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A single or short time repeated arsenic oral exposure in mice impacts mRNA expression for signaling and immunity related genes in the gut

Matthew G. Arnold[a], Kuppan Gokulan[a], Daniel R. Doerge[b], Michelle Vanlandingham[b], Carl E. Cerniglia[a], Sangeeta Khare[a,*]

[a] Division of Microbiology, National Center for Toxicological Research, US Food and Drug Administration, 3900 NCTR Rd, Jefferson, AR, 72079, USA
[b] Division of Biochemical Toxicology, National Center for Toxicological Research, US Food and Drug Administration, 3900 NCTR Rd, Jefferson, AR, 72079, USA

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ABSTRACT

Arsenic is prevalent in contaminated drinking water and affects more than 140 million people in 50 countries. While the wide-ranging effects of arsenic on neurological development and cancer draw the majority of concern, arsenic's effects on the gut mucosa-associated immune system are often overlooked. In this study, we show that 24 h after a single dose [low dose (50 μg/kg bw), medium dose (100 μg/kg bw) or high dose (200 μg/kg bw)] of arsenic by oral gavage, mice show significantly reduced gut mucosa-associated mRNA expression for the key genes involved in the signaling pathways central to immune responses, such as Nuclear factor κB (NFκB), Extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), p38 and Myeloid differentiation protein 88-dependent (Myd88) pathways. Additionally, mRNA expression of apoptosis, inflammasomes and inflammatory response genes are significantly downregulated in the animals exposed to arsenic. Comparisons of time-dependent effects (24 h vs 48 h) from low dose arsenic exposed animals showed a significant shift in expression of Myd88 alone, suggesting that the down regulation was sustained for the key genes/signaling pathway. An extended eight-day exposure to arsenic showed a decreased state of immune preparedness, though not as diminished as seen in the single dose exposure.

1. Introduction

Arsenic is a widely-distributed, noxious chemical found naturally in the soil, water, and air whose prevalence is a leading public health concern (https://www.who.int/ceh/capacity/heavy_metals.pdf, 2017). The World Health Organization (WHO) deems arsenic as the most common chemical contaminant of drinking water and the Substance Priority List from the Health and Human Services/Agency for Toxic Substances and Disease Registry names arsenic as the leading substance of significant threat to human health (ATSDR, 2017). Exposure to organic arsenic can occur through oral consumption of sea foods [(for example jelly fish and seaweeds) (Taylor et al., 2017);] contaminated food [mainly rice (Khan et al., 2010; Zhu et al., 2008)] or through inhalation of contaminated sawdust and tobacco products; but the most common exposure route is through ingestion of arsenic-contaminated drinking water. An estimated minimum of 140 million people worldwide are exposed to concentrations of arsenic in drinking water that exceed the WHO recommended guideline of 10 ppb (μg/L) (Ravenscroft et al., 2009). In the United States, regulatory agencies have set standards in place to better protect the public. The U.S. Environmental Protection Agency (EPA) has a maximum contaminant level of 10 ppb arsenic in drinking water (EPA, 2001), and the Food and Drug Administration (FDA) has proposed draft action levels of 100 ppb inorganic arsenic in infant rice cereal (FDA, 2016) and 10 ppb inorganic arsenic in bottled beverages (2018).

Arsenic exists in inorganic and organic forms, with inorganic arsenic generally recognized to be associated with the more severe health risk. Inorganic arsenic predominately exists in two oxidation states, As^{III} and As^{V}. Trivalent arsenite (As^{III}) is seen as more hazardous to human health as opposed to pentavalent arsenate (As^{V}) (Domingo, 1995). Arsenic has been associated with numerous health effects, predominantly cancer and skin lesions (Chen et al., 1992; Hopenhayn-Rich et al., 1998; https://monographs.iarc.fr/wp-content/uploads/2018/06/mono100C-6.pdf, 2018). Acute arsenic exposure can cause vomiting, abdominal pain, encephalopathy and diarrhea, while chronic exposure causes cancer of the skin, urinary bladder, and lungs, and is also positively

Abbreviations: APL, acute promyelocytic leukemia; GIT, gastrointestinal tract

* Corresponding author. Division of Microbiology, National Center for Toxicological Research, US Food and Drug Administration, 3900 NCTR Road, Jefferson, AR, 72079, USA.
E-mail address: sangeeta.khare@fda.hhs.gov (S. Khare).

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associated with cancer of the kidneys, prostate and liver (Campbell and Alvarez, 1989; IARC, 2012; Jomova et al., 2011). Other studies have shown clear links between arsenic and cardiovascular disorders such as atherosclerosis, hypertension and ischemic heart diseases, as well as diabetes and non-malignant lung diseases (Lu et al., 2014; Mazumder, 2007; Singh et al., 2011). *In utero* and early childhood exposure to arsenic is especially problematic in both animals and humans, as it is known to result in increased mortality rates (Fry et al., 2007), increased susceptibility to influenza A infection (Ramsey et al., 2013), reduced intelligence (Manju et al., 2017; Nahar et al., 2014), and permanent changes in gene expression in offspring (Dolinoy et al., 2006). Animal studies show that large dose arsenic exposure in pregnant females can cause low birth weight and premature birth (Kile et al., 2016), fetal malformations (Hood, 1972), or fetal death (Fry et al., 2007).

Arsenic's toxicity is fundamentally attributed to oxidative stress (Barchowsky et al., 1999) which leads to genotoxicity and modulation of signaling pathways and transcription factors (Faita et al., 2013; Fry et al., 2007). Arsenic undermines the immune system by altering the function and/or abundance of various immune cell populations including T cells and macrophages (Andrew et al., 2008; Argos et al., 2006; Banerjee et al., 2009; Maiti et al., 2012; Ostrosky-Wegman et al., 1991). Human subjects chronically exposed to arsenic-contaminated drinking water show significantly elevated serum antibody levels along with respiratory complications (Islam et al., 2007). Low level arsenic exposure is shown to suppress overall innate immune system health in zebrafish (Nayak et al., 2007). Chronic arsenic exposure significantly compromises the immune response of adult C57BL/6 J mice to influenza A infection (Kozul et al., 2009). Human bronchial epithelial cells exposed to arsenic exhibited decreased antimicrobial defense and increased susceptibility to *Pseudomonas aeruginosa* infection (Goodale et al., 2017).

Although arsenic clearly has an adverse effect on the immune system, less is known about its effect on the gastrointestinal immune response, particularly that relating to antibacterial function that is essential to maintaining gut homeostasis. The gastrointestinal tract must maintain a delicate balance of tolerance to harmless, commensal microflora, and immune activation against invasive pathogens. A leading mechanism in maintaining gut homeostasis is activation of the Toll-like receptors (TLRs) -pathway and TLR-mediated responses. Deficiency of TLRs has been shown to undermine the development of proper inflammatory responses in the gut to commensal microflora [reviewed in (Honda and Takeda, 2009)]. This raises the question about possible roles TLRs and TLR-mediated responses may play in arsenic-induced dysbiosis. Furthermore, MyD88-mediated signaling pathways in mice prevent development of azoxymethane/dextran sodium sulfate-induced adenocarcinomas in the colon (Salcedo et al., 2010). Triggering of MyD88 leads to the activation of inflammasomes and inflammatory responses that are important in controlling infections, autophagy and regulating gut homeostasis (Gagliani et al., 2014). Additional pathways such as NFκB, MEK, and JNK/p38 signaling pathways also lead to a variety of inflammatory and apoptotic responses essential to regulating gut homeostasis (Kucharczak et al., 2003; Lawrence, 2009). Our goal was to explore the effect of arsenic on immune system responses in three scenarios: 1) dose-dependent responses, 2) time-dependent responses, and 3) dose frequency responses (single *vs* repeated dose). Here we explore the signaling mechanisms underlying arsenic's exposure on an important barrier in the body.

2. Materials and Methods

2.1. Animals

Handling procedures for mice used in this study have been previously reported (Gokulan et al., 2018; Twaddle et al., 2018a, 2018b). In brief, all studies were approved by the National Center for Toxicological Research Animal Care and Use Committee. Female CD-1 mice (7–8 weeks of age) were obtained from Charles River Co. (Wilmington, MA). Mice were placed on a low-arsenic basal diet (5K96, Test Diets, Purina Mills, Richmond, IN) to reduce background arsenic levels prior to testing. The preference for the use of female animals in this study was due to two reasons, first, the prevalence of arsenicosis is more in females who are exposed to low doses of arsenic (Ahmad and Khan, 2015). Second, this study was part of the larger experimental plan, designed to evaluate arsenic toxicity during perinatal and postnatal development. During the first phase of the study (pilot study), mice were given a single oral gavage of low dose [LD24 (50 μg/kg bw)], medium dose [MD24 (100 μg/kg bw)] or high dose [HD24 (200 μg/kg bw)] of sodium arsenite (purchased from Lab Chem, Zelienople, PA). Ileum samples were collected 24 h and 48 h postexposure as described in our previous publication (Gokulan et al., 2018) and the terminal ileum was excised and frozen immediately at −80 °C for the mRNA expression of immune status related genes. Endpoint analysis during these 24 h and 48 h post exposure allowed us to assess if a single exposure to arsenite may have any impact on the immune related gene expression, as well as, if these changes stay beyond 24 h. The pilot study was conducted using two animals in each group to establish sample collection procedure and generate preliminary data for kinetic study for minimal set of time points for arsenic metabolism in few targeted tissues (Twaddle et al., 2018b). As the lowest dose (LD) itself showed significant metabolism by various tissues, a larger study was conducted using only low dose (n = 6); thus, LD had 8 animals, whereas MD and HD had only 2 animals in the experimental group. Moreover, arsenic contaminated drinking water has a level of arsenic that could be in the milligram-per-liter range, thus reaching a total daily intake of ~50 μg/kg body weight (b.w.), dose chosen in this study. A detailed schematic diagram of the arsenic exposure to animals, as well as numbers of animals used, is provided in Fig. 1A.

In next phase of study, mice were repeatedly exposed to arsenic through drinking water (1 mg/L) for 8 days continuously (RD) before sacrifice. Based on the drinking water consumption/day/mice, the average daily intake of arsenite in the RD animals was estimated to be 192 ± 24 μg/kg bw/day. In RD animals the level of consumed arsenite was 192 ± 24 μg/kg bw/day (n = 6) (Twaddle et al., 2018a). Some mice from the RD group were placed on clean water for 24 h instead of being sacrificed to view recovery after the cessation of arsenic (RDCE).

2.2. RNA extraction and cDNA conversion

The protocol for RNA extraction and subsequent cDNA conversion has been previously described (Gokulan et al., 2018). Briefly, mouse intestinal tissue was minced in TRIzol Reagent (Thermofisher, Waltham, MA) and homogenized using a handheld homogenizer and nuclease-free pestle. Samples were vortexed with chloroform, centrifuged, then the top aqueous layer transferred to a new tube. Samples were then mixed with an equal volume of isopropanol and rested at −20 °C for 2 h. Pellets were washed with ice-cold 70% isopropanol, air-dried to remove residual isopropanol and then resuspended in nuclease-free H_2O (Thermofisher, Waltham, MA). Extracted RNA was treated with TURBO DNase (Thermofisher, Waltham, MA) to remove potential contaminating genomic DNA. Superscript IV VILO Mastermix (Thermofisher, Waltham, MA) was then mixed with 2.5 μg of DNase-treated RNA for synthesis of cDNA to be used in qPCR analysis.

2.3. qPCR and analysis

Expression of antibacterial response genes was analyzed with PAMM-148 plates (Qiagen, Hilden, Germany) which assessed 84 immune response related genes and other housekeeping control genes. SYBRGreen Mastermix (Applied Biosystems, Waltham, MA) was mixed with sample cDNA and aliquoted into wells. PCR was conducted in an ABI 7500 Real-Time PCR System with assay conditions set as: 50 °C/2 min, 95 °C/10 min; 95 °C/15 s, 60 °C/1 min; (x45 cycles) with a final

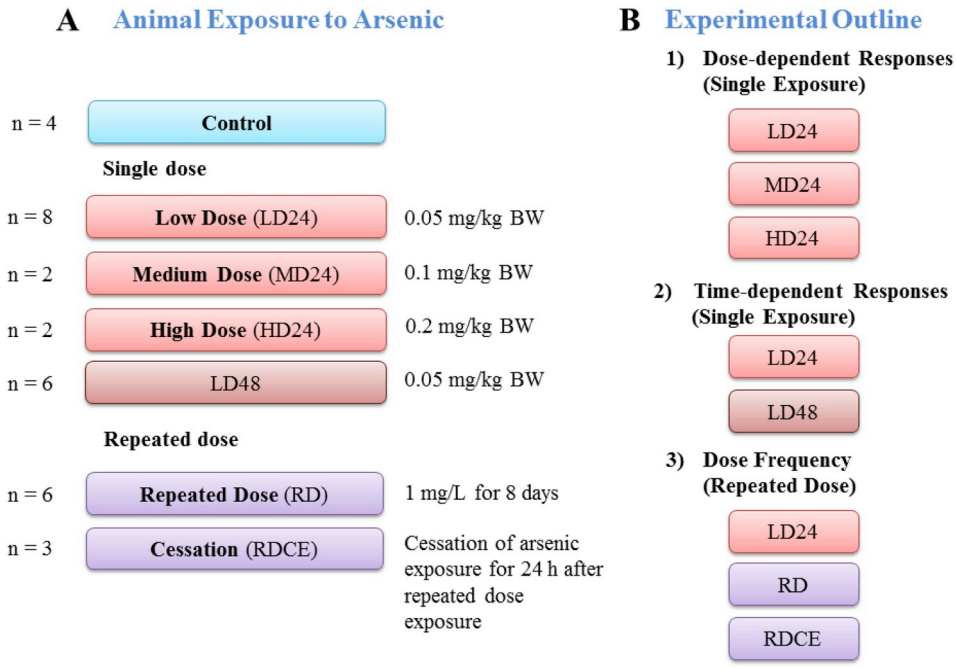

Fig. 1. Experimental strategy of studying how oral arsenic exposure affects mRNA expression of immune status related genes in the gastrointestinal tract A) How animals were exposed to arsenic. B) Experimental outline showing how samples were analyzed together.

melting curve from 60 °C–95 °C. Data was analyzed using the Qiagen Data Analysis Center with a threshold of 40 cycles. Arithmetic means of *β-actin, β-2 microglobulin, Gapdh* and *Hsp90ab* were used as the housekeeping genes to normalize data. The p-values were calculated in the Qiagen Data Analysis Center using a Student's t-test on the 2^(-Δ CT) values. Fold regulation values from Qiagen Data Analysis Center were used in data representation. P-values in graphs are depicted by either '*' (p-value ≤ 0.05) or '†' (p-value ≤ 0.005).

3. Results

Arsenic exposure causes significant downregulation of mRNA expression of immune response related genes in most of the experimental groups outlined in Fig. 1A. The results of each experimental group are provided below.

3.1. Dose-dependent responses (single exposure)

To investigate the effects of various arsenic dosage, mice were orally gavaged with a single dose of arsenic in three concentrations (as described in Materials and Methods) and the ileal tissue was collected 24 h after the single gavage (LD24, MD24, and HD24). Overall cursory analysis shows few genes were statistically significantly upregulated in any experimental group (Table 1). However, all experimental groups showed a variable number of genes that were downregulated. Interestingly, the HD24 group had noticeably fewer downregulated genes when compared to LD24 and MD24 (Table 1).

Table 1
Overall Gene Expression Changes in Arsenic-Exposed Experimental Groups. Only genes with fold changes greater than 2 or less than −2 are shown as upregulated or downregulated, respectively. Values shown in parenthesis are the number of genes below the p-value threshold of 0.05 in comparison to arsenic-free control mice.

	LD24	MD24	HD24	LD48	RD	RDCE
Upregulated	6(0)	5(4)	8(0)	5(0)	6(0)	10(1)
Downregulated	44(240)	40(11)	18(80)	39(22)	24(9)	28(8)

Our data showed that a single arsenic exposure caused significant differences in the mRNA expression of the cellular immune response genes representing NFκB, MEK, JNK/p38, and MyD88-dependent signaling pathways (Fig. 2A- C). It should be noted that some genes are involved in more than one function, thus some genes are reported in more than one pathway.

NFκB transcription factors are central to expression of pro-inflammatory cytokines and chemokines, as well as for lymphocyte proliferation and differentiation. Several critical components of the NFκB signaling pathway are significantly downregulated during arsenic exposure (Fig. 2A). In all three doses, positive regulator *Chuk* is significantly downregulated when compared to control mice. Low-arsenic dose mice (LD24) had significant downregulation of NFκB signaling genes *Ikbkb* and *Nfkb1* in addition to *Chuk*. Medium dose (MD24) and high dose (HD24) mice showed that expression of inhibitor *Nfkbia* (see Supplemental Fig. 1A) is significantly downregulated when compared to control mice.

Another mechanism for controlling cellular immune responses is through activation of the MEK, JNK/p38 signaling pathway. The mRNA expression of key genes that operate these signaling pathways is shown in Fig. 2B. Arsenic exposure in all three experimental groups caused significant downregulation of *Map2k4* (activates both JNK1 and p38) and *Mapk14* (p38). The LD24 group additionally showed significant downregulation of *Map2k1*, *Mapk1* (ERK2) and *Mapk8* (JNK1). Medium dose arsenic exposure caused significantly downregulation of *Mapk8* and *Jun* in addition to the previously mentioned *Map2k4* and *Mapk14*. The HD24 group showed downregulation of only the common *Map2k4* and *Mapk14* genes with no other significant differences.

Analysis of the MyD88-dependent pathway shows the HD24 group with the highest overall mRNA expression level among the three single arsenic dose groups (Fig. 2C). LD24 shows significant decreases in *Fadd, Map3k7, Myd88, Tlr5 and Tollip*. MD24 shows a significant decrease in the *Tollip* gene while showing a significant increase in *Irak1*. The HD24 group however, has no significant changes in gene expression.

Next, we compared the dose effects (LD24 vs MD24 vs HD24) for the mRNA expression of genes involved in these pathways. Analysis of experimental groups (dose response) revealed differences in the NFκB signaling pathway, with *Nfkb1* being significantly changed between

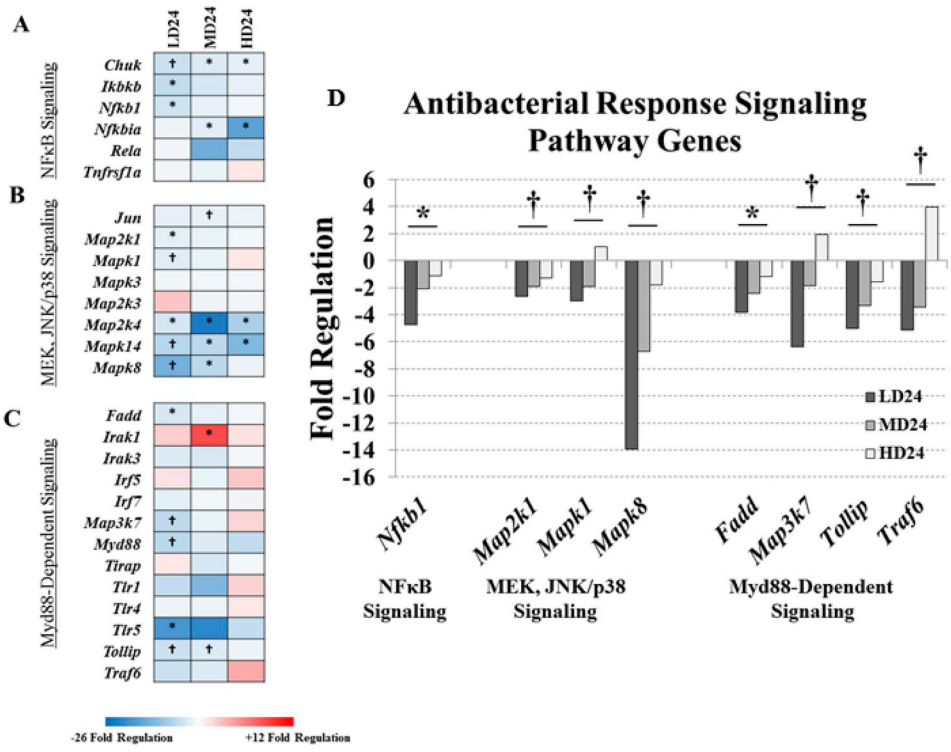

Fig. 2. Gene expression changes in antibacterial response signaling pathways in single dose arsenic groups. Heatmaps for A) NFκB, B) MEK, JNK/p38, and C) Myd88-dependent signaling pathways show gene expression changes as fold expression relative to the control. Only genes with fold changes greater than 2 or less than −2 are shown with significance. The bar graph in D) shows genes that are significantly altered between experimental groups LD24 and HD24. Statistical significance is shown as either "*" (p-value ≤ 0.05) or "†" (p-value ≤ 0.005).

LD24 and HD24 (Fig. 2D). While comparing the dose effect in MEK. JNK/p28 pathways, HD24 showed significant differences for the mRNA expression of *Mapk1, Map2k1* and *Mapk8* genes in comparison to LD24 (Fig. 2D). Furthermore, several MyD88-signaling genes such as *Fadd, Map3k7, Tollip* and *Traf6* have significantly higher expression in the HD24 group in comparison to the LD24 (Fig. 2D).

The previously described signaling pathways play important roles in regulating mucosal immune responses, which can be best exemplified through expression of genes involved in inflammasome components, inflammatory and apoptotic responses. However, a particular gene may be involved in more than one response/pathway. After arsenic exposure at LD24 and MD24, mRNA expression of most inflammasome component genes showed downregulation; while the HD24 group shows expression levels similar to the arsenic-free control mice (Fig. 3A and B). mRNA expression of *Casp1* and *Naip1* were significantly downregulated in the low dose animals (LD24) as compared to the controls. Medium dose animals showed significantly upregulated expression of *Pycard* and significant downregulation of the *Casp1* and *Nlrc4* genes as compared to control animals. Overall, the dose-dependent effect on various signaling cascades is best exemplified in expression changes of inflammatory response genes (Fig. 3B). LD24 shows inflammatory response genes *Lbp, Ly96, Rac1, Myd88*, *Tollip* and *Tlr5* are significantly downregulated. MD24 shows strong downregulation of several genes but only significantly for *Nlrc4* and *Tollip,* but also a significant increase in *Cd14* expression. HD24 shows significant decreases in expression of *Akt1* and *Rac1*. Intra-experimental group comparison for genes involved in the inflammasomes process show a significant increase in the expression of both *Casp1* and *Naip1* genes between HD24 and LD24 (Fig. 3A). While comparing the overall dose effect on inflammatory response genes, most significant differences in the expression of the genes were found between LD24 *vs* HD24. Similarly, significant differences were seen in the mRNA expression of inflammatory response genes *Lyz2, Mefv, Nfkb1, Ripk2, Tlr1* and *Tollip* in the HD24 when compared to the LD24 group (Fig. 3C).

Overall, expression of apoptotic genes is shown to be highest in HD24 relative to the other two experimental groups (Fig. 3D). LD24 has the lowest overall expression, with expression of *Casp1, Fadd, Ikbkb, Map3k7, Mapk1, Mapk8, Pik3ca, Rac1* and *Ripk1* all significantly downregulated compared to control mice. MD24 has fewer gene expression changes, showing *Casp1, Jun, Mapk8,* and *Nfkbia* significantly downregulated while *Cd14, Irak1* and *Pycard* are significantly upregulated. HD24 gene expression mostly falls within the range of the arsenic-free control mice, with only *Akt1, Nfkbia* and *Rac1* showing significant downregulation. While comparing between LD24 and HD24 (Fig. 3E), numerous genes in the apoptotic process, such as *Fadd, Map3k7, Mapk1, Mapk8, Nfkb1, Pik3ca, Ripk2* and *Traf6* show significantly higher expression in HD24 (in terms of either lowest level of downregulation or higher level of upregulation in the mRNA expression of a particular gene) from the LD24 to HD24.

3.2. Time-dependent responses (24 h vs 48 h)

To determine if changes in mRNA expression of immune system response genes during a single exposure to arsenic at the lowest dose would be sustained after a short recovery period of 48 h, murine mRNA expression 24 h after low dose arsenic exposure (LD24) was compared to expression 48 h post-exposure (LD48). The Venn diagram (Fig. 4A) depicts an overall summary of upregulated or downregulated immune response genes in LD24 and LD48. Of the six genes upregulated in either LD24 or LD48, five are upregulated in both groups while one is unique to LD24. Thirty-six genes are downregulated in common between LD24 and LD48. Genes involved in NFκB, MEK, p38/JNK and MyD88 signaling pathways were observed to be quite similar, with the notable exception of *Myd88* (Fig. 4B–D). Statistical analysis shows that the *Myd88* gene is the only significantly changed gene between these two groups; showing a significantly lesser downregulation in LD48 compared to LD24 (4.15 fold higher in LD48 than LD24, p-value = 0.001) (Supplemental Fig. 2). Overall, gene expression for inflammasome, inflammatory response and apoptosis gene groups between LD24 and LD48 was remarkably similar as gene expression for these gene groups was not significantly different between the two time points (Supplemental Fig. 3).

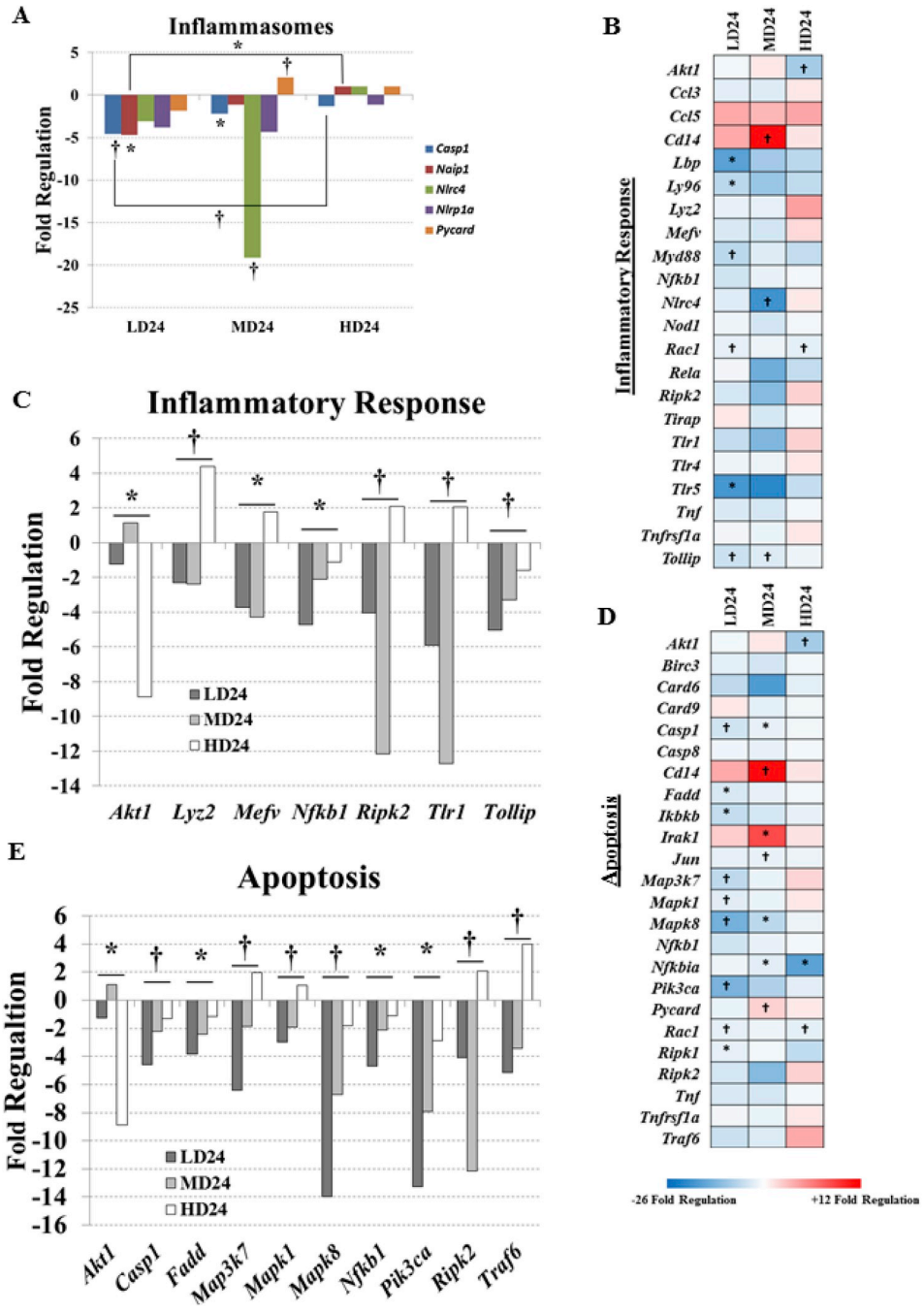

Fig. 3. Gene expression changes of antibacterial response genes in single dose arsenic groups. A) Genes encoding inflammasome components are shown in fold expression relative to control mice. Statistical significance between the LD24 and HD24 experimental groups is shown as well. B) Heatmap of inflammatory response genes shown as fold expression relative to control mice. C) Inflammatory response genes that are significantly different between experimental groups LD24 and HD24. D) Heatmap of apoptotic genes shown as fold expression relative to control mice. E) Apoptotic genes that are significantly different between experimental groups LD24 and HD24. Statistical significance is shown as either "*" (p-value ≤ 0.05) or "†" (p-value ≤ 0.005). Only genes with fold changes greater than 2 or less than −2 are shown with significance.

3.3. Effects of dose frequency

The final analysis investigated the potential differences between exposure to a single dose of arsenic (LD24) and a repeated dose of arsenic (RD). As mentioned earlier, on an average the daily exposure to arsenite in the RD animals was estimated to be 192 ± 24 μg/kg bw/day (n = 6) (Twaddle et al., 2018a). The RD experimental group totally had nine mice, six mice were used for RD group and 3 of them were used for cessation group. This analysis also included cessation mice (n = 3) (RDCE) which were repeatedly exposed to arsenic for 8 days, similar to the RD group, but then given arsenic-free water for an additional 24 h before sample collection. This allowed assessment of potential changes in the gene expression due to chronic exposure to arsenic, and whether these changes would normalize when arsenic exposure was stopped and replaced with normal water.

Analysis showed the repeated dose exposure had a variable impact on the gene expression level in the NFκB, MEK, JNK/p38 and MyD88-dependent pathways. Expression of *Chuk* was downregulated in both RD and RDCE groups, but the other NFκB pathway genes did not change significantly during the repeated exposure (Fig. 5A). Repeated exposure caused significant downregulation of *Mapk1* in both RD and RDCE, as well as *Map2k4* in RD (Fig. 5B). MyD88-dependent signaling pathway genes were not significantly changed in RD, while RDCE showed significant downregulation of *Map3k7* and *Tollip* (Fig. 5C). Inter-experimental group analysis showed a small number of signaling pathway genes significantly different between single dose exposure (LD24) and repeated dose exposure (RD) (Fig. 5D). For the MEK pathway *Mapk1* was shown to be significantly lower in RD relative to LD24. For MyD88-signaling genes *Irf7, Tollip* and *Traf6* showed significantly higher expression in RD than in LD24.

Expression of inflammasomal component genes is mostly downregulated, with *Casp1* significantly downregulated in both RD and

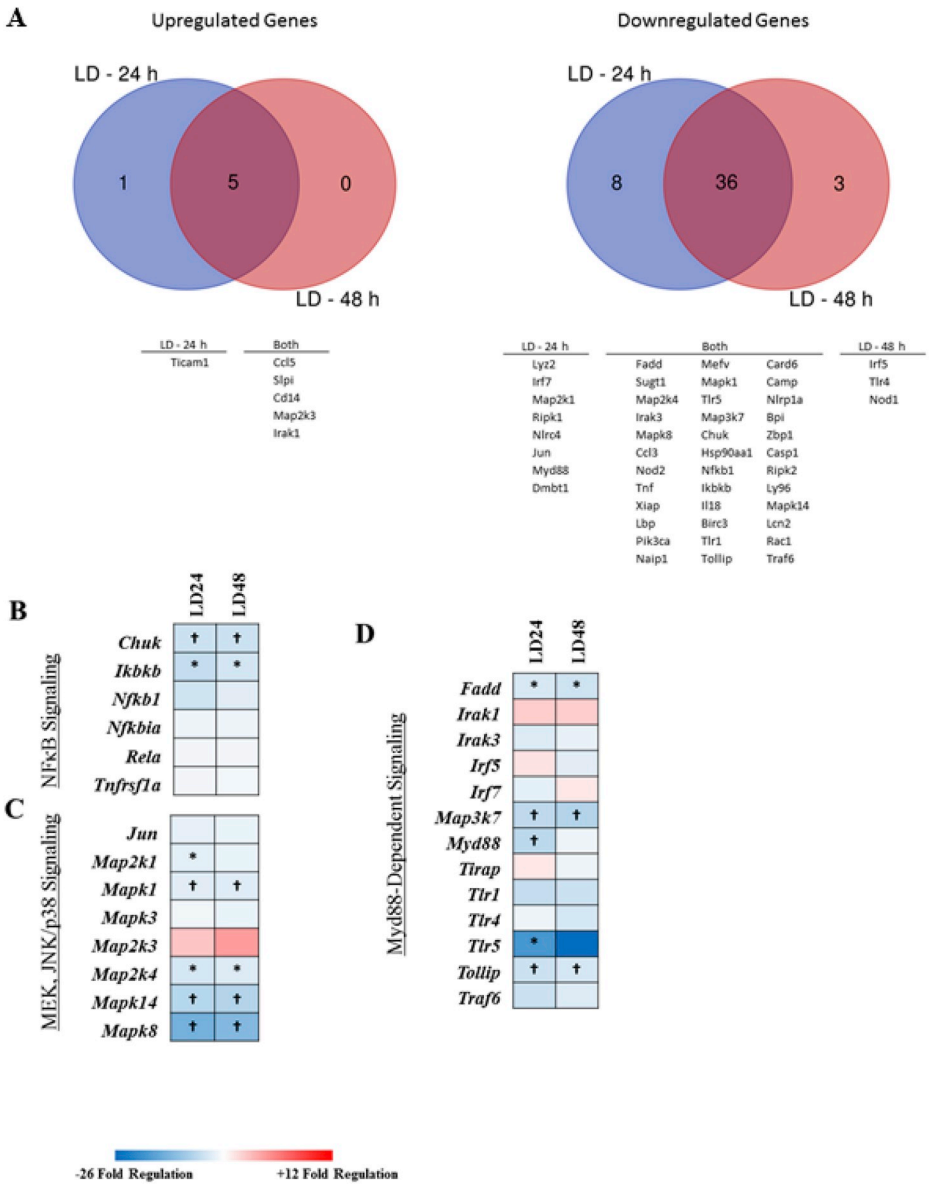

Fig. 4. A) Venn diagram demonstrating similarity in gene expression between LD24 and LD48 groups. Heatmaps for A) NFκB, B) MEK, JNK/p38, and C) Myd88-dependent signaling pathways show gene expression changes as fold expression relative to the control. Statistical significance is shown as either "*" (p-value $\leq$ 0.05) or "†" (p-value $\leq$ 0.005). Only genes with fold changes greater than 2 or less than -2 are shown with significance.

RDCE (Fig. 6A). Intra-experimental group analysis showed that only *Naip1* was significantly different between RD and LD24 (Fig. 6D). Inflammatory response genes surprisingly showed no significant difference, other than *Tollip* (an inhibitor for Irak1 activation), *Cd14* and *Lbp* (Fig. 6B). Overall expression of apoptotic genes was similar in LD24 and RD (Fig. 6C), with the exception of *Card9* and *Traf6* which were significantly upregulated in RD relative to LD24, while *Mapk1* as previously mentioned was downregulated (Fig. 6D). No significant differences were found between the RD and the RDCE groups in antibacterial response gene expression (data not shown).

4. Discussion

The dose-dependent impacts of a single arsenic gavage on gastrointestinal immune responses remains largely a mystery. Our work shows that a single arsenic gavage has a sizable impact on the critical NFκB, MEK, JNK/p38, and MyD88-dependent signaling pathways in mice, at all doses tested. NFκB transcription factors are central to expression of pro-inflammatory cytokines and chemokines, as well as for lymphocyte proliferation and differentiation. Several critical components of the signaling pathway are significantly downregulated. Increased arsenic exposure has been shown to modulate epigenetic changes at the methylation levels of various genes involved in the NFκB signaling pathway (Argos et al., 2014). In our work, all doses show significant downregulation of *Chuk* (a positive regulator of NFκB signaling), as well as *Ikbkb* at the low dose (LD24). *Nfkb1* was also significantly downregulated in the LD24. Inhibitor *Nfkbia* is significantly downregulated at both the medium and high dose. Together these results suggest that although NFκB elements are downregulated at every dosage, low dose would have the lowest activation due to significant decreases in expression of positive regulators *Chuk* and *Ikbkb*, as well as significant downregulation of *Nfkb1*. Medium and high doses would possibly have higher, albeit still downregulated, pathway activation due to decreases in both *Chuk* and inhibitor *Nfkbia* expression. Finally, the expression of *Nfkb1* was significantly higher in HD24 than in LD24, supporting the conclusion that the NFκB pathway may be overall more active in the HD24 than in the LD24 (Supplementary Fig. 1A).

It has been shown in neuronal cells that MAP2K1 activates ERK1

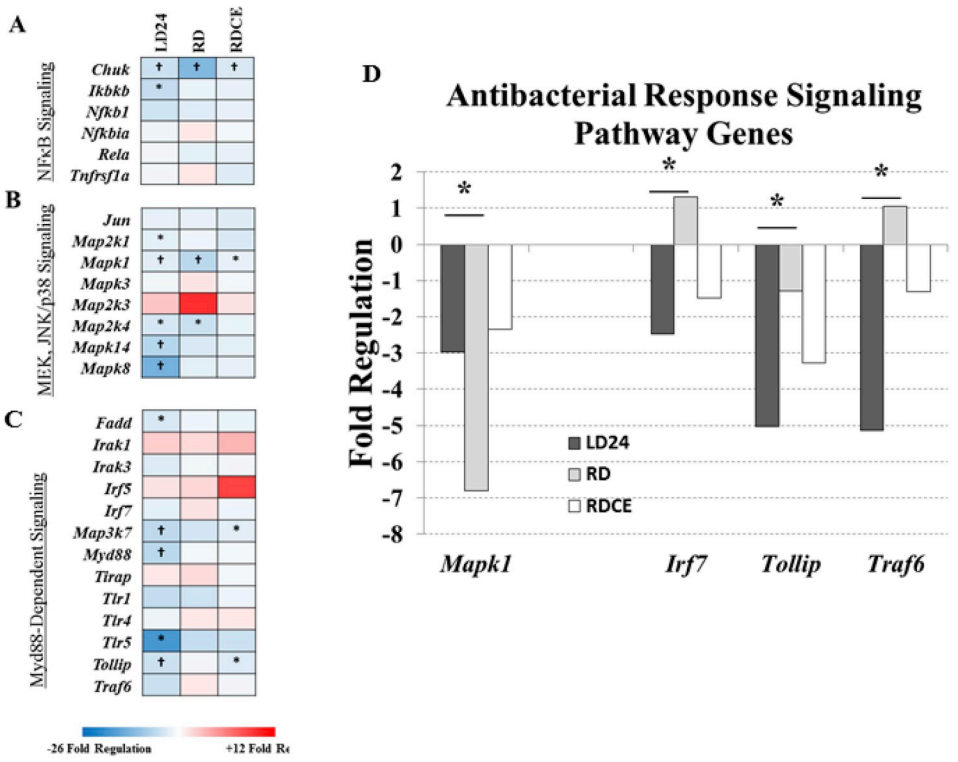

Fig. 5. Gene expression changes in antibacterial response signaling pathways in either single dose (LD24), repeated dose (RD) or cessation (RDCE) experimental groups. Heatmaps for A) NFκB, B) MEK, JNK/p38, and C) Myd88-dependent signaling pathways show gene expression changes as fold expression relative to the control. Only genes with fold changes greater than 2 or less than −2 are shown with significance. The bar graph in D) shows genes that are significantly altered between experimental groups LD24 and RD. Statistical significance is shown as either "*" (p-value ≤ 0.05) or "†" (p-value ≤ 0.005).

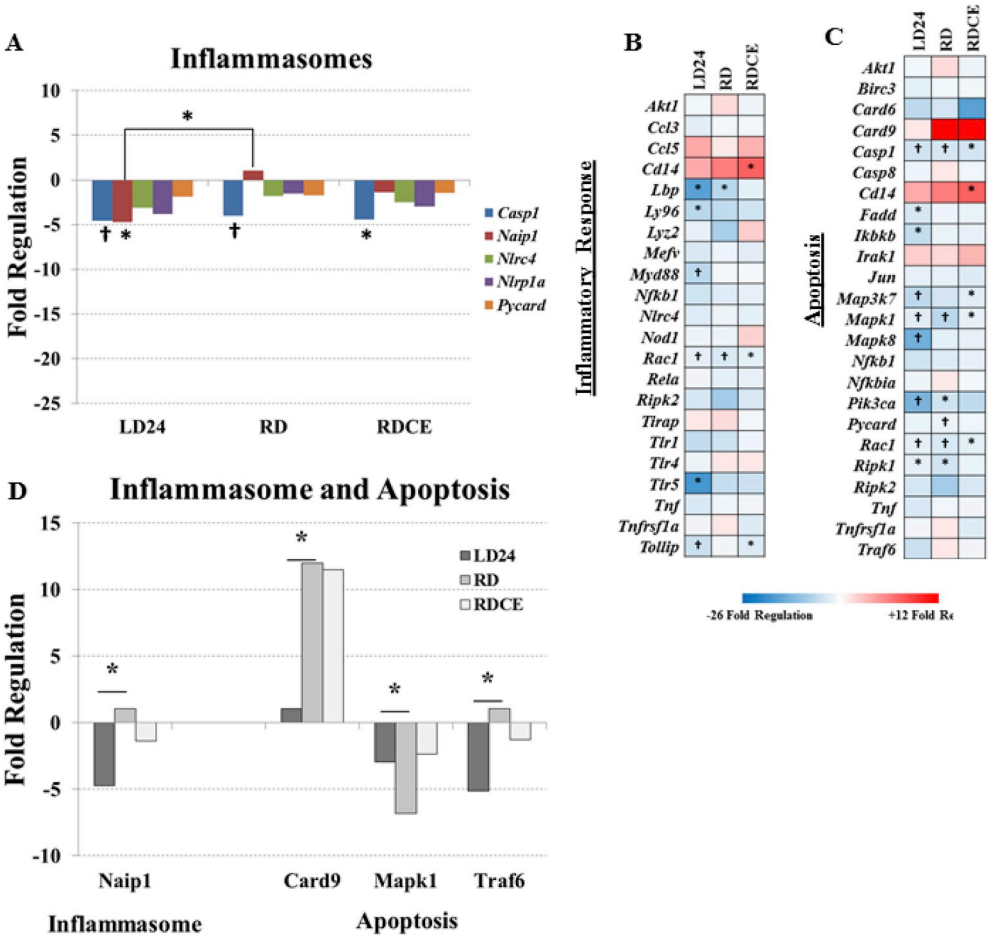

Fig. 6. Gene expression changes in antibacterial response signaling pathways in either single dose (LD24), repeated dose (RD) or cessation (RDCE) experimental groups. A) Genes encoding inflammasome components are shown in fold expression relative to control mice. Statistical significance between the LD24 and RD experimental groups is shown as well. B) Heatmap of inflammatory response genes shown as fold expression relative to control mice. C) Heatmap of apoptotic genes shown as fold expression relative to control mice. D) Inflammasome and apoptotic genes that are significantly different between experimental groups LD24 and RD. Statistical significance is shown as either "*" (p-value ≤ 0.05) or "†" (p-value ≤ 0.005). Only genes with fold changes greater than 2 or less than −2 are shown with significance.

and ERK2 (*Mapk3* and *Mapk1* respectively) which enter the nucleus to activate transcription factor c-Jun, mediating MAPK signaling (Eriksson et al., 2007). Intramucosal nerve cells are also present in the small intestine (Fang et al., 1993). These nerve cells are a main component of the enteric nervous system; the largest and most complex part of the peripheral nervous system (Nezami and Srinivasan, 2010). Our data shows that the MEK, p38/JNK pathway is mostly downregulated at all doses (Fig. 2B). These genes represent positive regulators in the kinase cascade, thus the pathway is downregulated at all doses (as shown in Supplementary Fig. 1B). However, LD24 and MD24 have more significantly downregulated genes, five and four genes, respectively; while the high dose only has two genes significantly downregulated. Additionally, numerous components such as *Map2k1, Mapk1*(ERK2) and *Mapk8* (JNK1) have significantly higher expression in HD24 than in the LD24 group, suggesting that while downregulated, HD24 again has the highest gene expression level among the experimental groups. The combination of higher *Mapk1* and *Mapk8* gene expression levels indicates an increased likelihood of activation of downstream transcription factor Jun, as well as others such as ATF-2 and SP-1 (Kumar et al., 2009; Zeke et al., 2016) (not assayed here), that could potentially lead to inflammation and apoptosis. The significant downregulation of *Mapk14* (p38) in all doses could be potentially compensated in HD24 by this increased expression of *Mapk1* and *Mapk8*.

Most importantly, we see in the Myd88-dependent pathway that the HD24 has no significantly changed genes, while LD24 has several significantly downregulated genes. Of particular interest is the *Map3k7/Traf6* link between the Myd88 pathway and activation of the NFκB and MEK, JNK/p38 pathways mentioned above. *Map3k7* encodes a kinase (TAK1) that activates NFκB and MAP2Ks (leading to activation of JNK and p38). Changes in *Traf6,* a central activator of various transcription factors including *Irf5, NFκB* and *Jun,* were not statistically significant in any of the arsenic exposed animals as compared to control. Together these two proteins form a kinase complex that is required for activation of NFκB (Landström, 2010). Single dose intra-experimental group analysis shows expression of *Map3k7* and *Traf6* genes are both significantly higher in the HD24 compared to LD24. However, a Myd88-pathway inhibitor *Tollip* also shows significantly higher expression in HD24 than in LD24, although still downregulated. This may be partially offset by the (non-significant) increased gene expression of Irak1, the target of Tollip's inhibition (Supplementary Fig. 1C). Taken together, these observations further support our conclusion that these signaling pathways are more active at higher doses of arsenic.

Arsenic undermines the human immune system at a cellular level, while interacting with peripheral blood mononuclear cells, natural killer cells, T-cell lymphocytes, eosinophils, monocytes, and macrophages, resulting in alterations in function and/or abundance (Andrew et al., 2008; Argos et al., 2006; Banerjee et al., 2009; Maiti et al., 2012; Ostrosky-Wegman et al., 1991). Macrophages have been proposed as a target of inorganic arsenic's toxic effects. In our study, *Cd14*, the pattern recognition receptor mainly expressed by macrophages, is upregulated in all arsenic-exposed groups, though this data was only statistically significant in MD24. However, expression of *Irak3*, a monocyte/macrophage-specific negative regulator of MyD88-dependent signaling, is slightly downregulated (non-significantly) in all arsenic-exposed groups including MD24. One possible explanation for the conflicting results in gene expression of macrophage-related *Cd14* and *Irak3* is the observation that arsenic exposure in drinking water causes an increase in $CD14^+$ monocytes (Dutta et al., 2015; Wu et al., 2003). Although a main macrophage chemotaxic protein *CCL3,* also known as macrophage inflammatory protein 1-alpha (MIP-1α), showed no significant changes in gene expression levels, in our previous work (Gokulan et al., 2018) we also showed significant increases in MCP1 (monocyte chemotactic protein; that is known to also interact with NFκB pathway (Ritchie et al., 2004)). This could lead to increased infiltration of monocytes and thus support increased expression of $CD14^+$. Our results suggest that a similar phenomenon may be occurring in the intestinal mucosa. Another study reports the de-differentiation of macrophages into CD14-expressing monocytic-like cells following a repeated arsenic exposure of 3–6 days (Lemarie et al., 2008), which may be relevant to our 8 day, repeated dose group results, where mRNA expression was higher for the CD14 gene. Finally, arsenic trioxide has been shown to inhibit monocyte differentiation into macrophages through repression of NFκB-related survival pathways (Lemarie et al., 2006), which we show are indeed downregulated. In fact, recently it has been shown that ileal tissue and the associated microbial population are highly proficient in conversion of arsenic into arsenic trioxide (Gokulan et al., 2018; Twaddle et al., 2018c).

Equally important as the signaling pathways mentioned earlier; inflammasomes, inflammatory genes and the apoptotic response play an essential role in maintaining mucosal integrity and gut homeostasis. The high dose group shows elevated levels of all three gene groups in comparison to the lower doses, though similarly expressed to the control. Of particular interest is the JNK, Akt and PI3K coordination in susceptibility to arsenic trioxide-induced apoptosis (Davison et al., 2004). *Pik3ca,* expressing the catalytic subunit of the PI3K enzyme, was downregulated in all arsenic exposed groups, though statistical significance was found only in LD24. *Mapk8* (gene encoding JNK) was downregulated in all arsenic-exposed groups; however, statistically significant differences were observed in LD24 and MD24 but not in HD24. HD24 shows significant downregulation of *Akt*, while *Mapk8* and *Pik3ca* are non-significantly downregulated. JNK, which inactivates Akt and PI3K through phosphorylation, has been linked to arsenic trioxide-dependent apoptosis in acute promyelocytic leukemia (APL) (Davison et al., 2004). This inactivation of Akt and PI3K leads to increased sensitivity to arsenic trioxide-dependent apoptosis in acute leukemia (Tabellini et al., 2005). Therefore, the observed significant downregulation *Akt* in HD24 could be directly related to increased susceptibility to apoptosis, which is indicated by the elevated expression of apoptotic gene in HD24 relative to the other lower doses. This downregulation of *Mapk8* (JNK gene) suggests these lower dose groups may be more protected from apoptosis than the HD24 group. The significant increased expression of apoptotic genes (*Casp1, Fadd, Map3k7, Mapk1, Nfkb1, Ripk2* and *Traf6*) in the HD24 group relative to LD24 support this theory.

Inflammasomes are part of the innate immune response which utilize PRRs (pathogen recognition receptors) from the Nod-like Receptors to initiate pro-inflammatory responses, including pro-inflammatory cytokines IL-1β and IL-18 and pyropoptosis via Caspase-1. Arsenic trioxide inhibits the response of NLRC4 inflammasomes to their ligands (Maier et al., 2014). Additionally, it inhibits both the autoproteolytic activity of caspase-1 and activity of pre-activated recombinant caspase-1. This, in turn, inhibits downstream processing of cytokines IL-1β and IL-18 by caspase-1 (Maier et al., 2014). *Casp1* gene expression, an important player in the apoptosis pathway, is significantly increased in HD24 compared to LD24. *IL-1β* gene expression level was often undetectable, but elevated expression was found in HD24 (4.9 fold increase), whereas *IL*-18 has a significant decrease in gene expression (−7.1 fold decrease, p-value > 0.05) (data not shown). Interestingly, although IL-18 is defined as pro-inflammatory, it has shown remarkable properties in tissue repair, epithelial restitution, and resolution of inflammation (Chen, 2017; Chen et al., 2011; Salcedo et al., 2010). Gene expression levels of the 'promiscuous' *Nlrp3* component go largely undetected in our study (data not shown). Increased amounts of arsenic may efficiently inhibit caspase-1 activity in high doses, however data showing the increased expression of inflammatory response genes does not support this. Our data suggests that the lowest level of arsenic exposure possible (in our study, 0.05 mg/kg bw) is the most effective in decreasing immune response gene expression.

Arsenic trioxide has also been shown to reduce protein levels of X-linked inhibitor of apoptosis protein (Xiap) in monocytes (Lemarie et al., 2006). In our results *Xiap* gene expression is significantly reduced in all arsenic-exposed groups (statistically significant; data not shown).

Reduced expression of Xiap, along with BCL-xL, and $cIAP_2$ (*Birc3* in our data), are suggested to be the causes of apoptotic induction in arsenic trioxide-exposed human APL NB-4 cells (Momeny et al., 2010). Our study also confirms the downregulation of the *Birc3* gene at all arsenic exposures tested, though only significantly in LD48.

The ability of arsenic to cause lasting damage is well illustrated through comparison of LD24 to LD48, as LD48 had time to recover from the single dose arsenic assault. Fig. 4A depicts the similarity of the two groups in gene expression. Interestingly, *Myd88* is the only gene significantly different between the two time points, though there is no clear reason why. Considering that MyD88-dependent signaling is indispensable for TLR-initiated responses, expression of TLR genes was analyzed. However, no significant differences in the mRNA expression of *Tlr* genes (*Tlr1, Tlr4* and *Tlr5*) were observed (data not shown). Moreover, expression of *Ly96* (Lymphocyte antigen 96 that interact with TLR 4), was also downregulated in LD24 and LD48 (data not shown). Probably activation of the MyD88 dependent signaling pathway may be TLR-independent in this case. It is likely that the similarity in gene expression between LD24 and LD48, and RD and RDCE, shows that arsenic exposure, whether a single instance or repeated, has a lasting impact on gut homeostasis and immune system function that is not easily repaired in only 24 h.

Our results clearly show that arsenic exposure suppresses a wide variety of immune response genes in the gastrointestinal tract. Previously, the immunosuppressive effects of arsenic trioxide have been utilized to induce remission of APL (Soignet et al., 1998), as well as to alleviate allograft rejection in mice (Zhao et al., 2018). Our gene expression results support these previous reports that a low dose of arsenic has an immunosuppressive effect as seen in APL treatment (Soignet et al., 1998), although it is unclear if this would have any benefits in the context of gastrointestinal health. It was postulated that the immunosuppressive effects of arsenic exposure would be best visualized through an increased presence of pathogens. Our previous study on the microbiome of these arsenic-exposed mice showed that the genus *Bilophila* was abundant in adult mice repeatedly-exposed (RD) to arsenite (Gokulan et al., 2018). *B. wadsworthia* is known to be an opportunistic pathogen that can cause intra-abdominal infections (Baron et al., 1992; Feng et al., 2017; Finegold et al., 1992) and is suspected to cause inflammation when found in abundance (Feng et al., 2017). This could help explain the relatively elevated immune state in RD, in comparison to other single-dose arsenic-exposed groups. This however, would not explain the overall similarity of the immune state between the controls and repeated exposure groups, suggesting that *B. wadsworthia* may be one of many factors. However, there are no significantly changed pathogenic species observed in the single-dose 24 h groups (LD24, MD24, and HD24), perhaps simply because not enough time has passed for such an infection to take place (Gokulan et al., 2018).

This study supports our previous work showing shifts in microbial population following arsenic exposure (Gokulan et al., 2018). The gut microbiome and host have co-evolved over millennia, and unsurprisingly have major impacts on one another. Previously, we investigated the changes in gut microbial populations in arsenic exposed mice (Gokulan et al., 2018). In the case of LD24 and LD48, little difference is observed aside from the expression of the *Myd88* gene, and accordingly the microbial populations are shown to be very similar. Dramatic changes in distal gut microbiota composition have been observed in *Myd88*$^{-/-}$ mice (Wen et al., 2008). Our data confirm previous work that has observed changes in MyD88 expression in response to arsenic. Our previous analysis of shifts in the microbial population correlates well with the overall degree of change observed in the MyD88-dependent signaling pathway. Additionally, *Nod1*$^{-/-}$ mice are also shown to have dramatic shifts in microbial populations of the gut (Bouskra et al., 2008), and NOD1-deficiency in mice causes susceptibility to *H. pylori* infection (Viala et al., 2004). *Nod1* is downregulated in all our arsenic-exposed groups except RDCE, although not significantly. RD and RDCE have no significant differences between the two groups, and again microbial population analysis showed a near identical population between the two groups (Gokulan et al., 2018). Earlier, we showed that repeated arsenic exposure caused a transient decrease in the recovery of intestinal bacteria, a shift in the bacterial population with abundance of arsenic resistance genes, and evidence for host metabolism of arsenite into less-reactive trivalent methylated species (Gokulan et al., 2018). Additionally, studies have also shown that ileal tissue efficiently metabolize arsenite into pentavalent and trivalent methylated metabolites. Methylation of arsenite (either due to bacterium-induced conversion or due to host factors) is generally viewed as a detoxification reaction because DMA^V is the predominant species excreted in urine and methylation appears to protect mice from acute toxicity of arsenite (Currier et al., 2016). There is an exception to this trend in the RD to control comparison, but this gene expression difference could perhaps be explained by long-term exposure to arsenic and its effect on the gastrointestinal homeostasis.

5. Conclusion

This study demonstrated that exposure to arsenic may have substantial effects on gastrointestinal health, possibly through the gene expression of the various immune response-related pathways. While the potential health effects of long-term arsenic exposure are well known, it is important to address the lack of knowledge of the impact of low doses of arsenic on gastrointestinal health. These data show that low-level arsenic exposure may have a dramatic impact on immune system preparedness, even after a single exposure.

Disclaimer

The findings/opinions presented here represent the views of the author. They do not reflect the views of the U.S. Food and Drug Administration.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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References

Ahmad, S.A., Khan, M.H., 2015. Groumd water arsenic contamination and its health effects in Bangladesh. In: Handbook of Arsenic Toxicology. Elsevier, pp. 51–68.

Andrew, A.S., Jewell, D.A., Mason, R.A., Whitfield, M.L., Moore, J.H., Karagas, M.R., 2008. Drinking-water arsenic exposure modulates gene expression in human lymphocytes from a US population. Environ. Health Perspect. 116, 524.

Argos, M., Kibriya, M.G., Parvez, F., Jasmine, F., Rakibuz-Zaman, M., Ahsan, H., 2006. Gene expression profiles in peripheral lymphocytes by arsenic exposure and skin lesion status in a Bangladeshi population. Cancer Epidemiology and Prevention Biomarkers 15, 1367–1375.

Argos, M., Chen, L., Jasmine, F., Tong, L., Pierce, B.L., Roy, S., Paul-Brutus, R., Gamble, M.V., Harper, K.N., Parvez, F., 2014. Gene-specific differential DNA methylation and chronic arsenic exposure in an epigenome-wide association study of adults in Bangladesh. Environ. Health Perspect. 123, 64–71.

Arsenic and Arsenic Compounds. https://monographs.iarc.fr/wp-content/uploads/2018/

06/mono100C-6.pdf.
ATSDR, 2017. Substance Priority List, Agency for Toxic Substances and Disease Registry. Arsenic CAS#: 7440-38-2, Public Health Statement; 4770 Buford Hwy NE, Atlanta, GA 30341.
Banerjee, N., Banerjee, S., Sen, R., Bandyopadhyay, A., Sarma, N., Majumder, P., Das, J.K., Chatterjee, M., Kabir, S.N., Giri, A.K., 2009. Chronic arsenic exposure impairs macrophage functions in the exposed individuals. J. Clin. Immunol. 29, 582–594.
Barchowsky, A., Klei, L.R., Dudek, E.J., Swartz, H.M., James, P.E., 1999. Stimulation of reactive oxygen, but not reactive nitrogen species, in vascular endothelial cells exposed to low levels of arsenite. Free Radic. Biol. Med. 27, 1405–1412.
Baron, E., Curren, M., Henderson, G., Jousimies-Somer, H., Lee, K., Lechowitz, K., Strong, C., Summanen, P., Tuner, K., Finegold, S., 1992. Bilophila wadsworthia isolates from clinical specimens. J. Clin. Microbiol. 30, 1882–1884.
Bouskra, D., Brézillon, C., Bérard, M., Werts, C., Varona, R., Boneca, I.G., Eberl, G., 2008. Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis. Nature 456, 507.
Campbell, J.P., Alvarez, J.A., 1989. Acute arsenic intoxication. Am. Fam. Physician 40, 93–98.
Chen, G.Y., 2017. Regulation of the gut microbiome by inflammasomes. Free Radic. Biol. Med. 105, 35–40.
Chen, C.J., Chen, C., Wu, M., Kuo, T., 1992. Cancer potential in liver, lung, bladder and kidney due to ingested inorganic arsenic in drinking water. Br. J. Canc. 66, 888.
Chen, G.Y., Liu, M., Wang, F., Bertin, J., Núñez, G., 2011. A functional role for Nlrp6 in intestinal inflammation and tumorigenesis. J. Immunol., 1100412.
Currier, J.M., Douillet, C., Drobna, Z., Styblo, M., 2016. Oxidation state specific analysis of arsenic species in tissues of wild-type and arsenic (+3 oxidation state) methyltransferase-knockout mice. J. Environ. Sci. (China) 49, 104–112.
Davison, K., Mann, K.K., Waxman, S., Miller, W.H., 2004. JNK activation is a mediator of arsenic trioxide-induced apoptosis in acute promyelocytic leukemia cells. Blood 103, 3496–3502.
Dolinoy, D.C., Weidman, J.R., Waterland, R.A., Jirtle, R.L., 2006. Maternal genistein alters coat color and protects Avy mouse offspring from obesity by modifying the fetal epigenome. Environ. Health Perspect. 114, 567.
Domingo, J.L., 1995. Prevention by chelating agents of metal-induced developmental toxicity. Reprod. Toxicol. 9, 105–113.
Dutta, K., Prasad, P., Sinha, D., 2015. Chronic low level arsenic exposure evokes inflammatory responses and DNA damage. Int. J. Hyg Environ. Health 218, 564–574.
EPA, 2001. Drinking Water Standard for Arsenic.
Eriksson, M., Taskinen, M., Leppä, S., 2007. Mitogen activated protein kinase-dependent activation of c-Jun and c-Fos is required for neuronal differentiation but not for growth and stress response in PC12 cells. J. Cell. Physiol. 210, 538–548.
Faita, F., Cori, L., Bianchi, F., Andreassi, M.G., 2013. Arsenic-induced genotoxicity and genetic susceptibility to arsenic-related pathologies. Int. J. Environ. Res. Public Health 10, 1527–1546.
Fang, S., Wu, R., Christensen, J., 1993. Intramucosal nerve cells in human small intestine. J. Auton. Nerv. Syst. 44, 129–136.
FDA, 2016. Draft Guidance for Industry: Inorganic Arsenic in Rice Cereals for Infants: Action Level.
Feng, Z., Long, W., Hao, B., Ding, D., Ma, X., Zhao, L., Pang, X., 2017. A human stool-derived Bilophila wadsworthia strain caused systemic inflammation in specific-pathogen-free mice. Gut Pathog. 9, 59.
Finegold, S., Summanen, P., Gerardo, S.H., Baron, E., 1992. Clinical importance ofBilophila wadsworthia. Eur. J. Clin. Microbiol. Infect. Dis. 11, 1058–1063.
Fry, R.C., Navasumrit, P., Valiathan, C., Svensson, J.P., Hogan, B.J., Luo, M., Bhattacharya, S., Kandjanapa, K., Soontararuks, S., Nookabkaew, S., 2007. Activation of inflammation/NF-κB signaling in infants born to arsenic-exposed mothers. PLoS Genet. 3, e207.
Gagliani, N., Palm, N.W., de Zoete, M.R., Flavell, R.A., 2014. Inflammasomes and intestinal homeostasis: regulating and connecting infection, inflammation and the microbiota. Int. Immunol. 26, 495–499.
Gokulan, K., Arnold, M.G., Jensen, J., Vanlandingham, M., Twaddle, N.C., Doerge, D.R., Cerniglia, C.E., Khare, S., 2018. Exposure to arsenite in CD-1 mice during juvenile and adult stages: effects on intestinal microbiota and gut-associated immune status. mBio 9.
Goodale, B.C., Rayack, E.J., Stanton, B.A., 2017. Arsenic alters transcriptional responses to Pseudomonas aeruginosa infection and decreases antimicrobial defense of human airway epithelial cells. Toxicol. Appl. Pharmacol. 331, 154–163.
Honda, K., Takeda, K., 2009. Regulatory mechanisms of immune responses to intestinal bacteria. Mucosal Immunol. 2, 187.
Hood, R.D., 1972. Effects of sodium arsenite on fetal development. Bull. Environ. Contam. Toxicol. 7, 216–222.
Hopenhayn-Rich, C., Biggs, M.L., Smith, A.H., 1998. Lung and kidney cancer mortality associated with arsenic in drinking water in Cordoba, Argentina. Int. J. Epidemiol. 27, 561–569.
IARC, 2012. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Arsenic, Metal, Fibres and Dust, vol 100C WHO Press.
Islam, L.N., Nurun Nabi, A., Rahman, M.M., Zahid, M.S.H., 2007. Association of respiratory complications and elevated serum immunoglobulins with drinking water arsenic toxicity in human. Journal of Environmental Science and Health Part A 42, 1807–1814.
Jomova, K., Jenisova, Z., Feszterova, M., Baros, S., Liska, J., Hudecova, D., Rhodes, C., Valko, M., 2011. Arsenic: toxicity, oxidative stress and human disease. J. Appl. Toxicol. 31, 95–107.
Khan, M.A., Stroud, J.L., Zhu, Y.-G., McGrath, S.P., Zhao, F.-J., 2010. Arsenic bioavailability to rice is elevated in Bangladeshi paddy soils. Environ. Sci. Technol. 44, 8515–8521.
Kile, M.L., Cardenas, A., Rodrigues, E., Mazumdar, M., Dobson, C., Golam, M., Quamruzzaman, Q., Rahman, M., Christiani, D.C., 2016. Estimating effects of arsenic exposure during pregnancy on perinatal outcomes in a Bangladeshi cohort. Epidemiology (Cambridge, Mass.) 27, 173.
Kozul, C.D., Ely, K.H., Enelow, R.I., Hamilton, J.W., 2009. Low-dose arsenic compromises the immune response to influenza A infection in vivo. Environ. Health Perspect. 117, 1441.
Kucharczak, J., Simmons, M.J., Fan, Y., Gelinas, C., 2003. To be, or not to be: NF-κB is the answer–role of Rel/NF-κB in the regulation of apoptosis. Oncogene 22, 8961.
Kumar, D., Hosse, J., von Toerne, C., Noessner, E., Nelson, P.J., 2009. JNK MAPK pathway regulates constitutive transcription of CCL5 by human NK cells through SP1. J. Immunol. 182, 1011–1020.
Landström, M., 2010. The TAK1–TRAF6 signalling pathway. Int. J. Biochem. Cell Biol. 42, 585–589.
Lawrence, T., 2009. The nuclear factor NF-κB pathway in inflammation. Cold Spring Harbor perspectives in biology, a001651.
Lemarie, A., Morzadec, C., Mérino, D., Micheau, O., Fardel, O., Vernhet, L., 2006. Arsenic trioxide induces apoptosis of human monocytes during macrophagic differentiation through nuclear factor-κB-related survival pathway down-regulation. J. Pharmacol. Exp. Ther. 316, 304–314.
Lemarie, A., Bourdonnay, E., Morzadec, C., Fardel, O., Vernhet, L., 2008. Inorganic arsenic activates reduced NADPH oxidase in human primary macrophages through a Rho kinase/p38 kinase pathway. J. Immunol. 180, 6010–6017.
Lu, K., Ryan, P.A., Schlieper, K.A., Graffam, M.E., Levine, S., Wishnok, J.S., Swenberg, J.A., Tannenbaum, S.R., Fox, J.G., 2014. Arsenic exposure perturbs the gut microbiome and its metabolic profile in mice: an integrated metagenomics and metabolomics analysis. Environ. Health Perspect. 122, 284.
Maier, N.K., Crown, D., Liu, J., Leppla, S.H., Moayeri, M., 2014. Arsenic trioxide and other arsenical compounds inhibit the NLRP1, NLRP3, and NAIP5/NLRC4 inflammasomes. J. Immunol. 192, 763–770.
Maiti, S., Chattopadhyay, S., Deb, B., Samanta, T., Maji, G., Pan, B., Ghosh, A., Ghosh, D., 2012. Antioxidant and metabolic impairment result in DNA damage in arsenic-exposed individuals with severe dermatological manifestations in Eastern India. Environ. Toxicol. 27, 342–350.
Manju, R., Hegde, A.M., Parlees, P., Keshan, A., 2017. Environmental arsenic contamination and its effect on intelligence quotient of school children in a historic gold mining area Hutti, north Karnataka, India: a pilot study. J. Neurosci. Rural Pract. 8, 364.
Mazumder, D.G., 2007. Arsenic and non-malignant lung disease. Journal of Environmental Science and Health Part A 42, 1859–1867.
Momeny, M., Zakidizaji, M., Ghasemi, R., Dehpour, A.R., Rahimi_Balaei, M., Abdolazimi, Y., Ghavamzadeh, A., Alimoghaddam, K., Ghaffari, S.H., 2010. Arsenic trioxide induces apoptosis in NB-4, an acute promyelocytic leukemia cell line, through up-regulation of p73 via suppression of nuclear factor kappa B-mediated inhibition of p73 transcription and prevention of NF-κB-mediated induction of XIAP, cIAP2, BCL-X L and survivin. Med. Oncol. 27, 833–842.
Nahar, M.N., Inaoka, T., Fujimura, M., 2014. A consecutive study on arsenic exposure and intelligence quotient (IQ) of children in Bangladesh. Environ. Health Prev. Med. 19, 194.
Nayak, A.S., Lage, C.R., Kim, C.H., 2007. Effects of low concentrations of arsenic on the innate immune system of the zebrafish (Danio rerio). Toxicol. Sci. 98, 118–124.
Nezami, B.G., Srinivasan, S., 2010. Enteric nervous system in the small intestine: pathophysiology and clinical implications. Curr. Gastroenterol. Rep. 12, 358–365.
Ostrosky-Wegman, P., Gonsebatt, M., Montero, R., Vega, L., Barba, H., Espinosa, J., Palao, A., Cortinas, C., Garcia-Vargas, G., Del Razo, L., 1991. Lymphocyte proliferation kinetics and genotoxic findings in a pilot study on individuals chronically exposed to arsenic in Mexico. Mutat. Res. Fund Mol. Mech. Mutagen 250, 477–482.
Ramsey, K.A., Foong, R.E., Sly, P.D., Larcombe, A.N., Zosky, G.R., 2013. Early life arsenic exposure and acute and long-term responses to influenza A infection in mice. Environ. Health Perspect. 121, 1187.
Ravenscroft, P., Brammer, H., Richards, K., 2009. Arsenic Pollution: a Global Synthesis. John Wiley & Sons.
Ritchie, M.H., Fillmore, R.A., Lausch, R.N., Oakes, J.E., 2004. A role for NF-κB binding motifs in the differential induction of chemokine gene expression in human corneal epithelial cells. Investig. Ophthalmol. Vis. Sci. 45, 2299–2305.
Salcedo, R., Worschech, A., Cardone, M., Jones, Y., Gyulai, Z., Dai, R.-M., Wang, E., Ma, W., Haines, D., O'hUigin, C., 2010. MyD88-mediated signaling prevents development of adenocarcinomas of the colon: role of interleukin 18. J. Exp. Med. 207, 1625–1636.
Singh, A.P., Goel, R.K., Kaur, T., 2011. Mechanisms pertaining to arsenic toxicity. Toxicol. Int. 18, 87.
Soignet, S.L., Maslak, P., Wang, Z.-G., Jhanwar, S., Calleja, E., Dardashti, L.J., Corso, D., DeBlasio, A., Gabrilove, J., Scheinberg, D.A., 1998. Complete remission after treatment of acute promyelocytic leukemia with arsenic trioxide. N. Engl. J. Med. 339, 1341–1348.
Tabellini, G., Tazzari, P.L., Bortul, R., Evangelisti, C., Billi, A.M., Grafone, T., Martinelli, G., Baccarani, M., Martelli, A.M., 2005. Phosphoinositide 3-kinase/Akt inhibition increases arsenic trioxide-induced apoptosis of acute promyelocytic and T-cell leukaemias. Br. J. Haematol. 130, 716–725.
Taylor, V., Goodale, B., Raab, A., Schwerdtle, T., Reimer, K., Conklin, S., Karagas, M.R., Francesconi, K.A., 2017. Human exposure to organic arsenic species from seafood. Sci. Total Environ. 580, 266–282.
Twaddle, N.C., Vanlandingham, M., Beland, F.A., Doerge, D.R., 2018a. Metabolism and disposition of arsenic species after repeated oral dosing with sodium arsenite in drinking water. II. Measurements in pregnant and fetal CD-1 mice. Food Chem. Toxicol. 115, 178–184.

Twaddle, N.C., Vanlandingham, M., Churchwell, M.I., Doerge, D.R., 2018b. Metabolism and disposition of arsenic species from controlled oral dosing with sodium arsenite in adult female CD-1 mice. I. Pilot study to determine dosing, analytical measurements, and sampling strategies. Food Chem. Toxicol. 111, 482–493.

Twaddle, N.C., Vanlandingham, M., Fisher, J.W., Doerge, D.R., 2018c. Metabolism and disposition of arsenic species from controlled dosing with sodium arsenite in adult female CD-1 mice. III. Toxicokinetic studies following oral and intravenous administration. Food Chem. Toxicol. 121, 676–686.

Viala, J., Chaput, C., Boneca, I.G., Cardona, A., Girardin, S.E., Moran, A.P., Athman, R., Mémet, S., Huerre, M.R., Coyle, A.J., 2004. Nod1 responds to peptidoglycan delivered by the Helicobacter pylori cag pathogenicity island. Nat. Immunol. 5, 1166.

Wen, L., Ley, R.E., Volchkov, P.Y., Stranges, P.B., Avanesyan, L., Stonebraker, A.C., Hu, C., Wong, F.S., Szot, G.L., Bluestone, J.A., 2008. Innate immunity and intestinal microbiota in the development of Type 1 diabetes. Nature 455, 1109.

Wu, M.M., Chiou, H.Y., Ho, I.C., Chen, C.J., Lee, T.C., 2003. Gene expression of inflammatory molecules in circulating lymphocytes from arsenic-exposed human subjects. Environ. Health Perspect. 111, 1429.

Zeke, A., Misheva, M., Reményi, A., Bogoyevitch, M.A., 2016. JNK signaling: regulation and functions based on complex protein-protein partnerships. Microbiol. Mol. Biol. Rev. 80, 793–835.

Zhao, B., Xia, J.J., Wang, L.M., Gao, C., Li, J.L., Liu, J.Y., Cheng, Q.J., Dai, C., Ma, Q.l., Qi, Z.-q., 2018. Immunosuppressive effect of arsenic trioxide on islet xenotransplantation prolongs xenograft survival in mice. Cell Death Dis. 9, 408.

Zhu, Y.-G., Williams, P.N., Meharg, A.A., 2008. Exposure to inorganic arsenic from rice: a global health issue? Environ. Pollut. 154, 169–171.