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MECHANISMS OF LOW DOSE ARSENIC LYMPHOID STEM CELL IMMUNOTOXICITY AND POTENTIAL INTERACTIONS WITH DIBENZO[def, p]CHRYSENE (DBC)

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by

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DEDICATION

This work is dedicated to my late mother Grace Ngozi Okechukwu, and my father Reuben Okechukwu Onuoha, who gave me their all, and taught me the importance of education, focus, and dedication to work. Thank you for teaching me how to succeed in life. To my late sister, Angela, who encouraged me, believed in me, wished the best for me and unceasingly prayed for me until her death. Akubueze, I wish you were here to take part in this accomplishment. To the rest of my siblings: Aunty Chinwe, IK, Ugboaku, Glory, Gabriel, Obi, Chimeze, and Nnanna whose tremendous support in various ways sustained me throughout this period.

I also dedicate this work to the East Ridge Ward Latter-day Saints whose love, care and support helped me to cope with several challenges that came my way during this period.

To Dr. Stella G. Uzogara, my long-time mentor, teacher and adviser, for her encouragement and contributions toward my academic successes. I am very grateful.

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Mechanisms of Low Dose Arsenic Lymphoid Stem Cell Immunotoxicity and Potential Interactions with Dibenzo[def, p]chrysene (DBC)

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ABSTRACT

In vivo assessment of the effects of inorganic arsenic, arsenite (As^{+3}), on the bone marrow (BM) immune progenitor cells in mice showed that 300 parts per billion (ppb) As^{+3} given over 30 days in drinking water, produced a decrease in total BM cells recovered and suppressed the lymphoid (pre-B) but not the myeloid (GM) progenitor cells colony forming units (CFUs). 75 and 300 ppb As^{+3} also suppressed spleen T-dependent antibody responses. There was no shift in the general markers of early hematopoietic stem cells, CD34 and CD38, or the mesenchymal stem cells, CD105, in the BM. Splenic $CD45^{+}$ B cells were unaltered. *In vitro* studies of three inorganic arsenic species and metabolites, As^{+3}, As^{+5}, and monomethylarsonous acid (MMA^{+3}) at low concentrations (5 - 500 nM) determined that As^{+3} selectively suppressed the lymphoid progenitors at 50 and 500 nM concentrations. MMA^{+3} (starting at 5nM) also demonstrated concentration-dependent lymphoid toxicity.

Dibenzo[def, p]chrysene (DBC) was also suppressive to the BM *in vivo,* producing about 90% suppression of the lymphoid progenitors at 10 mg/kg given orally over 5 days in

pill forms. At much lower (1 mg/kg), 5 day oral cumulative dose, DBC slightly decreased BM cell recovery, resulting in suppression of CFU-B but not CFU-GM.

As environmental co-contaminants, As^{+3} and DBC co-exposure could easily occur. Results from this research suggest that low dose DBC interacts with established no-effect doses (19 and 75 ppb) of As^{+3} to produce more BM lymphoid suppression that does not necessarily increase with dose *in vivo. In vitro* studies show similar pattern for As^{+3}-DBC interactions, occurring at lower (0.5 nM), but not at the higher (5 nM) concentration of As^{+3}.

The selective targeting of lymphoid progenitors by low dose As^{+3} and DBC suggest the involvement of an exclusive signaling pathway. B cells require IL-7 signaling for normal development. This study has uncovered the MMA^{+3} interference with phosphorylation of STAT5 in the IL-7 signaling pathway, and with the induction of *PAX5* gene which is required to maintain commitment to the B-lineage.

In summary, these studies contribute to our understanding of BM toxicity by environmentally relevant levels of arsenite.

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CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

The Bone Marrow, Hematopoiesis, Host Immunity and the Environment

Bone marrow (BM), the mass of soft tissue found in the inner core of axial and long bones, is a primary lymphoid and hematopoietic organ (Travlos, 2006). Having various structural components with specialized functions, the BM tissue consists mainly of the red marrow (red- colored hematopoietic tissue) and yellow marrow (yellow-colored adipose tissue). At birth, the BM is mostly red marrow and this continues to be replaced by yellow marrow through adulthood (Moore and Dawson, 1990). The function of hematopoietic tissue is to produce the immune cells by the process of hematopoiesis. This tissue is made up of hematopoietic stem cells (HSCs), stem cell derivatives including the immune cells and blood, and stromal cells which are not directly involved in the production of blood and immune cells. The stromal cells, however, provide the microenvironment necessary for maintenance and self-renewal of the stem cells (Gasper, 2000), and also produce vital growth factors, such as cytokines, which are required for lineage commitment, differentiation, maturation, viability and proliferation of the immune cells (Chen et al., 2001).

All immune cells originate from one pluripotent hematopoietic stem cell stage and then commit to specific lineages by responding to the lineage-appropriate growth factors. While in the BM, immune cell precursors express receptors for signaling the lineage-specific growth factors. From the pluripotent hematopoietic stem cells, the common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs), with their receptors, respond (in addition to other common factors) specifically to granulocyte monocyte cell stimulating factor (GM-CSF) and interleukin 7 (IL-7), respectively. They then undergo further

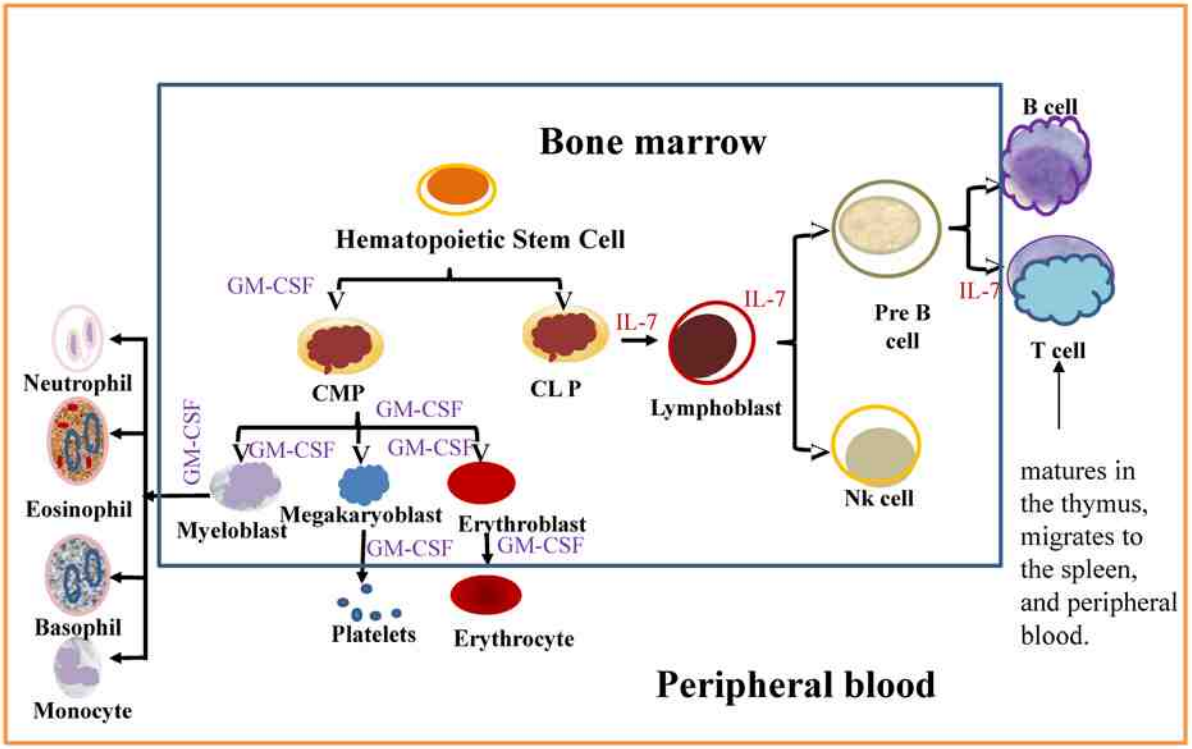


Figure 1.1. The Bone marrow and Hematopoiesis.

differentiation to produce the mature cells that are released into the blood and periphery (Figure 1.1). The BM has extensive vascularity, thus the red and yellow marrow have rich blood supply and could easily be exposed to endogenous and exogenous compounds that are transported in the blood. Studies have shown that the BM hematopoietic tissue and immune cell precursors can be very sensitive to substances that are carried in the blood. The immune cells have the primary responsibility of disease prevention and their differentiation through the lymphoid and myeloid lineages in the BM (Fig 1.1) could suffer the initial impact from environmental pollutants prior to disease manifestation. The myeloid and lymphoid progenitors produce the cells responsible for adaptive and innate immune responses respectively.

Lymphopoiesis and IL-7 Signaling

To understand the potential effects of environmental agents on adaptive and innate immunity, it is important to understand how the immune cells develop in the BM. The common lymphoid progenitors become differentiated under the specific influence of BM growth factors. For myeloid cells, GM-CSF is required for development, whereas lymphoid pre-B and pre-T cells develop in response to IL-7 (Figure 1. 2). IL-7 is a major cytokine of the immune system. It is vital for normal development of B cells and T cells, and is produced by the stromal cells of the BM and various other tissues, but lacking in normal lymphocytes (Golden-Mason et al., 2001; Gutierrez-Ramos et al., 1992; Sakata et al, 1990, Wiles, et al., 1992). In the various IL-7- producing tissues, it is known to bind to the molecules of extracellular matrix such as fibronectin and glycosaminoglycan and is therefore readily available in the microenvironment for signaling (Ariel et al., 1997). IL-7 receptors consist of the IL-7Rα (CD127) which is specific for IL-7, and IL-7Rγc chain that is shared by other

cytokines including IL-2, IL-4, IL-9, IL-15 and IL-21. Immature B cells express both components of IL-7R. When the ligand binds to CD127, it elicits a signaling response. Studies show that deletion of IL-7 inhibits the differentiation from pro-B cells to the pre-B cell stage (von Freeden-Jeffry et al., 1995), whereas deletion of the receptor, CD127, results in the inhibition of differentiation from the CLPs to the pre-pro B stage (Peschon et al., 1994). For signaling, IL-7 ligand binds to the CD127 of the IL-7- responsive immature lymphoid cells, and results to the dimerization of CD127 and IL-7Rγc chain. The Janus kinase 3 (JAK3) which is constitutively associated with the tyrosine of IL-7Rγc chain, phosphorylates the CD127-associated Janus kinase 1 (JAK1). It is JAK1 that recruits other intracellular signaling molecules, including the transcription factor, signal transduction activator of transcription 5 (STAT5), phosphotidylinositol 3 kinase (P13K), and Src kinase. CD127 does not have tyrosine kinase activity. Activated STAT5 molecules dimerize and translocate to the nucleus to regulate the transcription of downstream lymphoid development-associated genes (Figure 1.2).

Individual evaluation of the myeloid and lymphoid progenitor cell responses in the BM could therefore reveal an aspect of the immune system that may be compromised from environmental pollutant exposure. Alterations in BM differentiation of lymphoid and myeloid cells will likely adversely affect host immunity, resistance to infections, and could lead to cancer.

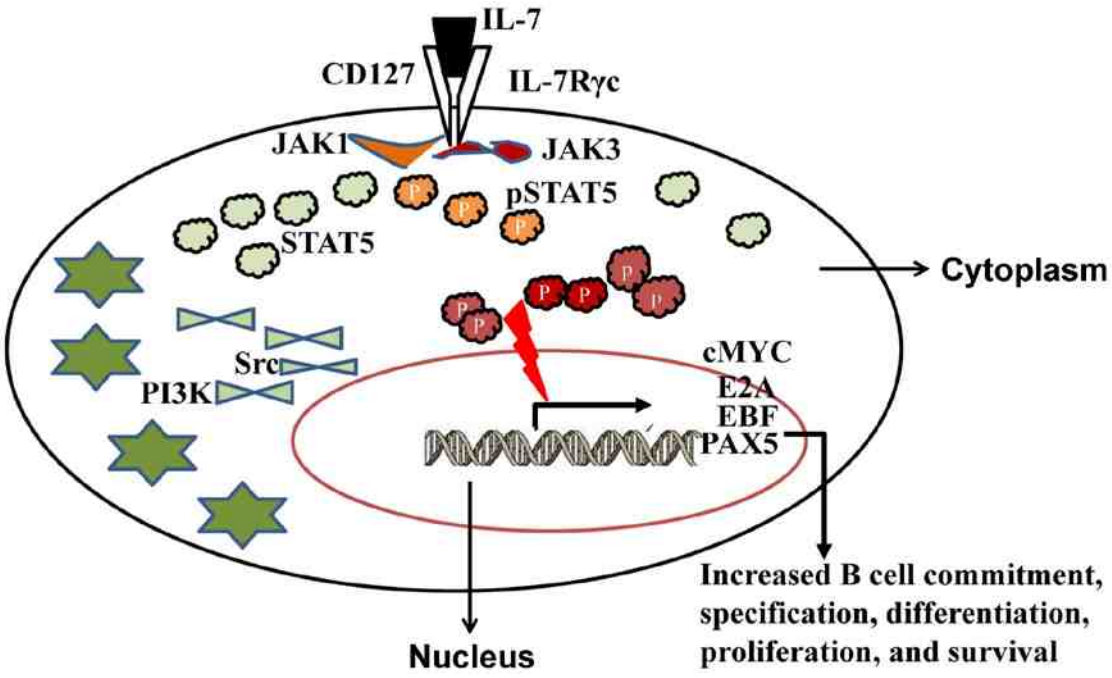


Figure 1.2. Scheme for IL-7 signaling in B cells

Environmental Chemicals Alter Host Immunity and BM Development

There is evidence that many environmental chemicals and drugs alter BM hematopoietic activity (Irons et al, 1985). Certain drugs, malnutrition, alcohol, and chemotherapy are known to suppress the BM (Cunha, et al., 2013, Fantuzzi and Faggioni, 2000; Scharf and Aul, 1988; Sommadossi and Carlisle, 1987). Exposure to pollutants such as benzene and PAHs (including benzo[a]pyrene, dimethylbenz[a]anthracene (DMBA)), or complex mixture of pollutants like cigarette smoke which contain PAHs, As^{+3} and other chemicals have been shown to produce bone marrow toxicity that manifest as alteration in peripheral blood cell populations (Burchiel et al., 1987; Galvan et al., 2003, 2005: Heidel et al., 1998; Irons, 1985; Page et al., 2003).

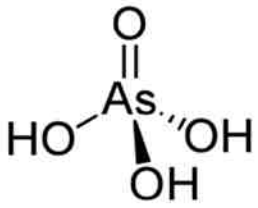
Polycyclic Aromatic Hydrocarbons (PAHs) and Immunotoxicity

PAHs are a class of compounds containing 2 or more benzene rings. They all differ in size, structure, and toxicity. The smaller size PAHs are more prevalent as they are formed at lower temperatures (300-650^{o}C) than the large PAHs which require much higher temperatures to produce. PAHs have also been detected in barbeque grilled foods.

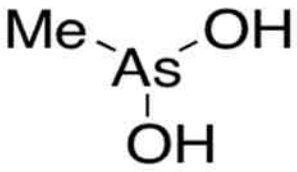
PAHs may leach into drinking water supplies, settle on the ground, and/or circulate in the air when they are released by activities such as forest fires, coal burning, and cigarette smoking. Some PAHs are known carcinogens, and as with arsenic, humans, mammals, plants, and aquatic life are exposed to PAHs. The average level of PAHs in urban drinking water supplies is in the range of 0.1-23 ng/L (Harvey et al., 1997). PAHs in the environment have been shown to produce BM toxicity through several mechanisms such as induction of pre B cell apoptosis due to the activation of caspases, interference with p53 signaling, and aryl hydrocarbon receptor (AHR) with cytochrome p450 1 (CYP1) enzymes induction.

(Heidel et al. 1998, 1999, 2000; Galvan et al., 2003, 2006; Page et al., 2003; N'jai et al, 2010) and systemic immunotoxicity (Burchiel et al., 2005; White Jr. et al., 1985). Our laboratory has shown that 7,12-dimethylbenz(a)anthracene (DMBA) produces immunotoxicity via the formation and binding of a DMBA-diol-epoxide to DNA, leading to the activation of ATM/ATR and p53 pathways (Gao et al., 2005, 2007, 2008).

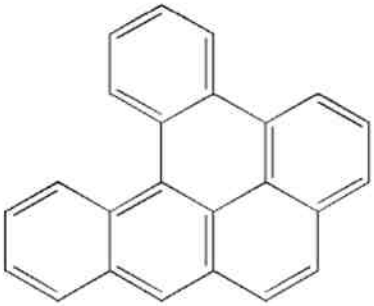
Although DMBA has been found to suppress pre-B cell formation, in mouse BM, its mechanism of action has not been well-defined.



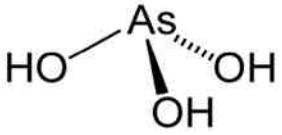
As^{+5}



MMA^{+3}



DBC



As^{+3}

Figure 1.3. Structures of Arsenate (As^{+5}), Monomethylarsonous Acid (MMA^{+3}), Dibenzo[def,p]Chrysene (DBC) and Arsenite (As^{+3}).

This study focuses on dibenzo[def,p]chrysene (DBC, also known as dibenzo[a,l]pyrene (DB[a,l]P)) as the PAH of interest because it is an important environmental PAH which occurs in wood smoke or smoky coal and is released into the air. In the United States, the typical range for few carcinogenic PAHs in the air is 0.1-1 ng/ m^3 (Menichini, 1992). High levels (about 5 ng/m^3) of DBC was detected in the indoor air in Xuan Wei homes in China, an area that also has very high incidence of lung cancer mortality (Mumford et al., 1995). DBC has not been previously studied for immunotoxicity on BM. However, our lab has recently shown that it is very immunotoxic to spleen cells (Lauer et al., 2013) and it is also known to be a potent carcinogen (Buters et al., 2002: Luch et al., 1998). DBC is a very large molecule consisting of six fused benzene rings (Fig 1.3), with an overall structure that is related to its toxicity in tissues. Its occurrence in nature is very limited due to its large size and the temperature requirement for its formation. DBC is thought to be the most potent carcinogenic PAH (Cavalieri et al., 1991).

Generally, it has been shown that long term or chronic exposures to PAHs produce decrease in immune function as measured by various immune parameters as shown in (Table 1.1). The suppression of humoral immune responses in mice (No. 2, Table 1.1) is supported by the observed decrease in immunoglobulin levels seen in PAH-exposed coke oven workers (No. 1 in Table 1.1). The DMBA –induced apoptosis in pre B cells is also suggestive of PAH toxicity to developing immune progenitors.

Table 1.1: Evidence for PAH immunosuppression

	Evidence/model	Parameter measured	Reference
1.	Decreased humoral immunity in PAH - exposed coke plant workers/ Human	Concentrations of Immunoglobulins G, A, M, and E	Szczeklik et al., (1994).
2.	Suppressed humoral immunity in mice exposed to various PAHs/ Mice	Antibody-forming cell response to sheep erythrocytes	White et al., (1985).
3.	Increased B cell death in mice BM following exposure to DMBA/ Mice- *in vitro,* cell line	B cell apoptosis	Mann et al., (1999).
4	Alteration in blood monocyte functional differentiation and maturation in PAH-exposed PBMC/ Human	Dendritic cell markers:CD1a, CD40, and CD80	Laupeze et al., (2002).

DBC Biotransformation and Toxicity

Studies indicate that DBC metabolism is catalyzed by two enzymes: Cytochrome P450 1B1 (CYP1B1) and microsomal Epoxide Hydrolase (mEH) (Amin et al., 1995; Buters et al., 2002; Chen et al., 2012; Crowell et al., 2014; Luch et al., 1998; Ralston et al., 1994), and sometimes by peroxidases (Cavalieri et al., 1991), (Figure 1.4). The action of CYP1B1 and Epoxide Hydrolase on DBC produces DBC-dihydrodiol which could be further oxidized in the presence of CYP1B1 to DBC-dihydrodiol epoxide (DBCDE), known to bind covalently to DNA resulting in DNA adduct formation, the genotoxicity property of DBC. DBC-dihydrodiol could also be reduced to catechol by the enzyme, aldoaketoreductase (AKR), for further breakdown to DBC-quinones (Courter et al., 2007). The formation of quinones is associated with redox cycling and generation of reactive oxygen species (ROS) with resultant oxidative stress. Activation of DBC by peroxidases result in the formation of a radical cation which forms depurinating DBC-dG or dA adducts on DNA. Exposure to DBC has been associated with immunosuppression, hematotoxicity, and carcinogenesis (Harvey et al., 1997; Li et al., 2010; N'jai et al., 2010).

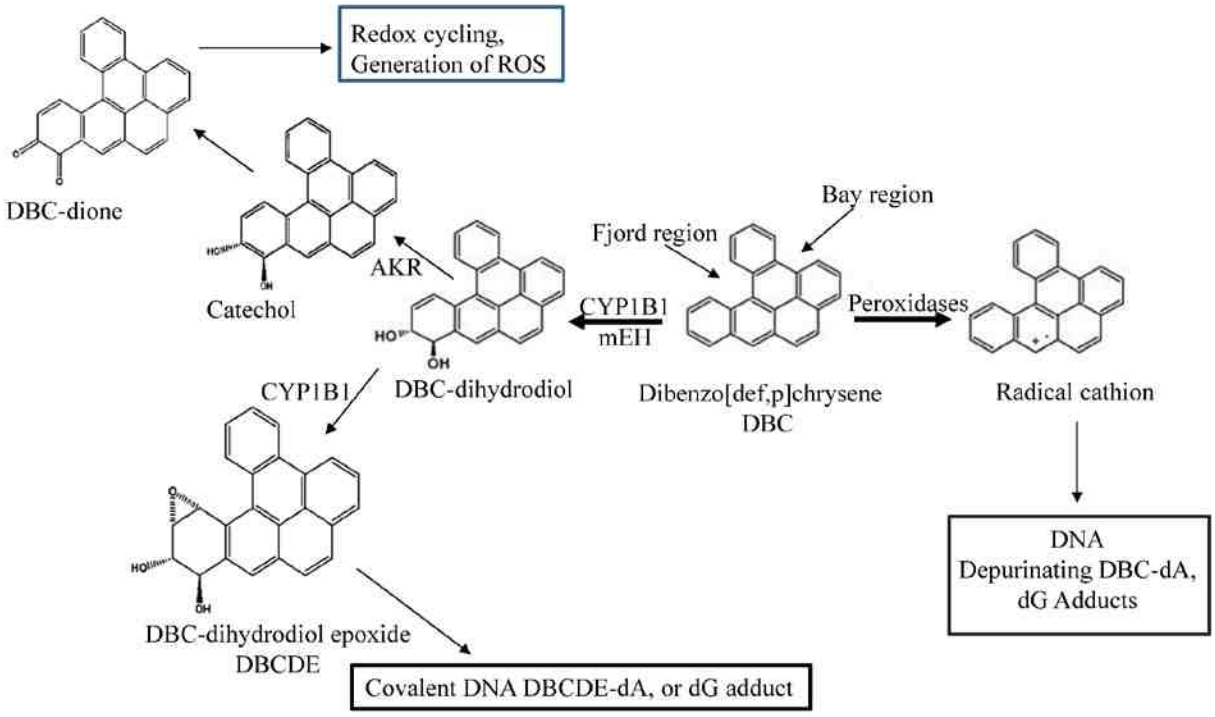


Figure 1.4. Proposed pathways for the biotransformation of DBC

Arsenic exposure compromises immunity

There is sufficient evidence that arsenic exposure causes immune dysfunction in mice and humans. Epidemiological studies reveal the association of arsenic exposure to various cancers and many other diseases including heart disease (Moon et al., 2013), diabetes (Khan, et al., 2011) and recurrent infections. Gradual erosion of the immune system in long term or chronic exposures to arsenite could also play a role in determining susceptibility to these diseases. Measurement of some immune parameters during arsenic exposure (Table 1.2), show the involvement of the immune system in arsenic association to diseases. Some studies on human immune suppression caused by arsenite exposure focus on gestational exposures, and data show that decreased immune cell counts, recurrent infections, and developmental abnormalities of peripheral lymphoid organs are among the manifestations of As^{+3} exposure. (No. 2, 4, and 6. in Table1.2). In adult human exposures, parameters of innate and adaptive immune responses are also shown to be altered. Mice showed alteration in the innate immune parameters, peripheral blood counts, increased viral titer which is an indication of reduced humoral immune responses, and increased morbidity.

Table 1.2: Evidence for As^{+3} immunosuppression

	Evidence/model/location	Parameter measured	Reference
1.	100 ppb As^{+3} during infection associated with higher influenza virus titer, and alteration of neutrophil and macrophage counts/ Mice/ USA	Viral titer, peripheral blood counts.	Kozul et al., (2009).
2.	Decreased $CD3^{+}$ T cells in placenta following in utero As^{+3} exposure/ Human fetus/ Bangladesh	Concentration of maternal urinary As^{+3} at GW 8 and GW 30	Ahmed et al., (2010).
3.	Altered expression of T lymphocyte immune response genes in humans exposed to >50 ppb As^{+3} /Adult human/ USA	Gene expression in peripheral blood lymphocytes by affymetrix microarray	Andrew et al., (2008).
4.	Increased Upper and lower respiratory infections, and total number of infections in infants following in utero exposure to As^{+3}/ Human fetus/ USA	Maternal urinary As^{+3} concentration, total number of infections and infection severity	Farzan et al., (2013).
5.	As^{+3} exposure correlates with respiratory complications, elevated immunoglobulins, decreased WBC counts with low neutrophil and high lymphocyte count/ Adult human/ Bangladesh	Serum immunoglobulins, WBC count and differential,	Islam et al., (2007).
6.	Impairment of infant thymic development following in utero exposure to As^{+3}/ Human fetus/ Bangladesh	Infant thymic size by sonography, maternal urine and breast milk immune marker, As^{+3} concentration, infant morbidity	Raqib et al., (2009).

Arsenic Metabolism, Speciation and Toxicity

Arsenic occurs as organic and inorganic species. The inorganic arsenic is harmful due to its ability to bind to sulfhydryl-containing compounds in the body. Biotransformation of inorganic arsenic is catalyzed by the enzyme arsenite methyltransferase (AS3MT), also known as CYT19. The enzyme is a 42 kD protein that catalyzes the methylation of inorganic arsenic to various trivalent and pentavalent methylated species (Aposhian et al., 2000; Thomas et al., 2004), using S-adenosylmethionine (SAM) from folic acid metabolism as the methyl group donor (Gamble et al., 2007) in the presence of glutathione (Fig 1. 5). The different species of inorganic arsenic differ in levels of toxicity (Styblo et al., 2000; Drobna et al., 2009). Humans can be directly exposed to two forms of inorganic arsenic: the trivalent arsenic (As^{+3}) and the pentavalent arsenic (As^{+5}). The pentavalent arsenic is found to be less toxic than the trivalent arsenic, but it is converted to the trivalent arsenite by arsenate reductase in the presence of inosine and lipoic acid. This is the first and only step in arsenic biotransformation that does not involve a methyltransferase. The methylation of trivalent arsenic to monomethylarsonous acid (MMA^{+3}) is known to be the rate limiting step in arsenic biotransformation.

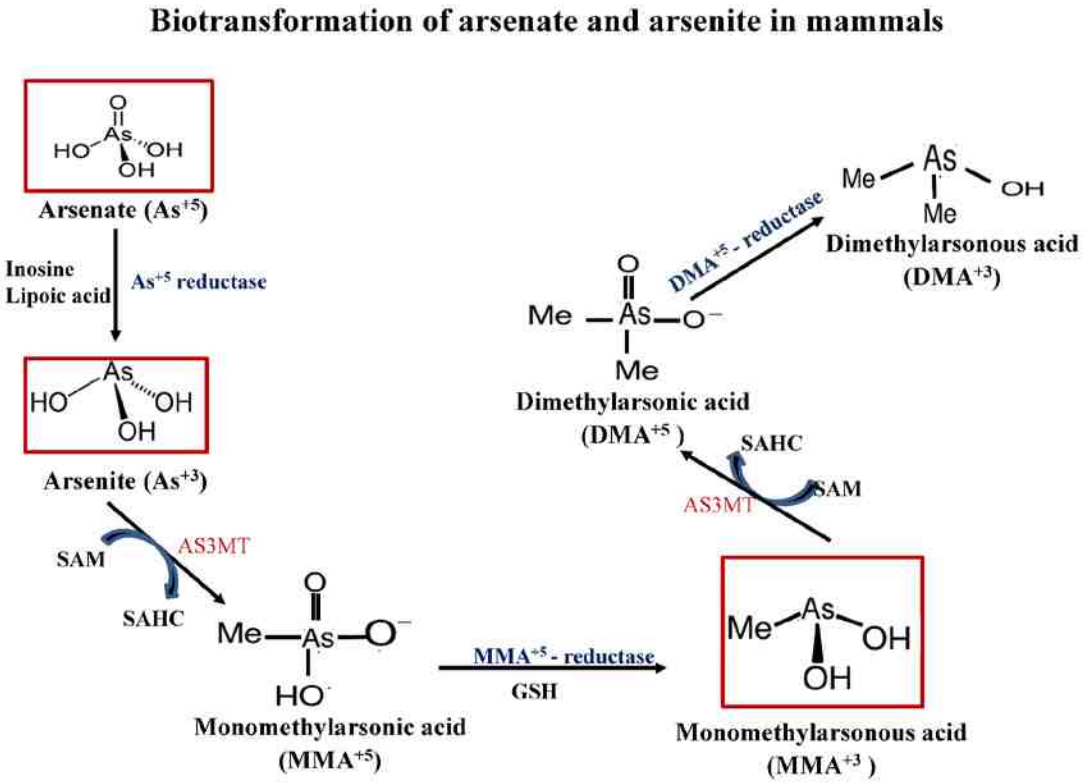


Figure 1.5. Biotransformation of arsenate and arsenite in mammals. AS3MT, Arsenite methyltranserase; SAM, S-adenosylmethionine; SAHC, S-adenosylhomocysteine. Structures in red box were evaluated *in vitro* in mice primary BM cells and 2E8 cell line in current study.

Arsenic and PAH Individual and Combined Exposures

Arsenite and PAHs are among the most ubiquitous environmental pollutants worldwide and they are also ranked among the top 10 in the most recent priority list for toxic substances compiled by the U. S. Agency for Toxic Substances and Disease Registry (ATSDR), 2013. As^{+3} has been implicated in the enhancement of the genotoxicity or mutagenicity of carcinogens, including PAHs and ultraviolet radiation, by inhibiting DNA repair (Chiang and Tsou, 2009; Wang et al., 2013).

Arsenic occurs naturally in the air, soil, and water. It is a threat to human health globally and is associated with numerous diseases. Due to its presence in the soil and water, arsenic is absorbed by plants and food crops (Carey et al., 2010; Zhao, 2012). Rice is known to be the major source of arsenic in diets as rice products are contained in many processed foods including fruit juices and baby formula (Davis et al., 2012), as reported in the United States Consumer Reports (Nov. 2012). Environmental regulations exist for arsenic in drinking water but not in food, in the United States (US). In this case, the Environmental Protection Agency (EPA) set a maximum contaminant level (MCL) for As^{+3} in drinking water as 10 ppb. Despite this, some people in the US continue to rely on private unregulated wells for drinking water supply and are thus exposed to dangerous levels of arsenic (Nielson et al., 2010). Given the pervading nature of arsenic and without regulation for arsenic levels in food, it is very challenging to control the level of arsenic exposure. Due to the uses of arsenic in the past and present, there has been intentional (for example, treatment of acute promyelocytic leukemia, APL) and unintentional exposures to As^{+3}. The lethal dose of As^{+3} is about 1-3 mg/kg (ATSDR, 2007).

Exposures to environmental chemicals seldom occur in isolation as many of them are found together naturally. The uptake of trace elements together with PAHs by fruits and vegetables from contaminated soils has been reported (Samsøe-Petersen et al., 2002). Arsenic and other pollutants like lead, are also known to be absorbed by vegetable crops that are grown on soils which have been contaminated by pesticides made with lead and arsenic (McBride, 2013). Elevated levels of arsenic, uranium, and selenium have been found to occur in Central Oklahoma Aquifer, which is a major source of drinking water for Central Oklahoma (USGS, 1998). The occurrence of arsenic in drinking water and food crops, and the presence of detectable levels of PAHs in cooked and uncooked food (Philip, 1999) is a public health concern. Table 1.3 provides some evidence for As^{+3} and PAH immunosuppressive interaction. Both the known detrimental effects of arsenite such as decrease in humoral immune responses, and the genotoxicity property of PAHs are modified in combined exposures to both chemicals.

Table 1.3: Evidence for As^{+3} and PAH immunosuppressive interaction

	Evidence/model	Parameter measured	Reference
1.	Synergistic suppression of immune responses in murine spleen cells exposed to low dose As^{+3} and PAHs/ Mice	T-dependent antibody responses	Li et al., (2010).
2.	Increased BaP genotoxicity in As^{+3}- exposed Hepa-1 cells and c37 cells/ cell lines	BaP-DNA adduct formation	Maier et al., (2002).
3.	Increased risk of lung cancer due to occupational exposure to As^{+3} and active smoking/Human/ Bangladesh	Examination of previous data on individuals exposures in relation to lung cancer risk	Hertz-Piccioto et al., (1992).
4.	Risk of As^{+3}-induced skin lesions modified by tobacco smoking/ Human/ Bangladesh	Baseline data from study subjects, information on well As^{+3} concentration, and history of well use.	Chen et al., (2006); Melkonian et al., (2010).

As^{+3} and PAH Interactions and Overall Goal of these Studies

The purpose of the present work is to determine whether PAHs, in this case DBC, interacts with As^{+3} *in vivo* and *in vitro* in mice models of BM development. These studies were proposed based on the knowledge that DMBA produces immunotoxicity in murine BM and spleen cells via genotoxicity due to the formation of DMBA-DE protein adducts and also that low doses of PAHs (DMBA, DBC, and BaP-diol) in combination with As^{+3} resulted in synergistic suppression of T-dependent antibody responses in murine spleen cells (Li et al., 2010).

The mechanism for synergistic immunosuppression has not been determined, but it is logical that inhibition of DNA repair pathways might be important. As^{+3} was known to inhibit DNA repair in many cells (Hartwig et al., 2003; Wang et al., 2013; Zhou et al., 2011). Inhibiting the repair of immune cell DNA adducts and strand breaks could result in immunosuppression. Based on this idea, it was postulated that low dose As^{+3} and PAH may produce synergistic immunosuppression of lymphoid cells in the BM. Thus, we proposed Specific Aims to look at the BM toxicity of low dose As^{+3} alone, and in combination with DBC, and then to investigate potential mechanisms.

Hypothesis and Specific Aims

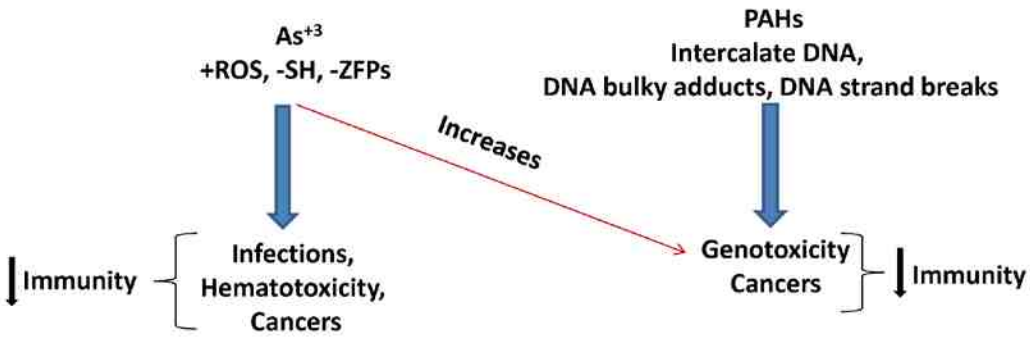
The overall hypothesis to be tested is that combined exposures to arsenite (As^{+3}) and dibenzo[def,p]chrysene (DBC), a polycyclic aromatic hydrocarbon (PAH), at environmentally relevant levels targets the immune progenitor cells in the bone marrow (BM) resulting in suppression of immune responses.

Specific Aims

Specific Aim 1: To determine whether oral exposure to environmentally relevant levels of arsenite leads to the suppression of immune progenitor cell responses in the bone marrow. The hypothesis to be tested is that As^{+3} suppresses the lymphoid and myeloid progenitor cells in the BM.

Specific Aim 2: To examine potential interactions between arsenite and DBC in BM immune cells suppression. The hypothesis is that mice simultaneously exposed to no-effect levels of arsenite and DBC orally, will show BM suppression due to the combined exposure.

Specific Aim 3: Based on findings obtained in Aim 1, we now hypothesize that IL-7 signaling pathways are selectively targeted by As^{+3} and MMA^{+3} in murine BM immune cells *in vitro*. The hypothesis to be tested is that As^{+3} and MMA^{+3} disrupts the cytokine signaling involved in the lymphoid lineage-specific differentiation of mouse bone marrow cells, leading to immunosuppression.



BM Myeloid Immune cells + Lymphoid Immune cells => Immunity

Hypothesis: Long term, low dose As^{+3} + low dose PAH => BM suppression

? 1: Does long term, oral low dose As^{+3} alone cause immunosuppression?

? 2: Could long term, oral no-effect dose As^{+3} interact with DBC to suppress immunity?

? 3: How does low dose As^{+3} exert its effect in the BM?

Figure 1.6. The Premise for Current Study

Rationale

All immune cells originate in the BM through the process of hematopoiesis (Figure 1.1). The myeloid and lymphoid progenitor cells together are responsible for all immune defenses, thus, individual evaluation of the myeloid and lymphoid progenitor cell responses in the BM may reveal an aspect of the immune system that may be compromised from low level arsenite and DBC individual and combined physiological exposures. The selective targeting of the lymphoid progenitors over myeloid progenitors in the BM by As^{+3} could utilize the lymphoid lineage-specific signaling in the BM.

Animal Model, Primary Cells and Cell Line; As^{+3} and DBC Doses and Concentrations; Experimental Design

Based on previous relevant studies, C57BL6/J male mice were used for the *in vivo* studies. Mice were fed environmentally relevant levels (0-300 ppb) of As^{+3} orally, via drinking water for 30 days to mimic long-term exposure. The As^{+3} doses were chosen after dose- range studies in order to establish maximum no-effect dose as to determine any interaction with DBC where applicable. DBC (0- 10 mg/kg, 5-day cumulative dose) was also administered orally in pill forms to arsenite- exposed (during the last 5 days of exposure) and unexposed mice. Primary BM cells from mice were exposed to the three inorganic arsenic species: As^{+5}, As^{+3}, and MMA^{+3} *in vitro* (at low levels, 0-500 nM) in order to determine which species was responsible for observed toxicity.

For the *in vivo* and *in vitro* studies, the pre-B and granulocyte-monocyte colony forming unit (CFU-B and CFU-GM respectively) assays were used as indicators of the bone marrow lymphoid and myeloid responses to immunotoxicity from the chemical exposures

and treatments. The IL-7 dependent mouse pre-B cell line, 2E8 cells (Ishihara, et al 1991) was used to elucidate the mechanism for the observed selective lymphoid toxicity. Because 2E8 cells were normally grown in 1 ng/ml mouse IL-7, they were starved of IL-7 overnight while in treatment media so that signaling could be stimulated and analyzed by immunoblotting, flow cytometry, or PCR after reintroduction and incubation with 10 ng/ml mIL-7 for 30 minutes.

The rationale for using the IL-7-dependent mouse pre-B cell line, 2E8, is that the BM cell population is highly heterogeneous and the progenitor cells constitute less than 1% of total BM cells. Thus, to obtain a pure pre-B cell population in large quantities for this study, the use of 2E8 cells as the *in vitro* model was necessary. The potential limitation to this approach, as with all *in vitro* work is the lack of heterogeneous cell-cell interaction obtained *in vivo* and with primary cells. However, the reduction in use of animals is added benefit. Furthermore, in addition to being IL-7-dependent, 2E8 cells were sensitive to the same concentrations of chemicals that were used for primary BM cells *in vitro.*

In contrast to most studies of As^{+3} and PAH toxicity involving high dose, short-term acute, and non-physiological exposures (Carbrey et al., 2009; N'jai et al., 2010; Noreault et a., 2005), this work focused on very low, environmentally relevant levels of these chemicals administered through physiological route of exposure. Thus the observations made are relevant to public health.

CHAPTER 2

Arsenite Selectively Inhibits Mouse Bone Marrow Lymphoid Progenitor Cell Development *In Vivo* and *In Vitro* and Suppresses Humoral Immunity *In Vivo*

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ABSTRACT

It is known that exposure to As^{+3} via drinking water causes a disruption of the immune system and significantly compromises the immune response to infection. The purpose of these studies was to assess the effects of As^{+3} on bone marrow progenitor cell colony formation and the humoral immune response to a T-dependent antigen response (TDAR) *in vivo*. In a 30 day drinking water study, mice were exposed to 19, 75, or 300 ppb As^{+3}. There was a decrease in bone marrow cell recovery, but not spleen cell recovery at 300 ppb As^{+3}. In the bone marrow, As^{+3} altered neither the expression of CD34+ and CD38+ cells, markers of early hematopoietic stem cells, nor CD45-/CD105+, markers of mesenchymal stem cells. Spleen cell surface marker CD45 expression on B cells (CD19+), T cells (CD3+), T helper cells (CD4+) and cytotoxic T cells (CD8+), natural killer (NK+), and macrophages (Mac 1+) were not altered by the 30 day *in vivo* As^{+3} exposure. Functional assays of CFU-B colony formation showed significant selective suppression ($p < 0.05$) by 300 ppb As^{+3} exposure, whereas CFU-GM formation was not altered. The TDAR of the spleen cells was significantly suppressed at 75 and 300 ppb As^{+3}. *In vitro* studies of the bone marrow revealed a selective suppression of CFU-B by 50 nM As^{+3} in the absence of apparent cytotoxicity. Monomethylarsonous acid (MMA^{+3}) demonstrated a dose-dependent and selective suppression of CFU-B beginning at 5 nM ($p < 0.05$). MMA^{+3} suppressed CFU-GM formation at 500 nM, a concentration that proved to be nonspecifically cytotoxic. As^{+5} did not suppress CFU-B and/or CFU-GM *in vitro* at concentrations up to 500 nM. Collectively, these results demonstrate that As^{+3} and likely its metabolite (MMA^{+3}) target lymphoid progenitor cells in mouse bone marrow and mature B and T cell activity in the spleen.

INTRODUCTION

Arsenic occurs ubiquitously in nature as an environmental pollutant found in the air, soil and water. The pervasive nature of arsenic pollution makes it a major concern globally despite environmental regulations in some countries. Humans are exposed to some level of arsenite (As^{+3}) via drinking water, food crops and air, and exposure is associated with many diseases (Argos et al., 2010). The US EPA set the maximum contaminant level (MCL) for arsenic in drinking water at 10 ppb. However, in many parts of the US and elsewhere in the world, people rely on private unregulated wells for their drinking water supply and therefore are exposed to higher levels of arsenic. Levels exceeding 500 ppb have been found in domestic well water in some parts of the US (Nielson et al., 2010). Humans are exposed to two primary forms of inorganic arsenic: trivalent arsenic (As^{+3}) and pentavalent arsenic (As^{+5}). Both of the forms undergo biotransformation involving reduction and oxidative methylation in the liver, to form methylated arsenicals such as MMA^{+5}, MMA^{+3}, DMA^{+5}, and DMA^{+3} (Drobna et al., 2009; Liu et al., 2006; Thomas et al., 2001). MMA^{+3} is thought to be the most toxic arsenic species *in vitro* (Styblo et al., 2000).

Previous studies have shown that exposure to arsenic via drinking water alters components of the innate and adaptive immune system in mouse lung and significantly compromises the immune response to infection (Kozul et al., 2009). Several mechanisms including interruption of cell signaling, altered expression of transcription factors, oxidative stress due to formation of reactive oxygen species, increased apoptosis, chromosomal aberration and inhibition of DNA repair and poly(ADP-ribose) polymerase (PARP) activity

have been proposed for arsenic toxicity (Argos et al., 2006; Cheng et al., 2004; Cooper et al., 2013; Flora et al., 2011; Hughes et al., 2011; King et al., 2012; Qin et al., 2012; Sun et al., 2013). The bone marrow is a vital organ of the immune system in which all immune cells originate through the process of hematopoiesis. In order to elucidate the role and mechanism of arsenic in immune suppression, it is necessary to study the effects of environmentally relevant levels of arsenic on the bone marrow and peripheral lymphoid organs.

Hematopoietic stem cells are able to commit to specific lineages in response to signal(s) from the microenvironment using the expressed receptor(s) (Carsetti et al., 2000). Modulation of the bone marrow microenvironment by arsenic may alter stem cell lineage populations. The pre-B and granulocyte-monocyte colony forming unit (CFU-B and CFU-GM) assays are indicators of the bone marrow lymphoid and myeloid responses to immunotoxicity and may define an aspect of immunity that may be compromised. Studies show that arsenic exposure impacts the immune system or alters immune responses in different ways. In humans, experiments show that chronic arsenic exposure causes decrease in T-cell proliferation and cytokine secretion (Biswas et al., 2008), impairment of macrophage functions (Banerjee et al., 2009), and increased apoptosis of peripheral blood mononuclear cells (Rocha-Amador et al., 2011). Given the association of arsenic exposure to immunosuppression, hematotoxicity, and associated diseases, one would expect that the immunosuppressive role of arsenic and its metabolites should be evident on the development of the progenitors as well as peripheral lymphoid organ activity.

Previous studies in our laboratory show that *in vitro* As^{+3} exposure produced immunosuppression at concentrations as low as 500 nM in mouse spleen cells (Li et al., 2010). The direct effect of As^{+3} and MMA^{+3} on bone marrow progenitor cells has not been

evaluated. To investigate the *in vivo* effects of As^{+3}, murine bone marrow and spleen cells were examined following a 30 day oral exposure of mice. CFU-B formation and spleen cell TDAR activity were suppressed by As^{+3}. The effects of direct exposure of As^{+3}, MMA^{+3}, and As^{+5} on *in vitro* progenitor cell cultures was monitored. Collectively, our results show that As^{+3} and MMA^{+3} selectively suppress the formation of lymphoid progenitors in murine bone marrow *in vitro*, demonstrating that lymphoid cell differentiation in the bone marrow and lymphoid cell activity in the spleen are important targets of arsenic action.

METHODS

Animals

C57BL/6J male mice were purchased at 8 to 10 weeks of age from Jackson Laboratory (Bar Harbor, ME) and were acclimated in our AAALAC-approved animal facility for at least one week before being used in experiments. All animals were handled in accordance to procedures and protocols approved by the Institutional Animal Use and Care Committee at the University of New Mexico Health Sciences Center. Following acclimation, mice (2-3 per cage) were exposed to arsenite (As^{+3}) at different concentrations in parts per billion (ppb) for 30 days via drinking water. Stock solutions were prepared using sterile double processed tissue culture water from Sigma-Aldrich. Drinking water bags were weighed to determine the amount of water consumed by each group of 2-3 mice. Drinking water contained less than 5 ppb total arsenic. For the *in vivo* experiments, the concentration of arsenic in drinking water was periodically checked and validated by the University of Arizona Laboratory for Emerging Contaminants (ALEC, Tucson AZ). Mouse 2020X Teklad Global Soy Protein-Free Extruded Rodent Diet, Harlan Laboratories Inc, Madison Wisconsin, USA, www.harlan.com contained 0.16 mg/kg (160 ppb) of total arsenic, of which approximately 10-15% may be considered to contain inorganic arsenic species (Kozul et al., 2008).

Each exposure or treatment group consisted of five mice and bone marrow cells from each mouse were analyzed in triplicate. *In vitro* studies were performed using bone marrow cells pooled together from three untreated C57BL/6J male mice femurs and each treatment was run in triplicate. All animals exposed to these chemicals were handled with caution,

using personal protective equipment, and disposed in accordance with the University of New Mexico's Risk and Safety Committee and the State of New Mexico guidelines.

Chemicals and Reagents

Sodium arsenite (CAS 774-46-5, Na AsO2) and sodium arsenate dibasic heptahydrate (CAS 10048-95-0, Na2HAsO4.7H2O) were purchased from Sigma-Aldrich (St. Louis, MO). Monomethylarsonous acid (MMA^{+3}) >98% purity, was prepared by the Synthesis Core of the Southwest Environmental Health Sciences Center at the University of Arizona (Tucson, AZ). MethoCult GF methylcellulose medium (Cat. No. M3534) with recombinant cytokines (without EPO) for mouse cells was purchased from Stem Cell Technologies (Vancouver, BC, Canada). Mouse methylcellulose complete media for pre-B cells (Cat. No.HSC009) was purchased from R&D Systems (Minneapolis, MN).

Isolation of Mouse Bone Marrow Cells

Bone marrow cells were isolated according to the procedure outlined in the Stem Cell Technologies Technical Manual version 3.1.1(http://www.stemcell.com/). Briefly, each mouse was sacrificed and 70% isopropyl alcohol was immediately used to wet the ventral fur to avoid contamination at site of dissection. Both femurs were collected sterilely and placed and held on ice in Hanks' Balanced Salt Solution (HBSS) purchased from Lonza (Walkersville, MD). To extract cells, femurs were placed in petri dish containing cold sterile RPMI 1640 Medium supplemented with 2% Fetal Bovine Serum (FBS). The ends of the femurs were trimmed to expose interior marrow shaft. Using a 1 cc syringe with a 25 gauge needle, approximately 1 ml cold sterile medium was flushed through the femur several times to release cells into a petri dish. The medium containing cells from both femurs was

immediately transferred to a 15 ml culture tube and placed on ice until needed. Cells were washed via centrifugation at 4^{o} C, 400 x g for 10 min and were resuspended in RPMI media (as above) for culturing or flow cytometry analysis as discussed below. Cell viability was determined by Acridine Orange/Propidium Iodide (AO/PI) staining and counting using the Nexcelom Cellometer 2000.

Isolation of Mouse Spleen Cells

Spleen cells were isolated following the procedure described by Lauer et al (Lauer et al., 2013). Briefly, harvested mouse spleens were weighed and placed in HBSS. Cells from each spleen were isolated and homogenized by placing the organ between the frosted ends of two microscopic glass slides and squeezing into a dish containing media. The cell suspension was centrifuged at 280 x g and 4^{o}C for 10 min. The cell pellets were collected and resuspended in complete media containing RPMI 1640 (Sigma-Aldrich) w/10 % FBS (Hyclone Logan, UT), 2 mM L-glutamine (GIBCO by Life Technologies, Grand Island, NY), 100 µg/ml streptomycin, 100 units/ml penicillin (GIBCO by Life Technologies) and placed on ice. Cell viability was determined by acridine orange/propidium iodide (AO/PI) staining and counting using the Nexcelom Cellometer 2000.

Bone Marrow and Spleen Cell Surface Markers by Flow Cytometry

For bone marrow cell surface marker subset analyses, antibody cocktails for CD34, CD38, CD45, and CD105 were obtained from BD Biosciences. CD34 and CD38 define early stages of hematopoietic cell development (Randall et al., 1996) and CD105 defines a population of CD45- mesenchymal stem cells and stromal cells (Dominici et al., 2006). Spleen cell surface markers were analyzed using a custom cocktail obtained from BD Biosciences (San Jose, CA) for CD45, CD3, CD4, CD8, CD19, NK and Mac1 for spleen cells as previously described, (Lauer et al., 2013). The control cocktail for the BM included isotype controls and CD45-PerCP (IgG2a-FITC; IgG2a-PE, CD45-PerCP;IgG2a-Alexa647), and the test cocktail included CD34-FITC; CD38-PE; CD45-PerCP; CD105-Alexa647. Briefly, 100 µl of media containing 1 $X10^6$ bone marrow cells were incubated with 20 µl of cocktail for 30 min at RT in the dark. Red blood cells were lysed by incubating with 2 ml of ammonium chloride lysing solution (0.15M ammonium chloride, Sigma A-4514; 10mM sodium bicarbonate, Sigma S-5716; 1mM disodium EDTA, Sigma-E7889; pH 7.4) for 10 min. The cells were centrifuged at 275 x g for 10 min, the supernatant was aspirated and the cells washed with 2 ml Dulbecco's phosphate buffered saline (DPBS) w/o Ca^{+2} and Mg^{+2} containing 1% FBS and 0.9% sodium azide (PBS/FBS). The washed cells were resuspended in 0.5 ml PBS/FBS and 20,000 cells were analyzed using the Accuri C6 Flow Cytometer (Becton Dickinson, San Jose, CA). The percent positive cell population was determined by gating on CD45+ cells and subtracting out background determined from the isotype controls for each sample. Spleen cell surface markers were analyzed as previously described (Lauer et al., 2013), using custom cocktail from BD Biosciences containing antibody reagents for

CD3(FITC)/CD8a(PE)/ CD45(PerCP)/CD4(APC) or CD3+CD19(FITC)/PanNK(PE)/ CD45(PerCP)/Mac-1(APC).

T-Dependent Antibody Assay

For the T-dependent antibody response (TDAR) assay, mice were immunized with 0.2 ml of 10% sheep red blood cells (Colorado Serum, Denver CO) in saline 4 days prior to euthanization. Following spleen harvesting, cells were washed and suspended at $4x10^6$ cell/ml in supplemented media. A mixture of 100 µl cells ($4x10^5$), 50 µl washed 1% sheep red bloods cells and 400 µl of 0.8% low-melting point SeaPlaque agarose (Lonza, Rockland, ME) solution (in RPMI held at 42°C) were combined and poured onto an agarose coated slide. Agarose was allowed to set-up before incubating face down on custom slide trays in a humidified plastic box at 37°C for 1.5 hour. Guinea pig complement (Colorado Serum, Denver, Colorado) was diluted 1:20 in Dulbecco's Phosphate Buffered Saline containing Ca^{2+} and Mg^{2+} (Sigma) and warmed to room temperature. Slides were flooded with diluted complement following the 1.5 hour incubation and then incubated, as described above for an additional 2 hrs. Slides were removed from the incubator and stored in a cold 0.85% sodium chloride solution. SRBC lysis was quantified by counting plaques in the SRBC/agar lawn using a dissecting microscope. Results are expressed as plaque forming cells (PFC) per culture of $4x10^5$ cells.

CFU-B Assay

This assay was performed as described in Stem Cell Technologies Technical Manual version 3.1.1 (http://www.stemcell.com/) for Mouse Colony-Forming Cell (CFC) Assays using MethoCult. Briefly, isolated bone marrow cells from each mouse (for *in vivo* studies)

or pooled from three mice (for *in vitro* studies) were suspended in RPMI 1640 Medium supplemented with 2% heat inactivated Hyclone Fetal Bovine Serum (Fisher Scientific, Pittsburgh, PA) at $1x10^6$ cells/ml. 400 µl ($4 x10^5$ cells) of the cell suspension was transferred to a 16 ml (17 x 100 mm) sterile culture tube which contained 4 ml Mouse Methylcellulose Complete Media for Pre-B Cells (R&D Systems, Minneapolis, MN).The tube was thoroughly mixed by vortexing and allowed to sit for 20 min to release air bubbles. 1 ml of the methylcellulose-cell mixture was dispensed into a 35 mm culture dish (Stem Cell Technologies) using a 3 cc syringe with a 16G x 11/2'' Monoject Aluminum Hub, blunt canula needle from Covidien (Mansfield, MA).Samples were run in triplicate. The mixture was evenly dispersed in the dish by rocking the dish. One sterile water dish and two sample dishes were placed in a 100 mm culture dish and incubated at 37^oC, 5% CO_2, in a humidified incubator for 10 days. CFU-B colonies were counted and recorded for statistical analysis.

CFU-GM Assay

The same initial methods as in the CFU-B assay described above were used for the CFU-GM assay except that the isolated bone marrow cells were suspended in Iscove's Modified Dulbecco's Medium (Sigma-Aldrich) supplemented with 2% heat inactivated Hyclone Fetal Bovine Serum at $2x10^5$ cells/ml. 400 µl ($8x10^4$ cells) of the cell suspension was transferred to a 16ml (17x100 mm) sterile culture tube which contained 4 ml MethoCult GF Methylcellulose Medium (Stem Cell Technologies). As described above for CFU-B, the mixture was evenly dispersed in the dish by rocking the dish. Sample dishes (35 mm) were placed in a 100 mm culture dish and put in the humidified incubator at 37^oC and 5% CO_2 for 14 days. CFU-GM colonies were counted and recorded for statistical analysis.

Annexin V Staining

Bone marrow cells from femurs of three male C57BL6/J mice were pooled together and incubated in RPMI media supplemented with 2% FBS, 2 mM L-glutamine, 100 units/ml penicillin and 100 units/ml streptomycin with treatment for 18 hrs. Following incubation the cells were washed in PBS by centrifuging at 300 x g. Cells were resuspended and stained in solutions and reagents provided in the FITC Annexin V Apoptosis Detection kit (BD Biosciences) according to manufacturer's procedure. Stained cells were analyzed using the Accuri C6 Flow Cytometer. Cells were selected using FSC and SSC and then analyzed based on percentage of stained cells.

Data Analysis and Statistics

Data were analyzed using the Sigma Stat version 3.5 and Sigma Plot 12.0 software, one-way analysis of variance (ANOVA) and Dunnett's t-test to determine differences between control and treatment groups. For *in vivo* studies, treatment groups consisted of five animals and each animal was analyzed in triplicate. Bone marrow cell recovery was expressed as the mean number of recovered bone marrow cells obtained from a pool of both femurs. In the *in vitro* experiments, a treatment group consisted of three replicates of one chemical treatment of pooled bone marrow cells or control. We calculated the number of CFU-B and CFU-GM formed per 10^6 cells plated at the beginning of the culture periods.

Table 2.1: 30 Day Arsenic Drinking Water Exposure and Effects on Mouse Body Weight, Spleen Weight, Bone marrow Cell and Spleen Cell recovery[1]

Arsenite treatment	Water consumed (ml)	Mean wt. of mice (g)	Mean Spleen wt. (g)	Mean BM cell recovery X 10^7	Mean Spleen cell recovery X 10^8
Control	125.1 ± 29.2	29.2 ± 1.0	0.107± 0.01	3.13 ± .64	1.63 ± .29
19 ppb	112.2 ± 13.4	29.3 ± 2.0	0.110 ± 0.02	3.22 ± .36	1.89 ± .45
75 ppb	136.0 ± 20.7	28.2 ± 2.0	0.10 ± 0.02	2.62 ± .28	2.05 ± .39
300 ppb	148.3 ± 13.8	29.0 ± 2.0	0.11 ± 0.02	2.46 ± .41 *	1.63 ± .20

[1]Five mice were examined for each group; results are Means ± SD with statistical significance at *$p \leq 0.05$

RESULTS

C57BL/6J male mice (5 per group) were given 0, 19, 75, or 300 ppb As^{+3} via drinking water for 30 days. As shown in Table 1, at the end of the 30 day period, there were no significant differences in body weights or spleen weights between groups and arsenic contained in the drinking water did not affect the amount of water consumed by the mice. There was an apparent trend towards a dose-dependent reduction in bone marrow cell recovery, which was significant at the 300 ppb As^{+3} exposure level ($p<0.05$). Spleen cell recovery was not affected by As^{+3} at any exposure level.

Flow cytometry analysis of the bone marrow cell surface markers demonstrated that the percentage of early stem cells CD45+/CD34+ and CD45+/CD38+ (which define early hematolymphoid cells, (Randall et al., 1996)) was not changed by As^{+3} consumption (Figure 2.1, top). Also, there was no significant difference in CD45-/CD105+ (markers of mesenchymal stem cells, (Dominici et al., 2006)) expression between control and treatment groups (Figure 2.1, bottom).

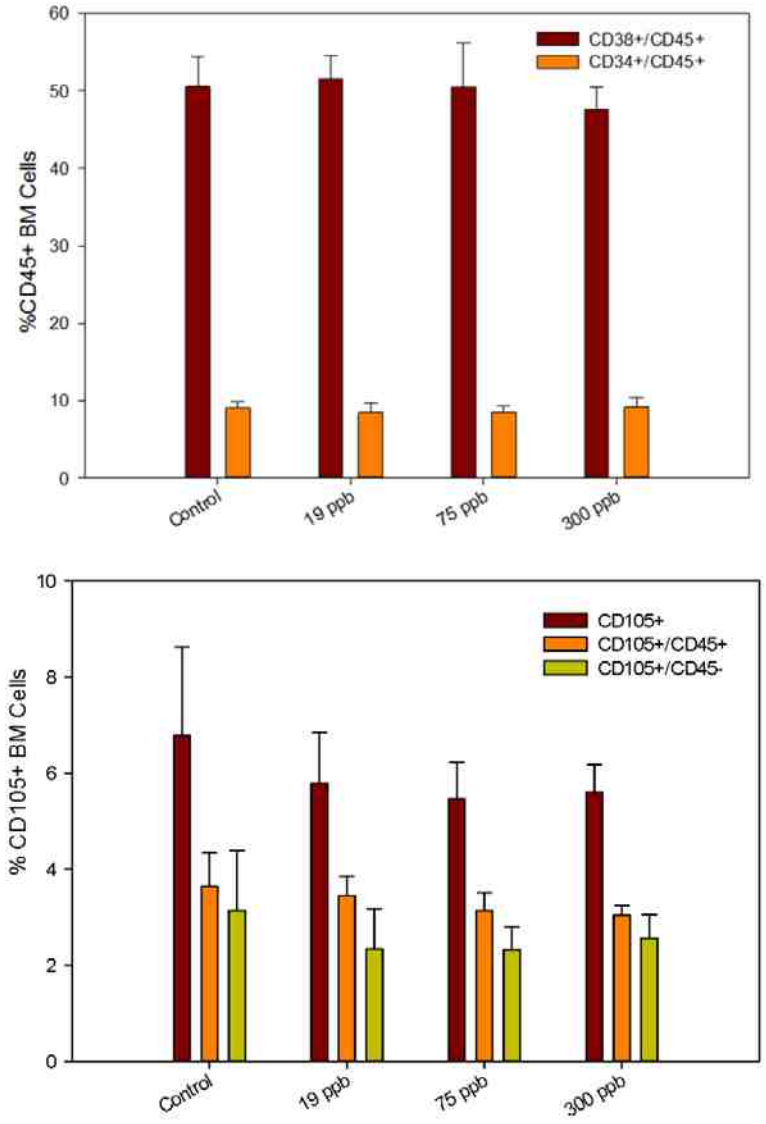

Figure 2.1. Bone marrow (BM) cell surface marker expression analyzed by flow cytometry. Percent CD34+ and CD38+ mice BM cells co-expressing CD45 [Top] and percent BM cells expressing CD105 [Bottom] in CD45+ and CD45- cells after 30 day consumption of As^{+3} in drinking water. Results are Means $\pm$ SD.

In the spleen, there were no differences in cell surface marker expression for mature B (CD45+/CD19+), T (CD45+/CD3+), helper T (CD45+/CD4+), cytotoxic T (CD45+/CD8+), natural killer (CD45+/NK+), and macrophage (CD45+/Mac-1+) populations between control and treatment groups (Figure 2.2, Top and bottom).

Since the purpose of these studies was to assess the effects of As^{+3} on lymphoid cell differentiation and function, we examined the colony forming unit (CFU) activity of pre-B cells and granulocyte-monocyte (GM) CFUs as well as peripheral lymphoid (spleen) cell function. As^{+3} was found to decrease the numbers of CFU-B measured in the femurs of the 300 ppb group (Figure 2.3, top), but it did not affect the number of CFU-GM detected (Figure 2.3, bottom). These results show a selective loss in lymphoid CFU activity, but not myeloid activity without altering the early progenitors (as measured by CD34/CD38) detected by flow cytometry.

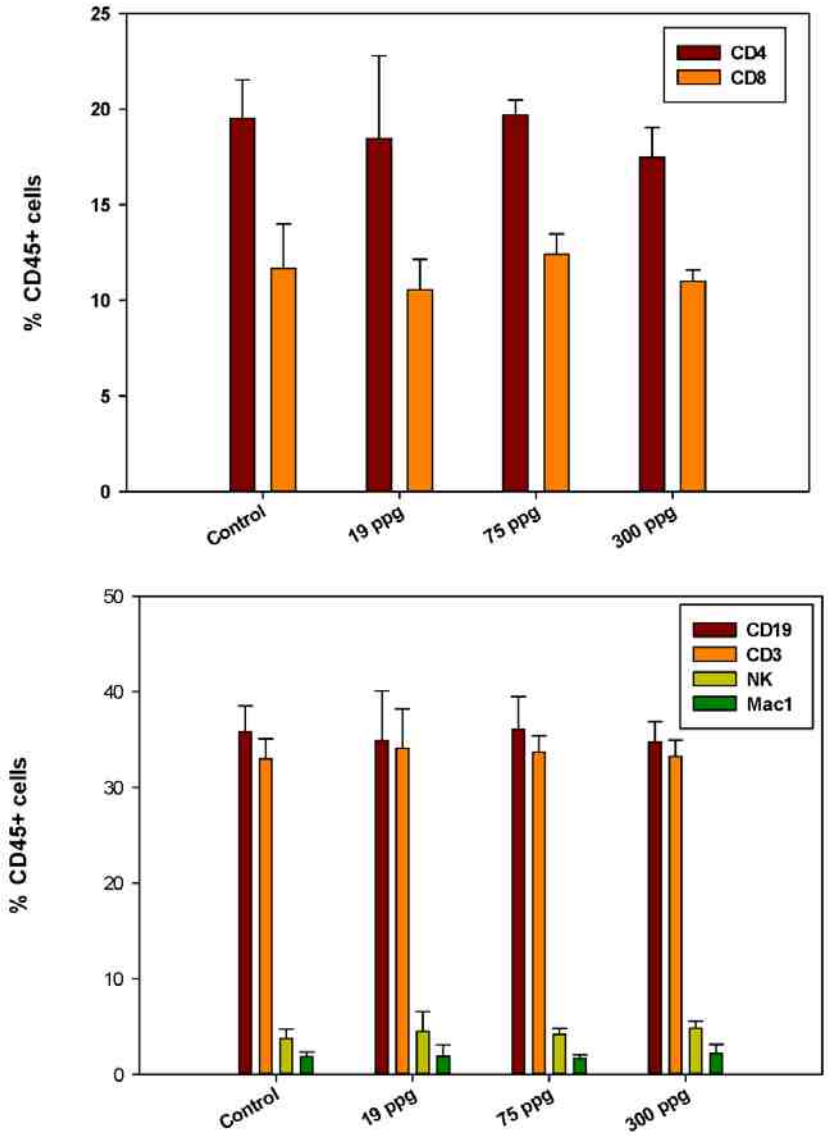

Figure 2.2. Spleen cell surface marker expression analyzed by flow cytometry. Percent CD19+, CD3+, NK and Mac 1 cells co-expressing CD45 [Top] and percent CD4+ or CD8+ cells co-expressing CD45 [Bottom] after 30 day consumption of As^{+3} in drinking water. Results are Means $\pm$ SD

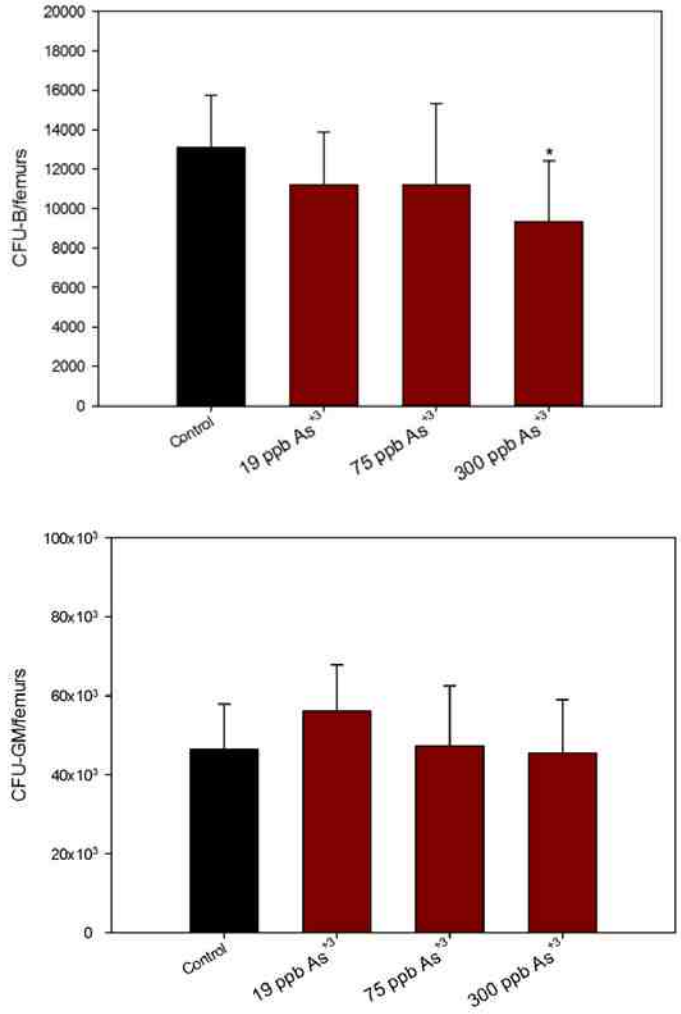

Figure 2.3. *In vivo* BM exposure to As^{+3} showing number of colonies per pair of mice femurs. Number of CFU-B colonies in a pair of mice femurs, 10 days post plating in mouse methylcellulose media for pre-B [Top]. Number of CFU-GM colonies in a pair of mice femurs 14 days post plating in Methocult media for GM selection [Bottom] after 30 day consumption of As^{+3} via drinking water. *Significantly different compared to control ($p<0.05$). Results are Means $\pm$ SD.

The T-dependent antibody response to sheep red blood cells was suppressed at both the 75 and 300 ppb exposure levels in the spleen (Figure 2.4) suggesting that immature and mature lymphoid lineages are sensitive to the effects of As^{+3}.

To determine whether As^{+3} has direct effects on bone marrow progenitor cells, we performed *in vitro* studies with known concentrations of As^{+3} over a wide concentration range. These studies also allowed us to examine the effects of MMA^{+3} on lymphoid and myeloid progenitor cultures, which is important because MMA^{+3} is a key and oftentimes more toxic metabolite of arsenite. In agreement with our *in vivo* studies, we found that pre-B cells were more sensitive than CFU-GM progenitors to *in vitro* exposure to As^{+3} (Figure 2.5). As^{+3} was found to suppress CFU-B formation at concentrations as low as 50nM, whereas, this concentration and 500 nM As^{+3} did not affect CFU-GM formation.

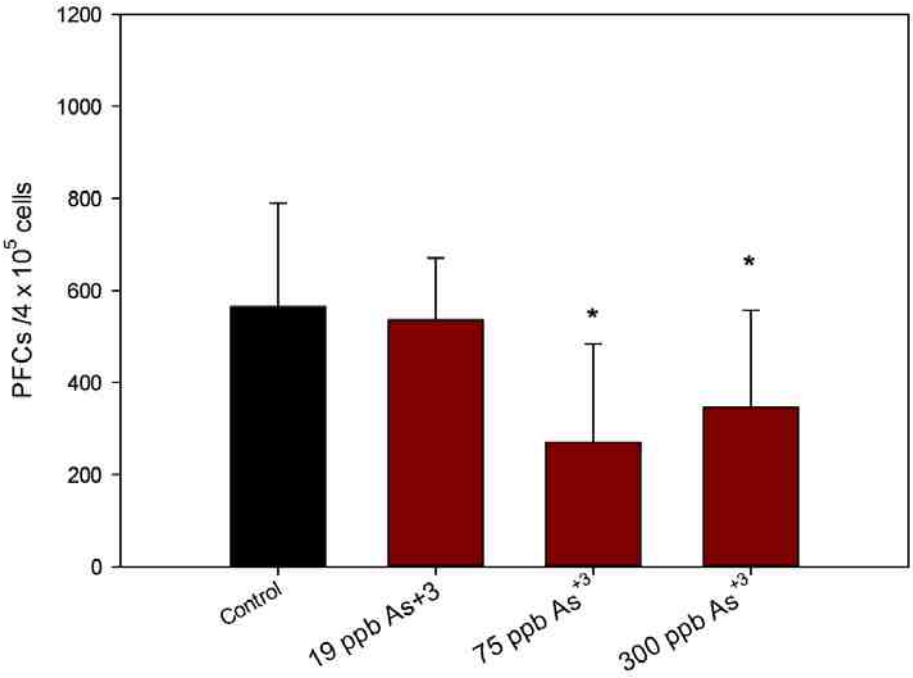

Figure 2.4. Suppression of T-dependent antibody response to sheep red blood cells post 30 day *in vivo* exposure of mice to As^{+3} via drinking water. After As^{+3} exposure, mice were immunized with SRBCs and examined for *in vitro* antibody production 4 days later. Results are Means $\pm$ SD.

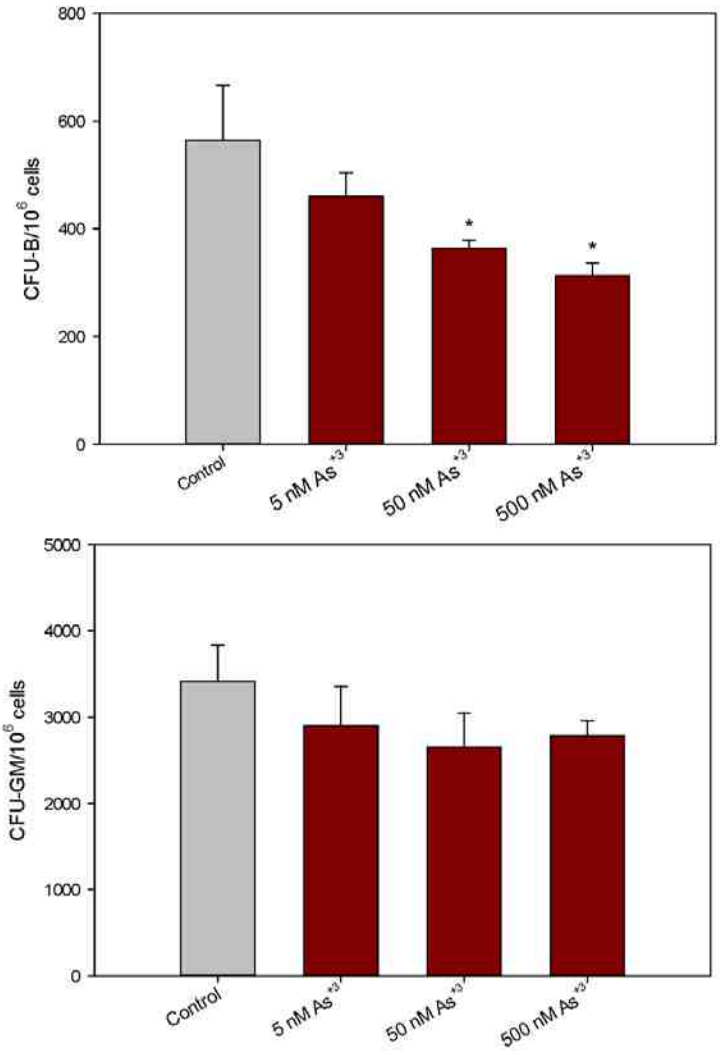

Figure 2.5. Number of colonies per million cells exposed to As^{+3} *in vitro*. Number of CFU-B colonies per million cells 10 days post plating in mouse methylcellulose media (containing As^{+3}) for pre-B cells [Top]. Number of CFU-GM colonies per million cells 14 days post plating in mouse methylcellulose media (containing As^{+3}) for GM cells [Bottom]. * Significantly different compared to control ($p<0.05$). Results are Means $\pm$ SD.

Exposure of murine bone marrow cells to MMA^{+3} was also found to selectively suppress pre-B colony formation. There was a concentration-dependent suppression of CFU-GM by MMA^{+3} (Figure 2.6, bottom). At concentrations as low as 5 nM MMA^{+3}, CFU-B formation was significantly suppressed (Figure 2.6, top), suggesting that MMA^{+3} may be approximately ten times more potent than As^{+3} in suppressing CFU-B formation. MMA^{+3} suppression of CFU-GM formation was significant at a concentration of 500 nM (Figure 2.6, bottom). The *in vitro* exposure to 500 nM MMA^{+3} proved to be nonspecifically cytotoxic to bone marrow cultures, as revealed by cell viability and Annexin V studies performed at 18 hrs after exposure (Table 2.2). Annexin V positive cells were found with both propidium iodide positive and negative cell populations, suggesting a mixed mode of cell death.

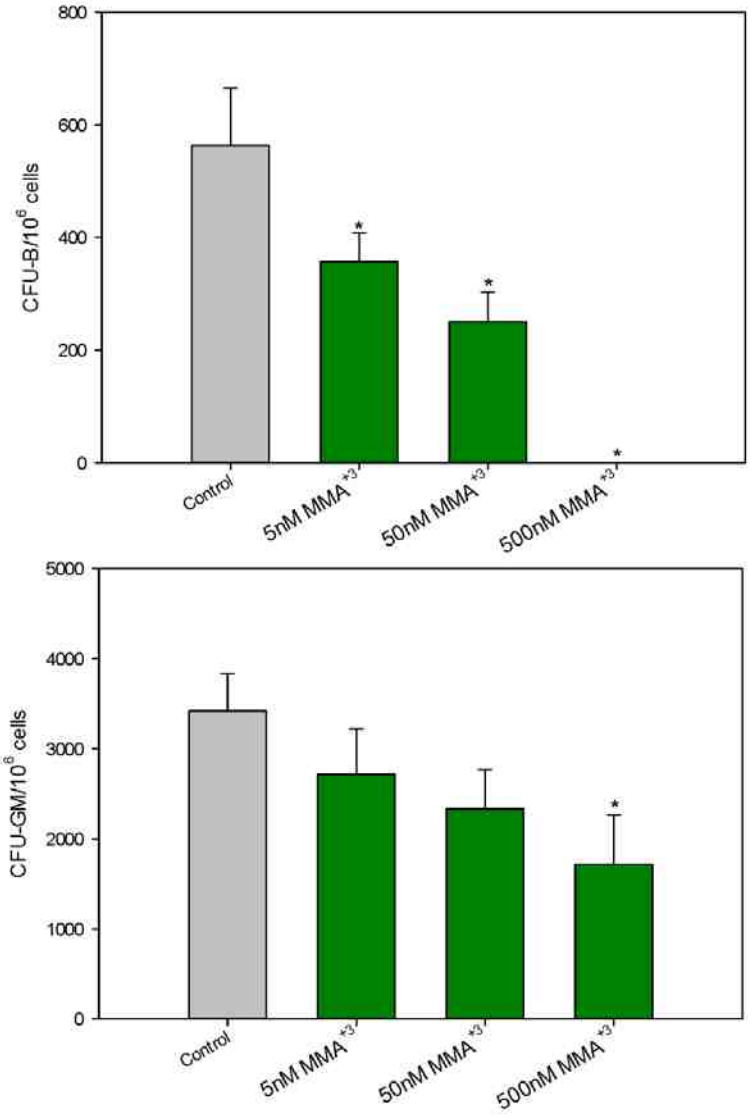

Figure 2.6. Number of colonies per million cells exposed to MMA^{+3} *in vitro*. Number of CFU-B colonies per million cells 10 days post plating in mouse methylcellulose media (containing MMA^{+3}) for pre-B cells [Top]. Number of CFU-GM colonies per million cells 14 days post plating in mouse methylcellulose media (containing MMA^{+3}) for GM cells [Bottom]. *Significantly different compared to control ($p<0.05$). Results are Means $\pm$ SD.

Table 2.2: *In vitro* (18 hrs.) As^{+3}, As^{+5} or MMA^{+3} Exposure and Effects on Mouse Bone marrow Cell Viability[1]

Treatments	% Viability (Cellometer)	% Annexin V negative/PI negative cells	% Annexin V positive/PI negative cells	% Annexin V negative/PI positive cells
Control	63.2 ± 1.4	78.1 ± 2.7	16.1 ± 2.5	5.7 ± 0.2
5 nM As^{+3}	63.3 ± 0.6	77.8 ± 2.2	16.3 ± 2.1	5.8 ± 0.8
50 nM As^{+3}	58.4 ± 3.5	76.2 ± 1.3	17.8 ± 0.6	5.8 ± 0.5
500 nM As^{+3}	59.8 ± 1.7	75.6 ± 2.0	18.4 ± 0.4	6.0 ± 1.7
5 nM As^{+5}	59.3 ± 0.1	77.8 ± 1.8	16.7 ± 0.3	5.4 ± 1.4
50 nM As^{+5}	58.8±3.7	75.9 ± 1.4	17.1 ± 1.5	6.7 ± 0.4
500 nM As^{+5}	62.7± 2.2	74.3 ± 0.8	19.2 ± 1.2	6.4 ± 0.5
5 nM MMA^{+3}	59.3 ± 3.5	76.5 ± 1.9	17.6 ± 2.0	5.8 ± 0.2
50 nM MMA^{+3}	59.9 ± 6.8	78.2 ± 0.5	16.6 ± 0.5	5.0 ± 0.6
500 nM MMA^{+3}	39.5 ± 10.0*	52.0 ± 2.5*	21.6 ± 3.4*	26.4 ± 2.2*

[1] Bone marrow cells from three mice femurs were pooled and exposed to each treatment in triplicate. Results are Means ± SD with statistical significance at *$p \leq 0.05$

The *in vitro* exposure to 500 nM MMA^{+3} proved to be nonspecifically cytotoxic to bone marrow cultures, as revealed by cell viability and Annexin V studies performed at 18 Finally, since many drinking water sources are contaminated with inorganic pentavalent arsenate (As^{+5}), we examined the effects of As^{+5} on mouse bone marrow progenitor cell activity *in vitro*. As^{+5} did not inhibit CFU-B or CFU-GM colony formation at concentrations as high as 500 nM (Figure 2.7). These results demonstrate that As^{+5} is far less toxic to the bone marrow than the trivalent arsenicals in mice.

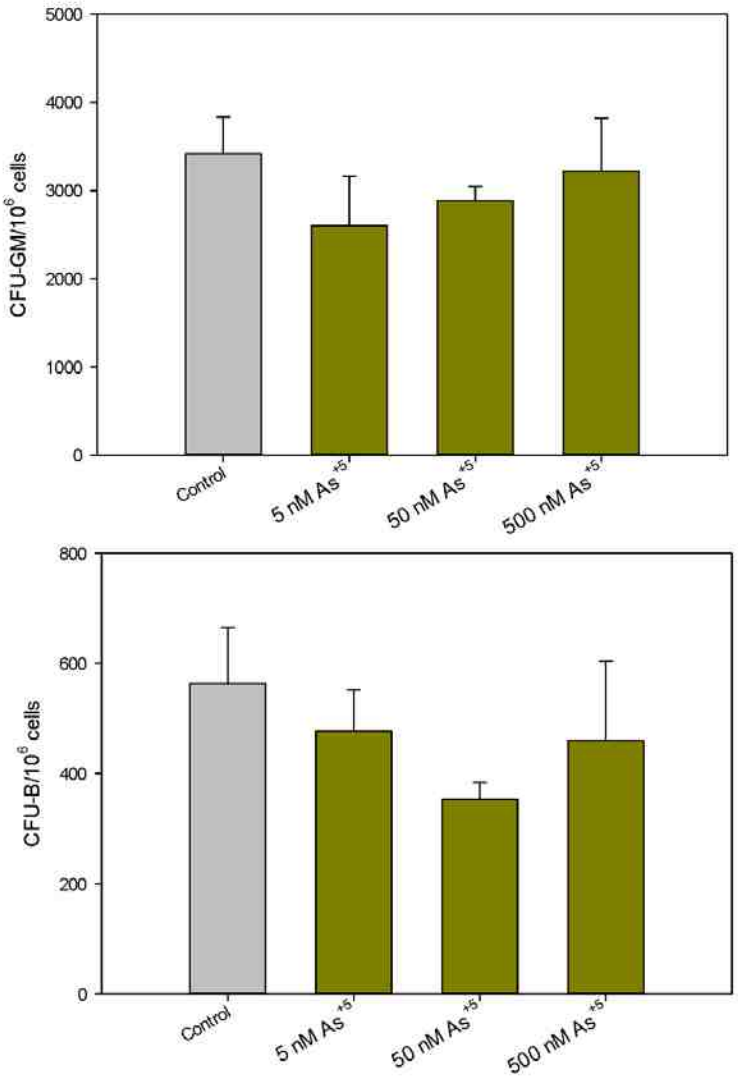

Figure 2.7. Number of colonies per million cells exposed to As^{+5} *in vitro*. Number of CFU-B colonies per million cells 10 days post plating in mouse methylcellulose media (containing As^{+5}) for pre-B cells [Top]. Number of CFU-GM colonies per million cells 14 days post plating in mouse methylcellulose media (containing As^{+5}) for GM cells [Bottom]. No significant differences between treatments and control ($p<0.05$) were found. Results are Means $\pm$ SD.

DISCUSSION

Arsenic is considered to be one of the most important global environmental risk factors for human diseases (Argos et al., 2010). There is increasing evidence that arsenic alters immune activity function in human populations. Many of the current findings rely on the use of peripheral blood and biomarkers to infer the cells and organs that are affected by arsenic (Andrew et al., 2006; Argos et al., 2006; Biswas et al., 2008; Soto-Pena et al., 2006). In addition, there have been *in vitro* studies with arsenite (As^{+3}) at micro molar concentrations in human leukocytes that demonstrate altered immune effects possibly by affecting T cells and macrophage/dendritic cells (Macoch et al., 2013; Morzadec et al., 2012a, 2012b). Also, it has been observed that micro molar concentrations of As^{+3} induce necrosis of human CD34+ bone marrow cells (Vernhet et al., 2008). These studies are interesting because arsenic trioxide is used clinically for the treatment of certain leukemia and myelodysplastic syndromes (Lengfelder et al., 2012).

Previous studies using environmentally relevant levels of exposure have shown that low levels of As^{+3} given in drinking water to mature mice increased their susceptibility to influenza virus (Kozul et al., 2009). Our present studies followed a similar design (30 day exposure, at concentrations up to 300 ppb As^{+3}). We found that As^{+3} produced a significant suppression of pre-B colony formation in the bone marrow. While the effects of As^{+3} on bone marrow could contribute to the immune suppression associated with influenza and other infections, there are numerous other immunologic abnormalities that could result from effects on the bone marrow. The hematopoietic system continually replenishes itself throughout life and is one of the most sensitive targets for cytotoxic and anti-proliferative agents, often being

the dose limiting organ/tissue for radiation and chemotherapy. The immune system that develops during embryogenesis has been found to be extremely sensitive to environmental chemical effects (Irons, 1985). There is evidence that the development of lymphoid tissues is altered by environmental arsenic exposure, as thymic defects have been detected in developmentally-exposed human populations (Ahmed et al., 2012).

To better understand the mechanisms of action associated with arsenic immunotoxicity, several labs have established animal models. Our own studies have shown that exposure of mice to arsenic trioxide via inhalation causes suppression of the T-dependent antibody response (Burchiel et al., 2009). The effect of As^{+3} may involve a complex action on the activation and differentiation of both lymphoid (B and T) and myeloid (macrophages and dendritic) cells. Therefore, in the present studies, we were interested in characterizing the effects of arsenic on lymphoid and myeloid cell development in the bone marrow following *in vivo* 30 day drinking water and *in vitro* exposures. Trivalent arsenite is known to be one of the toxic species of arsenic that can form in the body following *in vivo* drinking water exposure to arsenic. The toxicity of arsenic *in vivo* would depend on the type, quantity and stability of the specific arsenic metabolites formed through biotransformation (Styblo et al., 2000). A key organic metabolite of arsenite formed through metabolism by the enzyme arsenite 3-methyltransferase (AS3MT) is MMA^{+3} (Aposhian et al., 2000). To investigate the relative contribution of each arsenic species that could lead to bone marrow suppression, we directly exposed bone marrow cells to different arsenic species, including As^{+3}, MMA^{+3} and As^{+5}. As^{+3} significantly and selectively inhibited the lymphoid (CFU-B) progenitor formation at 50 nM, but had no effect on the myeloid progenitors (CFU-GM), even at 500 nM examined *in vitro*. The selective effects of As^{+3} on lymphoid progenitors are

therefore consistent between our *in vivo* and *in vitro* studies. The lymphoid progenitor CFU-B colony formation was significantly suppressed by 5 nM MMA^{+3} *in vitro*, whereas the myeloid progenitor cells (CFU-GM), were suppressed at 500 nM MMA^{+3}, indicating a hundred fold difference in sensitivity. The lack of significant changes in viability, Annexin V staining and PI staining 18 hrs following *in vitro* exposure to As^{+3} (up to 500 nM), MMA^{+3} (up to 50nM), and As^{+5} (up to 500 nM) may be an indication that cell death is not responsible for the BM suppression observed with the 300 ppb *in vivo* As^{+3} exposure. The 500 nM MMA^{+3} dose that caused the reduction in cell viability and increased Annexin V and PI staining also completely inhibited CFU-B colony formation, but only suppressed CFU-GM formation by 50%. The selectivity is thus clear but raises question in regard to mechanism.

In vitro exposure of murine bone marrow cells to As^{+5} showed that this agent did not significantly inhibit CFU-B or CFU-GM colony formation at concentrations up to 500 nM. MMA^{+3} has been found in the blood and urine of human subjects who were exposed to high levels of inorganic arsenite via drinking water (Aposhian et al., 2000; Gamble et al., 2007). It is produced in the liver and kidneys through the metabolism of arsenate or arsenite by the action of the enzymes arsenate/arsenite reductases, AS3MT, and MMA^{+5} reductase. AS3MT is a polymorphic enzyme in the human that has been shown to correlate with the toxicity of arsenic in drinking water (Engstrom et al., 2013; Fujihara et al., 2010; Meze et al., 2007). AS3MT has been detected in rodent tissues, primarily in liver and kidney (Healy et al., 1998) and is also expressed in human peripheral blood (Engstrom et al., 2011). Therefore, our studies demonstrate that it is important to understand the relative actions of important arsenite metabolites formed *in vivo*, such as MMA^{+3}, and the exposure levels received by the bone marrow and other lymphoid organs.

In summary, this study has demonstrated for the first time the sensitivity subpopulations of the bone marrow to inorganic and organic arsenicals. The fact that lymphoid progenitors are more sensitive to suppression than myeloid progenitors suggests that there may be novel targeting mechanisms associated with cell activation and cell differentiation that are affected by As^{+3} and MMA^{+3} in murine bone marrow cells. Future studies will address various mechanisms that may be associated with arsenite inhibition of CFU-B formation and will also examine the effects of arsenite and MMA^{+3} on early T cell development.

FUNDING

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CHAPTER 3

Arsenite interacts with Dibenzo[*def, p*]chrysene (DBC) at low levels to suppress bone marrow lymphoid progenitors in mice

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Key Words: immunotoxicity, bone marrow, lymphoid progenitors, arsenite, polycyclic aromatic hydrocarbons (PAHs)

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ABSTRACT

Arsenite and Dibenzo[*def,p*]chrysene (DBC), a polycyclic aromatic hyrdrocarbon (PAH), are found in nature as environmental contaminants. Both are known to individually suppress the immune system of humans and mice. In order to determine their potential interactive and combined immunosuppressive effects, we examined murine bone marrow (BM) immune progenitor cells response following combined oral exposures at very low levels of exposure to As^{+3} and DBC . Oral 5 day exposure to DBC at 1 mg/kg (cumulative dose) was found to suppress mouse BM lymphoid progenitor cells, but not the myeloid progenitors. Previously established no-effect doses of As^{+3} in drinking water (19 and 75 ppb for 30 days) produced more lymphoid suppression in the bone marrow when mice were concomitantly fed a low dose of DBC during the last 5-days. The lower dose (19 ppb) As^{+3} had more suppressive effect with DBC than the higher dose (75 ppb).Thus the interactive toxicity of As^{+3} and DBC *in vivo* could be As^{+3}-dose dependent. *In vitro,* the suppressive interaction of As^{+3} and DBC was also evident at low concentrations (0.5 nM), but not at higher concentrations (5 nM) of As^{+3}. These studies show potentially important interactions between As^{+3} and DBC on mouse BM at extremely low levels of exposure *in vivo* and *in vitro*.

INTRODUCTION

Arsenic and polycyclic aromatic hydrocarbons (PAHs) are environmental toxicants that are individually known to produce immunotoxicity in humans and mice (Argos et al., 2010; Burchiel et al., 2014; Kozul et al., 2009; Li et al., 2010; N'jai et al., 2010) . Our previous studies showed that As^{+3} given orally at low levels selectively suppressed bone marrow (BM) lymphoid progenitor cells response *in vivo* and *in vitro*, and spleen T-dependent antibody responses (TDAR) *in vitro* (Ezeh et al., 2014). Immunosuppression is one of the major adverse manifestations of As^{+3} and PAH exposures. Dibenzo[*def,p*]chrysene (DBC), like the highly toxic 7,12-diemthylbenz(a)anthracene (DMBA), is known to suppress T-dependent antibody responses (TDAR) of murine spleen cells following *in vivo* and *in vitro* exposures (Gao et al., 2005; Gao et al., 2007; Gao et al., 2008; Lauer et al., 2013) and, in combination with As^{+3}, these PAHs were found to produce synergistic immunosuppression of the TDAR *in vitro* (Li et al., 2010).

Our studies were motivated by the knowledge that PAHs produce immunosuppression in lymphoid organs, such as spleen and BM, through the formation of reactive metabolites that form DNA adducts with induction of p53 (Gao et al., 2005; Gao et al., 2007, 2008; Galvin et al., 2003, 2006). Since PAH-adducts can be repaired by both DNA Base Excision Repair (BER) and Nucleotide Excision Repair (NER) pathways that are sensitive to inhibition by As^{+3} (Qin et al., 2012; Zhou et al., 2011), we postulated that low concentrations of PAHs that are not cytotoxic to BM may produce increased immunotoxicity in the presence of As^{+3}.

We sought to investigate the possible influence of co-exposure to both pollutants and their possible interactions in the BM in mice at very low levels through physiologically relevant routes of exposure. Concentrations of 19 and 75 ppb As^{+3} in 30 day drinking water were previously established to be non-toxic to the BM (Ezeh et al., 2014), and 0.1 and 1 mg/kg cumulative doses of DBC in pill forms (Lauer et al, 2013) were chosen based on previous dose-range studies.

METHODS

Animals and Treatment Exposures

C57BL/6J mice were purchased from Jackson Laboratory, Bar Harbor, Maine, USA, at 8 -10 weeks old and housed in our AAALAC-approved facility, for use in this study at 12-16 weeks. These animals were handled according to procedures and protocols that have been approved by the Institutional Animal Care and Use Committee (IACUC) at the University of New Mexico Health Sciences Center. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. About 2-3 animals were housed per cage and exposed to different concentrations of arsenite (As^{+3}) in parts per billion (ppb) for 30 days via drinking water and/or exposed to Dibenzo[*def, p*]chrysene (DBC) by voluntary ingestion of a pill containing the chemical. Double processed tissue culture water (Sigma Aldrich), was used to prepare arsenite stock solutions. The cookie pill containing the DBC was prepared using Transgenic Dough Diet, DMSO, bromophenol blue and DBC according to the procedure previously described (Lauer et al., 2013).

Water samples were collected periodically and sent to University of Arizona Laboratory for Emerging Contaminants (ALEC, Tucson AZ) to determine the As^{+3} concentrations in the control (no As^{+3}) drinking water and to confirm As^{+3} levels in prepared solutions used for these studies. The concentration of As^{+3} in the control drinking water and that used to make solutions was found to be less than 5 ppb. For the water consumption of the mice in each cage, the water bags containing water were weighed prior to placement in the cage and again after water consumption by the mice. Mice were fed a Mouse 2020X Teklad Global Soy Protein-Free Extruded Rodent Diet purchased from Harlan Laboratories Inc, Madison Wisconsin, USA, www.harlan.com . The total arsenic content of this diet was

found to be 0.16 mg/kg (160 ppb), with approximately 10–15% estimated to be inorganic arsenic, As^{+3} and As^{+5}, as discussed previously (Kozul et al., 2009).

For *in vivo* studies, 45 male C57BL/6J mice were exposed to As^{+3} via drinking water for 30 days and/or exposed to DBC via pill ingestion. The mice were treated in groups of four or five for each treatment and bone marrow cells (BM) from each exposed mouse was isolated and analyzed in triplicate. For *in vitro* studies, bone marrow cells isolated from both femurs of three untreated C57BL/6J mice were pooled together, exposed to each treatment and analyzed in triplicate. All animals were handled and disposed of in accordance with the University of New Mexico's Department of Safety and Risk Services protocol.

Chemicals and Reagents

Sodium arsenite (CAS 774-46-5, $NaAsO_2$) was purchased from Sigma-Aldrich (St. Louis, MO). The PAHs were purchased or obtained at greater than 95% purity from the following sources: dibenzo[*def, p*]chrysene (DBC, Accustandard), DBC-11,12 dihydrodiol was provided by Dr. David Williams at Oregon State University. MethoCult GF Methylcellulose Medium (Cat. No. M3534) with recombinant cytokines (without EPO) for mouse cells was purchased from Stem Cell Technologies (Vancouver, BC, Canada). Mouse Methylcellulose Complete Medium for pre-B cells (Cat. No. HSC009) was purchased from R&D Systems (Minneapolis, MN).

Arsenic and PAH handling

Arsenic, DBC and DBC-diol and all animals exposed to these chemicals were handled and disposed according to the University of New Mexico's Department of Safety and Risk Services protocol.

Isolation of Mouse Bone Marrow Cells

Bone marrow cells were isolated according to the procedure outlined in the Stem Cell Technologies Technical Manual version 3.1.1(http://www.stemcell.com/) and as described in previous work (Ezeh et al., 2014). Briefly, each mouse was euthanized and the femurs were sterilely harvested, then placed and held on ice in Hanks' Balanced Salt Solution (HBSS) from Lonza Walkersville, MD, and transported to the laboratory. Bone marrow cells were extracted from the femurs using a 25 gauge needle with a 1 cc syringe, filled with approximately 1 ml cold sterile medium which was used to flush through the femur several times to release cells into a cell culture dish containing RPMI medium that was supplemented with FBS. The cells solution was immediately transferred to a 15 ml culture tube and placed on ice until needed. Cells were washed by centrifugation at 4°C, 300 *X g* for 10 min and were resuspended in RPMI Medium for culturing. Cell recovery and viability was determined by manual counting or automated counting using the Nexcelom Cellometer 2000.

CFU-B Assay

The procedure for Mouse Colony-Forming Cell (CFC) assays described in the Stem Cell Technologies Technical manual version 3.1.1(http://www.stemcell.com/) was used to determine the pre-B colony forming units (CFU) counts per pair of mice femurs and per million bone marrow cells. Briefly, bone BM cells marrow cells isolated from femurs of each

treated or untreated mouse (for *in vivo* studies) or pooled from femurs of three untreated mice (for *in vitro* studies), were suspended in RPMI 1640 Medium supplemented with 2% heat inactivated Hyclone Fetal Bovine Serum (Fisher Scientific, Pittsburgh, PA) at 1×10^6cells/ml. 400 µl (4×10^5 cells) of the cell suspension was transferred to a 16 ml (17×100 mm) sterile culture tube containing 4 ml Mouse Methylcellulose Complete Media for pre-B Cells. The tube was vortexed and left to sit for 20 min to release air bubbles. 1 ml of the methylcellulose-cell suspension was placed in a 35 mm culture dish (Stem Cell Technologies) using a 3 cc syringe with a 16G×11/2″ Monoject Aluminum Hub, blunt cannula needle (Covidien, Mansfield, MA). Samples were ran in triplicate. The mixture was evenly dispersed in the dish by rocking the dish. One sterile water dish and two sample dishes were placed into a 100 mm culture dish and incubated at 37°C, 5% CO_2, in a humidified incubator for 10 days. CFU-B colonies were counted and recorded for statistical analysis.

CFU-GM Assay

This assay was performed using the same procedure as in the CFU-B assay described above except that the isolated bone marrow cells were suspended in Iscove's Modified Dulbecco's Medium (Sigma-Aldrich) supplemented with 2% heat inactivated Hyclone Fetal Bovine Serum at 2×10^5 cells/ml, and 400 µl (8×10^4 cells) of the cell suspension was transferred to a 16 ml (17×100 mm) sterile culture tube containing 4 ml MethoCult GF Methylcellulose Medium (Stem Cell Technologies). Like in the CFU-B assay described above, the mixture was dispersed evenly in the dish and sample dishes (35 mm) were placed in a 100 mm culture dish and put in the humidified incubator at 37°C and 5% CO_2 for 14 days. CFU-GM colonies were counted and recorded for statistical analysis.

Data Analysis and Statistics

Sigma Stat version 3.5 and Sigma Plot 12.0 software were used for data analysis. One-way analysis of variance (ANOVA) and Dunnett's t-test were applied to determine differences between control and treatment groups. In the *in vivo* studies, treatment groups consisted of four or five animals and each animal was analyzed in triplicate. Bone marrow cell recovery was expressed as the mean number of recovered bone marrow cells obtained from both femurs, Results were reported as CFU-B and CFU-GM per million cells and also per pair of femurs. For the *in vitro* experiments, a treatment group consisted of three replicates of one chemical treatment of pooled bone marrow cells or control. CFU-B and CFU-GM colony counts were reported as per million cells.

Table 3.1 Effect of cumulative 5 day *in vivo* exposure to DBC on mice BM cell recovery and viability[a]

DBC (mg/kg)	BM Cell Count (x 10^7)	Viability (%)
0	5.9 ± 1.6	98.8 ± 2.4
0.01	6.8 ± 1.9	100 ± 0.0
0.1	7.4 ± 1.7	99.3 ± 1.5
1	4.8 ± 0.9	100 ± 0.0
10	5.8 ± 3.0	93.0 ± 8.6

[a]Results shown are the Means ± SD

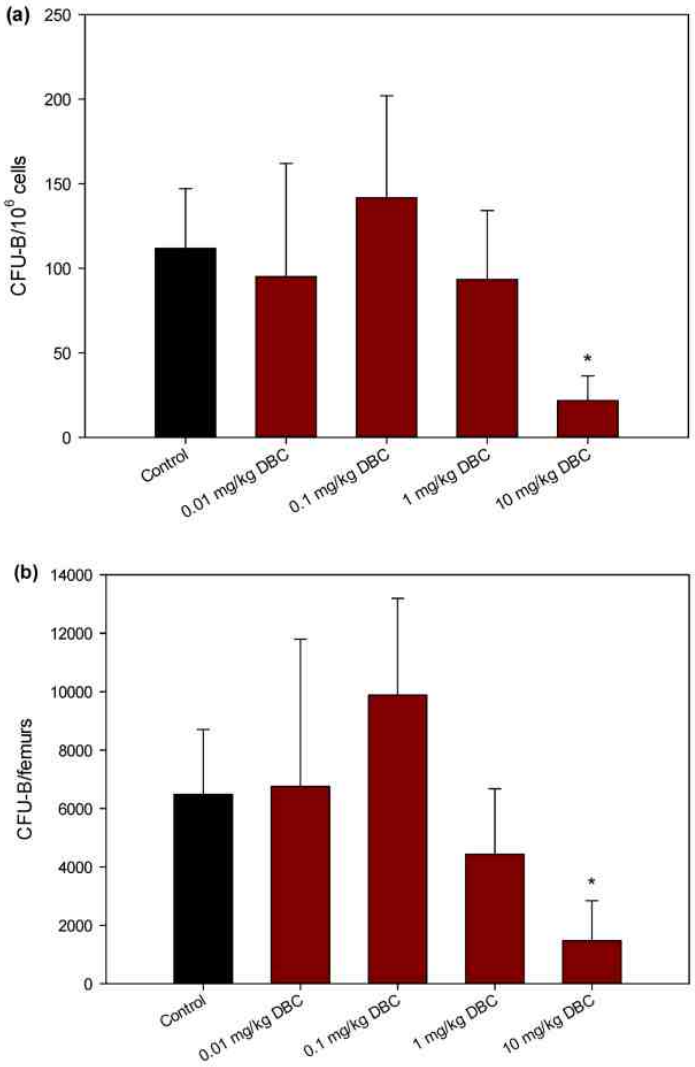

Figure 3.1. Pre-B colony formation in mice post 5 day *in vivo* cumulative exposure to DBC. Number of CFU-B colonies per million BM cells [a] or per set of femurs [b] in mice following 5 day *in vivo* exposure to DBC via oral pill ingestion, 10 days post plating in mouse methylcellulose media for pre-B cell selection. *Significantly different compared to control ($p < 0.05$). Data shown are Means $\pm$ SD.

RESULTS

C57BL/6J male mice, consisting of 4 mice per treatment dose, were exposed to 0, 0.01, 0.1, 1 or 10 mg/kg cumulative dose of DBC via voluntary ingestion of DBC pills daily for 5 days. As shown in Table 3.1, at these exposure concentrations, there was no effect on the recovery of viable cells from the BM. A dose-dependent decrease in CFU-B formation was detected when expressed on a per million cell basis (Fig. 3.1a) and on a per pooled femur basis (Fig. 3.1b). Greater than 90% inhibition of CFU-B colony formation was observed at the 10 mg/kg total cumulative dose of DBC given over 5 days.

Based on our previous work, we were interested in determining whether concomitant exposure of mice to low or no-effect doses of As^{+3} with low levels of DBC could produce significantly more suppression of the BM. In an *in vivo* experiment using 5 mice per dose group, mice were exposed to 0, 19, or 75 ppb As^{+3} in drinking water for 30 days in the absence or presence of DBC at 0.1 or 1 mg/kg given during the last 5 days of As^{+3} exposure. As expected, results showed that neither As^{+3} nor DBC suppressed CFU-B levels on their own when expressed on a per million cell basis (Fig. 3.2a), but the combined exposures to 19 ppb As^{+3} and 1 mg/kg DBC did show significant suppression which was more than that seen in the 75 ppb As^{+3} and DBC individual exposures . DBC produced a small decrease in BM cell recovery at the 1 mg/kg exposure (Table 3.2) which resulted in a significant effect on CFU-B formation when expressed on an individual mouse pooled femur basis (Fig. 3.2b). An additional finding was that these combinations of As^{+3} and DBC did not suppress the formation of CFU-GM colonies (Fig. 3.2c, 3.2d).

Table 3.2 *In vivo* low dose As^{+3} ± DBC has no effect on the cell recovery and viability of mice BM cells[a]

Treatment	Cell count (x 10^7)	Viability (%)
Control	3.2 ± 1.2	93.1 ± 3.6
19 ppb As^{+3}	3.0 ± 0.2	92.4 ± 1.6
75 ppb As^{+3}	3.0 ± 0.8	93.1 ± 2.8
0.1 mg/kg DBC	3.0 ± 0.7	94.0 ± 2.2
1 mg/kg DBC	2.3 ± 0.5	93.0 ± 1.1
19 ppb As^{+3} + 0.1 mg/kg DBC	3.3 ± 0.4	92.7 ± 2.8
19 ppb As^{+3} + 1 mg/kg DBC	3.2 ± 0.7	94.4 ± 1.4
75 ppb As^{+3} + 0.1 mg/kg DBC	3.4 ± 0.4	92.8 ± 2.2
75 ppb As^{+3} + 1 mg/kg DBC	3.2 ± 0.7	93.1 ± 2.5

[a]Results shown are the Means ± SD

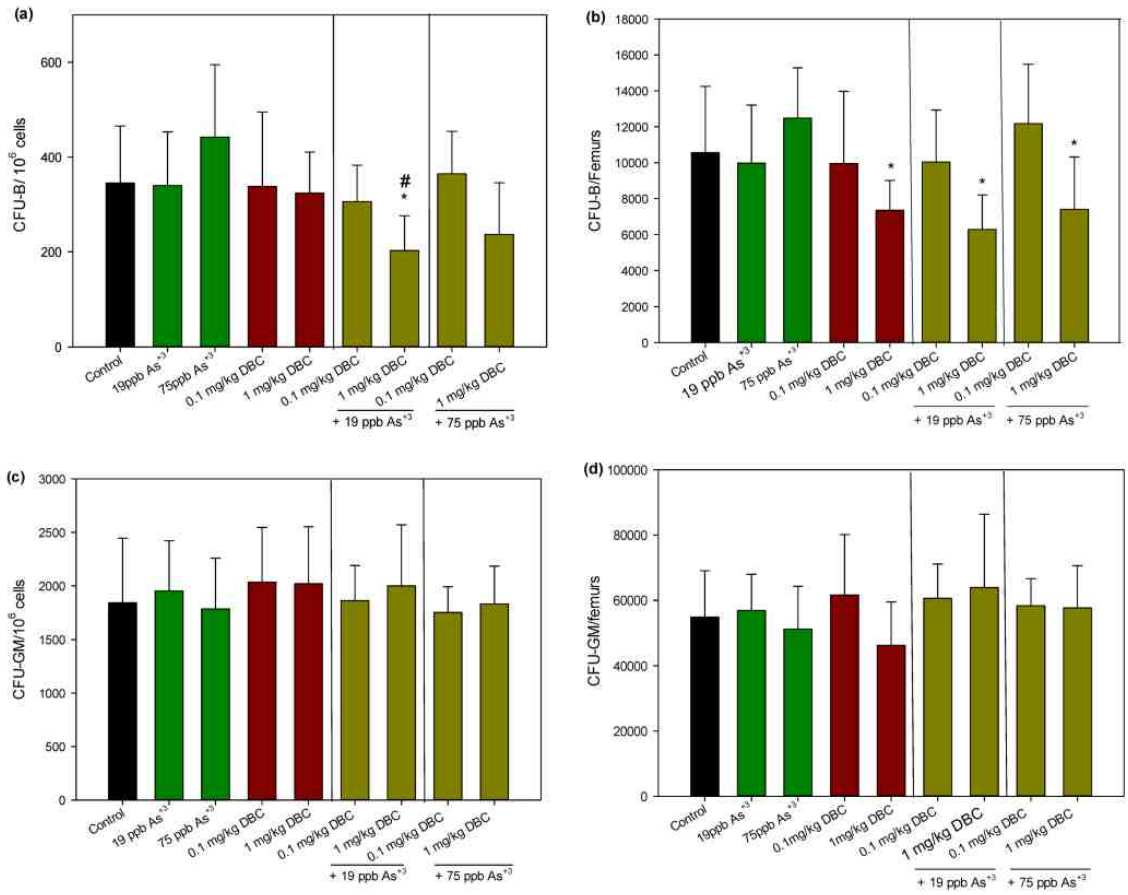

Figure 3.2. CFU-B and CFU-GM colony formation in mice post *in vivo* exposure to low level of As^{+3} ± DBC. Number of CFU-B colonies per million BM cells [a] or per set of femurs [b]; Number of CFU-GM colonies per million BM cells [c] or per set of femurs [d] in mice following 30 day *in vivo* exposure to As^{+3} via drinking water ± 5 day DBC exposure via oral ingestion. BM cells were plated in mouse methylcellulose media for pre-B cell selection for 10 days or in methocult media for GM selection for 14 days. *Significantly different compared to control ($p<0.05$). $^{\#}$Significantly different compared to effect of DBC or As^{+3} given as individual treatments at the selected dose. Data shown are Means ± SD.

These findings are consistent with our previous studies showing the selective suppression of CFU-B in mouse BM following 30 day low level As^{+3} exposures (Ezeh et al., 2014).

To further investigate the possibility of As^{+3} interaction with DBC in the BM, mice BM cells were exposed to various concentrations of As^{+3}, DBC, and DBC-diol (a DBC metabolite known to be the immediate precurosor of DBC-diol epoxide, which is thought to be important in DBC adduct formation in DNA) *in vitro*. Based on preliminary studies to determine no-effect doses of agents, we used 0.5 and 5 nM concentrations of As^{+3} and 0.1 and 1 nM concentrations of DBC (Fig. 3.3a). We found that these levels of As^{+3} and DBC given alone did not suppress CFU-B formation *in vitro.* However, low concentratiions of As^{+3} (0.5 nM) produced CFU-B suppression when combined with low level (0.1 nM) of DBC or DBC-diol (Fig. 3.3a). The colony count in the As^{+3}/DBC-diol combination was significantly different compared to both control and the individual parent compounds at the same doses, suggesting interaction. Increased As^{+3} concentration (5 nM) also produced suppression of CFU-B in the presence of 0.1 nM and 1 nM DBC, but not in the presence of DBC-diol. There were no interactions between As^{+3} and DBC at the higher concentrations of exposure.

Since we previously found that MMA^{+3} (an As^{+3} metabolite) may be responsible for the toxicity of As^{+3} on BM *in vivo* (Ezeh et al., 2014), we investigated possible interaction of MMA^{+3} with DBC or DBC-diol in *in vitro* cultures, using one concentration (5 nM) that was previously established to be minimally toxic to the BM and another ten times lower concentration (0.5 nM). Both concentrations of MMA^{+3} suppressed CFU-B formation in

mouse BM (Fig. 3.3b). However, we observed MMA^{+3}/DBC interacton at the higher concentrations of both chemicals but not at the lower concentrations nor with DBC-diol.

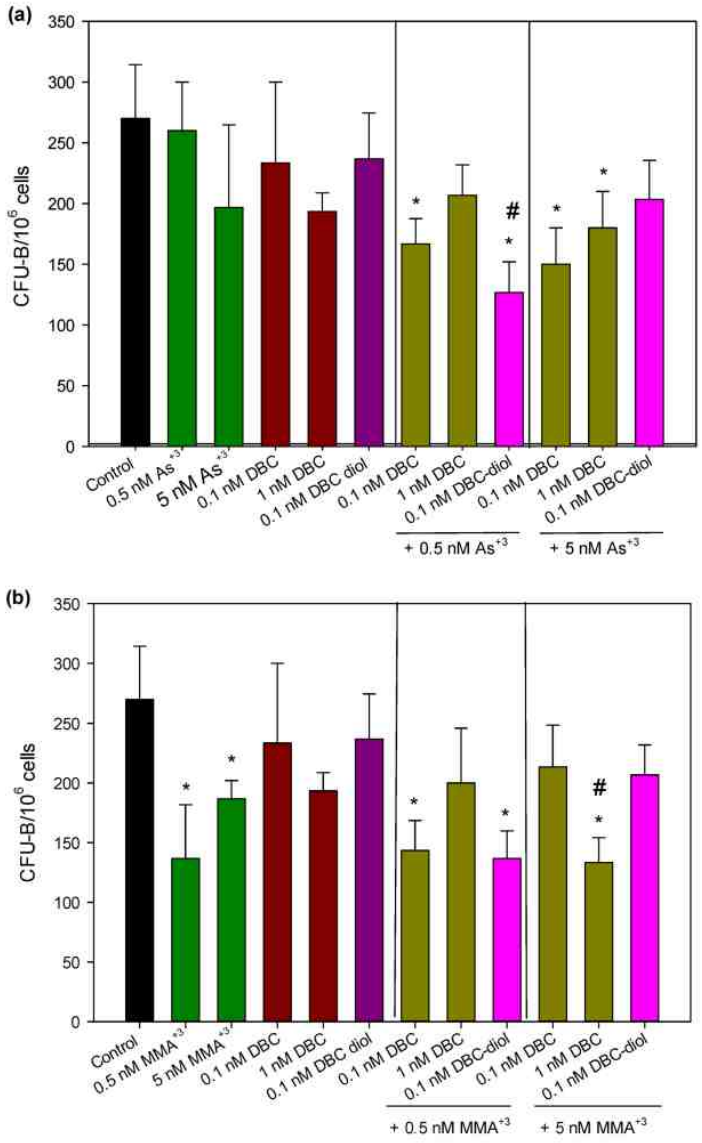

Figure 3.3. *In vitro* exposure of mice BM cells to As^{+3} ± DBC or DBC-diol.

Number of CFU-B colonies per million cells in untreated mice BM cells post 10 day plating in pre-B mouse methylcellulose media containing As^{+3} ± DBC or DBC-diol [a], or containing MMA^{+3} ± DBC or DBC-diol [b]. #Significantly different compared to effect of DBC or As^{+3} given as individual treatments at the selected dose. *Significantly different compared to control ($p<0.05$). Data shown are Means ± SD.

DISCUSSION

The present studies demonstrate for the first time that As^{+3} interacts with DBC when given orally at low doses *in vivo* and selectively suppresses pre-B formation in mouse BM. It was also shown that DBC alone is selectively toxic to BM CFU-B formation at a 1 mg/kg oral cumulative dose given over a five day period. In previous work, acute intraperitoneal and oral administration of 50 mg/kg DMBA, (a DBC-like, synthetic PAH) suppressed BM CFU-B and CFU-GM in mice (N'jai et al., 2010). The effect of the DBC following oral administration on the BM has not been reported previously, nor has the toxicity of DBC at the concentrations we examined been studied. . DBC toxicity is likely caused by the products of its biotransformation (Siddens et al., 2012). DBC-dihydrodiol epoxide (DBCDE) is formed through the action of two enzymes, CYP1B1 and epoxide hydrolase (Crowell et al., 2014). DBC radical cation is formed through the action of peroxidases (Cavalieri et al., 1991), and DBC-dione obtained through the breakdown of DBC-11, 12-dihydrodiol by aldoketoreductases (AKR), (Courter et al., 2007). Thus, in addition to the formation of DNA adducts by DBCDE, there is also potential redox cycling leading to the production of reactive oxygen species (ROS) with resultant oxidative stress.

As^{+3} and its metabolites are known to be toxic. The biotransformation of As^{+3} by arsenite methyltransferase (AS3MT) produces methylated species with varying toxicities, including monomethylated forms such as monomethylarsonous acid (MMA^{+3}) and monomethylasonic acid (MMA^{+5}), also dimethylated forms such as dimethylarsonous acid (DMA^{+3}) and dimetylarsonic acid (DMA^{+5}) (Styblo et al., 2000; Aposhian et al., 2000). MMA^{+3} is considered the most toxic arsenic species Styblo et al., 2000) and has been

previously implicated by our lab in BM toxicity (Ezeh et al., 2014). Thus, an increase in the formation of MMA^{+3} from *in vivo* As^{+3} is expected to produce more BM suppression than the parent compound.

The combination of As^{+3} and DBC *in vivo* showed significant interactions at low doses with slightly increased and selective suppression of CFU-B when 19 ppb, 30-day drinking water As^{+3} was given in combination with 1 mg/kg, 5-day oral cumulative dose of DBC . These doses did not significantly impact bone marrow cell recovery and viability in mice, nor did they suppress BM cell differentiation when given individually. The observed lack of significant effect on BM cell differentiation by individual exposures to these compounds may be due to a number of reasons, including: insufficient bioactive metabolites from the low doses administered, lack of induction of the enzymes responsible for their bioactivation or a redirection of metabolites to other pathways not associated with toxicity. It is, however, noteworthy that 1 mg/kg DBC showed a statistically significant suppression of pre-B cells, but not CFU-GM when results were averaged for mice and expressed on a pooled femur basis. The selective and significant suppression of CFU-B over CFU-GM colony formation observed with 19 ppb As^{+3} and 1 mg/kg DBC combination, suggest that there may be some interaction at these doses. Although we could not further define the precise parameters responsible pertaining to the dose and time responses for interactions, we know that As^{+3} and/or DBC metabolites could be involved in the toxicity observed. It is possible that the presence of both compounds in the system could influence their individual metabolism and resultant toxicity or lack of toxicity, such that ratio may also be important. It is known that in the liver, As^{+3} alters the regulation of enzymes that are responsible for PAH bioactivation (CYP1s), thereby decreasing PAH metabolite formation (Jacobs et al., 1999;

Vernhet et al., 2003). Other studies (Spink et al., 2002; Bessette et al., 2005) also indicate that As^{+3} diminishes the induction of the CYP1 enzymes. However, these studies were conducted at higher concentrations of As^{+3} (micro molar ranges) than used in our present work. As with DMBA (Gao et al., 2005), DBC is mostly metabolized by CYP1B1 in mouse lymphoid tissues (Crowell et al., 2014). Therefore, if high doses of As^{+3} interfere with the induction or activity of CYP1B1, this may explain the lack of interaction between As^{+3} and DBC at the higher concentration of As^{+3}.

For our *in vitro* studies, the combination of no-effect, low levels (0.5 and 5 nM) of As^{+3} with no-effect, low levels (0.1 and 1 nM) of DBC and DBC-diol, produced significant suppression of pre-B colonies that was greater than the individual parent compounds, but the suppression did not always increase with higher concentration of chemicals as expected.

We show evidence of complicated dose responses in As^{+3}/DBC combination that may be explained by the different direct and indirect effects of the individual compounds as well as their metabolites and associated enzymes. As expected, 0.5 and 5 nM MMA^{+3} were more potent than the same concentrations of As^{+3} *in vitro*. However, our studies demonstrate significant MMA^{+3} interactions with DBC at the higher MMA^{+3} and DBC doses *in vitro*. This suggests possible differences in the mechanisms of action between As^{+3} and MMA^{+3} in their interactions with DBC *in vitro*. Therefore further studies are needed to understand the reason for the lack of interaction between DBC and the higher doses/concentrations of As^{+3} and MMA^{+3} used in the present studies in murine BM.

In conclusion, we have observed that low doses of DBC selectively target the lymphoid progenitors in mice, and that *in vivo* and *in vitro* there is a complex non-linear

immunosuppressive interaction between As^{+3} and DBC. These findings are important because these interactions occur at environmentally relevant doses and via normal physiological routes of exposure.

ACKNOWLEDGEMENT

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CHAPTER 4

Monomethylarsonous acid (MMA^{+3}) Inhibits STAT5 Phosphorylation in Mouse Pre-B Cells

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Running title: Arsenite and its metabolite inhibit STAT5 phosphorylation in lymphoid cells.

ABSTRACT

Our previously published data show that As^{+3} *in vivo* and *in vitro,* at very low concentrations inhibit lymphoid, but not myeloid stem cell development in mouse bone marrow. We also showed that the As^{+3} metabolite, monomethylarsonous acid (MMA^{+3}) was responsible for the observed pre-B cell toxicity caused by As^{+3}. Interleukin-7 (IL-7) is the primary growth factor responsible for pre-lymphoid development in mouse and human bone marrow. Thus, we examined IL-7 signaling in a mouse pre-B cell line (2E8) that is dependent on IL-7 for growth. We found that MMA^{+3}, but not As^{+3} inhibited 2E8 cell proliferation at concentrations that do not produce cytotoxicity. MMA^{+3}, again in contrast to As^{+3}, inhibited IL-7 signaling in 2E8 cells as determined by a decrease in IL-7 induced STAT-5 phosphorylation. JAK phosphorylation was not affected by MMA^{+3}. We found that the induction of PAX5 gene expression, which is controlled by activated STAT5-associated transcription factors that are induced by IL-7, was also selectively inhibited by MMA^{+3}. Since 2E8 cells lack the enzymes responsible for the conversion of As^{+3} to MMA^{+3} *in vitro,* the results of these studies clearly show that MMA^{+3} is responsible for the lack of IL-7 signaling in 2E8 cells and offer a likely explanation for how As^{+3}, which is metabolized to MMA^{+3} *in vivo,* selectively targets the development of pre-lymphoid stem cells in mouse bone marrow.

INTRODUCTION

Arsenic exposure has been associated with many diseases including immunosuppression, hematotoxicity, and carcinogenesis (Kozul et al., 2009; Li et al., 2010). Trivalent inorganic arsenic (As^{+3}) and its metabolite, monomethylarsonous acid (MMA^{+3}), have been associated with many toxicities in mammalian systems (Styblo et al., 2000). Several studies have examined mechanisms associated with high dose acute exposures to As^{+3} with the resultant DNA damage and alteration of DNA repair system (Wei et al., 2009; Qin et al., 2012; Wang et al., 2013). In general, several factors including oxidative stress (Flora, 2011), chromosomal aberration (Oya Ohta et al., 1996), inhibition of DNA repair and PARP activity (Qin et al., 2012), and altered gene expression could collectively contribute to As^{+3} toxicity.

Only a few studies have attempted to address mechanisms associated with As^{+3} toxicity from physiological, long- term, low dose, and environmentally relevant exposures. Our lab previously observed that low doses of As^{+3} inhibit mouse bone marrow lymphoid but not myeloid progenitor cell development *in vivo* (Ezeh et al., 2014). We attributed the *in vivo* toxicity of As^{+3} to one of its primary organic metabolites, MMA^{+3}, which is formed by the liver and kidneys (Aposhian et al., 2000). We showed that MMA^{+3} inhibits pre-B development from hematopoietic stem cells at concentrations that can be achieved with environmental exposures.

For the selective targeting of mouse pre-lymphoid stem cells found in bone marrow (BM), we hypothesized that As^{+3} and MMA^{+3} could modulate the BM microenvironment, leading to alteration of IL-7 signaling which is critical for lymphoid cell formation and

expansion. The cytokine, IL-7 has been shown to specify B-cell fate from the common lymphoid to the pre-pro B stage (Kikuchi et al., 2005). It is also required for further differentiation of B and T lineage cells. Because of the tremendous heterogeneity of cell populations in the BM, we used 2E8 cells, an IL-7-dependent mouse pre-B cell line, to try to elucidate the mechanism by which As^{+3} and MMA^{+3} could target the lymphoid progenitors in the BM. Using similar concentration ranges of As^{+3} and MMA^{+3} that were previously studied in BM primary cells, we conducted studies to determine whether As^{+3} and/or MMA^{+3} alter IL-7 signaling in 2E8 cells.

MATERIALS and METHODS

Reagents and chemicals

Hanks Balanced Salt Solution (HBSS) from Lonza (Walkersville, MD), Dulbecco's phosphate buffered saline w/o Ca^{+2} or Mg^{+2} ($DPBS^{-}$), Iscoves Modified Dulbecco's Medium (IMDM) purchased from American Type Culture Collection (ATCC, Manassas, VA), Penicillin/Streptomycin 10,000 (mg/ml)/10,000 (U/ml) (Pen/Strep) Life Technologies (Grand Island, NY), β-Mecaptoethanol, Sigma Aldrich (St. Louis, MO), 1 mg/ml mouse IL-7 (mIL-7) purchased from Peprotech, Fetal Bovine Serum (FBS) Atlanta Biologicals (Flowery Branch, GA.), sodium arsenite (CAS 774-46-5, $NaAsO_2$) purchased from Sigma-Aldrich (St. Louis, MO); MMA^{+3} was the generous gift of Dr. Terry Monks at the University of Arizona Superfund Center and the Southwest Environmental Health Science Center. STAT5 (clone 3H7, Cat. No. 9358), Phospho-STAT5 (Tyr694) (clone C11C5, Cat No. 9359), phospho-JAK1 (Tyr1022/1023) (Cat. No.3331), PAX5 (clone D19F8, Cat. No. 11849), and anti-rabbit IgG –HRP secondary antibody (Cat. No. 7074) were purchased from Cell Signaling Technology (Danvers, MA,). Purified mouse anti-JAK1 monoclonal antibody was obtained from BD Transduction Laboratories (Franklin Lakes, NJ). Actin (clone I-19) goat polyclonal IgG (Cat. No. sc-1616) and donkey anti-goat IgG-HRP secondary antibody (Cat.No. sc-2020) were purchased from Santa Cruz Biotechnology (Dallas, TX).

Cell culture and treatments

The 2E8 cell line was purchased from ATCC and was first described by Ishihara et al., 1991. 2E8 cells were grown and maintained in our laboratory following ATCC's protocol using complete medium consisting of IMDM 1 ng/ml mouse IL-7 (mIL-7), 20% FBS, 1 %

Pen/Strep, and 0.05 μM β-mecaptoethanol. As^{+3} and MMA^{+3} were prepared in sterile water, as 1000X stock solutions and used to treat 2E8 cell preparations at final concentration ranges of 5 nM to 500 nM. Mouse IL-7 was prepared as 10 μg/ml stock solution and used to treat 2E8 cell preparations at a final concentration of 10 ng/ml in treatment medium or 1 ng/ml in culture medium. Cells were first seeded at 5 x 10^5 cells/ml, in T75 flasks using complete 2E8 medium with 1ng/ml mIL-7 for three days, then washed twice in $DPBS^-$ to remove all mIL-7. Cells were re-suspended in 2E8 medium without mIL-7 and treated with As^{+3} or MMA^{+3} overnight (~24 hrs.). Mouse IL-7 was re-introduced to treated cells at 10 ng/ml and cells were incubated for an additional 30 minutes to evaluate signaling. Post incubation, cells were harvested into 50 ml centrifuge tubes containing equal volume of ice cold $DPBS^-$ and washed twice by centrifugation at 400 x *g*, 4^{o}C for 10 minutes. Washed cells were used for Total RNA extraction or stored at -80^{o}C for protein extraction and immunoblotting.

Cell proliferation and viability

To determine cell proliferation and IL-7 dependence, 2E8 cells were grown at starting concentrations of 2.5 x 10^5 cells/ml in 2E8 medium with or without mIL-7 for 5 days. IL-7 was not replenished, and medium was not changed during the 5 day period. Cell counts were obtained in triplicate for each sample each day for 5 days, using the Nexcelom Cellometer Auto 2000 cell counter. Viability counts were also obtained using the same instrument with Acridine Orange/Propidium Iodide (AO/PI) staining. To test the sensitivity of 2E8 cells to treatment, proliferation and viability were also determined daily during the 5-day period for cells exposed to different concentrations of As^{+3} or MMA^{+3} in complete medium.

2E8 protein extraction and immunoblotting analyses

Treated and washed cell pellets were re-suspended at 2 x 10^5 cells/µl in radioimmune precipitation assay (RIPA) buffer (50 mM Tris, 150 mM sodium chloride, 0.5 % sodium deoxycholate, 1 % Triton X-100, 0.1 % sodium dodecyl sulphate (SDS), pH 8.0) with added protease inhibitors including one 25 mg protease inhibitor mini tablet (Roche Diagnostics), 1 % sodium orthovanadate (Na_3VO4), and 200mM phenylmethylsufonyl fluoride (PMSF).Samples were sonicated for 10 seconds on ice and centrifuged at 20,000 x *g*. Total protein (the supernatant) from each sample was quantified using the Bicinchoninic Acid (BCA) protein assay procedure (Pierce, Thermo Scientific). The protein extracts were resolved by electrophoresis on Criterion TGX precast gels (Bio-Rad) and transferred to nitrocellulose membranes. Antibodies against STAT5, pSTAT5, JAK1, pJAK1 or PAX5 were used to identify these proteins by immunoblotting. Using the Super Signal West Femto Maximum Sensitivity Substrate kit (Thermo Scientific), protein bands were resolved on the FLuorChem R ProteinSimple imager and analyzed with Image J (1.48v) software. Protein molecular weights were determined using the Precision Plus Protein Pre-stained Standards (Bio-Rad).

Total RNA isolation

Total RNA was isolated from treated and washed triplicate samples using RNeasy Mini Kit and Shredder (Qiagen, Valencia, CA.) following manufacturer's instructions. Briefly, treated and washed cells were pelleted in 15 ml centrifuge tubes, resuspended in 600 µl RLT buffer (contained in kit) with 10 µl/ml ß-mercaptoethanol and mixed by vortex. Each sample was transferred into a labeled QIA Shredder column and centrifuged at 20000 x *g* for

2 minutes. The isolation process was completed by following subsequent steps in the kit instructions, with final elution of total RNA using 50 µl, nuclease-free water. Total RNA concentration was calculated using the Nanodrop procedure for RNA quantitation. Results ranged from 3.7 - 7.5 µg/sample.

Synthesis of cDNA from total RNA

cDNA samples were synthesized from the isolated total RNA by reverse transcriptase (RT) reaction using High Capacity cDNA Archive Kit (Applied Biosystems) according to kit instructions. A 2X Master mix (MM) was prepared with 10X buffer, 25X dNTP, 10 Random primers, nuclease-free water and multiscribe and was placed into each PCR tube. The samples were placed in PCR tubes containing equal volumes of the 2X MM and centrifuged for ~10 seconds before incubating on to the Thermal Cycler DNA Machine set at 25^{o}C for 10 minutes, 37^{o}C for 2 hours. The cDNA samples were stored at -80^{o}C for later use.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Synthesized cDNA was used as template to amplify *PAX5* gene and measure the fold change in induction of PAX5 (Applied Biosystems, Assay ID: Mm00435501_m1). Using cDNA template (from 18 ng total RNA) and the TaqMan Universal PCR Master Mix (Applied Biosystems), the PCR reactions were set up for the detection and quantification of PAX5 mRNA. GAPDH was used as the endogenous housekeeping gene and the control or untreated samples as the calibrator. The parameters for the PCR reactions thermal profile were Activation 50^{o}C for 2 minutes, 95^{o}C for 10 minutes, 95^{o}C for 15 seconds, and 60^{o}C for 1 minute for 40 cycles. The qRT-PCR was carried out using the 7900 HT system (Applied Biosystems) with 384-well block. For relative mRNA quantification, we used the

Comparative C_T method. The ΔC_T values between the test and housekeeping genes and the fold difference ($2^{-\Delta\Delta CT}$) in the expressions for all the samples were determined and plotted as a bar or line graph in Sigma Plot.

Statistics and Data Analysis

All data were analyzed with SigmaPlot version 12.0, using one way analyses of variance (ANOVA) and Dunnetts t-test where applicable, for the determination of differences between control and treatment groups. For immunoblotting analysis, samples were treated and ran in triplicates as shown on the blots. Image J (1.48v) software (NIH download from website: http://rsb.info.nih.gov/ij/) was used to obtain band intensities for treatment and control samples.

For the qRT-PCR studies, samples were treated in triplicate and each replicate was ran in triplicate to obtain 9 data points per sample. The $\log_2 2^{-\Delta\Delta Ct}$ ratios (samples normalized to GAPDH and compared to control) were calculated from the average of samples run in triplicate. These ratios were compared to control via analysis of variance (ANOVA) with post-hoc comparisons using Dunnett's method to control for multiple comparisons. Differences were considered significant if the p-value for a given comparison was less than the type I error rate α=0.05.

RESULTS

The mouse 2E8 pre-B cell line is IL-7 dependent

Mouse 2E8 cells are a cloned and differentiation-limited pre-B cell line that maintains its responsiveness to IL-7 (Ishihara et al., 1991). To determine the suitability of 2E8 for the proposed IL-7 signaling studies, we first determined the IL-7 dependence of the cells grown in culture. As shown in Figure 4.1A, 2E8 cells were found to have an absolute growth factor dependence on IL-7. When IL-7 was removed from the media, 2E8 failed to grow and began to lose cell viability measured using AO/PI, 4 days after seeding cultures.

Comparative effects of As^{+3} and MMA^{+3} on 2E8 cell growth

As^{+3} did not modify the growth of 2E8 cells in the presence of IL-7 except at the high (500 nM) concentration (Figure 4.1B). However, MMA^{+3} was found to inhibit cell proliferation and viability at concentrations as low as 50 nM on days 3-5 (Figure 4.1C). 500 nM As^{+3} also inhibited proliferation and viability on days 3-5 (Figure 4.1B). The inhibition of proliferation and viability observed for As^{+3} and MMA^{+3} were comparable to that observed in the absence of IL-7. Since IL-7 was not replenished, and the medium was not changed during the 5-day culture period, we thought that the effects of MMA^{+3} and high concentration As^{+3} could be due to alterations in IL-7 action. This effect was not seen with lower levels of As^{+3} and MMA^{+3}. We therefore postulated that MMA^{+3} and As^{+3} were interfering with IL-7 signaling.

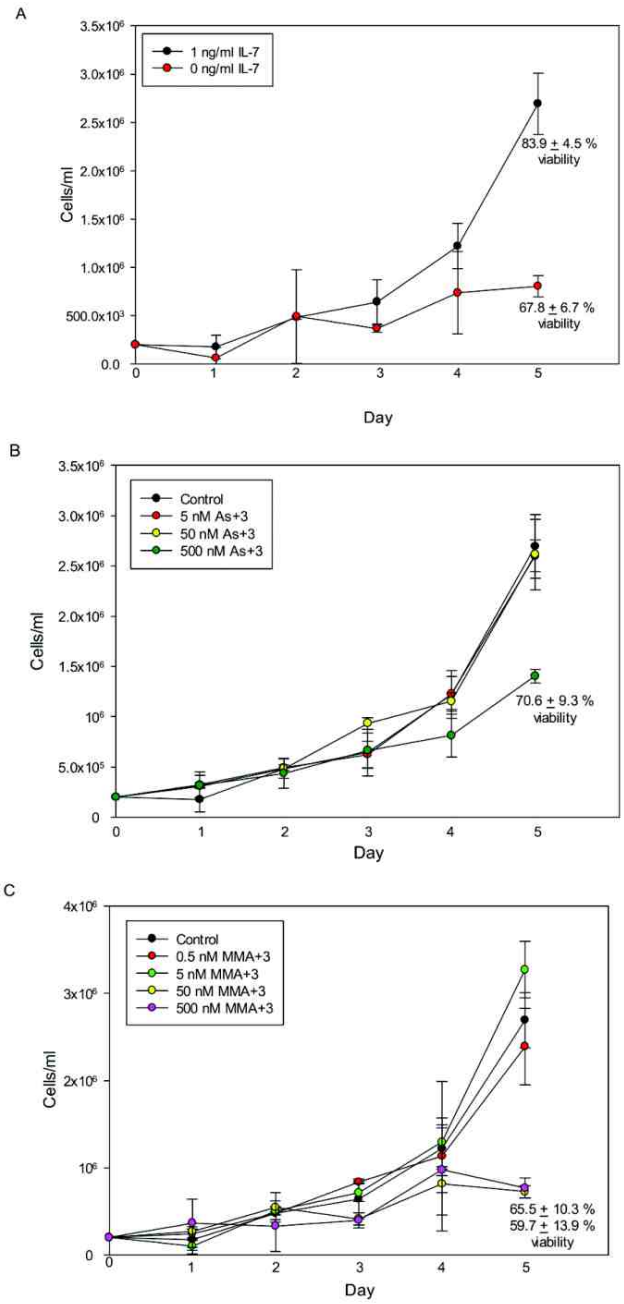

Figure 4.1. Five day cell proliferation (cells/ml) and viability (%) by automated cell counter and AO/PI staining. Cell culture in medium with or without 1 ng/ml IL-7 [A]; in medium containing IL-7 and different concentrations of As^{+3} [B]; in medium containingIL-7 and different concentrations of MMA^{+3} [C]. Error bars are ± SD.

MMA^{+3} decreases STAT5 activation in 2E8 cells, but does not inhibit JAK1 activation

IL-7 signals cell growth and differentiation through a STAT5 pathway involving phosphorylation of tyrosine 694 (Clark et al., 2014). We found that STAT5 activation was dependent on the presence of IL-7, as the levels of STAT5 phosphorylation were almost undetectable when cells were incubated overnight (~24 hr) in the absence of IL-7 (Figure 4.2A and 4.2C). To examine the effects of As^{+3} and MMA^{+3} on STAT5 activation, we analyzed total STAT5 protein expression as well as the phosphorylation of STAT5 via Western blots. MMA^{+3} decreased the ratio of phosphorylated STAT5 to total STAT5 protein. Thus, MMA^{+3} inhibited IL-7-induced STAT5 phosphorylation in a concentration-dependent manner (5-500 nM) when compared with total STAT5 protein, as determined by the density of band images obtained in triplicate (Figure 4.2A and 4.2C). STAT5 signaling was IL-7 dependent and 50 nM MMA^{+3} was unable to modify STAT5 phosphorylation in the absence of IL-7. The 500 nM concentration of MMA^{+3} almost abolished total STAT5 levels, (Figure 4.2B and 4.2C). Therefore, even though 500 nM MMA^{+3} showed comparable proliferation (live cell count) and percent viability (AO/PI stain), this concentration could involve additional mechanisms than just IL-7 signaling.

As^{+3} does not inhibit STAT5 phosphorylation

In contrast to MMA^{+3}, As^{+3} had no effect on total STAT5 or pSTAT in the concentration range of 5-500 nM (Figure 4.3A, 4.3B and 4.3C). These results clearly demonstrate that the effects of arsenic on IL-7 signaling *in vitro* is associated with MMA^{+3} and not As^{+3}.

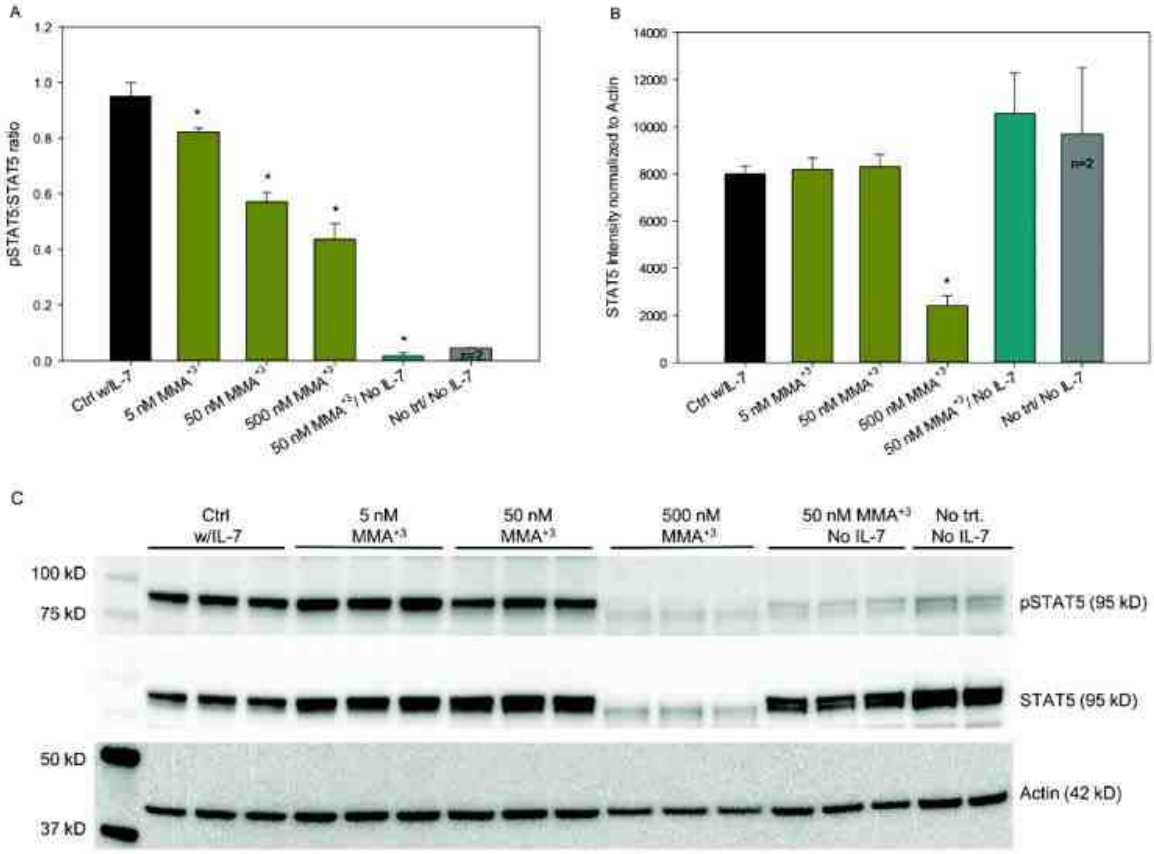

Figure 4.2. Effects of MMA^{+3} on STAT5 activation in 2E8 cells. Phosphorylated STAT5 compared to STAT5 total protein in MMA^{+3}- treated cells [A], total STAT5 protein band intensity normalized to Actin band intensity [B], blot of pSTAT5, total STAT5, and Actin for MMA^{+3}- treated cells [C]. Cells were incubated overnight (~24 hrs),in medium without IL-7, but containing 0-500 nM MMA^{+3}, then washed twice and treated with 0 ng/ml or 10 ng/ml IL-7 and incubated for 30 minutes. Error bars are $\pm$ SD. *Significantly different compared to control.

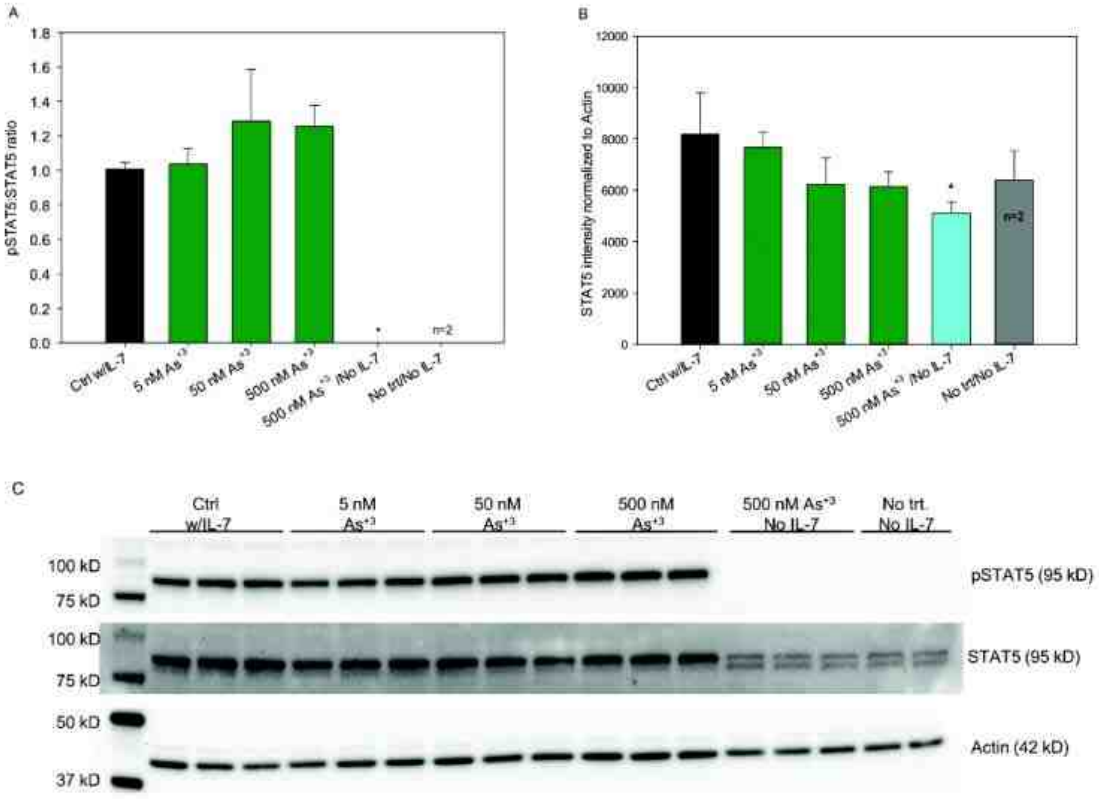

Figure 4.3. Effect of As^{+3} on STAT5 activation in 2E8 cells (by Western). Phosphorylated STAT5 compared to STAT5 total protein [A], total STAT5 protein band intensity normalized to Actin band intensity [B], blot of pSTAT5, total STAT5, and Actin for As^{+3}-treated cells [C]. Cells were incubated overnight (~24 hrs) in medium without IL-7, but containing 0-500 nM As^{+3}, then washed twice and treated with 0 ng/ml IL-7 or 10 ng/ml IL-7 and incubated for 30 minutes. Error bars are $\pm$ SD. *Significantly different compared to control.

MMA^{+3} and As^{+3} do not inhibit JAK1 activation

Activation of JAK1, the immediate upstream activator of STAT5, was also examined under identical conditions used to evaluate the effects of MMA^{+3} and As^{+3} on STAT5 activation. MMA^{+3} did not inhibit JAK1 phosphorylation (Figures 4.4A and 4.4C), and may have produced a small increase in total JAK1 level at 50 nM, compared to pJAK1 (Figures 4.4A and 4.4B). The 500 nM concentration of MMA^{+3} did inhibit total JAK1 (Figure 4.4C) and pJAK1 levels (Figure 4.4A), as it did for STAT5 and pSTAT5. Thus we decided to examine effect of lower concentrations of MMA^{+3} on the induction of PAX5, a STAT5 responsive gene. As^{+3} at concentrations of 5-500 nM did not inhibit JAK1 phosphorylation or total JAK1 (Figures 4.5A, 4.5B and 4.5C).

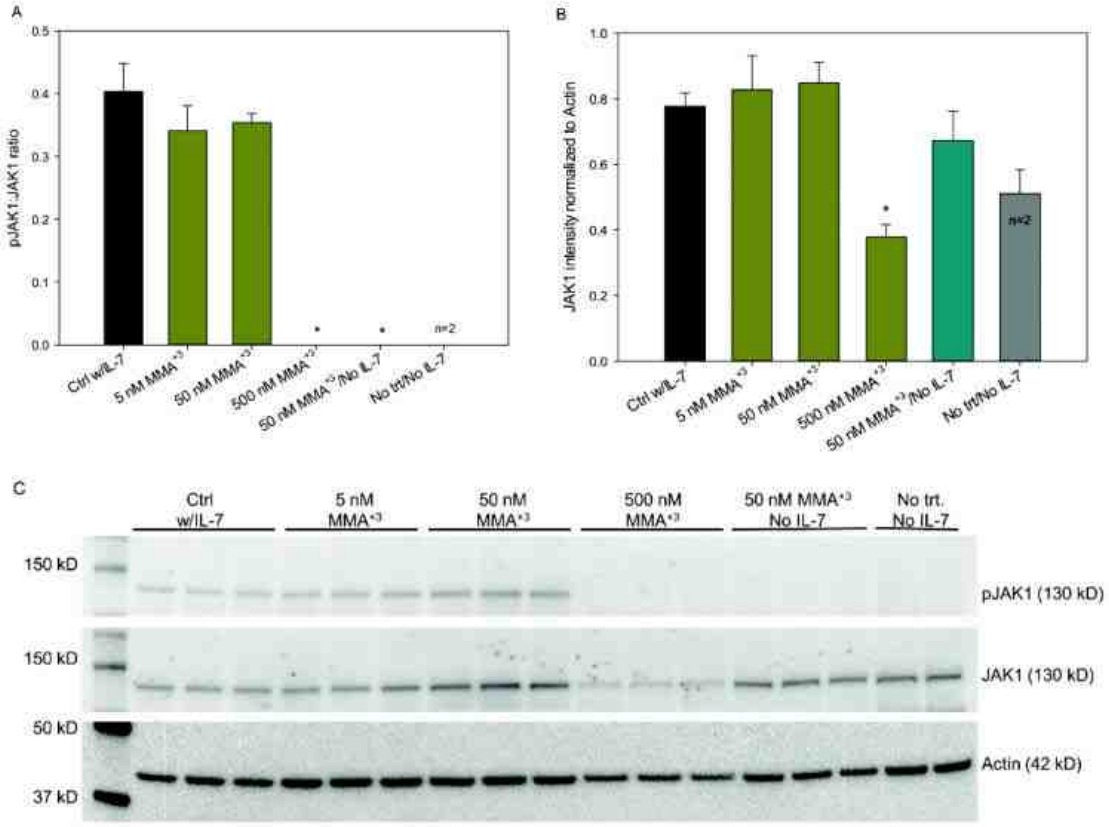

Figure 4.4. Effect of MMA^{+3} on JAK1 activation in 2E8 cells. Phosphorylated JAK1 compared to JAK1 total protein in MMA^{+3}- treated cells [A], total JAK1 protein band intensity normalized to Actin band intensity [B], blot of pJAK1, total JAK1, and Actin for MMA^{+3}- treated cells [C]. Cells were incubated overnight (~24 hrs) in medium without IL-7, but containing 0-500 nM MMA^{+3}, then washed twice and treated with 0 ng/ml or 10 ng/ml IL-7 and incubated for 30 minutes. Error bars are $\pm$ SD. *Significantly different compared to control.

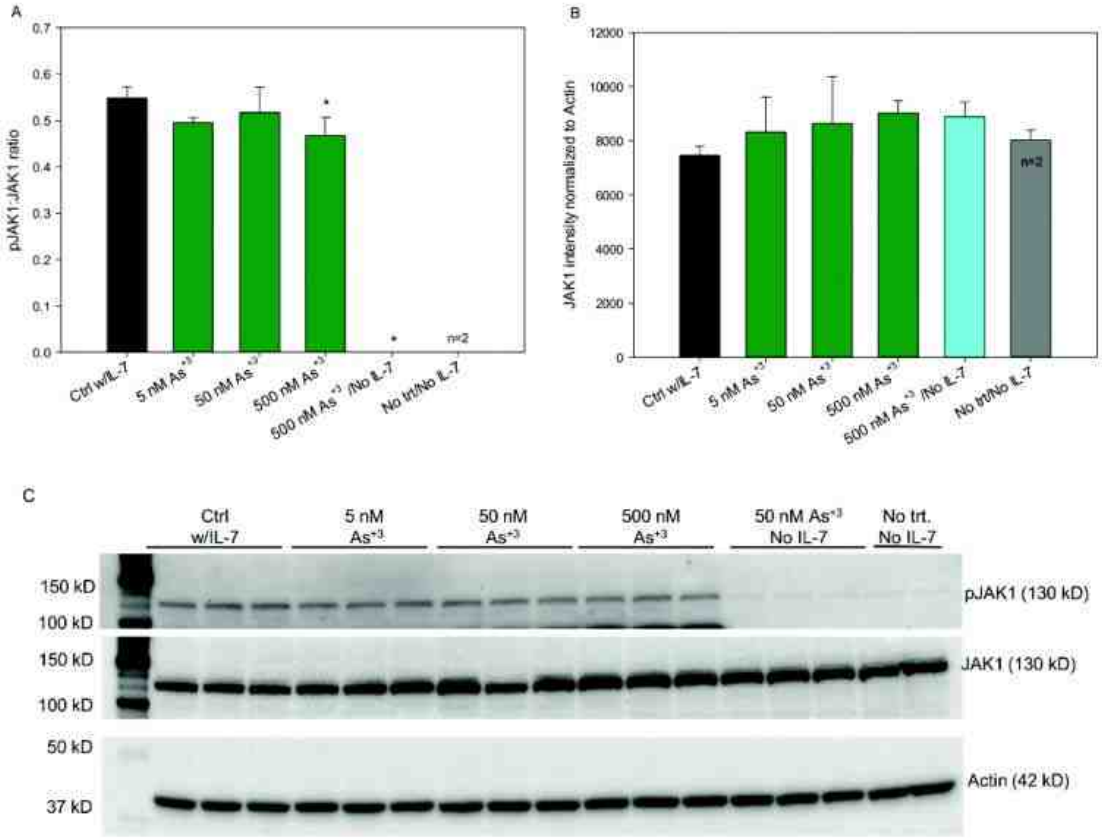

Figure 4.5 Effect of As^{+3} on JAK1 activation in 2E8 cells (by Western). Phosphorylated JAK1 compared to JAK1 total protein [A], total JAK1 protein band intensity normalized to Actin band intensity [B], blot of pJAK1, total JAK1, and Actin for As^{+3}-treated cells [C]. Cells were incubated overnight (~24 hrs) in medium without IL-7, but containing 0-500 nM As^{+3}, then washed twice and treated with 0 ng/ml IL-7 or 10 ng/ml IL-7 and incubated for 30 minutes. Error bars are $\pm$ SD. *Significantly different compared to control.

PAX5 message and protein were inhibited by MMA^{+3} but not by As^{+3}

As an additional test of altered pSTAT5 activity produced by MMA^{+3}, we examined the induction of PAX5, a pSTAT5 responsive gene associated with lymphoid differentiation (Liu et al., 2014). With IL-7 in the culture medium, we found a small but significant increase in the PAX5 mRNA levels in 2E8 cells compared to the IL-7 –deficient culture. The induction of the *PAX5* gene was inhibited by MMA^{+3} in a concentration-dependent manner in the concentration range of 100-200 nM (Figure 4.6A). These concentrations are slightly higher than those required to inhibit STAT5 phosphorylation; however, it is clear that PAX5 regulation is not as directly dependent on IL-7 signaling as STAT5. As^{+3} did not affect *PAX5* mRNA expression. We also examined PAX5 protein expression (Figure 4.6B). MMA^{+3} decreased PAX5 protein expression at 100-200 nM concentrations, but As^{+3} had no effect on PAX5 protein expression at concentrations up to 500 nM.

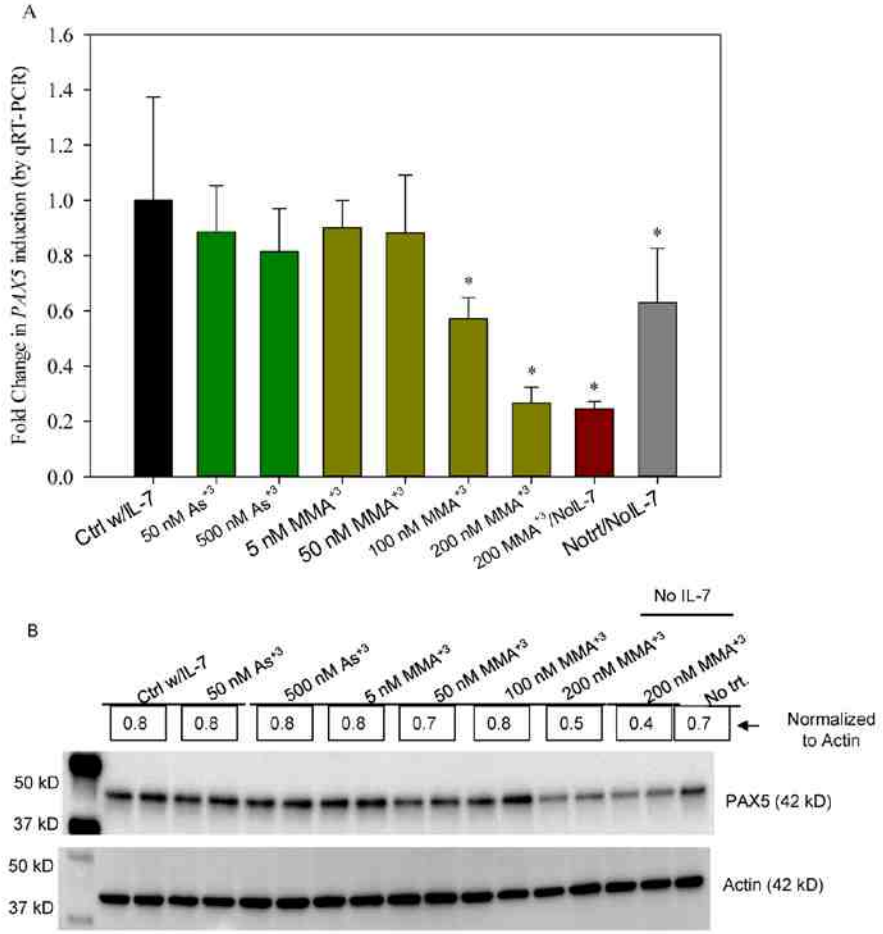

Figure 4.6. Fold change in PAX5 induction in As^{+3} or MMA^{+3}-treated cells (by RT-qPCR) [A]. Effect of As^{+3} or MMA^{+3} on PAX5 total protein (by Western) [B]. Cells were incubated overnight (~24 hrs) in medium without IL-7, but containing 0-500 nM As^{+3}, or 0-200 MMA^{+3}, then washed twice and treated with 0 ng/ml IL-7 or 10 ng/ml IL-7 and incubated for 30 minutes. Error bars for fold change in PAX5 are $\pm$ SD.

DISCUSSION

Numerous diseases including cancers, vascular diseases, lung diseases, and diabetes have been linked to environmental exposures to As^{+3} in drinking water and food (Argos et al., 2010, 2014; Chen et al., 2003; Chen Y, et al., 2011; Navas-Acien et al., 2005, 2006). While numerous mechanisms of As^{+3} toxicity in cells have been proposed, most studies have used extremely high levels of As^{+3} for *in vivo* and *in vitro* studies. At micro molar concentrations, As^{+3} is well known to produce oxidative stress that impacts many cellular pathways (Kitchin and Conolly, 2010; Flora et al., 2011). Previous studies have examined DNA damage and repair pathways that are associated with the genotoxicity of As^{+3} (King et al., 2012; Cooper et al., 2013). However, there have been few studies that have examined non-genotoxic pathways associated with cell signaling.

Immune and inflammatory diseases could play a role in many chronic diseases associated with arsenic exposures. Prenatal arsenic exposures have been linked to immune suppression and altered lymphoid cell development (Ahmed, et al., 2012; Nadeau et al., 2014). We have previously observed selective targeting of murine BM lymphoid progenitors by extremely low doses of As^{+3} *in vivo* administered through physiologically relevant route, and MMA^{+3} *in vitro* at environmentally relevant levels of exposure (Ezeh et al., 2014). MMA^{+3} is formed through a series of metabolic reactions via the action of arsenite-3-methyltransferase (AS3MT), which is expressed in the liver and a few other extrahepatic tissues (Aposhian et al., 2000). AS3MT is not found at appreciable levels in lymphoid cells. Thus, for *in vitro* studies, it was necessary to add MMA^{+3} directly to cell cultures to observe its effects on lymphoid cell function.

It is important to understand the effects of MMA^{+3} on lymphoid cells because they are very sensitive and are exposed to both As^{+3} and MMA^{+3} through blood circulation. Also, because AS3MT is polymorphic in humans and has been causally linked to many arsenic-induced environmental diseases (Pierce et al., 2012), it may play a role in differences in inter-individual toxicities in human populations.

The present studies assessed the role of As^{+3} and one of its key metabolites, MMA^{+3}, on lymphoid progenitor cell differentiation. Beginning from the Pre-pro B to the large B cell stage, lymphoid progenitors express the IL-7 receptor alpha (IL-7Rα, CD127) and respond to IL-7 (Clark et al., 2014; Peschon, et al., 1994). IL-7 is a cytokine growth factor produced by the BM stromal cells (Fry and Mackall, 2001) and it is associated with proliferation, survival, and differentiation of B and T cells (Corfe et al., 2012). Humoral immune responses, which we have shown are extremely sensitive to *in vivo* As^{+3} exposure (Ezeh et al., 2014), utilize the ability of the lymphoid progenitors to expand following antigen challenge. Inhibition of B cell differentiation and proliferation by environmental pollutants can negatively impact the humoral immune responses, leading to decreased immunity and increased susceptibility to disease.

The IL-7R consists of the IL-7Rα (CD127) which is specific for IL-7 ligand, and the IL-7Rγ_c chain (CD134) usually shared by other cytokines. Early B cells express CD127 for IL-7 signaling. Upon IL-7 binding to CD127 in a B cell, a cascade of phosphorylation initially begins with the receptor-associated Janus Kinases 1 and 3 (JAK1 and JAK3), and results in the phosphorylation of intracellular Signal Transduction and Activator of Transcription 5 (STAT5) molecules on tyrosine residues Tyr^{694} and Tyr^{699}. These

phosphorylated STAT5 molecules dimerize and translocate to the nucleus to drive the transcription of genes responsible for commitment maintenance, further differentiation, proliferation, and survival of the B lineage cells. It has been shown that deletion of total STAT5 protein (STAT5a/5b) in cultures of fetal liver cells inhibited IL-7- induced lymphoid expansion (Dai et al., 2007). Although STAT5 activation could occur with several other cytokines, it is predominantly activated by IL-7 (Snow et al., 2002). In our cloned pre-B 2E8 cell system, STAT5 is exclusively activated by IL-7. Downstream of STAT5, the transcription factor, early B factor (EBF) in concert with E2A upregulate *PAX5* gene expression (O'Riordan and Grosschedl, 1999). Whereas IL-7 and STAT5 signaling control the stepwise order of immunoglobulin gene rearrangement and also survival of B cells (Malin et al., 2010). Studies show that PAX5 is responsible for initiating and maintaining B lineage commitment (Mikkola et al., 2002). Due to the observed minimal levels of PAX5 in pre-pro B cells (Nutt et al., 1999; Rolink et al., 1999), it has been suggested that pre-pro B cells are not yet irreversibly committed to the B cells lineage, and do retain the potential to re-commit to T, NK, or DC-lineage. PAX5 protein has also been shown to repress commitment to the myeloid lineage. There is a complex relationship between STAT5 and PAX5. The promoter region of *PAX 5* contains a STAT5 binding motif (Goetz et al., 2005). STAT5 therefore maintains survival of B cells during the transition from the CLPs to the pro-B cell stage (Malin et al., 2010). Inhibition of STAT5 phosphorylation and PAX5 gene expressions could therefore lead to a diversion from the B lineage commitment and unbalanced immune responses.

Normal development of B and T cells rely on IL-7 signaling. Abnormal B and T cell function is associated with immunodeficiency which is marked by increased susceptibility to

infections, cancers, and other diseases. Many studies show that As^{+3} exposure produces immunosuppression and increased susceptibility to infections in children and adults (Burchiel et al., 2014; Nadaeu et al., 2014).The presence of MMA^{+3} in urine of individuals exposed to As^{+3} has been noted (Aposhian et al., 2000). MMA^{+3} is known to be associated with irreversible transformations that lead to disease. As^{+3} is also known to increase the genotoxicity of other carcinogens by inhibiting enzymes involved in DNA repair through various mechanisms, including the production of reactive oxygen species (Wang et al., 2013) and interaction with zinc finger proteins (Zhou et al., 2013).

This study shows that MMA^{+3} inhibited phosphorylation of STAT5 at low (5-50 nM) concentrations that are not cytotoxic to 2E8 cells. MMA^{+3} did not inhibit JAK1 activation, and also had no effect on IL-7Rα (CD127) expression, data not shown. The selective inhibition of STAT5 activation suggests that MMA^{+3} could be upregulating a STAT5-specific phosphatase or inhibitor. Protein inhibitors of activated stats (PIAS), also known as E3-SUMO ligase, reside in the nucleus and are known to specifically deactivate phosphorylated STATs that translocate to the nucleus (Arora et al., 2003; Chung et al., 1997; Liu et al., 2001). Furthermore, other proteins like the cytokine inducible SH2-containing protein (CIS), a suppressor of cytokine signaling (SOC), and phosphotyrosine phosphatases (PTPs) could dephosphorylate already phosphorylated STAT5; however, these proteins are not specific for STATs. We therefore believe that MMA^{+3}, but not As^{+3}, recruits PIAS upon activation of STAT5. Further studies will be necessary to confirm this speculation. We do not know the precise molecular structures of JAK1 and STAT5. However, we believe that 500 nM MMA^{+3} but not As^{+3} could target the thiol groups in the active site of these proteins

resulting in the observed toxicity, and that the lack of phosphorylation at this level may be due to toxicity to the total proteins.

Induction of the STAT5 responsive gene, PAX5 was also suppressed by MMA^{+3}, but not by As^{+3}. PAX5 protein levels were also reduced by MMA^{+3} in concentration-dependent manner in 2E8 cells. The different responses of STAT5 and PAX5 proteins to As^{+3} and MMA^{+3} could be due to a number of reasons, including differences in the cysteine residue content of the peptides, and the presence or absence of other thiol-containing amino acids in these proteins. As^{+3} is known to bind peptides that contain three (C3) and four (C4) cysteine residues, whereas MMA^{+3} is capable of binding peptides containing two (C2) or more cysteine residues (Zhou et al., 2011; Zhao et al., 2012). Thus, MMA^{+3} has a different spectrum of action than does As^{+3}.

In summary, our studies demonstrate important differences between the immunotoxicity of MMA^{+3} and As^{+3} on lymphoid cells. We demonstrate a new mechanism by which MMA^{+3} can inhibit pre-lymphoid cell development through inhibition of IL-7 signaling pathways. These effects occur at extremely low concentrations that may not involve a generalized oxidative stress and illustrate the importance of non-genotoxic pathways in the action of arsenic.

ACKNOWLEDGMENTS

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CHAPTER 5

GENERAL DISCUSSION AND SIGNIFICANCE

Our laboratory has previously shown in murine spleen cell models that most of the immunotoxicity of genotoxic PAHs, such as DMBA, result from their ability to metabolize PAHS to form reactive metabolites that covalently bind to DNA and activate ATM/ATR signaling pathways, resulting in p53-dependent inhibition of cell cycling (Gao et al., 2007, 2008). Proliferation is required for activation of lymphocytes. Therefore, such effects result in immunosuppression.

DNA adducts are repaired by NER and BER pathways (Crew et al., 2007; Gunz et al., 1996; Yu et al., 2012). Low dose exposures of cells to PAHs form a low level of adducts that are likely repaired without damage to the immune system; however, when DNA repair is compromised, we believe that these low dose exposures can result in significant immunosuppression. Therefore, we postulated that agents like As^{+3} that are known to inhibit NER and BER pathways (Ebert et al., 2011; Piatek et al., 2008), would block the repair of lymphoid adducts resulting in low dose immunosuppression by genotoxic PAHs. Previous *in vitro* studies in spleen cells supported this hypothesis, where it was found that DMBA, DBC, and other PAHs produced synergistic immunosuppression of the T-dependent antibody response (TDAR) in murine spleen cells (Li et al., 2010). Most of what is known about DBC is associated with its genotoxicity (Amin et al., 1995; Platt et al., 2004). As^{+3} has been implicated in the enhancement of the genotoxicity of carcinogens, including PAHs and

ultraviolet radiation (Hartwig et al., 2003; Wang et al., 2013) by inhibiting DNA repair (Cooper et al., 2013; Klein et al., 2007; Rossman et al, 2004).

BM stem cells play a critical role in the formation of lymphoid progenitors that are important in maintaining host immunity. Patients receiving cytotoxic drugs for cancer are immunosuppressed and often have serious morbidity and mortality resulting from increased infections (Mackall et al., 1994). The maximum tolerated dose for most cancer chemotherapeutic agents is often defined by BM toxicity. Therefore, it is somewhat surprising that more studies on characterizing the effects of environmental agents, such as PAHs, on mouse and human BM have not been performed. The limited amount of work that has been previously performed have used conditions that are neither physiologically or environmentally relevant (i.e., single high dose exposure given by the i.p, route, (Galvan et al., 2003, 2006; Heidel et al., 1998, 1999, 2000; N'jai et al., 2010; Page, et al., 2003).

Based on the knowledge of the genotoxic action of DMBA, it was decided to evaluate the hypothesis that As^{+3} may increase the immunotoxicity of PAHs *in vivo*. Previous work was performed *in vitro* with DMBA, BaP, and DBC (Li et al., 2010), knowing that many factors have the potential to influence these interactions *in vivo*. DBC, an environmentally relevant PAH (Mumford et al., 1995) that has been found to suppress spleen cells *in vitro* (Lauer et al., 2013), but has not been characterized for its effects on BM, was chosen. DBC was chosen over DMBA because DMBA is synthetic and not found in the environment. However, like DMBA, DBC is genotoxic and requires metabolic activation by P450 CYP1B1 to form adducts (Buters et al., 2002; Luch et al., 1998). CYP1B1 was found to be critical for DMBA suppression of pre-B cell colony formation (Heidel et al., 1998).

In several studies, an increase in susceptibility to infection following exposure to arsenite has been observed (Farzan et al, 2013; Kozul et al., 2009; Potera et al, 2013; Spivey et al, 2011). Thus, three Aims were developed to characterize the effects of As^{+3} *in vivo* and *in vitro* on murine BM cells, to evaluate the combined effects of As^{+3} and DBC *in vivo* and *in vitro*, and finally to explore the mechanisms by which As^{+3} and associated metabolites produce their effects on murine BM. The focus was on much lower doses of As^{+3} than have been previously studied in order to make predictions about the likely environmental relevance of our findings.

SUMMARY OF AIM 1 FINDINGS AND THEIR SIGNIFICANCE

In addressing the effects of low levels of exposure to As^{+3} *in vivo*, initial dose-range studies were performed with sodium arsenite in drinking water for 30 days at the levels of 75 and 300 ppb. These doses were chosen based on the work of Kozul et al. (2008), who found that 30 day exposure at 200 ppb resulted in significant morbidity and mortality to mice exposed to an influenza virus.

In this study, a novel observation that mouse BM lymphoid (CFU-B) progenitor cells activated by IL-7 were sensitive to suppression by As^{+3} at a concentration of 300 ppb, whereas the myeloid progenitors (CFU-GM) activated by GM-CSF were not. At the 300 ppb dose, there was also a slight but statistically significant ($p \leq 0.05$) decrease in BM cell recovery from femurs of exposed mice (Figure 2.3a; Table 2.1). For these initial studies, spleen cell TDAR responses were also examined and they were suppressed at 75 and 300 ppb exposure levels. Thus, there is differential toxicity of As^{+3} on different lymphoid organs, and these observations are currently being followed by other investigators in our lab. The

lymphoid progenitor suppression observed in this study occurred without any change in the markers of non-committed or stem cell populations or the hematopoietic and mesenchymal stem cells after 30 days exposure to As^{+3}. Cell surface markers on B cells in the spleen were also unaltered even though TDAR was suppressed.

For *in vitro* studies, a series of novel observations were made pertaining to the relative lack of effects of As^{+5} on murine BM, the selective toxicity of As^{+3} on BM lymphoid progenitors, and the extremely potent effects of MMA^{+3} on lymphoid progenitors (pre-B, but also likely to be observed in pre-T cells). MMA^{+3} was included in the *in vitro* studies because there are many papers that show that it is formed in vivo from As^{+3} in the presence of AS3MT (Aposhian et al., 2000; Chen et al., 2011; Hirano et al., 2004; Styblo et al., 2000). AS3MT is expressed predominantly in the liver and kidney, and the methylation of arsenic was initially thought to be important for rapid elimination of arsenicals from the body. In fact some investigators have performed clinical studies to try to hasten the elimination of arsenic by increasing metabolism to methylated species (Gamble et al., 2007). However, it is now known that MMA^{+3} has significant toxicological effects, and it may be responsible for many of the toxicities seen with As^{+3} (Chen et al., 2011; Drobna et al., 2009; Styblo et al., 2000) There is a strong correlation between AS3MT levels and cancer outcomes in several epidemiologic studies (Pierce et al., 2013). It is highly significant that we found that MMA^{+3} produced low dose selective suppression of CFU-B colony formation as it suggests, but does not prove, that the toxicity of As^{+3} to murine BM may in part be due to MMA^{+3}. Further, *in vivo* studies using, for example, AS3MT null mice, would be needed to provide further evidence (Chen et al., 2011; Drobna et al., 2009).

Given the low doses and/or concentrations of As^{+3}/MMA^{+3} at which pre-B cells were selectively targeted, important questions were raised as to the potential mechanism(s) of these effects. This idea was fully explored in Aim 3 following completion of studies to examine As^{+3} and PAH (DBC) interactions evaluated in Aim 2.

SUMMARY OF AIM 2 FINDINGS AND THEIR SIGNIFICANCE

The immunosuppressive properties of certain forms of arsenic (Burns et al., 1991; Harrison and McCoy, 2001; Szczeklik et al., 1994) and PAHs (Burchiel et al., 1988, 1990; White Jr., 1985) at high doses have been established. The PAH used in this study is DBC, which is naturally occurring and has been shown to be comparable in toxicity to the highly potent but synthetic DMBA (Cavalieri et al., 1991). Even though DMBA has been used as a model to demonstrate the carcinogenicity and overall toxicity of PAHs, it is not environmentally relevant like DBC. However, DBC's suppression of TDAR in spleen cells *in vivo* (Lauer et al., 2013) and *in vitro* (Li et al., 2010) provided a clue that low levels could synergize with low levels of As^{+3} to produce immunosuppression in the BM. Synergistic immunosuppression of DBC and As^{+3} was indeed found in spleen cells (Li et al., 2010) *in vitro*.

Although exposure to environmental pollutants is usually in combination, only a few studies address combination exposures to these pollutants. In contrast to the effect observed for As^{+3} and UV radiation exposure (Wang et al., 2013), this study showed that As^{+3} interaction with DBC at environmentally relevant doses is non-linear and may be dependent on dose and ratio of As^{+3} to DBC. Also, in an effort to establish no-effect doses of chemicals, this study showed for the first time that very low levels (1 mg/kg, 5-day cumulative dose) of DBC is selectively toxic to the lymphoid progenitors in the BM.

In vivo, As^{+3} doses as low as 19 and 75 ppb did not produce immunosuppression in murine BM following 30 day drinking water exposure (Figure 2.2). However, upon concomitant exposure to 1 mg/kg, 5 day cumulative dose of DBC, there is interaction with

the 19 ppb As^{+3} when CFU-B counts are expressed on per million cell basis (Figure 3.2a) and immunosuppression from both 19 and 75 ppb when CFU-B counts are expressed on per femur basis (Figure 3.2b). The expression of the results on per femur basis takes into consideration the biological and/or experimental BM cell loss that could have occurred in each animal due to exposure or during experiment. Increased doses did not necessarily produce increase in toxicity.

The *in vitro* studies confirmed the non-linear and complex nature of As^{+3} and DBC interactions. The combined exposure of murine primary BM cells, to very low, no-effect concentrations of As^{+3} with no-effect concentrations of DBC and DBC-diol *in vitro*, suppressed CFU-B colonies in a complex non-linear pattern as seen *in vivo* (Figure 3.3). Knowing that the metabolites of both compounds may be involved in the reactions, a detailed study of the metabolites and the enzymes responsible for the biotransformation will be important. Whereas the enzyme, AS3MT is responsible for As^{+3} biotransformation through a series of methylations and demethylations, the enzyme CYP1B1 and microsomal epoxide hydrolase (mEH) are known to catalyze the metabolism of DBC. It was previously suggested that As^{+3} influences PAH metabolism by altering the regulation of the CYP1 enzymes (Jacobs et al., 1999; Vernhet et al., 2003). It will be necessary to examine the influence of one chemical on the metabolism of the other, including the induction and activity of its enzyme in cases of combined exposure.

The interactive suppression of the lymphoid progenitors could be either by genotoxic or non-genotoxic mechanisms, or a combination of both. Our studies showed that MMA+3 was more effective in suppressing pre-B cells *in vivo* and *in vitro*. We know that MMA^{+3} can inhibit DNA repair leading to cell death (Piatek et al., 2008). However, we have seen an

inhibition of IL-7-induced pre-B cell formation in mouse BM at doses/concentrations that are not cytotoxic was observed in this study. This lead to the decision in Aim 3 to evaluate the effects of As^{+3} and MMA^{+3} on IL-7 signaling, which represents an important, potential non-genotoxic mechanism of action.

SUMMARY OF AIM 3 FINDINGS AND THEIR SIGNIFICANCE

The studies performed *in vitro* in Aims 1 and 2 implicate MMA^{+3} as important As^{+3} metabolite that may be responsible for inhibition of pre-B cell formation in murine BM. Aim 3 directly addressed the potential mechanism of action of MMA^{+3} utilizing a mouse pre-B cell line (2E8) that is IL-7 dependent. This cell line was chosen because it is a pure clone, which is advantageous for biochemical studies. Mouse BM, on the other hand, is extremely heterogeneous and makes data interpretation difficult. It was also found that the mouse 2E8 cells had minimal JAK/STAT signaling in the absence of IL-7, and is considered an appropriate and sensitive model to evaluate the effects of As^{+3} and MMA^{+3}.

Results clearly showed for the first time that MMA^{+3} at non-cytotoxic (5-50 nM) concentrations inhibited IL-7 dependent cell growth and signaling. As^{+3} only inhibited IL-7 cell growth and signaling at high concentrations (500 nM) in overnight incubations. The inhibition of IL-7 signaling occurred at the level of inhibition of STAT5 phosphorylation, but it did not interfere with JAK1 activation. These results suggest a novel mechanism of inhibition which further studies will need to explore (see below). It is interesting that As^{+3} does not affect STAT5 phosphorylation, and we know from preliminary PCR studies that 2E8 cells do not express significant, if any AS3MT. Thus, the *in vivo* formation of MMA^{+3}

by AS3MT could contribute to inhibition of IL-7 signaling in mouse bone marrow, but further studies will need to be performed to resolve this point.

Further support for the mechanism of inhibition of STAT5 phosphorylation by MMA^{+3} was obtained by examining the PAX5 gene promoter and protein which are induced by pSTAT5. The results showed that MMA^{+3} inhibited PAX5 gene induction and protein expression, but this gene and protein were not entirely turned off in the absence of IL-7 in control cells. The lack of complete IL-7 dependence make this gene and protein an imperfect model to study MMA^{+3}, but the literature suggests that this is the most sensitive gene to study. Nevertheless, the inhibitions at both the gene activation and protein level for PAX5 support our underlying hypothesis.

MMA^{+3} could possibly interact with STAT5 directly to prevent phosphorylation, or it could interact indirectly by modulating other enzymes. One or more of several processes could be involved in the selective dephosphorylation of activated STAT5 caused by MMA^{+3}, including: the involvement of protein inhibitors of activated STATs (PIAS), also known as E3-SUMO protein ligase; induction of cytokine inducible SH2 containing (CIS) proteins, which are suppressors of cytokine signaling (SOCs); and/or induction of protein tyrosine phosphatases (PTPs). Unlike the SOCs and PTPs, PIAS reside in the nucleus and are specific for activated STATs which translocate to the nucleus. They also require activation of STATs for interaction (Arora et al., 2003; Chung et al., 1997; Liu et al., 2001). Since phosphorylation of JAK1 was not inhibited, nor was the unphosphorylated STAT5 protein, it is reasonable to postulate that PIAS may be responsible for the selectivity in MMA^{+3} phospho-inhibitory effects. Immunoprecipitation and immunoblotting analysis to check for SUMO-attachment to activated STAT5 in MMA^{+3}-treated and control cells, and use of

phosphatase inhibitors with treatments, would have been helpful to determine by what mechanism MMA^{+3} inhibits STAT5 but not JAK1 activation. Also, STAT5 molecules are phosphorylated on Tyr^{694} (for STAT5a) and Tyr^{699} (for STAT5b). On a linear scale, these sites are very close to the DNA binding domain or active site of the proteins and would most likely be affected by MMA^{+3} binding to thiol groups in the active site. In contrast, JAK1 phosphorylation occurs on Tyr^{1022} and Tyr^{1023}, which are farther from the active site of the protein and less likely to be affected by MMA^{+3} binding to active site thiols. Overall, this study shows for the first time that MMA^{+3}, not As^{+3}, inhibits IL-7-induced STAT5 phosphorylation and decreases the transcription of important lymphoid lineage-specific genes.

OVERALL REFLECTIONS ON THE SIGNIFICANCE OF THESE STUDIES AND POTENTIAL AREAS FOR NEW STUDIES

These are the first studies to implicate a selective toxicity of MMA^{+3} on lymphoid stem cells and the IL-7 signaling pathway. B and T cell development relies on IL-7 signaling for immunocompetence. The common lymphoid progenitors (CLPs) which differentiate into all lymphoid lineages, including the B, T and NK cells, express IL-7R. These other subsets of cells derived from the CLP have not been evaluated, but will likely be affected.

Immunodeficiency marked by increased susceptibility to and recurrent infections is associated with abnormal B and T cell development. As cited earlier, numerous studies have shown strong associations between arsenic exposure and increased susceptibility to infections in children and adults. Occurrence of MMA^{+3} in urine of individuals exposed to arsenic is a marker for cancer and other diseases. The most enzymatically active AS3MT genotypes are associated with increased disease risk in humans exposed to arsenic (Pierce et al., 2013). Our own lab's work in human peripheral blood mononuclear cells (HPBMC) show that MMA^{+3} suppresses T cell activation at extremely low doses whereas As^{+3} does not (Burchiel et al., 2014)

Also for the first time, the possibility and nature of interaction between As^{+3} and DBC at very low levels to produce immunosuppression, was established even though our understanding of the mechanisms involved are incomplete. It was expected that low levels of As^{+3} in drinking water for 30 days may be toxic to the general BM populations, and this was probably shown by the dose-dependent decrease in BM cellularity observed post exposure (Table 2.1). However, the selective toxicity to the lymphoid progenitors over the myeloid

was unanticipated. It was this selectivity, and the fact that the hematopoietic and mesenchymal stem cell markers remained unchanged, that provided the clue to investigate the lymphoid lineage-specific signaling as a possible mechanism.

Using mice as the *in vivo* model, mice primary BM cells, and 2E8 cell line as the *in vitro* models, this study considered the two most ubiquitous environmental pollutants: arsenic and PAHs at extremely low levels and physiologically applicable route of exposure. It also considered the developing immune cells in their vital function of immune response. The observations made here are important and revealed what could be occurring in the immune system, even when there is no apparent disease. It could help focus attention to caring for the immune function, especially when addressing As^{+3} and PAH- associated diseases. Although the presence or lack of AS3MT in mouse BM myeloid and lymphoid cells has not been established, the cells are exposed to these chemicals through the blood. Thus, the observations made in this study are relevant.

Based on the observations made in this study, the following questions emerge that might be important for follow-up studies:

- At what specific step in lymphoid progenitor stem cell commitment do As^{+3}/MMA+3 act?
    - Possible Study: Evaluate the Lin/cKit/Sca-1 profile of BM immune cell progenitors from low dose, long-term arsenite– exposed mice
- Why are lymphoid cells more sensitive than many other cell types to arsenic exposures? Do they have altered transporters for uptake and export that cause the intracellular levels to be higher than other cells?

- Possible Study: Determine absolute arsenic levels in selected cells *in vivo* and *in vitro*.
- Possible Study: Evaluate the aquaglyceroporins (AQP7, mostly AQP9), a major As^{+3} passive water importer, for expression and activity in BM myeloid and lymphoid cells (Liu et al., 2002).
- Possible Study: Evaluate expression and activity of various exporters of arsenic in lymphoid and myeloid cells, especially the ABC family of exporters; Vernhet et al., (2011) have shown that blockade of MDR1 potentiates arsenic toxicity.
- Possible Study: Examine the role of AS3MT in lymphoid and myeloid BM cells using AS3MT null mice (Chen et al., 2008) compared to wild type.

- Why are only low doses or PAHs (DBC) associated with synergistic suppression of As^{+3} and MMA^{+3} in murine bone marrow cells
  - Possible Study: Study the influence of As^{+3} on DBC metabolism and activation of CYP1B1
  - Possible Study: Examine the effects and interactions of As^{+3}/MMA^{+3} with PAHs at the specific target protein or peptide level (e.g., STAT5, PARP, XPA).

LIST OF ABREVIATIONS

AAALAC Association for Assessment and Accreditation of Laboratory Animal Care

Ab Antibody

Ag Antigen

ANOVA Analysis of Variance

AO/PI Acridine Orange/Propidium Iodide

APL Acute Promyelocytic Leukemia

APC Accessory photosynthesis Pigment of the Cyanobacteria

AKR Alphaketoreductase

AQP Aquaglyceroporin

As^{+3} Arsenite

As^{+5} Arsenate

AS3MT Arsenite methyltransferase

ASTDR Agency for Toxic Substances and Disease Registry

ATCC American Type Culture Collection

ATM Ataxia Telangiectasia Mutated protein

ATR Ataxia Telangiectasia and Rad3-related protein

BaP Benzo[a]pyrene

2-ME Betamercaptoethanol

BCA Bicinchoninic Acid

BM Bone Marrow

Ca^{2+} Calcium ion

CD Cluster of Differentiation

CFC Colony Forming Cell

CFU-B Colony Forming Unit for B cells

CFU-GM Colony Forming Unit for GM cells

CIS Cytokine Inducible SH2 –containing protein

CYP Cytochrome P450

CYP1 Cytochrome P450 1

CYP1B1 Cytochrome P450 1B1

cDNA copy Deoxyribonucleic acid

CLP Common Lymphoid Progenitors

CMP Common Myeloid Progenitors

DBC Dibenzo[def,p]chrysene

DBC-dG Dibenzo[def,p]chrysene- deoxy Guanine adduct

DBC-dA Dibenzo[def,p]chrysene- deoxy Adenine adduct

DC Dendritic Cell

DMBA 7, 12-dimethylbenz[a]anthracene

DPBS Dulbecco's Phosphate Buffered Saline

$DPBS^-$ Dulbecco's Phosphate Buffered Saline without calcium and magnesium

EBF Early B cell Factor

EPO Erythropoietin

FITC Fluorescein Isothiocyanate

FSC Forward Scatter

GAPDH Glyceraldehyde-3-Phosphate Dehydrogenase

GF Granulocyte Factor

GM-CSF Granulocyte Monocyte- Cell Stimulating Factor

HBSS Hanks Balanced Salt Solution

HPBMC Human Peripheral Blood Mononuclear Cell

HSC Hematopoietic Stem Cell

IAUCC Institutional Animal Care and Use Committee

HRP Horse Radish Peroxidase

IgG Immunoglobulin G

IL Interleukin

IL-7R Ilterleukin-7 receptor

IL-7Rα Interleukin 7 receptor alpha

IL-7Rγc Interleukin 7 receptor gamma chain

JAK Janus Kinase

kD Kilo Dalton

Mac Macrophage

MCL Maximum Contaminant Level

MDR1 Multi Drug Resistance gene 1

mEH microsomal Epoxide Hydrolase

Mg^{2+} Magnesium

mg/kg milligram per kilogram

µl microliter

MMA^{+3} Monomethylarsonous acid

mRNA messenger Ribonucleic acid

NK Natural Killer cell

ng nanogram

ng/ml nanogram per milliliter

PAHs Polycyclic Aromatic Hydrocarbons

Pan NK Phenotypic Abnormality marker for NK cell

PARP Poly(ADP)ribose

PAX5 Paired Box-containing transcription factor 5

PBS Phosphate Buffered Saline

PCR Polymerase Chain Reaction

PE Phycoerythrin

PerCP Peridinin-Chlorophyll-Protein

PFC Plaque Forming Cell

PI Propidium Iodide

PIAS Protein Inhibitors of Activated STATs

ppb parts per billion

pSTAT5 phosphorylated STAT5

PTP Phosphotyrosine Phosphatases

pJAK1 phosphorylated JAK 1

qRT-PCR Quantitative Real Time Polymerase Chain Reaction

RPMI Roswell Park Memorial Institute

RIPA Radioimmunoprecipitation Assay

ROS Reactive Oxygen Species

SAM S-Adenosyl Methionine

SAHC S-Adenosyl Homocysteine

SDS Sodium DodesylSulphate

SH Sulfhydryl group

SRBC Sheep red blood cells

STAT5 Signal Tranducer and Activator of Transcription 5

TDAR T-Dependent Antibody Responses

Tyr Tyrosine

ZFPs Zinc Finger Proteins

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