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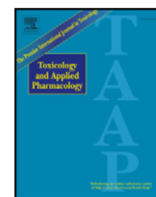

Activating transcription factor 4 underlies the pathogenesis of arsenic trioxide-mediated impairment of macrophage innate immune functions

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ABSTRACT

Chronic arsenic exposure to humans is considered immunosuppressive with augmented susceptibility to several infectious diseases. The exact molecular mechanisms, however, remain unknown. Earlier, we showed the involvement of unfolded protein response (UPR) signaling in arsenic-mediated impairment of macrophage functions. Here, we show that activating transcription factor 4 (ATF4), a UPR transcription factor, regulates arsenic trioxide (ATO)-mediated dysregulation of macrophage functions. In ATO-treated $ATF4^{+/+}$ wild-type mice, a significant down-regulation of CD11b expression was associated with the reduced phagocytic functions of peritoneal and lung macrophages. This severe immuno-toxicity phenotype was not observed in ATO-treated $ATF4^{+/-}$ heterozygous mice. To confirm these observations, we demonstrated in Raw 264.7 cells that ATF4 knock-down rescues ATO-mediated impairment of macrophage functions including cytokine production, bacterial engulfment and clearance of engulfed bacteria. Sustained activation of ATF4 by ATO in macrophages induces apoptosis, while diminution of ATF4 expression protects against ATO-induced apoptotic cell death. Raw 264.7 cells treated with ATO also manifest dysregulated Ca^{++} homeostasis. ATO induces Ca^{++}-dependent calpain-1 and caspase-12 expression which together regulated macrophage apoptosis. Additionally, apoptosis was also induced by mitochondria-regulated pathway. Restoring ATO-impaired Ca^{++} homeostasis in ER/mitochondria by treatments with the inhibitors of inositol 1,4,5-trisphosphate receptor (IP3R) and voltage-dependent anion channel (VDAC) attenuate innate immune functions of macrophages. These studies identify a novel role for ATF4 in underlying pathogenesis of macrophage dysregulation and immuno-toxicity of arsenic.

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1. Introduction

Arsenic exposure through contaminated drinking water is a worldwide public health problem (Brown et al., 2002; Abhyankar et al., 2012; McClintock et al., 2012). Its exposure has been associated with increased risk of cancer in kidney, skin, bladder, lung, prostate and other organs (Mink et al., 2008; Gibb et al., 2011; McClintock et al., 2012). In addition to its carcinogenic effects, arsenic also suppresses the immune system. As a consequence, elevated levels of arsenic in the environment can hinder innate immune responses against bacterial and viral infections. Illustrating this idea, a significant decrease in pulmonary antibacterial defense was noted in mice exposed to arsenic trioxide (ATO) (Aranyi et al., 1985; Burchiel et al., 2009). An exacerbated risk of influenza A (H1N1) infection and -associated lung function following arsenic exposure were also reported in other independent studies (Kozul et al., 2009a; Kozul et al., 2009b). ATO exposure enhanced human immunodeficiency virus type-1 (HIV-1) infection in an *in vitro* study (Nayak et al., 2007; Pion et al., 2007). Similarly, in a zebrafish model, 2–10 ppb arsenic levels in water augmented the viral load by 50-fold and bacterial load by 17-fold suggesting the immunosuppressive effects of arsenic (Nayak et al., 2007). Lower respiratory tract infections and diarrhea are more common in arsenic-exposed human populations, particularly among children from Bangladesh and other countries where high levels of arsenic in drinking water have been reported (Mazumder et al., 2000; Raqib et al., 2009; Rahman et al., 2011; Smith et al., 2011). Increased mortality from pulmonary tuberculosis has been reported in Chile from drinking arsenic-contaminated water (Smith et al., 2011).

The higher incidence of opportunistic infections, allergy and asthma in arsenic-exposed human populations likely results from failure to maintain an equilibrated immune response. In this regard, arsenic exposure has been shown to inhibit proliferative response of T cells and

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alters their cytokine secretion profiles (Biswas et al., 2008). Arsenic also reduces the proportion of T helper cell (CD4) relative to T cytotoxic cells (CD8) ratio (CD4/CD8) in exposed children (Soto-Pena et al., 2006). Prenatal exposure to arsenic significantly reduces thymic function via oxidative stress and apoptosis (Ahmed et al., 2012) and alters DNA methylation (Kile et al., 2014), which collectively are thought to contribute to immunosuppression in childhood. Furthermore, impaired T cell functions have also been reported in experimental animals subjected to arsenic (Burchiel et al., 2009; Martin-Chouly et al., 2011). Cutaneous contact hypersensitivity response is impaired in mice exposed to arsenic (Patterson et al., 2004; Zhou et al., 2006), and chronic arsenic exposure of mice significantly decreases adhesion property and phagocytic activity of splenic macrophages (Bishayi and Sengupta, 2003).

In addition to the deleterious effects of environmental arsenic, ATO is a food and drug administration (FDA) approved chemotherapeutic agent and is used for the treatment of promyelocytic leukemia (PML) (Lengfelder et al., 2012). ATO treatment of patients with multiple myeloma and colon cancer, as well as those with PML, has been reported to contribute to recurrent herpes simplex and herpes zoster virus infection (Tanvetyanon and Nand, 2004; Nouri et al., 2006; Yamakura et al., 2014; Cardenas et al., 2015). T cell mediated immunity is attenuated in these arsenic-treated cancer patients by induction of regulatory T cells (Tohyama et al., 2013).

The precise molecular mechanism by which arsenic impairs immune functions is yet to be defined. We demonstrated earlier that *in vitro* treatment of murine macrophage, Raw 264.7 cells with ATO diminished phagocytic functions. These effects were suggested to involve the unfolded protein response (UPR) signaling as 4-phenylbutyric acid (PBA), a chemical chaperone alleviated markers of UPR signaling, including GRP78, p-eIF2α, and CHOP, and afforded protection against ATO-mediated changes in these innate immune cells (Srivastava et al., 2013). ATF4 increases the expression of CHOP which is also a UPR transcriptional regulator that has been shown to play an important role in the pathogenesis and survival of mycobacterium in mouse macrophage cells (Lim et al., 2011). ATF4 plays key roles in diverse biological and patho-biological processes such as bone formation (Wang et al., 2012), hepatic steatosis (Jo et al., 2012) and glutamine-regulated cancer cell survival/apoptosis (Qing et al., 2012). Recent evidence indicates that ATF4 also participates in signaling of the toll like receptors 4 (TLR4), which in turn regulates cytokine production (Woo et al., 2009). In this study we determined that ATF4 is a central target involved in dampening of immune responses in arsenic exposed experimental animals. Our data provide novel *in vitro* and *in vivo* evidence for the involvement of ATF4 in ATO-mediated impairment of immune regulation.

2. Materials and methods

2.1. Cell culture

Mouse macrophage cell line Raw 264.7 (Cat no. TIB-71TM) were procured from American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM medium containing 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO) and 1% penicillin-streptomycin solution (Mediatech, Manassas, VA) at 37 °C in CO_2 incubator.

2.2. Animal studies

$ATF4^{+/+}$ wild-type (WT) and $ATF4^{+/-}$ heterozygous mice in the C57BL/6j background were purchased from Jackson laboratory (Bar Harbor, ME; http//www.jax.org). Selection of $ATF4^{+/-}$ heterozygous mice was based on the fact that $ATF4^{-/-}$ null mice are reported to be mostly neonatal lethal, and surviving animals are dwarf and display severe phenotypes in adulthood (Masuoka and Townes, 2002; Wang et al., 2009; Cornejo et al., 2013). To define the role of ATF4 on the susceptibility of animals for infection, twenty mice of each genotype (aged 5–6 weeks) were divided into two groups (10 mice/group). Group-1 received saline whereas group-2 received parental administration of ATO (50 μg/mouse in 200 μl PBS, intra-peritoneal; daily for 10 days). Each of these two groups was sub-divided into two subgroups. One subgroup received intratracheally (i.t.) saline, while the other subgroup received i.t. fluorescent *E. coli* bioparticles (2×10^7 *E. coli* in 30 μl PBS) as shown in Supplemental Fig. S1A. Fluorescent *E. coli* were administrated 3 h prior to euthanasia. Briefly, the tongue of mice was gently extended in isoflurane-anesthetized mice, and the fluorescent *E. coli* was deposited into the oropharynx as described earlier (Bae et al., 2011). All animal experiments were performed according to the protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Alabama at Birmingham.

2.3. siRNA transfection

Raw 264.7 cells were transfected with either ATF4 siRNA (Sigma, St. Louis, MO) or scrambled siRNA obtained from Ambion, Life Technologies, (Grand Island, NY) at final concentration 10 nM. Transfection with siRNA was carried out using Opti-MEM-I reduced-serum medium (Invitrogen, Grand Island, NY) and lipofectamine 2000 transfection reagent (Sigma, St. Louis, MO).

2.4. Inflammatory cytokine levels

Inflammatory cytokine mRNA expression was determined by real time PCR using SYBR green methodology as described earlier (Srivastava et al., 2013). Primers used in this study are listed in Supplementary Table-SI.

2.5. Latex beads phagocytosis assay

Latex beads coated with fluorescently labeled rabbit-IgG was used as a probe to assess the phagocytic capacity of murine macrophages. The experiment was performed using Cayman's phagocytosis assay kit (IgG FITC) (Ann Arbor, MI) according to manufacturer's instructions. Briefly, Raw 264.7 cells transfected with either ATF4 or scrambled siRNA were treated with either saline or ATO (2 μM for 14 h). Cells were then incubated with 30 μl of latex beads coated with fluorescently labeled rabbit-IgG for 45 min. at 37 °C and then washed three times with assay buffer followed by incubation with trypan blue quenching solution. The engulfed fluorescent beads were quantitated through ELISA-based microplate reader at excitation 485 and emission at 535 nm. Cells incubated with latex beads at 4 °C instead of 37 °C served as negative controls (Srivastava et al., 2013).

2.6. Bacterial clearance assay

For studying the kinetics of clearance of engulfed bacteria by Raw 264.7 cells, we used fluorescently labeled opsonized *E. coli* (K-12 strain) bioparticles (Invitrogen, Grand Island, NY). ATF4 siRNA transfected Raw 264.7 cells were treated with either saline or ATO (2 μM for 14 h). Following these treatments, cells were incubated with at 37 °C for 2 h with *E. coli* bioparticles (Bioparticles:cell 50:1 ratio). The fluorescence of bioparticles conjugates that are bound to the surface but not internalized were quenched by trypan blue and imaged by fluorescent microscopy to capture the base line fluorescence of engulfed *E. coli* by Raw 264.7 cells at 2 h. After this, these cells were further incubated at 37 °C in CO_2 incubator to study the clearance of these engulfed *E. coli*. Cells were imaged again at 24 h to capture the differences in fluorescence of engulfed *E. coli* between various treatments of Raw 264.7 cells.

2.7. ATO treatment studies using peritoneal macrophages isolated from WT and $ATF4^{+/-}$ heterozygous mice

Peritoneal macrophages were harvested from the peritoneal cavity of WT and $ATF4^{+/-}$ heterozygous mice by injecting 5 ml of sterile saline

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