacholine.

We have shown that the lung is highly sensitive to arsenic exposure in early life. Mice exposed to arsenic *in utero* and postnatal life had significant deficits in lung mechanics at two, four and eight weeks of age, and increased airways smooth muscle and hyper-responsive airways at eight weeks. We have shown previously that exposure to arsenic *in utero* can result in impaired lung growth, altered lung mechanics and lung structure early in life which resolved with age if the exposure to arsenic ceased after birth (Ramsey *et al.* 2013a; Ramsey *et al.* 2013b). The current study shows that continued exposure to arsenic after birth results in long term alterations to lung mechanics. In contrast, mice exposed to the same dose of arsenic for the same period of time beginning in adulthood had no deficits in lung mechanics or airway responsiveness. These data support previous work which showed that adult mice exposed to 100 µg/L arsenic for three months were not hyper-responsive to methacholine, whereas mice exposed to the same dose of arsenic *in utero* and early life had hyper-responsive airways (Lantz et al. 2009). We have shown that the lungs are highly sensitive to arsenic exposure during early life resulting in long term alterations to lung structure and function.

Conclusions

We have shown that *in utero* and postnatal exposure to arsenic can increase viral load and exacerbate the inflammatory response to influenza A infection in early life. Combined exposure to arsenic and infection with influenza resulted in additive deficits in lung mechanics in early life and additive effects on airway responsiveness in adulthood. Arsenic and influenza exposure resulted in remodeling of the airways through different mechanisms,

with arsenic increasing airway smooth muscle mass and influenza increasing the number of mucous producing cells in the airways. The lungs are particularly susceptible to arsenic in early life, as evident by the lack of abnormalities seen in mice exposed to arsenic in adulthood only. We postulate that infants exposed to arsenic in early life will have an exacerbated response to respiratory infections resulting in excess inflammation and structural damage to the lung. Recurrent and exacerbated responses to respiratory infections in early life are potential mechanisms for the increased risk of developing bronchiectasis in those exposed to arsenic in early life (Smith *et al.* 2006). Further research into the exacerbation of respiratory infections following arsenic exposure is needed to determine if arsenic exposed populations are at greater risk of morbidity and mortality from lower respiratory infections.

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Table 1: The number of mice tested at each time-point for each treatment group. Intranasal inoculation with $10^{4.2}$ pfu Influenza A/Mem71 (H3N1) or mock-infected cells occurred at one week postnatal age.

Number of days post-infection	3	7	21	49 (7 weeks)
Control	12	20	21	18
Arsenic	15	20	20	20
Influenza	13	17	16	19
Arsenic and influenza	13	20	16	17

Figure legends

Figure 1: Total cells, neutrophils, macrophages and lymphocytes in BALf of mice exposed to control water, arsenic (100 µg/L *in utero* and postnatal), influenza or combined arsenic and influenza at day 3, day 7 and day 21 and 7 weeks post-infection (mean ± SD). Two way ANOVA with Holm-Sidak post hoc analysis; bars with # indicates a significant effect of arsenic; brackets with * indicates a significant effect of influenza; brackets with δ indicates a significant interaction between arsenic and influenza treatments; $p < 0.05$.

Figure 2: Viral titer for influenza in mice exposed to influenza or combined arsenic and influenza at day 3 and 7 post-infection and inflammatory cytokines of mice exposed to control water, arsenic (100 µg/L *in utero* and postnatal), influenza or combined arsenic and influenza at day 3, 7 and 21 post-infection (mean ± SD). Two way ANOVA with Holm-Sidak post hoc analysis; bars with # indicates a significant effect of arsenic; brackets with * indicates a significant effect of influenza; $p < 0.05$.

Figure 3: Response to methacholine of male and female mice exposed to control water, arsenic (100 µg/L *in utero* and postnatal), influenza or both arsenic and influenza at 7 weeks post-infection (mean ± SD). Two way ANOVA with Holm-Sidak post hoc analysis; # indicates a significant effect of arsenic; * indicates a significant effect of influenza; $p < 0.05$.

Figure 4: Quantification of airway smooth muscle and airway mucous producing cells in large, medium and small airways of mice exposed to control water, arsenic (100 µg/L *in utero* and postnatal), influenza or both arsenic and influenza at 7 weeks post-infection (mean ± SD). Two way ANOVA with Holm-Sidak post hoc analysis; bars with # indicates a significant effect of arsenic; brackets with * indicates a significant effect of influenza; $p < 0.05$.

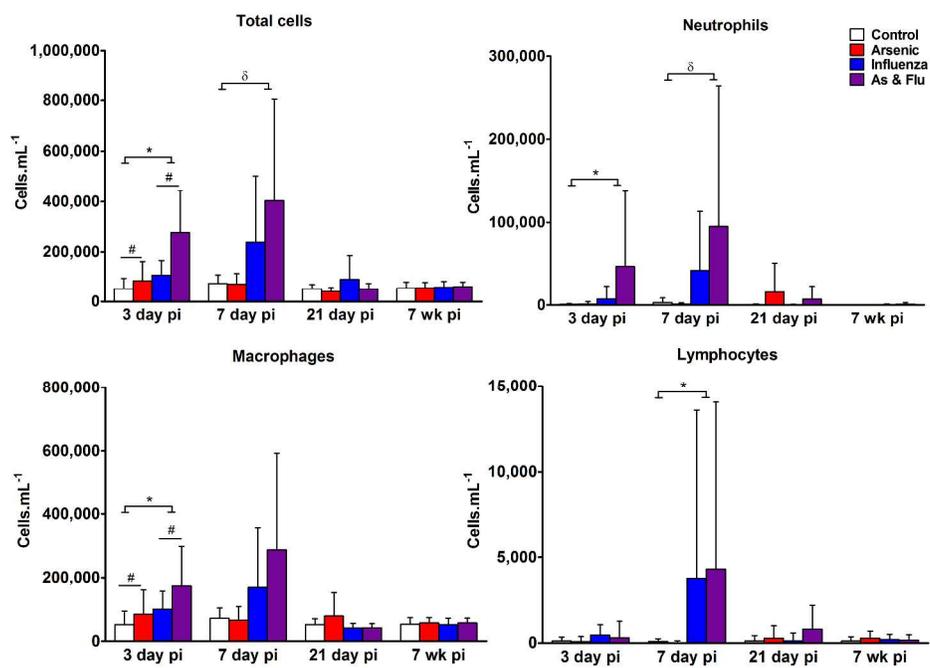


Figure 1
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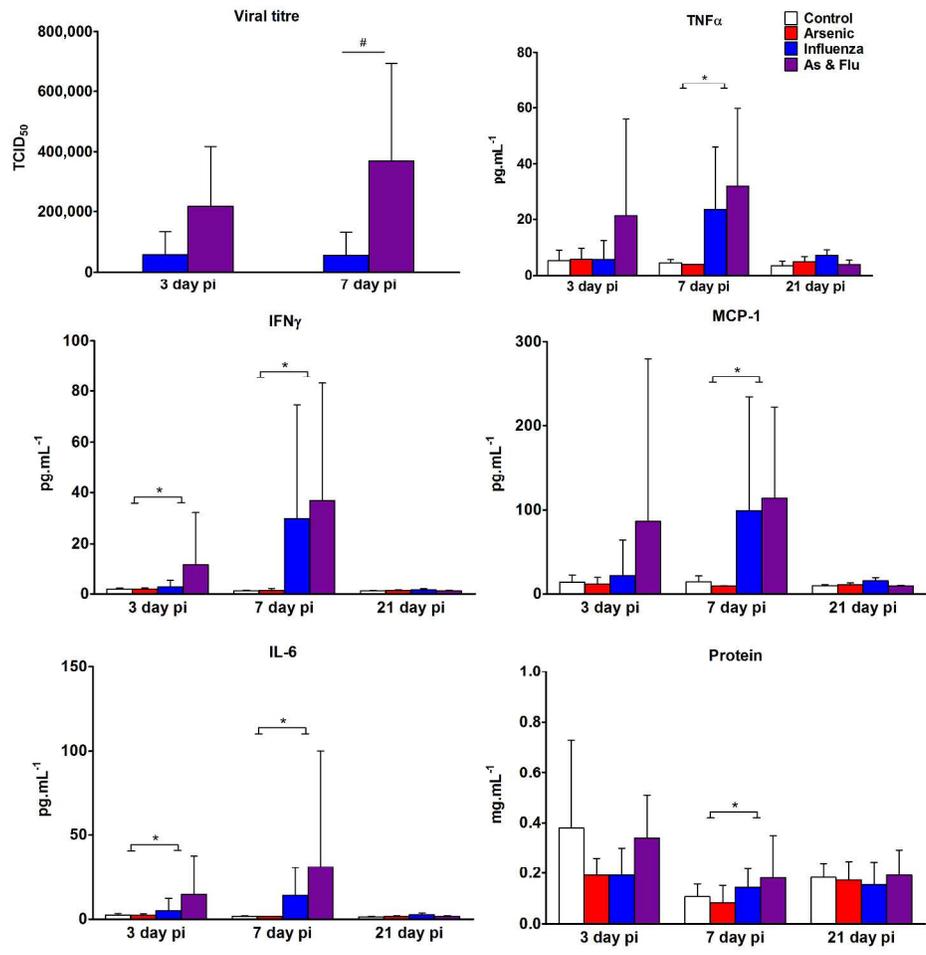


Figure 2
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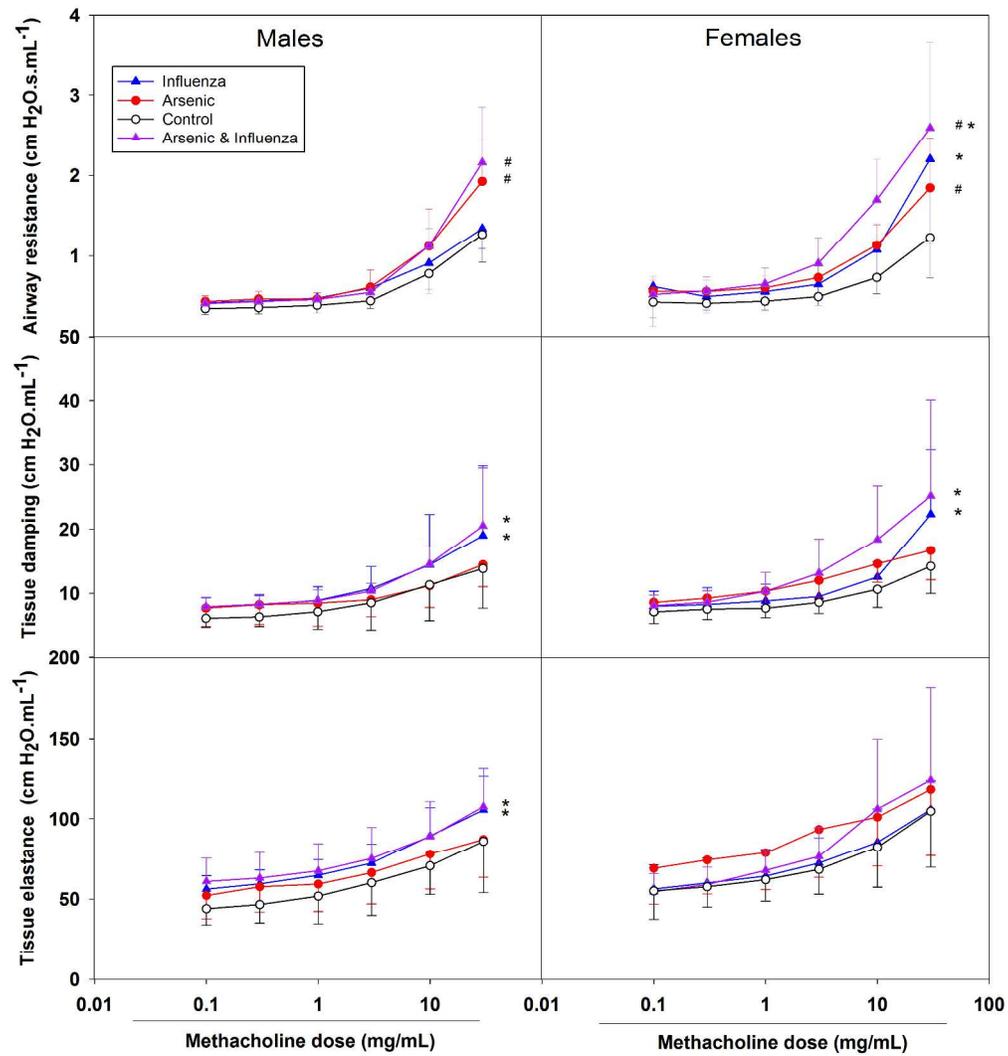


Figure 3
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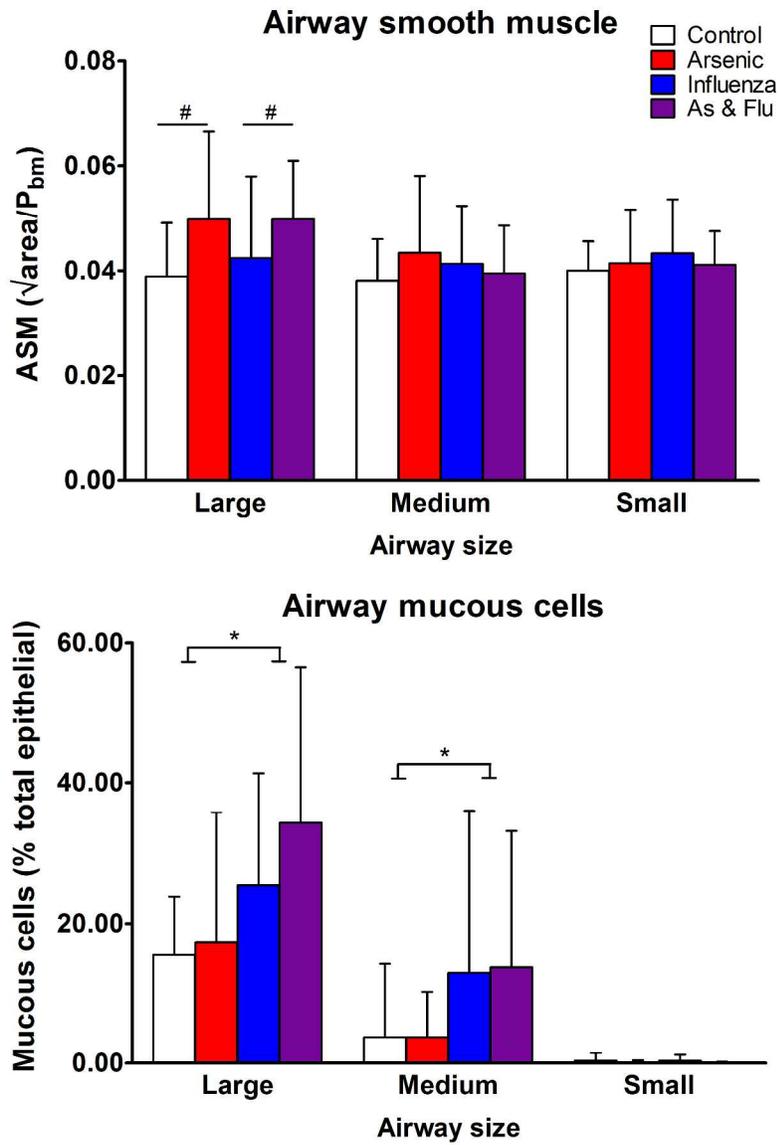


Figure 4
268x366mm (300 x 300 DPI)