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MECHANISMS OF ARSENIC-INDUCED SUPPRESSION OF EARLY RED BLOOD CELL DEVELOPMENT

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by

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B.S., Biology, New Mexico Highlands University, 2012

DISSERTATION

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DEDICATION

This work is dedicated to my wife, Crystal Medina. Thank you for standing with me through the highs and lows of my graduate studies and for your ability to listen and provide great advice, no matter the topic.

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ABSTRACT

Anemia adversely impacts the health of more than a billion people worldwide. A strong association between anemia and arsenic exposure has been reported in epidemiological studies from multiple countries. However, the mechanistic bases for the association between arsenic exposure and anemia is not fully understood. In this study, we found an inverse association between drinking water arsenic exposures and clinical indicators of anemia (i.e., red blood cell counts and hematocrit/packet cell volumes) in a cohort of men from rural Bangladesh enrolled in the Health Effects of Arsenic Longitudinal Study.

Follow-up studies in mice, revealed that 60-day exposure to trivalent inorganic arsenic (arsenite, As^{+3}) via drinking water resulted in anemia due to the impaired development of early erythroid progenitor cells in the bone marrow. To gain an understanding of the mechanisms of As^{+3}-induced suppression of erythroid progenitor cell development, we utilized an *in vitro* model of erythropoiesis using primary mouse bone marrow hematopoietic progenitor cells. As^{+3} and the As^{+3} metabolite,

monomethyarsonous acid (MMA^{+3}) suppressed erythropoiesis in the bone marrow, beginning at very early stages of erythroid differentiation. We found that As^{+3} selectively disrupted the function of an essential transcriptional regulator of erythropoiesis, GATA-1. Impairment of GATA-1 resulted from As^{+3} interaction with the zinc finger motifs of GATA-1, causing an inhibition of erythropoiesis and an imbalance of hematopoietic differentiation.

MMA^{+3} was also found to impair the differentiation of early erythroid progenitors through the loss of GATA-1 and by the suppression of erythropoietin (EPO)-activated signal transducer and activator of transcription 5 (STAT5) signaling. Disruption of GATA-1 and GATA-1/EPO-activated STAT5 by As^{+3} and MMA^{+3}, respectively compromised the regulation of a critical prosurvival factor, B-cell lymphoma-extra-large, resulting in the aberrant cell death of early erythroid progenitor cells.

Results from this study clearly demonstrate that early developing erythroid progenitors in the bone marrow are sensitive targets of As^{+3} and MMA^{+3} toxicity. MMA^{+3} was found to be more toxic than As^{+3} to early erythroid progenitor cells, likely by disrupting multiple pathways necessary for erythropoiesis. For the first time, this study provides novel mechanistic information of arsenic-induced dysregulation and inhibition of erythropoiesis, which is potentially a significant contributing factor to the high incidence of anemia in people chronically exposed to arsenic.

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LIST OF ABREVIATIONS

ANOVA	Analysis of variance
AO/PI	Acridine Orange/Propidium Iodide
As	Arsenic
As^{+3} or AsIII	Arsenite
As^{+5}	Arsenate
As3MT	Arsenite 3 Methyltransferase
Bcl-x_L	B-Cell Lymphoma-Extra-Large
BFU-E	Burst Forming-Unit Erythroid
BM	Bone Marrow
Caspase	Cysteinyl Aspartate-Specific Proteases
CD	Cluster of Differentiation
cDNA	Complementary Deoxyribonucleic Acid
CFU-E	Colony Forming-Unit Erythroid
CLP	Common Lymphoid Progenitor
CMP	Common Myeloid Progenitor
DMA^{+3}	Dimethylarsonous Acid
DMA^{+5}	Dimethylarsinic Acid
DNA	Deoxyribonucleic Acid
DPBS	Dulbecco's Phosphate Buffered Saline
$DPBS^{-}$	DPBS Without Calcium and Magnesium

EDTA	Ethylenediamine Teraacetate Acid
EPO	Erythropoietin
EryA	Basophilc Erythroblast
EryB	Polychromatophilic Erythroblast
EryC	Orthochromatophilic Erythroblast
FBS	Fetal bovine serum
FITC	Fluorescein Isothiocyanate
FSC-A	Forward Scatter Area
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GMP	Granulocyte Macrophage Progenitor
HBSS	Hanks Balanced Salt Solution
HCT/PCV	Hematocrit/Packed Cell Volume
HEALS	Health Effects of Arsenic Longitudinal Study
Hgb	Hemoglobin
HPBMC	Human Peripheral Blood Mononuclear Cell
HRP	Horse Radish Peroxidase
HSC	Hematopoietic Stem Cell
IgG	Immunoglobulin G
JAK	Janus Kinase
mAb	Monoclonal Antibody
MCH	Mean Corpuscular Hemoglobin
MCHC	Mean Corpuscular Hemoglobin Concentration

MCV	Mean Corpuscular Volume
MEP	Megakaryocyte Erythroid Progenitor
mL	Milliliter
MMA^{+3}	Monomethylarsonous acid
MMA^{+5}	Monomethylarsinic acid
mRNA	Messenger Ribonucleic acid
NIEHS	National Institutes of Environmental Health Sciences
NIH	National Institutes of Health
PCR	Polymerase Chain Reaction
PI	Propidium Iodide
ppb	Parts Per Billion (µg/L)
ppm	Parts Per million (mg/L)
Pre-GM	Pre-Granulocyte Macrophage Progenitor
Pre-MegE	Pre-Megakaryocyte Erythroid Progenitor
pSTAT5	Phosphorylated STAT5
qPCR	Quantitative Polymerase Chain Reaction
RBC	Red Blood Cell
Retic	Reticulocyte
SCF	Stem Cell Factor
STAT	Signal Transducer and Activator of Transcription
Zn	Zinc
µL	Microliter

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CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Arsenic Exposure and Toxicological Implications of Arsenic Metabolism

Arsenic is a pervasive environmental toxicant and common contaminant in soil, air, food, and water (Hughes 2011; WHO 2011). Chronic arsenic exposure is associated with numerous cancers and diseases, stemming from toxicity to nearly every major organ and organ system in the body, including the skin, liver, kidneys, bladder, cardiovascular system, respiratory system, nervous system, endocrine system, and immune system (Hughes et al., 2002; Heck et al., 2008; Naujokas et al., 2013; Tyler and Allan 2014; Ferrario et al., 2016).

A major route of human exposure to arsenic is through the consumption of contaminated drinking water (ASTDR 2007, 2016; WHO 2011). Millions of people worldwide are chronically exposed to arsenic in their drinking water at levels that exceed the World Health Organization and United States Environmental Protection Agency maximum contaminant level of 10 ppb (ATSDR 2007, 2016; U.S. EPA 2012; WHO 2011). Arsenic contamination of groundwater, which is the primary source of drinking water in many countries, results from natural geological sources as well as anthropogenic activities (Focazio et al., 1999; WHO 2011; Herath et al., 2016). In drinking water, arsenic is found primarily in the inorganic forms of arsenate (As^{+5}) and arsenite (As^{+3}) (ATSDR 2007, 2016).

As^{+3} is the most toxic inorganic arsenical (Styblo et al., 2000), and once ingested, is metabolized in the liver, kidneys, lungs, and testes through a series of reduction and oxidative methylation reactions catalyzed by the arsenite (+3 oxidation state)

methyltransferase (As3MT) enzyme to monomethylarsonic acid (MMA^{+5}), monomethylarsonous acid (MMA^{+3}), dimethylarsinic acid (DMA^{+5}), and dimethylarsinous acid (DMA^{+3}) (Thomas et al., 2001; Vahter, 2002). The *in vivo* metabolism of arsenic is depicted in Figure 1.1.

Arsenic metabolism has an important role in detoxification, but also generates bioactive intermediates with greater toxicity than the parent inorganic arsenicals (Vahter 2002; Douillet et al., 2017). Multiple *in vitro* and *in vivo* studies have found MMA^{+3} and DMA^{+3} to be more toxic than As^{+5} and As^{+3} (Petrick et al., 2000; Petrick et al., 2001; Thomas et al., 2001; Stýblo et al., 2002). Additional studies also indicate that MMA^{+5} and DMA^{+5} are less toxic than As^{+3} and MMA^{+3} (Vahter and Concha 2001; Stýblo et al., 2002; Gusman et al., 2013). The predominant forms of arsenic most readily excreted in urine are MMA^{+5} and DMA^{+5} (Vahter 2002).

The biotransformation of arsenic is contingent on the activity of As3MT (Lin et al., 2001; Thomas et al., 2007). Variations in the *As3mt* gene produced by single nucleotide polymorphisms result in interindividual differences in arsenic metabolism and are associated with differential sensitivities to arsenic toxicity (Vahter et al., 1995; Vahter 1999a; Engström et al., 2010). Epidemiological studies in populations chronically exposed to arsenic have shown that arsenic methylation capacity is associated with elevated cancer incidence and disease risk (Yu et al., 2000; Chen et al., 2003, 2009; Ahsan et al., 2007; Huang et al., 2008; Lin et al., 2018; Luo et al., 2018). The proportions of excreted arsenic metabolites are indicative of arsenic methylation efficiency (Vahter 1999a,b; Gamble et al., 2005). Higher levels of DMA^{+5} relative to MMA^{+5} in urine is

indicative of more efficient arsenic metabolism and is associated with decreased toxicity (Vahter and Concha 2001; Vahter 2002).

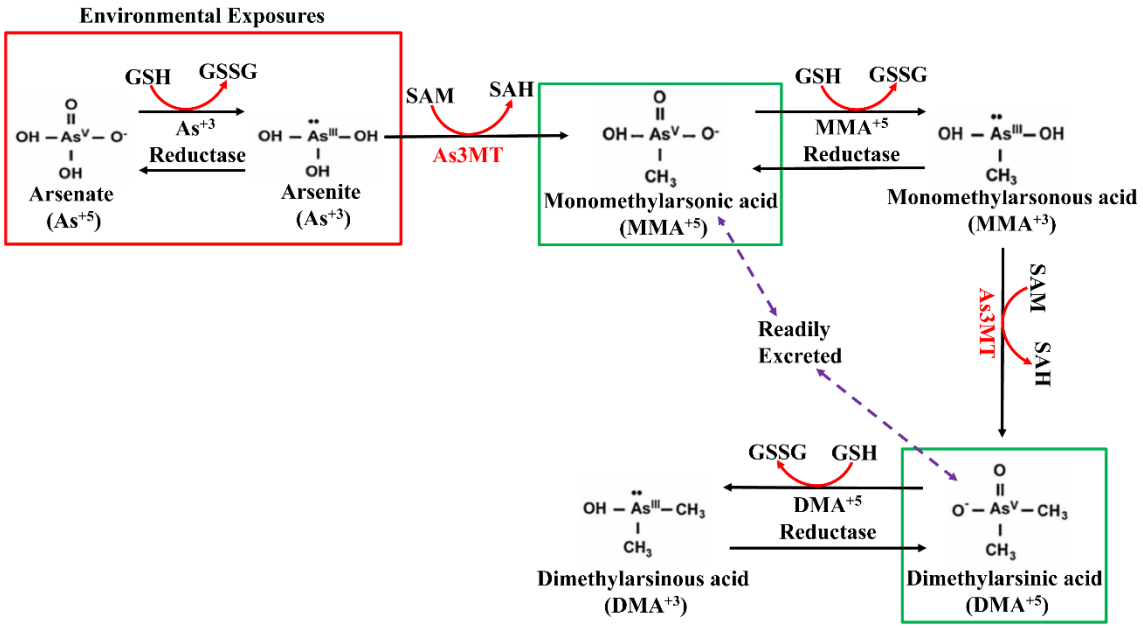

Figure 1.1. Biotransformation of arsenic in mammals. Arsenic (in the form of As^{+5} or As^{+3}) is metabolized *in vivo* through a series of reduction and oxidative methylation reactions catalyzed by As3MT using glutathione (GSH) as a reducing agent and S-adenosylmethionine (SAM) as a methyl donor (Gamble et al., 2005). As^{+3} is converted to di- and mono-methyl intermediates that are readily excreted from the body (MMA^{+5} and DMA^{+5}) and that have greater toxicity (MMA^{+3} and DMA^{+3}) than the inorganic arsenicals, As^{+5} and As^{+3} (Thomas et al., 2001; Vahter 2002; Stýblo et al., 2002). Glutathione disulfide (GSSG); S-adenosylhomocysteine (SAH)

Epidemiological Evidence of Arsenic-Associated Anemia

Anemia is a hematological disorder that negatively affects the health of more than a billion people worldwide (Koury 2014; Kassebaum et al., 2014; WHO 2015). Anemia is caused by insufficient quantities of circulating red blood cells (RBCs) and/or by reduced hemoglobin levels in circulating RBCs (Kassebaum et al., 2014; WHO 2015). Many deleterious health effects are associated with anemia, including fatigue, increased susceptibility to infection, cognitive and motor impairments, heart failure, pre-term birth, low birth weights, and risk of maternal and neonatal mortality (Balarajan et al., 2011; WHO 2015; Kassebaum et al., 2014).

The prevalence of anemia is disproportionately high in regions with high endemic arsenic exposures, even after consideration of important confounding risk factors of anemia (e.g., iron deficiency) (Kile et al., 2016). Epidemiological reports from these regions indicate a strong relationship between arsenic exposure and anemia (Brenton et al., 2006; Hopenhayn et al., 2006; Heck et al., 2008; Majumdor et al., 2009; Surdu et al., 2015; Kile et al., 2016). A summary of these studies and their major findings is provided in Table 1.1.

The frequency of arsenic-associated anemia is high among women, particularly during pregnancy (Hopenhayn et al., 2006; Surdu et al., 2015). Surdu et al., (2015), showed that low-level arsenic exposure (<10 ppb) was associated with an increased prevalence of anemia among pregnant women. Moreover, Hopenhayn et al., (2006), reported that not only was drinking water arsenic-exposure (33-53 ppb) associated with anemia during pregnancy, but the risk of anemia was exacerbated as pregnancy progressed, with 49% of arsenic exposed women being anemic in the third trimester

compared to 17% in an unexposed (<1 ppb) population. Arsenic-associated anemia in men has not been as thoroughly investigated as in females; however, all three studies which included men also found associations between arsenic exposure and anemia (Breton et al., 2006; Heck et al., 2008; Majumdor et al., 2009). Despite the strong epidemiological evidence of arsenic-associated anemias, the mechanistic bases for these associations remains to be elucidated.

Table 1.1. Epidemiological evidence of arsenic-associated anemia.

Exposure	Country	Cohort	Effects	Reference
Drinking Water (median 39 ppb)	Bangladesh	900 men and women	Arsenic exposed participants more likely to be anemic than controls (13.5% vs. 10.5 %), and arsenic exposed women more likely than men (18.2% vs. 8.2%) to be anemic. Odds of arsenic skin lesions inversely associated with hemoglobin in males, but not females.	Brenton et al., 2006
Drinking water (33-53 ppb)	Chile	810 pregnant women	Arsenic exposed women more likely to be anemic during pregnancy. Anemia prevalence among arsenic exposed women increased as pregnancy progressed (49% vs. 17%).	Hopenhayn et al., 2006
Urinary arsenic (>200 ppb)	Bangladesh	1954 men and women	Inverse association between arsenic exposure and hemoglobin in men and women with hemoglobin <12 g/dL.	Heck et al., 2008
Drinking water (>800 ppb)	India	7683 men and women	Increased frequency of anemia in arsenic exposed men and women.	Majumdor et al., 2009
Drinking water (<10 ppb)	Romania	217 women	Increased prevalence of anemia among arsenic exposed women, particularly during pregnancy.	Surdu et al., 2015
Drinking water (median 17 ppb)	Bangladesh	147 women	Higher risk of developing arsenic-caused skin lesions in anemic women. Anemia in 75% of participants was not caused by iron deficiency.	Kile et al., 2016

Hematopoiesis

The mammalian hematopoietic system is responsible for producing staggering numbers of blood cells consistently throughout life (Orkin and Zon 2008; Rieger and Schroeder 2012; Jagannathan-Bogdon and Zon 2013). Hematopoiesis is the process by which multipotent hematopoietic stem cells (HSCs) give rise to all major cell lineages of the blood, including lymphocytes, myeloid cells, monocytes, megakaryocytes, and RBCs (Figure 1.2.) (Metcalf 2007; Rieger and Schroeder 2012). In adult humans and rodents, the primary site of hematopoiesis is the bone marrow (Rieger and Schroeder 2012; Jagannathan-Bogdon and Zon 2013). Although HSCs are very rare among the total bone marrow cell population (approximately 0.06%), they are responsible for generating an enormous amount of lineage specialized cells on a daily basis (Rieger and Schroeder 2012).

The developmental fate of HSCs relies on extrinsic and intrinsic regulation for commitment and subsequent development of lineage specific cell types (Orkin and Zon 2008; Rieger and Schroeder 2012). Extrinsic regulation of HSC lineage commitment is dictated by a number of different cytokines and hormones, including interleukin (IL)-3, IL-6, IL-7, stem cell factor (SCF), erythropoietin (EPO), granulocyte-macrophage-colony stimulating factor, macrophage-colony stimulating factor, glucocorticoids, etc., (Rieger and Schroeder 2012). The presence or absence of these stimuli act as a mechanism by which the bone marrow microenvironment influences the differentiation fate of HSCs (Rieger and Schroeder 2012).

Additionally, a number of intrinsic regulatory factors, including transcription factors, signaling modulators, epigenetic modifiers, cell cycle regulators, microRNAs,

and RNA binding proteins have a critical role in regulating the differentiation of HSCs (Orkin and Zon 2008; Rieger and Schroeder 2012). Interplay between extrinsic and intrinsic regulators, combined with the plasticity of HSCs, provides the foundation of the mammalian blood system (Graf 2002; Rieger and Schroeder 2012).

One path of HSC lineage differentiation is commitment to multipotent common myeloid progenitors (CMPs), which give rise to myeloid cells, monocytes, megakaryocytes, and RBCs (Metcalf 2007; Seita and Weissman 2010; Rieger and Schroeder 2012). The lineage commitment choices of CMPs is under debate; however, the current hypothesis is that the fate of these cells is intrinsically regulated through the expression and subsequent functional antagonism of two major transcriptional regulators of erythro-megakaryopoiesis and myelopoiesis, GATA-1 and PU.1, respectively (Zhang et al., 1996; Rekhtman et al., 1999; Nerlov et al., 2000).

CMPs express low levels of GATA-1 and PU.1, but in response to stimulation from the bone marrow microenvironment, GATA-1 or PU.1 reach a threshold expression level and work reciprocally to interfere with the activity of the other transcription factor (Iwasaki and Akashi 2007). GATA-1 binds directly to PU.1 to impair its function, thereby reducing the expression of myeloid-specific genes (Nerlov et al., 2000; Rekhtman et al., 1999; Rhodes et al., 2005). In contrast, PU.1 binds to GATA-1 DNA binding sites, thereby reducing its ability to activate erythro-megakaryocytic-specific gene transcription (Zhang et al., 1996; Rekhtman et al., 1999).

As result of GATA-1 antagonism of PU.1, CMPs are pushed to the erythro-megakaryocytic lineage and undergo subsequent erythroid and megakaryocyte differentiation (Nerlov et al., 2000; Rekhtman et al., 1999; Rhodes et al., 2005). This

mechanism of functional interference acts as a regulatory control for dictating the lineage commitment of CMPs (Iwasaki and Akashi 2007). Once committed to erythro-megakaryocytic differentiation, CMPs further mature to megakaryocyte-erythroid progenitors (MEPs) (Iwasaki and Akashi 2007). In response to extrinsic and intrinsic regulation, MEPs undergo additional stages of differentiation to produce megakaryocytes and mature RBCs (Orkin and Zon 2008).

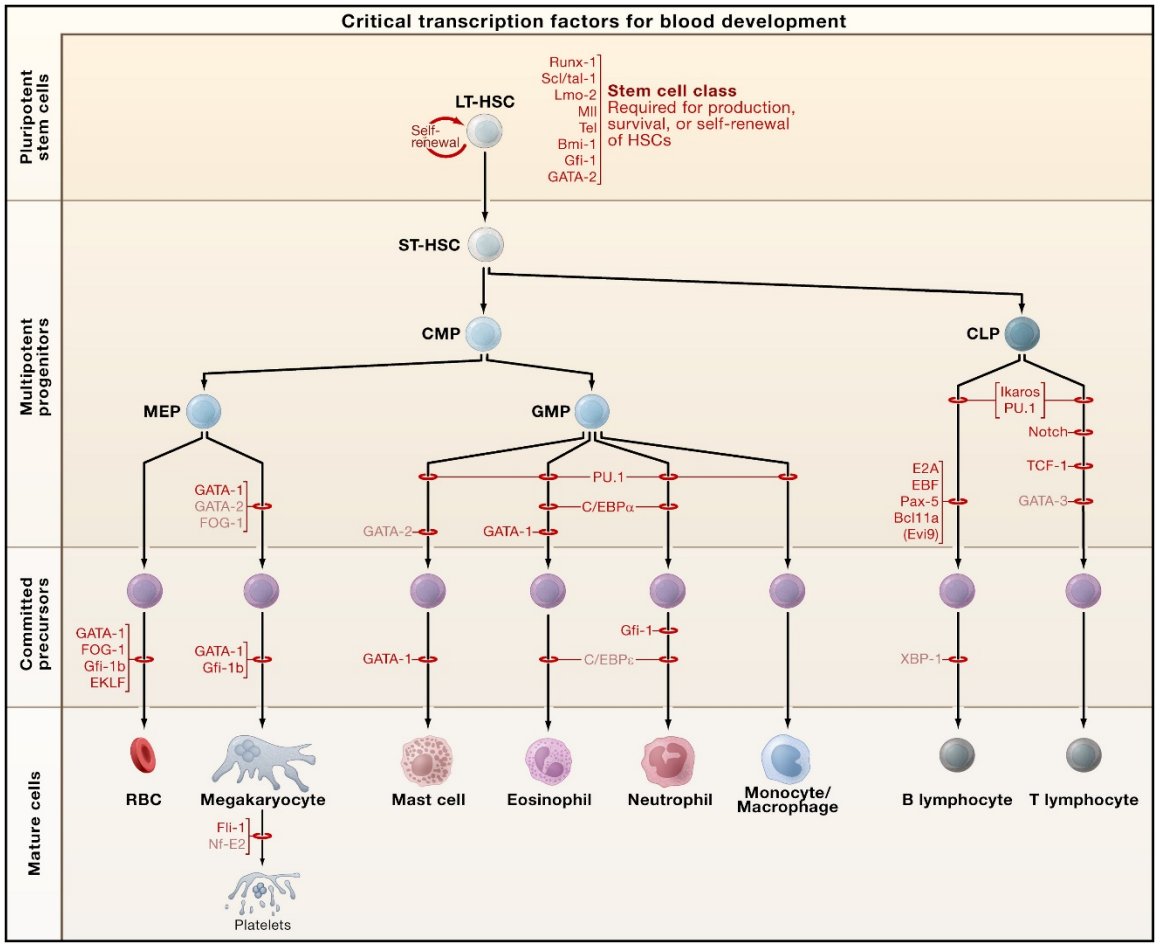

Figure 1.2. Hematopoietic hierarchy. Representation of the lineage commitment and differentiation of HSCs. In response intrinsic (pictured) and extrinsic stimuli, HSCs and downstream multipotent progenitors give rise to the major functionally specialized cell types of the blood (RBCs, megakaryocytes, myeloid and monocytes, and lymphocytes) (Orkin and Zon 2008). Reprinted from *Cell*, **132(4)**, Orkin and Zon, Hematopoiesis: An Evolving Paradigm for Stem Cell Biology, 631-644, Copyright 2008, with permission from Elsevier Inc.

Red Blood Cell Development

In adult humans, more than 2 million RBCs are produced every second and approximately 2×10^{11} every day to maintain normal physiological circulating levels (Dzierzak and Philipsen 2013; Kim et al., 2015a). The bone marrow is the primary site of RBC development (i.e., erythropoiesis) in adult humans and rodents (Tsiftsoglou et al., 2009; Dzierzak and Philipsen, 2013). However, in mice the spleen also has a critical function as an erythropoietic organ, especially if the bone marrow system is compromised (Dzierzak and Philipsen 2013). The stages and factors required for erythroid differentiation are largely conserved between humans and mice (Pishesha et al., 2014). The stages of erythropoiesis are depicted in Figure 1.3.

Erythropoiesis is controlled predominately by the hormone, erythropoietin (EPO), which is released from the kidneys in response to hypoxic conditions in the body (Hattangadi et al., 2011). EPO acts on early erythroid progenitor cells to promote survival, proliferation, and differentiation (Koury and Bondurant 1990; Hattangadi et al., 2011; Bunn 2013). The first detectable stage of committed erythroid differentiation is the slow proliferating, burst-forming unit erythroid cells (BFU-E), which were originally identified based on characteristic burst morphology colonies formed in methylcellulose-based medium (reviewed in Dzierzak and Philipsen 2013). BFU-E respond to elevated EPO along with other growth factors (glucocorticoids, SCF, IL-3, IL-6, etc.,) to mature to the highly proliferative and EPO-dependent, colony-forming unit erythroid cells (CFU-E) (Hattangadi et al., 2011; Dzierzak and Philipsen 2013).

CFU-Es were originally identified based on their ability to rapidly form colonies in methylcellulose-based medium (Dzierzak and Philipsen 2013). At the CFU-E stage,

hemoglobin production is initiated, and the cells undergo four additional stages of differentiation to proerythroblast, basophilic erythroblast, polychromatophilic erythroblast, and orthochromatophilic erythroblast (Migliaccio, 2010; Elliott and Sinclair, 2012).

During the latter four stages of differentiation (i.e., proerythroblast, basophilc, polychromatophilic, and orthochromatophilic), erythroid progenitors undergo a series of morphological and biochemical changes, such as size reduction, nuclear condensation, hemoglobin accumulation, and reduced proliferative capacity (Palis 2014; Moras et al. 2017). Following the orthrochromatophilic erythroblast stage, the nucleus is extruded forming short-lived pyrenocytes, and reticulocytes that are released from the bone marrow into circulation (Palis 2014).

Newly formed reticulocytes complete maturation in circulation (~1 week) by further reducing plasma membrane surface area and cell volume, clearing remaining organelles, and increasing cytoskeletal interactions with the outer plasma membrane, resulting in mature RBCs with characteristic biconcave morphology (Griffiths et al., 2012; Palis 2014). Mature RBCs have a circulating half-life of approximately 120 days in humans and 45 days in mice (Dzierzak and Philipsen 2013; Palis 2014); i.e., after 120 days in humans (or 45 days in mice) the total circulating red blood cell pool will be entirely replenished by newly produced cells.

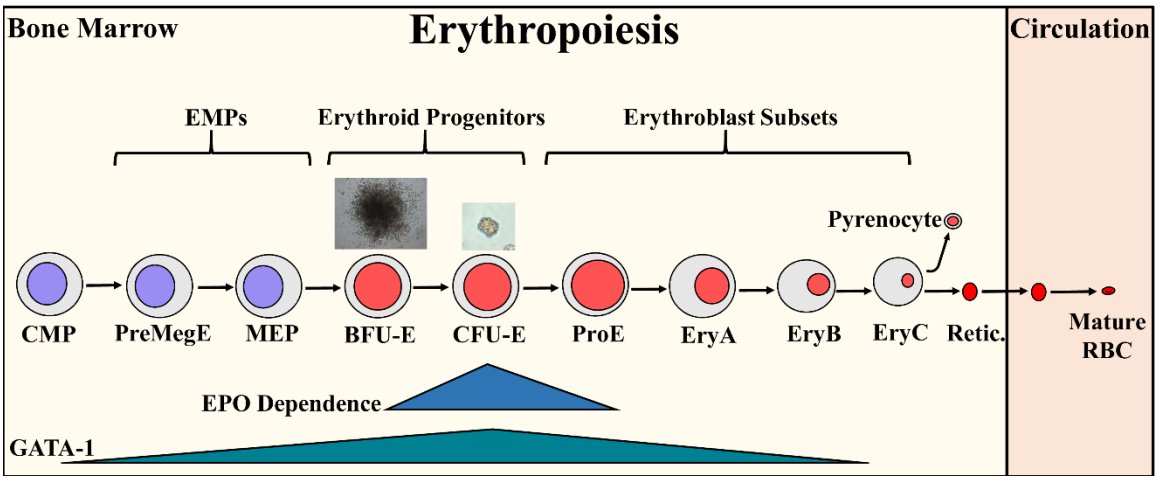

Figure 1.3. Stages of bone marrow erythropoiesis. In response to GATA-1, CMPs commit to the erythro-megakaryocytic lineage and differentiate to pre-megakaryocyte erythroid progenitors (Pre-MegE) (Iwasaki and Akashi 2007). Pre-MegE differentiate to MEPs, which give rise to RBCs and megakaryocytes (Pronk et al., 2007). The first identifiable stage of erythroid differentiation is BFU-E, which respond to increased EPO and other growth factors to mature to the EPO dependent CFU-E stage (Dzierzak and Philipsen 2013). CFU-E undergo additional stages of differentiation (i.e., proerythroblast (ProE); EryA, basophilicerythroblast; EryB, polychromatophilic erythroblast; and EryC, orthochromatophilic erythroblast), prior to enucleation and release from bone marrow into the circulation as a reticulocyte (Retic.) (Migliaccio, 2010; Elliott and Sinclair, 2012). Reticulocytes complete their development in circulation to become mature RBCs (Palis 2014).

Regulation of Erythropoiesis: GATA-1 and Erythropoietin Signaling

Erythropoiesis is an intricate process that relies on extrinsic and intrinsic regulation by many factors (e.g., hormones, cytokines, signal transduction pathways, cell cycle modulators, transcription factors, regulatory RNAs, epigenetic modifications, etc.,) for normal differentiation of erythroid progenitor cells (Hattangadi et al., 2011; Dzierzak and Philipsen, 2013). The development of early erythroid progenitors is dependent on the activities of two critical regulatory pathways mediated by GATA-1 and EPO signaling (Ferreira et al., 2005; Dzierzak and Philipsen, 2013).

GATA-1 is a zinc finger transcription factor that is regarded as the master regulator of erythropoiesis (Ferreira et al., 2005). GATA-1 is responsible for inducing and repressing many genes essential for erythroid differentiation and also has a critical role in the regulation of hemoglobin production (Evans et al., 1988; Pevny et al., 1991; Pevny et al., 1995; Welch et al., 2004; Ferreira et al., 2005; Katsumuara et al., 2013). GATA-1 expression levels vary throughout the stages of erythropoiesis, but the activity of this key transcription factor peaks at the late BFU-E and CFU-E stages of differentiation (Ferreira et al., 2005).

GATA-1 knockout studies revealed the necessity of this factor for erythropoiesis, as GATA-1 null embryos die from severe anemia, resulting from arrested differentiation and apoptosis of early stage erythroid progenitors (Weiss et al., 1994; Weiss and Orkin 1995; Pevny et al., 1995; Fujiwara et al., 1996). GATA-1 knockdown studies further confirmed the dependence of early erythroid progenitors on the activity of GATA-1, showing that graded loss of GATA-1 (i.e., 5 to 20% of normal GATA-1 levels) results in an expression-level dependent impairment of erythropoiesis (McDevitt et al., 1997).

Another critical regulatory mechanism in early erythroid cells is the EPO-activated signal transducer and activator of transcription 5 (STAT5) prosurvival pathway (Socolovsky et al., 1999; 2001; Liu et al., 2006). Expression of the EPO receptor is mediated by GATA-1 in early erythroid progenitors (Zon et al., 1991; Ferreira et al., 2005). The EPO receptor is a member of the type 1 cytokine receptor superfamily (Watowich 2011). EPO binding to the EPO receptor induces the dimerization and phosphorylation of tyrosine residues in the intracellular domain by Janus kinase 2 (JAK2), which is constitutively associated with the cytoplasmic domain of the EPO receptor (Watowich 2011).

EPO receptor phosphorylation provides docking sites for signaling molecules including, STAT5 (Watowich 2011). There are two isoforms of STAT5, STAT5a and STAT5b, which share 95% amino acid sequence identity (Liu et al., 1995). STAT5 recruitment and phosphorylation by the EPO receptor/JAK2 complex is essential for mediating downstream EPO-activated signaling events (Watowich 2011). Activated STAT5 is an essential regulatory factor that mediates signaling events critical for the survival, proliferation, and differentiation of early erythroid progenitor cells (Socolovsky et al., 1999; 2001; Watowich 2011).

Similar to GATA-1, EPO responsiveness also peaks at the late BFU-E and CFU-E stages of erythroid differentiation (Wu et al., 1995; Ferreira et al., 2005). EPO deprivation studies in erythroid progenitor cell models and STAT5 knockout studies in mice show that loss of STAT5 causes an inhibition of erythropoiesis by the loss of B-cell lymphoma-extra-large (Bcl-x_L) regulated survival of early erythroid progenitors and

results in severe anemia (Gregory et al., 1999; Silva et al., 1996; Socolovsky et al., 1999, 2001).

Follow-up studies demonstrate a role for GATA-1 in this EPO-activated STAT5 prosurvival pathway (Gregory et al., 1999). GATA-1 and EPO-activated STAT5 cooperate to promote optimal expression of the prosurvival factor Bcl-x_L, thereby preventing apoptosis of early erythroid progenitor cells (Figure 1.4) (Gregory et al., 1999). Under steady-state EPO conditions, most early erythroid progenitors (~60%) succumb to apoptosis via this mechanism; however, with elevated EPO levels, such as experienced with hypoxia, this mechanism promotes the survival and rapid expansion of early erythroid progenitors (Dzierzak and Philipsen, 2013).

This prosurvival mechanism controls erythropoietic rate and provides the basis for the plasticity of the bone marrow erythropoietic system, as it directly links EPO levels to the survival and differentiation of erythroid progenitors (Koulnis et al., 2012). It is important to note, that loss of either GATA-1 or EPO-activated STAT5 is sufficient to induce apoptosis and reduce the maturation of erythroid progenitors (Weiss and Orkin 1995; Socolovsky et al., 2001). Considering the importance of GATA-1 and STAT5 for the survival and differentiation of early erythroid progenitors, disruption of these pathways by arsenic may be a mechanism of arsenic-induced hematotoxicity.

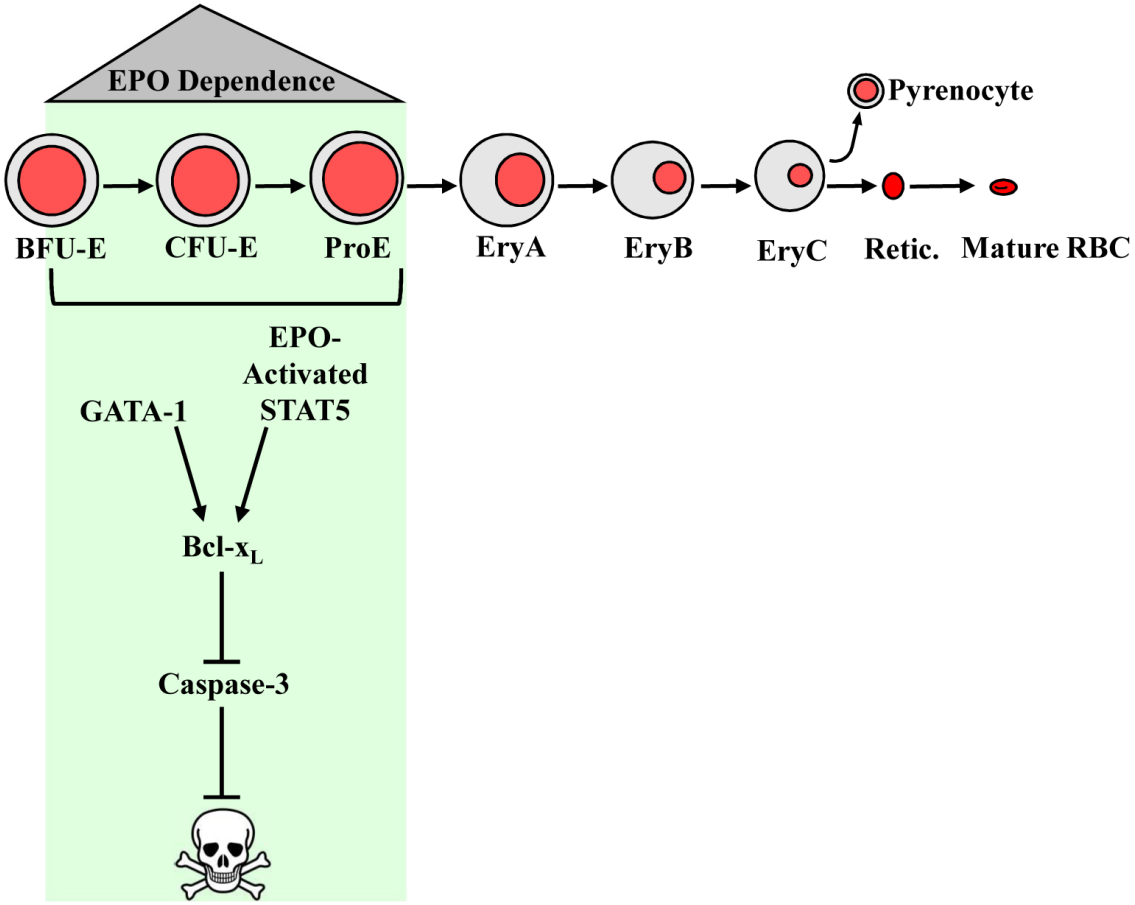

Figure 1.4. Schematic diagram of GATA-1 and EPO-activated STAT5 prosurvival pathway in early erythroid progenitors (late-BFU-E cells, CFU-E cells, and ProEs) (Gregory et al., 1999). GATA-1 and EPO-activated STAT5 cooperate to stimulate the optimal expression of the prosurvival factor B-cell lymphoma-extra-large (Bcl-x_L), which functions to prevent caspase-3 mediated death of early erythroid progenitors (Gregory et al., 1999).

Evidence of Arsenic-Induced Hematotoxicity

Despite the direct link between decreased RBC production and anemia (Koury 2014), the effects of arsenic on erythropoiesis have not been thoroughly investigated. Several studies report arsenic toxicity to mature circulating RBCs. Arsenic exposure causes oxidative stress, depletes intracellular ATP, and alters morphological characteristics of mature RBCs, resulting in compromised membrane integrity, eryptosis, and intra-and-extravascular hemolysis (Winski et al., 1997; Winski and Carter, 1998; Zhang et al., 2000; Biswas et al., 2008; Mahmud et al., 2009).

To date, very few studies have been performed to evaluate the effects of environmentally relevant arsenic exposures on early erythroid progenitor cells in the bone marrow. Acute exposure to high levels of arsenic has been reported to cause anemia in humans and rodents as a result of bone marrow depression (Szymańska-Chabowska et al., 2002), suggesting that the development of RBCs may be a target of arsenic toxicity. Studies focused on the chemotherapeutic actions of arsenic trioxide in erythroleukemia cell line models have also provided some evidence of arsenic toxicity to RBCs (Saulle et al., 2006; You et al., 2005). Despite the limitations of using erythroleukemia cells as models of normal erythroid cell biology, findings from these studies suggest that arsenic disrupts pathways important for differentiation and survival of erythroid cells.

Toxicological investigations of hematopoietic progenitors following environmentally relevant metal exposures are limited, likely resulting from the difficulty of studying these rare cell types. Ferrario et al., (2008), found that the development of myeloid progenitors (CFU-GM) was suppressed by *in vitro* As^{+3} and MMA^{+3} exposures. Additionally, previous studies from our lab showed that the development of pre-B cells in

the bone marrow and pre-T cells in the thymus is suppressed by low levels of As^{+3} and MMA^{+3} (Ezeh et al., 2014; Xu et al., 2016a; Xu et al., 2017). These studies show that hematopoietic progenitors in the bone marrow are sensitive to arsenic-induced toxicity and highlight the necessity for further investigations to understand the effects of environmental metal exposures on these vulnerable cell populations.

GATA-1 and Erythropoietin-Activated STAT5 as Targets of Arsenic Toxicity

Regulation of erythropoiesis is dependent on two critical pathways mediated by the activity of GATA-1 and STAT5. Interestingly, zinc finger proteins (such as GATA-1) and STAT5 signaling are known targets of arsenic toxicity (Cheng et al., 2004a; Wetzler et al., 2006; Ding et al., 2009; Zhou et al., 2011; Sun et al., 2014; Zhou et al., 2015; Ezeh et al., 2016a, Xu et al., 2016a). GATA-1 contains two C4 zinc fingers, one in the N-terminal domain and one in the C-terminal domain (Ferreira et al., 2005). The N-terminal zinc finger of GATA-1 is essential for protein-protein interactions and the C-terminal zinc finger is necessary for DNA binding (Fox et al., 1999; Liew et al., 2005; Bates et al., 2008). Congenital mutations in the N- or C-terminal zinc finger motifs of GATA-1 result in dyserythropoietic anemia (Del Vecchio et al., 2005; Ciovacco et al., 2008; Crispino and Horwitz 2017).

As^{+3} and MMA^{+3} are documented to inhibit the function of zinc finger proteins by displacing zinc from zinc finger motifs (Ding et al., 2009; Zhou et al., 2011; Sun et al., 2014; Zhou et al., 2014). As^{+3} selectively interacts with C3H1 and C4 zinc fingers; whereas, MMA^{+3}, is less selective and interacts with C2H2, C3H1, and C4 zinc fingers (Zhou et al., 2011; Zhou et al., 2014). Selective binding of As^{+3} to C3H1 and C4 zinc fingers renders cysteine residues in the zinc finger structure vulnerable to oxidation by As^{+3}-generated reactive oxygen species (Zhou et al. 2015). This selective cysteine thiol oxidation results in zinc loss and inhibits the function of zinc finger proteins (Zhou et al. 2015).

GATA-1 contains two zinc finger motifs that are structurally favorable for As^{+3} and MMA^{+3} binding (Piątek et al., 2008; Zhou et al., 2011; Zhao et al., 2012; Zhou et al.,

2014). As such, it is likely that As^{+3} and MMA^{+3}-induced disruption of the zinc finger motifs of GATA-1 may be an underlying mechanism of toxicity to early developing erythroid progenitor cells. Additionally, many other transcription factors and transcriptional coactivators involved in the development of erythroid progenitors are also zinc fingers with motifs that may be displaced by As^{+3} and MMA^{+3} (Hattangadi et al., 2011).

Since the maturation of erythroid progenitor cells is dependent on STAT5 (Socolovsky et al., 1999; 2001), disruption of EPO-activated STAT5 signaling is another possible pathway impacted by arsenic exposure. STAT5 activation is also a known target of arsenic toxicity in progenitor cells (Cheng et al., 2004a; Wetzler et al., 2006; Ezeh et al., 2016a, Xu et al., 2016a). Previous studies from our lab show that As^{+3} and MMA^{+3} inhibit the development of early B cells in the bone marrow and early T cells in the thymus by reducing IL-7-induced STAT5 activation (Ezeh et al., 2016a; Xu et al., 2016a). Additionally, studies in cancer cell line models also demonstrate that STAT5 activation is impaired by *in vitro* exposures to As^{+3} and arsenic trioxide (ATO) (Cheng et al., 2004a; Wetzler et al., 2006).

Arsenic trioxide is used as a therapy for treating patients with acute myeloid leukemia (AML) (Zhang et al., 1996; Shen et al., 1997; Soignet et al., 1998). Constitutive STAT5 activation is a hallmark of many hematologic cancers, including AML (Gouilleux-Gruart et al., 1996; Birkenkamp et al., 2001; Benekli et al., 2003). Multiple studies have demonstrated the utility of ATO-induced JAK2/STAT5 inhibition as an effective therapeutic intervention for treating AML (reviewed in Benekli et al., 2009). Arsenic trioxide impairs the phosphorylation of STAT5, thereby reducing cell growth and

inducing apoptosis of AML cells (Chen et al., 1996; Miller et al., 2002). Similar to ATO, JAK2/STAT5 inhibitors also show utility as therapeutic agents in the treatment of AML (Nelson et al., 2011; 2012; Oh et al., 2015; Wingelhofer et al., 2018); however, a major side effect of the pharmacological inhibition of JAK2/STAT5 is anemia (Harrison et al., 2012; Verstovsek et al., 2017). These studies not only highlight the utility of STAT5 inhibition as a target for therapeutic intervention in leukemia, but also provide indirect evidence for the role of STAT5 inhibition in the development of anemia.

To our knowledge, no studies have evaluated arsenic toxicity to early developing erythroid progenitors. Additionally, a clear mechanistic understanding of arsenic-associated anemia also remains to be elucidated. The goal of this study is to address these critical knowledge gaps by identifying whether the development of early erythroid progenitor cells is disrupted by As^{+3} and MMA^{+3} exposures and to identify whether such impairments are mediated through the inhibition of GATA-1 and EPO-activated STAT5 signaling.

Utilizing an *in vitro* Model of Erythropoiesis to Characterize Arsenic Toxicity to Early Erythroid Progenitors

Hematopoietic progenitor cells (HPC), such as erythroid progenitors, occur in very low abundance among the total bone marrow cell population (Rieger and Schroeder 2012; Dzierzak and Philipsen, 2013). The differentiation of these rare cell populations has been traditionally studied based on colony-forming ability in cytokine and growth factor supplemented methylcellulose-based medium (Bradley and Metcalf 1966; Pluznik and Sachs 1966; Iscove et al., 1974). This method allows for the expansion of a small number of cells into morphologically identifiable cell colonies. Colony formation is dependent on differentiation, proliferation, and cell viability; however, deciphering which of these factors is impacted and at what differentiation stage(s) such alterations occur as result of toxicant exposure is very difficult. Additionally, colony forming assays are not readily amendable to mechanistic studies.

To gain a detailed understanding of the effects of arsenic on erythroid progenitor development, an *in vitro* model of erythropoiesis was developed using primary mouse bone marrow HPC stimulated with a combination of EPO and SCF (as described by Shuga et al., (2007), with some modifications). Advancements in the field of HPC biology have identified and validated specific cell surface marker phenotypes of all hematopoietic progenitor lineages (Pronk and Bryder 2011; Grover et al., 2014). Dynamic changes in surface marker phenotypes can be assessed to monitor the progression of early HPC through the stages of lineage-specific differentiation using flow cytometry.

Stimulation of HPC with EPO and SCF promotes the lineage commitment and subsequent differentiation of erythroid progenitors. After 24 h of EPO and SCF stimulation, HPC demonstrate surface marker characteristics of early erythro-megakaryocytic and erythroid progenitors (Pre-MegE, MEP, BFU-E, CFU-E, proerythroblasts). After 48 and 72 h, nearly all major stages of erythroblast differentiation (e.g., basophilic erythroblasts, late basophilic erythroblasts and polychromatic erythroblasts, and orthochromatic erythroblasts are identifiable by CD71 and Ter119 surface marker expression and size characteristics (Socolovsky et al., 2001; Koulnis (2011).

This technique provides a model system to study the developmental progression of erythroid progenitor cells starting from very early stages of differentiation. This technique allows us to assess and identify the specific stages of erythroid progenitor differentiation most impacted by As^{+3} and MMA^{+3} exposures. The sensitivity of flow cytometry, in combination with the minimal cell numbers required for flow cytometry, allow for the mechanistic assessment of biochemical alterations induced by As^{+3} and MMA^{+3} in surface marker defined early erythroid progenitors.

HYPOTHESIS AND SPECIFIC AIMS

Overall Hypothesis

Exposure to environmentally relevant concentrations of arsenic impairs erythropoiesis through the suppression of GATA-1- and STAT5- regulated differentiation and survival pathways in early erythroid progenitors.

Specific Aims

Specific Aim 1: To evaluate the relationship between arsenic exposure and anemia in men chronically exposed to As^{+3}, and to determine whether *in vivo* drinking water exposures to low levels of As^{+3} result in the development of anemia in adult mice. Aim 1 is separated into two components, investigating the relationship between chronic arsenic exposures and anemia in humans (Chapter 1) and mice (Chapter 2). We hypothesize that hematological indicators of anemia (e.g., RBC counts and hematocrit/packed cell volumes) will decrease as a function of increasing As^{+3} exposures in men from rural Bangladesh. We also hypothesize that exposure of adult male mice to environmentally relevant levels of As^{+3} will result in the development of anemia via suppression of early erythroid progenitor cell development in the bone marrow. This study will reveal whether arsenic-associated anemia observed in human populations can be recapitulated in a mouse model and will also provide critical information regarding the *in vivo* effects of As^{+3} on early developing erythroid cells.

Aim 2: To examine the *in vitro* effects of As^{+3} and the As^{+3} metabolite, MMA^{+3} on the erythropoietin (EPO)-induced differentiation of primary mouse bone marrow erythroid progenitor cells. We hypothesize that As^{+3} and MMA^{+3} will suppress the *in vitro* differentiation of primary mouse bone marrow erythroid progenitors. This study (Chapter 3 and Chapter 4) will thoroughly characterize the effects of As^{+3} and MMA^{+3} on the differentiation of bone marrow erythroid progenitors, which will reveal the specific stages of erythroblast development most susceptible to arsenic-induced hematotoxicity.

Aim 3: To characterize the mechanisms responsible for As^{+3} and MMA^{+3}-induced suppression of primary mouse bone marrow erythroid progenitor cell development. We hypothesize that As^{+3} and MMA^{+3} suppress the development of early erythroid progenitors by combined effects on differentiation and survival caused by impairments of GATA-1 and EPO-activated STAT5 signaling. This aim (Chapter 3 and Chapter 4) will provide novel mechanistic insights critical for understanding the relationship between arsenic exposure and anemia reported in epidemiological studies.

CHAPTER 2

Arsenic Exposures Alter Clinical indicators of anemia in a Male Population of Smokers and Non-Smokers in Bangladesh

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KEY POINTS

- Arsenic exposure was negatively correlated with clinical indicators of anemia in men.
- Dose-dependent decreases in RBC and HCT/PCV were found with increasing drinking water arsenic levels.
- Correlations between arsenic exposure and clinical indicators of anemia were slightly stronger among smokers.
- Relationships between smoking and arsenic exposure suggest potential interactive effects on clinical indicators of anemia.

ABSTRACT

Drinking water arsenic (WAs) exposure has been linked to a number of detrimental health outcomes including anemia, primarily among pregnant women. Little is known about the effects of arsenic (As) on hematological disorders among men. We have examined the role of As exposure on hematological indicators of anemia in a group of men exposed to a wide range of As in their drinking water. We conducted a cross-sectional investigation among 119 healthy men in the Health Effects of As Longitudinal Study (HEALS) cohort, in rural Bangladesh. The participants are part of an ongoing study focused on evaluating the influence of As and smoking on immune function. Samples were collected at recruitment and analyzed for water As, urinary As (UAs) and UAs metabolites to assess As exposure. Blood samples were also collected at recruitment and assayed immediately for hematological parameters. We found that increased WAs was associated with decreased red blood cell counts [β= -0.13, p<0.0001] as well as hematocrit packed cell volume [β= -0.68, p=0.008] following adjustment for age, smoking, body mass index and polycyclic aromatic hydrocarbon-DNA adducts. Other measures of As exposure (UAs and its metabolites) demonstrated similar associations. Slightly stronger effects were observed among smokers. We also observed an effect of As on hemoglobin among smokers in relation to urinary As [β= -0.54, p<0.05]. Our analysis revealed effects of As exposure on hematological indicators of anemia in a group of healthy male smokers and non-smokers.

INTRODUCTION

As contamination in drinking water is a global public health problem affecting millions of people worldwide. The major arsenicals found in drinking water are the pentavalent and trivalent inorganic forms arsenate (As^{+5}) and arsenite (As^{+3}), respectively (Styblo et al., 2000; Naujokas et al., 2013). As a result, exposure to these inorganic arsenicals is of particular concern from a public health standpoint (Styblo et al., 2000). High-level As exposure has been linked with internal and external cancers, lung disease, immunosuppression, and cognitive impairment in children (Naujokas et al., 2013; Hughes et al., 2002; Tyler and Allan 2014; Ferrario et al., 2016). A few studies have also indicated an association between chronic As exposure and hematological disorders such as anemia, particularly among women during pregnancy (Hopenhayn et al., 2006; Heck et al., 2008; Surdu et al., 2015; Kile et al., 2016).

Anemia is characterized by reduced red blood cell counts (RBC) and decreased hemoglobin (Hgb) levels in the blood. Anemia has been linked with a number of deleterious health effects, including fatigue, increased susceptibility to infection, cognitive and motor impairments, low birth weights, and risk of maternal and neonatal mortality (Balarajan et al., 2011; WHO 2011). A cross-sectional study of 217 Romanian women, found higher prevalence of anemia [prevalence proportion ratio (PPR) =1.71, 95% (CI) 0.75-3.88], and particularly anemia during pregnancy [(PPR)= 2.87, 95% (CI) 0.62-13.26] associated with As exposure (Surdu et al., 2015). Similarly, a follow-up study of 810 pregnant women from two Chilean cities observed increased incidences of anemia related to As exposure. This study also revealed increasing prevalence of anemia

with the progression of pregnancy among As exposed as compared to unexposed women (49% versus 17%) (Hopenhayn et al., 2006).

We have reported that higher urinary As (>200 µg/L) was linked to low Hgb (<10 g/dL) among 1,954 men and women in the HEALS cohort in Bangladesh (Heck et al., 2008). Two studies from Bangladesh also reported an association of Hgb on arsenical-induced skin lesions. In a group of 147 women, Kile et al., (2016) observed that low Hgb (<12 g/dL) significantly increased risk [(OR) = 3.32, 95% (CI) 1.29, 8.52] of As-related skin lesions among women with normal Hgb levels after adjusting for As levels in drinking water and other covariates. Interestingly, the relationship of Hgb and arsenical skin lesions was found to be gender specific in a case-control study of 900 individuals from the same area of Bangladesh. Hgb was significantly associated with skin lesions only among males with a 40% reduction in the odds of skin lesions for every 1 g/dL increase in Hgb (OR= 0.60; 95% CI, 0.49-0.73). However, no direct associations (n=184) were observed between toenail As or UAs species and Hgb levels (Breton et al., 2006).

Although a link between smoking and anemia has yet to be established, multiple diseases caused by smoking can result in anemia (Leifert 2008). Several studies have found that smoking results in red blood cell hemolysis, which may be a contributing factor to anemia development (Minamisawa et al., 1990; Masilamani et al., 2016). Conversely, smoking has also been reported to complicate the identification of anemia by increasing Hgb and hematocrit packed cell volume (HCT/PCV) (Sagone and Balcerzak 1975; Nordenberg et al., 1990; Anandha, et al., 2014). These studies highlight the equivocal nature of the relationship between smoking and anemia and emphasize the need for further investigations.

There is strong evidence supporting an association between drinking water As exposure and anemia among women; however, very few studies have evaluated these relationships in men. To our knowledge, no studies have been conducted to determine the effects of smoking on anemia in As exposed individuals. Taking this into consideration, the present study assessed the relationships between chronic drinking water As exposure and hematological indicators of anemia among healthy male non-smokers and smokers in the HEALS cohort who live in rural Bangladesh.

METHODS

Study population

The study was implemented in the HEALS cohort, an ongoing population-based prospective cohort in Araihazar, Bangladesh, a rural sub-district, near the capital Dhaka (Parvez et al., 2006). The HEALS cohort was established to investigate the health effects of inorganic As (InAs) exposure from drinking water (Ahsan et al., 2006; Parvez et al., 2006). Briefly, between October 2000 and May of 2002 a total of 11,746 married (to reduce loss to follow-up) men and women were recruited between the ages of 18 to 75 years from a well-defined 25-km^2 geographical area where they had been residing for at least 3 years. The cohort was expanded to include an additional 8,287 participants using the same methodology between 2006 and 2008. Most recently an additional 15,000 participants were recruited to the HEALS cohort totaling slightly over 35,000 adults. Participants in the HEALS cohort underwent demographic and lifestyle data collection using standardized questionnaires. Information on As exposure is assembled by collecting water and urine samples at multiple time points. A detailed description of the study cohort has been previously published and described (Ahsan et al., 2006).

Recruitment procedure

We adopted a similar protocol for recruiting study participants based on our past procedures. The study protocol was approved by the Institutional Review Board of Columbia University and the Bangladesh Medical Research Council. Informed consent was obtained from all participants. The study team visited the potential study participants at their home, explained the study procedure, and ascertained their eligibility criteria

using a structured questionnaire. A potential list of participants for this study was formed based on As exposure, age, and smoking status from the HEALS central database. Upon further examination of the potential participants list a number of participants were deceased, migrated out of the study area, or had been suffering from a serious illness. Some eligible participants could not participate because they had taken employment out of the study area and visit home infrequently only during weekends. A large number of participants were not eligible to participate because they stopped drinking water from wells with As concentration (>50 µg/L). Persons identified with illnesses related to immune function and/or taking any medication that might have an impact on immune function, including medications for cardiovascular disease or diabetes were not enrolled in the study. If the participant was eligible, willing, and consented, an appointment was made for them at the field clinic. Our field team was able to identify 317 eligible participants to recruit for this study, out of which 267 participants visited our study clinic and completed the study procedure. We obtained and analyzed hematology and WAs data from 103 individuals.

Questionnaire data, clinical examination and anthropometric measurement

Following consent to participate in the study, baseline in-person interviews were performed by trained personnel with detailed questionnaires on lifestyle characteristics. Participants were asked to provide information on demographics, medical co-morbidities, and cigarette and betel-nut usage. Questions were also answered on socioeconomic status defined by television ownership, land ownership, years of education, and occupation. Anthropometric measurements including height, weight, and blood pressure were performed using standard techniques.

Sample collection, processing, and shipment

Information on drinking water was collected at the time of recruitment. If the study participants used the same water source since 2000 and it had been tested by Columbia University in the past, then the individuals were consented into the study. Following consent, trained physicians collected a 20 mL venous blood sample and 40 mL spot urine sample from each participant.

Blood samples were collected by venipuncture using a vein in the antecubital fossa into dipotassium EDTA or sodium heparin anticoagulant tubes. Blood samples in heparinized tubes were processed immediately to isolate peripheral blood mononuclear cells (PBMC) and blood collected in EDTA tubes was used for hematology. All hematological parameters were measured immediately at the Araihazar Laboratory, Columbia University As and Health Research in Bangladesh. All the biological samples were labeled with machine-readable barcodes to maintain privacy of study participants.

Automated complete blood count (CBC)

A CBC was performed using a MYTHIC 22, a fully automated (Microprocessor controlled) hematology analyzer used for the *in vitro* diagnostic testing of whole blood specimens. MYTHIC 22 is an optical measurement system for analyzing up to 22 hematological parameters.

Water sample collection and measurement of As

Procedures for field sample collection and laboratory analyses are described elsewhere in detail (Cheng et al., 2004b; Van Geen et al., 2005; Van Geen et al., 2007). Water samples were analyzed by high-resolution inductively coupled plasma mass spectrometry (HR ICP-MS) as previously described (Van Geen et al., 2007). The

analytical detection limit of the method is 0.1 µg/L; the standard deviation of a single measurement is conservatively estimated at 4 µg/L (Van Geen et al. 2005).

Urine collection, and UAs and creatinine assays

Spot urine samples were collected in 50 mL acid-washed tubes and stored at -80ºC until shipped to Columbia University on dry ice for analysis. UAs assays were performed with a graphite furnace atomic-absorption (GFAA) instrument using a Perkin-Elmer Analyst 600 graphite furnace system as described (Nixon et al., 1991). The detection limit for urinary As was 2 µg/L. Urinary creatinine was analyzed by a colorimetric method based on the Jaffe reaction.

Urinary As metabolites assay

UAs metabolites were assayed by inductively coupled plasma-mass spectrometry (ICP-MS-DRC) coupled to high performance liquid chromatography (HPLC). ICP-MS-DRC was used as a detector for As metabolites chromatographically separated on an Anion Exchange Column (Hamilton PRP-X100) with 10 mM ammonium nitrate/ammonium phosphate, pH 9.1, as the mobile phase (Van Geen et al., 2002). The excellent separation power of HPLC coupled with the very low detection limits of ICP-MS-DRC allowed us to detect InAs (i.e., As^{+3} and As^{+5}), total monomethylarsonic acid (MMA), and total dimethylarsinic acid (DMA).

PBMC isolation from peripheral blood

Blood from heparinized tubes was transferred into two 15 mL centrifuge tubes and was mixed with approximately 14 ml sterile phosphate buffered saline (PBS); 7 mL of the mixture was layered on top of 4 mL Ficoll-Paque Plus (at room temperature; RT), a ratio of approximately 2:1 (v/v) and samples were centrifuged at 2700 rpm at RT for 25

min. Following centrifugation PBMC were collected from the interface of the ficoll. Cells were diluted with sterile PBS and centrifuged this was repeated two more times. PBMC were cryopreserved and held in liquid nitrogen until shipped on dry ice to the U.S. for analysis of PAH-DNA adducts.

Polycyclic aromatic hydrocarbon-DNA (PAH-DNA) adducts

PAH diol epoxide-DNA adducts were analyzed by competitive ELISA, using methods described previously (Divi et al., 2002). Briefly, 96 microwell plates coated with 2 ng of benzo(a)pyrene diol epoxide (BPDE)-I-DNA (5 adducts/10^3 nucleotides) and rabbit antiserum #29 (Poirier 1980) was used with BPDE-DNA as a standard. DNA was isolated from frozen PBMC samples according to standard procedure using phenol/chloroform/isoamyl alcohol. DNA was assayed for PAH-DNA adducts after sonication and denaturation by laboratory technicians blinded to exposure status. For analytical purposes, those samples with <15% inhibition are considered non-detectable and assigned a value of 1 adduct /10^8 nucleotides, an amount midway between the lowest positive value and zero. A 5% blinded duplication was carried out using those subjects with the most DNA available. As an additional quality control, a DNA sample from an animal treated with BP was also assayed was run with the sample batch.

Statistical analysis

Summary statistics were calculated to describe the distribution of all variables for outliners. Similarly, we used frequency tables, crosstabs, box plots, and scatter plots to better understand the distribution of the data. T-tests and ANOVA were used to detect differences in categorical and continuous variables. Water and UAs as well as PAH-DNA adduct values were log- transformed to normalize their distributions to meet assumptions

of ANOVA and reduce the impact of extreme values in linear regression analysis. Spearman correlation coefficients were used to evaluate bivariate associations among hematological parameters and the exposure variables. We estimated associations between As exposure (water, urine and UAs metabolites) and different hematological parameters in separate linear regression models, with and without adjusting for socio-demographic characteristics, and other potential confounders (age, body mass index, smoking, length of education and PBMC PAH-DNA adduct). We repeated the same analysis among smokers and non-smokers in separate linear regression models adjusting for the same variables mentioned previously. We also examined interactions between smoking and PAH-DNA adducts for each measure of hematological parameters by including multiplicative interaction terms in the models. All statistical analyses were conducted using Statistical Analysis Software (SAS 9.4) (SAS Institute Inc.).

RESULTS

Characteristics of the study population

The characteristics of the study population are described in Table 2.1. A majority of the participants were more than 50 years old (60%) and had a BMI less than 25 (75%). The majority of participants had at least one or more years of schooling (70%). About half of the study participants were active smokers (46%) and more than 60% were exposed to WAs >50 µg/L, a drinking water standard for As in Bangladesh.

Table 2.1. Sociodemographic, exposure and hematology parameters of the study participants.[a]

Sociodemographic	**Mean (range)**
Age	52 (36-65)
Body Mass Index (BMI)	22.26 (14.50-30.00)
Education (years)	4.50 (0-12)
Exposure history	
Water As (µg/L)	103.50 (0.10-363.00)
Urinary As (µg/g)	1730.39 (100-11160)
Years of well use	15.12 (3-40)
Smoking in years	22.00 (5-30)
Cigarettes/per day	18.65 (10-30)
Packs of cigarettes/per year	188.62 (0-900)
PAH-DNA adduct (ng/mL)	1.99 (0.90-8.00)
Hematology parameters	
RBC ($x10^6$/µL)	4.98 (3.72-.8.98)
HCT/PCV (%)	42.32 (31.80-70.50)
MCV (fL)	85.47 (56.50-116.60)
Hgb (g/dL)	14.03 (10.20-18.20)
WBC (cell/cumm)	9,015.79 (4,200-14,700)
Lymphocytes (%)	34.59 (17.00-57.00)
Neutrophils (%)	56.18 (31.00-77.00)
Monocytes (%)	3.78 (1.00-10.00)
Eosinophils (%)	5.43 (1.00-13.00)

[a]Red blood cells counts (RBC), hematocrit packed cell volume (HCT/PCV), mean corpuscular volume (MCV), hemoglobin (Hgb), and total white blood cell counts (WBC).

Data provided as courtesy of Dr. Faruque Parvez.

Relationships among measures of exposure

The different measures of As exposure (WAs, UAs, and InAs, MMA and DMA) were moderately correlated with each other (r-values between 0.36 and 0.52 and $p<0.0001$, data not shown). However, the correlations among As exposures measures were relatively stronger among never smokers (r-values between 0.51 and 0.62 and $p<0.0001$, data not shown) as compared to smokers. A stronger significant correlation was observed between WAs and UAs among non-smokers (r= 0.61, $p< 0.0001$) as compared to smokers (r= 0.40, $p= 0.007$). Similar association were observed in relation to urinary As metabolites. On the other hand, PAH-DNA adducts were correlated only with WAs (r= 0.40, $p<0.001$) and did not differ appreciably due to smoking status (data not shown).

Relationships of As exposure and hematological parameters

In unadjusted models, significant inverse correlations were found between WAs and RBC and HCT/PCV (Figure 2.1A-B). Furthermore, a dose dependent decrease in RBC and HCT/PCV was also observed with increasing WAs levels among all participants (Figure 2.2A-B). Inverse relationships were also found between WAs and UAs and MCV (ß= 0.38, 95% CI: -0.05, 1.47; $p= 0.06$) and Hgb (ß= 0.03, 95% CI: -0.10, 0.16; $p= 0.66$, data not shown). Associations between UAs and RBC and HCT/PCV as well as other parameters were similar to those observed in relation to WAs (data not shown). However, a significant inverse correlation was observed between UAs and Hgb (ß= -0.38, 95% CI: -0.65, -0.12; $p= 0.005$, data not shown).

Table 2.2 shows associations of As exposure with hematological parameters in adjusted models. With adjustment for the covariates including age, BMI, educational

attainment, smoking, and PAH-DNA adducts, significant inverse associations were observed between WAs and RBC (ß= -0.13, 95% CI: -0.19, -0.07; p<0.0001) and HCT/PCV (ß= -0.68, 95% CI: -1.17, -0.19; p= 0.008). The relationship between RBC and HCT/PCV with UAs was found to be slightly stronger (Table 2.2). Similarly, a strong positive correlation was observed for total white blood cell counts (WBC) (ß= 516.11, 95% CI: 70.16, 962.06; p= 0.002) in relation to UAs.

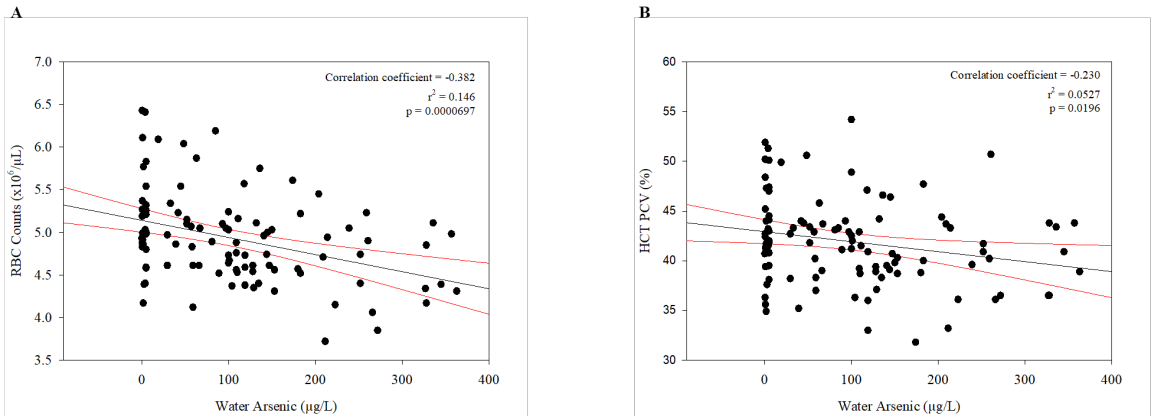

Figure 2.1. Correlations between red blood cell counts (RBC) and hematocrit packed cell volume (HCT/PCV) with water arsenic concentration in male participants (n=103). A. Relationship between RBC and water arsenic. B. Association between HCT/PCV and water arsenic. The red lines indicate 95% confidence intervals. Significance level is $p \leq 0.05$. Data provided as courtesy of Dr. Faruque Parvez.

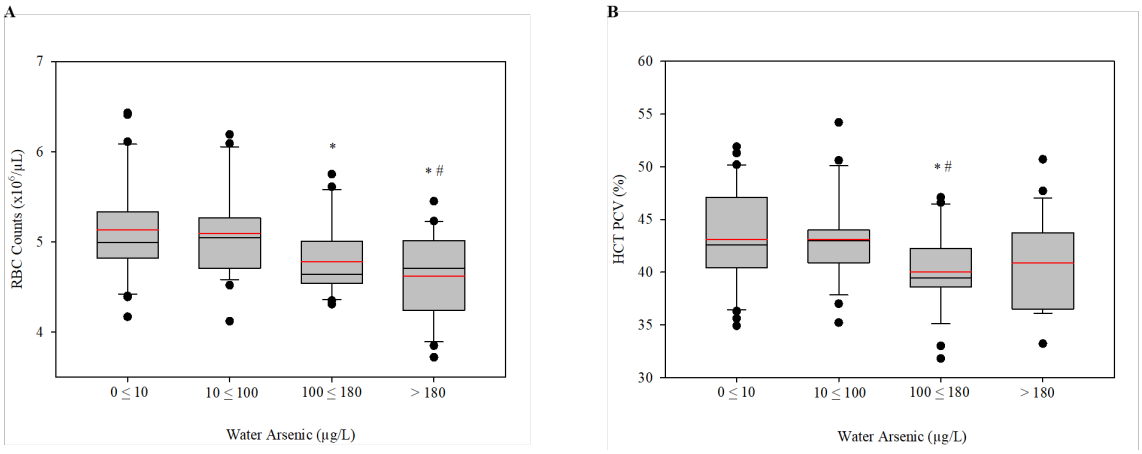

Figure 2.2. Changes in red blood cell counts (RBC) and hematocrit packed cell volume (HCT/PCV) as a function of increasing water arsenic levels in male participants. A. RBC and water arsenic. B. HCT/PCV and water arsenic. The red line indicates the mean and black line indicates the median (n=103). *Statistically significant difference compared to 0 ≤ 10 µg/L (p≤0.05). # Statistically significant difference compared to 10 ≤ 100 µg/L (p≤0.05). Data provided as courtesy of Dr. Faruque Parvez.

Table 2.2. Hematology parameters in arsenic-exposed men.[a,b]

Hematology Parameters	Water Arsenic (µg/L) β (95% CI), p-value	Urinary As (µg/gm) β (95% CI), p-value
RBC ($x10^6/\mu L$)	-0.13 (-0.19, -0.07), <0.0001*	-0.23 (-0.37, -0.09), 0.001*
HCT/PCV (%)	-0.68 (-1.17, -0.19), 0.008*	-1.08 (-2.2, 0.03), 0.06
MCV (fL)	0.67 (-0.16, 1.51), 0.11	1.43 (-0.41, 3.28), 0.13
Hgb (g/dL)	-0.06 (-0.18, 0.07), 0.33	-0.06 (-0.18, 0.07), 0.39
WBC (cell/cumm)	60.95 (-146.00, 267.9), 0.56	516.11 (70.16, 962.06), 0.02*

[a]Red blood cells counts (RBC), hematocrit packed cell volume (HCT/PCV), mean corpuscular volume (MCV), hemoglobin (Hgb), and total white blood cell counts (WBC).

[b]Parameter estimates are adjusted for age, BMI, smoking status, PAH-DNA adduct, and level of education *Significance level is $p \leq 0.05$.

Data provided as courtesy of Dr. Faruque Parvez.

Relationships of urinary As metabolites and hematological parameters

We further examined the role of urinary As metabolites on hematological parameters in adjusted models (Table 2.3). Associations between urinary As metabolites and hematological parameters were consistent with those observed for water and urinary As. As stated, we observed a significant inverse relationship between RBC and InAs (ß= -0.16, 95% CI: -0.26, -0.23; *p*= 0.004), MMA (ß=-0.12, 95% CI: -0.21, -0.02; *p*= 0.01), and DMA (ß= -0.11, 95% CI: -0.22, 0.11; *p*= 0.05). A significant inverse association of InAs (ß= -0.12 95% CI: -0.21, -0.02; *p*= 0.01) with HCT/PCV was also found. We observed a positive association of DMA with HCT/PCV (ß= 2.71, 95% CI: 0.62, 4.81; *p*=0.01). There was a marginally significant inverse relationship between InAs and Hgb (ß= -0.18, 95% CI: -0.39, 0.03; *p*=0.09) and this relationship was stronger among smokers (ß= -0.27, 95% CI:-0.50, -0.04; *p*=0.02, data not shown). Significant negative correlations were also found between percent lymphocytes and InAs (ß= -1.87, 95% CI:-3.22.50, -0.53; *p*= 0.007), MMA (ß= -1.37, 95% CI:-2.57.50, -0.16; *p*= 0.02), and DMA (ß= -1.45, 95% CI:-2.92, 0.02; *p*=0.05). Similarly, InAs was negatively correlated with percent neutrophils (ß= 1.60, 95% CI: 0.09, 3.11; *p*=0.04).

Table 2.3. Hematology parameters and urinary arsenic metabolites of the study participants.[a,b]

Hematology Parameters	In As (As^{+3} As^{+5}) (µg/L)	MMA (µg/L)	DMA (µg/L)
	β (95% CI), p-value	**β (95% CI), p-value**	**β (95% CI), p-value**
RBC ($x10^6$/µL)	-0.16 (-0.26, -0.05), 0.004*	-0.12 (-0.21, -0.02), 0.01*	-0.11 (-0.23, 0.11), 0.05*
HCT/PCV (%)	-0.77 (-1.6, 0.06), 0.07	-0.54 (-1.27, 0.19), 0.15	2.71 (0.62, 4.81), 0.01*
MCV (fL)	0.8 (-0.57, 2.18), 0.25	0.58 (-0.63, 1.79), 0.34	0.51 (-0.95, 1.97), 0.49
Hgb (g/dL)	-0.18 (-0.39, 0.03), 0.09	-0.06 (-0.25, 0.12), 0.48	-0.05 (-0.27, 0.17), 0.65
WBC (cells/cumm)	224.14 (-113.31, 561.58), 0.19	154.22 (-141.58, 450.02), 0.30	181.07 (-177.05, 539.18), 0.32
Lymphocytes (%)	-1.87 (-3.22, -0.53), 0.007*	-1.37 (-2.57, -0.16), 0.02*	-1.45 (-2.92, 0.02), 0.05*
Neutrophils (%)	1.6 (0.09, 3.11), 0.04*	1.13 (-0.2, 2.46), 0.09	1.29 (-0.32, 2.91), 0.11
Monocytes (%)	-0.18 (-0.46, 0.09), 0.19	-0.12 (-0.37, 0.12), 0.32	-0.22 (-0.52, 0.07), 0.13
Eosinophils (%)	0.43 (-0.02, 0.88), 0.06	0.36 (-0.04, 0.75), 0.08	0.38 (-0.1, 0.86), 0.12

[a]Red blood cells counts (RBC), hematocrit packed cell volume (HCT/PCV), mean corpuscular volume (MCV), hemoglobin (Hgb), and total white blood cell counts (WBC).

[b]Parameter estimates are adjusted for age, BMI, smoking status, PAH-DNA adduct, and level of education.*Significance level is $p \leq 0.05$.

Data provided as courtesy of Dr. Faruque Parvez.

Relationships of As exposure and hematological parameters by smoking status

Table 2.4 shows relationships between As exposure and hematological parameters among smokers and non-smokers. Among non-smokers, the negative correlation with RBC remained significant in relation to WAs. Interestingly, the magnitude of the associations of As exposure, particularly with UAs on RBC (ß= -0.4, 95% CI: -0.66, -0.14; *p*= 0.005) as well as HCT/PCV (ß= -1.98, 95% CI: -4.19, 0.23; *p*= 0.08) became stronger among smokers. Additionally, there was also a strong effect of UAs on Hgb among smokers in relation to urinary As (β= -0.54, 95% CI: -1.09, 0.00; *p*= 0.05). In contrast, a significant association was observed for WBC with WAs as well as UAs, which was non-significant among smokers. We have found a marginally significant interaction with water As and smoking (*p*=0.09) on RBC.

Table 2.4. Hematology parameters in arsenic-exposed men by smoking status.[a,b]

	Never Smokers		Smokers	
Hematology Parameters	**Water Arsenic (µg/L) β (95% CI), p-value**	**Urinary Arsenic (µg/gm) β (95% CI), p-value**	**Water Arsenic (µg/L) β (95% CI), p-value**	**Urinary Arsenic (µg/gm) β (95% CI), p-value**
RBC ($x10^6/\mu L$)	-0.09 (-0.15, -0.03), 0.003*	-0.1 (-0.24, 0.04), 0.17	-0.17 (-0.3, -0.04), 0.01*	-0.4 (-0.66, -0.14), 0.005*
HCT/PCV (%)	-0.38 (-0.9, 0.14), 0.15	-0.2 (-1.39, 0.99), 0.74	-0.87 (-1.96, 0.21), 0.12	-1.98 (-4.19, 0.23), 0.08
MCV (fL)	0.64 (-0.46, 1.73), 0.26	1.16 (-1.29, 3.61), 0.35	0.87 (-0.65, 2.4), 0.27	2.52 (-0.57, 5.61), 0.11
Hgb (g/dL)	-0.02 (-0.17, 0.13), 0.8	-0.02 (-0.34, 0.31), 0.92	-0.07 (-0.35, 0.21), 0.63	-0.54 (-1.09, 0.00), 0.05*
WBC (cells/cumm)	239.36 (-2.07, 480.79), 0.05*	776.17 (261.38, 1290.96), 0.004*	-279.95 (-677.24, 117.33), 0.17	337.84 (-495.65, 1171.32), 0.43

[a]Red blood cells counts (RBC), hematocrit packed cell volume (HCT/PCV), mean corpuscular volume (MCV), hemoglobin (Hgb), and total white blood cell counts (WBC).

[b]Parameter estimates are adjusted for age, BMI, smoking status, PAH-DNA adduct, and level of education. *Significance level is $p<0.05$.

Data provided as courtesy of Dr. Faruque Parvez.

DISCUSSION

The goal of this study was to evaluate the effects of chronic drinking water As exposure on clinical indicators of anemia among healthy male smokers and non-smokers in the HEALS cohort living in rural Bangladesh. To our knowledge, the present study is the first to investigate associations between As exposure (as indicated by WAs, UAs, and UAs metabolites) and clinical indicators of anemia among smokers. A dose-dependent reduction in RBC and HCT/PCV was found with increasing WAs levels. In models adjusted for age, BMI, educational attainment, smoking, and PAH-DNA adducts, inverse relationships were also found between measures of As exposure and RBC and HCT/PCV. The correlations between As exposure with RBC and HCT/PCV became stronger among smokers, suggesting potential interactive effects between As and smoking on clinical indicators of anemia. Additionally, associations between As exposure and WBC and white blood cell subsets were also identified.

A high prevalence of anemia exists among the general population in rural Bangladesh (Ahmed 2000; Jamil et al., 2008). Anemia in this region is often associated with nutritional deficiencies of iron, folate, and vitamins B_6 and B_{12} (Ahmed 2000), but a number of studies have also reported As exposure to be a contributing risk factor (Hopenhayn et al., 2006; Heck et al., 2008; Surdu et al., 2015; Kile et al., 2016). Nearly all of these referenced studies only included women, so there is a lack of information concerning the relationships between As exposure and anemia among men. A previous study by Heck et al. (2008), found that men exposed to elevated As were susceptible to anemia, whereas only women with predispositions (i.e., Hgb <10 g/dL) showed a

relationship between As exposure and anemia. These findings emphasize the need for gaining a deeper understanding of the influences of As exposure and anemia among men.

As exposure has been reported to contribute to anemia in humans and rodents by depressing the function of the bone marrow (Szymańska-Chabowska et al., 2002). A number of studies have indicated As-induced toxicity to red blood cells (Winski et al., 1997; Winski and Carter 1998; Zhang et al., 2000; Biswas et al., 2008; Mahmud et al., 2009). As has been shown to cause oxidative stress, deplete intracellular ATP, and to alter the morphological characteristics of red blood cells resulting in compromised membrane integrity, eryptosis, and intra- and-extravascular hemolysis (Winski and Carter 1998; Biswas et al., 2008; Mahmud et al., 2009). There is also a possibility that the inverse relationship between RBC and As exposure is related to reduced erythropoietic capacity of the bone marrow. In fact, we recently reported that drinking water exposure of adult male mice to low levels of As^{+3} (500 µg/L) disrupts erythropoiesis in the bone marrow by inhibiting the colony-forming ability of early erythroid progenitors and suppressing the differentiation of later stage erythroblasts (Medina et al., 2017).

The main form of As found in drinking water in Bangladesh is As^{+3} (Zheng et al., 2004), which is the most toxic inorganic arsenical (Styblo et al., 2000). In the body, As^{+3} is metabolized through a series of oxidative methylation and reduction reactions catalyzed by the As (+3 oxidation state) methyltransferase enzyme to monomethylarsonic acid (MMA^{+5}), monomethylarsonous acid (MMA^{+3}), dimethylarsinic acid (DMA^{+5}), and dimethylarsinous acid (DMA^{+3}) (Thomas et al., 2001; Vahter 2002). MMA^{+3} and DMA^{+3} are the most toxic forms of As and are considered more cytotoxic, genotoxic, and to exert greater inhibitory effects on the activity of certain enzymes than As^{+3} (Thomas et al.,

2001). MMA^{+5} and DMA^{+5} are the primary As metabolites excreted in urine and are less acutely toxic than As^{+3} (Vahter and Concha 2001; Drobna et al., 2009).

The relative amounts of excreted MMA to DMA are used as indicators to determine the efficiency of InAs metabolism and have been associated with severity of toxicological outcomes in a number of studies (Mäki-Paakkanen et al., 1998; Gamble et al., 2005; Steinmaus, et al., 2006; Kile et al., 2011; Li et al., 2013). Particularly, high levels of DMA in urine are indicative of more efficient As metabolism and are often associated with decreased toxicity (Vahter and Concha 2001; Vahter 2002). We found a positive relationship between DMA and HCT/PCV, which likely reflects a greater As metabolic capacity among some participants. Multiple As forms exist simultaneously in the body (Vahter 2002), so it is possible that the discrepancy between the direction of relationship with DMA and RBC was influenced by toxic effects caused by other As metabolites.

Cigarette smoke has been shown to cause red blood cell hemolysis in animal models and humans (Minamisawa et al., 1990; Masilamani et al., 2016). In a group of 62 men and women, Masilamani et al., (2016) recently found that smoking causes morphological abnormalities on the surface of red blood cells that result in hemolysis. The authors also indicate that such cell surface damage facilitates the entry of toxins into red blood cells (Masilamani et al., 2016). The stronger relationships identified between RBC and HCT/PCV in smokers may therefore be attributed to potential interactive effects on red blood cell hemolysis induced by combined exposure to As and cigarette smoke. However, this hypothesis requires further investigation. The inverse relationship between UAs and Hgb is consistent with previous findings that As binds to heme and

reduces heme metabolism resulting in decreased Hgb levels in red blood cells (Delnomdedieu et al., 1994; Hernández-Zavala et al., 1999; Lu et al., 2004). It is interesting to note that this association was detected only among smokers. Previous studies have indicated elevated RBC and HCT/PCV among smokers (Sagone and Balcerzak 1975; Nordenberg et al., 1990; Anandha, et al., 2014), but these studies did not consider the effects of combined exposures with other pollutants. This suggests that smoking may have differential effects on red blood cells when combined with other toxicants, such as As.

Interestingly, As exposure and smoking were correlated with hematological indicators of immune status (i.e., WBC and white blood cell subsets). The loss of positive associations between As exposure and WBC among smokers as well as the inverse relationships between all UAs metabolites and lymphocyte percentages suggest potential immune alterations among study participants and will be the topic of our future investigations.

In summary, results from this study provide evidence that chronic drinking water exposure to As results in hematological alterations that are consistent with anemia in a male cohort of smokers and non-smokers in rural Bangladesh. In addition, we also found that smoking may exacerbate As-induced effects on clinical indicators of anemia. Collectively, these findings show that men are sensitive to As-induced anemia and emphasize the necessity for further research concerning the mechanistic basis for the relationship between chronic As exposure and anemia.

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CHAPTER 3

Low Level Arsenite Exposures Suppress the Development of Bone Marrow Erythroid Progenitors and Result in Anemia in Adult Male Mice

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KEY POINTS

- Exposure to 500 ppb As^{+3} reduced MCH levels in adult male mice.
- BFU-E colony formation was attenuated by exposure to 500 ppb As^{+3}.
- Male mice exposed to 500 ppb As^{+3} had elevated serum levels of EPO.
- Erythropoiesis was impaired in 500 ppb As^{+3} exposed male mice.

ABSTRACT

Epidemiological studies report an association between chronic arsenic (As) exposure and anemia in men, and women who are predisposed to anemia. The purpose of these studies was to determine whether a 60-d drinking water exposure of adult male C57BL/6J mice to 0, 100, and 500 ppb arsenite (As^{+3}) results in anemia due to alterations in erythroid progenitor cell development in the bone marrow. Exposure to 500 ppb As^{+3} for 60 d resulted in a reduction of mean corpuscular hemoglobin (MCH) levels but did not significantly alter reticulocyte (Retic.) counts, red blood cell (RBC) counts, hemoglobin (Hgb) levels, mean corpuscular Hgb concentrations (MCHC), or mean corpuscular volumes (MCV). Attenuation of burst-forming unit-erythroid (BFU-E) colony formation was observed in bone marrow cells of mice exposed to 500 ppb As^{+3}. The differentiation of late stage bone marrow erythroblasts was reduced with the 500 ppb As^{+3} exposure. Mice exposed to 500 ppb As^{+3} also had elevated serum levels of erythropoietin (EPO). Collectively, these results show that exposure to environmentally relevant levels of As^{+3} attenuates the development of early BFU-E cells and reduces the differentiation of later stage erythroblasts. This suppression of bone marrow erythropoiesis may be a contributing factor to the mild hypochromic anemia observed in 500 ppb As^{+3} exposed mice.

INTRODUCTION

As is a widespread environmental toxicant and common contaminant in food and drinking water (WHO 2011; Naujokas et al., 2013). Many people are chronically exposed to levels of As in their drinking water that exceed the World Health Organization and United States Environmental Protection Agency maximum contaminant level of 10 ppb (U.S. EPA 2012; WHO 2011). As occurs in the environment in organic and inorganic forms with multiple valence states (i.e., +3 or +5) that have differential toxicological profiles (Petrick et al., 2001; Styblo et al., 2000; Szymańska-Chabowska et al., 2002). As^{+3} is commonly found in drinking water and is the most toxic inorganic form of As (Styblo et al., 2000; Naujokas et al., 2013). Exposure to elevated levels of As^{+3} has been documented to exert a multitude of detrimental health outcomes, including cancers, cardiovascular diseases, immunosuppression, and anemia (Hughes 2002; Heck et al., 2008; Naujokas et al., 2013; Ferrario et al., 2016).

Anemia is classified as a decrease in the number of RBCs and/or reduced Hgb levels in circulating RBCs (WHO 2015). Multiple epidemiological studies report an association between chronic As exposure and anemia (Heck et al., 2008; Surdu et al., 2015; Kile et al., 2016). Heck et al. (2008), found that low Hgb levels (<10 g/dL) were negatively associated with urinary As concentrations (>200 ppb) in men and women living in Bangladesh. Pregnant women exposed to elevated levels of As in their drinking water are particularly susceptible to developing anemia (Hopenhayn et al., 2006; Surdu et al., 2015). Findings from these studies emphasize the need to develop a clear

understanding of the relationship between environmentally relevant As^{+3} exposures and anemia.

The bone marrow is very sensitive to As-induced toxicity (Szymańska-Chabowska et al., 2002; Ezeh et al., 2014; Ezeh et al., 2016a; Xu et al., 2016b) and is the major site of erythropoiesis in adult humans and mice (Tsiftsoglou et al., 2009; Dzierzak and Philipsen, 2013). Erythropoiesis is regulated by EPO released from the kidney in response to hypoxic conditions in the body (Hattangadi et al., 2011). Increased EPO levels stimulate the proliferation and differentiation of early erythroid progenitor cells in the bone marrow (Hattangadi et al., 2011). The first stage of erythroblast differentiation is BFU-E, which respond to increased EPO and other growth factors (e.g., SCF, IL-3, IL-6) to proliferate and mature to the highly EPO responsive colony-forming unit-erythroid (CFU-E) stage (Hattangadi et al., 2011). At the CFU-E stage, Hgb production is initiated, and the cells undergo four additional stages of differentiation (i.e., proerythroblast, basophilc, polychromatophilic, and orthochromatophilic), prior to enucleation and release from bone marrow into the circulation (Migliaccio, 2010; Elliott and Sinclair, 2012).

The purpose of this study was to determine whether a 60-d drinking water exposure of adult male C57BL/6J mice to environmentally relevant levels of As^{+3} (0, 100, and 500 ppb) results in anemia. As a potential target for As^{+3}-induced toxicity with relevance to anemia, we evaluated the colony forming ability and differentiation of early erythroid progenitor cells in the bone marrow.

MATERIAL AND METHODS

Chemicals and reagents

Sodium meta-arsenite (CAS 774-46-5, Cat. No. S7400), Dulbecco's phosphate buffered saline w/o Ca^{+2} or Mg^{+2} ($DPBS^{-}$), thiazole orange (Cat. No. 390062), and Isocove's Modified Dulbecco's Medium were purchased from Sigma-Aldrich (St. Louis, MO). Hanks Balanced Salt Solution (HBSS) was purchased from Lonza (Walkersville, MD). Fetal Bovine Serum (FBS) was purchased from Atlanta Biologicals (Flowery Branch, GA). Penicillin/Streptomycin 10,000 (mg/ml)/10,000 (U/ml) and 200 mM L-Glutamine was purchased from Life Technologies (Grand Island, NY). Serum-free methylcellulose-based medium containing EPO for culture of mouse erythroid cells (Cat. No. SF M3436) was purchased from STEMCELL Technologies (Cambridge, MA). FITC rat anti-mouse Ter119 clone Ter119 (Cat. No. 557915) and PE rat anti-mouse CD71 clone C2 (Cat. No. 553267) antibodies were purchased from BD Biosciences (San Jose, CA). Cellometer acridine orange/propidium iodide (AO/PI) staining solution in PBS (Cat. No. CS2-0106-5ML) was purchased from Nexcelom Bioscience (Manchester, UK). Mouse EPO Quantikine® ELISA kit (Cat. No. MEP00B) was purchased from R&D Systems (Minneapolis, MN).

Mouse drinking water exposures

All experiments were performed in accordance with protocols approved by the Institutional Animal Use and Care Committee at the University of New Mexico Health Sciences Center. Male C57BL/6J mice were purchased at 8 weeks of age from Jackson Laboratory (Bar Harbor, ME) and allowed to acclimate in our animal facility for one

week prior to the onset of experiments. Mice were maintained on a 12:12 reverse light:dark cycle and were fed 2020X Teklad global soy protein-free rodent diet (Envigo, Indianapolis, IN) throughout the experiment.

Mice were housed 2-3 individuals per cage and exposed to 0 (control), 100, or 500 ppb As^{+3} in their drinking water for 60 d (n = 5 mice/group). As^{+3} doses were prepared fresh weekly by weighing each water bag and determining the appropriate volume of stock As^{+3} to add into each bag to yield 100 or 500 ppb As^{+3}. Water bags were collected and weighed at the end of each week and the change in weight was used to estimate water consumption by mice in each cage.

Primary bone marrow cell isolation

Bone marrow cells were isolated as previously described (Ezeh et al., 2016b). Both femurs from each mouse were harvested and placed into cold HBSS. Femurs were then transferred into a 60 mm dish with HBSS to trim excess tissue from the bone. Trimmed femurs were placed into a 60 mm dish containing 5 mL cold colony-forming unit (CFU) medium (Isocove's Modified Dulbecco's Medium supplemented with 2% heat inactivated FBS, 2 mM L-glutamine, and 100 mg/ml streptomycin and 100 units/ml penicillin) and the ends of each femur were carefully cut to reveal the interior marrow shaft. Bone marrow cells were flushed from each femur by passing approximately 6-9 mL of CFU medium through the marrow shaft using a 1cc syringe and 25-G needle. The cell suspensions were then transferred to a 15 mL centrifuge tube, centrifuged at 200 x*g* for 10 mins, and resuspended in 5 mL of CFU medium. Cell viabilities and concentrations were determined using AO/PI staining and a Nexcelom Cellometer® Auto 2000 (Nexcelom Bioscience, Manchester, UK).

Blood collection, serum preparation, and hematological analysis

Whole blood and blood for serum preparation was collected at the time of sacrifice by cardiac puncture into EDTA coated 250µL tubes or 1.5 mL microcentrifuge tubes, respectively. Hematological analysis of whole blood was performed using an Abaxis VetScan HM5 hematology analyzer (Abaxis, Union City, CA). For serum preparation, blood was clotted for 2 h at room temperature (RT). Clotted blood was centrifuged at 2000 x*g* for 30 mins and serum was carefully removed and transferred to a clean 1.5 mL microcentrifuge tube for storage at -80˚C.

BFU-E assay

Mouse BFU-E assays were setup following manufacturer's instructions described in version 3.4.0 of STEMCELL Technologies Technical Manual for Mouse Colony-Forming Unit Assays using MethoCult™. Bone marrow cells from both femurs of each mouse were pooled and resuspended to 1×10^6 cells/mL in CFU medium. 400 µL of the 1×10^6 cells/mL solution (4×10^5 cells) was transferred into 4 mL SF M3436 methylcellulose-based medium containing EPO to promote BFU-E development and expansion. Samples were then mixed thoroughly by vortexing and held for approximately 10 mins to allow bubbles to dissipate. 1 mL (1×10^5 cells) of each sample solution was then transferred in triplicate to treated 35 mm culture dishes (STEMCELL Technologies, Cambridge, MA) using a 5cc syringe and a 16-G blunt-end needle. Each plate was then gently rocked back and forth to evenly distribute the media across the surface of the dish. Two culture dishes and one uncovered 35 mm dish containing 3 mL of sterile water were placed into a covered 100 mm dish and incubated at 37˚C in humidified incubator with 5% CO_2 for 14 d. After 14 d of culture, BFU-E colonies containing at least 30 cells were

counted based on morphology using a dissecting microscope. Colony counts are reported as the number of BFU-E colonies per million bone marrow cells.

Flow cytometry

Whole blood (non-coagulated) from each mouse was stained with 0.01 mg/mL thiazole orange at RT for 30 mins (as described by BD Biosciences ReticCount procedure, available: http://www.bdbiosciences.com/ds/is/tds/23-1789.pdf) and reticulocytes were measured by flow cytometry using an Accuri™ C6 flow cytometer. Total reticulocyte counts (ARC) were obtained using the following formula: TRC = (% reticulocytes/100) x RBC counts/L.

Bone marrow erythroblast subsets were evaluated based on CD71 and Ter119 surface marker expression. 1×10^6 bone marrow cells from each mouse were transferred to 12x75 mm tubes and stained in 100 µL of flow stain/wash buffer ($DPBS^-$ with 2% heat inactivated FBS and 0.09% sodium azide) with 0.5 µg of rat anti-mouse CD71-PE and rat anti-mouse Ter119-FITC monoclonal antibodies at RT for 30 mins. Samples were then washed twice with flow stain/wash buffer. After the final wash, samples were resuspended in 0.5 mL flow stain/wash buffer and analyzed using an Accuri™ C6 flow cytometer (BD Biosciences, San Jose, CA).

Mouse EPO ELISA

Serum EPO levels were measured using the Mouse EPO Quantikine® ELISA kit according to manufacturer's instructions. Briefly, serum samples and EPO standards were diluted two-fold and 50 µL of each sample was added in duplicate to the appropriate wells of a microplate pre-coated with an EPO specific monoclonal antibody. The plate was then covered and incubated at RT for 2 h on a microplate shaker. Following

incubation, the plate was washed five times. After the last wash, the plate was emptied and 100 µL of mouse EPO monoclonal antibody conjugated to horse radish peroxidase was added to each well and incubated at RT for 2 h on a microplate shaker. The plate was washed again and 100 µL of substrate solution (hydrogen peroxide and tetramethylbenzidine) was added to each well and incubated at RT for 30 mins. Colorimetric reactions were stopped by adding 100 µL of 0.25 N hydrochloric acid to each well and the absorbance was read immediately at 450 nm and 540 nm using a SpectraMax® 340PC microplate reader (Molecular Devices, Sunnyvale, CA). Readings at 540 nm were subtracted from those at 450 nm to correct for optical imperfections in the plate and the corrected values were used for subsequent analysis. Sample concentrations were determined using a four-parameter logistic standard curve.

Statistics

Data was analyzed using Sigma Plot 12.5 software. Five mice ($n = 5$) were assigned to each exposure group and unless otherwise specified in figure legends were utilized in statistical analysis. Differences between control and As^{+3} exposure groups were determined using a Student's *t*-test at a significance level of $p<0.05$.

RESULTS

Hematological effects of drinking water exposure in adult mice

Adult male C57BL/6J mice were exposed to 0, 100, and 500 ppb As^{+3} via their drinking water for 60 d. There were no differences in body weights in any of the As^{+3} exposure groups. A slight decrease in water consumption in the 100 ppb As^{+3} exposure group was observed, but this effect was not dose-dependent (Table 3.1). To determine whether As^{+3} exposure results in the development of anemia, RBC counts, Hgb levels, MCHCs, MCH levels, and MCVs were measured in whole blood using an Abaxis VetScan hematology analyzer. Mice exposed to 500 ppb As^{+3} had significantly lower MCH levels than control mice (Table 3.2). Although not statistically significant, a trend of decrease in MCHCs and Hgb levels was also noted in mice exposed to 500 ppb As^{+3} (Table 3.2). There were no changes in total retic counts, RBC counts, or MCVs with either of the As^{+3} doses (Table 3.2). These results imply that exposure to 500 ppb As^{+3} for 60 d resulted in a moderate Hgb reduction consistent with hypochromic anemia.

Table 3.1. Body weights and drinking water consumption of male C57BL/6J mice exposed via drinking water to 0, 100, and 500 ppb As^{+3} for 60 d.[a]

Treatment	Body Weight (g)	Water Intake (mL/day)
Control	34.42 ± 2.36	3.66 ± 0.29
100 ppb	32.76 ± 3.41	3.10 ± 0.13*
500 ppb	33.30 ± 1.36	3.77 ± 0.57

[a]Mice were 9-week old at the start of the As^{+3} exposure. Water consumption was monitored weekly based on weight change of the water bags. Data are expressed as mean ± SD (n = 5 mice/group).

*Statistically significant difference compared to control (p<0.05).

Table 3.2. Retic. counts, RBC counts, Hgb levels, MCH levels, MCHCs, and MCVs in whole blood collected from mice exposed via drinking water to 0, 100, and 500 ppb As^{+3} for 60 d.[a]

Parameter	Control	100 ppb As^{+3}	500 ppb As^{+3}
Retic. ($x10^9$ cells/L)	352.09 ± 16.96	359.01 ± 23.63	379.38 ± 35.86
RBC ($x10^{12}$/L)	10.85 ± 0.35	11.11 ± 0.27	10.99 ± 0.23
Hgb (g/dL)	17.16 ± 0.52	17.26 ± 0.18	16.76 ± 0.38
MCH (pg)	15.80 ± 0.16	15.56 ± 0.27	15.24 ± 0.46*
MCHC (g/dL)	35.92 ± 0.36	35.26 ± 0.63	34.94 ± 1.20
MCV (fL)	43.80 ± 0.45	44.20 ± 0.45	43.80 ± 0.45

[a]Hematology analysis was performed using an Abaxis VetScan hematology analyzer. Data are expressed as mean ± SD (n = 5 mice/group).

*Statistically significant difference compared to control (p<0.05).

Bone marrow BFU-E colony formation was attenuated by As^{+3} exposure

To determine whether alterations in erythroid progenitor cell development is a target of As^{+3} toxicity, we evaluated the colony-forming ability of BFU-E cells from the bone marrow of mice exposed to 0, 100, and 500 ppb As^{+3} for 60 d. Bone marrow cells were cultured *ex vivo* in serum free methylcellulose-based medium containing EPO for 14 days and the number of BFU-E colonies was determined based on morphology using a dissecting microscope. A significant reduction in the number of BFU-E colonies was observed in mice exposed to 500 ppb As^{+3} (Figure 3.1A). Another interesting observation was BFU-E colonies from the bone marrow of 500 ppb As^{+3} exposed mice were not as dense as control colonies, which is indicative of suppressed proliferation, delayed differentiation, and/or reduced cell viability (Figure 3.1B). There were no statistically significant changes in BFU-E colony formation with the 100 ppb As^{+3} exposure (Figure 3.1A).

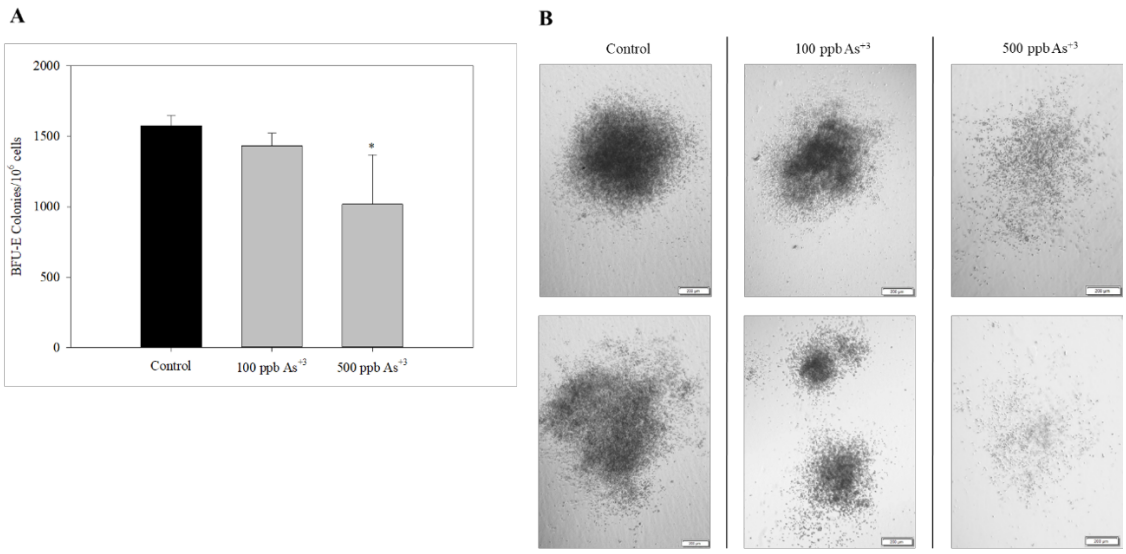

Figure 3.1. BFU-E colony formation in bone marrow of male C57BL/6J mice exposed to 0, 100, and 500 ppb As^{+3} for 60 d. A. Number of BFU-E colonies per million bone marrow cells cultured *ex vivo* in serum free methylcellulose-based medium containing EPO for 14 days. B. Representative BFU-E colony images from 0, 100, and 500 ppb As^{+3} exposed mice. Data are expressed as mean ± SD of triplicate cultures per mouse (n = 4-5 mice/group). *Statistically significant difference compared to control (p<0.05).

Suppressed differentiation of late-stage erythroid progenitors in the bone marrow of mice exposed to As^{+3}

Based on the finding that As^{+3} attenuates BFU-E development, we investigated whether the downstream bone marrow erythroblast population subsets were also altered. Stages of erythroblast differentiation can be defined based on CD71 and Ter119 surface marker expression using flow cytometry (Socolovsky et al., 2001; Lau et al., 2012). Erythroblast subsets in bone marrow were defined as follows: (I) proerythroblast: $CD71^{high}/Ter119^{med}$, (II) basophilic erythroblast: $CD71^{high}/Ter119^{high}$, (III) = late basophilic and polychromatophilic erythroblasts $CD71^{med}/Ter119^{high}$, and (IV) = orthochromatophilic erythroblasts $CD71^{low/-}/Ter119^{high}$ (Figure 3.2A). As^{+3} exposure at 500 ppb caused a significant decrease in the percentage of late stage orthochromatophic erythroblasts (Figure 3.2B). No differences in other erythroblast subsets (I-III) were detected for either of the As^{+3} exposures (Figure 3.2B). These results indicate that As^{+3} also alters the differentiation of late stage erythroid progenitors in the bone marrow.

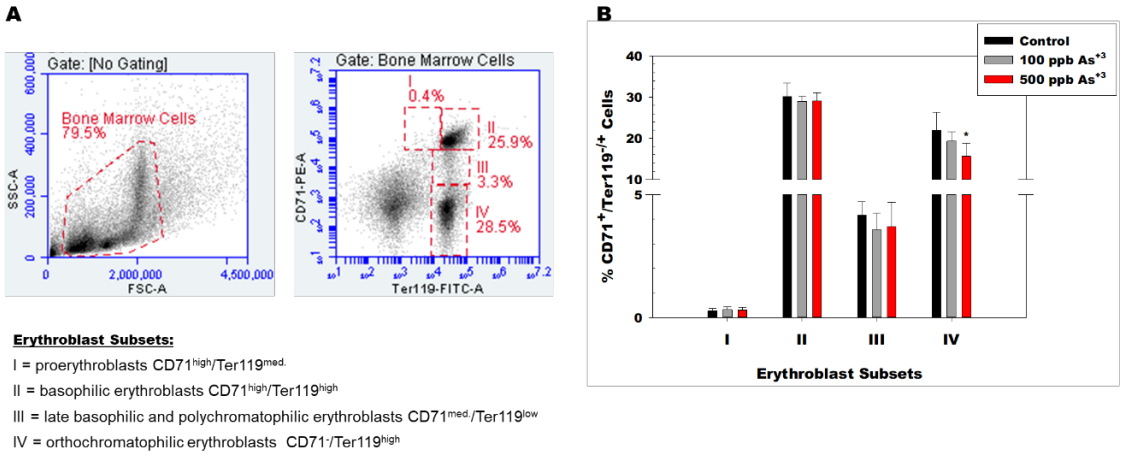

Figure 3.2. Erythroblast subsets in bone marrow of male C57BL/6J mice exposed to 0, 100, and 500 ppb As^{+3} in drinking water for 60 d. Bone marrow cells were stained with CD71 and Ter119 surface markers and analyzed on a flow cytometer. A. Flow cytometry gating strategy used to define erythroblast subsets (I-IV) in mouse bone marrow cells. B. % erythroblast subsets defined based on CD71 and Ter119 surface marker expression. Data are expressed as mean ± SD (n = 5 mice/group). *Statistically significant difference compared to control (p<0.05).

Serum EPO levels were elevated in As^{+3} exposed mice

To evaluate whether disrupted erythropoiesis in the bone marrow (i.e., attenuated BFU-E colony-formation and suppressed differentiation of later stage erythroblasts) was accompanied by an increase in circulating EPO levels in As^{+3} exposed mice, the concentration of EPO in serum was analyzed using a mouse EPO ELISA kit. A significant increase in EPO concentrations was detected in serum from 500 ppb As^{+3} exposed mice (Figure 3.3). There was no difference in serum EPO levels in 100 ppb As^{+3} exposed mice (Figure 3.3). These findings suggest that suppressed erythropoiesis in the bone marrow of 500 ppb As^{+3} exposed mice stimulated EPO release from the kidneys.

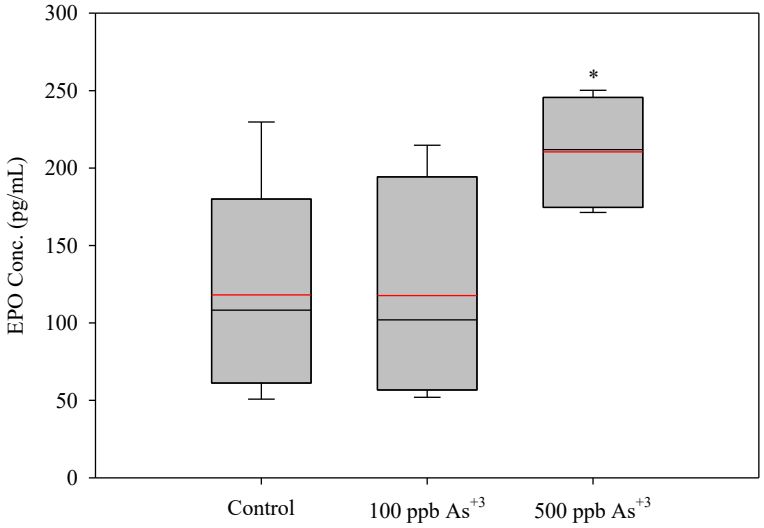

Figure 3.3. EPO concentrations (pg/mL) in serum from male C57BL/6J mice exposed to 0, 100, and 500 ppb As^{+3} via drinking water for 60 d. Serum EPO concentrations were determined using a mouse EPO ELISA. Red line indicates the mean and black line indicates the median (n = 4-5 mice/group). *Statistically significant difference compared to control (p<0.05).

DISCUSSION

In the present study, we evaluated whether a 60-d drinking water exposure of adult male C57BL/6J mice to environmentally relevant levels of As^{+3} (0, 100, and 500 ppb) would result in anemia. Mice exposed to 500 ppb As^{+3} had significantly lower MCH levels and also showed a non-statistically significant trend of decrease in MCHCs and Hgb levels (Table 3.2). RBC counts and MCVs were not altered with either of the As^{+3} exposures (Table 3.2). This is consistent with previous findings that As reduces heme metabolism and can bind to Hgb, which results in decreased Hgb concentrations in RBCs (Delnomdedieu et al., 1994; Hernández-Zavala et al., 1999; Lu et al., 2004). Acute high level As^{+3} exposure in humans and rodents has been reported to result in anemia as a result of bone marrow depression (Szymańska-Chabowska et al., 2002). Morse et al. (1980), showed an inhibition of iron incorporation in circulating RBCs along with suppressed proliferation of early erythrocytes from the bone marrow of mice injected with high levels of As^{+3}. These studies provide evidence that acute high level As exposures can cause anemia in mice.

The bone marrow is the major site of erythropoiesis in humans and mice (Tsiftsoglo et al., 2009; Dzierzak and Philipsen, 2013). Several studies have shown that hematopoietic progenitor cells in the bone marrow are sensitive to low levels of As^{+3} *in vivo* and *in vitro* (Ferrario et al., 2008; Ezeh et al., 2014; Ezeh et al., 2016a). As a potential target for As^{+3}-induced toxicity, we evaluated the development of BFU-E cells in the bone marrow. BFU-E cells are the earliest stage of erythroid cell development and they can be readily assessed based on colony formation in methylcellulose-based

medium. BFU-E colony formation was only attenuated in bone marrow cells from 500 ppb As^{+3} exposed mice (Figure 3.1). BFU-E colonies from 500 ppb As^{+3} exposed mice were also less dense than control colonies, indicating compromised development of the cells likely as result of decreases in cell viability, proliferation, and/or differentiation capacity (Figure 3.1B).

To assess whether As^{+3} altered downstream erythroblast differentiation, we evaluated erythroblast subsets in bone marrow based on CD71 and Ter119 surface marker expression. The stages of erythroblast differentiation were identified based on the degree of CD71 and Ter119 surface marker expression using flow cytometry (Figure 3.2A). Interestingly, only the latest stage of erythroblast differentiation (i.e., stage IV, orthrochromatophilic erythroblasts) was reduced in 500 ppb As^{+3} mice (Figure 3.2B). This provides evidence that As^{+3} not only attenuates the development of early BFU-E cells, but it also reduces the differentiation of later stage erythroblasts. Taken together, these results show that different stages of erythroid cell development may have differential sensitives to As^{+3}.

Our group previously reported that mice exposed to 500 ppb As^{+3} in their drinking water for 30 d had elevated As levels in the bone marrow (Xu et al., 2016b). It has also been shown that As^{+3} and MMA^{+3} can inhibit the development of pre-B cells in the bone marrow by reducing STAT5 activation (Ezeh et al., 2016a). Since the maturation of erythroid progenitor cells is also dependent on STAT5 signaling (Socolovsky et al., 1999; 2001), it is likely that the attenuation of BFU-E colony formation may be, at least in part, attributed to interference with this signaling pathway. As^{+3} has also been shown to inhibit the function of C3 and C4 zinc finger proteins by

replacing zinc on the zinc fingers (Zhou et al., 2011; Zhou et al., 2014). Many of the transcription factors that regulate the expression of genes critical for development of early erythroid progenitors are zinc fingers with motifs that can be displaced by As^{+3} (Hattangadi et al., 2011). It is therefore possible that As^{+3} may be suppressing the development of early erythroid progenitors by inhibiting the function of these essential zinc finger transcription factors.

Mice exposed to 500 ppb As^{+3} also exhibited increased levels of EPO in their serum (Figure 3.3). Increased circulating EPO levels is likely a physiological attempt to cope with the loss of erythropoietic function in the bone marrow. In mice the spleen plays a critical role in maintaining erythropoiesis during conditions of hypoxic stress, such as experienced when the bone marrow is not capable of producing adequate amounts of RBCs to meet physiological demands (Socolovsky 2007; Paulson et al., 2011; Dzierzak and Philipsen, 2013). Compensation from the spleen for the loss of erythropoietic capacity in the bone marrow is likely responsible for the stable RBC counts observed with the 60 d As^{+3} exposure. In support, As^{+3} exposed mice not only exhibited elevated circulating EPO levels, but also showed slight trends of increase in total retic and RBC counts. However, further studies are needed to determine whether As^{+3}-induced disruption of bone marrow erythropoiesis causes activation of stress erythropoiesis in the spleen. Taking findings from epidemiological studies into consideration, it is also possible that a longer exposure duration, such as that experienced by chronically exposed human populations, may result in reduced RBC counts and further diminished Hgb levels.

Results from this study show that 60 d drinking water exposure to environmentally relevant levels of As^{+3} in adult male mice results in a moderate Hgb reduction that is consistent with hypochromic anemia. In addition, it was found that 500 ppb As^{+3} disrupts erythropoiesis in the bone marrow by attenuating the colony-forming ability of early BFU-E cells and reducing the differentiation of later stage erythroblasts. Collectively, these findings provide initial insights into a possible means by which As^{+3} exposures can contribute to the development of anemia in chronically exposed individuals.

FUNDING

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CHAPTER 4

Selective Inhibition of Erythropoiesis by Environmental Arsenic Exposure through GATA-1 Zinc Finger Disruption

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KEY POINTS

- Arsenic, a widespread environmental toxicant, impairs bone marrow erythropoiesis by selective disruption of GATA-1 function.
- Dysregulation of erythropoiesis is a likely mechanism underlying the high prevalence of anemia in people chronically exposed to arsenic.

ABSTRACT

Anemia is a hematological disorder that adversely affects the health of millions of people worldwide. Although many variables influence the development and exacerbation of anemia, one major contributing factor is the impairment of erythropoiesis. Normal erythropoiesis is regulated by the zinc finger (ZF) transcription factor GATA-1. Disruption of the ZF motifs in GATA-1, such as produced by germline mutations, compromises the function of this critical transcription factor and cause dyserythropoietic anemia. Utilizing a combination of in vitro and in vivo studies, we demonstrate that arsenic, a widespread environmental toxicant, selectively inhibits erythropoiesis through the disruption of GATA-1 ZF motifs. We found that arsenic selectively interacts with ZF motifs of GATA-1, causing zinc loss, and inhibition of DNA and protein binding activities, resulting in dyserythropoiesis and an imbalance of hematopoietic differentiation. For the first time, we show that exposures to a prevalent environmental contaminant compromise the function of a key regulator of erythropoiesis, producing effects functionally similar to inherited GATA-1 mutations. These findings provide insights into a novel mechanism by which arsenic exposure can cause anemia and provide critical insights into potential prevention and intervention for arsenic-related anemias.

INTRODUCTION

Anemia is a hematological disorder that affects approximately 25% of the population globally (WHO 2015). A major cause of anemia is decreased red blood cell (RBC) production (Hodges et al., 2007; Gangat and Wolanskyj 2013; Koury 2014), which occurs primarily in the bone marrow (BM). RBCs are produced through erythropoiesis starting from hematopoietic stem cells (HSC) (Fujiwara et al., 2009; Dzierzak and Philipsen 2013). HSCs differentiate to common myeloid progenitors (CMP), which depending on expression levels and subsequent functional antagonism of two key transcriptional regulators, GATA-1 and PU.1, commit to either erythro-megakaryocytic or myeloid lineages, respectively (Zhang et al., 1996; Nerlov et al., 2000; Hsu et al., 2001; Rhodes et al., 2005; Iwasaki and Akashi 2007). Once committed to the erythro-megakaryocytic lineage, megakaryocyte-erythroid progenitors (MEPs) differentiate to megakaryocyte and erythroid progenitors (Dzierzak and Philipsen 2013). The earliest committed erythroid progenitors are detected as slowly proliferating burst-forming unit erythroid cells (BFU-E). BFU-Es differentiate into rapidly dividing colony-forming unit erythroid cells (CFU-E) (Dzierzak and Philipsen 2013). CFU-Es further differentiate through multiple stages and undergo many substantial changes to finally produce mature RBCs (Dzierzak and Philipsen 2013).

Erythropoiesis is a dynamic and precisely regulated process under the control of many regulatory factors (Pevny et al., 1991; Fujiwara et al., 1996; Hattangadi et al., 2011), among which GATA-1 is recognized as the master regulator of erythropoiesis and its activity is necessary for the normal differentiation of erythroid progenitors (Weiss and

Orkin 1995; Fujiwara et al., 1996; Ferreira et al., 2005). GATA-1 is the founding member of the GATA family of transcription factors and is a crucial regulator of genes encoding hemoglobin subunits and heme biosynthetic enzymes (Evans et al., 1988; Pevny et al., 1991; Pevny et al., 1995; Welch et al., 2004). GATA-1-dependent mechanisms constitute an essential regulatory core that achieves physiological control of hemoglobin synthesis (Katsumura et al., 2013). The function of GATA-1 depends on two zinc finger (ZF) domains (Liew et al., 2005). The N- and C-terminal ZFs are responsible for binding to an important co-factor, friend of GATA-1 (FOG-1) and DNA, respectively (Fox et al., 1999; Liew et al., 2005; Bates et al., 2008). Mutation, protein level alteration, or dysfunction of GATA-1 results in the inhibition of erythropoiesis (Pevny et al., 1991; Pevny et al., 1995; Del Vecchio et al., 2005; Ciovacco et al., 2008; Crispino and Horwitz 2017). Zinc finger dysfunction caused by mutations in the ZF motifs of GATA-1 is a characteristic phenotype of inherited dyserythropoietic anemia (Ciovacco et al., 2008; Del Vecchio et al., 2005; Crispino and Horwitz 2017).

Arsenic (As) is a widespread environmental toxicant that poses a significant threat to public health. Millions of people worldwide are chronically exposed to inorganic trivalent As (arsenite, AsIII) in drinking water at levels exceeding the World Health Organization and United States Environmental Protection Agency maximum contaminant level of 10 μg/L (WHO 2011; U.S. EPA 2012). Arsenic levels up to 1700 μg/L have been measured in surface water in the United States (ATSDR 2016) and 12% of water supplies in the north central and western regions have levels >20 μg/L (0.26 μM), with much higher levels occurring in unregulated well water (ATSDR 2007, 2016). Exposure to As is well documented to produce many acute and chronic health effects including, anemia

(Hopenhayn et al., 2006; Heck et al., 2008; US EPA 2012; Vigeh et al., 2015; Surdu et al., 2015; ATSDR 2016; Kile et al., 2016; Parvez et al., 2017). We reported that AsIII exposure correlates with a decrease in RBC counts in a cohort of men from rural Bangladesh exposed to a wide range of AsIII concentrations in their drinking water (Parvez et al., 2017). Our previous work also showed that low-level AsIII exposure causes anemia through inhibition of erythropoiesis in mice (Medina et al., 2017), but the molecular mechanism of As-caused anemia is still unknown.

Our work on AsIII carcinogenesis demonstrated that ZF DNA repair proteins, such as poly (ADP-ribose) polymerase 1 (PARP-1), are highly sensitive molecular targets of AsIII (Ding et al., 2009; Zhou et al., 2011; Sun et al., 2014; Zhou et al., 2015). AsIII interacts with the ZF domain, causing zinc loss, and dysfunction of the PARP-1 protein (Ding et al., 2009; Sun et al., 2014). AsIII selectively interacts with ZFs containing ≥3 cysteine residues (Zhou et al., 2011), making ZF proteins containing at least 3 cysteines, which are a minority in the ZF protein family, extremely sensitive to AsIII exposure. Inspired by our previous work, we hypothesized that As-induced inhibition of erythropoiesis may be attributed to loss of GATA-1 function, resulting from selective interaction of AsIII with the ZFs on GATA-1, which are C4 (4 cysteines) configuration.

Here, we report that GATA-1 is a sensitive molecular target of AsIII exposure at environmentally relevant concentrations. Our findings demonstrate, for the first time, that exposure to a widespread environmental metal disrupts the function of GATA-1, the key regulator of RBC development, resulting in the inhibition of erythropoiesis. The results presented here highlight a novel mechanism by which AsIII exposure causes anemia, and

also illustrate that arsenic influences the lineage commitment of hematopoietic progenitor cells (HPC) by differential interactions with key regulatory proteins.

MATERIALS AND METHODS

Chemicals

Sodium meta-arsenite (>95% purity), hemin, phorbol 12-myristate 13-acetate (PMA), and benzidine stain were purchased from Sigma-Aldrich.

Cell culture and chemical-induced differentiation of K562 cells

Human leukemic cell line K562 was purchased from ATCC. K562 cells were maintained in DMEM with 10% FBS according to instructions from ATCC. Erythroid and megakaryocytic, myelocytic, and monocytic differentiation of K562 cells was induced by 24 h treatment with 5 μM hemin or 5 ng/mL PMA as described previously, respectively (Villeval et al., 1983; Shelly et al., 1998; Palma et al., 2012; Huang et al., 2014). Differentiation status of K562 cells was determined based as follows: erythropoietic (CD71, and benzidine staining) (Hafner et al., 1995), megakaryocytic (CD41), myelocytic (CD11b), and monocytic (CD11b and forward scatter). Surface marker antibodies were purchased from ThermoFisher Scientific. See below for a detailed description of flow cytometry staining procedure.

Mice and i*n vivo* drinking water exposures

Experiments involving mice were performed in accordance with protocols approved by the Institutional Animal Use and Care Committee at the University of New Mexico Health Sciences Center. Male C57BL/6J mice were purchased from Jackson Laboratory and acclimated in our facility for one week prior to *in vivo* and *in vitro* experiments. Mice were maintained on a 12:12 reverse light:dark cycle and fed 2920X Teklad global soy protein-free rodent diet (Envigo, Indianapolis, IN) *ad libitum*. Mice

were exposed via their drinking water to 0 µg/L (control), 20 µg/L, 100 µg/L, and 500 µg/L or to 0 µg/L (control), 500 µg/L, and 1 mg/L As^{+3} for 30 days (n = 5 mice/group) or 2 weeks (n = 6 mice/group), respectively. Stock As^{+3} solutions were prepared at the onset of each experiment at a concentration 1000 times greater (1000X) than the desired dose. The volume of stock 1000X As^{+3} added to drinking water pouches to yield the desired exposure doses was determined by weighing each pouch and subtracting the total weight of the pouch and injection port to estimate the total water volume (1 g water ≈ 1 mL).

Primary mouse bone marrow cell isolation

BM cells were isolated from femur bones as described in detail by Ezeh et al., (2016b). Femurs from each mouse were flushed using a 1 mL syringe and 25-G needle. The cell suspension was transferred to a 15 mL centrifuge tube, centrifuged at 200 x*g* for 10 mins, and resuspended in 20 mL of Isocove's Modified Dulbeccos Medium (IMDM) with 2 or 10% FBS, 2 mM L-glutamine, and 100 mg/ml streptomycin and 100 units/ml penicillin). Cell viabilities and concentrations were then determined using acridine orange/propidium iodide (AO/PI) staining and a Nexcelom Cellometer Auto 2000 (Manchester, UK).

CFU-E assay

Mouse CFU-E assays was performed according to manufacturer's instructions described in detail in version 3.4.0 of STEMCELL Technologies Technical Manual for Mouse Colony-Forming Unit Assays using MethoCult™. BM cells (4×10^5 cells) in IMDM with 2% FBS were transferred into 4 mL MethoCult M3334 methylcellulose-based medium containing EPO (STEMCELL Technologies, Cambridge, MA). Samples were vortexed thoroughly and transferred in triplicate (1×10^5 cells) to treated 35 mm

culture dishes (STEMCELL Technologies, Cambridge, MA). Samples were incubated for 72 h and CFU-E colonies were enumerated using an inverted microscope. CFU-E colony counts are reported as number of colonies/million BM cells.

Hematopoietic progenitor cell isolation and *in vitro* erythropoiesis model

HPC were isolated from BM using the EasySep Mouse HPC Isolation Kit (STEMCELL Technologies, Cambridge, MA) according to manufacturer's instructions. BM cells were concentrated to 1×10^8 cells/mL in Easy Sep Buffer (DPBS without calcium and magnesium ($DPBS^-$) containing 2% FBS and 1 mM EDTA) and stained with a cocktail of biotinylated lineage- specific (CD5, CD11b, CD19, CD45R/B220, Ly6G/C(Gr-1), TER119) for 15 mins at 4°C. Streptavidin-coated magnetic particles were added to each sample and incubated for 10 mins. Samples were brought up to 2.5 mL in EasySep buffer and placed into an EasySep magnet (STEMCELL Technologies) for isolation. Supernatant containing HPC was collected and utilized to develop an *in vitro* model of erythropoiesis as described by Shuga et al., (2007). HPC were cultured in SF StemSpan hematopoietic progenitor expansion media (STEMCELL Technologies) supplemented with 100 ng/mL murine SCF and 5 IU/mL (31.25 ng/mL) human recombinant EPO (Peprotech) to stimulate erythroid lineage commitment and differentiation.

Flow cytometry

Erythroid and myeloid progenitors were evaluated based on surface marker phenotype as defined by Pronk et al., (2011) and Grover et al., (2014): PreMegE (Lin^-, $cKit^+$, $SCA\text{-}1^-$, $CD16/32^-$, $CD150^+$, $CD105^{-,\,low}$); MEP (Lin^-, $cKit^+$, $SCA\text{-}1^-$, $CD16/32^-$, $CD34^-$), BFU-E (Lin^-, $cKit^+$, $SCA\text{-}1^-$, $CD16/32^-$, $CD150^+$, $CD105^+$); CFU-E (Lin^-, $cKit^+$,

SCA-1$^-$, CD16/32$^-$, CD150$^-$, CD105$^+$); pre-Granulocyte Macrophage (pre-GM; Lin$^-$, cKit$^+$, SCA-1$^-$, CD16/32$^-$, CD150$^-$, CD105$^-$); Granulocyte Macrophage Progenitor (GMP; Lin$^-$, cKit$^+$, SCA-1$^-$, CD16/32$^+$, CD150$^-$). Erythroblast differentiation was evaluated based on CD71 and Ter119 surface marker expression and cell size characteristics subsets (early erythroblasts, CD71$^{low/high}$, Ter119$^{-,low}$; EryA, CD71^{high}Ter119^{high}FSC^{high}; EryB, CD71^{high}Ter119^{high}FSC^{low}; EryC, CD71^{low}Ter119^{high}FSC^{high}) previously described by Socolovsky et al., (2001) and Koulnis et al., (2011).

For surface marker analysis, 0.5-1×10^6 cells were stained with 0.5 µg of monoclonal antibodies (BD Biosciences, San Jose, CA): CD117 clone 2B8 (APC-R700), CD34 (PE-Cy7), SCA-1 clone D7 (BV605), CD16/32 clone 2.4G2 (BV510), CD150 clone Q38-480 (BV421), CD105 clone MJ7/18 (BB515), CD71 clone C2 (PE) and Ter119 clone TER-119 (FITC) in 100 µL BD Horizon Brilliant Stain Buffer at room temperature in the dark for 30 mins. Samples were washed twice and resuspended in 0.5 mL FACS buffer (DPBS$^-$ with 2% FBS and 0.09% sodium azide) prior to analysis using a BD Accuri C6 or BD LSRFortessa flow cytometer.

Immunoprecipitation and co-immunoprecipitation

GATA-1 or PU.1 protein was isolated by immunoprecipitation and GATA-1/FOG-1 complex was co-immunoprecipitated as previously described (Zhou et al., 2011). Briefly, proteins were purified with corresponding antibodies (Cell Signaling Technologies) using Dynabeads Protein-A Immunoprecipitation Kit (ThermoFisher Scientific) from cell extracts according to manufacturer's instructions. To prepare samples for inductively coupled plasma mass spectrometry (ICP-MS), a non-denaturing method was used to elute GATA-1 or PU.1 from beads, and the solution adjusted to

pH>7 using the neutralizing buffer provided in the immunoprecipitation kit.

Inductively coupled plasma-mass spectrometry

Immunoprecipitated GATA-1 or PU.1 protein was collected, and protein content determined using BCA assay (ThermoFisher Scientific). Protein samples were diluted in trace metal grade nitric acid, and zinc and arsenic contents in GATA-1 or PU.1 were measured by ICP-MS. Blank samples, internal standards and standard curves were included with experimental samples as additional quality control for preparation and analyses. Results were normalized to immunoprecipitated protein content.

Statistics

Flow cytometry data was processed using FlowJo version 10 software (FlowJo LLC, Ashland, Oregon). Data were analyzed with GraphPad Prism 7 software. Differences between untreated control and treatment groups were determined using a one-way analysis of variance and Tukey's multiple-comparison tests at a significance level of $p<0.05$. Three replicates were performed and analyzed for each As^{+3} dose and at least three independent experiments were conducted with comparable results attained

RESULTS

AsIII inhibits erythropoiesis *in vivo*

To demonstrate the inhibition of erythropoiesis by arsenic exposure through drinking water, we exposed male C57BL/6J mice to 20, 100, and 500 µg/L AsIII via drinking water for 30 days. Early erythroid cells ($CD71^{+}/Ter119^{-/low}$: late BFU-E, CFU-E, and pro-erythroblasts) were assessed in bone marrow (BM) by CD71 and Ter119 surface marker expression using flow cytometry (Socolovsky et al., 2011; Koulnis et al., 2011). We found that AsIII exposure reduced the percentage of early erythroid cells in a dose-dependent manner, starting at 20 µg/L (Figure 4.1A). Additionally, to determine if AsIII compromised the differentiation ability of early erythroid progenitors, we assessed the colony-forming ability of CFU-E from BM of AsIII exposed mice. CFU-E colony formation was reduced by AsIII, starting at 20 µg/L and was suppressed over 60% with the 500 µg/L AsIII dose (Figure 4.1B). These results provide evidence that *in vivo* drinking water exposure to environmentally relevant levels of AsIII suppresses the differentiation of early BM erythroid progenitors.

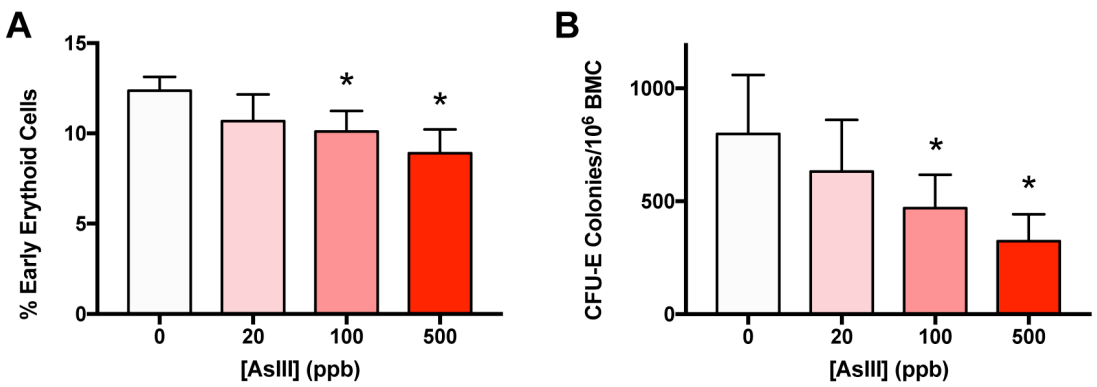

Figure 4.1. Inhibition of bone marrow erythropoiesis *in vivo* by AsIII expsosure. Male C57BL/6J mice (9 weeks of age) were exposed to 0, 20, 100, and 500 µg/L AsIII in drinking water for 30 days. (A) Reduction of early erythroid progenitor cells measured by flow cytometry of CD71 and Ter119 surface markers. (B) Attenuation of bone marrow CFU-E colony formation (showed as number of colonies/million bone marrow cells). Bar charts show mean ± SD, $n = 5$, *, $p < 0.05$ in one-way ANOVA, Tukey's multiple-comparison tests compared to untreated group.

AsIII inhibits erythropoiesis, but not myelopoiesis.

To further demonstrate the inhibitory effect of AsIII on erythropoiesis and investigate whether AsIII is able to inhibit other hematopoietic pathways, we developed an *in vitro* model of erythropoiesis using primary mouse BM HPC stimulated with EPO and stem cell factor (SCF). The progression of HPC through the stages of hematopoiesis were assessed based on surface marker phenotype using flow cytometry as previously described (Pronk and Bryder 2011; Grover et al., 2014). We identified a significant reduction in the percentage of erythro-megakaryocytic progenitor subsets (MEP, BFU-E, and CFU-E) after 24 h exposure to 0.5 μM AsIII (Figure 4.2A-B). In contrast, the percentage of early myeloid progenitors (pre-GM) was significantly increased with exposure to 0.1 and 0.5 μM AsIII (Figure 4.2C).

These results showed that AsIII inhibition of erythropoiesis starts from very early stages of differentiation, starting from MEPs. Interestingly, the suppression of erythroid differentiation introduces a lineage differentiation imbalance, skewing erythropoiesis in favor of myelopoiesis, despite EPO stimulation and the lack of myeloid supportive growth factors.

To determine whether the suppressive effects of AsIII on early erythroid progenitor differentiation persist to later-stages of maturation, we evaluated the progression of erythroblast differentiation every 24 h for 72 h based on CD71 and Ter119 surface marker expression and cell size (Socolovsky et al., 2011; Koulnis et al., 2011). A significant suppression of all discernable erythroblast subsets (late BFU-E, CFU-E, and EryA) was observed after 24 h exposure to 0.5 μM AsIII (Figure 4.2D). These effects were persistent up to 72 h (Figure 4.2F), with significantly fewer AsIII-exposed

erythroblasts properly transitioning to late-stages erythroblast maturation (EryB and EryC) (Figure 4.2D-F).

Collectively, these results suggest that AsIII inhibits early-stages of erythropoiesis and these effects are persistent throughout erythroblast differentiation, resulting in decreased production of mature erythroblasts. However, the suppressive effects of AsIII were selective to erythro-megakaryocytic progenitors, as myeloid progenitors were not reduced by AsIII exposure.

To further demonstrate that AsIII selectively inhibits erythropoiesis, we tested the effects of AsIII on the differentiation of the K562, human erythroleukemia cell line. We treated K562 cells with 1 μM AsIII for 48 h, during which the cells were induced with hemin (5 μM) or PMA (5 ng/mL) for 24 h to induce erythroid or megakaryocytic, myelocytic, and monocytic differentiation, respectively (Villeval et al., 1983; Shelly et al., 1998; Palma et al., 2012; Huang et al., 2014). In hemin-induced K562 cells, we measured erythropoietic differentiation using surface marker expression of CD71 by flow cytometry. AsIII reduced the percentage of erythroid differentiated ($CD71^{+}$) K562 cells (Figure 4.3A). Hemoglobin production was also measured as an additional measure of erythroid differentiation using benzidine staining (Hafner et al., 1995). AsIII treatment at 1 μM significantly reduced the percentage of benzidine positive K562 cells by more than 50% (Figure 4.3B).

Megakaryocytic differentiation of K562 cells was determined using CD41 surface marker expression by flow cytometry after exposure to 1 μM AsIII. AsIII significantly reduced the percentage of PMA differentiated megakaryocytic ($CD41^{high}$) K562 cells (Figure 4.3C). Further evaluation of forward scatter and CD11b surface marker

expression, characteristics of myelopoiesis and monopoiesis, showed no significant differences with AsIII (Figure 4.3D-E). These results showed that in the K562 human erythroid cell model, AsIII inhibits erythropoiesis and megakaryopoiesis, but not myelopoiesis or monopoiesis. The selective inhibition of erythroid and megakaryocytic differentiation and the lack of effects on myelocytic progenitors in two independent cell models (primary mouse BM and K562 cell line model) motivated us to follow-up with mechanistic studies focused on understanding the effects of AsIII on the master regulators of erythropoiesis and myelopoiesis, GATA-1 and PU.1, respectively.

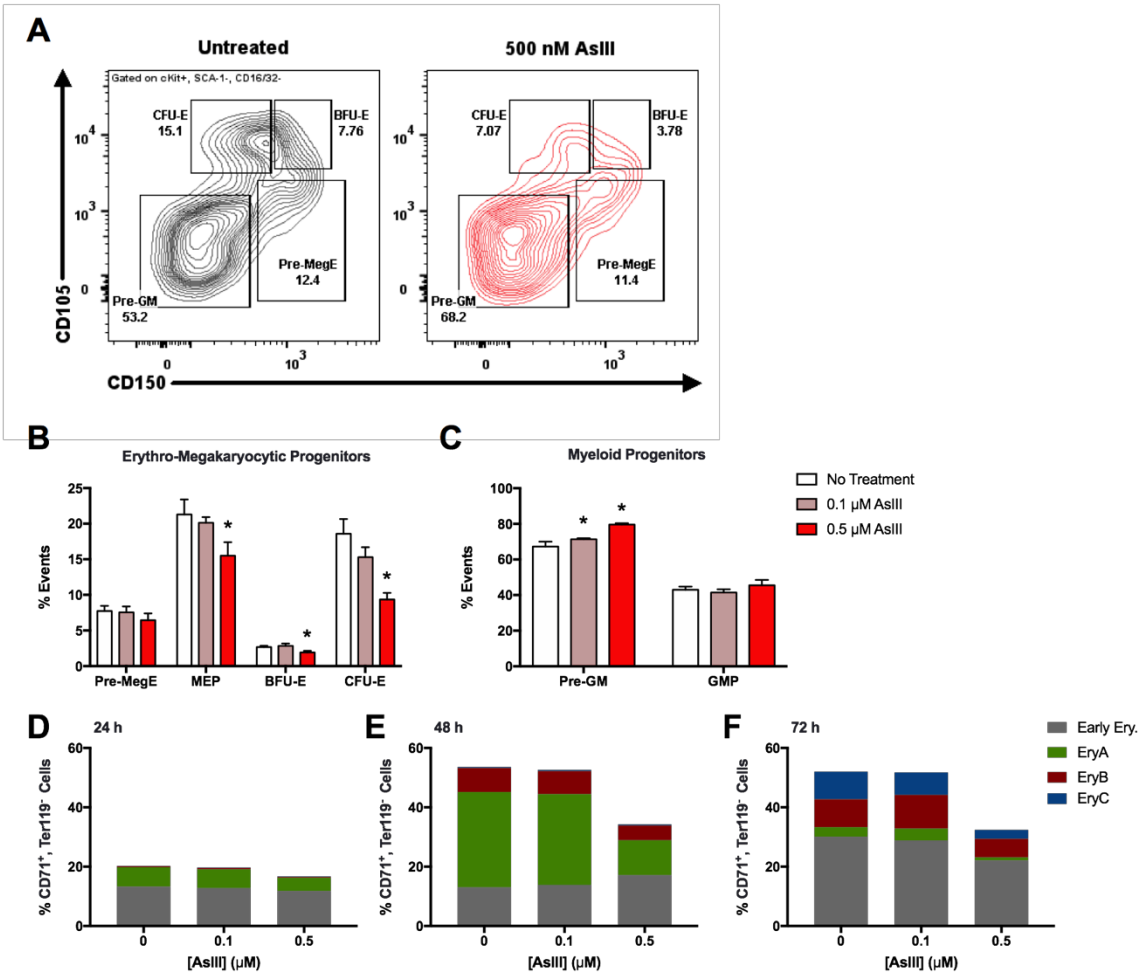

Figure 4.2. AsIII suppresses erythropoiesis, not myelopoiesis, of primary mouse bone marrow hematopoietic progenitor cells. Primary mouse bone marrow cells were stimulated with EPO and SCF for 24 h to promote erythroid differentiation. (A) Representative flow cytometry plot depicting effects of 0 and 0.5 μM AsIII on erythro-megakaryocytic and myeloid progenitor cell subsets. (B) Percentages of surface marker defined erythro-megakaryocytic progenitors: PreMegE (Lin^-, $cKit^+$, $SCA\text{-}1^-$, $CD16/32^-$, $CD150^+$, $CD105^{-,\ low}$); MEP (Lin^-, $cKit^+$, $SCA\text{-}1^-$, $CD16/32^-$, $CD34^-$), BFU-E (Lin^-, $cKit^+$, $SCA\text{-}1^-$, $CD16/32^-$, $CD150^+$, $CD105^+$); CFU-E (Lin^-, $cKit^+$, $SCA\text{-}1^-$, $CD16/32^-$, $CD150^-$, $CD105^+$) and (C) myeloid progenitors: pre-Granulocyte Macrophage (pre-GM; Lin^-, $cKit^+$, $SCA\text{-}1^-$, $CD16/32^-$, $CD150^-$, $CD105^-$); Granulocyte Macrophage Progenitor (GMP; Lin^-, $cKit^+$, $SCA\text{-}1^-$, $CD16/32^+$, $CD150^-$) after 24 h exposure to 0, 0.1, and 0.5 μM AsIII. (D, E, & F) Percentage of erythroblast subsets (early erythroblasts, $CD71^{low/high}$, $Ter119^{-,low}$; EryA, $CD71^{high}Ter119^{high}FSC^{high}$; EryB, $CD71^{high}Ter119^{high}FSC^{low}$; EryC, $CD71^{low}Ter119^{high}FSC^{high}$) after 24, 48, and 72 h exposure to 0, 0.1, and 0.5 μM AsIII. Data are expressed as mean ± SD, n =3, *, $p < 0.05$ in one-way ANOVA, Tukey's multiple-comparison tests compared to untreated control group.

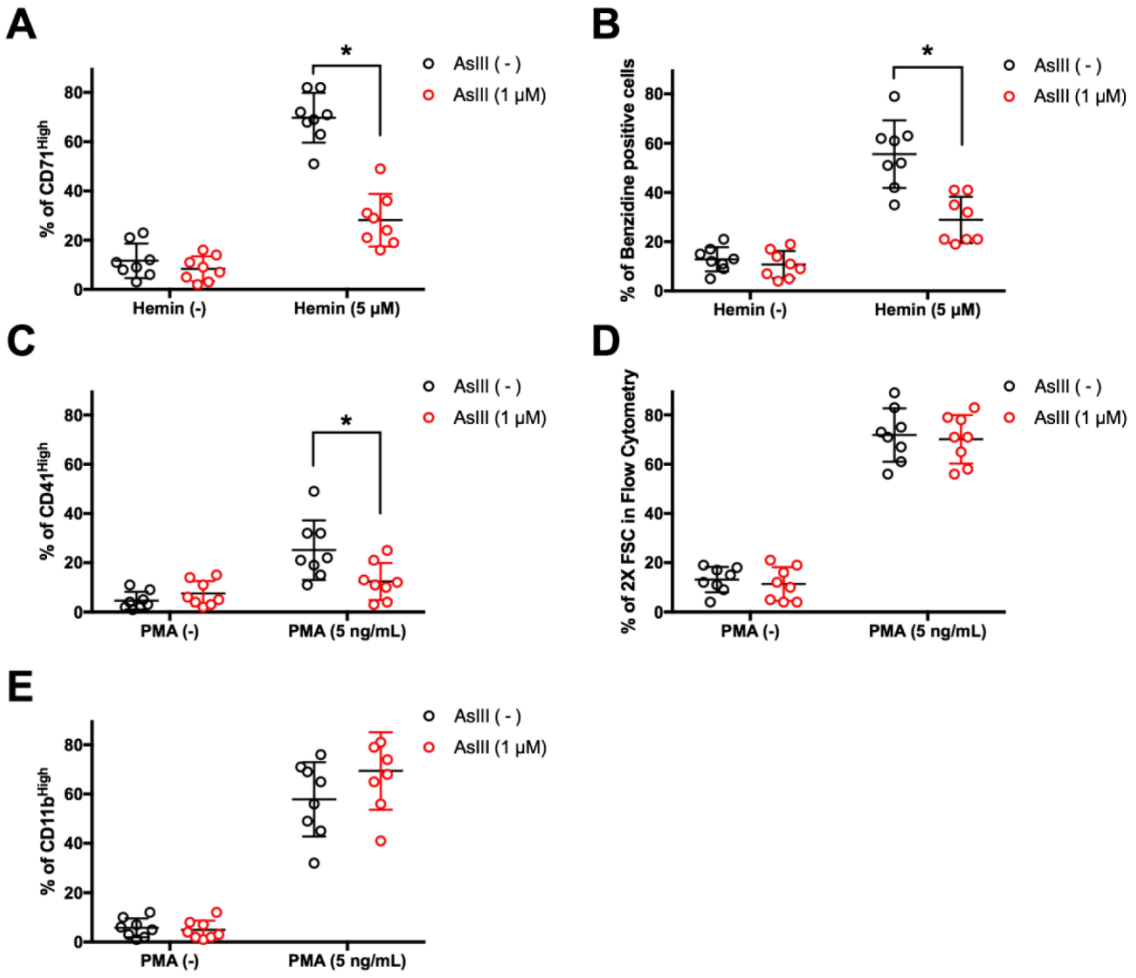

Figure 4.3. AsIII disrupts erythroid and megakaryocytic differentiation, not monocyte or myeloid differentiation of K562 cells. Human K562 erythroleukemia cells were treated with 1 μM AsIII for 48 h. Erythroid or megakaryocyte, monocyte, and myeloid differentiation was induced with hemin or PMA for 24 h, respectively. (A) Percentage of erythropoietic differentiated K562 cells ($CD71^{+}$) and (B) percentage of benzidine positive (hemoglobin positive) K562 cells. (C) PMA-induced megakaryocyte differentiation of K562 cells ($CD41^{high}$). (D) PMA-induced monocyte ($CD11b^{+}$ and 2X forward scatter) and (E) myeloid differentiation $CD11b^{high}$. Data are expressed as mean ± SD, n =3, *p<0.05 in one-way ANOVA, Tukey's multiple-comparison tests compared to untreated control group. Data provided as courtesy of Dr. Xixi Zhou.

AsIII inhibits GATA-1 activities *in vitro*

Since erythroid and megakaryocyte differentiation depend on the activity of GATA-1 (Fujiwara et al., 1996; Shirdasani et al., 1997), we tested the impact of AsIII on GATA-1 DNA and protein binding. GATA-1 DNA binding activity was determined by immunoprecipitating GATA-1 from K562 cells or erythroid differentiated mouse BM HPC and assessing the ability of GATA to bind a GATA consensus sequence using the EpigentikTM fluorescent protein-DNA interaction kit. In 1 μM and 2 μM AsIII treated K562 cells, GATA-1 DNA binding activity was dramatically decreased by over 50% and 70%, respectively (Figure 4.4A). GATA-1 DNA binding activity was even more sensitive in primary mouse BM erythroid cells and an inhibition of GATA-1 DNA binding was found with 0.1 and 0.5 μM AsIII (30% and 50%, respectively) (Figure 4.4B).

In addition to DNA binding activity, GATA-1 interaction with FOG-1 is also critical for its function (Tsang et al., 1998). We tested AsIII effects on the interaction between GATA-1 and FOG-1 using co-immunoprecipitation followed by western blotting. We found that AsIII treatment in K562 cells significantly inhibited GATA-1 and FOG-1 interaction, starting from 0.5 μM (Figure 4.4C). At 2 μM concentration, AsIII reduced the interaction between GATA-1 and FOG-1 by ~75% (Figure 4.4C-D). Since zinc ion is critical to GATA-1 ZF structure and function, to demonstrate that AsIII inhibiting GATA-1 is through a zinc dependent mechanism, we used the specific zinc chelator N,N,N′,N′-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine (TPEN) to demonstrate the necessity of zinc to GATA-1/FOG-1 interaction. We found that treating the K562 cells with TPEN dramatically decreased GATA-1/FOG-1 interaction (Figure 4.4C-D), suggesting that AsIII treatment is equivalent to zinc chelation in terms of

removing zinc from GATA-1 protein, leading to inhibition of GATA-1/FOG-1 interaction.

Because AsIII treatment caused decreased GATA-1 DNA binding and interaction with FOG-1, these findings indicate that AsIII disrupts the function of both the N- and C-terminal ZF domains of GATA. Together, our results show that AsIII inhibits GATA-1 DNA binding and protein-protein interaction activities and clearly demonstrate that GATA-1 is a sensitive molecular target of AsIII.

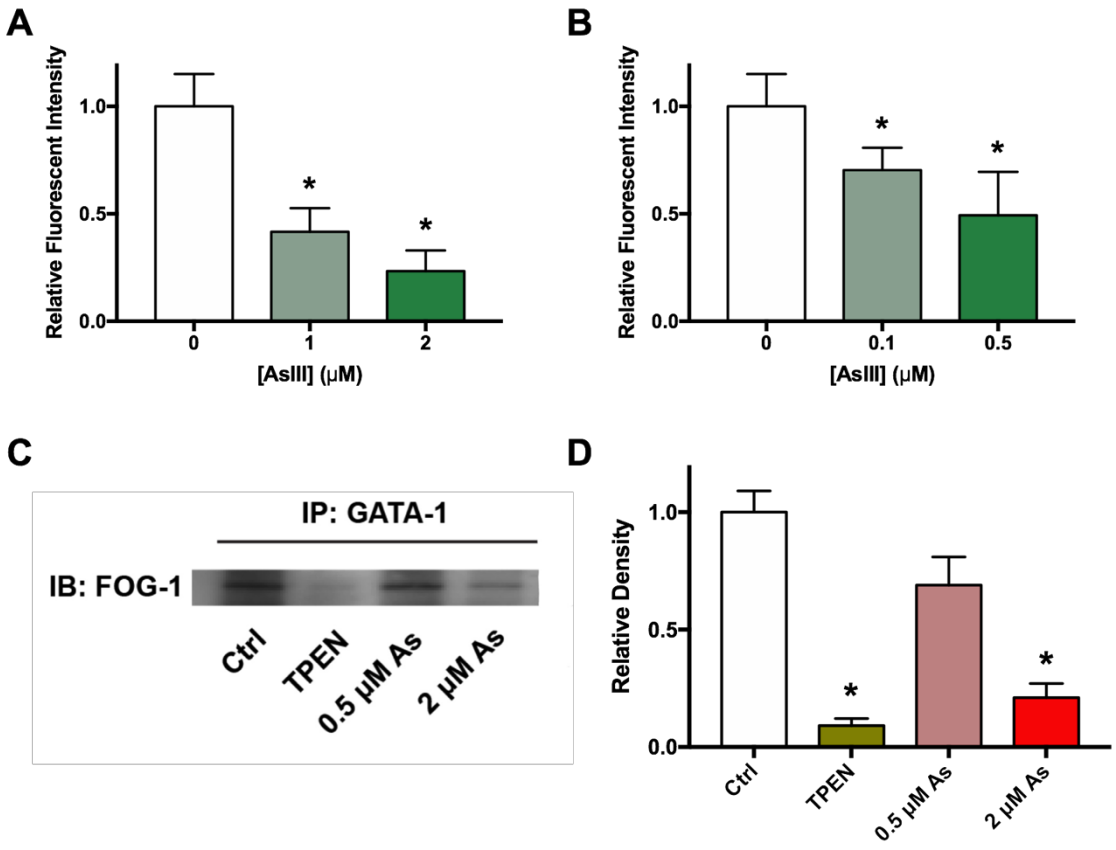

Figure 4.4. AsIII impairs DNA binding and FOG-1 interaction activities of GATA-1. GATA-1 DNA binding was measured using an ELISA DNA binding kit after AsIII treatment in (A) K562 cells for 48 h and (B) primary mouse bone marrow erythroid cells after 24 h. (C) GATA-1 and FOG-1 were co-immunoprecipitated from K562 cell lysate using GATA-1 antibody after AsIII treatments. FOG-1 interaction with GATA-1 after 48 h treatment with 0.5 and 1 μM AsIII. (D) Densitometry of western blotting. Data are expressed as mean ± SD, n =3, *p<0.05 in one-way ANOVA, Tukey's multiple-comparison tests compared to untreated control group. Experiment (B) performed in collaboration with Dr. Xixi Zhou. Data (A,C,D) provided as courtesy of Dr. Xixi Zhou.

AsIII interacts with GATA-1, not PU.1 through zinc finger disruption.

We demonstrated that AsIII compromises erythropoiesis through the inhibition of GATA-1 activity (DNA binding and protein-protein interaction) resulting from impairments of the ZF motifs on GATA-1. To provide further evidence that the AsIII-induced inhibition of GATA-1 was through ZF disruption, we treated K562 cells and primary mouse BM erythroid cells with AsIII for 48 and 24 h, respectively. GATA-1 and PU.1 protein was purified from cell lysates using immunoprecipitation and the Zn and As contents were measured by ICP-MS. In 1 μM and 2 μM AsIII treated K562 cells, zinc contents were decreased by over 50% (Figure 4.5A) and arsenic content was dramatically increased (Figure 5B). GATA-1 from primary mouse BM erythroid cells, showed greater sensitivity to AsIII (Figure 5D-E). At 0.1 μM and 0.5 μM AsIII concentrations, zinc contents in GATA-1 were decreased (Figure 5D) and with 0.5 μM were reduced by 50% (Figure 4.5D). Similar to K562 cells, a dramatic increase in GATA-1 As content was also found (Figure 4.5E).

These findings indicate that arsenic replaces zinc in GATA-1 ZF motifs. In contrast, using the same approach, AsIII did not bind to PU.1 in either K562 or primary mouse BM cells (Figure 4.5C, F), indicating that PU.1 was not a molecular target of AsIII.

Collectively, these results demonstrated that AsIII binds to GATA-1 ZF motifs, causing zinc loss, and dysfunction of DNA and protein binding activities (Figure 4.4). More importantly, the interaction of AsIII with GATA-1 was selective, as no changes to PU.1 were found.

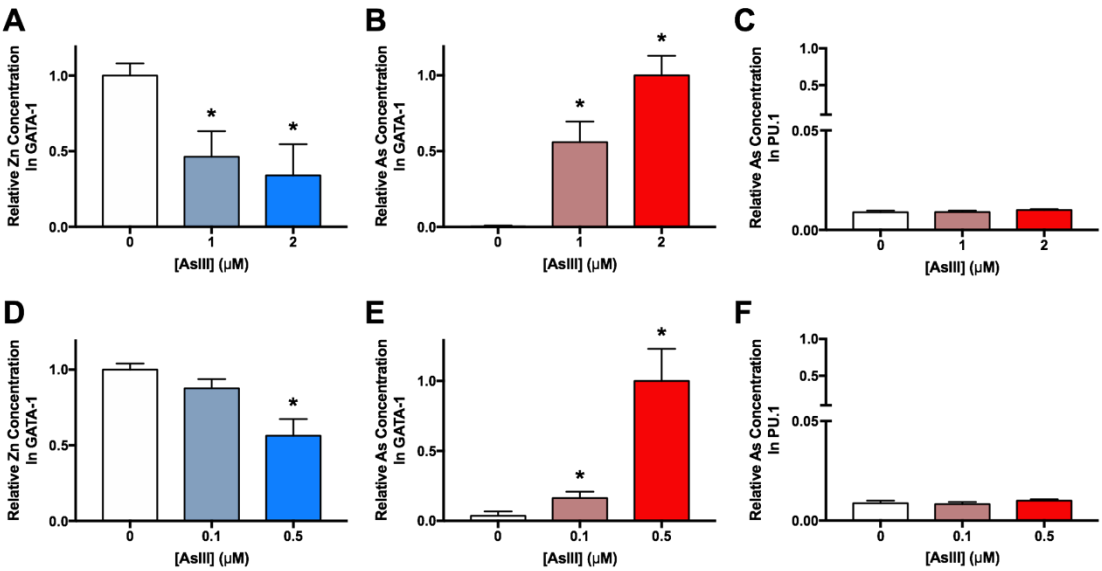

Figure 4.5. AsIII binds to GATA-1 causing zinc loss but does not bind to PU.1. GATA-1 and PU.1 were immunoprecipitated and Zn/As contents were analyzed by ICP-MS. (A, B, & C) K562 cells were treated by AsIII for 48 h. (A) Zinc content in GATA-1 from K562 cells treated with 1 and 2 μM AsIII. (B) Arsenic bound to GATA-1 protein in AsIII treated K562 cells. (C) Arsenic content in PU.1 from AsIII treated K562 cells. (D, E, & F) Primary mouse bone marrow erythroid cells were treated with 0.1 or 0.5 μM AsIII for 24 h. (D) Zinc content in GATA-1 from primary mouse bone marrow erythroid cells. (E) Arsenic bound to GATA-1 from primary mouse bone marrow erythroid cells. (F) Arsenic content in PU.1 from primary mouse bone marrow erythroid cells. Data are expressed as mean ± SD, n =3, *p<0.05 in one-way ANOVA, Tukey's multiple-comparison tests compared to untreated control group. Data from A-C provided as courtesy of Dr. Xixi Zhou. Experiments (D-F) were performed in collaboration with Dr. Xixi Zhou.

AsIII causes GATA-1 zinc finger dysfunction *in vivo*.

To demonstrate that GATA-1 is a sensitive molecular target of AsIII *in vivo*, we tested arsenic binding and zinc loss using GATA-1 protein collected from BM of C57BL/6J mice exposed to 500 μg/L and 1 mg/L AsIII via drinking water for 2 weeks. BM cells were isolated from the femurs of each mouse, GATA-1 protein was immunoprecipitated, and ICP-MS was performed to measure the zinc and arsenic content in GATA-1. Consistent with findings from our *in vitro* studies, we found the zinc content in GATA-1 was significantly decreased with 1 mg/L AsIII (Figure 4.6A). We also found a dramatic increase of arsenic content in GATA-1 following 1 mg/L AsIII exposure (Figure 4.6B). These results corroborated our *in vitro* findings and provided additional evidence that AsIII interacts with GATA-1 to cause zinc loss and protein dysfunction *in vivo*.

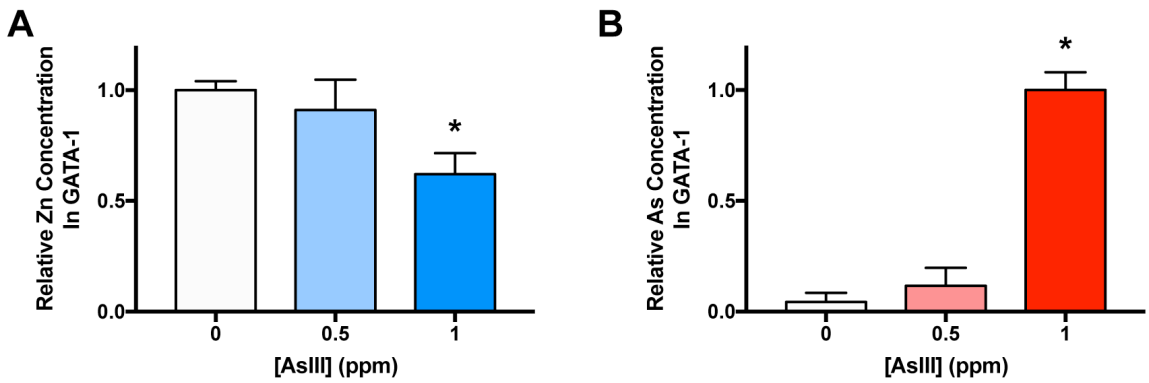

Figure 4.6. AsIII disrupts GATA-1 zinc finger *in vivo*. Male C57BL/6J mice (12 weeks of age) were exposed to 500 µg/L and 1 mg/L AsIII in drinking water for 2 weeks. (A) Zinc content and (B) arsenic content were measured in immunoprecipitated GATA-1 by ICP-MS. Data are expressed as mean ± SD, $n = 6$, *$p<0.05$ in one-way ANOVA, Tukey's multiple-comparison tests compared to untreated control group. Experiment performed in collaboration with Dr. Xixi Zhou.

DISCUSSION

The lineage commitment and differentiation of HPC is a sophisticated process regulated by series of factors. Erythropoiesis is largely regulated by the activity of the key transcription factor, GATA-1 (Fujiwara et al., 1996; Ferreira et al., 2005). GATA-1 binds to the DNA consensus sequences, T/A(GATA)A/G and is responsible for regulating the expression of a large family of erythropoiesis required genes (Ko and Engel 1993; Ferreira et al., 2005). Similarly, myelopoiesis is regulated primarily by the actions of another key transcription factor, PU.1. PU.1 binds to purine-rich DNA consensus sequences (GAGGAA), and is responsible for activating the expression of genes required for myelopoiesis and cellular communications in the immune system (Klemsz et al., 1990; Rhodes et al., 2005; Iwasaki and Akashi 2007). In this study, we found that AsIII selectively inhibited erythropoiesis, but not myelopoiesis (Figure 4.2, 4.3). This differential inhibition most likely resulted from the structural differences between GATA-1 and PU.1.

GATA-1 is a ZF protein with two ZF domains, whereas PU.1 is not a ZF protein without any zinc in the structure. The N-terminal ZF of GATA-1 is responsible for binding with FOG-1 and the C-terminal ZF is responsible for DNA binding (Ferreira et al., 2005). Our previous work in DNA repair inhibition by AsIII demonstrated that ZF proteins such as PARP-1 are sensitive molecular targets of AsIII (Ding et al., 2009). We found that AsIII selectively interacts with ZF motifs with ≥3 cysteine residues (Zhou et al., 2011), which are a minority of ZF proteins. Both C- and N-terminal ZFs of GATA-1 contain 4 cysteine residues, making GATA-1 structurally favorable for arsenic binding.

In contrast, PU.1 contains only two spatially separated cysteine residues and does not have zinc in its structure. As a result, PU.1 molecule does not interact with and is insensitive to AsIII.

Our results showed that AsIII exposure not only impaired GATA-1 DNA binding (Figure 4.4A-B), but also inhibited protein-protein interaction of GATA-1 with the FOG-1 (Figure 4.4C-D), indicating that AsIII is capable of binding to both ZFs of the GATA-1 protein. Interaction with FOG-1 and DNA binding are both necessary for the function of GATA-1 (Tsang et al., 1998; Ferreira et al., 2005); i.e., erythroid lineage commitment and promotion of erythropoiesis. Previous studies reported that a single germ-line mutation on just one ZF of GATA-1 leads to dyserythropoietic anemia (Ciovacco et al., 2008; Del Vecchio et al., 2005). Additionally, a mutation in the N-terminal ZF of GATA-1 disrupts DNA binding and results in X-linked thrombocytopenia and β-thalassemia (Yu et al., 2002). These reports suggest that disruption of either ZF on GATA-1 can cause dysfunction resulting in hematological disorders. Our results indicate that AsIII impairs both ZF motifs on GATA-1, which explains why such low concentrations of AsIII may be able to cause inhibition of erythropoiesis.

The selective interaction of AsIII with GATA-1 ZF vs. PU.1, produced a shift from erythropoiesis in favor of myelopoiesis (Figure 4.7). The lineage commitment of CMPs regulated by the functional antagonism of GATA-1 and PU.1 (Zhang et al., 1996; Rekhtman et al., 1999; Nerlov et al., 2000; Iwasaki and Akashi 2007). Studies have shown that ectopic expression of GATA-1 or PU.1 block erythroid or myeloid differentiation, respectively, through the functional repression of gene activation (Rekhtman et al., 1999; Zhang et al., 1996). The C-terminal ZF of GATA-1 is essential

for suppressing the function of PU.1 by direct interaction with the ETS domain on PU.1 (Nerlov et al., 2000). The myeloid shift observed in this study (Figure 4.2C) is likely a consequence of AsIII-induced disruption of the C-terminal ZF of GATA-1. The hypothesis that AsIII disrupts the lineage commitment of HPC resulting from effects on lineage specific transcription factors requires further investigation and will be the topic of our future studies.

In this work, the concentrations of AsIII that produced an inhibition of erythropoiesis started at 20 μg/L (20 ppb), which is lower than 12% of the AsIII concentrations measured in water supplies in north central and western regions of the United States (ATSDR 2007, 2016). Some unregulated water sources in rural areas around the world contain much higher AsIII concentrations (ATSDR 2007, 2016). Many regions with high endemic AsIII levels have also been reported to be disproportionately affected by anemia, even after common risk factors of anemia are considered and adjusted for (Hopenhayn et al., 2006; Heck et al., 2008; Surdu et al., 2015; Kile et al., 2016). These studies emphasize the necessity for gaining a mechanistic understanding of arsenic-caused anemia.

Findings from the present study showed that GATA-1 is a sensitive target of AsIII. GATA-1 ZF disruption was observed with environmentally relevant concentrations of AsIII in vitro and in vivo. We identified a clear molecular mechanism by which AsIII causes GATA-1 ZF disruption that produces effects functionally equivalent to germ-line mutations of GATA-1. Such mutations of GATA-1 ZF result in severe dyserythropoietic anemia (Ciovacco et al., 2008; Del Vecchio et al., 2005). Although inherited dyserythropoietic anemia is rare, millions of people worldwide are chronically exposed to

AsIII through drinking water. This presents a very important environmental health issue that requires further attention from the scientific community.

Our work demonstrated for the first time that AsIII, a widespread environmental toxicant, selectively inhibits erythropoiesis by disruption of the GATA-1 ZF transcription factor. AsIII selectively interacts with GATA-1 ZF motifs, causing zinc loss and inhibition of DNA and protein binding activities, resulting in dyserythropoiesis and an imbalance of HPC differentiation. These findings present a novel mechanism by which AsIII can cause anemia and provide critical insights into potential prevention and intervention for arsenic and other environmental exposure related anemias.

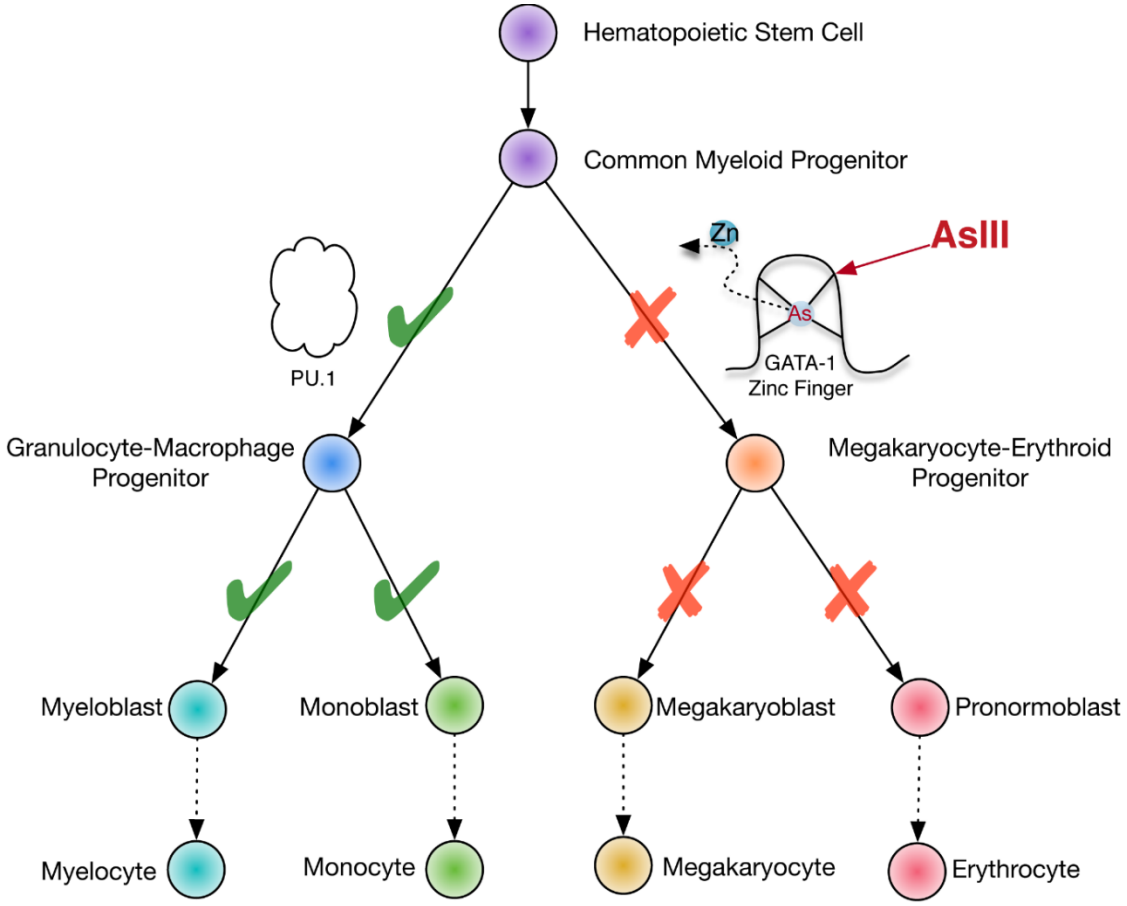

Figure 4.7. AsIII inhibits erythropoiesis, but not myelopoiesis through selective interactions with the zinc finger transcription factor, GATA-1. AsIII disrupts GATA-1 zinc finger, causing zinc loss and inhibition of DNA/protein binding activities. However, AsIII does not interact with PU.1, a non-zinc finger transcription factor. The selective effect of AsIII on GATA-1 vs. PU.1 result in dyserythropoiesis and produce a shift in the differentiation fate of progenitor cells from erythropoiesis in favor of myelopoiesis.

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AUTHORSHIP CONTRIBUTIONS

X.Z., S.M., S.W.B., and K.J.L. designed experiments, analyzed, and interpreted results. X.Z. and S.M. performed experiments. X.Z., S.M., S.W.B., and K.J.L. wrote and edited the paper. A.B., H.X., F.T.L., and S.C.W. provided intellectual input to the design of experiments and with reviewing and editing the paper. K.J.L. and S.W.B. conceived the study.

DISCLOSURE OF CONFLICTS OF INTEREST

The authors declare no competing financial interests.

CHAPTER 5

Arsenic Disrupts Erythropoiesis by Combined Effects on Differentiation and Survival of Early Erythroid Progenitors

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KEY POINTS

- Development of early stage erythroid progenitors was impaired by As^{+3} and MMA^{+3}.
- As^{+3} and MMA^{+3} disrupt GATA-1 and STAT5 regulated differentiation and survival of early erythroid cells.
- MMA^{+3} is more toxic than As^{+3} to early developing erythroid cells.

ABSTRACT

Strong epidemiological evidence demonstrates an association between chronic arsenic (As) exposure and anemia. However, the underlying mechanisms remain largely unknown. The goal of this study was to evaluate the effects of arsenite (As^{+3}) and the As^{+3} metabolite, monomethyarsonous acid (MMA^{+3}) on two critical regulatory pathways that control the differentiation and survival of early erythroid progenitor cells. We found that 500 nM As^{+3} and 100 and 500 nM MMA^{+3} suppress erythropoiesis by impairing the differentiation of early stage erythroid progenitors (BFU-E and CFU-E). The suppression of early erythroid progenitor development was due to combined effects on differentiation and survival pathways mediated by disruption of GATA-1 and STAT5. Exposure to 500 nM As^{+3} and 100 and 500 nM MMA^{+3} reduced the expression of GATA-1 responsive genes critical for erythroid differentiation. Additionally, the phosphorylation of STAT5 (pSTAT5) was suppressed by exposure to 100 and 500 nM MMA^{+3}. Impairment of GATA-1 and pSTAT5 by As^{+3} and MMA^{+3} decreased a critical prosurvival factor, Bcl-x_L resulting in the loss of early erythroid progenitors by cell death. Our results show that As^{+3} primarily disrupted GATA-1 function; whereas, MMA^{+3} suppressed GATA-1 and STAT activation, suggesting that MMA^{+3} is more toxic than As^{+3} to early developing erythroid cells. Collectively, these findings provide novel mechanistic insights into arsenic-induced dyserythropoiesis.

INTRODUCTION

Chronic exposure to arsenic (As) in drinking water has been linked to a multitude of detrimental health outcomes, including cancer, immunosuppression, cardiovascular disease, neurological disorders, and anemia (Hughes, 2002, Heck et al., 2008, Naujokas et al., 2013, Tyler and Allan 2014; Ferrario et al., 2016). The primary form of As in drinking water is the trivalent inorganic arsenical, arsenite (As^{+3}). As^{+3} is metabolized through a series of oxidative methylation and reduction reactions catalyzed by the As (+3 oxidation state) methyltransferase enzyme to monomethylarsonic acid (MMA^{+5}), monomethylarsonous acid (MMA^{+3}), dimethylarsinic acid (DMA^{+5}), and dimethylarsinous acid (DMA^{+3}) (Thomas et al., 2001, Vahter, 2002). Numerous in vitro and in vivo studies report MMA^{+3} to be most toxic arsenical (Styblo et al., 2000; Petrick et al., 2001). We found that MMA^{+3} accumulated in the bone marrow of mice exposed to As^{+3} in their drinking water for 30 days (Xu et al., 2016b), suggesting that progenitor cells developing at this site are at an increased risk of MMA^{+3} exposure and toxicity.

Anemia is a hematological disorder that adversely affects the health of millions of people worldwide (Koury 2014; WHO 2015). In a cross-sectional study of clinical indicators of anemia, we found a strong inverse relationship between measures of As exposure (water and urine) and urinary As metabolites (MMA and DMA) with RBC counts and hematocrits among a subset of male Health Effects of Arsenic Longitudinal Study cohort volunteers from rural Bangladesh (Parvez et al., 2017). Other epidemiological studies also report similar associations between As exposure and anemia (Hopenhayn et al., 2006, Heck et al., 2008, Surdu et al., 2015, Kile et al., 2016).

However, the mechanistic basis of such arsenic-induced anemia is not well understood. Our recent findings show that the development of erythroid progenitors in the bone marrow is impaired by in vivo and in vitro As^{+3} exposures (Medina et al., 2017; Zhou et al., submitted). Therefore, we hypothesize that the inhibition of erythropoiesis may be a plausible mechanism underlying As-associated anemias.

Red blood cells develop in the bone marrow in response to the hormone, erythropoietin (EPO). EPO acts on early erythroid progenitors (burst-forming-unit erythroid (BFU-E) and colony-forming-unit erythroid (CFU-E) to promote survival, proliferation, and differentiation (Hattangadi et al., 2011; Dzierzak and Philipsen, 2013). Erythropoiesis is a dynamic and highly regulated process that relies on coordination of multiple pathways for normal differentiation of erythroid progenitors (Tsiftsoglou et al., 2009, Hattangadi et al., 2011; Dzierzak and Philipsen, 2013). GATA-1 is an essential transcriptional regulator of erythropoiesis and is responsible for inducing and repressing many genes critical for differentiation (Ferreira et al., 2005). Another important regulatory mechanism in early erythroid progenitors is the EPO-activated Signal Transducer and Activator of Transcription 5 (STAT5) prosurvival pathway (Socolovsky et al., 1999; 2001; Liu et al., 2006).

EPO-activated STAT5 signaling utilizes both GATA-1 and STAT5 to regulate and maintain the expression of the prosurvival factor, B-cell lymphoma-extra-large (Bcl-x_L), thereby preventing apoptotic cell death of early erythroid progenitors (Gregory et al., 1999). Under basal EPO conditions, erythroid progenitors mature to the CFU-E stage, but most (~60%) succumb to cell death via this mechanism (Wu et al., 1995; Dzierzak and Philipsen, 2013). During hypoxic conditions, EPO levels are elevated and this prosurvival

mechanism is fully activated, thereby allowing for the survival and rapid expansion of the early erythroid progenitor pool (Dzierzak and Philipsen, 2013). This survival mechanism represents a critical regulatory pathway that directly links EPO levels to the control of erythropoietic rate by regulating the survival and differentiation of early erythroid progenitors (Koulnis et al., 2012).

We recently found that As^{+3} selectively interacts with the zinc finger motifs of GATA-1 impairing both DNA binding and protein-protein interactions of this critical transcription factor (Zhou et al., submitted). Additionally, studies from our lab as well as others have shown that STAT5 phosphorylation is sensitive to As^{+3} and MMA^{+3} exposures (Cheng et al., 2004a; Ezeh et al., 2016a; Xu et al., 2016a;). Considering the importance of GATA-1 function in regulating erythroid differentiation, along with the importance of this factor in combination with STAT5 for promoting the survival of early erythroid progenitors, we postulated that these pathways may be responsible for As-induced suppression of erythropoiesis. In this study, the effects of As^{+3} and MMA^{+3} on GATA-1-regulated differentiation of erythroid progenitors were evaluated. We also investigated the effects of As^{+3} and MMA^{+3} on STAT5 and GATA-1 mediated survival of early erythroid progenitors.

METHODS

Chemicals and reagents

Sodium meta-arsenite (NaAsO2, ≥95% purity, CAS 774-46-5, Cat. No. S7400) was purchased from Sigma Aldrich. Monomethylarsonous acid (CH5AsO2, ≥95% purity, CAS 25400-23-1, Cat. No. M565100) was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Dulbecco's phosphate buffered saline w/o Ca+2 or Mg+2 (DPBS-), and Isocove's Modified Dulbecco's Medium (IMDM) were purchased from Sigma-Aldrich (St. Louis, MO). Fetal Bovine Serum (FBS) was purchased from Atlanta Biologicals (Flowery Branch, GA). Penicillin/Streptomycin 10,000 (mg/ml)/10,000 (U/ml) and 200 mM L-Glutamine were purchased from Life Technologies (Grand Island, NY). Serum-free (SF) StemSpan medium for expansion of mouse hematopoietic progenitor cells (Cat. No. 09650) and mouse hematopoietic progenitor cell isolation Kit (Cat. No. 19856) were purchased from STEMCELL Technologies (Cambridge, MA). Recombinant murine stem cell factor (SCF) (Cat. No. 250-03) and recombinant human EPO (Cat. No. 100-64) were purchased from Peprotech (Rocky Hill, NJ). FITC rat anti-mouse TER119 clone TER-119 (Cat. No. 557915), APC-R700 rat anti-mouse CD117 clone 2B8 (Cat. No565476), BV605 rat anti-mouse SCA-1 clone D7 (Cat. No563288), BV510 rat anti-mouse CD16/32 clone 2.4G2 (Cat. No. 740111), BV421 rat anti-mouse CD150 clone Q38-480 (Cat. No. 562811), BB515 rat anti-mouse CD105 clone MJ7/18 (Cat. No. 564744), BD Phosflow™ PE Mouse Anti-Stat5 (pY694) Clone 47/Stat5(pY694) (Cat. No. 612567), BD Horizon™ Brilliant Stain Buffer (Cat. No. 566394), Transcription Factor Buffer Kit (Cat. No. 562574), and Annexin V Apoptosis

Detection Kit I (Cat No. 556547) were purchased from BD Biosciences (San Jose, CA). PE GATA-1 (D52H6) XP® rabbit anti-mouse (Cat. No. 13353S) and PE Rabbit (DA1E) mAb IgG XP® Isotype Control (Cat. No. 5742S) were purchased from Cell Signaling Technologies (Danvers, MA). AlexaFluor 647 Annexin-V (Cat. No. 640912) was purchased from Biolegend (San Diego, CA). Acridine orange/propidium iodide (AO/PI) staining solution in PBS (Cat. No. CS2-0106-5ML) was purchased from Nexcelom Bioscience (Manchester, UK). RNeasy Mini Kit and QIAshredder were purchased from Qiagen (Valencia, California). High capacity cDNA reverse transcription kit (Cat. No. 4368814), Gapdh (Mm99999915_g1), Gata-1 (Mm01352636_m1), Gata-2 (Mm00492300_m1), Klf-1 (Mm00516096_m1), EpoR (Mm000833882_m1), Alas2 (Mm00802083_m1), Alad (Mm00476255_m1), Abcb10 (Mm00497926_m1), and Bcl2l1 (Bcl-xL) (Mm01245982_g1) TaqMan gene expression assays, and TaqMan universal PCR master mix (Cat. No. 4304437) were purchased from Applied Biosystems (Foster City, CA).

Primary bone marrow cell isolation

All experiments were performed in accordance with protocols approved by the Institutional Animal Use and Care Committee at the University of New Mexico Health Sciences Center. Male C57BL/6J mice were purchased at 11 weeks of age from Jackson Laboratory (Bar Harbor, ME) and allowed to acclimate in our animal facility for one week. All experiments were performed using male C57BL/6J mice between 12 and 16 weeks of age.

Bone marrow cells from each femur set of three mice were pooled together for all experiments. Primary mouse bone marrow cells were isolated as previously described

(Ezeh 2016b). Briefly, bone marrow cells were flushed from both femurs of each mouse by passing approximately 6-9 mL of media through the marrow shaft using a 1cc syringe and 25-G needle. The cell suspension was transferred using a 9 in Pasteur pipette to a 15 mL centrifuge tube, centrifuged at 200 x*g* for 10 mins, and resuspended in 20 mL of medium IMDM supplemented with 10% heat inactivated FBS, 2 mM L-glutamine, and 100 mg/ml streptomycin and 100 units/ml penicillin). Cell viabilities and concentrations were then determined using AO/PI staining and a Nexcelom Cellometer® Auto 2000.

Hematopoietic progenitor cell isolation and *in vitro* erythropoiesis model

Hematopoietic progenitor cells (HPC) were isolated from total bone marrow cells using the EasySep mouse HPC isolation kit according to manufacturer's instructions. Briefly, bone marrow cells were concentrated to 1×10^8 cells/mL in Easy Sep Buffer (DPBS- w/ 2% FBS and 1 mM EDTA) and stained with a cocktail of biotinylated lineage specific antibodies (CD5, CD11b, CD19, CD45R/B220, Ly6G/C(Gr-1), TER119) for 15 mins at 4°C. After 15 mins, streptavidin-coated magnetic particles were added to each sample and incubated for an additional 10 mins. Samples were brought up to 2.5 mL with EasySep buffer and placed into the EasySep™ magnet (STEMCELL Technologies, Cambridge, MA) for 3 mins. After two rounds of isolation, supernatant containing HPC was utilized to develop an *in vitro* model of erythropoiesis as described by Shuga et al. (2007). HPC were cultured in SF StemSpan hematopoietic progenitor expansion media supplemented with 100 ng/mL murine SCF and 5 IU/mL human recombinant EPO (31.25 ng/mL) to stimulate erythroid lineage commitment and differentiation.

Growth of erythroid progenitor cells was determined based on the concentration of viable cells after 48 h of erythroid differentiation. The concentration of viable cells in

was measured using AO/PI staining (20 µL of cells + 20 µL of AO/PI stain) and a Nexcelom Cellometer.

RNA isolation and quantitative real-time PCR

RNA was isolated from erythroid progenitor cells using the QIAshredder and RNeasy kit according to manufacturer's instructions. RNA concentrations were determined using an Agilent Nanodrop Spectrophotometer. A total of 100 ng of RNA was utilized for cDNA synthesis using the High Capacity Reverse Transcription Kit. Samples were diluted 1:4 (v/v) in RNase/DNase free water and stored at -80°C. Quantitative real-time PCR (qPCR) was performed in 10 µL reactions consisting of RNase/DNase free water, TaqMan universal PCR master mix, and TaqMan gene expression assay probes for *Gapdh, Gata-1*, *Gata-2*, *EpoR*, *Klf-1*, *Alas2*, *Alad*, or *Bcl-x*$_L$ using a BioRad CFX384 Touch Real-Time PCR Detection System (BioRad, Hercules, CA). The comparative CT method was used for relative quantification of gene expression using *Gapdh* as an endogenous control.

Flow cytometry

Early Erythroid progenitor subsets (i.e., BFU-E and CFU-E) were classified as Lin-, cKit$^+$, SCA-1$^-$, CD16/32$^-$, CD150$^{+,-}$, CD105$^+$ based on cell surface marker phenotypes previously described by Pronk and Bryder (2011) and Grover et al., (2014) (Figure 1A). Late-stages of erythroblast differentiation were measured based on Ter119 surface marker expression as previously described by Socolovsky et al., (2001); Koulnis (2011) (Figure 1C).

For surface marker analysis, 0.5-1×10^6 cells were stained with 0.5 µg of monoclonal antibodies with the following antigen and fluorochrome conjugations:

CD117 (APC-R700), SCA-1 (BV605), CD16/32 (BV510), CD150 (BV421), CD105 (BB515), Ter119 (FITC) in 50 µL BD Horizon Brilliant Stain Buffer at room temperature in the dark for 30 mins. Samples were washed twice and resuspended in 0.5 mL wash/stain buffer (DPBS$^-$ with 2% FBS and 0.09% sodium azide) prior to analysis using a BD Accuri™ C6 flow cytometer or BD LSRFortessa flow cytometer (BD Biosciences, San Jose, CA).

Intracellular staining was performed using the Transcription Factor buffer kit (BD Biosciences, San Jose, CA) according to manufacturer's instructions. Briefly, 0.5-1 x 10^6 cells were stained with surface markers for 30 mins at 4°C in the dark. Cells were washed and resuspended in 1 mL of 1X Fixation/Permeabilization reagent and incubated at 4°C for 45 min. After 45 min, 1 mL of 1X Permeabilization/Wash reagent was added to each tube and the samples are centrifuged at 350 x*g* for 6 min. Cells were resuspended in 50 µL BD Horizon Brilliant Stain Buffer containing 0.024 µg GATA-1 (PE) or 20 µL phosphoSTAT5 (pY694; PE), or concentration, fluorochrome, and isotype matched controls and incubated at room temperature for 1 h. Cells were washed twice and resuspended in 0.3 mL wash/stain buffer prior to analysis using a BD LSRFortessa flow cytometer.

Apoptosis was measured using the BD Annexin-V Apoptosis Detection Kit I according to manufacturer's instructions. Briefly, 0.5 x 10^6 erythroid progenitor cells were stained with surface markers. After surface marker staining, cells were resuspended in 100 µL 1X annexin-V binding buffer and stained with 5 µL of AlexaFluor 647 annexin-V and 5 µL propidium iodide for 15 mins at room temperature in the dark. An additional 400 µL of annexin-V binding buffer and samples were analyzed using a BD

LSRFortessa flow cytometer. Erythroid progenitor cells were treated with 5 µM etoposide for 4 h as a positive control.

Data analysis and statistics

All flow cytometry data was processed using FlowJo version 10 software (FlowJo LLC, Ashland, Oregon). Data were analyzed with Excel 2010 and Sigma Plot version 12.5 software. Differences between control and treatment groups were determined using a one-way analysis of variance (ANOVA) and Dunnett's t-test at a significance level of $p<0.05$. Three replicates were performed and analyzed for each As^{+3} and MMA^{+3} dose and at least three independent experiments were conducted, and comparable results attained.

RESULTS

As^{+3} and MMA^{+3} impair erythroid differentiation of primary mouse bone marrow hematopoietic progenitor cells

To determine the effects of As^{+3} and MMA^{+3} on erythroblast differentiation, we utilized an *in vitro* model of erythropoiesis using primary mouse bone marrow HPC stimulated with EPO and SCF to promote erythroid lineage commitment and differentiation (Shuga et al., 2007). Utilizing this *in vitro* model of erythropoiesis, we assessed erythroblast differentiation based on cell surface marker phenotype using multiparameter flow cytometry (Pronk and Bryder 2011; Grover et al., 2014) after 48 h As^{+3} and MMA^{+3} exposure (Figure 5.1A).

A significant reduction in the percentage of early erythroid progenitors (i.e., CFU-E and BFU-E) was found with 500 nM As^{+3} (Figure 5.1B). A similar suppression of early erythroid progenitors was found with MMA^{+3} at 100 nM; however, early erythroid differentiation was nearly abolished with 500 nM MMA^{+3} (Figure 5.1B). Interestingly, the suppressive effects of As^{+3} and MMA^{+3} were selective for erythroid progenitors, as no significant decreases were observed in other cell lineages (i.e., lymphoid progenitors and myeloid progenitors; Supplemental Figure 5.S1).

To determine if the reduction of early erythroid progenitors persisted throughout erythroblast differentiation, we evaluated later-stage erythroblasts based on Ter119 cell surface marker expression using flow cytometry (Socolovsky et al., 2001; Koulnis 2011) (Figure 5.1C). Similar to the effects observed on early erythroid progenitors, the percentage of later-stage erythroblasts ($Ter119^{+}$) was strongly reduced by 500 nM As^{+3}

and 100 nM MMA^{+3}, respectively (Figure 5.1D) and very few erythroblasts reached later stages of erythroblast differentiation with 500 nM MMA^{+3} (<5% of cells) (Figure 5.1D). The differentiation of erythroid progenitors is highly dependent on proliferation (Kinross et al., 2006; Tallack et al., 2007; Pop et al., 2010). As such, we utilized AO/PI staining and a Nexcelom Cellometer to evaluate cell growth after 48 h exposure to As^{+3} and MMA^{+3}. 500 nM As^{+3} was found to produce a significant suppression of cell growth and a similar level of inhibition of cell proliferation was seen with 100 nM MMA^{+3} (Figure 5.1E). MMA^{+3} at 500 nM inhibited growth to the level of non-EPO and SCF stimulated HPCs (Figure 5.1E).

Collectively, these results show that As^{+3} and MMA^{+3} impair erythropoiesis by suppressing very early stages of erythroid differentiation. Additionally, both As^{+3} and MMA^{+3} were found to suppress the growth of erythroid progenitors, likely resulting from the failure to properly differentiate. Our findings also show that MMA^{+3} produced more potent suppression of erythropoiesis than As^{+3}.

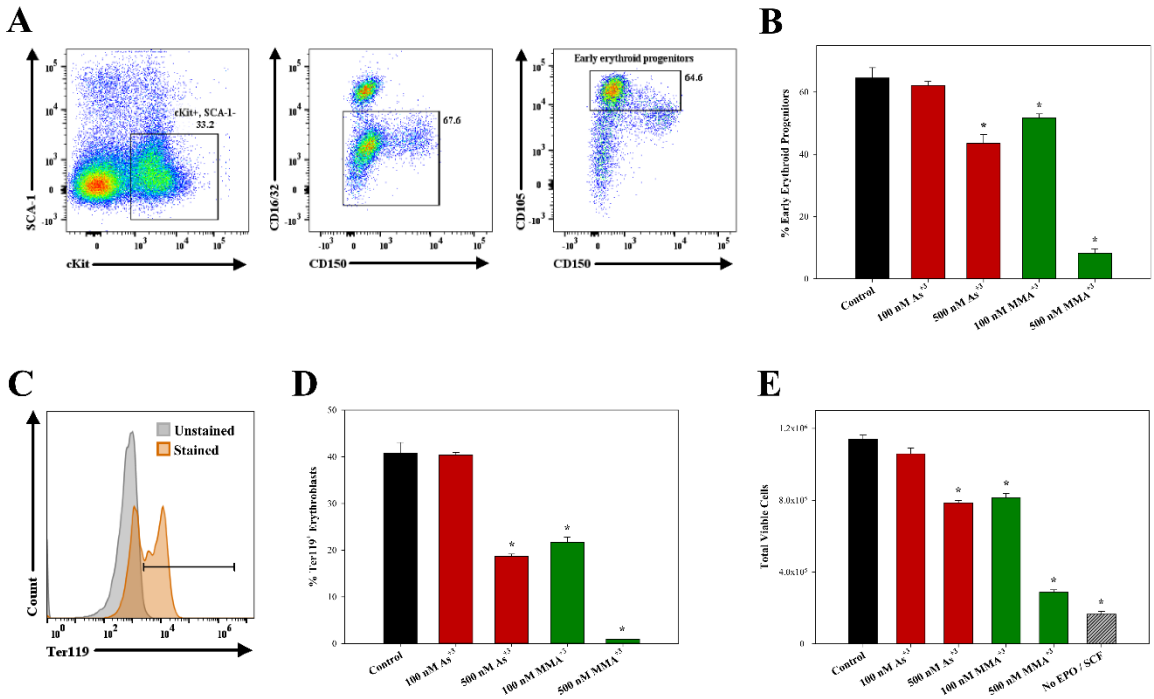

Figure 5.1. As^{+3} and MMA^{+3} suppress the differentiation and growth of erythroid progenitor cells. Erythroid differentiation of primary bone marrow hematopoietic progenitor was induced *in vitro* with erythropoietin and stem cell factor in the presence of 100 and 500 nM As^{+3} or MMA^{+3} for 48 h. Erythroid differentiation was assessed by cell surface marker phenotype using flow cytometry. (A) Flow cytometry gating strategy used to define early erythroid cells (BFU-E and CFU-E; $cKit^{+}$, $SCA\text{-}1^{-}$, $CD16/32^{-}$, $CD150^{+,-}$, $CD105^{+}$. (B) Percentage of early erythroblasts after treatment with As^{+3} or MMA^{+3}. (C) Flow cytometry gating strategy used to define late-stage ($Ter119^{+}$) erythroblasts. (D) Percentage of late-stage erythroblasts ($Ter119^{+}$) following exposure to As+3 or MMA+3. (E) Erythroid progenitor cell growth measured by acridine orange and propidium iodide staining using a Nexcelom Cellometer following 48 h exposure to 100 and 500 nM As^{+3} or MMA^{+3}. Data are expressed as mean ± SD. *Statistically significant difference compared to control ($n = 3$; $p<0.05$).

Differential suppressive effects of As^{+3} and MMA^{+3} on GATA-1 protein and downstream gene expression in erythroid progenitor cells

We recently showed that the master regulator of erythropoiesis, GATA-1, is a sensitive molecular target of As^{+3}. As^{+3} was found to selectively interact with the zinc finger motifs of GATA-1 disrupting DNA binding and protein-protein interactions (Zhou et al., submitted). In an effort to determine whether disruption of GATA-1 is responsible for the suppressed differentiation of early erythroid progenitors, we measured GATA-1 levels in early erythroid progenitors using intracellular flow cytometry and qPCR. We also assessed the expression of GATA-1-regulated genes important for erythroid differentiation using qPCR following 24 h As^{+3} or MMA^{+3} exposure.

The percentage of $GATA\text{-}1^{+}$ early erythroblasts, the fluorescence intensity of GATA-1 in early erythroblasts, and *Gata-1* mRNA expression were significantly reduced after 24 h exposure to 100 nM and 500 nM MMA^{+3} (Figure 5.2A-D). Interestingly, As^{+3} did not significantly modulate GATA-1 protein in early erythroid progenitors. However, As^{+3} did significantly reduce *Gata-1* mRNA expression (Figure 5.2A-D). The expression of several GATA-1-regulated genes critical for erythroid differentiation were significantly reduced following 500 nM As^{+3} exposure, including *Gata-2*, Krüppel-like factor-1 (*Klf-1*), and Epo receptor (*EpoR*) (Figure 5.2D). The effects of MMA^{+3} on GATA-1-regulated gene expression were more pronounced. All genes measured (*Gata-2*, *Klf-1*, *EpoR*, 5- aminolevulinate synthase 2 (*Alas2*), 5-aminolevulinate dehydratase (*Alad*), ATP binding cassette mitochondrial erythroid (*Abcb10*) were significantly reduced with 100 and 500 nM MMA^{+3} (Figure 5.2D).

Taken together, these findings show differential effects of As^{+3} and MMA^{+3} on GATA-1 in early erythroid progenitor cells. Whereas As^{+3} primarily disrupts GATA-1

function, MMA^{+3} disrupts both expression and function of GATA-1, as evidenced by decreased GATA-1 protein and mRNA levels in early erythroid progenitors as well as reductions of GATA-1-regulated gene expression.

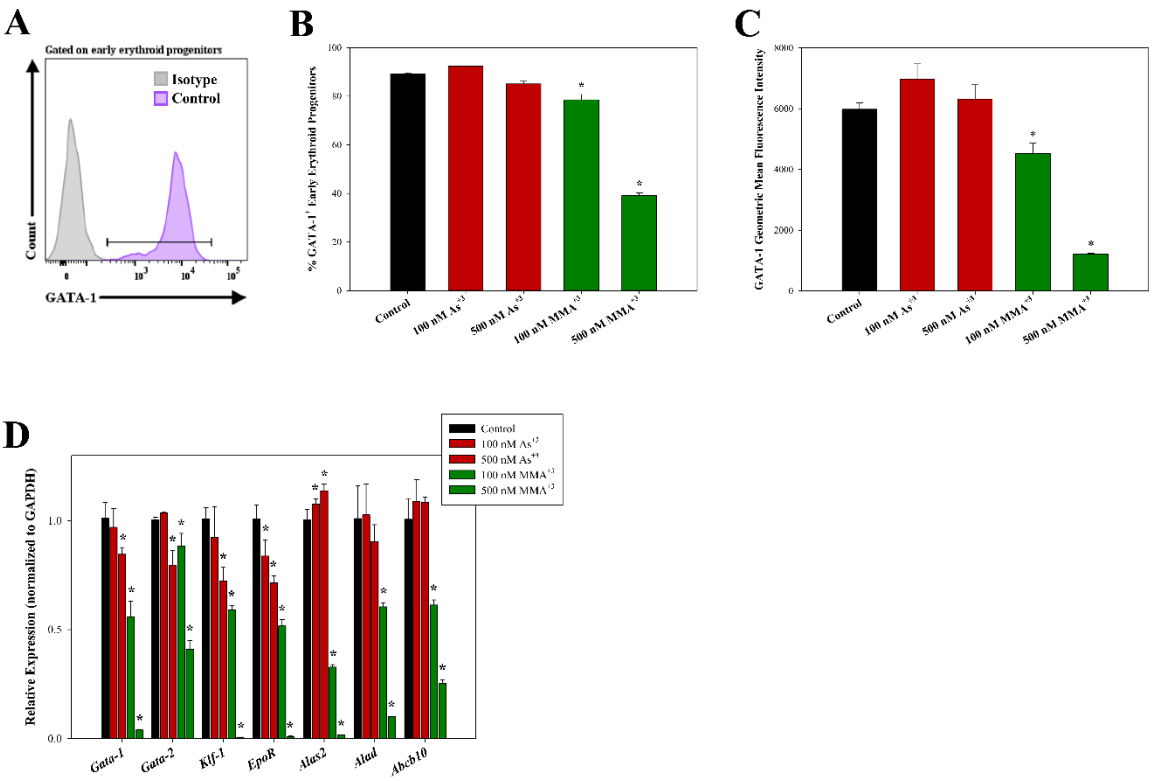

Figure 5.2. As^{+3} and MMA^{+3} produce differential suppressive effects on GATA-1 protein and GATA-1-regulated gene expression in erythroid progenitor cells. Erythroid progenitor cells were treated *in vitro* with 100 and 500 nM As^{+3} or MMA^{+3} for 24 h and GATA-1 and GATA-1-regulated genes expression was measured in early erythroid progenitors (BFU-E and CFU-E) by flow cytometry or qPCR, respectively. (A) GATA-1 gating showing isotype control and stained control sample. (B) Percentage of $GATA\text{-}1^{+}$ early erythroid progenitors following 24 h exposure to As^{+3} or MMA^{+3}. (B) GATA-1 geometric mean fluorescence intensity in early erythroid progenitors. (C) Relative expression of *Gata-1* and GATA-1-regulated genes (normalized to *Gapdh*) in erythroid progenitor cells after As^{+3} or MMA^{+3} exposure. Data are expressed as mean ± SD. *Statistically significant difference compared to control ($n = 3$; $p<0.05$).

MMA^{+3} suppresses STAT5 phosphorylation in early erythroid progenitors

A downstream pathway regulated by GATA-1 in coordination with EPO-activated STAT5 is a critical prosurvival pathway in early erythroid progenitors. As^{+3} and MMA^{+3} have been reported to inhibit the development of early B cells in the bone marrow and early T cells in the thymus by reducing STAT5 activation (Ezeh et al., 2016a; Xu et al., 2016a). Since the survival and subsequent maturation of erythroid progenitors is dependent on STAT5 signaling (Socolovsky et al., 1999; 2001), and taking into account that this pathway is partially regulated by GATA-1, we hypothesized that the suppression of early erythroid progenitor cell development may also be influenced by inhibition of STAT5 signaling.

To determine if As^{+3} or MMA^{+3} alter the activation of STAT5, we evaluated the phosphorylation of STAT5 (pSTAT5) in early erythroid progenitors using multiparameter intracellular flow cytometry, a method that we have previously validated by Western Blot (Ezeh et al., 2016a; Xu et al., 2016a). A significant reduction in the percentage of $pSTAT5^{+}$ early erythroid progenitors and the fluorescence intensity of pSTAT5 in early erythroid progenitors was detected following 24 h exposure to 100 and 500 nM MMA^{+3} (Figure 5.3A-C). A slight reduction of pSTAT5 was also observed with 500 nM As^{+3}; however, it was not statistically significant (Figure 5.3B-C).

These findings show that MMA^{+3}, but not As^{+3} significantly disrupted the activation of STAT5 in early erythroid progenitor cells. These findings suggest that; whereas, As^{+3} acts dominantly through the inhibition of GATA-1 function, MMA^{+3} impairs multiple pathways (GATA-1 and pSTAT5) important for erythroid progenitor differentiation.

As^{+3} and MMA^{+3} impaired GATA-1 and pSTAT5 regulated survival of early erythroid progenitors

To determine if the suppression of GATA-1 and STAT5 activation resulted in disruption of STAT5 and GATA-1 regulated survival of early erythroid progenitors, we measured two downstream factors in the prosurvival pathway (i.e., Bcl-x_L and cleaved caspase-3) using multiparameter intracellular flow cytometry. A significant reduction of the percentage of Bcl-x_L^+ erythroid progenitors was found after 24 h exposure to 500 nM As^{+3} and 100 and 500 nM MMA^{+3} (Figure 5.4A-B). Additionally, MMA^{+3}, but not As^{+3}, significantly decreased Bcl-x_L gene expression after 24 h exposure (Figure 5.4C).

Cleaved caspase-3, the terminal factor downstream of Bcl-x_L in the STAT5 and GATA-1 regulated survival pathway, was significantly increased in early erythroid progenitors treated with 500 nM As^{+3}, and 100 and 500 nM MMA^{+3} for 24 h (Figure 5A-B). In further support of the As^{+3} and MMA^{+3}-induced impairment of the GATA-1 and STAT5 mediated survival pathway, we measured cell death using annexin-V and PI staining in early erythroid progenitors by flow cytometry. At 500 nM As^{+3} and MMA^{+3}, there was a reduction of early apoptotic erythroid progenitors in favor of late apoptosis/necrosis (Figure 5.5C). The percentage of cell death (late apoptosis/necrosis, annexin-V^+, PI^+) among early erythroid progenitors was significantly increased by approximately 2-fold with 500 nM As^{+3} and 100 nM MMA^{+3} and approximately 20-fold with 500 nM MMA^{+3} (Figure 5.5C-D).

Taken together, these findings provide support that As^{+3} and MMA^{+3} impair GATA-1 and STAT5 regulated survival of early erythroid progenitors, as indicated by the loss of Bcl-x_L, increased caspase-3 activation, and increased death of this population.

Additionally, as observed in our previous experiments, the effects of MMA^{+3} on early erythroid progenitor cell survival were greater compared to As^{+3}.

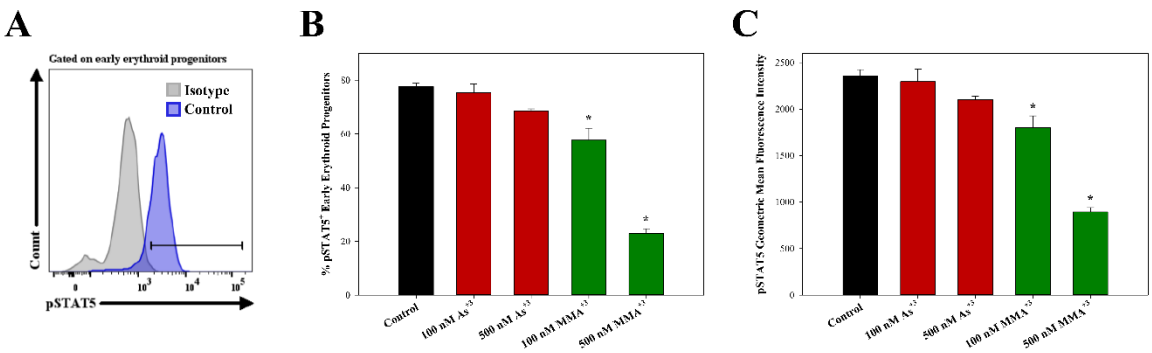

Figure 5.3. Effects of As^{+3} or MMA^{+3} on STAT5 activation in early erythroid progenitors. Erythroid progenitor cells were treated *in vitro* with 100 and 500 nM As^{+3} or MMA^{+3} for 24 h and STAT5 phosphorylation (pY694) (pSTAT5) was measured in early erythroid progenitors (BFU-E and CFU-E) by flow cytometry. (A) pSTAT5 gating showing isotype control and stained control sample. (B) Percentage of $pSTAT5^{+}$ early erythroid progenitors following exposure to As^{+3} or MMA^{+3}. (C) pSTAT5 geometric mean fluorescence intensity in early erythroid progenitors. Data are expressed as mean ± SD. *Statistically significant difference compared to control ($n = 3$; $p<0.05$).

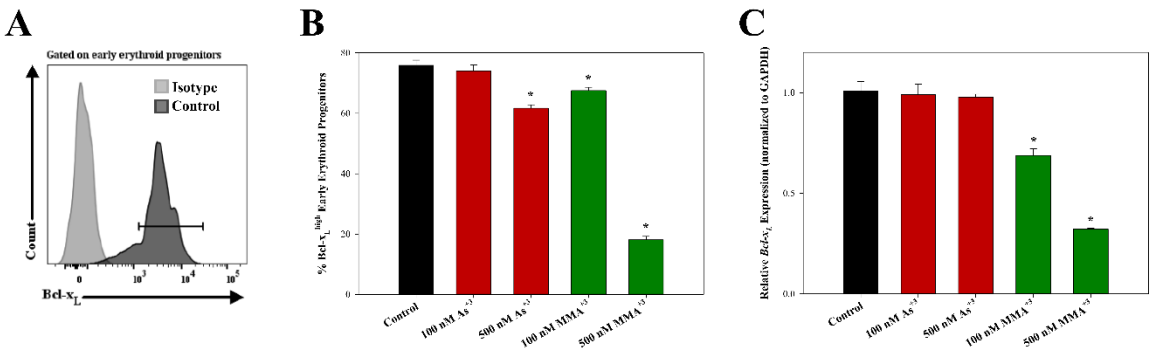

Figure 5.4. Effects of As^{+3} or MMA^{+3} on Bcl-x_L in early erythroid progenitors. Erythroid progenitor cells were treated *in vitro* with 100 and 500 nM As^{+3} or MMA^{+3} for 24 h and Bcl-x_L was measured in early erythroid progenitors (BFU-E and CFU-E) by flow cytometry. (A) Bcl-x_L gating showing isotype control and stained control sample. (B) Percentage of Bcl-x_L^+ early erythroid progenitors following treatment with As^{+3} or MMA^{+3}. (C) Relative *Bcl-x$_L$* mRNA expression (normalized to *Gapdh*) in erythroid progenitor cells following exposure to As^{+3} or MMA^{+3} for 24 h. Data are expressed as mean ± SD. *Statistically significant difference compared to control (n = 3; p<0.05).

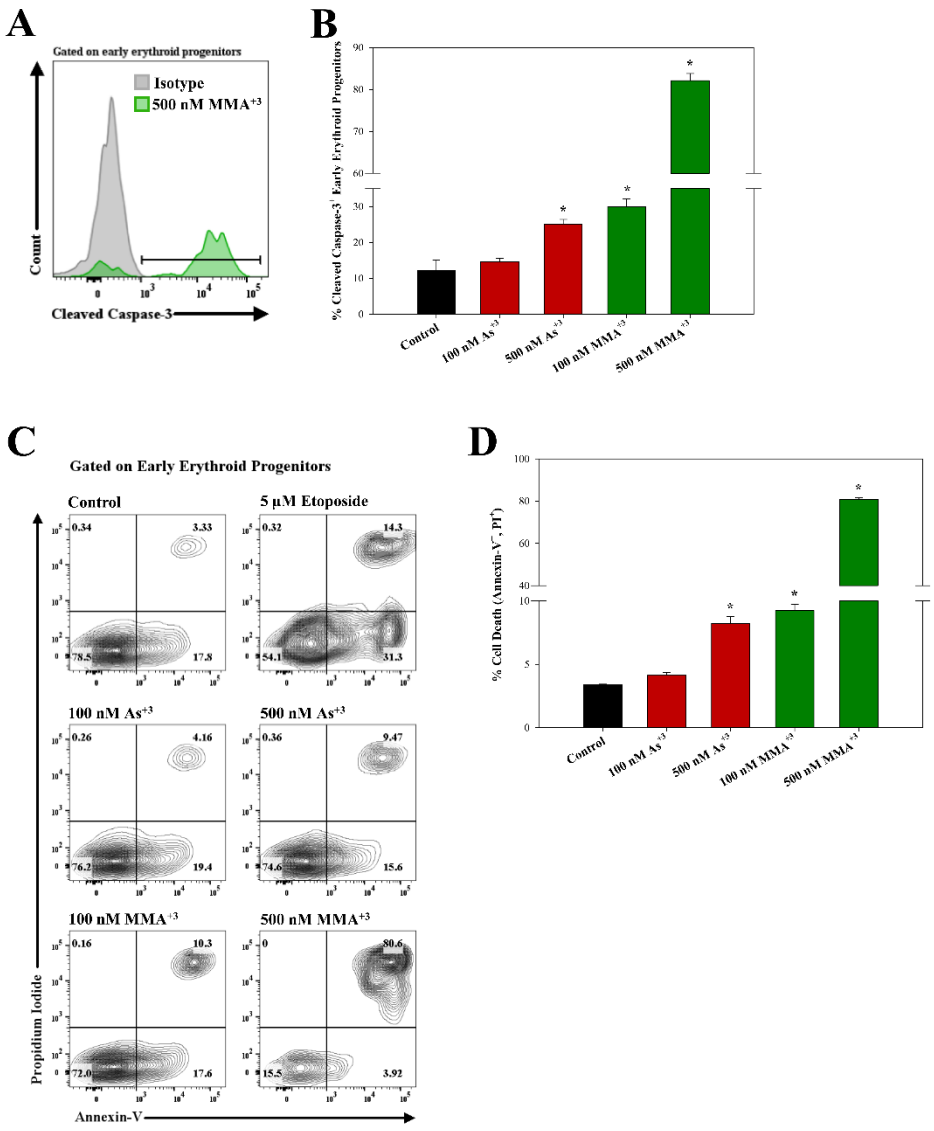

Figure 5.5. As^{+3} and MMA^{+3} increases cleaved caspase-3 and late stage apoptosis/necrosis of early erythroid progenitors. Erythroid progenitor cells were treated *in vitro* with 100 and 500 nM As^{+3} or MMA^{+3} for 24 h and cleaved caspase-3 and apoptosis (annexin-V and propidium iodide (PI) staining) were measured in early erythroid progenitors (BFU-E and CFU-E) by flow cytometry. (A) Cleaved-caspase 3 gating showing isotype control and 500 nM MMA^{+3} treated sample. (B) Percentage of cleaved-caspase 3 in early erythroid progenitor cells following exposure to As^{+3} or MMA^{+3}. (C) Apoptosis gating strategy showing representative annexin-V and PI staining in early erythroid progenitors treated with control (untreated), positive control (5 µM etoposide), 100 nM As^{+3}, 500 nM As^{+3}, 100 nM MMA^{+3}, or 500 nM MMA^{+3}. (D) Percentage of early apoptotic erythroid progenitors following 24 h As^{+3} or MMA^{+3} treatment. (E) Percentage of late apoptotic/necrotic early erythroid progenitors following 24 h As^{+3} or MMA^{+3} treatment. Data are expressed as mean ± SD. *Statistically significant difference compared to control ($n = 3$; $p<0.05$).

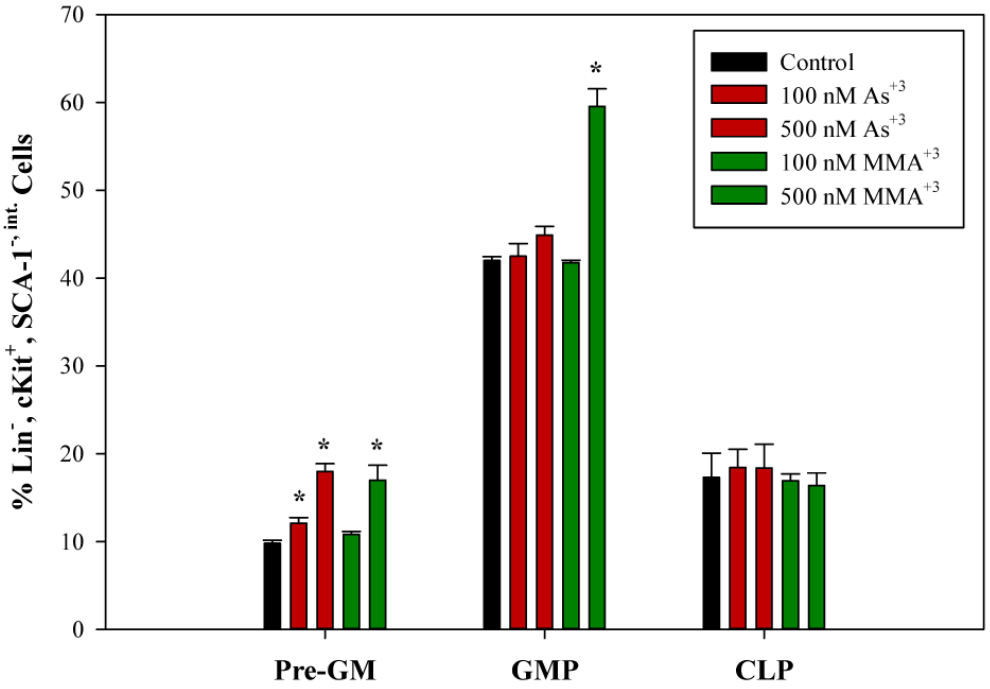

Figure 5.S1. As^{+3} and MMA^{+3} do not significantly reduce the percentages of other major progenitor cell lineages (myeloid and lymphoid). Effects of As^{+3} and MMA^{+3} on myeloid and lymphoid progenitors following *in vitro* stimulation of primary bone marrow hematopoietic progenitors with erythropoietin and stem cell factor in the presence of 100 and 500 nM As^{+3} or MMA^{+3} for 48 h. Myeloid and lymphoid progenitor differentiation was assessed by cell surface marker phenotype using flow cytometry as follows: Myeloid progenitors, pre-granulocyte macrophage (Pre-GM; $cKit^{+}$, $SCA\text{-}1^{-}$, $CD16/32^{-}$, $CD150^{-}$, $CD105^{-}$) and granulocyte macrophage progenitor (GMP; $cKit^{+}$, $SCA\text{-}1^{-}$, $CD150^{-}$, $CD16/32^{+}$) and common lymphoid progenitors (CLP; $cKit^{+}$, $SCA\text{-}1^{int}$, IL-7Rα^{+}). Data are expressed as mean ± SD. *Statistically significant difference compared to control ($n = 3$; $p<0.05$).

DISCUSSION

Erythropoiesis is a dynamic and highly regulated process that requires the coordination of many molecular regulators for normal development of erythroid progenitors (Dzierzak and Philipsen, 2013). In this study, we report that As^{+3} and MMA^{+3} impair erythropoiesis by disrupting two critical regulatory pathways controlling the differentiation and survival of early erythroid progenitor cells. As^{+3} and MMA^{+3} were found to suppress GATA-1 regulated erythroid differentiation and impair the GATA-1 and STAT5 regulated survival of early erythroid progenitor cells. We previously reported that the development of early erythroid progenitor cells is sensitive to As^{+3} exposure *in vivo* and *in vitro*, likely resulting from functional impairment of GATA-1 (Medina et al., 2017; Zhou et al., submitted).

In this study, As^{+3} and MMA^{+3} produced differential effects on GATA-1. As^{+3} primarily impaired GATA-1 function; whereas, MMA^{+3} also impaired GATA-1 regulation, as evidenced by decreased protein expression. As a result, MMA^{+3} produced stronger suppressive effects on the expression of erythroid-specific differentiation genes than equimolar doses of As^{+3}. The decrease of GATA-1-regulated genes with As^{+3} is consistent with our previous findings demonstrating that As^{+3} disrupts the zinc finger motifs of GATA, thereby inhibiting DNA binding ability and interactions with the transcriptional coactivator friend of GATA-1 (Zhou et al., submitted).

The functional impairment of GATA-1 caused by As^{+3} was also evident in this study, as the expression of GATA-1-regulated genes critical for erythroid differentiation were significantly reduced. Disruption of GATA-1 function by MMA^{+3} has not been

thoroughly evaluated. MMA^{+3} also disrupts the function of zinc finger proteins (Zhou et al., 2014), so there is a strong likelihood that it also compromises GATA-1 activity via a similar inhibitory mechanism as As^{+3}. The interaction of MMA^{+3} with the zinc finger motifs of GATA-1 requires further evaluation and will be the topic of our future investigations. Collectively, the suppression of GATA-1-regulated genes important for the development of early erythroid progenitors highlights a potential mechanism by which As^{+3} and MMA^{+3} compromise erythropoiesis.

Early erythroid cells are dependent on EPO for survival and differentiation (Wu et al., 1995; Dzierzak and Philipsen, 2013). The EPO receptor stimulates activation of STAT5, which in combination with GATA-1, functions to regulate the expression of the prosurvival factor, Bcl-x_L (Gregory et al., 1999). STAT5 knockout studies in mice and EPO deprivation studies in erythroid progenitor cell models show that loss of STAT5 corresponds to reduced Bcl-x_L, increased cell death, and inhibition of erythropoiesis (Gregory et al., 1999; Silva et al., 1996; Socolovsky et al., 1999, 2001). Similarly, GATA-1 knockout studies in erythroid progenitor cell models have also demonstrated that erythroid cells lacking this factor undergo maturation arrest and apoptosis (Weiss et al., 1994, 1995; Fujiwara et al., 1996).

In this study, we found that As^{+3} and MMA^{+3} reduced EPO receptor mRNA expression, the upstream regulator of STAT5. Additionally, we found that MMA^{+3}, but not As^{+3} significantly disrupted STAT5 activation. The suppression of EPO-activated STAT5 phosphorylation, along with the disruption of GATA-1 expression/function reduced Bcl-x_L in early erythroid progenitors. The loss of Bcl-x_L in early erythroid progenitors resulted in increased activation of caspase-3 mediated cell death. The greater

cell death response observed with MMA^{+3}, likely resulted from the combined loss of pSTAT5 and GATA-1 regulation of Bcl-x_L.

Several studies have found the phosphorylation of STAT5 is impaired by As^{+3} and MMA^{+3} exposure (Cheng et al., 2004a; Ezeh et al., 2016a; Xu et al., 2016a). We previously reported that As^{+3} and MMA^{+3} inhibit the development of pre-B cells in the bone marrow and pre-T cells in the thymus by reducing STAT5 activation (Ezeh et al., 2016a). In both of these studies, As^{+3} had a limited ability to disrupt STAT5 phosphorylation, in comparison with MMA^{+3} (Ezeh et al., 2016a; Xu et al., 2016a). Similarly, in the present study, phosphorylation of STAT5 was only significantly reduced by MMA^{+3}. Considering that the impairment of STAT5 activation has been observed in two separate progenitor cell lineages (lymphoid and erythroid), it may represent a common mechanism of As-induced toxicity among HPC.

Our results show that MMA^{+3} is more toxic than As^{+3}, which is consistent with many previous in vitro and in vivo studies (Petrick et al., 2001; Stýblo et al., 2000; Ezeh et al., 2016a; Xu et al., 2017). This is significant because we previously found that the predominant arsenical in the bone marrow of mice exposed to As^{+3} in their drinking water for 30 days was MMA^{+3}, reaching concentrations of approximately 80 nM (Xu et al., 2016b). Interestingly, there was no detectable As^{+3} in the bone marrow of these mice and only minimal levels of other As species (Xu et al., 2016b). As such, when evaluating As toxicity on bone marrow endpoints, it is important to perform comparative studies using both As^{+3} and MMA^{+3}.

In summary, we identified that two important erythropoiesis regulatory pathways are compromised by As^{+3} and MMA^{+3}. As^{+3} and MMA^{+3} were found to suppress the

expression of GATA-1-regulated genes important for promoting the differentiation of early erythroid progenitors. Additionally, As^{+3} and MMA^{+3} decreased the Bcl-x_L-mediated survival of early erythroid progenitor cells through impairments of GATA-1/STAT5 and GATA-1, respectively. These findings provide evidence that As disrupts both differentiation and survival of early erythroid progenitors, resulting in dyserythropoiesis. Collectively, results from the present study provide novel mechanistic insights by which As exposures can contribute to the development or exacerbation of anemia.

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CHAPTER 6

GENERAL DISCUSSION AND SIGNIFICANCE

GENERAL DISCUSSION

Anemia is a widespread public health issue (WHO 2015). Recent evidence indicates that the global prevalence of anemia is approximately 33% (Kassebaum et al., 2014). Many regions with high anemia prevalence are also disproportionately affected by arsenic contamination (Majumdor et al., 2009; Surdu et al., 2015; Kile et al., 2016). The high prevalence of anemia in these regions is undoubtedly influenced by socioeconomic determinants; however, even in the absence of common risk factors of anemia, associations between chronic arsenic exposure and anemia are still reported (Kile et al., 2016). Other epidemiological reports also highlight arsenic exposure as an important risk factor for anemia (Breton et al., 2006; Hopenhayn et al., 2006; Heck et al., 2008; Majumdor et al., 2009; Surdu et al., 2015; Kile et al., 2016). Despite the clear evidence of arsenic-associated anemia in chronically exposed human populations, the mechanisms of arsenic-induced hematotoxicity have not been elucidated.

A major cause of anemia is decreased RBC production in the bone marrow (Hodges et al., 2007; Gangat and Walanskyj 2013; Koury 2014). Erythropoiesis is a dynamic, multi-stage process responsible for producing staggering numbers of RBCs to maintain steady-state physiological conditions (Dzierzak and Philipsen 2013; Kim et al., 2015a). Although there is a direct correlation between reduced RBC production and anemia, very few studies have investigated the effects of arsenic on early RBC development. The present study provides evidence of arsenic-associated anemia in humans and also demonstrates that this phenotype can be recapitulated in mice following drinking water exposures to environmentally relevant levels of As^{+3} (Parvez et al., 2017;

Medina et al., 2017). For the first time, this work revealed novel mechanistic insights of how As^{+3} exposures impair the development of early RBCs, resulting in the inhibition of erythropoiesis (Zhou et al., submitted; Medina et al., in preparation). Collectively, these findings suggest that inhibition of erythropoiesis may be a significant contributing factor to the development and/or exacerbation of anemia in people chronically exposed to arsenic.

Chronic exposure to arsenic is associated with numerous detrimental health outcomes (Hughes, 2002; Heck et al., 2008; Naujokas et al., 2013; Tyler and Allan 2014; Ferrario et al., 2016). However, the hematotoxicity of arsenic is not well understood. Several studies have shown that arsenic is toxic to mature RBCs in circulation (Winski et al., 1997; Winski and Carter, 1998; Zhang et al., 2000; Biswas et al., 2008; Mahmud et al., 2009), but to our knowledge, no studies have been performed to understand the effects of environmentally relevant arsenic exposures on immature RBCs in the bone marrow.

In this research, we found that exposure of mice to As^{+3} at environmentally relevant levels for 60 days suppressed the development of early erythroid progenitor cells in the bone marrow (Medina et al., 2017). In contrast to expectations, we did not observe a decrease in circulating RBC counts in response to the observed reduction of erythroid progenitors in the bone marrow of As^{+3} exposed mice. There are two potential explanations for this outcome, the first is that the terminal time point of the study (60 days) may have been too short to observe systemic effects on RBC levels due to the fact that the circulating half-life of RBCs in mice is approximately 45 days. Secondly, anemic conditions, such as those caused by impairment of bone marrow erythropoiesis, induces

a physiological response mechanism known as stress erythropoiesis to cope with the loss of RBCs (Socolovsky 2007; Paulson et al., 2011).

An important distinction between mice and humans is that the primary site of stress erythropoiesis in mice is the spleen; whereas, in humans it occurs predominately in the bone marrow (Socolovsky 2007; Paulson et al., 2011; Kim et al., 2015b). Although we did not measure splenic erythropoiesis endpoints in this study, the contribution of stress erythropoiesis to the lack of RBC decrease cannot be fully excluded and should be evaluated in future investigations. In our study, it is likely that the As^{+3}-incuded suppression of bone marrow erythropoiesis resulted in the activation of stress erythropoiesis in the spleen, thereby producing a compensatory increase in RBC production. Consistent with this hypothesis, mice in our 60 day As^{+3} study demonstrated distinguishing factors of stress erythropoiesis, including elevated circulating EPO levels and slight trends of increase in total reticulocyte and RBC counts.

Differences in arsenic deposition and exposure provide insights to why erythropoiesis was compromised in the bone marrow, but likely not the spleen. We previously found minimal accumulation of arsenic species in the spleen; whereas, MMA^{+3} was found to accumulate in the bone marrow (Xu et al., 2016b). Collectively, results from this study were the first to show that chronic exposure to arsenic in mice caused anemia and our findings revealed that the differentiation of early erythroid progenitors is sensitive to *in vivo* As^{+3} exposures.

In an effort to elucidate the mechanistic basis for arsenic-induced suppression of early erythroid differentiation, we developed an *in vitro* model of erythropoiesis utilizing primary mouse bone marrow hematopoietic progenitor cells. Utilizing this model system,

we found that the suppressive effects of As^{+3} observed *in vivo* could be recapitulated *in vitro* using this erythropoiesis model. Additionally, we assessed the effects on MMA^{+3} on erythroid progenitor cell maturation. As^{+3} and MMA^{+3} were found to impair erythroid differentiation at environmentally relevant and physiologically achievable concentrations, respectively.

Similar to our *in vivo* findings, the differentiation of erythroid progenitors was impaired by *in vitro* exposures to As^{+3} and MMA^{+3}, beginning at very early stages of maturation. Several studies from our lab and others have reported that the differentiation of lymphoid progenitor cells in the bone marrow is sensitive to low levels of As^{+3} and MMA^{+3} (Ferrario et al., 2008; Ezeh et al., 2014; Ezeh et al., 2016a). Consistent with these observations, we found that another hematopoietic progenitor cell lineage was also sensitive to As^{+3} and MMA^{+3}. However, these findings were the first to show that exposure to environmentally relevant levels of arsenic disrupts the development of early RBCs.

Erythroid progenitors are dependent on the activity of a key transcription factor, GATA-1 (Fujiwara et al., 1996; Ferreira et al., 2005). GATA-1 is a zinc finger protein containing N- and C-terminal zinc finger domains of the C4 configuration (Ferreira et al., 2005). Both the N-and C-terminal zinc fingers are critical for the protein-protein interaction and DNA binding activities of GATA-1, respectively (Fox et al., 1999; Liew et al., 2005; Bates et al., 2008).

In this project, As^{+3} was found to interact with GATA-1 causing zinc loss and functional impairment of both zinc finger motifs of GATA-1 (Zhou et al., submitted). Previous studies found that zinc finger proteins, such as PARP-1 are sensitive molecular

targets of arsenic (Ding et al., 2009; Zhou et al., 2011; Sun et al., 2014; Zhou et al., 2015). Particularly, zinc finger motifs containing ≧3 cysteine residues have been found to be disrupted by As^{+3} (Zhou et al., 2011); whereas, MMA^{+3} is less selective and interacts with C2H2, C3H1, and C4 zinc fingers (Zhou et al., 2014).

Another important function of GATA-1 is hemoglobin production (Evans et al., 1988; Pevny et al., 1991; Pevny et al., 1995; Welch et al., 2004; Katsumuara et al., 2013). Consistent with our findings showing As^{+3}-induced impairment of GATA-1, in our 60-day drinking water study, blood hemoglobin levels were decreased in As^{+3} exposed mice, which is indicative of hypochromic anemia (Ali 1976). Since hemoglobin production is largely controlled by the actions of GATA-1, it is likely that the decreased hemoglobin levels were produced by suppressive effects on GATA-1.

This was the first study to identify that a critical regulator of erythropoiesis, the zinc finger transcription factor, GATA-1, is impaired by arsenic via the displacement of zinc, in a similar manner as described for PARP-1 (Zhou et al., 2011). The inhibition of GATA-1 resulted in dyserythropoieisis, caused by the loss of early erythroid progenitor differentiation. These findings were the first to identify a mechanism by which arsenic exposure selectively disrupts the development of RBCs and also provide insights into potential prevention and intervention strategies for arsenic-associated anemias.

Additionally, these studies revealed that arsenic influenced the lineage commitment choices of hematopoietic progenitors by selective interference with lineage-specific transcriptional regulators. Functional antagonism between GATA-1 and PU.1 dictates the lineage commitment patterns of early hematopoietic progenitors (Zhang et al., 1996; Rekhtman et al., 1999; Nerlov et al., 2000; Iwasaki and Akashi 2007). The

selective effects on GATA-1 vs. a non-zinc finger regulator of myeloid differentiation, PU.1, disrupted the lineage commitment of hematopoietic progenitors, causing a shift from erythropoiesis in favor of myelopoiesis.

We previously found that the predominant form of arsenic found in the bone marrow of mice exposed to As^{+3} via drinking water for 30 days was MMA^{+3} (Xu et al., 2016b). Interestingly, these mice did not have any measurable As^{+3} and only minimal levels of other arsenicals (Xu et al., 2016b). Since MMA^{+3} accumulates in the bone marrow, it is possible that the effects of As^{+3} *in vivo* may be mediated through the toxicity of MMA^{+3}. *In vivo* and *in vitro* studies show that MMA^{+3} is more toxic than As^{+3} (Petrick et al., 2001; Thomas et al., 2001; Stýblo et al., 2002). Consistent with these reports, in this study, MMA^{+3} was at least 5 times more toxic than As^{+3} to early developing erythroid progenitors. In comparison to As^{+3}, MMA^{+3} has a greater affinity for interactions with sulfhydryl groups in proteins, exerts greater inhibitory effects on enzymes, and is more cytotoxic than As^{+3} (Styblo et al., 1995; Styblo et al., 1997; Lin et al., 1999; Petrick et al., 2000; Shen et al., 2013). As such, it is likely that MMA^{+3} disrupts a broader spectrum of factors important for erythroid development than As^{+3}.

As^{+3} and MMA^{+3} were both found to disrupt the development of early erythroid progenitors through impairment of two critical regulatory pathways mediating differentiation and survival. Early erythroid progenitors are dependent on GATA-1 for the induction and repression of many genes critical for normal erythropoiesis (Ferreira et al., 2005). In addition, GATA-1 functions in cooperation with EPO-activated STAT5 to control the survival of early erythroid progenitors by promoting the optimal expression of the prosurvival factor, Bcl-x_L (Gregory et al., 1999).

In this study, As^{+3} and MMA^{+3} produced suppressive effects on GATA-1 and the induction of GATA-1 regulated genes necessary for normal erythroid differentiation, including the EPO receptor. As^{+3} primarily alters the function of GATA-1; whereas, MMA^{+3} disrupts the regulation and function of GATA-1, and also impairs the EPO-induced activation of STAT5. Previous studies from our lab have found that STAT5 activity is more sensitive to MMA^{+3} than As^{+3} exposures in early developing B and T cells. (Ezeh et al., 2016a; Xu et al., 2016a.). Consistent with previous studies, we also found that MMA^{+3} produced stronger suppressive effects on *Epo receptor* mRNA expression and STAT5 activation than As^{+3}. The suppressive effects of As^{+3} and MMA^{+3} on GATA-1 and GATA-1/EPO-activated STAT5 signaling, respectively, resulted in the loss of Bcl-x_L regulation, causing caspase-3 activation, and death of early erythroid progenitors.

These findings provide mechanistic support for the arsenic-caused inhibition of erythropoiesis observed *in vivo* by identifying that two critical regulatory pathways important for the differentiation and survival of early erythroid progenitors are disrupted by As^{+3} and MMA^{+3}. Loss of differentiation signaling, in combination with the aberrant death of early erythroid progenitor cells, results in decreased output of mature erythroid cells, and provides a likely mechanism by which arsenic can cause or contribute to anemia. Figure 6.1 provides a schematic representation summarizing the effects of As^{+3} and MMA^{+3} on early erythroid progenitor cell development.

In summary, this project provided critical insights into arsenic associated anemia by revealing that environmentally relevant arsenic exposures compromise the development of early erythroid progenitors in the bone marrow. This project also

characterized mechanisms responsible for As^{+3} and MMA^{+3}-induced suppression of erythropoiesis. Collectively, these findings elucidated novel mechanisms of arsenic-induced hematotoxicity directly relevant to anemia associated with chronic arsenic exposure. These results provide essential information for understanding the link between environmental arsenic exposures and anemia.

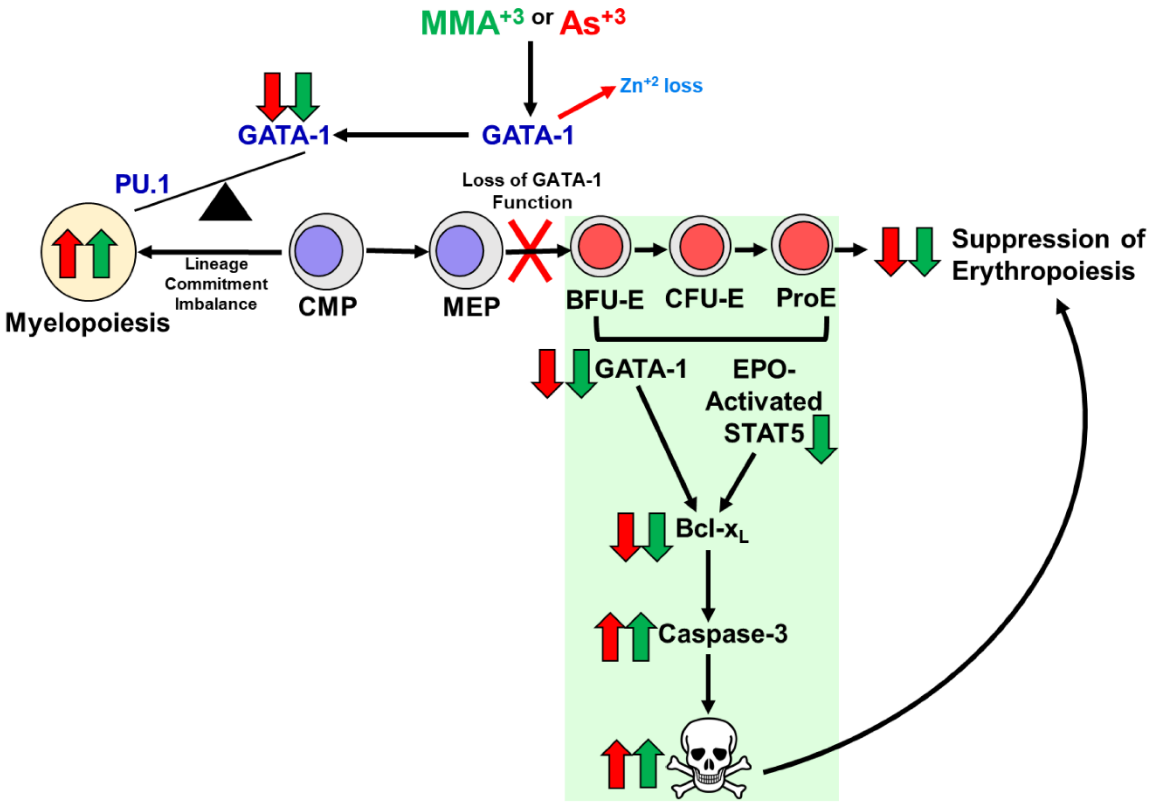

Figure 6.1. Summary of As^{+3} and MMA^{+3} effects on the development of early erythroid progenitors (EryP; BFU-E, CFU-E, ProE). As^{+3} and MMA^{+3} impair the function of GATA-1 in MEP and EryP causing a suppression of differentiation. As^{+3} binds to GATA-1 causing zinc loss and dysfunction. MMA^{+3} disrupts GATA-1 by reducing expression and activity. The loss of GATA-1 function and expression caused by As^{+3} and MMA^{+3}, respectively, also results in an imbalance between GATA-1 and PU.1, thereby shifting the lineage commitment of CMPs from erythropoiesis in favor of myelopoiesis. MMA^{+3}, but not As^{+3} reduces the EPO-induced pSTAT5. Loss of GATA-1 and GATA-1/pSTAT5 caused by As^{+3} and MMA^{+3}, respectively, reduces the expression of Bcl-x_L, resulting in increased caspase-3 mediated death of early erythroid progenitors. Overall, the disruption of GATA-1 regulated differentiation and erythroid lineage commitment, combined with the loss of GATA-1/pSTAT5-mediated survival of early erythroid progenitors, results in an overall suppression of erythropoiesis. Red and green arrows indicate As^{+3} or MMA^{+3} effects, respectively.

SUMMARY AND SIGNIFICANCE OF AIM 1

Studies in Aim 1 were separated into two components. The first part of Aim 1 was focused on understanding the association between arsenic exposure and anemia in a cohort of healthy male volunteers from rural Bangladesh enrolled in the HEALS. The purpose of our study was to provide additional evidence of the relationship between arsenic exposure and anemia in men. In this study we found dose-dependent decreases in RBC counts and hematocrit/packed cell volumes with increasing drinking water arsenic exposure levels. In an effort to gain an understanding of how arsenic exposures may interact with other environmental insults, we evaluated the influence of smoking status and arsenic exposure on clinical indicators of anemia.

These analyses showed that inverse relationships between arsenic exposure and hematological indicators of anemia were slightly stronger among smokers, indicating potential interactive effects of smoking and arsenic exposure on anemia. However, further studies are needed to provide further clarification of these observations. Collectively, findings from the first part of Aim 1 clearly demonstrated that chronic drinking water exposure to arsenic was associated with decreases in hematological indices consistent with clinical manifestations of anemia.

The second part of Aim 1 was focused on determining whether environmentally relevant As^{+3} exposure in mice could produce anemia. In these studies, we exposed male C57BL/6J mice to 0, 100, and 500 ppb As^{+3} via their drinking water for 60 days. After the 60-day exposure we evaluated hematological indicators of anemia and also assessed the development of early erythroid progenitors in the bone marrow using a combination

of colony forming assays and flow cytometry. We showed for the first time that chronic drinking water exposure to low-levels of As^{+3} caused anemia in mice, resulting from a suppression of early erythroid progenitor cell development in the bone marrow. The suppression of early erythroid progenitor maturation is consistent with previous studies from our lab and others that also report the development of hematopoietic progenitors is sensitive to As^{+3} and MMA^{+3}-induced toxicity (Ferrario et al., 2008; Ezeh et al., 2014; Ezeh et al., 2016a).

Collectively, studies from Aim 1 confirmed our hypotheses that arsenic exposure is associated with indicators of anemia in chronically exposed men and also that exposure to environmentally relevant levels of As^{+3} resulted in the development of anemia via suppression of early erythroid progenitor development in the bone marrow of mice. This is significant because not only did these findings provide additional epidemiological evidence of arsenic-associated anemia, but we also showed that anemia could be recapitulated in a mouse model following exposures to environmentally relevant concentrations of As^{+3}. These results also identified early erythroid progenitors (i.e., cell populations directly relevant to anemia) are sensitive to As^{+3}-induced toxicity.

SUMMARY AND SIGNIFICANCE OF AIM 2

Findings from Aim 1 reveled that early developing erythroid cells in the bone marrow are sensitive to *in vivo* As^{+3} exposures. Studies in Aim 2 were focused on understanding which specific stages of early erythroid differentiation were impacted by As^{+3} and MMA^{+3} exposure. In these studies, we utilized an *in vitro* model of erythropoiesis to assess the effects of As^{+3} and MMA^{+3} on the progression of hematopoietic progenitor cells through the stages of erythropoiesis.

Consistent with *in vivo* findings from Aim 1, we found that *in vitro* exposures to As^{+3} and MMA^{+3} in the nanomolar concentration range, suppressed the development of erythroid progenitors starting from very early stages of differentiation. The compromised differentiation of these early erythroid progenitors (i.e., MEP, BFU-E, CFU-E) persisted throughout erythropoiesis, resulting in significantly fewer erythroid progenitors reaching late stages of maturation. These studies confirmed our observations from Aim 1 that early erythroid progenitor subsets were disrupted by As^{+3} and MMA^{+3} exposures and provided additional information on the specific subsets impacted by arsenic exposure.

These findings also showed that MMA^{+3} produced suppressive effects to early erythroid progenitor differentiation that were at least 5 times greater than As^{+3}. This is significant because we recently found that the predominant species of arsenic that accumulates in the bone marrow of mice exposed to As^{+3} *in vivo* is MMA^{+3} (Xu et al. 2016b). This suggests that the suppressive effects on early erythroid differentiation observed in Aim 1 may be significantly mediated by MMA^{+3}-induced toxicity.

Overall, findings from these studies confirmed our hypothesis that As^{+3} and MMA^{+3} suppress the *in vitro* differentiation of primary mouse bone marrow erythroid progenitors. Our results clearly identified early erythroid progenitors as sensitive targets of As^{+3}- and MMA^{+3}-induced toxicity and also revealed differential sensitivities of these cells to As^{+3} and MMA^{+3}.

SUMMARY AND SIGNIFICANCE OF AIM 3

The development of early erythroid progenitors is dependent on the activity of the zinc finger transcription factor GATA-1 (Fujiwara et al., 1996; Ferreira et al., 2005). GATA-1 is responsible for orchestrating the expression of a wide range of erythroid specific genes critical for normal erythropoiesis. Additionally, in early erythroid progenitors, GATA-1 functions in cooperation with EPO-activated STAT5 to promote survival and differentiation (Gregory et al., 1999). Zinc finger proteins and STAT5 activation have been reported as targets of As^{+3} and MMA^{+3} toxicity (Cheng et al., 2004a; Ding et al., 2009; Zhou et al., 2011; Sun et al., 2014; Zhou et al., 2015; Ezeh et al., 2016a, Xu et al., 2016a).

Aims 1 and 2 demonstrated that early erythroid progenitors are sensitive to As^{+3} and MMA^{+3} exposures *in vivo* and *in vitro,* respectively. The development of early erythroid progenitor cells is dependent on the activities of GATA-1 and STAT5. Therefore, in Aim 3 we evaluated the hypothesis that As^{+3} and MMA^{+3} suppress the development of early erythroid progenitors by combined effects on differentiation and survival, caused by impairments of GATA-1 and EPO-activated STAT5 signaling.

Utilizing a combination of *in vivo* and *in vitro* studies, we found that As^{+3} selectively interacts with GATA-1, causing zinc loss, and inhibition of DNA and protein-protein binding activities. The loss of GATA-1 function resulted in dyserythropoiesis and an imbalance of hematopoietic differentiation. This was the first study to demonstrate that an essential regulator of erythropoiesis, is selectively impaired by As^{+3} exposure through zinc finger disruption. The inhibition of GATA-1 produced by As^{+3} was

independent of changes in expression, as no changes in GATA-1 protein levels were found in early erythroid progenitors. Follow up studies revealed that the loss of GATA-1 function reduced the expression of GATA-1 responsive genes critical for the differentiation of early erythroid progenitor cells.

In contrast to As^{+3}, MMA^{+3} disrupted the expression and function of GATA-1. Additionally, MMA^{+3}, but not As^{+3}, reduced the EPO-induced activation of STAT5. Loss of GATA-1 and GATA-1/STAT5 compromised the regulation of Bcl-x_L, which resulted in increased caspase-3-mediated death of early erythroid progenitors. This is significant because it demonstrated for the first time that As^{+3} and MMA^{+3} disrupt two critical regulatory pathways in early erythroid progenitors that control differentiation and survival. Collectively, As^{+3}- and MMA^{+3}-mediated effects on GATA-1 result in fewer cells differentiating to the erythroid lineage and cells that do differentiate to early stages of erythropoiesis are susceptible to death resulting from the loss of GATA-1 and STAT5 mediated survival. This presents a novel mechanism of arsenic-induced suppression of erythropoiesis.

These studies confirmed our hypothesis that As^{+3} and MMA^{+3} impair early erythroid progenitor development by combined effects on differentiation and survival signaling pathways. These findings were the first to clearly demonstrate a mechanistic basis of arsenic-induced suppression of early erythroid progenitor development. Taken together, these findings suggest that dysregulation of erythropoiesis is a probable mechanism of anemia in people chronically exposed to arsenic.

OVERALL SIGNIFICANCE

This is the first comprehensive study assessing the underlying mechanisms of arsenic-associated anemia reported in human populations chronically exposed to arsenic (Brenton et al., 2006; Hopenhayn et al., 2006; Heck et al., 2008; Majumdor et al., 2009; Surdu et al., 2015; Kile et al., 2016). In this study, we show a relationship between arsenic exposure and anemia in humans and this effect was recapitulated in mice, clearly establishing the role of arsenic exposure in the development of anemia. We report for the first time, that early stages of erythroid progenitor development are sensitive to arsenic-induced toxicity. This study also identified mechanisms of arsenic-induced suppression of erythropoiesis. These are the first findings to show that arsenic exposure compromises the function of the master regulatory factor of erythropoiesis, GATA-1.

Selective interaction of As^{+3} with the zinc finger motifs of GATA-1 was identified as a mechanism of As^{+3}-induced suppression of early erythroid progenitor differentiation. Not only did we identify that direct effects of As^{+3} on GATA-1 compromised the differentiation of early erythroid progenitors, but we also found that loss of GATA-1 impaired the regulation of a critical prosurvival factor, Bcl-x_L resulting in aberrant death of the early erythroid progenitor cell pool. This is the first report to link As^{+3} exposure to a molecular mechanism of impaired erythropoiesis, which is directly relevant for understanding the basis of anemia and other hematological disorders in human populations chronically exposed to arsenic.

Additionally, this was the first study to evaluate the effects of MMA^{+3} on erythroid progenitor development, which is significant because our studies revealed an

inverse association between urinary MMA and RBC counts in a cohort of men from rural Bangladesh (Parvez et al., 2017), highlighting the importance of arsenic metabolism in arsenic-associated anemias. People with variants of As3MT that reduce As^{+3} methylation capacity may be at an elevated risk of anemia resulting from MMA^{+3}-induced suppression of erythropoiesis. Our previous studies indicate that MMA^{+3} is the predominant form of arsenic in the bone marrow following *in vivo* As^{+3} exposures in mice (Xu et al., 2016b), suggesting that cells at this site are at an increased risk of MMA^{+3} exposure and toxicity. This study revealed for the first time that early stages of erythroid progenitor differentiation are more sensitive to MMA^{+3} than As^{+3} and also provided mechanistic information for the observed differences in toxicity.

MMA^{+3} produced differential effects on GATA-1 by reducing regulation and function, as indicated by decreased protein levels and by the suppression of GATA-1 responsive gene expression, respectively. In contrast to As^{+3}, MMA^{+3} also disrupted STAT5 activation. Similar to As^{+3}, but at concentrations 5 times lower, we found MMA^{+3} also disrupted GATA-1 regulated differentiation and caused a substantial loss of early erythroid progenitors by cell death resulting from the combined loss of GATA-1 and EPO-activated STAT5 regulation of Bcl-x_L. Inhibition of STAT5 activation has been reported in other progenitor lineages following MMA^{+3} exposure (Ezeh et al., 2016a; Xu et al., 2016a), and therefore, may represent a common mechanism of MMA^{+3} toxicity to hematopoietic progenitor cells.

For the first time, this study provides mechanistic evidence of arsenic-induced suppression of early erythroid progenitor development. Selective suppression of erythropoiesis by arsenic is a probable means by which environmental arsenic exposures

cause anemia or exacerbate existing anemia. Dysregulation of erythropoiesis is a potential mechanism underlying the high prevalence of anemia in people chronically exposed to arsenic. Findings of this project not only elucidated a mechanistic basis of arsenic-associated anemia, but also provide critical information for developing prevention and intervention strategies for arsenic and potentially other environmental metal exposure related anemias and hematological disorders.

FUTURE DIRECTIONS

The present study provided novel mechanistic insights into arsenic-induced suppression of erythropoiesis, which may be an underlying factor in arsenic associated anemias. However, the following questions should be addressed in future studies to provide further support for this phenomenon:

- Is the toxicity of As^{+3} and MMA^{+3} to early developing erythroid progenitors the same in males and females? The present study only focused on understanding the effects of As^{+3} and MMA^{+3} on erythropoiesis in males, therefore whether RBC development in females is impacted to the same extent and through the same mechanisms remains to be explored. This is a very important consideration, because many epidemiological studies have identified women as having a high risk of arsenic-associated anemia (Brenton et al., 2006; Hopenhayn et al., 2006; Heck et al., 2008; Majumdor et al., 2009; Surdu et al., 2015; Kile et al., 2016). To gain this understanding, the following hypothesis should be addressed: As^{+3} and MMA^{+3} suppress the development of early erythroid progenitors by combined effects on differentiation and survival caused by impairments of GATA-1 and EPO-activated STAT5 signaling in females. To test this hypothesis, a series of experiments similar to those performed in Aims 1-3 of this study can be performed.

- In the absence of erythropoietic contributions from the spleen, is loss of bone marrow erythropoiesis caused by arsenic sufficient to produce anemia in a mouse model? Since the primary erythropoietic and stress erythropoietic organ in humans is

the bone marrow (Socolovsky 2007; Paulson et al., 2011; Dzierzak and Philipsen 2013; Kim et al., 2015b), these studies would provide information more reflective of environmental arsenic exposures in human populations. Findings from the present study show that arsenic impairs bone marrow erythropoiesis; however, further *in vivo* studies are required to provide direct evidence that compromised bone marrow erythropoiesis is responsible for anemia development. This can be determined by exposing splenectomized (or sham control) male and female mice to As^{+3} at environmentally relevant levels in their drinking water for 90 days (twice the half-life of circulating RBCs) and inducing anemia by periodic bleeding (Moreau et al., 2012) or iron deficiency (Asperti et al., 2018). This would test the hypothesis that As^{+3}-induced inhibition of bone marrow erythropoiesis impairs the ability to recover from anemia, which in the absence of splenic contributions, would be dependent on the bone marrow.

- Is there a relationship between arsenic exposure, iron deficiency, and anemia? The vast majority of anemia worldwide is resultant of iron deficiency (WHO 2015), and many of the regions most affected by this type of anemia are also areas with high endemic arsenic exposures. Many of the aforementioned epidemiological studies of anemia in arsenic exposed women show exacerbations during pregnancy, suggesting potential interactive effects between arsenic exposure and pregnancy-related anemia, which is generally caused by iron deficiency (McMahon 2010). As such, it is important to understand whether arsenic exposures influence the severity of anemia in instances of iron deficiency. To address this, the following hypothesis should be tested: Iron deficiency anemia is exacerbated in males and females by

environmentally relevant exposures to As^{+3}. To test this hypothesis, the relationship between the presence of anemia and anemia severity can be determined in iron deficient men and women who are chronically exposed to arsenic. Many epidemiological cohorts, such those in rural Bangladesh, are in regions disproportionately affected by iron deficiency anemia (Ahmed 2000; Jamil et al., 2008) and could serve as ideal populations to understand the association between iron deficiency, chronic arsenic exposure, and anemia. Additionally, iron deficiency anemia is a common comorbidity among heart failure patients (Anand and Gupta 2018). As such, heart failure patients who have chronic arsenic exposures may also be good population for these studies.

Experiments can also be performed in the laboratory using *in vivo* mouse models. Male and female mice can be exposed to environmentally relevant levels of As^{+3} via drinking water and iron deficiency anemia can be simultaneously induced by feeding iron deficient chow. Anemia can be measured in unexposed and As^{+3} exposed mice based on hematological parameters. The development of RBCs can also be assessed using a combination of flow cytometry and colony forming assays. Pending the outcomes of these investigations more detailed follow-up hypotheses can be generated using *in vitro* and *in vivo* mouse models to better understand the mechanisms of these effects.

- Does MMA^{+3} disrupt cell cycle regulation in early erythroid progenitor cells? In early erythroid cells, KLF-1 expression is regulated by GATA-1. In this study we found that As^{+3} and MMA^{+3} disrupt GATA-1, resulting in decreased *Klf-1* mRNA expression (Figure 6.2A). KLF-1 regulates the activation of E2F2, which promotes

cell cycle entry (i.e., transition to S phase) and subsequent differentiation of early erythroid progenitor cells (Tallack et al., 2007, 2009; Pilon et al., 2008). Loss of KLF-1 and subsequent E2F2 regulation results in an accumulation of early $cKit^{+}$ progenitors that fail to undergo erythroid differentiation due to failure of successful cell cycle entry (Tallack et al., 2007, 2009; Pilon et al., 2008; Gnanapragasan et al., 2016). MMA^{+3} at 100 and 500 nM reduced *E2f2* mRNA expression in erythroid differentiated hematopoietic progenitor cells (HPC) after 24 h exposure (Figure 6.2B) and caused an increase in $cKit^{+}$, $SCA\text{-}1^{-}$ progenitors (Figure 6.2C). This suggests that the accumulation of HPC may be caused by the loss of E2F2-mediated cell cycle entry and failure to subsequently undergo erythroid differentiation.

These preliminary findings provide support for the following hypothesis: MMA^{+3} disrupts KLF-1 (possibly through the impairment of GATA-1), resulting in loss of E2F2 mediated cell cycle entry and failure of HPC to undergo erythropoiesis. This hypothesis can be tested using a combination of *in vitro* and *in vivo* mouse studies. Using the *in vitro* erythropoiesis model outlined in this study, erythroid progenitors can be exposed to MMA^{+3} at 0, 100, and 500 nM and the effects on cell cycle and cell cycle regulators (e.g., cyclins, cyclin dependent kinases, cyclin dependent kinase inhibitors) can be measured by flow cytometry. Additionally, effects on KLF-1 and E2F2 as well as on cell cycle and cell cycle regulators can be measured in early bone marrow erythroid progenitors following *in vivo* exposures of male and female mice to environmentally relevant levels of As^{+3}.

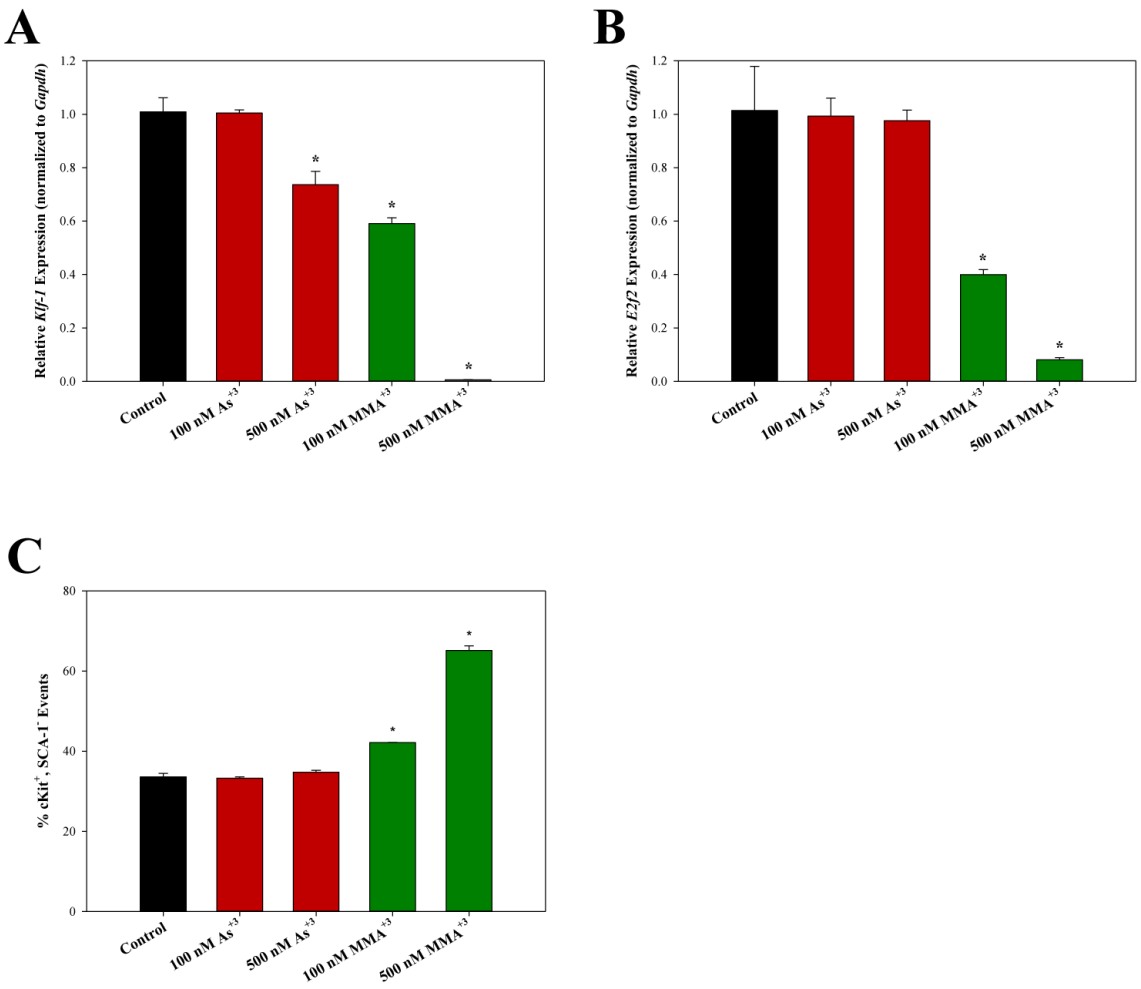

Figure 6.2. Effects of As^{+3} or MMA^{+3} on *KLf-1* and *E2f2* mRNA expression and $cKit^{+}$, $SCA\text{-}1^{-}$ progenitors. Hematopoietic progenitor cells were treated *in vitro* with 100 and 500 nM As^{+3} or MMA^{+3} for 48 h and *Klf-1* and *E2f2* mRNA expression was measured by quantitative real-time PCR. The percentage of $cKit^{+}$, $SCA\text{-}1^{-}$ progenitors was assessed by flow cytometry. (A) Klf-1 and (B) *E2f2* mRNA expression (normalized to *Gapdh*) following exposure to As^{+3} or MMA^{+3}. (C) Percentage of $cKit^{+}$, $SCA\text{-}1^{-}$ progenitors following treatment with As^{+3} or MMA^{+3}. Data are expressed as mean ± SD. *Statistically significant difference compared to control ($n = 3$; $p<0.05$).

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