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**Low Dose Arsenic Compromises the Immune Response to Influenza A Infection *in vivo***

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**Abbreviations:**

APC	Antigen presenting cell
As	Arsenic
BALF	Bronchoalveolar lavage fluid
DC	Dendritic cell
GM-CSF	Granulocyte macrophage colony-stimulating factor
IL1b	Interleukin 1 beta
IL10	Interleukin 10
IL-6	Interleukin 6
M-CSF	macrophage colony stimulating factor
MCP-1	monocyte chemoattractant protein 1
MIP	macrophage inflammatory protein
MLN	Mediastinal lymph Node

p.i. post infection  
SpO<sub>2</sub> peripheral blood oxygen saturation  
TNF $\alpha$  tumor necrosis factor alpha

## **Outline**

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## **Abstract**

*Background:* Arsenic (As) exposure is a significant worldwide environmental health concern. We have recently reported that 5-week exposure to environmentally relevant levels (10 and 100 ppb) of As in drinking water significantly altered components of the innate immune response in mouse lung, which we hypothesize are an important contributor to the increased risk of lung disease in exposed human populations.

*Objectives:* Our findings led us to investigate the effects of As exposure on respiratory Influenza A (H1N1) virus infection, a common and potentially fatal disease.

*Methods:* In this study, C57BL/6J mice were exposed to 100 ppb As in the drinking water for 5 weeks, followed by inoculation intra-nasally with a sub-lethal dose of Influenza A/PuertoRico/8/34 (H1N1) virus. Multiple endpoints were assessed post infection.

*Results:* Arsenic was associated with a number of significant changes in response to influenza, including an increase in morbidity and higher pulmonary influenza virus titers on day 7 post-infection. There were also many alterations in the immune response relative to As-unexposed controls, including a decrease in the number of dendritic cells in the mediastinal lymph nodes early in the course of infection.

*Conclusions:* Our data indicate that chronic As exposure significantly compromises the immune response to infection. Alterations in response to repeated lung infection may also contribute to other chronic illnesses, such as bronchiectasis, which is elevated by As exposure in epidemiology studies.

## Introduction

Chronic exposure to arsenic (As) is a significant worldwide environmental health concern (ASTDR 1999; NRC 1999). Contamination of drinking water by natural geological sources of As is the primary route of exposure. The EPA standard for drinking water As exposure was recently reduced to 10 ppb (0.13  $\mu\text{M}$ ). However, this standard does not cover private wells. In many areas of the United States, As is naturally found at levels higher than the federal guidelines and a significant portion of the population may be drinking excess As chronically (Karagas et al. 2002). In the U.S., this may represent as many as 25 million people, and worldwide there are hundreds of millions of people who are exposed to levels of As far above 10 ppb. In addition, significant biological effects of As have been observed in cell culture and in animal models at and below the current 10 ppb EPA standard (Andrew et al. 2007; Kozul et al. 2009; Lantz et al. 2007; Straub et al. 2008).

Chronic exposure to As has been associated with many diseases, including lung, liver, skin, kidney, and bladder cancer; cardiovascular disease; diabetes; and reproductive and developmental defects (Abernathy et al. 1999; NRC 1999; Smith et al. 1992; Tapio and Grosche 2006; Watanabe et al. 2003). Multiple mechanisms have been associated with As induced disease risk, including endocrine disruption, oxidative stress, alterations in cell signaling, DNA repair and others (Andrew et al. 2006; Aposhian and Aposhian 2006; Kaltreider et al. 2001; Rossman 2003). However, differences in dose, time, and tissue, as well as co-exposures, can result in differing mechanisms and complicate the interpretation of disease risk under varying exposure conditions. Recent reports have indicated that chronic As exposure in human populations results in an increased risk of a

variety of lung diseases, including impaired lung function, bronchiectasis, lung cancer, and increased risks of other respiratory illnesses (Ghosh et al. 2007; Raqib et al. 2009; Smith et al. 2006). The ability of As to increase the risk of lung disease through ingestion, as opposed to inhalation, make it a unique and intriguing lung toxicant.

Arsenic has been identified as a potent immuno-modulatory agent in many experimental models and epidemiological studies (Andrew et al. 2008; Hernandez-Castro et al. 2009; Lemarie et al. 2006; Nayak et al. 2007; Zhou et al. 2006). We have recently shown that chronic low dose As exposure can profoundly alter the gene and protein expression of many regulators of the innate immune system in a mouse model of exposure (Kozul et al. 2009). We have hypothesized that As-induced alterations on the immune system in the lung will lead to a compromised response to a subsequent immune challenge. In this study, we tested this hypothesis by investigating whether chronic low dose As exposure could affect the severity of influenza A (H1N1) infection in a murine model. Based on our previous studies, we were specifically interested in the innate immune response to infection, including the migration capability of innate immune cells, such as dendritic cells (DC). Dendritic cells are antigen presenting cells (APCs) that activate naïve T cells and their functioning is critical to the initiation of a primary immune response. With particular respect to the lung, where there is persistent exposure to environmental pathogens, DCs constantly sampling the environment, processing antigen and controlling tolerance, demonstrating their indispensable role in immune regulation.

Respiratory infections with influenza are a significant public health concern and a major cause of morbidity and mortality worldwide (Fauci 2005). It is estimated that 5-



15% of the population will contract influenza infection annually, resulting in over 3-5 million hospitalizations and between 250,000 and 500,000 deaths worldwide. Identifying risk factors, including environmental exposures, such as As, could have a substantial and immediate impact on public health (Lawrence 2007). Additionally, the impact of As exposure on the potential for a pandemic flu outbreak is of particular concern, considering that many of the geographic areas with confirmed human cases of avian flu or H1N1 (swine) flu are known to include populations that also have significantly elevated As exposures, such as in Southeast Asia and Mexico. The recent outbreak of H1N1 flu in Mexico and across the world has demonstrated the impact a pandemic flu can have on global populations. Age and underlying medical conditions have an influence on the susceptibility and severity of infection; however, these factors cannot account for the extreme variability observed in response to influenza infection. Many questions have been raised concerning the increased mortality as a result of H1N1 infection in Mexico, while the disease has been relatively mild in other infected populations. There is growing evidence that exposure to environmental toxicants can dramatically alter anti-viral responses, including dioxin and cigarette smoke (Burleson et al. 1996; Gualano et al. 2008; Warren et al. 2000). In this current study, we show that chronic low dose As exposure can profoundly alter the response to influenza A (H1N1) infection. Understanding the role of As in response to such viral challenges will be important in the overall assessment of the public health risk.

## Methods

*Animal Husbandry.* All animal studies were conducted in accordance with AALAC approved guidelines using a protocol approved by IACUC at Dartmouth Medical School. All animals were treated humanely and with regard for alleviation of suffering. Seven week-old C57BL/6J male mice (Jackson Laboratories, Bar Harbor, ME) were housed on AIN-76A diet (ad lib) (Harlan-Teklad, Madison, WI) and Carefresh bedding in autoclaved cages. Background As concentrations in the diet were less than 20 ppb (Kozul et al. 2008). At the start of the experiment, animals were given drinking water (ad lib, changed weekly) with or without addition of 100 ppb sodium arsenite. Mice were maintained on control or 100 ppb As water throughout the course of infection.

*Influenza Virus Infection.* Following 5 weeks of As exposure, Mice were anesthetized with 9:1 ketamine:xylazine mix at 0.1ml/30 g body weight and inoculated intranasally with 0.5LD<sub>50</sub> of standard laboratory influenza A virus A/PuertoRico/8/34 (H1N1) strain. Morbidity (measured as weight loss) was monitored daily over the course of infection. Body weight losses of greater than 20% was the endpoint for termination of the study, based on IACUC compliance.

*Viral Titer.* Whole lungs from infected mice were homogenized, snap frozen and stored at -80°C. Influenza virus titers in whole lung homogenate were quantified using TCID<sub>50</sub> determination. Briefly, 10x serial dilutions of lung samples were added in triplicate to Madin-Darby canine kidney (MDCK) cells in a 96 well plate. Plates were incubated at 37°C for 5 days. Infected cells were identified by chicken RBC hemagglutination.

*Bronchoalveolar lavage fluid.* Lungs were lavaged *in situ* with 1 ml of PBS. Remaining cells were collected with 4 sequential washes. Bronchoalveolar lavage fluid (BALF) was

centrifuged and cells retrieved from all 5 washes were combined for total cell counts. Total cell counts (excluding non-nucleated cells) were obtained using a hemocytometer with trypan blue exclusion. Cytospin preparations were stained with Protocol Hema 3 stain set (Fisher, Houston, TX). Measurements of cytokine and albumin levels in the BALF were assessed from the first 1 ml lavage only. Albumin ELISA kit (Bethyl labs, Montgomery, TX) was used to assay albumin concentrations in BALF per manufacturer's instructions.

*MLN cell suspensions.* Cell suspensions were prepared from the mediastinal lymph nodes (MLN) by gentle mincing and digestion in collagenase (1 mg/ml, type II, Worthington Biochemical Corp., Lakewood, NJ) and DNase (0.02 mg/ml, grade II bovine pancreatic, Roche, Manheim, Germany) for 45 minutes at room temperature, followed by the addition of EDTA (0.1 M) for an additional 5 minutes.

*Peripheral Blood Oxygen Saturation.* SpO<sub>2</sub> was measured using the MouseOx system (Starr Life Sciences Corp., Allison Park, PA) according to manufacturer's protocol. Conscious mice were restrained and the sensor was placed at the base of the tail. Mice were acclimated in the restraint and data was collected for a period of 10 seconds per mouse.

*Flow Cytometry.* BALF and MLN single cell suspensions were collected as described above. Red blood cells were lysed by incubation with buffered ammonium chloride solution. FITC conjugated CD4 antibody and PerCpCy5.5 conjugated CD8 antibody, APC conjugated CD11c, and PerCpCy5.5 conjugated B220 were purchased from eBioscience (San Diego, CA). Biotin conjugated CD103 was purchased from BioLegend (San Diego, CA). Samples were run on FACSCalibur cytometer (BD Biosciences, San

Jose, CA). All data were analyzed with FlowJo software (Tree Star, Ashland, OR).

*Cytokine Profile Analysis.* Cell-free BALF was analyzed using the Bio-plex suspension array system using fluorescently dyed Luminex microspheres beads (Bio-Rad, Hercules, CA). Cytokines and chemokines measured in this study were Interleukin 1 beta (IL-1b), Interleukin 6 (IL-6), macrophage inflammatory protein 1-alpha (MIP-1a), RANTES, monocyte chemoattractant protein 1 (MCP-1), Interleukin 10 (IL-10), macrophage colony stimulating factor (M-CSF), macrophage inflammatory protein-2 (MIP-2), macrophage inflammatory protein 1-beta (MIP-1b), and tumor necrosis factor alpha (TNF $\alpha$ ). BALF samples were assayed in duplicate. Cytokine/chemokine standards were prepared in PBS and were assayed in triplicate. Assay was performed according to manufacturer protocol.

*DC isolation and culture.* The DC culture protocol is described previously (Castellino et al. 2000) and is a modification of Inaba et al. (Inaba et al. 1993). Briefly, bone marrow cells were collected from the femurs of C57BL/6J mice exposed to control of 100 ppb As in the drinking water for five weeks (in the absence of infection). Cells were resuspended at  $10^6$  cells/ml in RPMI 1640 media (10% heat-inactivated fetal/bovine serum, 100 U/ml penicillin/streptomycin, 50 mM  $\beta$ -mercaptoethanol, 5% cell culture supernatant from X63 cells secreting granulocyte macrophage colony-stimulating factor (GM-CSF)). Cells were plated at 2 ml/well into 12 well plates. All cells were cultured in the absence of As. On days 2, 4 and 6, the cells were washed, non-adherent cells were removed and fresh media was applied. On day 7, cells were scraped and collected for transwell migration assays.

*Transwell migration Assay.* On day 7, bone marrow cells were counted with trypan blue exclusion using a hemocytometer to assess survival. No differences in cell survival were

observed between cells isolated from control and As exposed mice. Costar Transwell plates (6.5 mm diameter insert, 8.0  $\mu\text{m}$  pore size, polycarbonate membrane, Corning Costar, Corning, NY) were precoated with 2  $\mu\text{g}/\text{ml}$  fibronectin and 500  $\mu\text{L}$  of 10  $\mu\text{M}$  ADP was added to the bottom well. 100,000 cells were added in serum free media to the top chamber of a transwell plate and allowed to migrate for 2 hr. Following migration period, any cells remaining on top of the membrane were removed and the membranes were then rinsed with PBS. Migrated cells were fixed, permeabilized with 0.01% Triton X-100 (Sigma, St Louis, MO) and stained with crystal violet (Sigma, St Louis, MO). The membranes were mounted on microscope slides for analysis. Q-Fired cooled CCD camera attached to an Olympus microscope was used to capture 10 random fields per membrane. Migrated cells were counted with SigmaScan Pro imaging analysis software. Counts for all 10 fields were averaged to give a mean cell count for each membrane. All experiments were completed at least three times with  $n = 3$  per trial.

*Statistical Analysis* Statistical analysis was performed GraphPad Prism Version 5.0a for Macintosh using a two-tailed unpaired t-test with 95% confidence interval and one way ANOVA (\*  $p < 0.05$ ; \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ). Error bars represent mean + SEM.

## Results

As shown in Figure 1, following a five-week exposure to 100 ppb As in the drinking water, mice infected with influenza A (H1N1) displayed a significant increase in morbidity. By day 8 post-infection (p.i.), the As-exposed mice displayed such severe morbidity (e.g., body weight decrease of 20% or greater) that those experimental groups were euthanized in compliance with institutional IACUC standards. Due to the severity of these responses, subsequent analyses focused on day 3 and day 7 p.i.. In contrast, a parallel group of control mice infected with influenza but not exposed to As displayed a moderate weight loss, but then began to recover weight by day 10 p.i., with complete weight recovery by day 16 p.i. (Figure 1). Exposure to As alone in the absence of viral infection did not influence weight or growth over the five-week period, nor did anesthesia alone, with or without respiratory exposure vehicle, in either the control or As-exposed mice (data not shown). Thus, the increased morbidity was due to the combination of As in drinking water and influenza infection at an infectious dose at which mice not exposed to As recover.

Given that the inability to properly clear virus from the lung is positively correlated with increased risk of adverse outcomes, we examined the levels of influenza A virus in the whole lung homogenates using TCID<sub>50</sub> determination at day 7 p.i.. Relative to As-unexposed controls, the As-exposed mice exhibited a significant 10-fold increase in viral titers at this time point, correlating with their relative increase in morbidity (Figure 2).

At day 7 p.i., obvious gross histology changes in the As exposed mice, including edema and hemorrhaging, could be observed by visual inspection of the whole lung

(Figure 3A). Capillary leakage into the lungs was measured by assaying albumin concentrations in the BALF. Albumin concentrations were found to be significantly increased in the As-exposed vs. unexposed mice at day 7 p.i.. Albumin concentrations did not differ between control or As-exposed mice prior to infection or at day 3 p.i. (Figure 3B). Since an increased severity of influenza infection often correlates with a decrease in peripheral blood oxygen saturation ( $SpO_2$ ), the oxygen saturation levels in infected control and As-exposed conscious mice at day 7 p.i. were measured. The As-exposed mice displayed a significant decrease in  $SpO_2$  levels compared to control mice (Figure 3C). The average  $SpO_2$  reading for the control mice infected with influenza was 95.2% (range 93-97.5%) and the average  $SpO_2$  reading for As-exposed mice infected with influenza was 82.9% (range 75-90%). Three of the six As-exposed mice had  $SpO_2$  readings considered to be dangerously low, i.e., at or below 80%. Exposure to As in the absence of infection had no effect on peripheral oxygen saturation levels.

Cellular infiltration into the lungs was investigated at 36 hr and at day 3 and day 7 p.i.. We previously reported that exposure to 100 ppb As for 5 weeks in uninfected mice did not induce changes in the total number of cells recovered from BALF, nor did it alter gross changes in lung histology (Andrew et al. 2007; Kozul et al. 2009). However, in the current experimental model, significant differences in the total number of cells recovered post-infection from the BALF of control and As-exposed mice were observed. At 36 hr and day 3 p.i., As-exposed mice had a significant decrease in the total number of cells recovered from the BALF compared with control mice. Conversely, at day 7 p.i., As-exposed mice had a significant increase in the number of cells in the BALF (Figure 4A) relative to controls.

To assess whether changes in total cell populations were accompanied by changes in the specific cell types recruited to the lung, we conducted a morphological analysis of the cells after staining of cytospin preparations. Representative cytospin preparations are shown in Figure 4E-J. The absolute numbers of macrophages and neutrophils within the lung were significantly and profoundly affected by As exposure (Figure 4B-C). The total number of macrophages (Figure 4B) and neutrophils (Figure 4C) were significantly decreased at the early stages of infection and increased at day 7 p.i. in As-exposed mice vs. controls, whereas lymphocytes were decreased in the As-exposed mice at day 3 but were similar to controls at day 7 p.i. (data not shown). In addition to these differences in cell number, the percentages of these different cell types within the total cells recovered from the BALF also changed, as shown in Figure 4D.

Although the absolute number of lymphocytes was largely unaffected at day 7 p.i., when subtypes within the lymphocyte populations were analyzed by flow cytometry, CD8<sup>+</sup> cells were found to be significantly increased in terms of both cell percentages (Figure 5B) and total cell numbers (Figure 5C) in As-exposed mice relative to controls. The percentage of CD4<sup>+</sup> T cells did not differ by As exposure (data not shown).

We previously reported that As exposure decreased basal cytokine levels in the lungs of uninfected mice (Kozul et al. 2009). We used a Bioplex assay to examine cytokine production in the BALF at day 3 and day 7 p.i.. We examined a panel of 10 cytokines, including IL1b, IL-6, MIP-1a, RANTES, MCP-1, IL-10, M-CSF, MIP-2, MIP-1b, and TNF $\alpha$ . With the exception of MIP-1a, cytokine production had the same general pattern. No significant differences were observed between the control and As-exposed mice at day 7 p.i.. However, relative to controls, a significant decrease was observed in



the As-exposed mice in 9 out of the 10 cytokines at day 3 p.i. (Figure 6). In some cases, such as IL-1b and TNF $\alpha$ , cytokine production was below the limits of assay detection in the As-exposed mice. Mip-1a levels were below detection at day 3 p.i. in all mice, but indicated a trending increase in the As-exposed animals relative to controls at day 7 p.i. (data not shown).

The early recruitment of DCs to the lymph node is essential for the proper initiation of an immune response and we have previously reported that As exposure decreases the expression of genes involved in cell adhesion and migration (Kozul et al. 2009). Therefore, to determine whether the migration of DCs to the lymph node was impaired by As exposure, we analyzed the mediastinal lymph nodes (MLN) for DC populations at day 3 p.i.. Single cell suspensions from MLN were assessed for DC markers CD11c<sup>+</sup>/CD103<sup>+</sup> and CD11c<sup>+</sup>/B220<sup>+</sup>. Relative to controls, a significant decrease was observed in the total number of cells recovered from the MLN in the As-exposed mice at day 3 p.i. (Figure 7A). Flow cytometry staining for CD11c<sup>+</sup>/CD103<sup>+</sup> and CD11c<sup>+</sup>/B220<sup>+</sup> DC types also indicated that these DC populations were significantly decreased in the lymph node of As-exposed mice vs. controls at day 3 p.i. (Fig 7B and C). No differences were observed in the intensity of the staining (data not shown).

We then assessed effects of As on DC migration capacity, using primary bone marrow DCs isolated from control mice and mice exposed to 100 ppb As for five weeks (in the absence of influenza infection). Cells were cultured for 7 days in the presence of GM-CSF to produce immature DCs. Relative to controls, cells isolated from As-exposed mice had a significant decrease in migration capability towards ADP in a transwell assay (Fig 7D), indicating that As alone can compromise aspects of immune cell function and

that this is then manifest as a significantly altered innate immune response following viral infection.

## Discussion

To determine whether chronic low dose drinking water As exposure compromises the immune response to respiratory infection, we analyzed the outcome of influenza A (H1N1) infection in mice drinking water containing 100 ppb As. Our studies demonstrated that As exposure had a significant impact on the immune system, resulting in a severely compromised response to influenza A infection and increased morbidity. We also observed increased viral titers, capillary leakage, altered cellular responses and decreased cytokine production and oxygen saturations in the As exposed mice relative to controls. At early time points post infection, we observed that As exposed mice had an attenuated response. Conversely, by the later time points, the As exposed mice displayed an excessive cellular inflammatory response to infection, indicated by significant increases in the number of cells within the lung and increases in markers of lung injury, such as BALF albumin concentrations and decreased oxygen saturations compared with controls.

The initial attenuated immune response in the As exposed mice was expected based on our previous results (Kozul et al. 2009). It is clear that As exposure affects a variety of aspects of host defense, including responses of macrophages (Lemarie et al. 2006), lymphocytes (Andrew et al. 2008; Hernandez-Castro et al. 2009), and airway epithelial cells (Olsen et al. 2008). Additionally, an appropriate and sufficient response to an infectious challenge requires integration of the innate and adaptive immune defenses. Given that As exposure has important impacts on both components, it is not unexpected that the effects of As exposure on the immune response to viral infection are complex. It

is therefore likely that several mechanisms contributed to the adverse outcomes observed in the As exposed mice.

The switch from an inadequate innate immune response at early time points to an excessive response at later time points is intriguing, and the underlying mechanism for this biphasic response warrants future investigation. The excessive response at late time points was also accompanied by considerable hemorrhaging and leakage within the lung. It does not appear that this switch was principally chemokine driven, since we observed that chemokine levels were not increased at day 7 p.i.. Moreover, it should be noted that at this time point there were 2-3 times more cells in the lungs of the As mice compared with controls, suggesting that cytokine and chemokine production may still be decreased on a per cell basis. However, given that cytokine and chemokine production is a dynamic process, we cannot exclude that an increase in cytokine production may have occurred, but was not detected at the time points investigated in this study. It is possible that the increased number of macrophages and neutrophils at day 7 p.i. may have been the result of a compensatory innate response that was not properly initiated early in the course of infection. An overly exuberant macrophage and neutrophil response has also been observed with highly pathogenic strains of influenza, such as H5N1 (Perrone et al. 2008), and the 1918 strain (Kobasa et al. 2007). Nevertheless, the later response was ineffective in controlling levels of virus within the lung, since the titers were increased by ten-fold in the As exposed mice relative to control mice at day 7 p.i. The increased viral titers in conjunction with the increase in CD8+ cells in the As exposed mice suggest the possibility that the CD8+ cells may not have been fully capable of expressing antiviral effector activities. Alternatively, the impaired innate response may have resulted in

unhindered viral replication that even an exaggerated CD8<sup>+</sup> T cell response could not effectively control. It is difficult to assess the role of CD8<sup>+</sup> T cells in the response using this model, because of the high morbidity in the As exposed mice.

We have also shown that As decreased cell migration, which suggests that the critical components of this response were defective. With respect to respiratory viral infection, it has been shown that DC migration to the regional lymph nodes early in the course of infection is a critical component for initiating an appropriate immune defense against influenza infection (Legge and Braciale 2003). The CD11c<sup>+</sup>/B220<sup>+</sup> DC in the mouse lymph node are presumptively immature plasmacytoid dendritic cells, which are capable of producing large amounts of type I interferon (Nakano et al. 2001; Siegal et al. 1999). The CD11c<sup>+</sup>/CD103<sup>+</sup> DC have been shown to reside in lung mucosa (Sung et al. 2006). The relative decrease in this population of DCs in the lymph nodes may have contributed to the initial attenuated response of As exposed mice and suggests that antigen presentation within the lymph node from this subset of resident lung DCs may have been compromised. Defective migration by bone marrow derived DCs isolated from uninfected As exposed mice demonstrated that As exposure alone compromised critical functional components of the innate host defense mechanisms, which would likely have consequences in response to many pathogens and other pathological conditions.

The immune response against pathogens is a tightly regulated equilibrium, requiring precise management of effectively clearing pathogen while minimizing immunopathologic damage to the host (Bachmann and Kopf 2002). Therefore, it is not unexpected that the initial impairment in an appropriate immune response due to As exposure could have significant consequences on the ability to clear influenza infection,

while prolonged viral carriage may predispose to enhanced tissue injury. To our knowledge, this is the first report showing that chronic low-dose As exposure significantly impairs the immune system and, specifically dendritic cells, in response to a respiratory virus infection. Further investigation into mechanisms and the effects at lower doses of As, such as 10 ppb, will be the basis of future studies. We also hypothesize that As exposure will predispose individuals to aberrant responses to other types of infection as well. In addition to the potential acute effects as in this study, these aberrant responses may place exposed individuals at risk for development of other chronic lung diseases, such as bronchiectasis, which has been reported to be significantly elevated in As-exposed populations (Smith et al. 2006). These results also suggest that chronic As exposure, particularly in areas of Southeast Asia and Mexico, may be a factor that could enhance the potential impact of a pandemic strain of influenza, should this emerge in the human population.

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### Figure Legends

Figure 1: Chronic drinking water As exposure increased the morbidity of influenza infection. Mice were exposed to control drinking water (open circles) or drinking water containing 100 ppb As (closed circles) for five weeks. Following exposure, mice were infected with a sub-lethal dose of influenza A and morbidity (measured by weight loss) was recorded daily. One experiment was conducted for days 0-16 (n=6-8 per group), and three additional experimental repeats were conducted for days 0-7 (n=6 per group per experiment). Error bars represent mean  $\pm$  SEM. The p value for overall significance between groups exposed to flu alone or As plus flu is  $p < 0.0001$  (Two way ANOVA). Significance for individual time point exposures is detailed in the graph. (\*\*  $p < 0.01$ , \*\*\* $p < 0.001$ ).

Figure 2: Chronic drinking water As exposure increased viral titers on day 7 post infection. Mice were exposed to control drinking water (white bars) or drinking water containing 100 ppb As (black bars) prior to inoculation with influenza A. Whole lung homogenates were assessed for viral titers by TCID<sub>50</sub> method. Data represent mean  $\pm$  SEM from two experimental repeats (n=3-6 per group). (\*  $p < 0.05$ , two tailed student's t-test).

Figure 3: Chronic drinking water As exposure increased capillary leakage at day 7 post infection. (A.) Representative lungs from control drinking water and As drinking water exposures at day 7 post infection. Lungs were perfused with PBS and inflated. (B.) BALF was assessed for albumin by ELISA at the indicated time points. Mice were exposed to control drinking water (white bars) or drinking water containing 100 ppb As (black bars)

prior to inoculation with influenza A. (C.) Oxygen saturations were measured with the MouseOx® system at day 0 and day 7 post infection. Data represent mean  $\pm$  SEM from one representative experiment (n=6 per group). (\*\* p < 0.01, \*\*\*p < 0.001, two tailed student's t-test).

Figure 4: Chronic drinking water As exposure altered cellular numbers into the BALF.

Mice were exposed to control drinking water (white bars) or drinking water containing 100 ppb As (black bars) prior to inoculation with influenza A. (A.) Viable nucleated cells recovered from BALF were counted by hemocytometer with trypan blue exclusion at 36 hr, day 3 and day 7 post infection. Uninfected control and As exposed mice are shown for reference. Data represent mean  $\pm$  SEM from one representative experiment (n=5-6 per group). (\*\* p < 0.01, \*\*\*p < 0.001, two tailed student's t-test). Extrapolation of total number of (B.) macrophages and (C.) neutrophils recovered from BALF. Uninfected control and As exposed mice are shown for reference. (D.) Neutrophils (dark gray), macrophages (light gray), and lymphocytes (medium gray) are represented as percentages of the total cells recovered. Equal volumes of BALF were prepared onto cytopsin slides, stained and ten random fields per slide were counted by visualization of cell morphology (40x magnification). Representative cytopsin preparations are shown for: 36 hours p.i. (E.) flu alone (F.) flu and arsenic; 3 days p.i. (G.) flu alone (H.) flu and arsenic; 7 days p.i. (I.) flu alone (J.) flu and arsenic. Data represent mean  $\pm$  SEM from one representative experiment (n=5-6 per group). (\* p < 0.05, \*\* p < 0.01, \*\*\*p < 0.001, two tailed student's t-test).

Figure 5: Chronic drinking water As exposure increased the percentages and total number of CD8<sup>+</sup> cells in the BAL. Mice were exposed to control drinking water (white bars) or drinking water containing 100 ppb As (black bars) prior to inoculation with influenza A. (A.) Isolated BALF cells were stained with fluorochrome-conjugated antibodies against CD4 and CD8. Representative FACs plots are shown. (B.) The percentage of CD8<sup>+</sup> cells and (C.) the average number of total CD8<sup>+</sup> cells recovered from BALF at day 7 p.i. are shown. Data represent mean  $\pm$  SEM from one representative experiment (n=5-6 per group). (\* p < 0.05, two tailed student's t-test).

Figure 6: Chronic drinking water As exposure decreased cytokine and chemokine levels in the BALF at day 3 post infection. Mice were exposed to control drinking water (white bars) or drinking water containing 100 ppb As (black bars) prior to inoculation with influenza A. BALF was collected as described in methods at day 3 and day 7 post infection. BALF was analyzed using a luminex system for levels of IL1b, IL-6, MIP-1a, RANTES, MCP-1, IL-10, M-CSF, MIP-2, MIP-1b, and TNF $\alpha$ . Results for (A.) Rantes, (B.) MIP-2, and (C.) MCP-1 are shown. Data represent mean  $\pm$  (n=5-6 per group). (\* p < 0.05, \*\* p < 0.01, \*\*\*p < 0.001 two tailed student's t-test).

Figure 7: Chronic drinking water As exposure decreased the number of dendritic cells in the MLN. Mice were exposed to control drinking water (white bars) or drinking water containing 100 ppb As (black bars) prior to inoculation with influenza A. (A.) Lymph nodes were collected, digested and single cell suspensions were counted using trypan blue exclusion with a hemocytometer. MLN cells were stained with fluorochrome-

conjugated antibodies against CD11c, B220, and CD103. The total number of (B.) CD11c<sup>+</sup>/CD103<sup>+</sup> and (C.) CD11c<sup>+</sup>/B220<sup>+</sup> are shown. Data represent mean  $\pm$  SEM from three representative experiments (n=5 per group) (D.) Bone marrow derived DCs were collected and cultured from uninfected control and As exposed mice. Following differentiation in culture in the presence of GM-CSF, cells were migrated in a transwell assay using ADP as a chemoattractant. Data represent mean  $\pm$  SEM from three representative experiments (n=3 per experiment) and are expressed as mean cell migration relative to control mice. (\* p < 0.05, \*\* p < 0.01, \*\*\*p < 0.001 two tailed student's t-test).

Figure 1

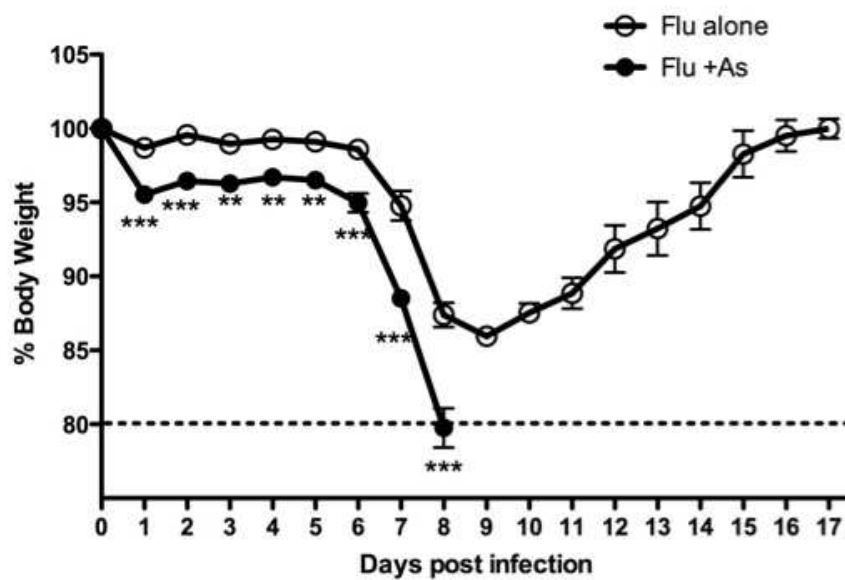


Figure 1: Chronic drinking water As exposure increases the morbidity of influenza infection.  
190x254mm (72 x 72 DPI)



Figure 2

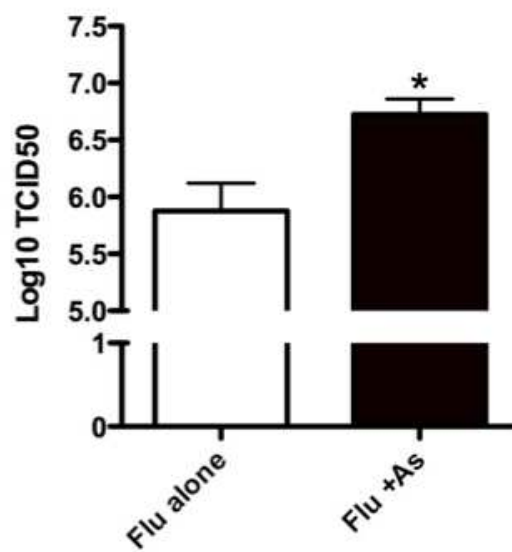


Figure 2: Chronic drinking water As exposure increases viral titers on day 7 post infection.  
190x254mm (72 x 72 DPI)

Figure 3

A

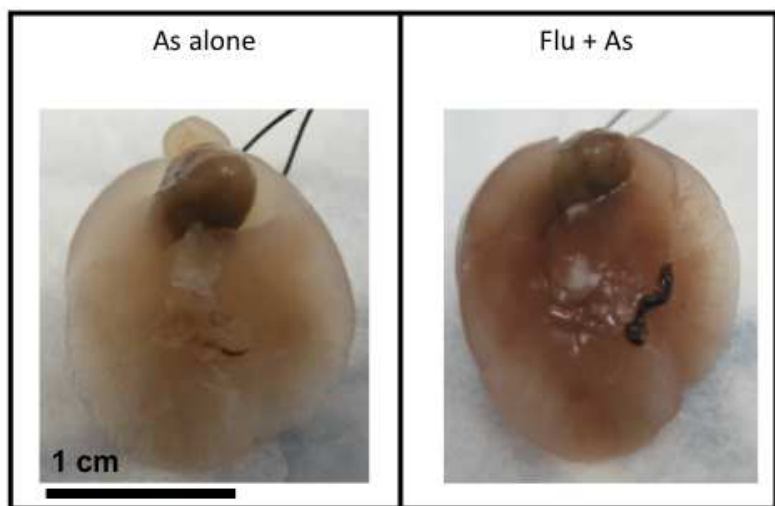


Figure 3A: Chronic drinking water As exposure increases capillary leakage at day 7 post infection.  
190x254mm (72 x 72 DPI)

Figure 3

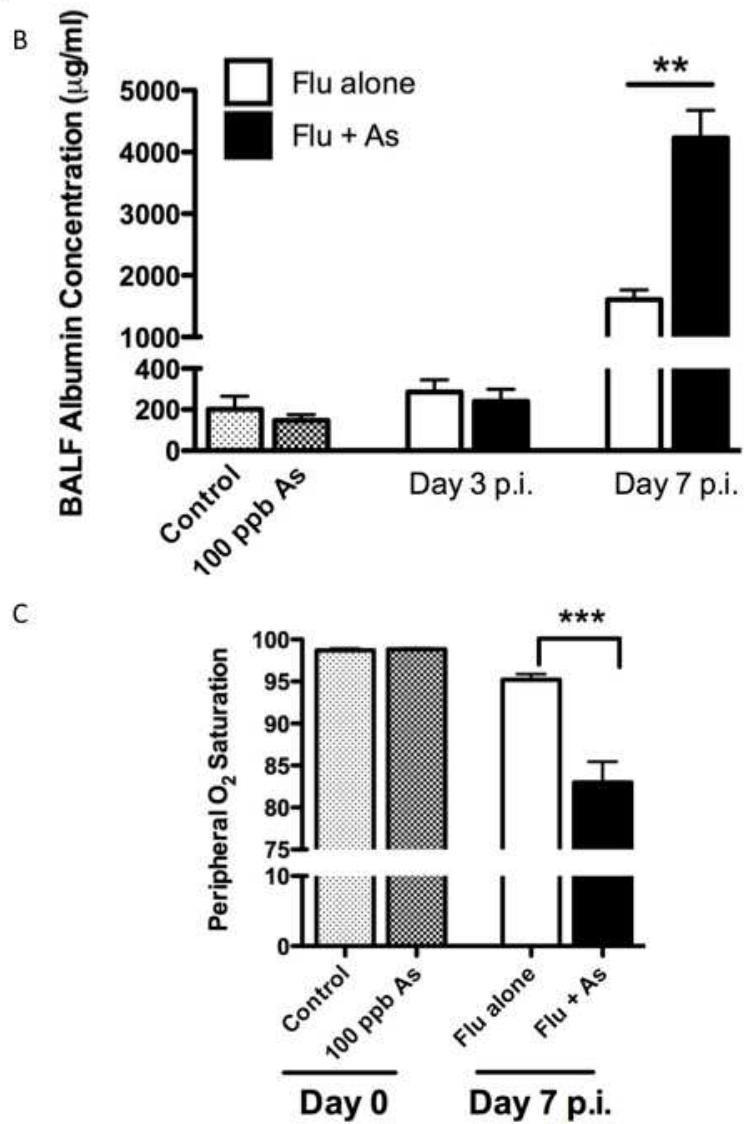


Figure 3B and C: Chronic drinking water As exposure increases capillary leakage at day 7 post infection.

190x254mm (72 x 72 DPI)

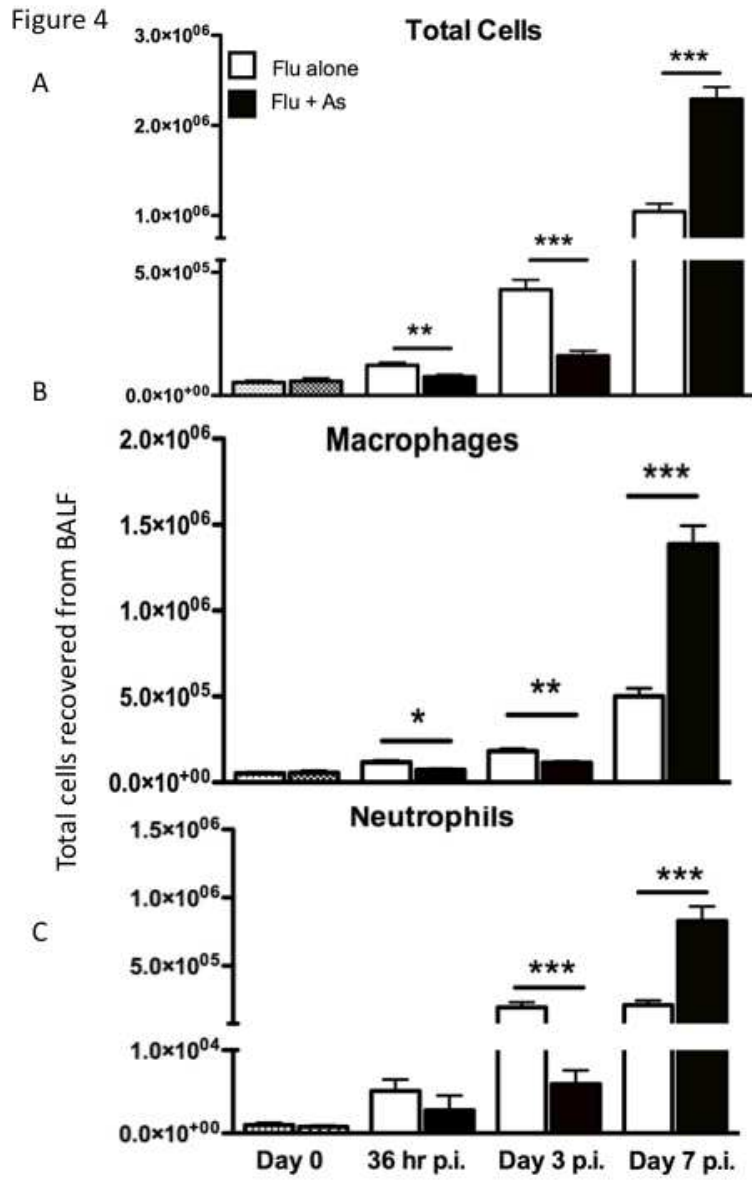


Figure 4A-C: Chronic drinking water As exposure alters cellular numbers in the BALF. 190x254mm (72 x 72 DPI)

Figure 4

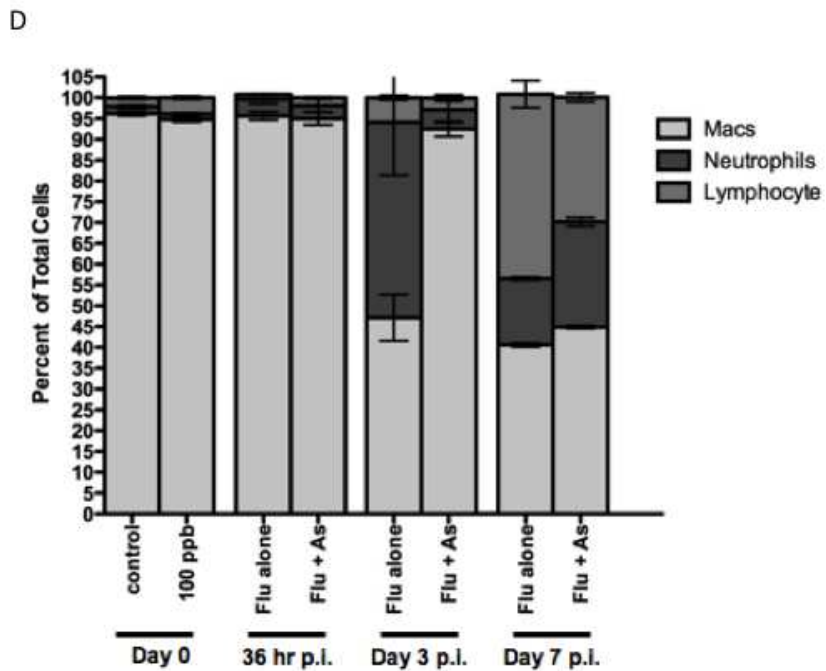
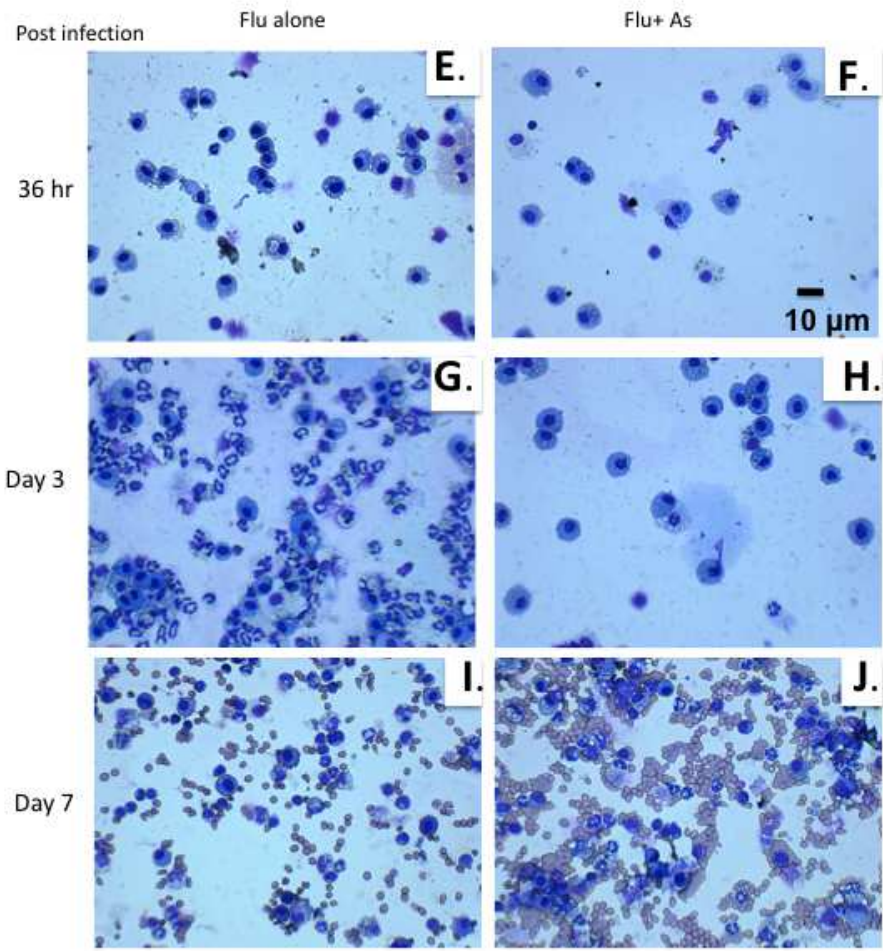


Figure 4D: Chronic drinking water As exposure alters cellular numbers in the BALF.  
190x254mm (72 x 72 DPI)

Figure 4 cont'd



190x254mm (72 x 72 DPI)

Figure 5

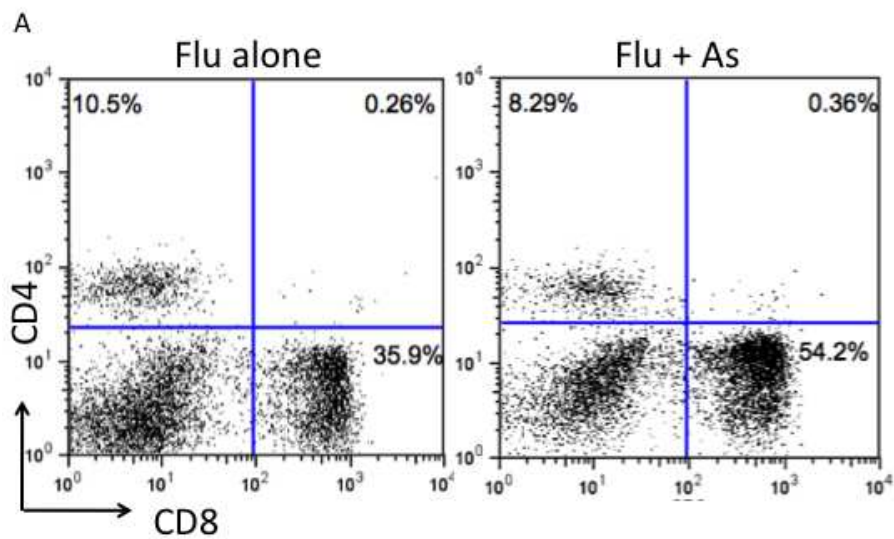


Figure 5: Chronic drinking water As exposure increases the percentages and total number of CD8+ cells in the BAL.  
190x254mm (72 x 72 DPI)

Figure 5

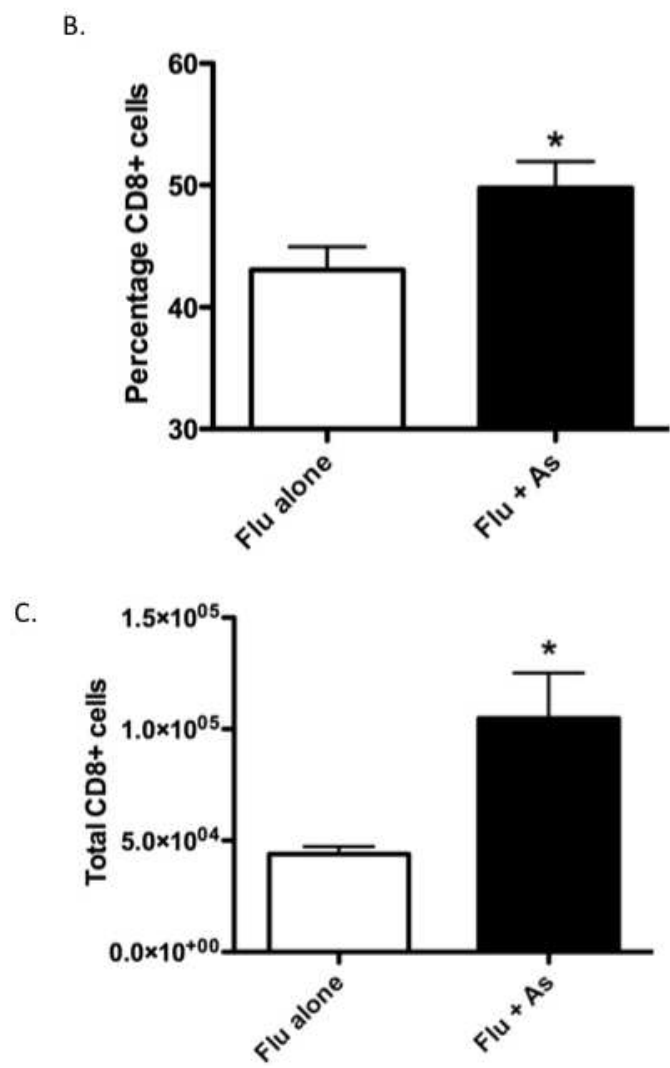


Figure 5B and C: Chronic drinking water As exposure increases the percentages and total number of CD8+ cells in the BAL.  
190x254mm (72 x 72 DPI)



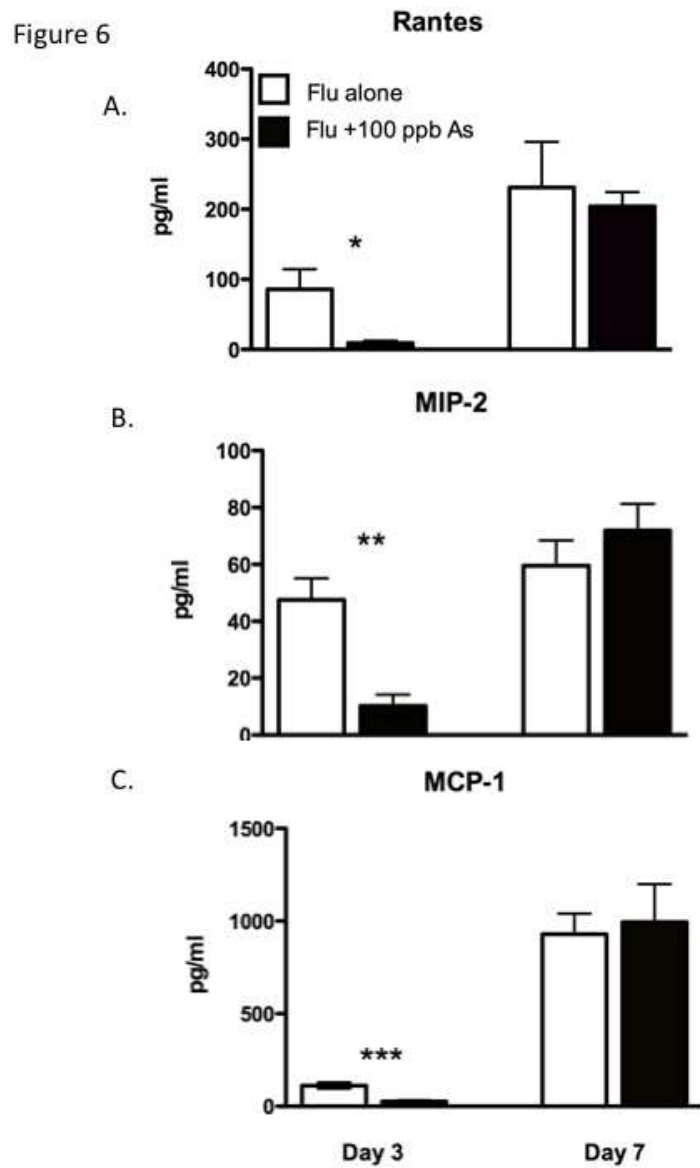


Figure 6: Chronic drinking water As exposure decreases cytokine and chemokine levels in the BALF at day 3 post infection.  
190x254mm (72 x 72 DPI)

Figure 7

A.

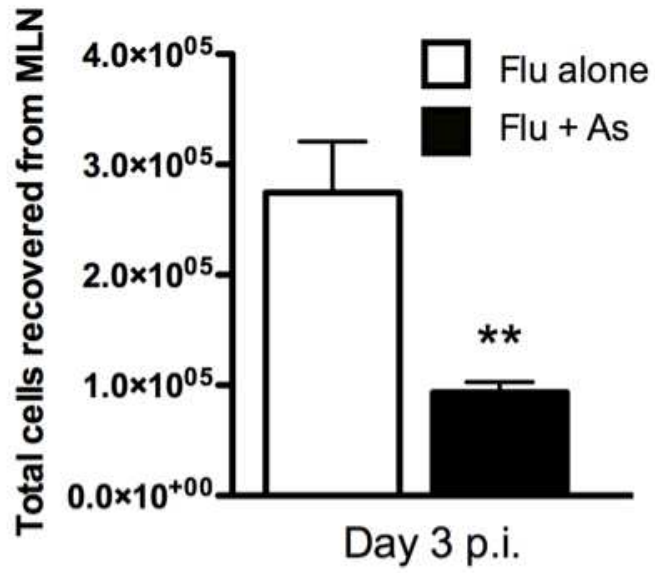


Figure 7: Chronic drinking water As exposure decreases the number of dendritic cells in the MLN.  
190x254mm (72 x 72 DPI)

Figure 7

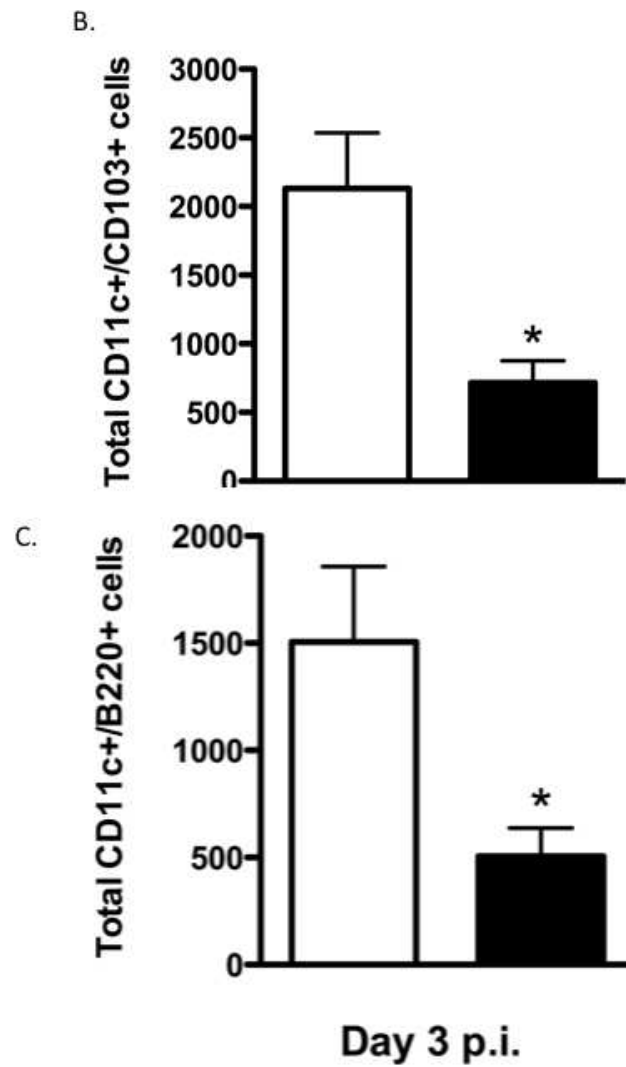


Figure 7B and C: Chronic drinking water As exposure decreases the number of dendritic cells in the MLN.  
190x254mm (72 x 72 DPI)

Figure 7D.

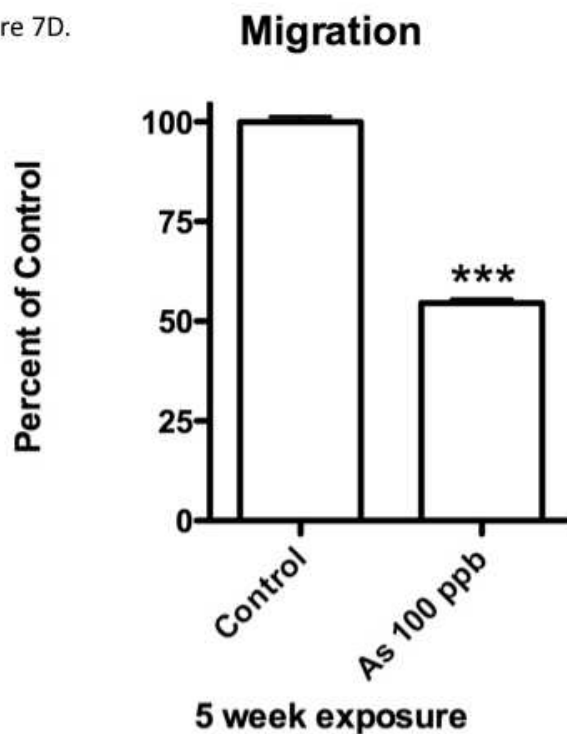


Figure 7D: Chronic drinking water As exposure decreases the number of dendritic cells in the MLN.  
190x254mm (72 x 72 DPI)