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Environmentally Relevant Concentrations of Arsenite and Monomethylarsonous Acid Inhibit IL-7/STAT5 Cytokine Signaling Pathways in Mouse CD3+CD4–CD8– Double Negative Thymus Cells

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Abstract

Environmental arsenic exposure is a public health issue. Immunotoxicity induced by arsenic has been reported in humans and animal models. The purpose of this study was to evaluate mechanisms of As⁺³ and MMA⁺³ toxicity in mouse thymus cells. Because we know that MMA⁺³ inhibits IL-7 signaling in mouse bone marrow pre-B cells, we studied the influence of As⁺³ and MMA⁺³ on T cell development in the thymus at the earliest stage of T cell development (CD4–CD8–, double negative, DN) which requires IL-7 dependent signaling. We found in a DN thymus cell line (D1) that a low concentration of MMA⁺³ (50 nM) suppressed IL-7 dependent JAK1, 3 and STAT5 signaling. As⁺³ suppressed STAT5 and Jak3 activations at higher concentrations (500 nM). Cell surface expression of the IL-7 receptor (CD127) was also suppressed by 50 nM MMA⁺³, but was increased by 500 nM As⁺³, indicating possible differences in the mechanisms of action of these agents. A decrease in cyclin D1 protein expression was observed in D1 cells exposed to As⁺³ at 500 nM and MMA⁺³ starting at 50 nM, suggesting that arsenic at these environmentally-relevant doses suppresses early T cell development through the inhibition of IL-7 signaling pathway.

Keywords

arsenite; monomethylarsonous acid; double negative T cells; IL-7 signaling; Jak/STAT; cyclin D1; selective toxicity

1. INTRODUCTION

Arsenic exposure in food and drinking water is a worldwide public health issue. Arsenic is known to cause suppression of the immune system in multiple systems (Ahmed et al., 2014; Biswas et al., 2008; Nadeau et al., 2014; Soto-Peña et al., 2006; Vahter et al., 2008).

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However, the mechanism of arsenic induced immunosuppression is still poorly understood. Previous studies in our laboratory found that environmentally-relevant doses of arsenite (As^{+3}) and its metabolite, monomethylarsonous acid (MMA^{+3}) suppressed T cell function and early B cell proliferation at extremely low concentrations (Burchiel et al., 2014; Ezeh et al., 2014), indicating that immune cells are very sensitive to As^{+3} and MMA^{+3} exposure.

Early T cells are originally produced in bone marrow and transported into the thymus for development and selection. T cells in thymus go through double negative (CD4-CD8- , DN), double positive (CD4+CD8+ , DP) and single positive ($\text{CD4+}/\text{CD8+}$, SP) stages, committing to either CD4+ T helper cells or CD8+ cytotoxic T cells (Germain, 2002). The conversion of early pre-T DN cells to DP cells is controlled by IL-7 signaling (Boudil et al., 2015). Suppression of DN T cells has been documented to have detrimental effects on immune functions (Ohm et al., 2003; Seinen and Penninks, 1979). IL-7 is secreted by stromal cells in bone marrow and thymus and is a critical cytokine for the proliferation of T cells during DN stage (Namen et al., 1988). The IL-7 receptor (IL-7R) is a heterodimer consisting both an α chain and a common γ chain (γc). The binding of IL-7 to IL-7R leads to a cascade of signals of Janus Kinases (JAKs) and Signal Transducers and Activators of Transcription (STATs), inducing the transcription of multiple genes such as Pim1 and Cyclin D1 (de Groot et al., 2000; Linowes et al., 2013). STAT5 is phosphorylated by phospho-JAK1 (pJAK1) and phospho-STAT5 (pSTAT5) is stabilized by phospho-JAK3 (pJAK3) (Haan et al., 2011). Once pSTAT5 is dimerized and translocated into the nucleus, it binds to STAT5 response elements on genes that control proliferation and differentiation (Nosaka et al., 1999).

Arsenic-induced alterations in cell signaling, including the inhibition of tyrosine kinase activation or ROS production, can have potent non-genotoxic effects on cells (Qian et al., 2003). The inhibitions on STATs and JAKs in γc receptor signaling by arsenic were observed by different groups (Cheng et al., 2004; Hayashi et al., 2002). However, all these existing studies were conducted with high doses of arsenic. Since there is evidence showing that low concentrations of As^{+3} and MMA^{+3} inhibit the proliferation of IL-7 dependent lymphoid progenitors (Ezeh et al., 2014), the present study was focused on the effects of environmentally-relevant non-cytotoxic doses of As^{+3} and MMA^{+3} within nanomolar ranges on IL-7 signaling pathway of DN mouse thymus cells, the early developing T cell population which affects the whole immune system.

2. MATERIAL AND METHODS

2.1 Chemicals and reagents

Sodium arsenite (CAS 774-46-5, NaAsO_2) was purchased from Sigma-Aldrich (St. Louis, MO). MMA^{+3} was a gift from Dr. Terry Monks at Southwest Environmental Health Science Center of the University of Arizona. Hanks Balanced Salt Solution (HBSS) was purchased from Lonza (Walkersville, MD). Penicillin/Streptomycin 10,000 (mg/ml)/10,000 (U/ml) (Pen/Strep) and 200 mM L-Glutamine were purchased from Life Technologies (Grand Island, NY). β -Mercaptoethanol (2-ME), Dulbecco's phosphate buffered saline w/o Ca^{+2} or Mg^{+2} (DPBS⁻), and base medium RPMI 1640 were purchased from Sigma Aldrich. Mouse IL-7 was purchased from Peprotech (Rocky Hill, NJ). Fetal Bovine Serum (FBS) was

purchased from Atlanta Biologicals (Flowery Branch, GA.), Alexa Fluor® 488 Rat Anti-Mouse CD127 (Cat. No. 561533), Alexa Fluor® 488 Rat IgG2b, κ Isotype Control (Cat. No. 557726), FITC Rat Anti-Mouse CD8a (Cat. No. 553031), APC Rat Anti-Mouse CD4 (Cat. No. 553051), PE-Cy™7 Hamster Anti-Mouse CD3e (Cat. No. 552774), purified mouse anti-JAK1 monoclonal antibody (Cat. No. 610231) and purified mouse anti-STAT5 (Cat. No. 610191) were purchased from BD Biosciences (San Jose, CA). PE Anti-Hu/Mo pSTAT5 (Y694) (Cat. No. 12-9010-42), PE Mouse IgG1 κ Isotype Control (Cat. No. 12-4714-42) and IC fixation buffer (Cat. No. 00-8222-49) were purchased from eBioscience (San Diego, CA). Cyclin D1 (DCS-6) PE (Cat. No. sc-20044 PE) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-STAT5 (Tyr694) (Cat. No. 9359), anti-phospho-JAK1 (Tyr1022/1023) (Cat. No. 3331), anti-phospho-JAK3 (Tyr980/981) (Cat. No. 5031), anti-JAK3 (Cat. No. 3775), anti-rabbit IgG –HRP secondary antibody (Cat. No. 7074) and anti-mouse IgG-HRP secondary antibody (Cat. No. 7076) were purchased from Cell Signaling Technology (Danvers, MA).

2.2 Thymus cells in vitro treatments and intracellular flow cytometry assays

Thymus were harvested from three C57BL/6J male mice purchased at 8 weeks of age from Jackson Laboratory (Bar Harbor, ME), cells were isolated and pooled. Mice were handled in accordance with protocols approved by the Institutional Animal Use and Care Committee at the University of New Mexico Health Sciences Center in our AAALAC-approved animal facility. One million cells were treated with different doses of As⁺³ or MMA⁺³ in the medium consisting of RPMI-1640, 10% FBS, 1% Pen/Strep, and 2mM L-Glutamine at 37 °C for 18 h. For pSTAT5 intracellular staining, cells were washed and resuspended in 100 μ l of DPBS⁻ and incubated with 5 μ l cocktail consisting of 0.5 μ g of CD8, CD4 and CD3 antibodies or DPBS⁻ (unstained) for 30 min at RT in dark and fixed by adding 100 μ l of IC fixation buffer and incubated for 30 min at RT in dark. Following incubation, cells were washed twice with DPBS⁻ and then incubated in 100 μ l of 90% –20 °C methanol for 30 min 4 °C in dark. Washed cells were resuspended in 100 μ l of DPBS⁻ and stained with 5 μ l of intracellular staining antibody (pSTAT5, cyclin D1) or isotype control and incubated for 30 min at RT in dark. Cells were washed twice with DPBS⁻ before being analyzed on an AccuriC6 Flow Analyzer (BD Biosciences).

2.3 D1 cells pSTAT5 and pJAKs Western Blot assays

The D1 cell line was a gift from Dr. Scott K. Durum (Center for Cancer Research, National Institute of Health, Frederick, MD). It is a CD3+CD4–CD8–, IL-7 dependent pre-T cell line established from p53–/– mouse thymocytes (Kim et al., 2003). Cells were maintained in RPMI 1640 with 2 mM L-glutamine, 1% Pen/Strep, 55 μ M 2-ME and 50 ng/mL IL-7. Cells were arrested in G1 phase by 18 h of IL-7 deprivation and treated with As⁺³ or MMA⁺³ at the same time. 50 ng/mL of IL-7 was added to induce IL-7 signaling for 30 min before harvesting. Cell lysates (20 μ g protein/well) were separated by SDS-PAGE electrophoresis and transferred onto nitrocellulose membranes for 15 min using semi-dry transferring system (Bio-Rad, Hercules, CA). Membranes were blocked with 5% Blotting Grade Blocker (Bio-Rad) in TBST (50 mM Tris, 150 mM NaCl and 0.05% Tween 20), and incubated with antibodies for pSTAT5, pJAK1 or pJAK3, followed by HRP conjugated antibodies. The membranes were stripped with pH 2.2 glycine stripping buffer (15 g glycine, 1 g SDS and

10 ml Tween 20 in a total of 1 L) and probed for total STAT5, JAK1 and JAK3. Signals were detected using SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific, Rockford, IL) and FluorChem™ M System (ProteinSimple, San Jose, CA).

2.4 D1 cells CD127 staining

After treatments with As⁺³ or MMA⁺³, D1 cells were washed, resuspended in 100 µl DPBS- and stained with 0.5 µg of anti-CD127 or IgG2b κ Isotype Control for 30 min at RT in the dark. Cells were washed twice with DPBS⁻ before analyzed on an AccuriC6 Flow Analyzer

2.5 Statistics and Data Analysis

All data were analyzed with Excel 2010 or SigmaPlot version 12.5, one way analyses of variance (ANOVA) and Dunnetts t-test were used to determine the differences between control and treatment groups. For flow cytometry experiments, each single dose was done in triplicate. For Western Blot experiments, three independent experiments were performed for each assay. Image J (1.48v) software (NIH, downloaded from website: <http://rsb.info.nih.gov/ij/>) was used to obtain band intensities for treatments and control samples.

3. RESULTS

3.1 As⁺³ and MMA⁺³ suppressed STAT5 phosphorylation in mouse DN T cells *in vitro*

Mouse thymus cells were isolated and exposed to 5, 50 and 500 nM As⁺³ and MMA⁺³ *in vitro* for 18 h. The DN T cell population was defined as negative for both CD4 and CD8 markers in CD3⁺ thymocytes (Fig. 1A) using flow cytometry. STAT5 phosphorylation was measured by intracellular staining and found to be inhibited by As⁺³ at 500 nM and MMA⁺³ from 50 nM (Fig. 2B), indicating a suppression of the IL-7 signaling pathway at these doses *in vitro*.

A DN, IL-7 dependent mouse thymic cell line, D1, was utilized to confirm our observations in the primary mouse DN T cells. STAT5 activation is IL-7 responsive in D1 cells, and neither As⁺³ or MMA⁺³ induced STAT5 phosphorylation without IL-7 (Fig. 1C and 1D). A significant decrease in STAT5 phosphorylation was seen in 500 nM As⁺³ and 50 nM MMA⁺³ treatments (Fig. 1C and 1D), confirming the observations in primary mouse DN T cells.

3.2 As⁺³ and MMA⁺³ suppressed JAK1 and JAK3 phosphorylation in D1 cells *in vitro*

Since the suppression of STAT5 phosphorylation was observed in DN T cells, phosphorylation of JAK1 and JAK3, upstream tyrosine kinases which phosphorylate STAT5 in the IL-7 signaling pathway, was examined by Western Blot after IL-7 stimulation in D1 cells. As we can see in Fig. 2, the absence of IL-7 also depleted JAK1 and JAK3 phosphorylation. 50 nM MMA⁺³ exposure suppressed JAK1 phosphorylation (Fig. 2A and 2B) and 500 nM As⁺³ or 50 nM MMA⁺³ exposures inhibited JAK3 phosphorylation (Fig. 2C and 2D) in IL-7 stimulated D1 cells. These results indicate that suppression of STAT5 phosphorylation by As⁺³ and MMA⁺³ exposures may be due to JAK1 and JAK3 inhibition.

3.3 MMA⁺³ suppressed IL-7R (CD127) expression on cell surface

In order to see if IL-7R expression is affected by As⁺³ and MMA⁺³ treatments, D1 cells exposed to 50 and 500 nM As⁺³ or 5 and 50 nM MMA⁺³ for 18 h *in vitro* were stained with CD127 antibody. A decrease in cell surface expression of IL-7R was observed in D1 cells exposed to 50 nM MMA⁺³ (Fig. 3). Interestingly, 500 nM As⁺³ increased the expression of IL-7R, indicating that As⁺³ and MMA⁺³ may have differential mechanisms in IL-7 signaling suppression. The results are also confirmed by Western Blot (Supplementary Fig. 1).

3.4 As⁺³ and MMA⁺³ selectively targeted DN T cells and decreased cell cycle protein expression

Since the suppression of IL-7 signaling pathways in mouse DN T cells by As⁺³ and MMA⁺³ was confirmed, we wanted to determine whether the DN subset is selectively affected and evaluate a potential mechanism. We performed intracellular staining for cyclin D1, an indicator of cell cycle progression which is transcriptionally regulated by STAT5 activation (Matsumura et al., 1999). These studies were performed in primary mouse DN thymus cells exposed to As⁺³ and MMA⁺³ for 18 h *in vitro*. For MMA⁺³ exposures, we also applied doses between 50 nM and 500 nM to get more information on dose response within the nanomolar range. MMA⁺³ significantly decreased the number of DN cells, but had no effect on the DP cell populations (Fig 4A, 4B). We have previously shown that these concentrations did not affect cell viability, therefore the fact that fewer cells were being recovered suggested that MMA and As⁺³ might affect the entry of cells into this compartment by blocking IL-7 dependent proliferation and differentiation. We therefore examined cyclin D1 expression in the DN cells treated with MMA⁺³ (50-200 nM) and As⁺³ (500 nM). A statistically significant inhibition of cyclin D1 was seen at 100 nM MMA⁺³ and 500 nM As⁺³ indicating that these STAT5-dependent proteins was affected by these treatments (Fig. 4C, D).

4. DISCUSSION

Environmental exposures to arsenic via drinking water and food are known to be associated with vascular diseases, lung diseases, and different types of cancers (Argos et al., 2010; Chen, et al., 2011; Navas-Acien et al., 2005). Immunological suppression caused by arsenic has been related to the occurrence of several diseases and has been addressed by many research groups (Dangleben et al., 2013). However, most of the studies on arsenic immunotoxicity used high concentrations within low to high micromolar. Since the accessible doses of arsenic in the environment are usually low (Josyula et al., 2006), studying the immunotoxic effects of nanomolar doses of arsenic are important for environmentally-relevant exposures. Previously, we demonstrated that As⁺³ had differential genotoxic effects in mouse DN thymus cells at low concentrations that did not induce apoptosis (Xu et al., 2016). Therefore, the present study shows that As⁺³ and MMA⁺³ inhibits a critical signaling pathway in the same cell population via nongenotoxic mechanism resulting in inhibition of pre-T cell differentiation.

IL-7 signaling is critical for early T cell development in thymus. It has been showed that high concentrations of As⁺³ significantly inhibited γ c receptor signaling such as IL-2 and

IL-6 (Cheng et al., 2004). Previously, we showed that MMA^{+3} had a strong inhibitory effect on IL-7 signaling in pre-B cells within nanomolar range (Ezeh et al., 2016). The observation of a similar pattern of suppression by low concentrations of As^{+3} and MMA^{+3} in bone marrow Pre-B and thymic DN T cells suggests a common mechanism of arsenic induced immunosuppression in lymphoid progenitor cells. Because IL-7R (CD127) expression was decreased by MMA^{+3} in both systems, it is possible that receptor modulation, in addition to actions on downstream targets is responsible for inhibition of STA5 signaling. Because 500 nM As^{+3} increased the receptor expression in D1 cells, and did not affect CD127 expression in a pre-B cell model (Ezeh et al., 2015), it is likely that As^{+3} suppression of IL-7 signaling occurs downstream of the receptor by actions on JAK or STAT by modulating protein phosphorylation (Xue et al., 2011; Paul et al., 2007). Therefore, the possibility that inorganic and organic forms of arsenic act by different mechanisms in γc receptor signaling should be further explored.

As^{+3} is the prevalent form of arsenic in nature. After the intake of As^{+3} , MMA^{+3} is formed *in vivo* through the action of arsenite-3-methyltransferase, which is highly expressed in the liver (Aposhian et al., 2000). Previous studies have indicated that MMA^{+3} is more toxic than As^{+3} (Orihuela et al., 2013; Petrick et al., 2000). In this study, MMA^{+3} was found to be 10 times more effective in suppressing STAT5 and JAK1, 3 phosphorylation (Fig 1 and 2). Trivalent arsenicals are known to induce toxicity by binding to thiol-containing molecules (Styblo et al., 1995). The differences between As^{+3} and MMA^{+3} in protein binding is that As^{+3} requires a structure with 3 cysteines, while MMA^{+3} needs only 2, which significantly increases the numbers of its binding targets (Shen et al., 2013). Therefore, it is possible that MMA^{+3} reacts directly on the IL-7R, resulting in suppression of the downstream activation pathways. As^{+3} is known to interact with JAK kinase proteins directly (Cheng et al., 2004). Since the activation of the downstream transcriptional factor, STAT5, is required for early T cell proliferation, it is likely that the cells would express more IL-7R on their surfaces as compensation.

Since DN thymus cells are sensitive to As^{+3} and MMA^{+3} at low doses, the immunosuppression induced by arsenic exposure may occur at very early stages of T cell differentiation in the thymus. In this study, we found that the percentage of DN subset was decreased following As^{+3} and MMA^{+3} *in vitro* exposures, and the percentage of the DP subset was not affected (Fig. 4). The selection suppression on the DN early T cell subset may results in a decrease of the total thymus cells. We found a trend for a decrease in thymus weight and total cell recovery in male mice after 30 d low dose drinking water exposure to As^{+3} (Supplemental Fig. 2). There are also reports showing that prenatal arsenic exposures are related to the altered lymphoid cell development and thymic atrophy (Ahmed et al., 2012; Raqib et al., 2009). It is very possible that these early life developmental suppressions are associated with the high sensitivity of the DN T cell population. Therefore, studies addressing the differences between the DN population and other populations such as the DP T cells or mature T cells are also important for understanding the mechanisms of arsenic induced immunosuppression in environmental exposures.

Collectively, As^{+3} and MMA^{+3} inhibit IL-7 signaling in mouse DN thymus cells at environmentally relevant concentrations. The two forms of arsenic have differential

effective doses and may act through distinct mechanisms. The suppression of the IL-7 signaling result in the decrease of cyclin D1, a STAT5 transcriptionally-regulated cell cycle protein, that controls cell proliferation, suggesting a mechanistic explanation of the reduction of the DN T cell population, which exhibits high sensitivity to arsenic exposures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

- As^{+3} and MMA^{+3} suppress IL-7 signaling as measured by pSTAT5 in DN thymocytes.
- JAK1 was inhibited by MMA^{+3} , while JAK3 was inhibited by MMA^{+3} and As^{+3} .
- MMA^{+3} decreased but As^{+3} increased cell surface expression of the IL-7 receptors.
- MMA^{+3} and As^{+3} exposures selectively decreased the DN cell population *in vitro*.
- In DN cells, MMA^{+3} (50 nM) and As^{+3} (500 nM) inhibited cyclin D1 expression.

Fig. 1.

Suppression of STAT5 phosphorylation in DN primary thymus T cells exposed to As³⁺ and MMA³⁺ *in vitro*. Primary mouse thymus cells were treated with 5, 50 and 500 nM As³⁺ and MMA³⁺. A, DN T cell population defined by CD3, CD4 and CD8 cell surface markers and isotype control for pSTAT5 intracellular staining. B, intracellular pSTAT5 levels were measured by multiparameter flow cytometry. In panels 1C and 1D, D1 cells were starved 18 h for IL-7 with As³⁺ and MMA³⁺ treatments. pSTAT5 levels were measured 30 min after re-adding IL-7, three independent experiments were performed. C, one representative pSTAT5 and STAT5 blot. D, pSTAT5 to STAT5 band intensities from three independent experiments. *Significantly different compared to control (p < 0.05). Results are Means $\pm$ SD.

Fig. 2. Suppression of JAK1 and JAK3 phosphorylation in a mouse DN cell line (D1) exposed to As³⁺ and MMA³⁺ *in vitro*. D1 cells were starved 18 h for IL-7 with As³⁺ and MMA³⁺ treatments. pJAK1 and JAK3 levels were measured 30 min after re-adding IL-7, three independent experiments were performed. A, one representative pJAK1 and JAK1 blot is shown. B, pJAK1 to JAK1 band intensities from three independent experiments are shown. C, one representative pJAK3 and JAK3 blot is shown. D, pJAK3 to JAK3 band intensities from three independent experiments are shown. *Significantly different compared to control (p<0.05). Results are Means $\pm$ SD.

Fig. 3. IL-7R (CD127) cell surface expression in D1 cells exposed to As³⁺ and MMA³⁺ in vitro. D1 cells were exposed to As³⁺ and MMA³⁺ for 18 h and stained with anti-CD127 antibody. A, isotype and staining control. B, surface IL-7R expression measured by CD127 mean channel fluorescence.*Significantly different compared to control (p<0.05). Results are Means $\pm$ SD.

Fig. 4.

A, Decrease in the percentage of CD3+ DN (CD4-, CD8-) cells recovered from primary thymus cells treated for 18 h with MMA⁺³ or As⁺³. B, lack of effect of MMA⁺³ and As⁺³ on the CD3+ DP (CD4+, CD8+) population of thymus cells. C, cyclin D1 fluorescence detected by flow cytometry is shown for a specific antibody (left) and an isotype control (right) for primary mouse thymus cells. D, cyclin D1 expression measured by multiparameter flow cytometry in CD3+ DN primary mouse thymocytes treated with 50 and 500 nM As⁺³ or 50, 100 and 200 nM MMA⁺³ for 18 h *in vitro*. *Significantly different compared to control ($p < 0.05$). Results are Means $\pm$ SD.