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Studies on Effects of Arsenic on Human Beta-Defensin-1

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Studies on Effects of Arsenic on Human Beta-Defensin-1

By

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Professor Martyn T. Smith, Co-Chair
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Abstract

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Doctor of Philosophy in Molecular Toxicology

University of California, Berkeley

Professor Martyn T. Smith, Co-Chair

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Arsenic (As) is a well established cause of cancer in humans, and increasing evidence indicates that As has deleterious effects on the immune system that are not directly related to carcinogenesis. However, the mechanisms of As toxicity remain poorly understood. Our laboratory previously reported decreased urinary levels of human β-defensin-1 (HBD1) peptides in As-exposed individuals from two cross-sectional studies based in Nevada and Chile, and confirmed *in vitro* that As exposure suppressed HBD1 mRNA expression which is encoded by the *DEFB1* gene. *DEFB1* is constitutively expressed in epithelial tissues, plays a role in both the innate and adaptive branches of the immune system, and is implicated in anti-tumor immunity. Therefore, the objectives of this dissertation are to review the immunotoxicological effects of As, characterize the effects of As on *DEFB1* gene and protein expression in relevant *in vitro* model systems, investigate the molecular mechanisms mediating these effects, and explore the influence of other metals on HBD1 levels.

A comprehensive review of the literature on the immune-related effects associated with As exposure in humans, animals and *in vitro* models reveals that chronic exposure to As can severely impair various aspects of immune function and consequently result in elevated risk of infections and chronic diseases. However, further investigation is needed to better understand the relationship between As exposure and the development of disease, and several recommendations are discussed to help bridge the gaps in knowledge.

The current research investigated the effects of As exposure on *DEFB1* in cells derived from target tissues of toxicity using immortalized non-tumorigenic human HOK-16B keratinocytes and HK-2 kidney epithelial cells. *DEFB1* mRNA levels were more abundant in HK-2 cells than in HOK-16B cells, and were suppressed by exposure to arsenite (As^{III}) or monomethylarsonous acid (MMA^{III}), the postulated more toxic metabolite. The suppressive effect of As^{III} and MMA^{III} treatments on *DEFB1* transcript levels continued for several passages after removal of As. HBD1 peptide levels were significantly reduced following exposure to As^{III}, but were not affected by treatment with lead, cadmium or chromium, suggesting that decreased HBD1 may be a specific response to As. Finally, As^{III} treatment was found to suppress *DEFB1* promoter activity,

indicating that the inhibition of *DEFB1* mRNA by As is likely due to transcriptional down-regulation. Taken together, the research presented here provides evidence that our previous findings of decreased urinary HBD1 levels are likely due to a direct effect of As on the kidney, and suggest a novel mechanism by which As exposure may promote cancer development.

This dissertation summarizes the known *in vivo* and *in vitro* effects of As on the immune system, characterizes the effects of As on *DEFB1* using relevant cell culture models, and establishes *DEFB1* as a potentially relevant biomarker of response to As. Future studies should address the role of *DEFB1* inhibition in As immunotoxicity and carcinogenicity.

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Chapter 1
Introduction

Occurrence of As and sources of exposure

Arsenic is a metalloid whose elemental symbol is "As," atomic number is 33, atomic mass is 74.9, and place is in the fourth period and Va group of the periodic table of elements. As occurs ubiquitously on the earth's crust and is easily dissolved from mineral deposits in the soil or bedrock into surrounding water, sometimes resulting in elevated concentrations in groundwater (NRC, 2001; IARC, 2004).

The main source of human exposure is through ingestion of naturally contaminated drinking water. Additional exposure from food consumption also occurs due to As contamination of soil (Meharg and Rahman, 2003; Kile *et al.*, 2007). According to the latest estimates, well over 100 million people are exposed to As-contaminated groundwater in India, Bangladesh, Inner Mongolia, Taiwan, Chile, and the United States (US) (Nordstrom, 2002; IARC, 2012). The most devastated countries are India and Bangladesh, where millions of hand-pumped tube wells yield drinking water with As concentrations well above the World Health Organization drinking water guideline of 10 µg/L (WHO, 2004). Although the maximum contaminant level (MCL) of 10 µg/L for As adopted by the US Environmental Protection Agency took effect in 2006, many people in the US continue to be at risk because they obtain drinking water from private wells, which are not covered by the new As standard, and high As levels have been documented in private wells in several states including California, Nevada, Utah, Washington and Oregon (Steinmaus *et al.*, 2003).

Another route of exposure to As is through inhalation, which can occur in occupational settings. This is because the distribution of As in the environment has increased due to human activities such as pesticide and wood preservative application, fossil fuel combustion, metal smelting and mining, and in glass manufacturing and semiconductor materials processing (Viren and Silvers, 1994; Tomasek and Darby, 1995; Tseng, 2007). Consequently, the Agency for Toxic Substances and Disease Registry has ranked As as the number one toxic agent on its priority list of hazardous substances at industrially contaminated sites (ATSDR, 2005).

Metabolism of As

Inorganic As (iAs) exists in drinking water as arsenate (As^{V}) or arsenite (As^{III}), and is metabolized via methylation which facilitates urinary excretion (Buchet *et al.*, 1981). Methylation of iAs involves one-carbon metabolism with S-adenosylmethionine (SAM) as the methyl donor (Vahter, 2002). Although most tissues appear to have the capacity to methylate As (Vahter and Concha, 2001), the liver is implicated as the major site of As methylation, given its mass and the first-pass effect of ingested As (Marafante *et al.*, 1985; Thomas *et al.*, 2001; Vahter, 2002). Once ingested, As^{V} is reduced to As^{III}, followed by methylation to monomethylarsonic acid (MMA^{V}), which is reduced to monomethylarsonous acid (MMA^{III}). MMA^{III} is then methylated to dimethylarsinic acid (DMA^{V}), which is reduced to dimethylarsinous acid (DMA^{III}) (Figure 1). This process is incomplete in humans, and some of the ingested As is excreted as iAs and MMA along with DMA in the urine (Vahter, 2002). Since urinary excretion is the primary pathway of elimination of ingested As, the distribution of urinary As species is used as an indicator of the efficiency of As metabolism in individuals (NRC, 1999). Although there are large differences among individuals (Vahter, 1999), ingested iAs is usually excreted as 10-20% iAs, 10-15% MMA, and 60-75% DMA (Hopenhayn-Rich *et al.*, 1993).

The importance of a complete second methylation step is implicated in the following observation: unlike humans, most mammals that do methylate As are extremely efficient at this

and excrete virtually no MMA in the urine; these animals exhibit an overall rapid excretion of As (Vahter, 1999) and are less susceptible to As-induced toxicity (NRC, 1999). In humans, an efficient transformation of inorganic arsenic to DMA (i.e. high DMA, and low iAs and MMA in urine) is associated with a higher rate of excretion, decreased tissue concentrations and therefore lower toxicity (Vahter, 1999). Therefore, the large variation in the distribution of urinary As metabolites among individuals might be associated with differences in tissue distribution and retention of toxic metabolites (Vahter, 2002). Likewise, variation in metabolism is likely to correspond to variation in susceptibility.

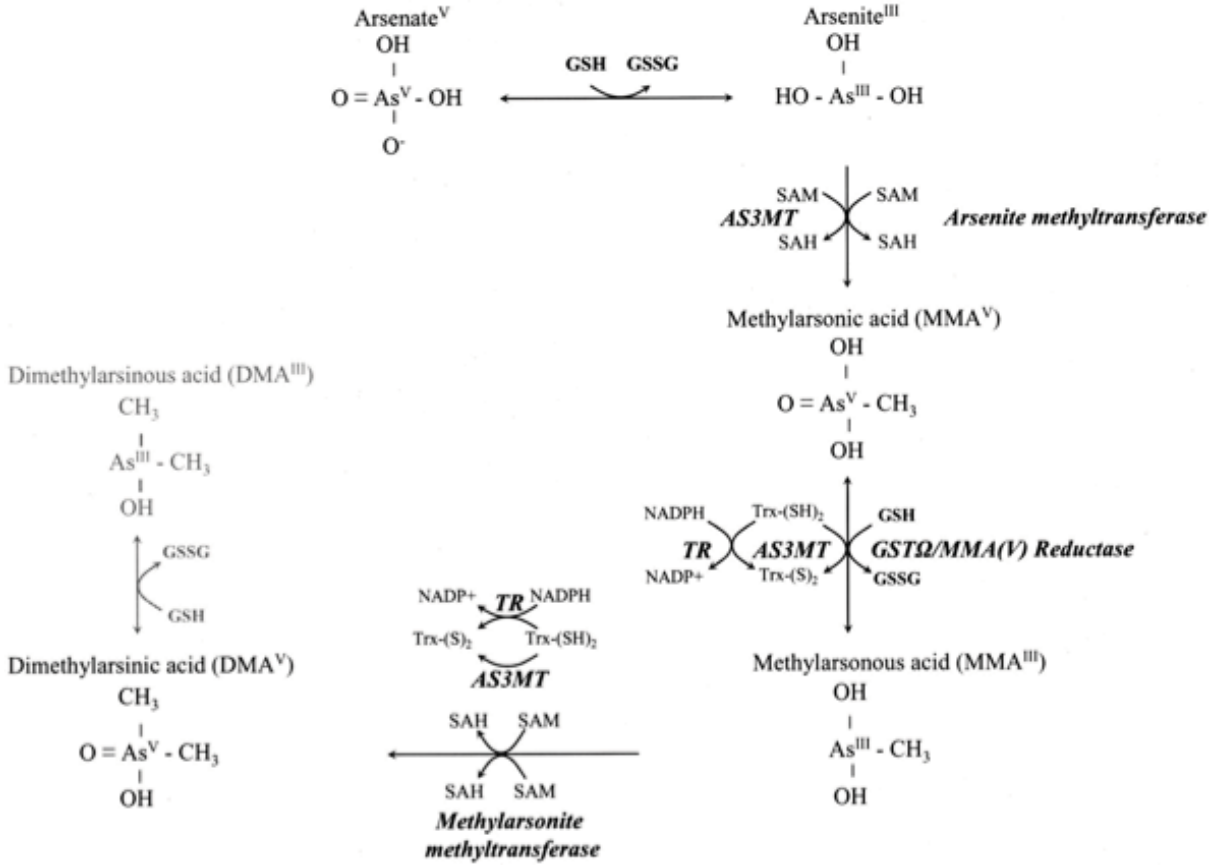


Figure 1. The As metabolic pathway. Arsenate (As^{V}) is reduced to arsenite (As^{III}), which then undergoes an oxidative methylation, with *S*-adenosylmethionine (SAM) as the methyl donor, forming monomethylarsonic acid (MMA^{V}). MMA^{V} is reduced to MMA^{III} before a subsequent oxidative methylation step yielding dimethylarsinic acid (DMA^{V}). Little is known about the *in vivo* reduction of DMA^{V} to DMA^{III}. Enzymes capable of catalyzing the illustrated reactions include arsenic-3-methyltransferase (AS3MT), and MMA^{V} reductase (also known as GST omega). GSSG, glutathione disulfide; GST, glutathione-*S*-transferase; TR, thioredoxin; Trx, thioredoxin reductase. Taken from Gamble *et al.*, 2006.

Toxicity induced by As

The International Agency for Research on Cancer has classified As as a group 1 carcinogen because of its association with increased risk of cancers of the skin, lung and bladder

(IARC, 2012). Other pathologies related to chronic As exposure include dermatological manifestations such as hyperpigmentation and palmar and plantar hyperkeratoses, diabetes, hypertension, cardiovascular disease, and respiratory, neurological, hepatic and urological disorders (NRC, 2001; IARC, 2004). There is growing evidence indicating that some of the toxic effects of As may be mediated by its effects on the immune system (Selgrade, 2007; Vahter, 2008). These alterations of immune responses mediated by As exposure will be discussed in detail in Chapter 2.

At the cellular level, As exposure has been associated with oxidative stress, inhibition of DNA repair, chromosomal aberrations, micronuclei formation, modification of cellular signaling via altered activation, expression and DNA binding activity of transcription factors, and epigenetic alterations (NRC, 2001; IARC, 2004; Reichard *et al.*, 2007). Several studies have reported associations between elevated urinary levels of MMA and increased risks of As toxicity, including skin lesions and increased rates of chromosomal aberrations (Del Razo *et al.*, 1997; Hsueh *et al.*, 1997; Maki-Paakkanen *et al.*, 1998; Yu *et al.*, 2000; Chen *et al.*, 2003a; Chen *et al.*, 2003b; Chen *et al.*, 2005; Steinmaus *et al.*, 2006; Pu *et al.*, 2007). Further, *in vitro* studies indicate that MMA^{III} is the most toxic of the As species (Petrick *et al.*, 2000; Styblo *et al.*, 2000; Mass *et al.*, 2001; Petrick *et al.*, 2001). Thus, the increased prevalence of toxic effects with higher urinary percentage of MMA could be attributed to the presence of MMA^{III} in tissues (Lindberg *et al.*, 2008).

Association of HBD1 and As exposure

To gain mechanistic insight into As toxicity and identify novel markers of early biological effect, our laboratory conducted urinary proteomic analyses in individuals exposed to As in drinking water (Hegedus *et al.*, 2008). Study participants included 14 men and 16 women from Nevada and a validation study of 31 men and 26 women from Chile (Table 1). A significant 36% decrease was found in levels of a 4.37 kDa peptide in men having high urinary As levels (>100 μg/L) compared to those with low levels (<100 μg/L) in the Nevada population (Figure 2). Results from the Chile population confirmed the decrease in the 4.37 kDa peptide, which was reduced by 43%, and identified a 32% reduction in a 4.76 kDa peptide in highly exposed men (Figure 3). No statistically significant difference was observed in women.

The differentially expressed peptides were sequenced and identified as human β-defensin-1 (HBD1) peptides, which are discussed in detail below. Subsequent *in vitro* analyses demonstrated the potential of As to decrease HBD1 gene expression, which is encoded by the *DEFB1* gene. Exposure of human HeLa cervical carcinoma and 293T embryonic kidney cell lines to As^{III} or MMA^{III} for 48 h resulted in dose-dependent decreases in *DEFB1* gene expression (Figure 4), providing evidence that HBD1 may be a biomarker of early effect to As. MMA^{III} was more potent than As^{III} in decreasing *DEFB1* expression, in agreement with its postulated role as the most toxic As species *in vitro* (Petrick *et al.*, 2000; Styblo *et al.*, 2000). The relevance of these findings will be the subject of the next section of this chapter.

Table 1. Subjects included in urinary proteomic analysis

Nevada	**High Exposure**	**Low Exposure**
Number of subjects	16	14
Number of males (current / former smokers)	8 (0/2)	6 (0/4)
Number of females (current / former smokers)	8 (4/0)	8 (4/0)
Average age	37	39
Average urinary As levels (μg/L) (range μg/L)	670 (114-2500)	33 (11-80)
Chile	**High Exposure**	**Low Exposure**
Number of subjects	34	23
Number of males (current / former smokers)	19 (1/3)	12 (0/2)
Number of females (current / former smokers)	15 (1/1)	11 (0/0)
Average age	24	27
Average urinary As levels (μg/L) (range μg/L)	454 (105-1025)	37 (8-64)

Taken from Hegedus *et al.*, 2008.

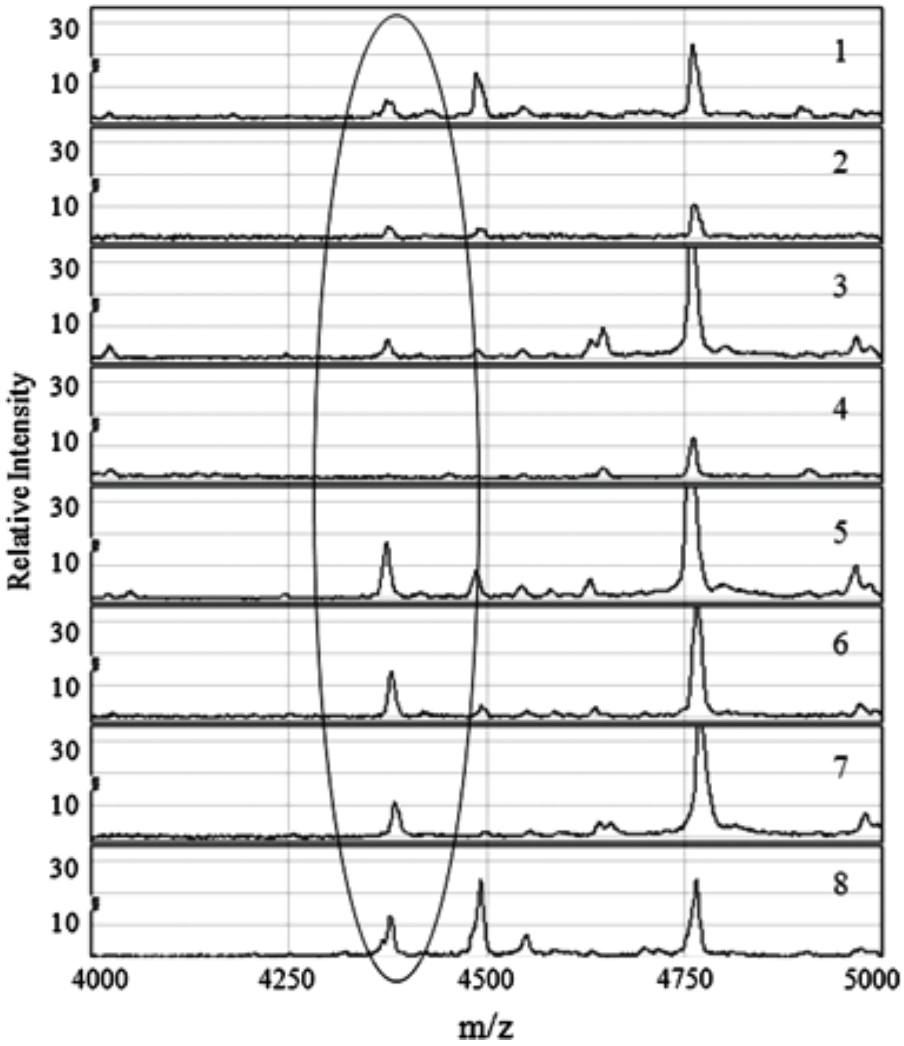


Figure 2. Differential expression of the 4.37 kD peptide in urine from the Nevada study. Representative spectra from analysis of urine from men with high (1-4) and low (5-8) As exposure levels. The circled peak represents the differentially expressed 4.37 kD peptide, which was reduced by 36%. Taken from Hegedus *et al.*, 2008.

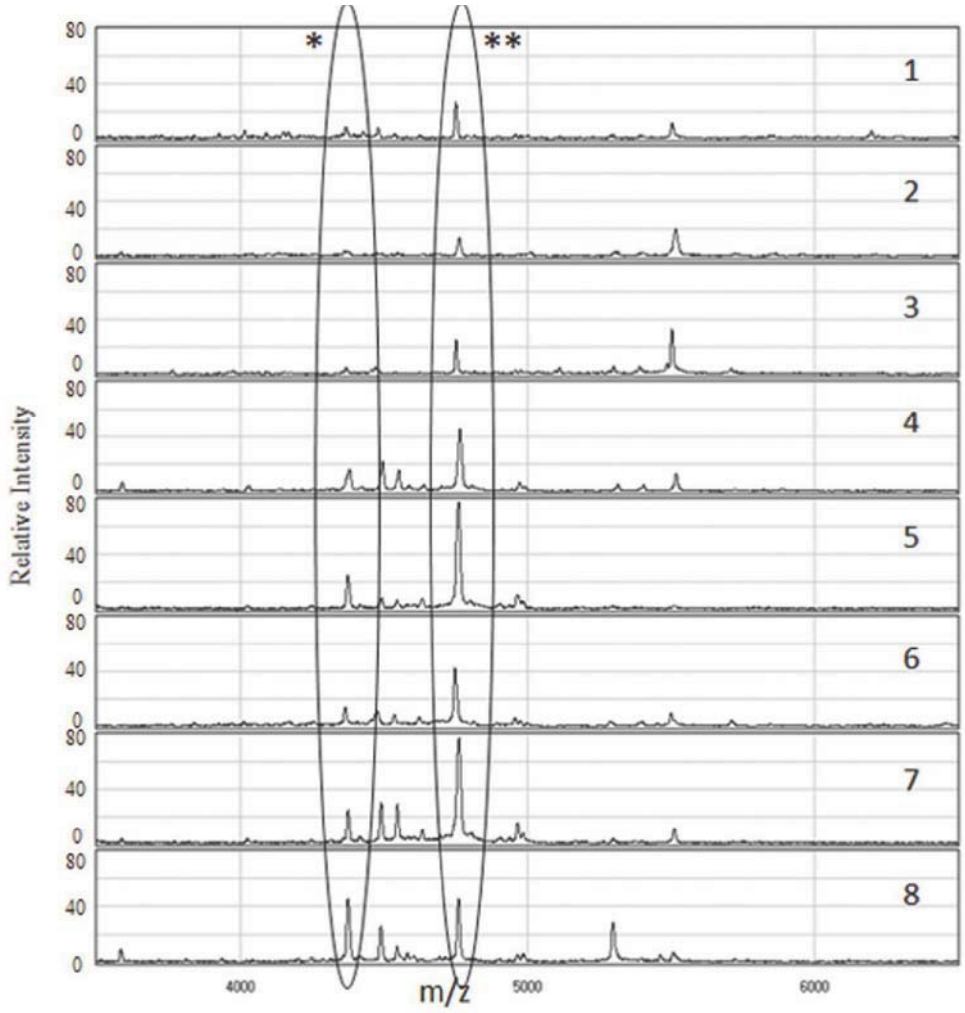


Figure 3. Differential expression of the 4.37 and 4.76 kD protein peaks in urine from the Chile study. Representative spectra from analysis of urine from men with high (1-4) and low (5-8) As exposure levels. The circled peak (*) represents the differentially expressed 4.37 kD peptide and the circled peak (**) represents the differentially expressed 4.76 kD peptide, which were reduced by 43% and 32%, respectively. Taken from Hegedus *et al.*, 2008.

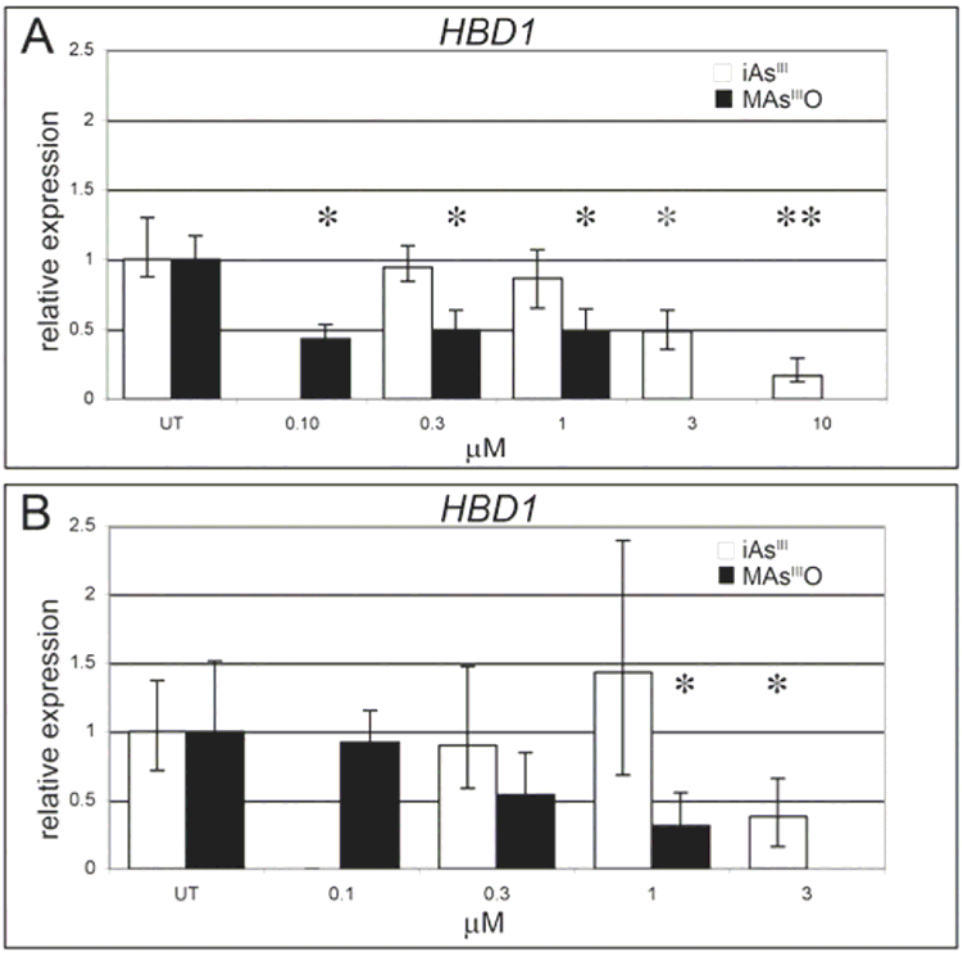


Figure 4. Suppression of *DEFB1* mRNA expression by As^{III} and MMA^{III} in HeLa cells (A) and 293T cells (B). *DEFB1* mRNA expression was normalized to *ACTB* mRNA and is shown following 48 h treatment with As^{III} (gray bars) or MMA^{III} (black bars) relative to the respective untreated control (UT). Separate controls are shown for As^{III} and MMA^{III} treatments because experiments were not conducted simultaneously. * $p<0.05$, ** $p<0.01$. Taken from Hegedus *et al.*, 2008.

Biological significance of HBD1

Expression

HBD1 is encoded by the *DEFB1* gene on chromosome 8p23. It is an antimicrobial peptide that is constitutively expressed in epithelial tissues, but is most abundant in the skin, oral cavity, and respiratory and urogenital tracts (Valore *et al.*, 1998; Zucht *et al.*, 1998; Pazgier *et al.*, 2006). It is a polycationic peptide consisting of six cysteine residues that form three disulfide bonds, and is synthesized as a 68 amino acid (aa) precursor peptide that undergoes N-terminal truncation giving rise to mature peptides ranging in size from 36 to 47 aa (Valore *et al.*, 1998; Zucht *et al.*, 1998). Urinary HBD1 peptides are thought to originate primarily from the kidney, and the 40 and 44 aa peptides have been identified as the most abundant variants in urine (Valore *et al.*, 1998). These variants correspond to the reported 4.37 and 4.76 kD peptides that were

reduced in As-exposed men in the two case-control populations based in Nevada and Chile (Hegedus *et al.*, 2008).

Regulation

The regulation of *DEFB1* expression has not been completely elucidated. Its promoter region does not contain consensus sites for NFκB, which could explain why, unlike other defensin molecules, its expression is not induced by infection or inflammation (Valore *et al.*, 1996; Liu *et al.*, 1997). Evidence from *in vitro* studies indicates that c-Myc and CLOCK:BMAL1 heterodimer activate *DEFB1* expression, whereas CRY1 and PAX2 repress it (Bullard *et al.*, 2008; Sherman and Froy, 2008; Bose *et al.*, 2009). c-Myc and CLOCK:BMAL1 heterodimer are responsible for regulating transcription of a large number of genes (Reppert and Weaver, 2002) and CRY proteins have strong inhibitory effects on CLOCK:BMAL1-mediated transcriptional activity (Kume *et al.*, 1999). PAX2 is a transcriptional regulator that is normally expressed early in development (Eccles *et al.*, 2002), but has been implicated as an oncogene that mediates evasion of cell death in tumors of the kidney, prostate, and other tissues (Maulbecker and Gruss, 1993; Muratovska *et al.*, 2003).

Role in immunity

Innate immunity. HBD1 is believed to play an important role in the innate immune response due to its antimicrobial properties and constitutive secretion at epithelial surfaces (Lehrer and Ganz, 2002; Ganz, 2003). This is likely mediated through electrostatic interactions owing to the strong cationic charges that disrupt the cytoplasmic membranes of microorganisms leading to pore formation (Lehrer and Ganz, 2002; Ganz, 2003).

Adaptive immunity. There is also evidence of a role for HBD1 in adaptive immunity by serving as a chemotactic attractant to recruit immature dendritic cells and memory T-cells through the CCR6 chemokine receptor (Yang *et al.*, 1999). Dendritic cells are powerful antigen-presenting cells that are able to prime naïve T-cells against tumors or invading microbes (Thomas-Kaskel *et al.*, 2007). Thus, HBD1 is primed to play a critical role in host defense by linking both the innate and adaptive immune responses.

Anti-tumor immunity. Several lines of evidence suggest that *DEFB1* may be a putative tumor suppressor gene. First, *DEFB1* is encoded on chromosome 8p23, which is frequently deleted in renal and prostate cancer and believed to contain multiple tumor suppressor genes (Ichikawa *et al.*, 1996; Knuutila *et al.*, 1999; Perinchery *et al.*, 1999).

Second, HBD1 down-regulation has been reported for several cancers. A large-scale gene expression profiling study of renal neoplasms was the first to identify *DEFB1* as one of the most significantly down-regulated transcripts in renal cell carcinoma (RCC) (Young *et al.*, 2001). An expanded analysis of this gene revealed a cancer-specific loss of HBD1 protein in 90% of RCC and 82% of prostate carcinomas with either complete loss of expression or weak staining whereas the adjacent benign epithelium maintained high expression, consistent with a tumor suppressor protein (Donald *et al.*, 2003). Additional analysis of a subset of the prostate carcinomas showed a correlation between protein and mRNA levels. Loss of *DEFB1* expression has also been reported in oral cancer. Gene expression analyses of oral squamous cell carcinoma cell lines and patient biopsies have identified significant reductions of *DEFB1* mRNA compared to healthy gingival keratinocytes (Wenghoefer *et al.*, 2008; Joly *et al.*, 2009).

Third, studies addressing the potential role of HBD1 in cancer provide direct evidence that HBD1 can function as a tumor suppressor protein for urological cancers (Sun *et al.*, 2006;

Bullard *et al.*, 2008). Sun *et al*. (2006) showed that purified HBD1 peptide inhibited bladder cancer cell line (TSU-Pr1) proliferation compared to cells receiving a random sequence control peptide, and that over-expression of HBD1 in SW156 renal cancer cells induced caspase-mediated apoptosis. The tumoricidal effects of HBD1 were also demonstrated in DU145 and PC3 prostate cancer cells that underwent rapid cytolysis and caspase-mediated apoptosis when transfected with HBD1 (Sun *et al.*, 2006; Bullard *et al.*, 2008). Taken together, these data strongly suggest a putative role for *DEFB1* in anti-tumor immunity.

Implications of *DEFB1* inhibition by As

As is a potent human carcinogen that is associated with numerous cancers. The potential role of HBD1 in kidney, bladder and prostate cancers and the carcinogenic effect of As in these tissues suggest that *DEFB1* may be a target of As toxicity. Given its cancer-specific loss in urological cancers, cytotoxic potential toward tumor cells and ability to induce migration of dendritic cells and T-cells, it is plausible that As-mediated down-regulation of *DEFB1* could play a role in As carcinogenicity.

Hypotheses and specific aims

Specific Aim 1: To establish a relevant *in vitro* model system to investigate effects of As on *DEFB1* expression.
Hypothesis: As suppresses DEFB1 mRNA expression in cells derived from target tissues of As toxicity.
Rationale. In our previous study (Hegedus *et al.*, 2008), *in vitro* validation of the observed decrease in urinary HBD1 peptides in two As-exposed populations was done using human HeLa cervical carcinoma cells and 293T embryonic kidney cells because the cervix and kidney are reported to have abundant *DEFB1* expression (Valore *et al.*, 1998; Zucht *et al.*, 1998; Pazgier *et al.*, 2006). In this dissertation, immortalized non-tumorigenic cells derived from human kidney, bladder and keratinocytes will be utilized for the analysis of effects of As^{III} and its metabolite MMA^{III} on *DEFB1* expression.

Specific Aim 2: To examine effects of As on HBD1 peptide expression.
Hypothesis: As suppresses HBD1 peptide levels in vitro.
Rationale. Because our case-control population studies identified decreased levels of urinary HBD1 peptides in men exposed to As in their drinking water, we needed to confirm in our *in vitro* model system whether As also suppresses the peptide levels. Since HBD1 is a secreted peptide, both its intra- and extra-cellular levels will be quantified following As treatment of cells.

Specific Aim 3: To elucidate the mechanism responsible for As-induced *DEFB1* suppression.
Hypothesis: As down-regulates DEFB1 mRNA expression through transcriptional and/or post-transcriptional inhibiton.
Rationale. mRNA abundance of any gene is dependent upon both the rate of transcription and the stability of the mRNA (Maier *et al.*, 2000; Lehmann and McCabe, 2007). Therefore, As treatment could suppress *DEFB1* transcript levels by decreasing its transcription and/or mRNA stability. Analysis of the influence of As exposure on *DEFB1* transcription will be conducted via a gene reporter assay using cells transfected with a luciferase construct whose expression is directly driven by activity of the *DEFB1* promoter. Decreased luciferase expression in As-treated cells relative to untreated cells would be indicative of transcriptional inhibition. To query the effect of As treatment on *DEFB1* transcript stability, mRNA stability assays will be performed in cells co-treated with As and the transcriptional inhibitor actinomycin D to measure the rate of *DEFB1* mRNA degradation. If As treatment destabilizes *DEFB1* mRNA, then *DEFB1* mRNA from cells treated with both As and actinomycin D should have a shorter half-life than *DEFB1* mRNA isolated from cells treated with actinomycin D alone.

Specific Aim 4: To investigate effects of other metals on HBD1 peptide levels.
Hypothesis: Other metals do not affect HBD1 peptide expression.
Rationale. To begin to address whether HBD1 may be suitable for use as a specific marker of early biological effect of As, the influence of other compounds will need to be tested. Therefore, this dissertation will examine the effects of several other metal treatments on HBD1 expression using the in *vitro* model system developed in Specific Aim 1.

Chapter 2
Arsenic Immunotoxcity: A Review

Abstract

Exposure to arsenic (As) is a global public health problem because of its association with cancer and numerous other pathological effects, and millions of people worldwide are exposed to As on a regular basis. Increasing lines of evidence indicate that As may adversely affect the immune system, but its specific effects on immune function are poorly understood. Therefore, we conducted a literature search of non-cancer immune-related effects associated with As exposure and summarized the known immunotoxicological effects of As in humans, animals and *in vitro* models. Overall, the data show that chronic exposure to As has the potential to impair vital immune responses which could lead to increased risk of infections and chronic diseases. Although animal and *in vitro* models provide some insight into potential mechanisms of the As-related immunotoxicity observed in human populations, further investigation, particularly in humans, is needed to better understand the relationship between As exposure and the development of disease.

Introduction

Exposure to arsenic (As) is a worldwide public health concern because of its widespread distribution and association with a multitude of adverse health effects. As is a well-established cause of skin, lung and urinary bladder cancers in humans (IARC, 2012), and its exposure has also been associated with other disorders, including skin lesions, diabetes, and cardiovascular disease, to name a few (Liu and Waalkes, 2008; Hughes *et al.*, 2011; IARC, 2012). Well over 100 million people worldwide are exposed to As, particularly through ingestion of As-contaminated food and drinking water in countries such as India, Bangladesh, Taiwan, Chile, and the United States (Nordstrom, 2002; IARC, 2012). Exposure to As can also occur through inhalation, especially in agricultural and industrial occupational settings (Nordstrom, 2002).

Inorganic As is found in the environment as arsenite (As^{III}) or arsenate (As^{V}) and its metabolism in humans involves conversion of As^{V} to As^{III} with subsequent methylation to mono- and di-methylated arsenicals (MMA and DMA, respectively) (Drobna *et al.*, 2009). Proposed mechanisms of As toxicity include oxidative stress, inhibition of DNA repair, induction of apoptosis and modification of cellular signaling (NRC, 2001; IARC, 2004; Flora, 2011).

Although extensive research efforts have focused on investigating As carcinogenicity, growing evidence indicates that As may also have deleterious effects on the immune system (Selgrade, 2007; Vahter, 2008). This may potentially play a role in As carcinogenesis through reduced immune surveilance. However, the specific effects of As on immune function remain poorly understood. Therefore, we considered that further investigation of As immunotoxicity is warranted and conducted a PubMed search of arsenic exposure and non-cancer immune-related effects through October 2012. Here, we summarize the known toxicological effects of arsenic on immune function in humans, laboratory animals and *in vitro* models, and identify possible future research directions to help close the gaps in knowledge.

Epidemiological findings

Effects in adults

Gene expression. Microarray-based assays are highly sensitive and useful for identification of differentially expressed genes, and are widely used in investigations of As toxicity, particularly related to As effects in carcinogenicity. However, a very limited number of reported epidemiological studies have employed this powerful method to investigate the toxic effects of As exposure on immune cells from otherwise healthy persons. Results from a microarray-based genome-wide expression study of peripheral blood lymphocytes from 21 subjects in New Hampshire whose drinking water As levels averaged 0.7 μg/L (range 0.007-5.3 μg/L, $n = 10$) and 32 μg/L (range 10.4-74.7 μg/L, $n = 11$) showed significant differences between exposure groups in transcripts with functions in multiple pathways, including T-cell receptor signaling, cell cycle regulation and apoptosis, but most strikingly in defense and immune response (Andrew *et al.*, 2008). Notably, higher As exposure was associated with increased expression of killer cell immunoglobulin-like receptors that inhibit natural killer (NK) cell-mediated cytotoxicity, as well as decreased expression of MHC class II molecules, including *HLA-DQB1*, *HLA-DPA1*, and *HLA-DRB1*, defense response genes, including *CD69*, *HSPA9B* and *MALT1*, and inflammatory genes, *IL2RA* and *IL1B* (Andrew *et al.*, 2008). As exposure was determined by drinking water As levels combined with urinary or toenail As levels as internal markers of exposure, and control and exposed subjects were matched for age, sex and smoking status.

Down-regulated *IL1B* was also identified in a genome-wide expression profiling study of circulating lymphocytes from a Bangladeshi population (Argos *et al.*, 2006). This study compared expression profiles of individuals with As-associated skin lesions (n = 11) to As-exposed persons without skin lesions (n = 5), and found an overall suppression of 467/468 differentially expressed genes. These findings contradict those from a microarray analysis of peripheral blood lymphocytes from 24 individuals in Taiwan with low (0–4.32 μg/L), intermediate (4.64–9 μg/L), and high (9.6–46.5 μg/L) blood As levels (Wu *et al.*, 2003). Among 62 out of 708 significantly altered genes were several inflammatory molecules that were up-regulated, including *IL1B*, *IL6*, *CCL2* and *CD14*, indicating that prolonged human exposure to As may induce ongoing inflammation that could contribute to development of As-associated disease (Wu *et al.*, 2003).

More recently, a cDNA microarray study of peripheral blood mononuclear cells (PBMC) from 10 individuals in Mexico with urinary As levels between 117.23 and 435.12 mg/g creatinine (n = 5) revealed significant differences in expression of apoptosis and inflammation-related genes compared to unexposed subjects (n = 5) (Salgado-Bustamante *et al.*, 2010). Overall, As exposure was associated with down-regulation of several inflammatory genes, including *TNF*, *IL11*, *IL10RB*, *CCR1*, and *CXCL2* (Salgado-Bustamante *et al.*, 2010), which is in stark contrast with the study reporting up-regulated expression of inflammatory genes in chronically exposed persons in Taiwan (Wu *et al.*, 2003). However, the finding of decreased *TNF* is in agreement with that of Argos *et al*. (2006). Some genes involved in apoptosis were significantly up-regulated, including *BCL2L1* and *CASP2*, whereas others, including *TRAIL* and *FASLG*, were down-regulated (Salgado-Bustamante *et al.*, 2010). These contradictory results in humans could be influenced by differences in exposure levels, sample size, methodology, population genetics and/or environmental factors. For instance, Salgado-Bustamante *et al*. (2010) analyzed gene expression of individual RNA samples from each subject, whereas Wu *et al*. (2003) analyzed pooled RNA samples. In addition, the small number of study participants in these studies may not be large enough to draw definitive conclusions. Clearly, more epidemiological investigations with larger sample sizes are needed to identify patterns of differentially expressed genes in humans exposed to As to help characterize effects and outcomes of As exposure and thereby help elucidate its mechanism(s) of immunotoxicity.

Lymphocyte activation. Several studies have reported impaired T-cell activation and functional responses associated with As exposure in humans. A pilot study of 11 As-exposed individuals and 13 controls in Mexico found that chronic exposure to As was associated with a significant decrease in proliferation of peripheral blood lymphocytes as evidenced by decreased tritiated thymidine incorporation (Ostrosky-Wegman *et al.*, 1991), which is further supported by a later study identifying delayed lymphocyte cell cycle progression from S- to M-phase in chronically exposed persons whose drinking water levels averaged 412 μg As/L compared with persons consuming drinking water that averaged 37.2 μg As/L (Gonsebatt *et al.*, 1994). Consistent with these earlier reports, a cross-sectional study in West Bengal, India of 18 unexposed individuals and 20 As-exposed individuals with skin lesions found significant reductions in Concanavalin A (ConA)-stimulated T-cell proliferation and Th1/Th2 cytokine secretion of IL-2, IL-4, IL-5, IL-10, IFN-γ and TNF-α in As-exposed individuals compared with unexposed individuals (Biswas *et al.*, 2008). In contrast, lymphocytes from Chilean copper smelter workers exposed to As-contaminated air (n = 40) displayed higher proliferation rates in response to phytohemaglutinin (PHA) stimulation than lymphocytes of As-unexposed individuals from the same region (n = 32) (Escobar *et al.*, 2010). While the reasons for these

observed differential effects on T-cell proliferation remain to be determined, it is plausible that differences in the nature of the stimulation (ConA versus PHA), population genetics, metabolism, and duration and level of exposure may be contributing factors. This study also demonstrated that urinary As levels were positively correlated with oxidative stress markers, specifically serum superoxide dismutase (SOD) activity and lymphocyte malondialdehyde (MDA), and negatively correlated with serum vitamin E levels, suggesting that chronic As exposure induces oxidative damage in lymphocytes (Escobar *et al.*, 2010).

Lymphocyte subpopulations. Increasing evidence in humans indicates that exposure to As affects numerous immune cell subpopulations. Hematopoietic data of subjects exhibiting dermatological symptoms ($n = 30$) including pigmentation and hyperkeratosis from exposure to > 100 µg/L of As for at least 10 years in Eastern India, demonstrated a significant increase in eosinophil numbers and decrease in monocyte counts compared to unexposed persons ($n = 25$) (Maiti *et al.*, 2012). There is also evidence that As exposure in humans can disrupt macrophage function. Monocyte-derived macrophages from As-exposed individuals with skin lesions ($n = 70$) demonstrated cell rounding and significant reductions in adhesion, nitric oxide anion (NO^-) production and phagocytic capacity compared to macrophages from non-exposed persons ($n = 64$) (Banerjee *et al.*, 2009). Moreover, the authors found decreased expression of CD54 adhesion molecule and F-actin, and alterations in Rho A-ROCK signaling that likely contributed to impaired macrophage function.

A study investigating the influence of As exposure on the regulation of the immune system found that in chronically-exposed but otherwise healthy individuals ($n = 47$), urinary As levels (range, 8.1 - 448 µg/g creatinine) were significantly inversely correlated with the number and inhibitory function of natural T regulatory (nTreg) lymphocytes but not other regulatory T-cells (Hernandez-Castro *et al.*, 2009). nTreg lymphocytes are $CD25^+Foxp3^+$ T-cells that constitute approximately 10% of circulating $CD4^+$ T-cells and play a critical role in immune homeostasis by suppressing the immune response (Miyara and Sakaguchi, 2007). Although increased levels of apoptotic PBMC were evident in exposed subjects, no significant correlation was found with urinary As levels, suggesting that the effect on nTreg cells was not mediated by induction of apoptosis (Hernandez-Castro *et al.*, 2009). On the other hand, this could be due to individual differences in susceptibility to As. Additionally, As exposure was positively correlated with innate immune receptor complex TLR4/CD14 and TNF-α secretion by monocytes (Hernandez-Castro *et al.*, 2009), which may be causally associated with the effect of As on nTreg lymphocytes given the inhibitory effect of regulatory cells on release of pro-inflammatory TNF-α (Ehrenstein *et al.*, 2004). These results are in agreement with previous findings that chronic human exposure to As induces inflammation, including *CD14* expression (Wu *et al.*, 2003).

Humoral immunity. Conflicting results come from studies evaluating serum antibody (Ab) levels in As-exposed individuals. On one hand, one study observed no changes in the serum levels of IgM, IgA or IgG in 47 adult male workers exposed to As in a coal-burning power plant compared to 27 workers in another plant in the same district where the As coal content was more than 10 times lower (Bencko *et al.*, 1988). It should be noted that the duration of exposure and internal doses of As (in blood or urine) are unknown; thus, these negative results could be due to acute or low-dose internal levels of As. On the other hand, subjects ($n = 125$) in rural villages of Bangladesh chronically exposed to As through drinking water demonstrated significantly elevated serum levels of IgA, IgG and IgE compared to unexposed persons (Islam *et al.*, 2007). Levels of IgG and IgE were significantly higher during initial stages of skin manifestations, and

IgE levels continued to increase with prolonged As exposure. Moreover, increased prevalence of respiratory complications including cough associated with As toxicity, chest sound, bronchitis and asthma were evident in exposed individuals, and mean serum IgE levels were higher in subjects with respiratory symptoms relative to exposed subjects without (Islam *et al.*, 2007). No effect on eosinophil numbers was observed in this study (Islam *et al.*, 2007), in contrast with the increased eosinophil counts associated with chronic As exposure reported by Maiti *et al*. (2012), suggesting that the observed increase in serum IgE levels may be due to direct inflammatory effects of As exposure rather than allergic disease (Islam *et al.*, 2007).

Pulmonary effects. The health outcomes of the immunosuppressive effects of As are becoming more evidenced by the increased prevalence of opportunistic infections, such as tuberculosis, fungal infections and respiratory tract infections, observed in populations with chronic exposure to As (Biswas *et al.*, 2008; Banerjee *et al.*, 2009; Smith *et al.*, 2011). A recent report from Chile revealed increased mortality from pulmonary tuberculosis associated with chronic As exposure (Smith *et al.*, 2011). These findings of As-associated pulmonary effects (Islam *et al.*, 2007; Biswas *et al.*, 2008; Banerjee *et al.*, 2009; Smith *et al.*, 2011) add to growing evidence indicating that long-term As exposure increases the risk of reduced lung function and non-malignant lung disease (Tsai *et al.*, 1999; Mazumder *et al.*, 2000; Milton and Rahman, 2002; Milton *et al.*, 2003; De *et al.*, 2004; von Ehrenstein *et al.*, 2005). Moreover, investigations in humans provide compelling evidence that As exposure significantly increases the incidence of bronchiectasis (Mazumder *et al.*, 2005; Smith *et al.*, 2006), a localized pulmonary disease characterized by chronic infection, inflammation, irreversible bronchial damage, cough, and respiratory failure (King *et al.*, 2006; Ilowite *et al.*, 2008). In a study from West Bengal, India, subjects with As-related skin lesions (n = 108) had a 10-fold increased prevalence of bronchiectasis related to long-term ingestion of As compared with subjects without lesions (n = 150) (Mazumder *et al.*, 2005). Shortly thereafter, a report from Chile indicated very high mortality rates for bronchiectasis in young adults aged 30-49 resulting from early-life exposure to As; compared with unexposed persons, the mortality rate for those with childhood As exposure was 12-fold higher, whereas that for those exposed *in utero* was 46-fold higher (Smith *et al.*, 2006). The authors also found 6- to 7-fold increases in lung cancer mortality rates resulting from *in utero* and early childhood As exposures, respectively. Further, studies on this As-exposed population from Chile indicate long latency patterns of increased mortality from lung, kidney and bladder cancers continuing for more than 25 years after the As exposure period ended (Marshall *et al.*, 2007; Yuan *et al.*, 2010). Overall, these studies indicate that exposure to As not only exerts severe respiratory effects, but that early-life exposure to As may have pronounced long-term consequences. Intriguingly, women appear to be somewhat protected from skin manifestations and respiratory effects related to As exposure (von Ehrenstein *et al.*, 2005; Rahman *et al.*, 2006), possibly due to sex hormone-related increased methylation capacity of As in women than in men (Lindberg *et al.*, 2008).

HBD1 involvement. Interestingly, our group has previously reported in two As-exposed human populations a significant inverse correlation in men between urinary levels of As and the antimicrobial peptide human β-defensin-1 (HBD1) (Hegedus *et al.*, 2008), which is implicated in respiratory infections. Specifically, studies suggest a primary role for HBD1 in the lung against specific pathogens, particularly the ones relevant to bronchiectasis (King *et al.*, 2006; Ilowite *et al.*, 2008), and evidence indicates that inactivation of HBD1 antimicrobial activity contributes to recurrent airway infections in the lungs of cystic fibrosis patients (Goldman *et al.*, 1997; Weinberg *et al.*, 1998). Further, *in vivo* observations from a transgenic mouse model deficient in

the mouse ortholog of HBD1 indicate that β-defensin 1 serves as an initial barrier to bacterial colonization in the lung (Moser *et al.*, 2002). Moreover, given growing evidence that *DEFB1* may be a tumor suppressor gene whose inactivation may be involved in tumorigenesis of multiple tissues (Ichikawa *et al.*, 1996; Knuutila *et al.*, 1999; Perinchery *et al.*, 1999; Young *et al.*, 2001; Donald *et al.*, 2003; Sun *et al.*, 2006; Bullard *et al.*, 2008; Wenghoefer *et al.*, 2008; Joly *et al.*, 2009), it is tempting to speculate that perhaps HBD1 down-regulation may contribute to As-induced carcinogenesis or pathogenesis of bronchiectasis in individuals chronically exposed to As. Although our ongoing *in vitro* studies demonstrate As-induced reductions in *DEFB1* mRNA and protein levels in keratinocyte and kidney cell lines (unpublished data), confirmatory evidence of HBD1 inhibition is needed from other As-exposed populations. Thus, it remains to be determined whether HBD1 is suppressed in the lungs of As-exposed individuals, and further investigations are needed to elucidate the role of down-regulated HBD1 in As immunotoxicity and carcinogenicity.

Effects in children and infants

It has long been known that the fetus, infant and young child, each at critical stages in development, are particularly sensitive or vulnerable to stressors that could have short-term as well as long-term effects. Yet, there are few epidemiological studies on the influence of early-life As exposures on immunological outcomes in children and even fewer in newborns and infants. Evidence indicates that exposure to As early in life may have consequences that manifest much later in the adult (Vahter, 2008; Vahter, 2009), as evidenced by the increased prevalence of and mortality from bronchiectasis and lung cancer in young adults (Smith *et al.*, 2006). Therefore, it is plausible that early signs indicative of future disease following early-life exposure to As may be evident in young subjects.

Induction of apoptosis. Indeed, studies of early-life exposure to As have reported detectable markers of immune dysfunction in infants and children. For example, studies of Mexican children between the ages of 4 and 13 have reported a higher incidence of apoptotic PBMC in As-exposed children compared with non-exposed children (de la Fuente *et al.*, 2002; Rocha-Amador *et al.*, 2011). Although apoptosis is important in immune system homeostasis, abnormal apoptosis of immune cells can contribute to dysregulation of immune function, which may result in immunodeficiency, autoimmune disease or malignant transformation (Thompson, 1995); thus, induction of apoptosis may be an important mechanism of As-mediated immunosuppression. In the more recent study, which consisted of 20 children with high urinary As levels (mean = 46.3 ug/g creatinine) and 20 children with lower urinary As levels (mean = 14.2 ug/g creatinine), a significant positive association was found between As exposure and the percentage of apoptotic PBMC (Rocha-Amador *et al.*, 2011). However, despite the high rate of basal apoptosis observed in PBMC from chronically exposed children in the earlier study consisting of 7 highly exposed (mean urinary As levels = 143.9 μg/g creatinine) and 5 non-exposed children (mean urinary As levels = 24.8 μg/g creatinine), no significant correlation was observed between urinary As levels and the proportion of apoptotic cells (de la Fuente *et al.*, 2002), in agreement with a study in adults (Hernandez-Castro *et al.*, 2009), possibly because of small sample size or individual differences in susceptibility to As (de la Fuente *et al.*, 2002).

Lymphocyte activation. Consistent with findings from As-exposed adults (Ostrosky-Wegman *et al.*, 1991; Gonsebatt *et al.*, 1994; Biswas *et al.*, 2008), significant reductions in PBMC IL-2 secretion and proliferative response to mitogenic stimulation were observed in 6 to 10 year-old children exposed to As in drinking water (n = 90, mean urinary As levels of low

exposure group = 29.3 ug/L, mean urinary As levels of high exposure group = 194.9 ug/L) (Soto-Pena *et al.*, 2006). Also noteworthy were increased granulocyte-macrophage colony stimulating factor (GM-CSF) secretion by mononucleated cells and a reduction in the $CD4^+$ cells and CD4/CD8 ratio without any alteration in the proportion of $CD8^+$ cells (Soto-Pena *et al.*, 2006). Because a low CD4/CD8 ratio is considered a surrogate marker of immunosuppression (Schofer and Roder, 1995; Hernberg *et al.*, 1998; Wikby *et al.*, 1998), the observed As-related decrease in CD4/CD8 ratio may be an early indicator of As-mediated immunosuppression. Furthermore, the increased GM-CSF secretion identified by Soto-Pena *et al*. (2006) may be indicative of chronic inflammation given growing lines of evidence of a role for elevated GM-CSF levels in initiating or mediating chronic inflammation (Zhan *et al.*, 2012), and is consistent with a previous study of an As-exposed adult population demonstrating increased expression of several inflammatory mediators (Wu *et al.*, 2003).

ROS production. Production of reactive oxygen species (ROS) such as NO^- and superoxide anion (O_2^-) by activated mononuclear cells is an important innate immune response to help destroy invading microbes. Cross-sectional studies assessing the influence of As exposure on ROS production by monocytic cells in children have yielded conflicting results. A recent study of 87 children living in Mexico exposed to As through drinking water found that As exposure was positively associated with O_2^- production by activated monocytes as well as basal levels of NO^- and O_2^- in PBMC and monocytes (Luna *et al.*, 2010). This is inconsistent with an earlier study of 65 children living near a primary smelter in Mexico in which As exposure was negatively associated with NO^- and O_2^- production by activated monocytes (Pineda-Zavaleta *et al.*, 2004). The discrepancy may be due to differences in exposure levels; in the earlier study, children had lower urinary As levels (range 16.7-465.7 µg/g creatinine) (Pineda-Zavaleta *et al.*, 2004) than children in the more recent study (range 12.3-1411 µg/g creatinine) (Luna *et al.*, 2010). Regardless of the source of the variation in results, these studies indicate that exposure to As could alter the ability of circulating blood cells to respond to immunological challenge. For example, elevated ROS levels in un-stimulated PBMC indicate As-induced oxidative stress, concurrent with findings from a study on copper smelter workers by Escobar *et al*. (2010); overproduction of ROS by activated PBMC could result in oxidative damage to surrounding host tissues, whereas diminished ROS production could weaken PBMC defense against pathogens.

Prenatal exposure. As readily crosses the placenta (Concha *et al.*, 1998) and could potentially alter prenatal development. Indeed, gestational exposure to As is linked to increased fetal loss and infant mortality (Rahman *et al.*, 2007; Rahman *et al.*, 2010). However, reports on immune-related effects of prenatal As exposure in newborns and infants are scarce. In one mother-child cohort study in Bangladesh (n = 140), maternal urinary As levels showed a significant negative correlation with child thymic index and the breast milk trophic factors IL-7 (needed for thymic and T-cell development) and lactoferrin (an antioxidant and factor in innate immunity), as well as a positive correlation with maternal morbidity (fever and diarrhea during pregnancy) and acute respiratory infections only in male infants (Raqib *et al.*, 2009). These findings are supported by a more recent prospective population-based cohort study of 1,552 infants born in Bangladesh, which revealed dose-dependent increases of up to 69% and 20% in infant lower respiratory tract infection and diarrhea, respectively, related to maternal As exposure during pregnancy (maternal urinary As levels, lowest quintile < 39 µg/L; highest quintile = 262-977 µg/L) (Rahman *et al.*, 2011). The increased prevalence of infant respiratory illness observed in these studies is not only consistent with As-associated non-malignant lung disease in adults (Mazumder *et al.*, 2005; Smith *et al.*, 2011), but also with the marked increased

risk of such disease resulting from early-life exposure to As (Smith *et al.*, 2006). Moreover, the enhanced susceptibility of male infants to respiratory infections is consistent with earlier findings of increased As-related pulmonary effects in men and not in women (von Ehrenstein *et al.*, 2005).

Another study of women delivering babies in Bangladesh (n = 130) found that gestational exposure to As induced inflammation (IL-1β, TNF-α and IFN-γ) in the placenta via oxidative stress (8-oxoguanine), reduced placental $CD3^+$ T-cell numbers, and increased cytokine levels of IL-8, IL-1β, TNF-α and IFN-γ in umbilical cord blood (Ahmed *et al.*, 2011). These findings are in agreement with previous reports of increased expression of oxidative stress markers (Escobar *et al.*, 2010) and inflammatory mediators (Wu *et al.*, 2003) in adults with chronic exposure to As. In a follow-up study (n = 44), levels of As in maternal urine and placental and cord blood were positively associated with cord blood 8-hydroxy-2'-deoxyguanosine and inversely associated with infant thymic function at birth, as measured by signal-joint T-cell receptor-rearrangement excision circles in cord blood mononuclear cells (CBMC) (Ahmed *et al.*, 2012). Further, prenatal As exposure was associated with down-regulation of oxidative-stress defense genes, including *SOD3*, as well as up-regulation of apoptosis-related genes in CBMC, including *CASP2* (Ahmed *et al.*, 2012), the latter consistent with previous results from adults (Salgado-Bustamante *et al.*, 2010).

Overall, these data indicate that *in utero* As exposure leads to reduced infant thymic size and function, likely through inhibition of breast milk trophic factors IL-7 and lactoferrin and/or induction of apoptosis and oxidative stress. These effects may contribute to immune suppression or deficiency in infants as evidenced by an increased prevalence of respiratory illness. The lack of data supporting a relationship between early-life exposure to As and non-pulmonary infections suggests that the developing lung is specifically targeted by As. Furthermore, given increasing evidence of the association between As exposure and adverse immune-related outcomes, it is likely that immune disruption resulting from early-life As exposure will have long-term detrimental consequences well into adulthood, as seen in the increased prevalence of bronchiectasis and lung cancer, for example.

Experimental animal studies

Gene expression. In various animal models, As exposure is associated with altered expression of genes involved in immune response. In lungs of mice exposed to As^{III} (up to 100 ppb) for 5-6 weeks, significant changes were identified in transcripts encoding humoral immune response, antigen binding, TLRs, cytokines, cytokine receptors and genes involved in cell adhesion and migration (Andrew *et al.*, 2007; Kozul *et al.*, 2009b). Specifically, down-regulated expression of several genes encoding the TLR/IL1R signaling pathway was identified, including *Il1b* (Kozul *et al.*, 2009b). Studies using zebrafish embryos also found that As exposure significantly inhibited induction of genes involved in regulating innate immune responses against viral and bacterial infection, including *il1b* (Mattingly *et al.*, 2009), *tnfa*, *ifnphi1* (type1 interferon) and *mx* (interferon-inducible Mx) (Hermann and Kim, 2005; Nayak *et al.*, 2007). As treatment also disrupted the JAK/STAT pathway, which is critical in cytokine regulation (Hermann and Kim, 2005). These effects are in agreement with epidemiological findings of As-associated decreased expression of *IL1B* (Argos *et al.*, 2006; Andrew *et al.*, 2008) and *TNF* (Salgado-Bustamante *et al.*, 2010).

Lymphocyte subpopulations. Direct evidence that chronic As exposure can suppress the weight, index and/or cellularity of the major immunocompetent organs, including the spleen and

thymus, comes from studies in rats (Flora and Kumar, 1996; Schulz *et al.*, 2002; Xia *et al.*, 2009), mice (Sikorski *et al.*, 1991b), catfish (Ghosh *et al.*, 2006) and chickens (Aggarwal *et al.*, 2008). Chronic *in vivo* exposure to As also alters lymphocyte subpopulations, as evidenced by reduced $CD4^+$ T-cell populations, concomitant decreased CD4/CD8 T-cell ratio and increased percentage of total monocytes in splenic mononuclear cells (SMC) in mice (Soto-Pena and Vega, 2008). These results support previous observations of reduced $CD4^+$ T-cell populations and CD4/CD8 ratio in As-exposed children (Soto-Pena *et al.*, 2006). In catfish, As exposure induced the presence of atypical lymphocytes and depleted lymphoid and melano-macrophage populations in the head kidney (HK), a major immunocompetent organ (Ghosh *et al.*, 2006; Ghosh *et al.*, 2007). Interestingly, a single intra-tracheal exposure of mice to 200 mg/kg gallium arsenide (GaAs) markedly decreased peritoneal lymphocyte counts (Sikorski *et al.*, 1989) and the total number of splenic T-cells, B-cells and macrophages by 58, 61 and 30%, respectively, without affecting their proportions (Sikorski *et al.*, 1991b). In contrast, an evaluation of acute exposure (four oral doses of 1.625 g/kg over the course of 7 days) of mice to arsenobetaine (AsBe), a common contaminant of seafood, revealed no toxicity to splenocytes, thymocytes, Peyer's patch lymphocytes or peritoneal macrophages (Sakurai *et al.*, 2004). Despite the 8-fold higher concentration administered to mice multiple times by Sakurai *et al*. (2004), it is possible that the AsBe formulation and/or oral delivery were not as potent as pulmonary GaAs exposure administered by Sikorski *et al*. (Sikorski *et al.*, 1989; Sikorski *et al.*, 1991b).

Lymphocyte activation. Consistent with epidemiological observations (Ostrosky-Wegman *et al.*, 1991; Gonsebatt *et al.*, 1994; Soto-Pena *et al.*, 2006; Biswas *et al.*, 2008), chronic *in vivo* animal exposure to As inhibits mitogen-stimulated proliferation of peripheral blood and splenic lymphocytes in broiler chickens (Aggarwal *et al.*, 2008) and SMC in mice (Soto-Pena and Vega, 2008), as well as proliferation of T-cells and B-cells in the spleen and HK of catfish (Ghosh *et al.*, 2006; Ghosh *et al.*, 2007). Consequently, decreases have been observed in mitogen-stimulated cytokine secretion of IFN-γ, IL-2, IL-6 and IL-12 in mice (Soto-Pena and Vega, 2008), and "IL-4-like factors" from HK T-cells in catfish (Ghosh *et al.*, 2007). One important consideration regarding animal studies is that concentrations of As administered typically far exceed human exposure levels, which may account for differences observed between human populations and experimental animals.

Humoral and hypersensitivity responses. As exposure has been shown to inhibit humoral immune responses, as evidenced by a suppression in the *in vitro* primary (IgM) and/or secondary (IgG) Ab-forming cell (AFC) response of rodent splenocytes to sheep red blood cells (SRBC) (Blakley *et al.*, 1980; Sikorski *et al.*, 1989; Sikorski *et al.*, 1991b; Flora and Kumar, 1996; Burchiel *et al.*, 2009; Nain and Smits, 2010). Studies of GaAs exposure in mice have demonstrated that IL-2 is a primary target of the As-mediated inhibition of T-cell-dependent humoral immune responses of splenocytes (Burns and Munson, 1993). Further, *in vivo* As exposure suppressed the delayed-type hypersensitivity reaction, an *in vivo* cell-mediated response to cutaneous sensitization, in mice (Sikorski *et al.*, 1989; Patterson *et al.*, 2004), rats (Savabieasfahani *et al.*, 1998; Schulz *et al.*, 2002) and chickens (Aggarwal *et al.*, 2008). Compared to control sensitized mice, As^{III}-exposed sensitized mice demonstrated reductions in lymph node cell proliferation, ear swelling, activated Langerhans cells (LC) in cervical lymph nodes, and thioglycollate-induced peritoneal macrophages and circulating neutrophils (Patterson *et al.*, 2004). These findings suggest that As inhibits LC migration to the lymph nodes and subsequent T-cell activation, which could be partly due to As-induced alterations in the secretion

of pro-inflammatory and growth-promoting factors that regulate LC migration and maturation following intradermal challenge (Patterson *et al.*, 2004).

Macrophages. As seen in humans (Banerjee *et al.*, 2009), *in vivo* animal exposure to As significantly suppresses macrophage production of NO^- and/or O_2^- (Sengupta and Bishayi, 2002; Arkusz *et al.*, 2005; Aggarwal *et al.*, 2008; Chakraborty *et al.*, 2009), release of TNF-α (Lantz *et al.*, 1994), and phagocytosis (Sengupta and Bishayi, 2002; Ghosh *et al.*, 2006; Chakraborty *et al.*, 2009). In chronically exposed animals, such effects may be long-term. For example, exposure of the freshwater bivalve *L. marginalis* to As^{III} (1 to 5 ppm) for up to 30 days resulted in time- and dose-dependent decreases in phagocytic efficiency and NO^- production in haemocytes, the major phagocytes and immune effector cells in bivalves (Chakraborty *et al.*, 2009). In a recovery assay, animals were maintained in As-free water for the same duration as the As exposure period for evaluation of immune activity. The mollusks demonstrated partial recovery of phagocytic potential in As-free water, but the effect of inhibition was still apparent; whereas NO^- production was restored to control levels in animals exposed to 1 ppm As for up to 4 days, NO^- generation remained suppressed in the high-dose- and long-term-exposed animals (Chakraborty *et al.*, 2009). As can also induce apoptosis in macrophages, as seen in a 3-fold increase in DNA fragmentation in splenic macrophages from As^{III}-exposed mice (Sengupta and Bishayi, 2002). Further, splenic macrophages isolated from As-treated mice have demonstrated reductions in cell adhesion property and chemotactic index (Bishayi and Sengupta, 2003), as well as cell surface expression of $I\text{-}A^k$ (MHC) class II molecules and antigen presentation to T-cells (Sikorski *et al.*, 1991a). Similar observations have been made in humans exposed to As, specifically macrophage cell rounding and decreased adhesion, NO^- production and phagocytic capacity (Banerjee *et al.*, 2009), as well as reduced expression of MHC class II molecules by peripheral blood lymphocytes (Andrew *et al.*, 2008), thus giving biological plausibility to these results.

Immune surveillance. Taken together, these findings suggest that *in vivo* As exposure could potentially disrupt normal innate and humoral immune responses. Indeed, studies on the influence of As on the allogeneic immune response, i.e. rejection of MHC mismatched allografts, provide direct evidence that As can disrupt the ability of the immune system to distinguish "self" from "non-self" (Yan *et al.*, 2009; Kavian *et al.*, 2012). In a mouse heart transplantation model, allograft rejection was significantly reduced in recipients receiving arsenic trioxide (As_2O_3), especially in combination with cyclosporine A, compared to those receiving PBS treatment (Yan *et al.*, 2009). The altered Th1/Th2 cytokine mRNA expression pattern observed in the As_2O_3-cyclosporine A allografts may have likely contributed to the increased graft survival (Yan *et al.*, 2009). Similarly, As_2O_3 treatment radically reduced the severe clinical symptoms of graft-versus-host disease displayed in mice following allogeneic hematopoietic stem cell transplantation (Kavian *et al.*, 2012). These effects of As_2O_3 were mediated by glutathione depletion and H_2O_2 overproduction leading to death of activated ROS-producing plasmacytoid dendritic cells and $CD4^+$ T-cells triggered by minor MHC incompatibilities following transplantation (Kavian *et al.*, 2012). Together with the previously observed decrease in macrophage $I\text{-}A^k$ class II expression and antigen processing and/or presentation (Sikorski *et al.*, 1991a), these findings provide potential mechanisms whereby As exposure can suppress the ability of the immune system to discriminate between self and non-self antigens.

Interestingly, in a study of the influence of As on the regulatory cells of the immune system, 3-week exposure to As^{III} resulted in redistribution of nTreg lymphocytes in a rat model of the autoimmune disease multiple sclerosis, which is characterized by decreased function and number of nTreg cells (Hernandez-Castro *et al.*, 2009). Whereas low doses of As^{III} increased the

number of nTreg cells in the spleen and alleviated the severity of the autoimmune condition, high concentrations (> 100 μg/L) reduced the number of these cells in both peripheral blood and spleen, consistent with the epidemiological findings in this study of decreased levels of peripheral nTreg cells in an As-exposed population (Hernandez-Castro *et al.*, 2009). Since most Ab-forming cells in rodents are localized in the spleen, it is likely that the increased number of splenic nTreg lymphocytes induced by low As concentrations inhibit generation of (auto-) immune responses, hence the beneficial effect of immune suppression by low doses of As in this animal model of autoimmune disease. Evidently, there are at least two possible scenarios whereby As exposure can interfere with self/non-self recognition: i) by inducing failure of the immune surveillance to recognize "non-self" from "self", leading to increased survival of non-self antigens, as in the case of allograft transplantation; or ii) by inhibiting recognition of self antigens as "self", which could arise from As-induced reduction of the inhibitory activity of nTreg cells, leading to "anti-self" Ab production indicative of autoimmune disease. Such effects would likely render the host immunocompromised and may therefore have detrimental health consequences.

Increasing evidence indicates that *in vivo* exposure to As also compromises the ability of the immune system to rid the host of pathogens and tumor cells, as demonstrated by several animal models. In As-exposed mice demonstrating depressed humoral and cellular immune responses, a significant impairment was seen in their resistance against B16F10 melanoma, which resulted in a 7-fold increase in tumor burden (Sikorski *et al.*, 1989). As^{III} exposure of zebrafish embryos and larvae resulted in significant 57- to 80-fold increases in viral titers and 17- to 19-fold increases in bacterial loads (Nayak *et al.*, 2007), as well as decreased induction of ROS production (Hermann and Kim, 2005; Nayak *et al.*, 2007). As exposure-challenge studies in catfish revealed efficient colonization of pathogens in distant host tissues (Ghosh *et al.*, 2007) and increased susceptibility to ulcer development and septicemia following infection with *A. hydrophilia* (Ghosh *et al.*, 2006). In mice, there are conflicting results on the influence of As on the host's ability to clear bacterial or parasitic infection. Whereas in one study As exposure was found to delay splenic clearance of *S. aureus* (Bishayi and Sengupta, 2003), in another study As exposure appeared to enhance host resistance to gastrointestinal infection by *G. muris* cysts (Escudero-Lourdes *et al.*, 2005). While the reasons for these inconsistencies are unclear, it should be noted that in the earlier study As exposure resulted in greater than 50% reductions in cell adhesion and chemotaxis of splenic macrophages (Bishayi and Sengupta, 2003), which could explain the increased survival of bacteria. However, in the later study (Escudero-Lourdes *et al.*, 2005), no other immune functional parameters were examined; thus, it stands to reason that the concentrations of As used in this mouse model were insufficient to observe the immunosuppressive effects of As observed in other studies.

A more recent study found that chronic low-dose As^{III} exposure (up to 100 ppb) aggravated the severity of H1N1 influenza A infection in mice, increasing both morbidity and respiratory viral titers (Kozul *et al.*, 2009a). Early in the course of infection, As suppressed recruitment of lymphocytes, macrophages and neutrophils to the lungs as well as migration of dendritic cells to lymph nodes, and inhibited production of 9 out of 10 cytokines examined, including TNF-α and IL-1β (Kozul *et al.*, 2009a), consistent with previously reported decreased expression of cytokines and genes involved in cell adhesion and migration in the lungs of uninfected As-exposed mice (Andrew *et al.*, 2007; Kozul *et al.*, 2009b). Exposure to As directly depressed the migration capacity of dendritic cells, as demonstrated by an *in vitro* assay of bone marrow-derived dendritic cells isolated from uninfected As-exposed and unexposed mice (Kozul

et al., 2009a). Although the levels of cell counts and cytokines in the lungs of As-exposed mice were similar to or higher than those of the unexposed controls by day 7 post-infection (Kozul *et al.*, 2009a), these results show that prolonged As exposure could significantly impair the immune response against infection, and suggest that impaired response to repeated infections could contribute to chronic diseases in humans such as bronchiectasis.

***In vitro* studies**

Lymphocyte activation. Consistent with findings from epidemiological and experimental animal studies, *in vitro* exposure to As suppresses IL-2 secretion and proliferative responses of activated splenic or peripheral blood lymphocytes from humans (Martin-Chouly *et al.*, 2011; Morzadec *et al.*, 2012), mice (Conde *et al.*, 2007; Cho *et al.*, 2012) and chickens (Das *et al.*, 2011). *In vitro*, As^{III} treatment inhibited lymphoproliferation through decreased G_2/M phase of the cell cycle in 11B7501 harbor seal lymphoma B-cells (Frouin *et al.*, 2010) and increased proportion of non-dividing cells blocked in G_1 phase via inhibition of cyclin D3 and CDC25A in primary human T-cells (Morzadec *et al.*, 2012). A biphasic dose-dependent response was observed following *in vitro* As^{III} or As^{V} exposure of activated human and bovine peripheral blood lymphocytes; whereas low concentrations enhanced proliferation, high doses completely inhibited it (McCabe *et al.*, 1983). Thus, the immunosuppressive effects of As are dependent upon the exposure level. As treatment also markedly suppressed lymphocyte secretion and/or mRNA levels of IFN-γ (Das *et al.*, 2011; Cho *et al.*, 2012; Morzadec *et al.*, 2012), IL-4 and IL-10 (Cho *et al.*, 2012) in different *in vitro* models. In contrast with reports of reduced $CD4^+$ and unaltered $CD8^+$ T-cell populations in As-exposed children (Soto-Pena *et al.*, 2006) and mice (Soto-Pena and Vega, 2008), *in vitro* As^{III} treatment of mitogen-stimulated mouse T-lymphocytes resulted in a decrease in the $CD8^+$ cell count without affecting the $CD4^+$ cell count (Conde *et al.*, 2007). Because the authors used concentrations of As^{III} (1 and 10 μM) within the range of urinary As levels reported in the exposed children, the reasons for this discrepancy remain unclear. Additionally, As^{III} treatment inhibited early activation of mouse $CD4^+$ and $CD8^+$ cells, as evidenced by reduced surface CD69 expression (Conde *et al.*, 2007), an effect that was reported at the protein level in human $CD4^+$ and $CD8^+$ lymphocytes exposed to As *in vitro* (Tenorio and Saavedra, 2005) and also at the mRNA level in lymphocytes of As-exposed humans (Andrew *et al.*, 2008).

Induction of apoptosis and humoral immunity. In agreement with observations from As-exposed humans and laboratory animals, the immunosuppressive effects of As are further demonstrated *in vitro* by the increased rate of apoptosis in multiple immune cell subtypes, including B- and T-lymphocytes, macrophages and neutrophils (Bustamante *et al.*, 1997; Harrison and McCoy, 2001; de la Fuente *et al.*, 2002; Gonzalez-Rangel *et al.*, 2005; Lemarie *et al.*, 2006b; Binet *et al.*, 2009). Prior to inducing apoptosis in TA3 mouse antigen-presenting B-cells, As^{III} treatment inhibited enzymatic activity of lysosomal protease cathepsin L, which is important in antigen processing/presentation for elicitation of T-cell responses (Harrison and McCoy, 2001). This could possibly potentiate As-induced suppression of the humoral immune response, for *in vitro* As treatment has been shown to inhibit the AFC response of mouse splenocytes to SRBC antigen (Yoshida *et al.*, 1987; Li *et al.*, 2010), consistent with findings from animal studies.

Macrophages. *In vitro* studies support evidence from epidemiological and animal studies that exposure to As can disrupt the survival, development and function of monocytes/macrophages. As_2O_3 exposure of human blood monocytes and U937 promonocytic

cells led to marked induction of apoptosis during macrophagic differentiation, an important event in the immune response, likely through inhibition of NF-κB-related survival pathways (Lemarie *et al.*, 2006b). Further, As exposure was shown to inhibit the differentiation of human monocytes into macrophages (Lemarie *et al.*, 2006a; Sakurai *et al.*, 2006; Bourdonnay *et al.*, 2009a; Wang *et al.*, 2011), and reverse macrophage-specific features, essentially leading to macrophagic "de-differentiation." For example, As-treated macrophages demonstrated cell rounding, reorganized F-actin cytoskeleton resembling that of monocytes, reduced expression of several macrophage surface markers (Lemarie *et al.*, 2006a), loss of adhesion and enhanced expression of monocytic marker CD14 (Lemarie *et al.*, 2006a; Sakurai *et al.*, 2006), and impaired endocytosis and phagocytosis via activated Rho A-ROCK signaling (Lemarie *et al.*, 2006a). Furthermore, As-treated macrophages could be differentiated into dendritic-like cells, similar to monocytes (Lemarie *et al.*, 2006a; Sakurai *et al.*, 2006).

Additional studies identified altered expression of macrophage-specific genes, including suppressed macrophage differentiation transcription factor *EGR2*, via redox-sensitive signaling unrelated to ROS production following As_2O_3 exposure of human PBMC-derived macrophages (Bourdonnay *et al.*, 2009a; Bourdonnay *et al.*, 2009b). Interestingly, As_2O_3 enhanced LPS-induced mRNA and secretion levels of TNF-α and IL-8 by monocyte-derived macrophages (Lemarie *et al.*, 2006a), suggesting that the toxicity of As toward macrophages is complex, and supporting the epidemiological findings of As-associated inflammation in chronically exposed individuals (Wu *et al.*, 2003). Thus, it appears that As can suppress the number of functional human macrophages by i) inhibiting differentiation of monocytes into macrophages, ii) disrupting differentiation, morphology and function of monocyte-derived macrophages, specifically endocytosis and phagocytosis, and iii) inducing apoptosis of monocytes/macrophages. Together, these data support *in vivo* evidence of disrupted macrophage function in chronically exposed humans (Banerjee *et al.*, 2009) and reduced phagocytic activity and antimicrobial response of As-exposed mice (Bishayi and Sengupta, 2003).

Pulmonary effects. Studies that address the influence of As exposure on pulmonary function *in vitro* provide some mechanistic insight into how As exposure *in vivo* compromises respiratory function and immunity in humans and animals. Exposure of primary rat pulmonary alveolar macrophages (PAM) to As^V or As^{III} (up to 300 μg/mL) for 24 h led to marked inhibition of LPS-induced TNF-α release, consistent with PAM exposed *in vivo* (Lantz *et al.*, 1994), which is in contrast with results from monocyte-derived macrophages exposed *in vitro* to As_2O_3 (Lemarie *et al.*, 2006a). Exposure to As^V or As^{III} also suppressed O_2^- production, but As^{III} was more potent than As^V, inhibiting O_2^- production and LPS-induced TNF-α release at concentrations 10-fold lower (Lantz *et al.*, 1994). Whereas LPS-induced PGE2 production was inhibited by As^V, As^{III} treatment had no effect, suggesting different potencies and/or mechanisms between the arsenicals. More importantly, these results indicate that altered PAM function resulting from As exposure could contribute to impaired host defense against pathogens.

Several other mechanisms have been proposed for the As-associated increased incidence of respiratory infections, including decreased airway epithelial chloride secretion. This is mediated through As-induced degradation of cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel that is essential for mucociliary clearance of respiratory pathogens, as seen in human airway epithelial cells (Bomberger *et al.*, 2012) and in the gills of killifish (Shaw *et al.*, 2010). In human bronchial epithelial cells, As exposure restricted the airway wound response through up-regulation of MMP9 expression and activity (Olsen *et al.*, 2008) and inhibition of paracrine ATP-dependent Ca^{2+} signaling, which is essential to innate

immune functions such as bactericide production and wound repair (Sherwood *et al.*, 2011). These findings of decreased airway epithelial cell migration and wound repair are consistent with decreased expression of genes involved in cell adhesion and migration in the lungs of As-exposed mice (Andrew *et al.*, 2007; Kozul *et al.*, 2009b), as well as altered airway proteins in mice and humans chronically exposed to low-dose As (10-50 ppb) in drinking water (Josyula *et al.*, 2006; Lantz and Hays, 2006; Lantz *et al.*, 2007). Collectively, these studies suggest that As exposure could disrupt pulmonary defense through various mechanisms, including i) altered PAM function, ii) decreased chloride secretion resulting in depressed mucociliary clearance of respiratory pathogens, and iii) airway remodeling due to impaired epithelial wound response, ultimately promoting the development of chronic lung diseases such as bronchiectasis.

Summary

An efficient immune response requires functional innate and adaptive immune defenses. The studies reviewed in this chapter show that As has significant impact on both components of the immune system, as demonstrated both *in vivo* and *in vitro* by the induction of apoptosis, oxidative stress and inflammation in circulating lymphocytes, impaired lymphocyte activation and macrophage function, and alterations in cellular and humoral immunity (Table 2). These effects of As can result in immunosuppression, as evidenced by reduced microbial clearance in animal studies and increased rates of opportunistic infections in humans, particularly of the respiratory tract. Furthermore, epidemiological data suggest significant susceptibility of the lung to perturbation by As, especially during prenatal and childhood development, which results in unprecedented rates of chronic lung diseases, notably lung cancer and bronchiectasis. The pleiotropic effects of As on the immune system, including specific examples of compromised immune surveillance (Table 3), lend biological plausibility to the increased rates of infection, cancer and other immune-related illnesses observed in As-exposed human populations, and are illustrated in Figure 5.

Conclusions and Future Directions

Numerous *in vitro* and *in vivo* studies support the role of As as an immunomodulator that could render the host immunocompromised. Such immune alterations could help explain the increased risk of infections and cancers observed in human populations chronically exposed to As. The As-mediated alterations of cellular and humoral immunity reported in animal and *in vitro* models are generally consistent with As-associated immunological outcomes in humans. However, more work is needed to close the gap between experimental data and risk of human immunotoxicity. Moreover, there are some inconsistencies in epidemiological findings, possibly due to differences in dose, sampling, genetic background, and environmental and nutritional factors. Therefore, epidemiological investigations consisting of larger numbers of participants as well as different ethnic populations are urgently needed. Due to differential effects of exposure levels, human populations having low, intermediate and high As exposures must be evaluated to better understand dose-dependent relationships. Furthermore, there is strong evidence for an association between developmental exposure to As and elevated risk of infectious, malignant and non-malignant diseases in humans. Hence, more investigations of early-life As exposures should be conducted, especially follow-up studies to monitor the health status of participants who have already been enrolled in such studies. Finally, comprehensive molecular analyses involving genomic, proteomic and metabolomic profiling will be necessary for the identification and

validation of potential molecular targets of As in order to monitor the progression of As-associated diseases to help elucidate the mechanisms of As immunotoxicity.

Table 2. Summary of major findings of immune-related effects associated with As exposure that are consistent across multiple studies

Immune parameter	Major findings	Study model	Description	References
Defense genes/proteins	↓ MHC class II	Humans	PBMC mRNA levels	Andrew *et al.*, 2008
		Animals	Mouse macrophage surface expression	Sikorski *et al.*, 1991a
	↓ CD69	Humans	PBMC mRNA levels	Andrew *et al.*, 2008
		Human cells	PBMC surface expression	Tenorio and Saavedra, 2005
		Animal cells	Mouse SMC surface expression	Conde *et al.*, 2007
	↓ IL-1β	Humans	PBMC mRNA levels	Andrew *et al.*, 2008; Argos *et al.*, 2006
		Animals	Mouse lung mRNA & protein levels	Kozul *et al*., 2009a; Kozul *et al*., 2009b
			Zebrafish mRNA levels	Mattingly *et al.*, 2009
	↑ CD14	Humans	PBMC mRNA & surface expression	Wu *et al.*, 2003; Hernandez-Castro *et al*., 2009
		Human cells	Macrophage surface expression	Lemarie *et al*., 2006a; Sakurai *et al*., 2006
	↓ TNF-α	Humans	PBMC mRNA levels	Salgado-Bustamante *et al.*, 2010; Argos *et al.*, 2006
			PBMC secretion	Biswas *et al.*, 2008
		Animals	Zebrafish mRNA levels	Hermann and Kim, 2005
			Rat PAM secretion	Lantz *et al.*, 1994
			Mouse lung fluid protein levels	Kozul *et al.*, 2009a
Inflammation	↑ Expression of inflammatory mediators	Humans	↑ PBMC *IL1B, IL6, CCL2* & *CD14* mRNA in adults	Wu *et al.*, 2003
			↑ PBMC CD14 surface expression & TNF-α secretion in adults	Hernandez-Castro *et al*., 2009

Immune parameter	Major findings	Study model	Description	References
			↑ Stimulated PBMC GM-CSF secretion in children	Soto-Pena *et al.*, 2006
			↑ Placental & cord blood IL-1β, TNF-α and IFN-γ in neonates	Ahmed *et al.*, 2011
		Human cells	↑ Stimulated macrophage mRNA & secretion of TNF-α & IL-8	Lemarie *et al.*, 2006a
Lymphocyte activation	↓ Stimulated proliferation	Humans	PBMC in adults	Ostrosky-Wegman *et al.*, 1991; Gonsebatt *et al.*, 1994; Biswas *et al.*, 2008
			PBMC in children	Soto-Pena *et al.*, 2006
		Animals	Chicken SMC & PBMC	Aggarwal *et al.*, 2008
			Mouse SMC	Soto-Pena and Vega, 2008
			Catfish SMC	Ghosh *et al.*, 2006; Ghosh *et al.*, 2007
		Human cells	PBMC	Martin-Chouly *et al.*, 2011; Morzadec *et al.*, 2012
		Animal cells	Mouse SMC	Conde *et al.*, 2007; Cho *et al.*, 2012
			Chicken SMC	Das *et al.*, 2011
	↓ Stimulated IL-2 secretion	Humans	PBMC in adults	Biswas *et al.*, 2008
			PBMC in children	Soto-Pena *et al.*, 2006
		Animals	Mouse SMC	Soto-Pena and Vega, 2008; Burns and Munson, 1993
			Catfish SMC	Ghosh *et al.*, 2007
		Human cells	PBMC	Martin-Chouly *et al.*, 2011; Morzadec *et al.*, 2012
		Animal cells	Mouse SMC	Conde *et al.*, 2007; Cho *et al.*, 2012
			Chicken SMC	Das *et al.*, 2011

Immune parameter	Major findings	Study model	Description	References
			Harbor seal 11B7501 lymphoma B-cells	Frouin *et al*., 2010
Humoral immunity	↓ AFC response to antigen	Animals	Mouse SMC	Blakley *et al.*, 1980; Sikorski *et al.*, 1989; Sikorski *et al.*, 1991b; Burchiel *et al*., 2009
			Rat SMC	Flora and Kumar, 1996; Nain and Smits, 2010
		Animal cells	Mouse SMC	Yoshida *et al.*, 1987; Li *et al.*, 2010
Hypersensitivity reaction	↓ Response to cutaneous sensitization	Animals	↓ LC migration to lymph nodes & subsequent T-cell activation in mice	Sikorski *et al.*, 1989; Patterson *et al.*, 2004
			Rats	Savabieasfahani *et al.*, 1998; Schulz *et al.*, 2002
			Chickens	Aggarwal *et al.*, 2008
Monocytes/ macrophages	↓ Number/survival	Humans	↓ Monocyte count	Maiti *et al.*, 2012
		Animals	↓ Mouse splenic macrophage count	Sikorski *et al.*, 1991b
			↓ Catfish HK macrophage count	Ghosh *et al.*, 2006; Ghosh *et al.*, 2007
			↑ Apoptosis of mouse splenic macrophages	Sengupta and Bishayi, 2002
		Human cells	↑ Apoptosis of blood monocytes & U937 promonocytic cells	Lemarie *et al.*, 2006b
	Impaired development	Human cells	↓ Differentiation of monocytes into macrophages	Lemarie *et al.*, 2006a; Sakurai *et al.*, 2006; Bourdonnay *et al.*, 2009a; Wang *et al*., 2011
			Induced differentiation of macrophages into dendritic-like cells	Lemarie *et al.*, 2006a; Sakurai *et al.*, 2006

Immune parameter	Major findings	Study model	Description	References
	Diminished function	Humans	Cell rounding; ↓ adhesion/CD54 adhesion molecule, F-actin, NO^- production & phagocytosis; altered Rho A-ROCK signaling	Banerjee *et al.*, 2009
		Animals	↓ Rat PAM stimulated TNF-α secretion	Lantz *et al.*, 1994
			↓ Mouse peritoneal macrophage NO^- & O_2^- production	Arkusz *et al.*, 2005
			↓ Mouse splenic macrophage adhesion, chemotactic index, phagocytosis, NO^- production, MHC class II surface expression & antigen presentation	Bishayi and Sengupta, 2003; Sengupta and Bishayi, 2002; Sikorski *et al.*, 1991a
			↓ Chicken SMC & PBMC NO^- production	Aggarwal *et al.*, 2008
			↓ Molluscan haemocyte phagocytosis & NO^- production	Chakraborty *et al.*, 2009
		Human cells	Cell rounding; ↓ adhesion & macrophage-specific markers; reorganized F-actin cytoskeleton resembling that of monocytes; ↑ monocytic marker CD14; ↓ endocytosis & phagocytosis via activated Rho A-ROCK signaling	Lemarie *et al.*, 2006a; Sakurai *et al.*, 2006
Survival	↑ Induction of apoptosis	Humans	PBMC in adults	Hernandez-Castro *et al*., 2009
			↑ PBMC *BCL2L1* & *CASP2* mRNA in adults	Salgado-Bustamante *et al.*, 2010
			PBMC in children	de la Fuente *et al.*, 2002; Rocha-Amador *et al*., 2011
			↑ CBMC *CASP2* mRNA in neonates	Ahmed *et al.*, 2012
		Animals	Mouse splenic macrophages	Sengupta and Bishayi, 2002
		Human cells	Blood monocytes & U937 promonocytic cells	Lemarie *et al.*, 2006b
			PBMC	de la Fuente *et al.*, 2002

Immune parameter	Major findings	Study model	Description	References
			B-cells, T-cells, macrophages & neutrophils	Binet *et al.*, 2009
		Animal cells	Mouse TA3 antigen-presenting B-cells	Harrison and McCoy, 2001
			Rat T-cells	Bustamante *et al.*, 1997
ROS production	Induced oxidative stress	Humans	↑ Serum SOD & PBMC MDA in adults	Escobar *et al.*, 2010
			↑ Basal PBMC/monocyte NO^- & O_2^- in children	Luna *et al.*, 2010
			↑ Placental 8-oxoguanine in neonates	Ahmed *et al.*, 2011
			↑ Cord blood 8-hydroxy-2'-deoxyguanosine in neonates	Ahmed *et al.*, 2012
	↓ Stimulated ROS production	Humans	↓ Macrophage NO^- in adults	Banerjee *et al.*, 2009
			↓ Monocyte NO^- & O_2^- in children	Pineda-Zavaleta *et al.*, 2004
		Animals	↓ Mouse peritoneal macrophage NO^- & O_2^-	Arkusz *et al.*, 2005
			↓ Mouse splenic macrophage NO^-	Bishayi and Sengupta, 2003; Sengupta and Bishayi, 2002; Sikorski *et al.*, 1991a
			↓ Chicken SMC & PBMC NO^-	Aggarwal *et al.*, 2008
			↓ Molluscan haemocyte NO^-	Chakraborty *et al.*, 2009
			Zebrafish embryos & larvae	Hermann and Kim, 2005; Nayak *et al.*, 2007
Microbial challenge	↓ Clearance of pathogens	Animals	↑ Viral & bacterial loads in zebrafish embryos and larvae	Nayak et al., 2007
			↑ Pathogen colonization & ulcers/septicemia following bacterial infection in catfish	Ghosh *et al.*, 2007; Ghosh *et al.*, 2006
			↓ Splenic clearance of *S. aureus* in mice	Bishayi and Sengupta, 2003
			↑ Morbidity & respiratory viral titers	Kozul *et al.*, 2009a

Immune parameter	Major findings	Study model	Description	References
			following H1N1 viral infection in mice	
Pulmonary health	Altered lung features	Humans	Altered airway protein expression in adults	Josyula *et al.*, 2006; Lantz *et al.*, 2007
		Animals	Altered mouse airway protein expression	Lantz and Hays, 2006; Lantz *et al.*, 2007
			↓ Rat PAM stimulated TNF-α secretion	Lantz *et al.*, 1994
			↓ Mouse lung expression of genes involved in cell adhesion/migration	Andrew *et al.*, 2007; Kozul *et al.*, 2009b
			↓ Killifish gill chloride secretion via ↑ CFTR degradation	Shaw *et al.*, 2010
		Human cells	↓ CFBE41o- airway epithelial cell chloride secretion via ↑ CFTR degradation	Bomberger *et al.*, 2012
			↓ 16HBE14o- bronchial epithelial cell migration and wound repair	Olsen *et al.*, 2008; Sherwood *et al.*, 2011
		Animal cells	↓ Rat PAM stimulated TNF-α secretion & NO^- & O_2^- production	Lantz *et al.*, 1994
	↑ Risk of infection/disease	Humans	↑ Respiratory tract infections & tuberculosis in adults	Biswas *et al.*, 2008; Banerjee *et al.*, 2009; Smith *et al.*, 2011
			↓ Lung function; ↑ prevalence/mortality from lung cancer and non-malignant lung disease, including bronchitis & bronchiectasis in adults	Tsai *et al.*, 1999; Mazumder *et al.*, 2000; Milton and Rahman, 2002; Milton *et al.*, 2003; De *et al.*, 2004; von Ehrenstein *et al.*, 2005; Islam *et al.*, 2007
			↑ Respiratory tract infections in infants	Raqib *et al.*, 2009; Rahman *et al.*, 2011
		Animals	↑ Morbidity & respiratory viral titers following H1N1 infection in mice	Kozul *et al.*, 2009a

Note: all cells are primary cells unless otherwise stated. Subjects of human studies are adults unless otherwise stated.
As, arsenic; ↓ decreased; ↑ increased; PBMC, peripheral blood mononuclear cells; SMC, splenic mononuclear cells; PAM, pulmonary alveolar macrophages; GM-CSF, granulocyte-macrophage colony stimulating factor; Ab, antibody; AFC, Ab-forming cell; NO^-, nitric oxide anion; O_2^-, superoxide anion; HK, head kidney; LC, Langerhans cells; CBMC, cord blood mononuclear cells; ROS, reactive oxygen species; SOD, serum superoxide dismutase; MDA, malondialdehyde; CFTR, cystic fibrosis transmembrane conductance regulator.

Table 3. Summary of specific observations of immune-related effects associated with As exposure

Major findings	Biological relevance	References
↓ nTreg lymphocyte number & function in adults; redistribution in a rat model of autoimmune disease	nTreg cells play a critical role in immune homeostasis; alterations could affect self-recognition or influence autoimmune disease	Hernandez-Castro *et al.*, 2009
Prenatal As exposure ↓ infant thymic size & function	Thymus is site of T-cell development; impaired function may account for ↑ prevalence of As-associated respiratory, cancer & other immune-related effects in adulthood	Raqib *et al.*, 2009; Ahmed *et al.*, 2012
↓ CD4/CD8 T-cell ratio in children & mice	Indicator of immune suppression	Soto-Pena *et al.*, 2006; Soto-Pena and Vega, 2008
↓ Rejection of MHC mismatched heart/bone marrow allografts in mice	↓ Immune surveillance could lead to immunocompromised state & ↓ ability to fight infection/cancer cells	Yan *et al.*, 2009; Kavian *et al.*, 2012
↓ Resistance in mice against B16F10 melanoma resulted in 7-fold ↑ tumor burden	↓ Anti-tumor immunity could lead to cancer development	Sikorski *et al.*, 1989
↓ Migration of lymphocytes, macrophages & neutrophils to lungs/dendritic cells to lymph nodes early in the course of H1N1 influenza infection in mice	↓ Immune surveillance could lead to immunocompromised state & ↓ ability to fight infection/cancer cells	Kozul *et al.*, 2009a
↓ Urinary HBD1 peptides in men; ↓ *DEFB1* mRNA in human 293T renal and HeLa cervical cells	HBD1 is an antimicrobial peptide implicated in host anti-tumor & pulmonary immunity; its down-regulation could contribute to As-induced cancers & respiratory illnesses observed in humans	Hegedus *et al.*, 2008

As, arsenic; ↓ decreased; ↑ increased; nTreg, natural T regulatory; HBD1, human β-defensin 1.

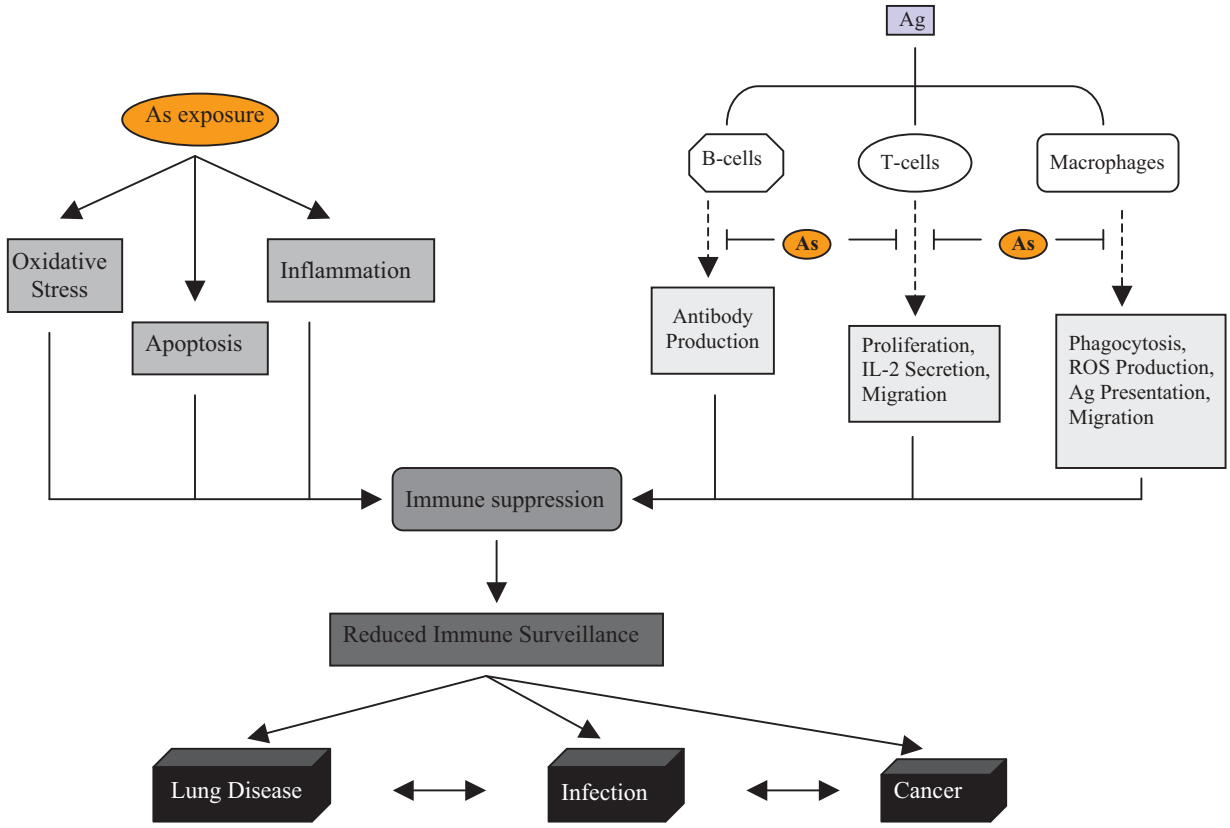


Figure 5. Simplified scheme of select arsenic (As)-induced alterations of immune responses and how such effects might contribute to development of As-associated infections, diseases, and cancer. Antigen (Ag) can be foreign or tumor cell.

Chapter 3
Arsenic Down-Regulates Human Beta-Defensin-1

Abstract

Arsenic (As) is a potent human carcinogen and a major drinking water contaminant for millions of people worldwide. We previously reported decreased urinary levels of human β-defensin-1 (HBD1) peptides in men exposed to As in drinking water in two cross-sectional studies based in Nevada and Chile, and confirmed *in vitro* that As exposure suppressed HBD1 mRNA expression which is encoded by the *DEFB1* gene. In the current study, we investigated effects of arsenite (As^{III}) and the postulated more toxic metabolite, monomethylarsonous acid (MMA^{III}), on *DEFB1* mRNA and protein levels in immortalized human keratinocytes and kidney epithelial cells. *DEFB1* mRNA expression was more abundant in HK-2 kidney epithelial cells than in HOK-16B keratinocytes, and was suppressed by As^{III} and MMA^{III} treatments. The suppressive effect of As^{III} and MMA^{III} exposures on *DEFB1* transcript levels continued for several passages in As-free media after treatment. HBD1 peptide levels were significantly reduced following exposure to As^{III}, but not to lead, cadmium or chromium, indicating that decreased HBD1 may be a specific response to As. Finally, we found that As^{III} treatment inhibited *DEFB1* promoter activity, suggesting that As-induced decreased *DEFB1* mRNA is likely due to transcriptional down-regulation. The current data indicate that our previous findings of decreased urinary HBD1 levels are likely the result of a direct effect of As on the kidney, and suggest a novel mechanism by which As exposure may promote cancer development.

Introduction

Arsenic (As) is classified as a group 1 carcinogen by the International Agency for Research on Cancer based on its association with skin, lung and urinary bladder cancers (IARC, 2012), and its exposure has also been associated with cancer of the kidney, liver and prostate, as well as diabetes, hypertension, cardiovascular disease, and respiratory, neurological, hepatic and urological disorders (Hughes *et al.*, 2011; Liu and Waalkes, 2008; IARC, 2012). As occurs naturally in the environment but its distribution has further increased due to human activities, including agricultural and industrial processes (Nordstrom, 2002). Well over 100 million people worldwide are exposed to As-contaminated groundwater, particularly in Bangladesh, India, Chile, and the United Sates (Nordstrom, 2002; IARC, 2012). As exposure is therefore a serious threat to public health in many parts of the world.

Inorganic As is found in drinking water as arsenite (As^{III}) or arsenate (As^{V}). Human metabolism involves conversion of As^{V} to As^{III} with subsequent methylation to mono- and di-methylated arsenicals (MMA and DMA, respectively) (Drobna *et al.*, 2009). MMA^{III} is considered the most toxic arsenical *in vitro* (Petrick *et al.*, 2000; Styblo *et al.*, 2000; Mass *et al.*, 2001; Petrick *et al.*, 2001) and individuals who excrete a higher proportion of ingested As as urinary MMA have an increased risk of As-associated cancers (Chen *et al.*, 2003; Steinmaus *et al.*, 2006), suggesting a key role for MMA in As toxicity. At the cellular level, As exposure has been associated with oxidative stress, inhibition of DNA repair, chromosomal aberrations, micronuclei formation, and modification of cellular signaling via altered activation, expression and DNA binding activity of transcription factors (Flora, 2011; NRC, 2001; IARC, 2004). Despite extensive research on the adverse effects of As exposure, the mechanisms of As toxicity remain poorly understood.

We previously reported lower urinary levels of human β-defensin-1 (HBD1) peptides in men exposed to As in drinking water in two case-control populations based in Nevada and Chile, and demonstrated *in vitro* that As suppressed HBD1 mRNA which is encoded by the *DEFB1* gene, providing evidence that decreased HBD1 may be a biomarker of response to As (Hegedus *et al.*, 2008). By virtue of its antimicrobial properties and constitutive expression in skin and epithelia of most tissues, HBD1 serves as a first line of defense against invading microbes (Valore *et al.*, 1998; Zucht *et al.*, 1998; Pazgier *et al.*, 2006). HBD1 also plays a role in the adaptive branch of the immune system by inducing migration of immature dendritic cells and memory T-cells (Yang *et al.*, 1999). Interestingly, a cancer-specific loss of *DEFB1* expression has been reported for various carcinomas (Young *et al.*, 2001; Donald *et al.*, 2003; Young *et al.*, 2003; Wenghoefer *et al.*, 2008; Joly *et al.*, 2009), and several lines of evidence indicate that *DEFB1* is a putative tumor suppressor gene for urological cancers (Sun *et al.*, 2006; Bullard *et al.*, 2008).

In the current study, we tested the hypothesis that As exposure inhibits *DEFB1* in human cells derived from target tissues of toxicity, including skin and renal epithelia. Because As^{III} and MMA^{III} are excreted in urine, we evaluated the effects of both arsenicals on *DEFB1* mRNA expression and addressed whether a transcriptional mechanism was involved in the As-induced effects. We also determined whether exposure of kidney epithelial cells to As or other metals could affect HBD1 peptide levels. Taken together, we provide evidence that our previous findings of decreased urinary HBD1 levels are likely the result of a direct effect of As on the kidney, and suggest a novel mechanism by which As exposure may promote cancer development.

Materials and Methods

Cell cultures

With the exception of HeLa cells, all cell lines used in the current study were immortalized, non-tumorigenic cells derived from normal, primary human cells. HK-2 kidney epithelial cells, HeLa cervical carcinoma cells, and SV-HUC-1 uroepithelial cells were purchased from ATCC (Manassas, VA), and HOK-16B keratinocytes and UROtsa uroepthelial cells were kindly provided by Professors No-Hee Park (UCLA) and Petia Simeonova (NIOSH, CDC), respectively. HK-2, HeLa, and UROtsa cells were cultured in DMEM medium containing 4.5 g/L glucose and supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin. HOK-16B cells were maintained in KGM-2 keratinocyte growth medium containing calcium and a supplementary bullet kit that includes growth factors and antibiotics (Lonza, Walkersville, MD). SV-HUC-1 cells were grown in Ham's F12 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. For all cultures, media were routinely renewed every 2 days.

Chemicals

Sodium arsenite ($NaAsO_2$), potassium dichromate ($K_2Cr_2O_7$), cadmium chloride ($CdCl_2$), lead nitrate ($Pb(NO_3)_2$), and actinomycin D were purchased from Sigma (St. Louis, MO). Monomethylarsonous acid, in the form of diiodomethylarsine (MMA^{III} iodide, CH_3AsI_2), was a generous gift from Professor A. Jay Gandolfi (University of Arizona, Tucson). Each metal compound was dissolved in sterile water to make stock solutions and then serially diluted to the desired concentrations in the respective cell culture media.

Treatments

Several experiments were performed for each cell line to establish dose-response curves for a sub-cytotoxic dose range relevant to levels of As detected in urine of exposed human subjects (Hegedus *et al.*, 2008). For each metal(loid) compound, the highest dose used in experiments represents the concentration that induced approximately 50% reduction in cell viability (IC_{50}) for each respective cell line. Cell viability was determined as the percentage of live cells relative to control and assessed using the trypan blue exclusion assay.

Subconfluent HK-2 kidney epithelial and HOK-16B keratinocyte cell cultures were seeded in 6-well plates and allowed to reattach for at least 24 h before dosing. While still in log phase growth, at about 65% confluence, cells were incubated with either As-free medium or medium containing As^{III} or MMA^{III}. Following 24 or 48 h treatment, cells were washed with ice-cold phosphate-buffered saline (PBS) and harvested for RNA isolation. For RNA stability analysis, HeLa cells and HK-2 kidney epithelial cells were incubated with actinomycin D with or without As^{III} or MMA^{III} for up to 24 h prior to RNA isolation.

Separate experiments were conducted to analyze effects of As treatment on gene expression in cells subsequently cultured in As-free medium. At approximately 25% confluence, HK-2 kidney epithelial cells were incubated for 6 days with either As-free medium or medium containing As^{III} or MMA^{III}. Media were renewed every 2 days. Following treatment, cells were washed with PBS, seeded at equal densities, and cultured in As-free medium for up to three weeks for gene expression analysis.

For HBD1 peptide quantification, HK-2 kidney epithelial cells were incubated with or without As^{III}, Cd, Cr or Pb for up to 72 h in 12-well plates. Cells were given fresh medium every 2 days. Following treatment, media samples were aliquoted, cell pellets were collected and

thoroughly washed in PBS, and all samples were immediately stored at –80ºC until assayed. All experiments consisted of three or more replicates each.

RNA extraction and RT-PCR analysis

Total cellular RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions and digested with TURBO DNase (Applied Biosystems, Foster City, CA) to eliminate contaminating genomic DNA. RNA concentration and purity were assessed using the SmartSpec 3000 spectrophotometer (Bio-Rad, Hercules, CA), and all samples used for analysis had an A_{260}/A_{280} ratio between 1.8 and 2.1 in 10 mM Tris-HCl, pH 7.5. RNA was reverse transcribed into cDNA using the Superscript III First-Strand Synthesis System (Invitrogen). Negative reverse transcription (RT) controls, which consisted of nuclease-free water instead of RT, were included to confirm absence of genomic DNA in samples.

Relative quantitative-RT-PCR was performed using Power SYBR Green PCR Master Mix in an ABI Prism 7300 thermocycler (Applied Biosystems) in a final volume of 20 µL containing 0.5 µM primers. Primers were designed to span adjacent exons to avoid amplification of contaminating genomic DNA sequences. For verification of amplification product specificity, the final dissociation stage was run to generate a melting curve, and negative RT controls were included for all experiments. Oligonucleotide sequences used as primers for PCR are as follows: *ACTB* forward, 5'-GGA CTT CGA GCA AGA GAT GG-3'; *ACTB* reverse, 5'-AGC ACT GTG TTG GCG TAC AG-3'; *DEFB1* forward, 5'-TCC CCA GTT CCT GAA ATC CTG-3'; *DEFB1* reverse, 5'-TCC CTC TGT AAC AGG TGC CTT G-3'. For quantification of transcripts, relative *DEFB1* expression was normalized to *ACTB* expression and calculated using the $\Delta\Delta C_T$ method. All experiments consisted of three replicates each and PCR reactions were run in triplicate for each sample.

Cell lysate preparation and ELISA analysis of peptide levels

Cells were lysed with ice-cold lysis buffer (150 mM NaCl, 50 mM HEPES, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, pH 7.4 supplemented with Roche protease inhibitor cocktail). Cell solutions were vortexed, freeze-thawed, vortexed again and centrifuged. Supernatants were collected in fresh tubes, aliquoted and used immediately or stored at –80ºC prior to subsequent analysis. Total protein concentrations of all lysate samples were determined using the DC Protein Assay (Bio-Rad).

Concentrations of HBD1 peptides in cell lysates and cell culture media were determined using sandwich ELISA according to manufacturer's instructions (Pepro Tech, Rock Hill, NJ) in 96-well plates (MaxiSorp, Nunc, Rochester, NY). Briefly, HBD1 was captured by rabbit anti-HBD1 antibody and detected with rabbit biotinylated anti-HBD1 antibody followed by avidin-HRP. The assay was developed by ABTS Liquid Substrate Solution (Sigma) and color development was monitored and measured with an ELISA plate reader (PowerWave XS, BioTek, Winooski, VT) at 405 nm with wavelength correction set at 650 nm. HBD1 peptide levels were normalized to cell lysate total protein concentration or to cell density of each lysate or medium sample, respectively, and presented as a fold change relative to HBD1 peptide expression in the respective untreated controls. All experiments consisted of three or more replicates, and ELISA was performed in triplicate.

Transfections and luciferase reporter assay

The -1140 bp *DEFB1* promoter-containing firefly luciferase reporter construct (DEFB1-luc) was kindly provided by Professor John A. Petros (Emory University), and the renilla luciferase construct, pRL-TK (Promega, Madison, WI), served as an internal control. Transient transfections were performed using FuGENE HD reagent (Promega) according to manufacturer's instructions. Briefly, HeLa cells were seeded into 12-well plates 24 h prior to transfection to give 70% confluence on the day of transfection. Cells were co-transfected with 100 ng of DEFB1-luc and 10 ng of pRL-TK plasmids and incubated for 24 h prior to treatment with 0, 3, or 10 μM As^{III}. Following treatment, cells were washed with PBS and harvested using passive lysis buffer (Promega). Firefly and renilla luciferase activities were assayed with the Dual-Luciferase Reporter Assay System (Promega) using a Centro XS^3 LB 960 microplate luminometer (Berthold Technologies, Germany). Firefly luciferase activity was normalized to renilla luciferase activity and reported as a fold change relative to the untreated control samples. All experiments consisted of three replicates assayed three times.

Statistical analyses

Data are representative of two or three independent experiments consisting of two to six samples. Error bars represent the standard error of the mean. Results were analyzed by unpaired two-tailed Student *t*-test to compare two groups, one-way ANOVA to compare effect of treatment versus control, or two-way ANOVA to estimate effects of dose and time followed by Bonferroni post hoc tests using Prism 4.0 for Mac software (GraphPad, San Diego, CA). Statistical significance was determined by $p<0.05$.

Results

DEFB1 is differentially expressed in immortalized human cells

Relative quantitative-RT-PCR analysis was performed to quantify *DEFB1* transcript levels in immortalized cells derived from human skin, kidney and bladder epithelia. Cell lines analyzed included HOK-16B keratinocytes, HK-2 kidney cells, and UROtsa and SV-HUC-1 uroepithelial (bladder) cells. *DEFB1* mRNA levels were normalized to those of *ACTB*, and expression of *DEFB1* in HeLa cells, which we used in our previous study (Hegedus *et al.*, 2008), was selected as the reference for comparison with all other cell lines. *DEFB1* mRNA levels were most abundant in HK-2 kidney epithelial cells followed by HOK-16B keratinocytes (Table 4). Specifically, *DEFB1* gene expression was 86-fold ($p<0.0001$) and 5-fold ($p<0.01$) that of HeLa in the HK-2 kidney epithelial cells and HOK-16B keratinocytes, respectively. *DEFB1* mRNA expression was not detectable in either UROtsa or SV-HUC-1 bladder cells, which were therefore excluded from further analyses.

Table 4. Quantification of *DEFB1* mRNA expression in human cell lines

Cell line	Tissue derivation	Relative *DEFB1* mRNA expression	P-value
HeLa	Cervical carcinoma	1	N/A
HK-2	Renal epithelium	85.9	3.56×10^{-5}
HOK-16B	Keratinocyte	4.9	2.80×10^{-3}
UROtsa	Uroepithelium	Not detectable	N/A
SV-HUC-1	Uroepithelium	Not detectable	N/A

Relative quantitative-RT-PCR analysis was performed to quantify *DEFB1* transcripts in immortalized human cells. Relative expression of *DEFB1* mRNA was normalized to *ACTB* and presented as relative to *DEFB1* expression in HeLa cells, which were selected as the reference for comparison. Unpaired two-tailed Student *t*-test was used to compare each cell line to HeLa cells. N/A, not applicable.

Arsenical treatments suppress DEFB1 gene expression in target cells

We examined the effect of As treatment on *DEFB1* mRNA expression in HOK-16B keratinocytes and HK-2 kidney epithelial cells to test the hypothesis that As suppresses *DEFB1* expression in cells derived from target tissues of toxicity. Because As^{III} in drinking water is metabolized to the postulated more toxic metabolite, MMA^{III}, and both species are excreted in urine, we investigated the role of both arsenicals to better understand As toxicity. Therefore, relative quantitative-RT-PCR analysis was performed following exposure of cells to increasing concentrations of either As^{III} or MMA^{III} for 24 or 48 h.

In HOK-16B keratinocytes, As^{III} induced a significant dose-dependent decrease in *DEFB1* mRNA levels ($p<0.0001$), but there was no statistically significant effect of time in reducing transcript levels (Figure 6A). The highest concentration of As^{III} (3 μM or 225 μg/L) resulted in 50% and 75% reductions at 24 h and 48 h, respectively (Figure 6A, black bars) relative to untreated control (white bars). Although exposure of HOK-16B cells to MMA^{III} appeared to reduce transcript levels, there was no statistically significant effect of dose or time

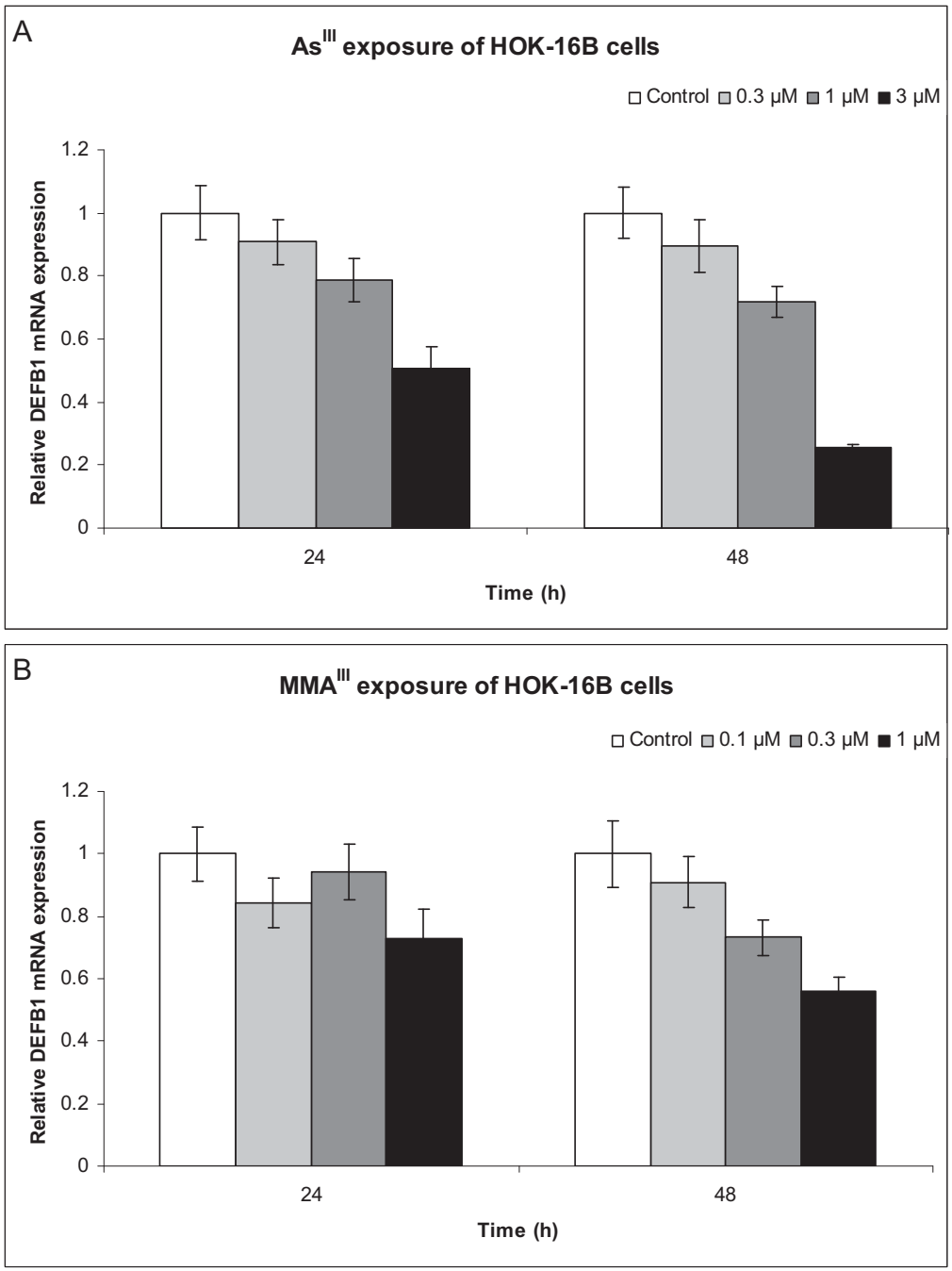


Figure 6. Arsenical-induced suppression of *DEFB1* mRNA expression in HOK-16B keratinocytes. *DEFB1* transcripts were quantified by relative quantitative-RT-PCR analysis following 24 h and 48 h exposure of HOK-16B keratinocytes to 0, 0.3, 1 or 3 μM As^{III} (A) or 0, 0.1, 0.3 or 1 μM MMA^{III} (B). Relative expression of *DEFB1* mRNA was normalized to *ACTB* and presented as a fold change relative to *DEFB1* expression in the respective untreated controls. Data represent the mean of two independent experiments consisting of three biological replicates (wells of cells), and PCR was performed in triplicate for each sample. Error bars represent SEM. Two-way ANOVA was used to estimate effects of dose and time followed by Bonferroni post hoc tests for multiple comparisons. As^{III} induced a significant dose-dependent decrease

(p<0.0001), but had no time-dependent effect (A). MMA^{III} had no dose- or time-dependent effect (B), although there was a trend toward decrease with dose (p=0.0654).

on *DEFB1* mRNA expression but there was a trend toward decrease with dose (p=0.0654, Figure 6B). Significant time- and dose-dependent decreases were observed in *DEFB1* gene expression following exposure of HK-2 kidney epithelial cells to As^{III} (p=0.0387 and p<0.0001, respectively; Figure 7A). Reductions of 46% and 70% were observed at the highest concentration (1 μM or 74.9 μg/L) at 24 h and 48 h, respectively (Figure 7A, black bars) relative to untreated control (white bars). MMA^{III} induced a significant dose-dependent decrease (p=0.0327) in *DEFB1* mRNA levels in HK-2 cells, with a 40% reduction at 0.3 μM (32 μg/L) compared with control, but did not suppress expression in a time-dependent manner (Figure 7B).

DEFB1 down-regulation persists for several passages after termination of arsenical exposure

To determine whether cells could restore *DEFB1* expression to pre-exposure levels after removal of As from the medium, we performed separate experiments in which HK-2 kidney epithelial cells were cultured for up to three weeks in As-free medium following an initial exposure period to 1 μM As^{III} or 0.3 μM MMA^{III} for 6 days (Figure 8A). At the end of treatment, cells were seeded at equal densities and cultured in the absence of As. The As^{III} and MMA^{III} lineage cells did not have any altered appearance or growth properties relative to the control lineage cells when visualized by microscopic inspection (data not shown).

Relative quantitative-RT-PCR analysis showed gradual restoration of transcripts to control levels in both As^{III} and MMA^{III} lineage cells (Figure 8B, C). In the As^{III} lineage cells, *DEFB1* transcript levels were significantly suppressed on days 3, 6 and 9 following cessation of treatment by 62% (p<0.001), 54% (p<0.001) and 24% (p<0.05), respectively, relative to the respective control cells (Figure 8B). *DEFB1* mRNA levels were also suppressed in the MMA^{III} lineage cells on days 3, 6 and 9 after termination of treatment by 68% (p<0.001), 46% (p<0.01) and 42% (p<0.001), respectively, compared to control cells on the corresponding day (Figure 8C). By day 12, *DEFB1* expression in both As^{III} and MMA^{III} lineage cells was restored to that of control cells.

Arsenite exposure reduces intra- and extra-cellular HBD1 peptide levels

Since HBD1 is a secreted peptide, we measured its intra- and extra-cellular levels using ELISA following exposure of HK-2 kidney epithelial cells to As^{III} for 24, 48 or 72 h (Figure 9). As^{III} treatment significantly decreased intra-cellular HBD1 peptide levels in a dose-dependent (p=0.0032) but not time-dependent manner (Figure 9A). Exposure of HK-2 cells to 1 μM (black bars) resulted in up to 48% reductions in cell lysate levels of HBD1 peptides relative to control (white bars). Extra-cellular HBD1 peptide levels were significantly suppressed by the effect of dose, time and the interaction of dose and time (p<0.0001, p<0.0001, and p=0.0018, respectively; Figure 9B). The most substantial inhibition was observed with 1 μM at 72 h, when extra-cellular HBD1 peptides underwent a substantial 51% reduction (Figure 9B, black bars).

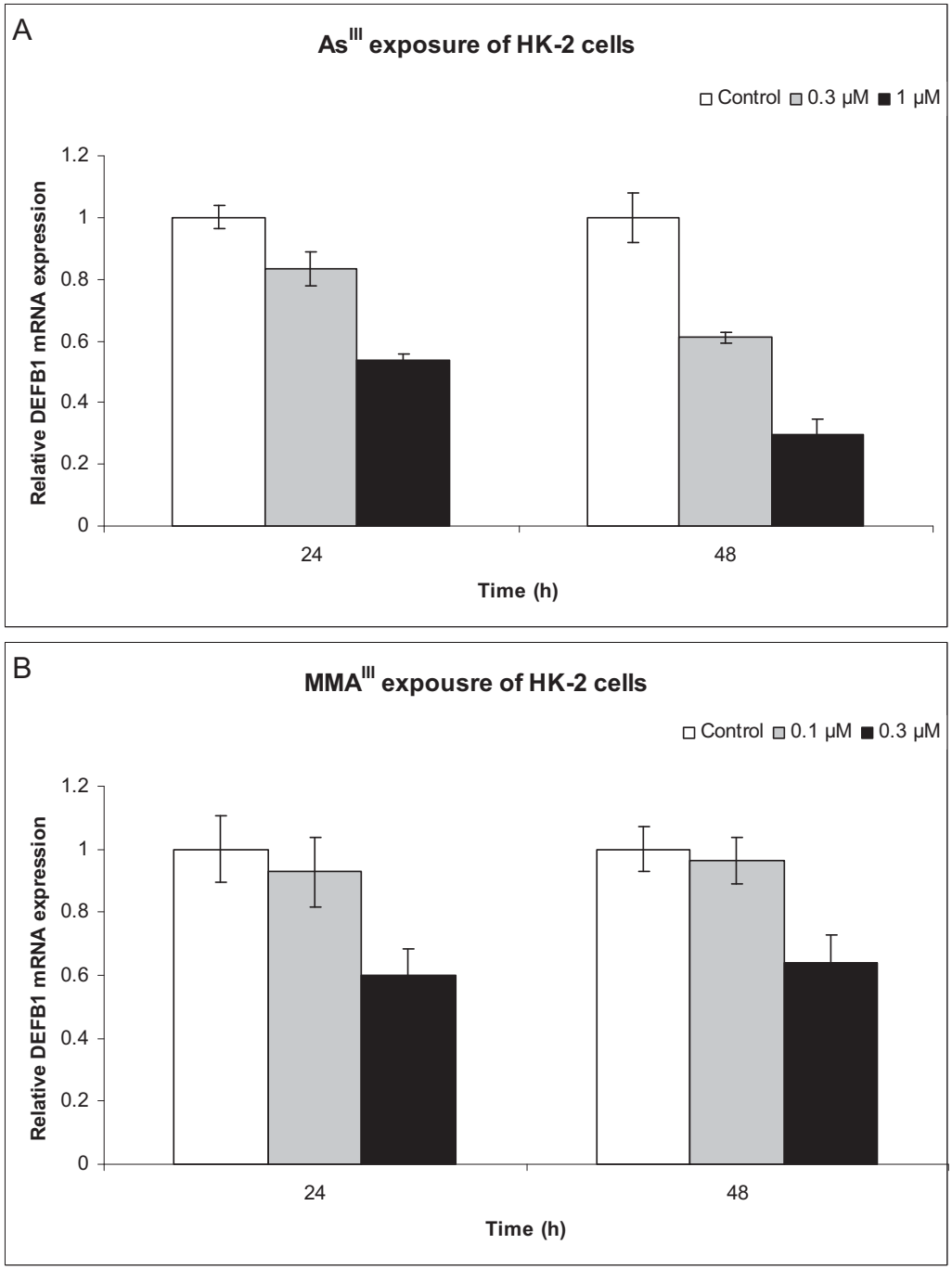
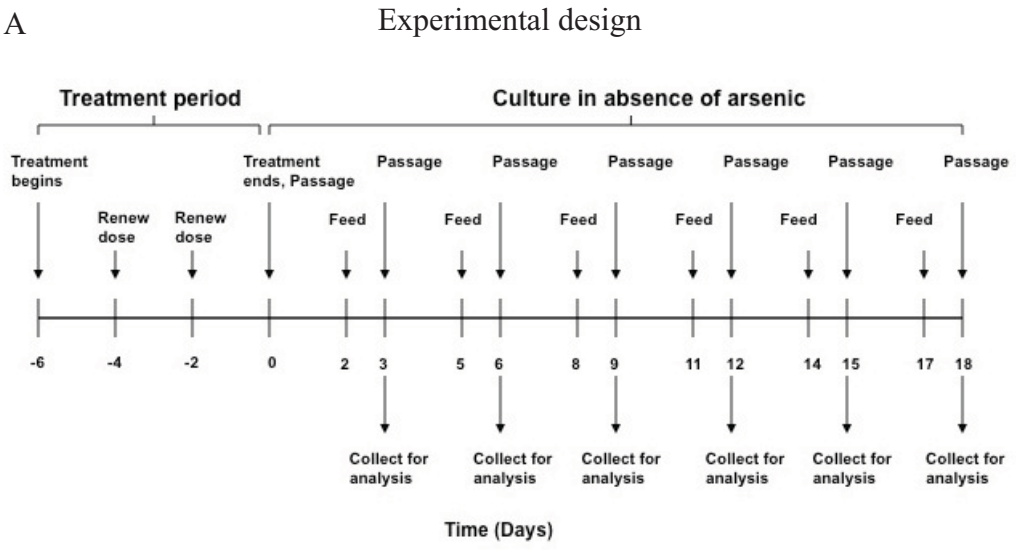
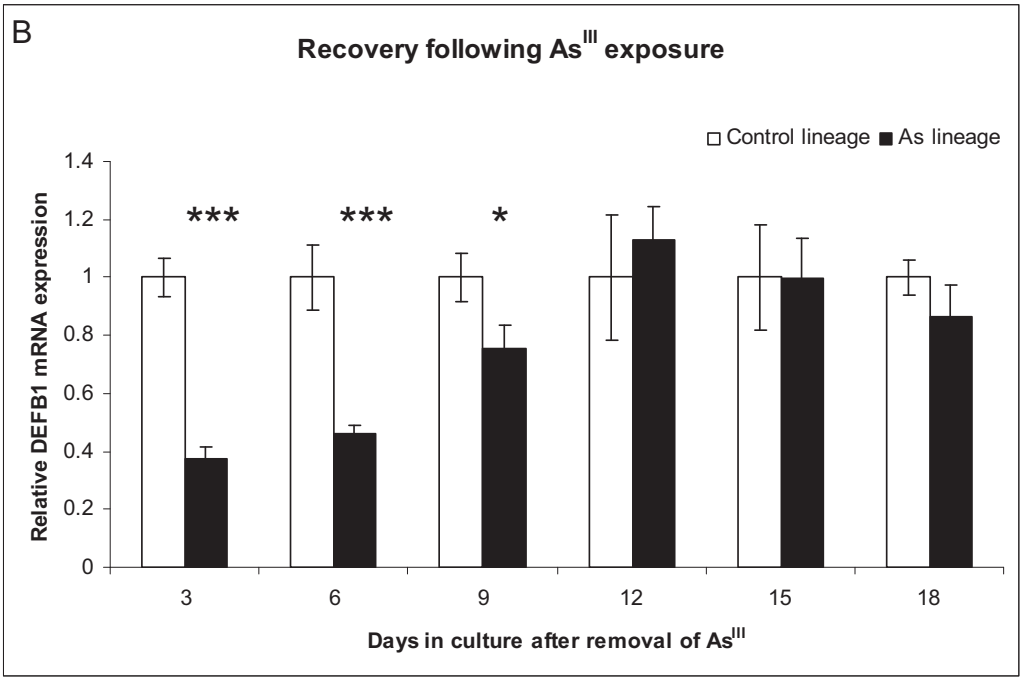


Figure 7. Arsenical-induced suppression of *DEFB1* mRNA expression in HK-2 kidney epithelial cells. *DEFB1* transcripts were quantified by relative quantitative-RT-PCR analysis following 24 h and 48 h exposure of HK-2 kidney epithelial cells to 0, 0.3 or 1 μM As^{III} (A) or 0, 0.1 or 0.3 μM MMA^{III} (B). Relative expression of *DEFB1* mRNA was normalized to *ACTB* and presented as a fold change relative to *DEFB1* expression in the respective untreated controls. Data represent the mean of two independent experiments consisting of three biological replicates (wells of cells), and PCR was performed in triplicate for each sample. Error bars represent SEM. Two-way ANOVA was used to estimate effects of dose and time followed by Bonferroni post

hoc tests for multiple comparisons. As^{III} induced significant time-dependent (p=0.0387) and dose-dependent (p<0.0001) decreases (A). MMA^{III} induced a significant dose-dependent decrease (p=0.0327) but had no time-dependent effect (B).

A





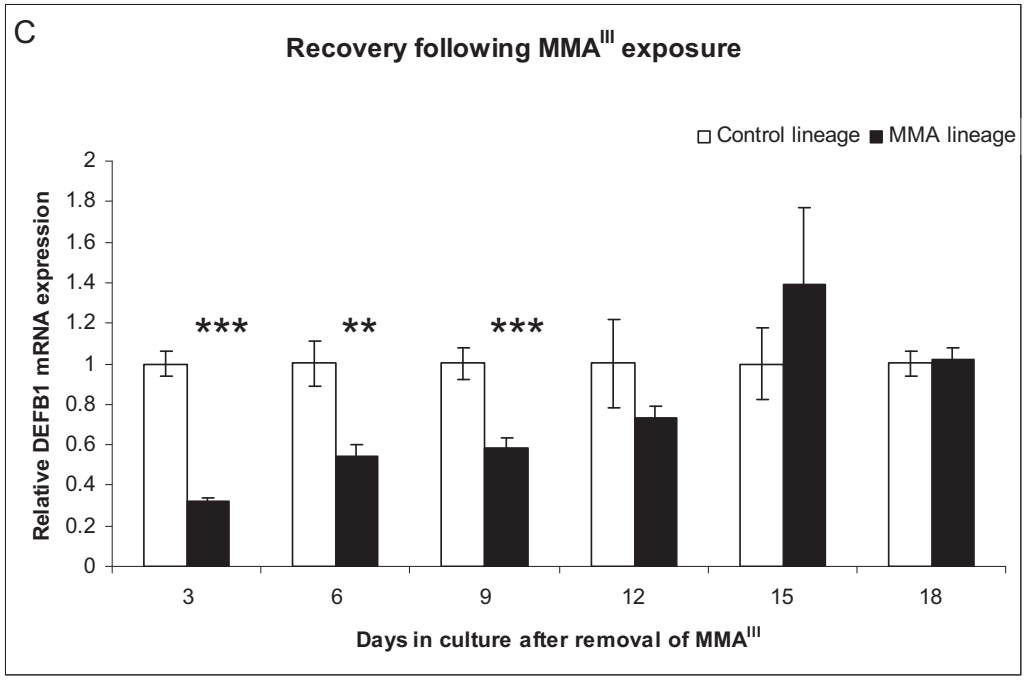


Figure 8. Persistence of *DEFB1* down-regulation in HK-2 kidney epithelial cells following cessation of 6-day arsenical treatments. Summary of experimental design for 6-day arsenical treatments and subsequent culture in the absence of As (A). HK-2 kidney epithelial cells were exposed to As for 6 days, with medium renewed every 2 days. At the end of treatment, As-containing medium was removed and cells were washed, seeded at equal densities and cultured in the absence of As for up to three weeks. Medium was renewed every 2 days, and on the third day cells were passaged or harvested. Relative quantitative-RT-PCR analysis was performed to quantify *DEFB1* transcript levels in HK-2 cells grown in As-free medium for several weeks following 6-day exposure to 1 μM As^{III} (B) or 0.3 μM MMA^{III} (C). Relative expression of *DEFB1* mRNA levels in As^{III} or MMA^{III} lineage cells (black bars) was normalized to *ACTB* and presented as a fold change relative to *DEFB1* expression in passage-matched untreated control cells (white bars). Data represent two experiments consisting of three replicates (wells of cells), and PCR was performed in triplicate for each sample. Error bars represent SEM. Unpaired two-tailed Student *t*-test was used to compare differences between control and As^{III} lineage cells or control and MMA^{III} lineage cells at each time point; $*p<0.05$, $**p<0.01$, $***p<0.001$.

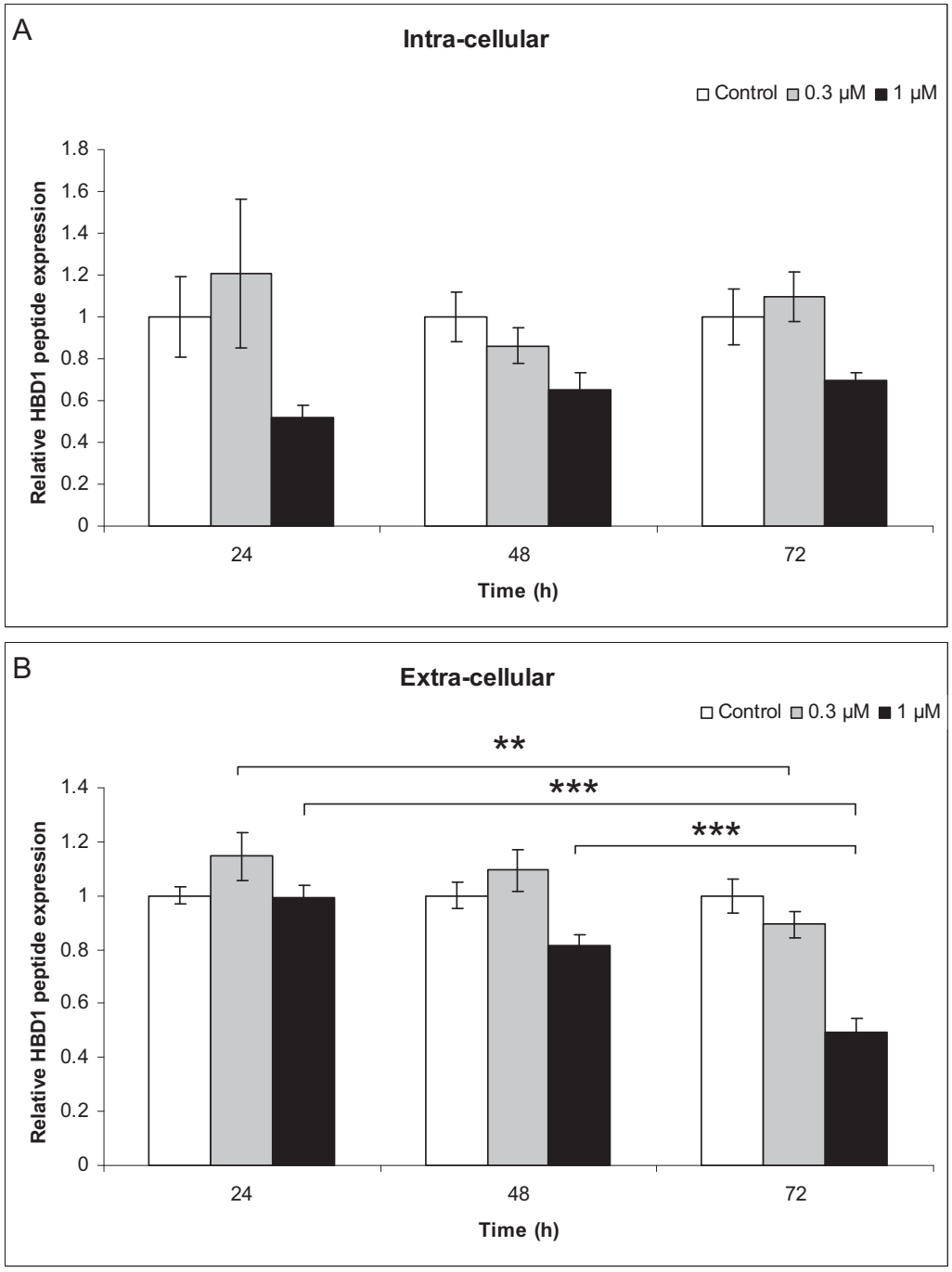


Figure 9. Arsenite-induced suppression of HBD1 peptide levels in HK-2 kidney cell lysates and cell culture media. HBD1 peptide expression in cell lysates (A) and cell culture media (B) was quantified by ELISA analysis following 24, 48 or 72 h exposure of HK-2 kidney epithelial cells to 0, 0.3 or 1 μM As^{III} (white, gray, and black bars, respectively). HBD1 peptide levels were normalized to cell lysate protein concentration or to cell density of each lysate (A) or medium (B) sample, respectively, and presented as a fold change relative to HBD1 peptide expression in the respective untreated controls. Data represent the mean of two or three independent experiments consisting of three or six replicates (wells of cells), and ELISA was performed in

triplicate for each sample. Error bars represent SEM. Two-way ANOVA was used to estimate effects of dose and time followed by Bonferroni post hoc tests for multiple comparisons; $^{**}p<0.01$, $^{***}p<0.001$. As^{III} significantly decreased intra-cellular HBD1 peptide levels in a dose-dependent ($p=0.0032$) but not time-dependent manner (A). Extra-cellular HBD1 peptide levels were significantly suppressed by the effect of dose ($p<0.0001$), time ($p<0.0001$) and the interaction of dose and time ($p=0.0018$) (B).

No observed effect of other metals on HBD1 peptide levels

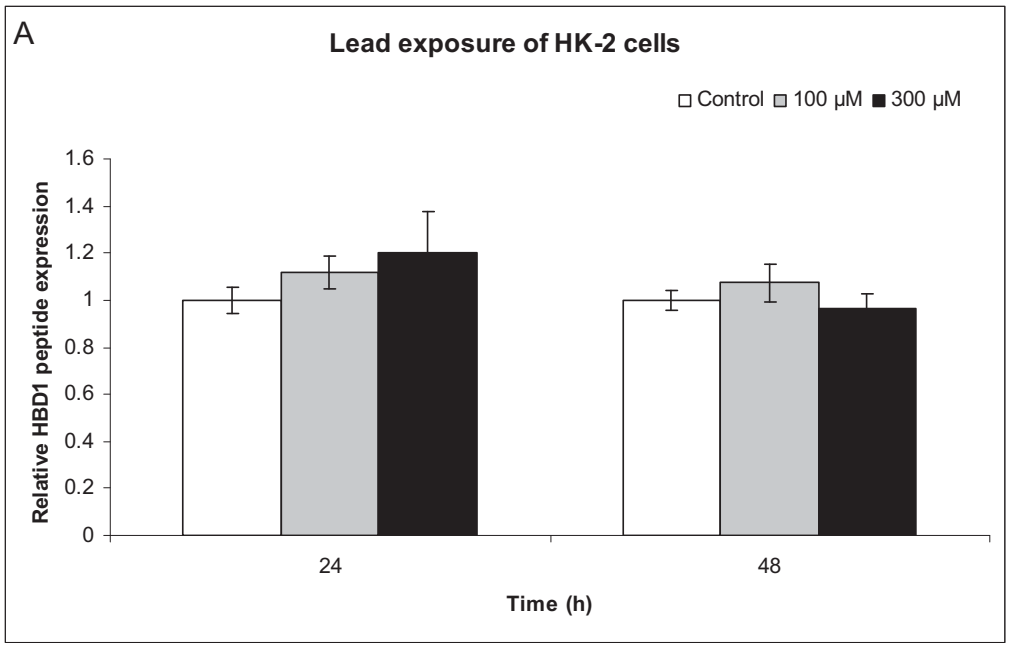
ELISA analysis was also performed to investigate the effects of other metals on HBD1 peptide levels. Exposure of HK-2 kidney epithelial cells for 24 or 48 h to chromium, cadmium or lead, at concentrations up to 1.5, 10 or 300 μM, respectively, had no effect on HBD1 intra-cellular peptide levels (Figure 10). These concentrations of chromium, cadmium and lead demonstrated the same capacity as 1 μM As^{III} to reduce viability of HK-2 kidney epithelial cells by 50% (data not shown). Because we used effective concentrations having similar effects as As^{III} with regard to cell viability and observed no effect on intra-cellular levels of HBD1 peptides, we did not proceed to quantify extra-cellular levels.

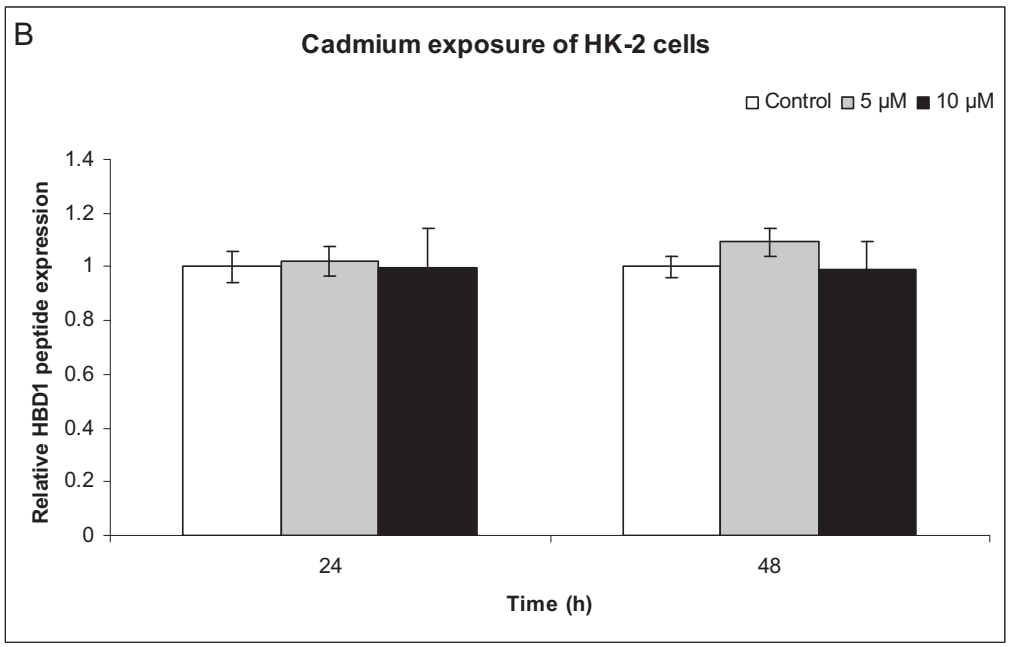
Arsenite exposure inhibits DEFB1 promoter activity

The effect of As^{III} treatment on *DEFB1* promoter activity was investigated to determine whether As suppressed *DEFB1* gene expression through transcriptional inhibition. For this, we transfected cells with an artificial construct containing the intact *DEFB1* promoter region upstream of the luciferase gene, which allowed us to test the ability of As to alter expression of the luciferase reporter driven by activity of the *DEFB1* promoter. Since our preliminary experiments indicated that transfection was cytotoxic to HK-2 cells, we were unable to use them for these experiments. Therefore, HeLa cells were transfected with the luciferase reporter construct and treated with As^{III} to assay luciferase activity. *DEFB1* promoter activity was normalized to renilla luciferase activity of the pRL-TK reporter plasmid, which was co-transfected and used as a loading control. There was a significant effect of dose, time and the interaction of dose and time on the inhibition of *DEFB1* promoter activity following exposure of HeLa cells to As^{III} ($p<0.0001$, $p<0.0001$ and $p=0.0027$, respectively; Figure 11). Treatment with 10 μM As^{III} (black bars) reduced *DEFB1* promoter activity by 14% at 3 h and 26% at 6 h compared to control (white bars).

Effect of arsenical exposure on DEFB1 transcript stability is inconclusive

RNA stability assays were performed to assess whether As also down-regulates *DEFB1* expression post-transcriptionally. For this, we incubated cells with actinomycin D, a potent nonselective inhibitor of transcription, to measure the stability of *DEFB1* mRNA in As-treated cells to investigate whether the decrease in *DEFB1* mRNA abundance is due to a decrease in mRNA stability. Relative quantitative-RT-PCR analysis was performed following exposure of HeLa cells or HK-2 kidney epithelial cells to actinomycin D, at the standard recommended concentration of 1 μg/mL, with or without As^{III} for up to 24 h. However, the ability of As to affect *DEFB1* transcript stability could not be determined because the cells could not withstand actinomycin D treatments long enough to calculate the half-life of *DEFB1* mRNA (Figure 12).


A
Lead exposure of HK-2 cells
Control
100 µM
300 µM
Relative HBD1 peptide expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
24
48
Time (h)


B
Cadmium exposure of HK-2 cells
Control
5 µM
10 µM
Relative HBD1 peptide expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
24
48
Time (h)

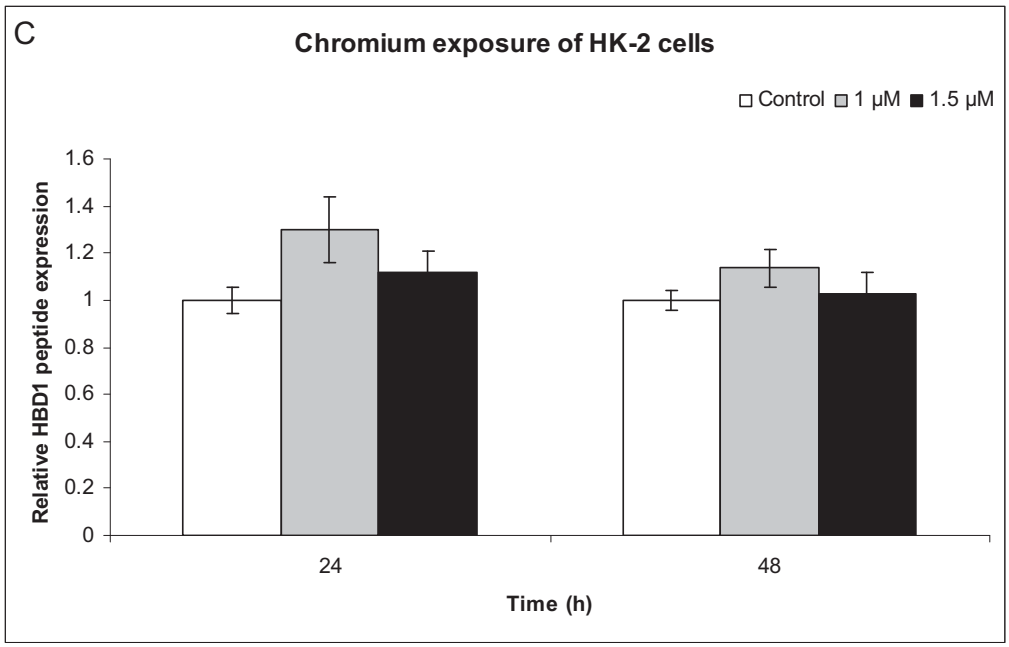


Figure 10. HBD1 peptide levels in HK-2 kidney epithelial cells are not affected by exposure to other metals. HBD1 peptide expression in cell lysates was quantified by ELISA analysis following 24 or 48 h exposure of HK-2 kidney epithelial cells to increasing concentrations of lead (A), cadmium (B), or chromium (C). HBD1 peptide levels were normalized to cell lysate protein concentration and presented as a fold change relative to HBD1 peptide expression in the respective untreated controls. Data represent the mean of two independent experiments consisting of two to six replicates, and ELISA was performed in triplicate for each sample. Error bars represent SEM. Two-way ANOVA was used to estimate effects of dose and time for each metal followed by Bonferroni post hoc tests for multiple comparisons. HBD1 peptides were not affected by exposure to lead, cadmium or chromium.

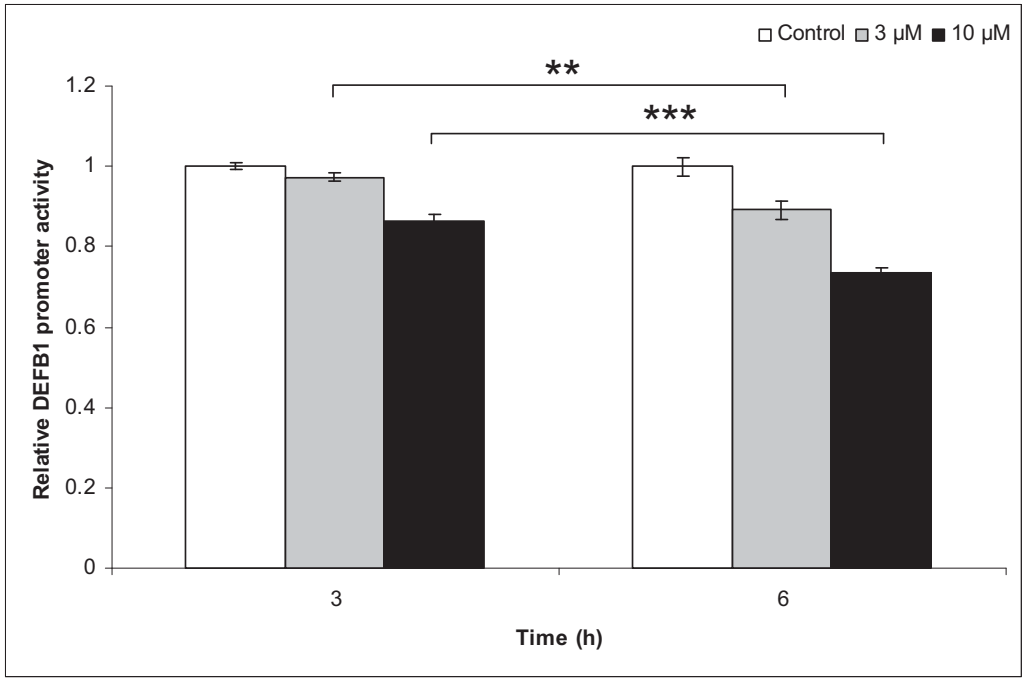


Figure 11. Arsenite-induced suppression of *DEFB1* promoter transcription. *DEFB1* promoter activity was measured via dual-luciferase analysis in HeLa cells co-transfected with DEFB1-luc and renilla pRL-TK plasmids following 3 or 6 h exposure to 0, 3 or 10 μM As^{III} (white, gray, and black bars, respectively). Relative DEFB1-luc firefly luciferase values were normalized to renilla luciferase values, and presented as a fold change relative to promoter activity in the respective untreated controls. Data represent the mean of two independent experiments consisting of three replicates, and dual-luciferase assay was performed in triplicate for each sample. Error bars represent SEM. Two-way ANOVA was used to estimate effects of dose and time followed by Bonferroni post hoc tests for multiple comparisons; $^{**}p<0.01$, $^{***}p<0.001$. There was a significant effect of dose ($p<0.0001$), time ($p<0.0001$) and the interaction of dose and time ($p=0.0027$) on the inhibition of *DEFB1* promoter activity following exposure to As^{III} (A).

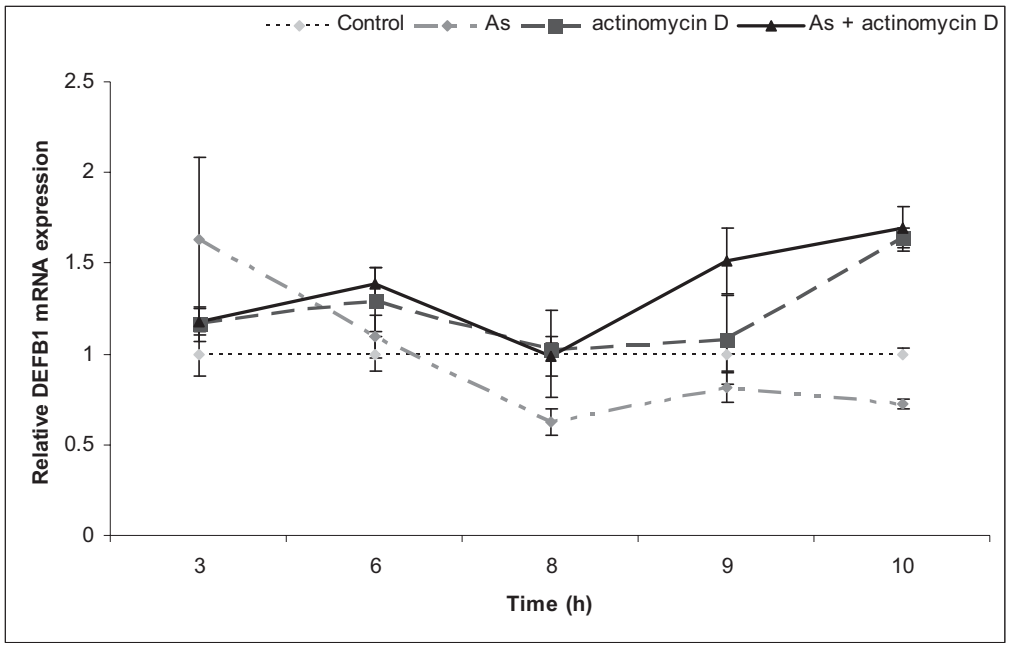


Figure 12. Effect of arsenical exposure on *DEFB1* mRNA stability is undetermined. *DEFB1* transcripts were quantified by relative quantitative-RT-PCR analysis following exposure of HK-2 kidney epithelial cells to 1 μg/mL actinomycin D with or without 1 μM As^{III} for up to 10 h. Relative expression of *DEFB1* mRNA levels was normalized to *ACTB* and presented as a fold change relative to *DEFB1* expression in the respective untreated controls. Data are representative of three independent experiments, and PCR was performed in triplicate for each sample. Error bars represent SEM. One-way ANOVA was used to compare effect of each treatment versus control followed by Bonferroni post hoc tests for multiple comparisons. There was no significant effect of any treatment relative to control. The half-life of *DEFB1* transcripts could not be calculated due to high stability following inhibition of transcription by actinomycin D, and exposures longer than 10 h were cytotoxic to cells. Thus, the effect of As on *DEFB1* mRNA stability could not be determined.

Discussion

The current study provides evidence that As down-regulates *DEFB1*, resulting in decreased mRNA and protein expression, through a mechanism involving transcriptional inhibition. Abundant *DEFB1* expression observed in the kidney cells compared to all other cell lines studied is consistent with reports of very high *DEFB1* expression in intact kidney (Zucht *et al.*, 1998; Hiratsuka *et al.*, 2000), providing further evidence that kidneys are the primary source of urinary HBD1 peptides (Valore *et al.*, 1998). This confirmation, together with the observed effects of As exposure on *DEFB1* mRNA and peptide secretion, suggests that kidney cells constitute sensitive targets of As toxicity and that the decrease in urinary HBD1 levels reported previously (Hegedus *et al.*, 2008) is likely the result of a direct effect of As on the kidney. However, it cannot be ruled out that As may similarly affect *DEFB1* expression in the bladder *in vivo*. Despite extremely weak expression of *DEFB1* in both bladder cell lines analyzed in our experiments, we note that *DEFB1* is expressed throughout the urinary tract, including the bladder (Zucht *et al.*, 1998; Hiratsuka *et al.*, 2000).

The marginal effect of As on HBD1 secretion during the first 48 hours of treatment is likely due to a lag time in turnover from the intra-cellular reductions in HBD1 peptides. The observed lack of effect of the other metals tested (lead, cadmium and chromium) on HBD1 peptide levels suggests that decreased HBD1 production may not merely be an indiscriminate effect of any metal(loid) or an oxidative stress or general stress response, and that HBD1 may indeed serve as a useful biomarker of response specific to As exposure. Other compounds will need to be tested to further examine the specificity of the response.

The association between As exposure and decreased *DEFB1* expression is intriguing given that HBD1 is constitutively expressed and secreted by epithelia of the skin, lung, and urogenital tract, all of which are targets of As carcinogenicity. Increasing evidence indicates that *DEFB1* may be a tumor suppressor gene: *DEFB1* is encoded on chromosome 8p23, which is frequently deleted in urological cancers and purported to contain multiple tumor suppressor genes (Ichikawa *et al.*, 1996; Knuutila *et al.*, 1999; Perinchery *et al.*, 1999); a significant cancer-specific loss of *DEFB1* mRNA and protein has been observed in oral squamous cell and urological carcinomas, respectively (Wenghoefer *et al.*, 2008; Joly *et al.*, 2009); and HBD1 has demonstrated cytotoxic and tumoricidal potential toward urological cancer cells (Sun *et al.*, 2006; Bullard *et al.*, 2008). HBD1 can also serve as a chemotactic attractant for immature dendritic cells and memory T-cells (Yang *et al.*, 1999). Collectively, these data suggest that HBD1 may contribute to host anti-tumor immunity and that loss of *DEFB1* expression may enhance tumor formation. It is therefore plausible that *DEFB1* down-regulation could play a role in As-induced carcinogenesis.

Our observation that acute exposure to As can have relatively long-term effects on gene expression is novel. Whereas other *in vitro* studies have shown that cells chronically exposed to As have altered transcriptional profiles and undergo malignant transformation (Su *et al.*, 2006; Jensen *et al.*, 2009a; Jensen *et al.*, 2009b; Wnek *et al.*, 2010), we show here that short-term, low-dose exposure to As has profound effects on *DEFB1* gene expression long after the exposure without any alterations in cell growth or morphology. Epidemiological studies in Chile indicate long latency patterns of increased mortality from kidney, bladder and lung cancers continuing for 25 or more years after As exposures began to decline (Marshall *et al.*, 2007; Yuan *et al.*, 2010). Early-life exposure to As also results in markedly higher mortality in adults from malignant and nonmalignant diseases (Smith *et al.*, 2006; Yuan *et al.*, 2010). Together with our data, these findings suggest that alterations in cellular function induced by As may be permanent (or semi-

permanent as shown in the current study) even after subsequent termination of the exposure and have long-term health consequences later in life.

The mechanisms responsible for heritable changes in gene expression involve either genetic or epigenetic phenomena (Arita and Costa, 2009). Growing evidence indicates that some of the toxicity and carcinogenicity of As is mediated by epigenetic mechanisms, including altered methylation patterns in histones and DNA (Reichard and Puga, 2010; Marsit *et al.*, 2006; Arita and Costa, 2009; Ren *et al.*, 2011). Gene silencing resulting from As exposure has been widely associated with hypermethylation of promoter regions, particularly in tumor suppressor genes (Chanda *et al.*, 2006; Marsit *et al.*, 2006; Chai *et al.*, 2007). Because DNA methylation plays a role in *DEFB1* down-regulation in urological cancer cell lines (Sun *et al.*, 2006), it is possible that As-induced decreases in *DEFB1* expression could involve a DNA methylation-dependent effect in the promoter region of *DEFB1* or its transcription factors. Although regulation of *DEFB1* transcription has not been fully elucidated, it is known that c-Myc and CLOCK:BMAL1 heterodimer promote its transcription and that CRY1 and PAX2 repress it (Sherman and Froy, 2008; Bose *et al.*, 2009). Hence, it is plausible that As may suppress *DEFB1* gene expression by altering the protein abundance and/or *DEFB1* promoter binding activity of one or more of these transcriptional regulators. As has been shown to both induce and suppress c-Myc expression (Deng *et al.*, 1999; Zhang *et al.*, 2011), making it difficult to hypothesize whether this factor may play a role here. Despite a lack of published reports of As down-regulating gene expression through decreased RNA stability, this possibility cannot as yet be ruled out. However, investigation of the mechanism(s) involved is beyond the scope of the current study since numerous possibilities exist. Thus, additional studies are warranted to elucidate *DEFB1* down-regulation by As.

In conclusion, we show that exposure to As down-regulates *DEFB1* expression through a mechanism involving transcriptional inhibition, and that short-term, low-dose As exposure has relatively long-term effects on *DEFB1* mRNA expression. We also demonstrate that the decrease in HBD1 peptides may be a response specific to exposure to As and not other metals, including lead, chromium and cadmium. Further investigation into the underlying mechanisms involved will be the basis for future study.

General conclusions

As discussed in Chapter 1, As exposure is a serious global public health concern associated with a multitude of adverse health effects, including cancer, and over 100 million people worldwide are chronically exposed to As through ingestion of contaminated drinking water. Although the toxic effects of As exposure are well studied and a growing body of evidence implicate its capacity as a potent immunomodulator, its mechanisms remain poorly understood. A previous study from our laboratory reported decreased urinary levels of HBD1 peptides in two independent As-exposed populations. Therefore, this dissertation contributes to the field a thorough literature review of As immunotoxicity and direct investigation of the molecular toxicity of As by establishing an *in vitro* model system to characterize its effects on *DEFB1*, a putative tumor suppressor gene and early biomarker of As effect.

Chapter 2 describes the immune-related effects of As exposure in humans, animals and *in vitro* models. The data reveal that chronic exposure to As can severely impact functional immune responses and compromise overall immune health. As exposure has been shown to induce inflammation, oxidative stress and apoptosis in lymphocytes, suppress T-cell activation and proliferation, impair macrophage function, and alter various other aspects of cellular and humoral immunity. Such compromises in immune function could lower host resistance to infections and chronic diseases, as seen in reduced clearance of microbial pathogens in animal studies and elevated risk of infections and cancers in human populations chronically exposed to As. However, inconsistencies in epidemiological studies illustrate the need for further investigation to better understand the relationship between As exposure and disease progression, as outlined in several recommendations proposed in Chapter 2.

In Chapter 3, characterization of the relationship between As exposure and *DEFB1* is described. Extensive analyses of the influence of As on *DEFB1* were performed using immortalized non-tumorigenic human cells derived from target tissues of As toxicity, namely skin and kidney epithelia. *DEFB1* mRNA levels were more abundant in HK-2 kidney epithelial cells than in HOK-16B keratinocytes, consistent with reports of very high *DEFB1* expression in the intact kidney, and were suppressed by treatments with As^{III} and its metabolite MMA^{III}. *DEFB1* transcript levels remained suppressed for several passages after removal of As^{III} or MMA^{III} from the cell culture media, suggesting a relatively long-term effect of As on *DEFB1*. MMA^{III} did not appear to be more potent than As^{III} at inhibiting *DEFB1*, despite reports of its greater toxicity. HBD1 peptides were significantly reduced following exposure to As, but were not affected by treatment with lead, cadmium or chromium, suggesting that decreased HBD1 may be a specific response to As. As^{III} treatment was found to suppress *DEFB1* promoter activity, indicating that the inhibition of *DEFB1* mRNA by As likely involves transcriptional down-regulation. However, it is undetermined whether As-induced suppression of *DEFB1* gene expression also involves a post-transcriptional mechanism because the transcript stability could not be measured.

Taken together, this dissertation establishes HK-2 kidney epithelial cells as a relevant *in vitro* model system to investigate effects of As on *DEFB1*, strengthens the development of HBD1 for use as an early marker of biological effect of As, and proposes a potential role for HBD1 in As toxicity within the broader context of host anti-tumor immunity. Therefore, the goal of this research is to help increase our understanding of the biological mechanisms of As toxicity and carcinogenicity.

Future perspectives

The findings from this dissertation open up several lines of inquiry. For example, what are the molecular targets of As-induced transcriptional down-regulation? Is epigenetic modification involved? Is HBD1 consistently decreased in target tissues of As-exposed human subjects? How specific is the response to As? What role does decreased *DEFB1* expression play in As toxicity? Many of the molecular mechanisms can be further explored using the *in vitro* model system established in this dissertation.

Further investigation is needed to address the molecular targets involved in *DEFB1* inhibition by As. Such studies would be required to characterize the As-responsive region within the *DEFB1* promoter leading to transcriptional down-regulation. This could be facilitated via luciferase reporter assays using constructs containing truncated regions of the *DEFB1* promoter. Such analyses have been used to identify DNA binding sites that regulate *DEFB1* transcription (Sherman and Froy, 2008; Sun *et al*., 2006). The mutant constructs for which As treatment fails to inhibit promoter activity would reveal the site(s) in the promoter responsible for As down-regulation of *DEFB1* transcription. These steps would help identify candidate transcription factors based on the consensus sequence sites within the responsive region of the promoter. Chromatin immunoprecipitation (ChIP) analyses could then be used to determine the transcription factors specifically targeted by As treatment by characterizing the transcription factor-DNA binding interactions within the As-regulated region in the *DEFB1* promoter. Although regulation of *DEFB1* transcription is not well understood, it is known that c-Myc and CLOCK:BMAL1 heterodimer promote its transcription and that CRY1 and PAX2 repress it (Sherman and Froy, 2008; Bose *et al.*, 2009). Thus, it is plausible that As may inhibit *DEFB1* gene expression by altering the *DEFB1* promoter binding activity of any of these regulators.

Epigenetic modification is emerging as an important mechanism by which As alters DNA binding of transcription factors and hence gene expression. Hypermethylation of promoter regions, particularly in tumor suppressor genes, is widely associated with As-induced gene silencing (Mass and Wang, 1997; Chanda *et al.*, 2006; Marsit *et al.*, 2006a; Chai *et al.*, 2007). Furthermore, DNA methylation has been shown to play a role in the down-regulation of *DEFB1* in urological cancer cell lines (Sun *et al.*, 2006). Therefore, it is possible that As-mediated suppression of *DEFB1* could involve an epigenetic-dependent effect. Use of chromatin-modifying agents could help address these questions. For example, the ability of the demethylating agent, 5-aza-deoxycytidine, to restore *DEFB1* gene expression in As-treated cells could be tested to examine whether DNA methylation is involved. If results are positive, then methylation analysis of the *DEFB1* promoter could be performed following As exposure of cells. Likewise, histone deacetylase inhibitors could be used to examine whether alterations of histones are involved, and ChIP analysis could be used to identify loss of active marks or appearance of silencing marks in histones. Knowledge of the molecular mechanisms involved in the down-regulation of *DEFB1* by As could be potentially powerful in the development of diagnostic and therapeutic tools for exposed subjects.

Confirmatory evidence of As-associated reduced levels of HBD1 peptides is needed from other population-based studies with larger sample sizes. Other studies have reported decreased serum or plasma levels of peptides in the 3-5 kDa range but, unlike Hegedus *et al*. (2008), did not identify the proteins (Zhai *et al.*, 2005; Harezlak *et al.*, 2008). Samples from subjects having low, intermediate and high exposure levels should be analyzed to determine whether there is a dose-response relationship between As exposure and HBD1 levels. It would also be useful to monitor the relationship between changes in As exposure and HBD1 levels to determine the temporal nature of the association given that our *in vitro* recovery experiments suggest a

somewhat long-term effect of As exposure on *DEFB1* expression and epidemiological observations indicate long latency patterns of increased mortality from cancers for several decades after exposures ended. Further, various biological specimens should be analyzed to determine whether As inhibits HBD1 in tissues and fluids besides urine, including saliva, blood and respiratory secretions.

The previous findings by Hegedus *et al*. (2008) of decreased urinary HBD1 peptide levels in men but not women in both study populations are consistent with previous reports of higher bladder cell micronuclei frequency in males than in females with high versus low As exposure (Warner *et al.*, 1994; Moore *et al.*, 1996; Moore *et al.*, 1997). Further evidence supporting increased susceptibility to As toxicity in men is provided by reports of much greater frequency of skin lesions in males relative to females (Haque *et al.*, 2003; von Ehrenstein *et al.*, 2005), and pronounced reductions in pulmonary function related to long-term ingestion of As in men compared to women (von Ehrenstein *et al.*, 2005). Higher urinary levels of HBD1 peptides have been observed in men than in women (Valore *et al.*, 1998), thus it is possible that there may be a sex-specific effect of As on HBD1. However, because the samples analyzed by Hegedus *et al*. (2008) were relatively small, the association between As and HBD1 in men but not women must be further evaluated. Although we found no apparent difference in potency between As^{III} and MMA^{III} in our cell culture systems, further investigation should look into the influence of metabolism of As on HBD1 expression given epidemiological and *in vitro* evidence supporting MMA^{III} as the most toxic species. For example, studies could look for correlation between urinary As metabolites and HBD1 levels.

To address whether decreased HBD1 is a biomarker exclusive to As, a battery of other compounds would need to be tested. Various classes of chemicals could be assayed *in vitro*, including metals, immunotoxic agents and carcinogens, for example. Such testing could therefore help characterize the HBD1 response. These findings could then be confirmed in epidemiological studies of persons exposed to various chemicals or contaminants. Regardless of the specificity of the response to As, *DEFB1* could serve as a potential therapeutic target given evidence of its potential role in pulmonary and anti-tumor immunity.

Ultimately, functional studies (in animals) and monitoring studies (in human subjects) would be needed to address the consequence of loss of *DEFB1* expression with respect to the prevalence of As-associated toxicity and disease.

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