Inhibition of early T cell cytokine production by arsenic trioxide occurs independently of Nrf2

Kelly R. VanDenBerg1,2☯, Robert A. Freeborn1☯, Sheng Liu1, Rebekah C. Kennedy1, Joseph W. Zagorski2,3, Cheryl E. Rockwell1,2,3*

1 Department of Pharmacology & Toxicology, Michigan State University, East Lansing, Michigan, United States of America, 2 Institute for Integrative Toxicology, Michigan State University, East Lansing, Michigan, United States of America, 3 Cell and Molecular Biology Program, Michigan State University, East Lansing, Michigan, United States of America

☯ These authors contributed equally to this work.

* rockwelc@msu.edu

Abstract

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a stress-activated transcription factor that induces a variety of cytoprotective genes. Nrf2 also mediates immunosuppressive effects in multiple inflammatory models. Upon activation, Nrf2 dissociates from its repressor protein, Keap1, and translocates to the nucleus where it induces Nrf2 target genes. The Nrf2-Keap1 interaction is disrupted by the environmental toxicant and chemotherapeutic agent arsenic trioxide (ATO). The purpose of the present study was to determine the effects of ATO on early events of T cell activation and the role of Nrf2 in those effects. The Nrf2 target genes Hmox-1, Nqo-1, and Gclc were all upregulated by ATO (1–2 μM) in splenocytes derived from wild-type, but not Nrf2-null, mice, suggesting that Nrf2 is activated by ATO in splenocytes. ATO also inhibited IFNγ, IL-2, and GM-CSF mRNA and protein production in wild-type splenocytes activated with the T cell activator, anti-CD3/anti-CD28. However, ATO also decreased production of these cytokines in activated splenocytes from Nrf2-null mice, suggesting the inhibition is independent of Nrf2. Interestingly, ATO inhibited TNFα protein secretion, but not mRNA expression, in activated splenocytes suggesting the inhibition is due to post-transcriptional modification. In addition, c-Fos DNA binding was significantly diminished by ATO in wild-type and Nrf2-null splenocytes activated with anti-CD3/anti-CD28, consistent with the observed inhibition of cytokine production by ATO. Collectively, this study suggests that although ATO activates Nrf2 in splenocytes, inhibition of early T cell cytokine production by ATO occurs independently of Nrf2 and may instead be due to impaired AP-1 DNA binding.

Introduction

Inorganic arsenic is an environmental contaminant and is also used clinically as a chemotherapeutic in the form of arsenic trioxide. As an environmental contaminant, it is often found in
water sources and has been found to elicit numerous toxic effects. It is present in two valence states, arsenite (As³⁺) or arsenate (As⁵⁺), with arsenite considered to be the most toxic form [1,2]. Exposure to inorganic arsenic has been linked to an increased incidence of gastroenteritis, cardiovascular disease, diabetes, and various forms of cancer [1,3–5]. Other evidence also suggests chronic exposure to arsenic causes liver injury, immunotoxicity, peripheral neuropathy and other neurotoxic effects. The toxic effects of arsenic are likely mediated through multiple mechanisms, including induction of oxidative stress, alteration of various sulfhydryl-containing proteins, disruption of mitochondria energy generation, and DNA adducts, among others.

Numerous studies have shown that chronic exposure to inorganic arsenic impairs the human immune response [6,7]. Arsenite alters the abundance of particular immune cell populations—such as those of eosinophils and monocytes—and modulates expression of genes associated with immune response in peripheral blood lymphocytes [8,9]. Of relevance to the present studies, arsenic has been shown to inhibit events associated with early T cell activation, including T cell proliferation, as well as early cytokine secretion [10]. These effects of arsenic can ultimately impact functional immunity [7,11,12]. In addition, arsenic impairs immunosurveillance, which may ultimately contribute to its carcinogenicity [7,12]. However, the mechanism through which arsenic affects these immune responses is not fully understood.

Inorganic arsenic activates the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) in a variety of different cell types, including osteoblasts, keratinocytes, and bladder epithelial cells, among others [13–15]. Nrf2 is a stress-activated transcription factor that is responsible for inducing a battery of cytoprotective genes [16,17]. Under homeostatic conditions, Nrf2 is sequestered in the cytoplasm and degraded by the proteasome due to association with its repressor protein Kelch-like ECH-associated protein 1, Keap1 [18,19]. Under stressful conditions, such as those induced by oxidative or electrophilic stimuli, Nrf2 is no longer degraded by the proteasome and consequently translocates to the nucleus to induce numerous genes involved in antioxidative responses, xenobiotic detoxification, and glutathione homeostasis [20–22]. In murine leukocytes, Nrf2 upregulates genes such as heme oxygenase-1 (Hmox1), NAD(P)H quinone oxidoreductase-1 (Nqo1), glutamate-cysteine ligase catalytic subunit (Gclc), and others [23,24]. As such, these genes serve as useful markers for Nrf2 activation in immune cells.

In addition to its cytoprotective capabilities, Nrf2 also plays an important role in modulating immune responses. Nrf2 deficiency is associated with worsened inflammation in a number of different models of inflammatory disease, including sepsis, experimental autoimmune encephalomyelitis (a model of multiple sclerosis), autoimmune hepatitis, and airway inflammation [25–28]. An anti-inflammatory role for Nrf2 is further supported by the development of multi-organ autoimmune disease in Nrf2-null mice that resembles systemic lupus erythematosus (SLE) in humans [29,30]. Furthermore, our previous studies have shown that the Nrf2 signaling pathway modulates Th1/Th2 differentiation and early events of T cell activation in both mice and humans [31–33].

Because published studies suggest arsenic trioxide (ATO) may affect T cell function, the purpose of the present study was to determine the effects of ATO on the early events of T cell activation in anti-CD3/anti-CD28-activated murine splenocytes, specifically regarding the potential role of Nrf2 in these events.

Materials and methods
Materials
Arsenic(III) oxide (arsenic trioxide, ATO) was purchased from Sigma-Aldrich (St. Louis, MO), along with all other reagents, unless otherwise stated.
Nrf2-null mice

Nrf2-null mice on a mixed C57BL/6 and AKR background were generated as previously described and were a generous gift from Dr. Jefferson Chan [16]. The mice were subsequently backcrossed 8 generations onto the C57BL/6 background, were 99% congenic (analysis performed by Jackson Laboratories, Bar Harbor, ME) and their female progeny were used for these studies. Age-matched female wild-type (C57BL/6) control mice were purchased from Charles River Laboratories (Wilmington, MA). Food and water were provided ad libitum. All animal studies were conducted in accordance with the Guide for Care and Use of Animals as adopted by the National Institutes of Health, and were approved by the Institutional Animal Care and Use Committee at Michigan State University.

Cell culture

Splenocytes from C57BL/6 (wild-type) mice and Nrf2-null mice were isolated and cultured at 5x10⁶ cells/mL in DMEM media supplemented with 10% fetal bovine serum (Biowest LLC, Kansas City, MO), 25 mM HEPES, 50 μM 2-mercaptoethanol, nonessential amino acids (1X final concentration from 100X stock solution), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were left untreated or treated with a vehicle, phosphate-buffered saline (PBS), 1 μM ATO, or 2 μM ATO for 30 min. Samples were subsequently activated with 1.5 μg/mL anti-CD3 (clone eBio500A2, Affymetrix/eBioscience, San Diego, CA) crosslinked with 1.5 μg/mL anti-CD28 (clone 37.51, Affymetrix/eBioscience) by an F(ab')₂ fragment species for anti-Syrian hamster IgG (Jackson ImmunoResearch, West Grove, PA) or left unactivated (BKG) for varying time-points.

CD4⁺ T cell isolation and culture

Splenocytes were isolated as above. CD4⁺ T cells were isolated by negative selection using a commercially available kit for magnetic separation (Miltenyi Biotec, Auburn, CA). Isolated T cells were cultured at 5 x 10⁶ cells/mL in DMEM media supplemented with 10% fetal bovine serum (Biowest LLC, Kansas City, MO), 25 mM HEPES, 50 μM 2-mercaptoethanol, nonessential amino acids (1X final concentration from 100X stock solution), 100 U/mL penicillin, and 100 μg/mL streptomycin. The isolated CD4⁺ T cells were left untreated (BKG) or treated with vehicle (PBS), 0.1 μM arsenic trioxide, or 0.5 μM arsenic trioxide for 30 min. Cells were then activated with 1.5 μg/mL anti-CD3 (clone eBio500A2, Affymetrix/eBioscience, San Diego, CA) crosslinked with 1.5 μg/mL anti-CD28 (clone 37.51, Affymetrix/eBioscience) by an F(ab')₂ fragment for anti-Syrian hamster IgG (Jackson ImmunoResearch, West Grove, PA) or left unactivated (BKG) for 24 h.

mRNA quantification: RT-PCR

6 h post-activation, cells were collected and total RNA was isolated via TRIzol Reagent per the manufacturer’s protocol (Life Technologies, Grand Island, NY). Isolated RNA was quantified by the NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). For reverse transcription, 5 μL of diluted RNA (50 ng/μL) was added to 7.5 μL of reverse transcription master mix, which was prepared as follows: for each sample, 4 μL nuclease-free water, 2.5 μL M-MLV 5X RT Buffer (Promega, Madison, WI), 0.5 μL 10 mM dNTPs (Promega, Madison, WI), 0.16 μL random primers (Promega, Madison, WI), 0.16 μL rRNAsin (Promega, Madison, WI), and 0.16 μL M-MLV Reverse Transcriptase (Promega, Madison, WI). After reverse transcription, real-time PCR SYBR green analysis was performed to quantify IL-2, IFNγ, TNFα, GM-CSF, CD25, CD69, Hmox-1, Nqo1, and Gclc mRNA expression.
Ribosomal protein L13A (RPL13A) served as the endogenous control and relative mRNA expression was calculated using the ΔΔCt method. The primers (Integrated DNA Technologies, Coralville, IA) were as follows: RPL13A forward (5′-GTT GAT GCC TTC ACA GCG TA-3′), RPL13A reverse (5′-AGA TGG CGG AGG TGC AG-3′), IL-2 forward (5′-GTC AAA TCC AGA ACA TGC CG-3′), IL-2 reverse (5′-AAC CTG AAA CTC CCC AGG AT-3′), IFNγ forward (5′-TGA GCT CAT TGA ATG TTT GG-3′), IFNγ reverse (5′-AGA GAG GGC AGA AGG ACC AT-3′), TNF-α forward (5′-ATG AGG AGG CAG TCC CCT ATG G-3′), TNF-α reverse (5′-AGA GAG GGC AGA AGG ACC AT-3′), GM-CSF forward (5′-TGC ATG CCT TCA CAT TGA ATG AA-3′), GM-CSF reverse (5′-ATG AGG GCG CTT TGA ATG TG-3′), CD25 forward (5′-TTG CTG ATG TTG GGG TTT CT-3′), CD25 reverse (5′-TTG CTG ATG TTG GGG TTT CT-3′), CD69 forward (5′-AGA GAG GGC AGA AGG ACC AT-3′), CD69 reverse (5′-AGA GAG GGC AGA AGG ACC AT-3′), Hmox-1 forward (5′-GGA CAG AAC CAG CCT GAA CTA-3′), Hmox-1 reverse (5′-GGA CAG AAC CAG CCT GAA CTA-3′), Nqo1 forward (5′-GGA CAG AAC CAG CCT GAA CTA-3′), Nqo1 reverse (5′-GGA CAG AAC CAG CCT GAA CTA-3′), Gclc forward (5′-GGA CAG AAC CAG CCT GAA CTA-3′), and Gclc reverse (5′-GGA CAG AAC CAG CCT GAA CTA-3′).

Flow cytometry

24 h post activation, cells were labeled with anti-CD4/FITC (Affymetrix/eBioscience, San Diego, CA), anti-CD25/APC (Affymetrix/eBioscience, San Diego, CA) and anti-CD69/PE-Cy7 (BioLegend, San Diego, CA). After washing and resuspending the cells in FACS buffer, fluorescence was detected by a BD Accuri C6 flow cytometer and quantified by C-Flow software (BD Accuri, San Jose, CA).

IL-2, IFNγ, TNFα, and GM-CSF ELISAs

Cell supernatants were collected from splenocytes and isolated CD4+ T cells 24 h post-activation. IL-2, IFNγ, TNFα, and GM-CSF were quantified from the supernatants using commercially available kits following the manufacturer’s protocols (BioLegend, San Diego, CA and Affymetrix/eBioscience, San Diego, CA). Absorbance was quantified at 450 nm using the Infinite M1000 Pro Microplate (Tecan, San Jose, CA).

Quantification of c-fos DNA binding

4h post-activation, splenocytes were collected and nuclear protein was isolated using a commercially available kit (Active Motif, Carlsbad, CA). Nuclear protein was then quantified by Bradford Assay (Bio-Rad, Hercules, CA). 5 μg of nuclear protein was used to determine binding of c-fos to the AP-1 consensus binding site, as determined by quantification with an ELISA-based assay (TransAM kit, Active Motif, Carlsbad, CA).

Statistical analysis

The mean ± SE was determined for each treatment group in individual experiments. Homogeneous data were analyzed by two-way parametric ANOVA. When significant differences were observed, the Holm-Sidak post hoc test was used to compare treatment groups using SigmaPlot 12.3 (Systat Software, San Jose, CA).
Results

Upregulation of Nrf2 target genes by ATO in murine splenocytes

Previous studies suggested that inorganic arsenic activates Nrf2 in numerous cell types. To determine the effect of ATO on Nrf2 activation in murine splenocytes, freshly isolated splenocytes were treated with clinically-relevant concentrations of ATO (1–2 μM) and activated with a T cell-specific activator, anti-CD3 and anti-CD28. Quantification of Nrf2 target gene expression revealed that Hmox-1, Nqo1, and Gclc mRNA levels were upregulated in the wild-type splenocytes when exposed to concentrations of ATO as low as 1 μM (Fig 1). However, this trend was significantly less pronounced, and often unobserved, in Nrf2-null splenocytes. Collectively, these data indicate that ATO upregulates Hmox-1, Nqo1, and Gclc through a largely Nrf2-dependent mechanism, demonstrating that Nrf2 is activated by ATO in murine splenocytes.

Inhibition of IL-2, IFNγ, TNFα, and GM-CSF production by ATO

In addition to Nrf2 target gene expression, T cell immune parameters were also quantified. ATO treatment significantly inhibited IL-2 production by anti-CD3/anti-CD28-activated splenocytes at both the mRNA and protein levels (Fig 2). In addition, ATO inhibited induction of IFNγ and GM-CSF mRNA and protein in anti-CD3/anti-CD28-activated splenocytes (Figs 3 and 4). Notably, IFNγ production was significantly greater in splenocytes derived from Nrf2-null mice compared to wild-type, which is consistent with our previously published observations [31]. The same genotype effect appears to exist for GM-CSF in Nrf2-null which is also consistent with the literature [28]. Surprisingly, TNFα mRNA remained unaffected by treatment with ATO, though TNFα protein expression was inhibited in a similar fashion to the other measured cytokines (Fig 5). Interestingly, the inhibition of each cytokine’s production by ATO was observed in both the wild-type and the Nrf2-null splenocytes, suggesting that the effect of ATO on these cytokines is independent of Nrf2. Decreased production of IL-2, IFNγ by ATO treatment also occurred in activated CD4⁺ T cells; production of TNFα and GM-CSF was reduced in the Nrf2-null, but not wild-type, CD4⁺ cells. Reduced ATO concentrations were used in isolated CD4⁺ T cells due to increased toxicity compared with splenocytes (Fig 6).

ATO does not alter CD25 and CD69 expression by activated splenocytes

The suppression of cytokine production by ATO prompted us to investigate the induction of other genes that are upregulated during T cell activation. In contrast to the aforementioned cytokines, induction of CD25 and CD69 mRNA and protein expression was not impacted by ATO treatment in activated splenocytes from either wild-type or Nrf2-null mice (Fig 7). Overall, these data suggest that ATO has differential effects on early events following T cell activation.

Inhibition of AP-1 binding by ATO in activated splenocytes

Because the effect of ATO on cytokine expression appeared to be Nrf2-independent, we considered alternative mechanisms. We identified the AP-1 and NFκB transcription factors as key to promoting the expression of all the cytokines quantified in this study. Previous studies established that NFκB is also critical for the induction of CD25, while regulation of CD69 and CD25 by AP-1 is more complex and may involve other transcription factors [34–39]. Since we observed no effects of ATO on CD25 or CD69 expression, we focused on AP-1 as a potential
Fig 1. ATO upregulates the Nrf2 target genes Hmox-1, Nqo1, and Gclc in wild-type, but not Nrf2-null, splenocytes. Wild-type and Nrf2-null splenocytes were isolated and either left untreated (BKG) or treated with the vehicle (VH, PBS), 0 (activator alone), 1, or 2 μM ATO for 30 min. The cells were then either left unactivated (BKG) or activated with anti-CD3/anti-CD28 for 6 h. Real-time PCR was used to quantify the mRNA expression of (A) Hmox-1, (B) Nqo1, and (C) Gclc. * denotes p<0.05 compared to the wild-type VH group. † denotes p<0.05 compared to the Nrf2-null VH group. ‡ denotes p<0.05 between the wild-type and Nrf2-null genotypes.

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Fig 2. ATO markedly inhibits IL-2 production by anti-CD3/anti-CD28-activated splenocytes from wild-type and Nrf2-null mice. Wild-type and Nrf2-null splenocytes were isolated and either left untreated (BKG) or treated with vehicle (VH, PBS), 0 (activator alone), 1, or 2 μM ATO for 30 min. The cells were then either left unactivated (BKG) or activated with anti-CD3/anti-CD28 for (A) 6 h prior to quantification of IL-2 mRNA by real-time PCR, or (B) 24 h prior to quantification of IL-2 protein in cell supernatants by ELISA. * denotes p<0.05 compared to the wild-type VH group. † denotes p<0.05 compared to the Nrf2-null VH group. ‡ denotes p<0.05 between the wild-type and Nrf2-null genotypes.

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Fig 3. ATO markedly inhibits IFNγ production by anti-CD3/anti-CD28-activated splenocytes from wild-type and Nrf2-null mice. Wild-type and Nrf2-null splenocytes were isolated and either left untreated (BKG) or treated with the vehicle (VH, PBS), 0 (activator alone), 1, or 2 μM ATO for 30 min. The treatment groups were then either left unactivated (BKG) or activated with anti-CD3/anti-CD28 for (A) 6 h prior to quantification of IFNγ mRNA by real-time PCR, or (B) 24 h prior to quantification of IFNγ protein in cell supernatants by ELISA. * denotes p<0.05 compared to the wild-type VH group. † denotes p<0.05 compared to the Nrf2-null VH group. ‡ denotes p<0.05 between the wild-type and Nrf2-null genotypes.

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Fig 4. ATO markedly inhibits GM-CSF production by anti-CD3/anti-CD28-activated splenocytes from wild-type and Nrf2-null mice. Wild-type and Nrf2-null splenocytes were isolated and either left untreated (BKG) or treated with the vehicle (VH, PBS), 0 (activator alone), 1, or 2 μM ATO for 30 min. The treatment groups were then either left unactivated (BKG) or activated with anti-CD3/anti-CD28 for (A) 6 h prior to quantification of GM-CSF mRNA by real-time PCR, or (B) 24 h prior to quantification of GM-CSF protein in cell supernatants by ELISA. * denotes p<0.05 compared to the wild-type VH group, † denotes p<0.05 compared to the Nrf2-null VH group, ‡ denotes p<0.05 between the wild-type and Nrf2-null genotypes.

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Fig 5. ATO markedly inhibits protein secretion, but not gene expression, of TNFα by anti-CD3/anti-CD28-activated splenocytes from wild-type and Nrf2-null mice. Wild-type and Nrf2-null splenocytes were isolated and either left untreated (BKG) or treated with the vehicle (VH, PBS), 0 (activator alone), 1, or 2 μM ATO for 30 min. The treatment groups were then either left unactivated (BKG) or activated with anti-CD3/anti-CD28 for (A) 6 h prior to quantification of TNFα mRNA by real-time PCR, or (B) 24 h prior to quantification of TNFα protein in cell supernatants by ELISA. * denotes p<0.05 compared to the wild-type VH group. † denotes p<0.05 compared to the Nrf2-null VH group. ‡ denotes p<0.05 between the wild-type and Nrf2-null genotypes.

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mechanism. Accordingly, the effect of ATO on c-fos DNA binding was evaluated. Consistent with our cytokine data, activation of isolated splenocytes with anti-CD3/anti-CD28 markedly decreased c-fos binding to the AP-1 consensus binding site in a Nrf2-independent fashion (Fig 8). Taken together, these data suggest that the inhibition of cytokine induction by ATO in anti-CD3/anti-CD28-activated splenocytes may be due to inhibition of c-fos activation, independent of Nrf2.

**Discussion**

It has been previously shown that chronic exposure to inorganic arsenic has immunosuppressive effects that correlate with altered cytokine production [6,7,10]. In addition, inorganic arsenic has been shown to activate the stress-induced transcription factor Nrf2. Furthermore, studies by our lab and others have established that Nrf2 alters cytokine production by activated T cells and other leukocytes [16,17,40]. Thus, this study aimed at determining whether ATO affects early events following T cell activation, including cytokine production. In addition, we
Fig 7. ATO does not affect expression of CD69 or CD25 in anti-CD3/anti-CD28-activated splenocytes. Wild-type and Nrf2-null splenocytes were isolated and either left untreated (BKG) or treated with the vehicle (VH, PBS), 0 (activator alone), 1, or 2 \( \mu M \) ATO for 30 min. The cells were then either left unactivated (BKG) or activated with anti-CD3/anti-CD28 for either (A-B) 6 h prior to quantification of (A) CD25 mRNA and (B) CD69 mRNA by real-time PCR or (C-E) 24 h for quantification of CD25+ and CD69+ cells by flow cytometry. The CD4+ T cells were gated on prior to analysis of CD25 and CD69 expression. (E) Representative dot plots of CD25 and CD69 cell surface protein expression in CD4 T cells. * denotes p<0.05 compared to the wild-type VH group. † denotes p<0.05 compared to the Nrf2-null VH group. ‡ denotes p<0.05 between the wild-type and Nrf2-null genotypes.

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sought to determine whether Nrf2 is activated by ATO in primary mouse splenocytes and to identify the role of Nrf2 in the effects of ATO on the early events following T cell activation.

The current study demonstrates that ATO activates Nrf2 in murine splenocytes, as evidenced by induction of Nrf2 target gene expression in wild-type, but not Nrf2-null splenocytes. Whereas ATO markedly inhibits the upregulation of IL-2, IFNγ TNFα, and GM-CSF in anti-CD3/anti-CD28-activated splenocytes, it has little effect on the induction of CD25 and CD69, cell surface molecules that are rapidly upregulated during T cell activation. Interestingly, ATO inhibited IL-2, IFNγ, and GM-CSF, but not TNFα, mRNA production in both wild-type and Nrf2-null splenocytes, whereas protein secretion of IL-2, IFNγ, GM-CSF, and TNFα, was inhibited by ATO exposure. These results suggest that the inhibition of cytokine secretion by ATO occurs independently of Nrf2 and further suggest post-transcriptional modulation of TNFα by ATO. In support of this, the literature has shown TNFα to be both transcriptionally and post-transcriptionally regulated by various mechanisms, including microRNA and metabolic state, which could explain why we observed an alteration on TNFα protein levels, but not mRNA [41–44]. In addition, we found that ATO significantly inhibits c-fos binding to the AP-1 consensus binding site in a Nrf2-independent manner, which correlates with the decrease in cytokine expression. Collectively, the present study is the first to demonstrate that ATO has differential effects on T cell activation in which CD25/CD69 expression is unaffected, but IL-2, IFNγ, TNFα, and GM-CSF production are markedly decreased. Furthermore, the inhibition of cytokine production by ATO is independent of Nrf2.
and correlates with decreased c-fos DNA binding, suggesting the effect may be due to impaired AP-1 activity.

In agreement with splenocytes, isolated CD4+ T cells showed no changes in CD25 or CD69 expression (data not shown) along with decreased IL-2 and IFNγ secretion that was independent of Nrf2. Interestingly, secretion of TNFα and GM-CSF by wild-type CD4+ cells were unaffected by ATO, while CD4+ cells from Nrf2-null animals remained sensitive. Our results indicate that CD4+ T cells are directly affected by ATO exposure, but results differ from the splenocyte preparation depending on cytokine, indicating that antigen-presenting cells have some effect on cytokine secretion in the presence of ATO.

CD25, the alpha chain of the IL-2 cell receptor, and CD69, a C-type lectin protein, are rapidly upregulated during T cell activation. As such, they are useful markers of early T cell activation. Our data indicate that at sub-cytotoxic levels of ATO (≤2μM for splenocytes, ≤0.5μM for CD4+ T cells), neither CD25 nor CD69 expression is significantly altered in anti-CD3/anti-CD28-activated splenocytes from either wild-type or Nrf2-null mice. Whereas previously published data indicate that sodium arsenite decreases CD69 surface expression in PBMCs isolated from C57BL/6 mice and humans, the current study demonstrates that ATO does not affect CD69 expression on splenic CD4 T cells [45,46]. Overall, these studies suggest that trivalent arsenic may have differential effects on CD69 expression, which may be dependent on the specific congener of trivalent arsenic.

The current study also demonstrates that ATO markedly inhibits early production of IL-2, IFNγ, TNFα, and GM-CSF in anti-CD3/anti-CD28-activated splenocytes, as well as IL-2 and IFNγ in isolated CD4+ T cells, which is consistent with previous studies [7,12,45,47,48]. By utilizing Nrf2 knockout mice in which Nrf2 expression is completely ablated, we found that lack of Nrf2 expression did not rescue the inhibition of cytokine production by ATO. Similar to our findings, a previous study conducted in human T cells isolated from PBMCs revealed that while inorganic arsenic exposure (2μM) decreased the expression of certain cytokines—including IL-2, IFNγ, and TNFα—partial silencing of Nrf2 (~45% knockdown) by transient siRNA transfection did not rescue the inhibition [49]. Collectively, these studies demonstrate that inhibition of IL-2, IFNγ, TNFα, and GM-CSF induction by inorganic arsenic in activated T cells occurs independently of Nrf2.

T cell activation results in the initiation of multiple kinase cascades that lead to the activation of several transcription factors that subsequently upregulate cytokine production [50,51]. One such transcription factor is AP-1, which is composed of a c-fos/c-jun heterodimer, and is a known regulator of IL-2, IFNγ, TNFα, and GM-CSF transcription [52–56]. The present study shows that ATO impairs c-fos binding to the AP-1 binding site. There are conflicting reports with respect to the effect of ATO on c-fos expression and activity. Whereas several studies show that ATO upregulates c-fos mRNA expression in a number of different cell types, ATO has also been shown to decrease c-fos expression in HL-60 cells, an immune cell line [57]. Our results are consistent with the latter report. The reason for the differential effects of ATO on c-fos expression in different cell types is not yet clear. Although Nrf2 can directly interact with AP-1 family members and thereby affect AP-1 activity, the inhibition of c-fos by ATO was observed in both wild-type and Nrf2-null splenocytes, suggesting the effect is independent of Nrf2 [58]. The decrease in c-fos DNA binding correlates with the reduction in cytokine production and thus is a likely mechanism for the observed decrease in IL-2, IFNγ, TNFα, and GM-CSF induction by ATO in activated T cells.

Altogether, this study suggests that while Nrf2 is activated by ATO in murine splenocytes stimulated with the T cell activator anti-CD3/anti-CD28, the effects of ATO on cytokine production are independent of Nrf2 activation. Furthermore, this study demonstrates that ATO has differential effects on the early events of T cell activation in which it markedly inhibits
production of IL-2, IFNγ, TNFα, and GM-CSF but does not affect induction of CD69 and CD25. Lastly, this study is the first to show that the inhibition of IL-2, IFNγ, TNFα, and GM-CSF production by ATO correlates with decreased c-fos DNA binding, which suggests the effect of ATO on cytokine production is due to impaired AP-1 activity.

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Author Contributions

Conceptualization: Kelly R. VanDenBerg, Cheryl E. Rockwell.
Data curation: Kelly R. VanDenBerg, Robert A. Freeborn, Cheryl E. Rockwell.
Formal analysis: Kelly R. VanDenBerg, Robert A. Freeborn, Cheryl E. Rockwell.
Funding acquisition: Cheryl E. Rockwell.
Investigation: Kelly R. VanDenBerg, Robert A. Freeborn, Sheng Liu, Rebekah C. Kennedy, Joseph W. Zagorski.
Methodology: Sheng Liu, Rebekah C. Kennedy, Joseph W. Zagorski, Cheryl E. Rockwell.
Project administration: Cheryl E. Rockwell.
Supervision: Joseph W. Zagorski, Cheryl E. Rockwell.
Writing – original draft: Kelly R. VanDenBerg.
Writing – review & editing: Robert A. Freeborn, Rebekah C. Kennedy, Cheryl E. Rockwell.

References

1. Abernathy CO, Thomas DJ, Calderon RL. Toxicity and Risk Assessment of Trace Elements. J Nutr. 2003; 133: 1536S–1538S. PMID: 12730460
2. Abernathy CO, Thomas DJ. United Nations Synthesis Report on Arsenic in Drinking Water. 2001; 68–160.
3. Chen C-J, Wang S-L, Chiou J-M, Tseng C-H, Chiou H-Y, Hsueh Y-M, et al. Arsenic and diabetes and hypertension in human populations: A review. Toxicol Appl Pharmacol. 2007; 222: 298–304. https://doi.org/10.1016/j.taap.2006.12.032 PMID: 17307211
4. Emadi A, Gore SD. Arsenic trioxide—An old drug rediscovered. Blood Rev. 2010; 24: 191–199. https://doi.org/10.1016/j.bire.2010.04.001 PMID: 20471733
5. Schuhmacher—Wolz U, Dieter HH, Klein D, Schneider K. Oral exposure to inorganic arsenic: evaluation of its carcinogenic and non-carcinogenic effects. Crit Rev Toxicol. Taylor & Francis; 2009; 39: 271–298. https://doi.org/10.1080/1040840802291505 PMID: 19235533
6. Ostrosky-Wegman P, Gonsebatt ME, Montero R, Vega L, Barba H, Espinosa J, et al. Lymphocyte proliferation kinetics and genotoxic findings in a pilot study on individuals chronically exposed to arsenic in Mexico. Mutat Res Mol Mech Mutagen. 1991; 250: 477–482. https://doi.org/10.1016/0027-5107(91)90204-2
7. Soto-Peña GA, Luna AL, Acosta-Saavedra L, Conde-Moo P, López-Carrillo L, Cebrián ME, et al. Assessment of lymphocyte subpopulations and cytokine secretion in children exposed to arsenic. FASEB J. 2006; https://doi.org/10.1096/fj.05-4660fje PMID: 16461332
8. Andrew AS, Jewel DA, Mason RA, Whitfield ML, Moore JH, Karagas MR. Drinking-water arsenic exposure modulates gene expression in human lymphocytes from a U.S. population. Environ Health Perspect. National Institute of Environmental Health Science; 2008; 116: 524–31. https://doi.org/10.1289/ehp.10861 PMID: 18414638
9. Maiti S, Chattopadhyay S, Deb B, Samanta T, Maji G, Pan B, et al. Antioxidant and metabolic impairment result in DNA damage in arsenic-exposed individuals with severe dermatological manifestations in Eastern India. Environ Toxicol. Wiley Subscription Services, Inc., A Wiley Company; 2012; 27: 342–350. https://doi.org/10.1002/tox.20647 PMID: 20925122

10. Biswas R, Ghosh P, Banerjee N, Das J, Sau T, Banerjee A, et al. Analysis of T-cell proliferation and cytokine secretion in the individuals exposed to arsenic. Hum Exp Toxicol. 2008; 27: 381–386. https://doi.org/10.1177/09603770108094607 PMID: 18715884

11. Dangleben NL, Skibola CF, Smith MT. Arsenic immunotoxicity: a review. Environ Health. BioMed Central; 2013; 12: 73. https://doi.org/10.1186/1476-069X-12-73 PMID: 24004508

12. Wang X-J, Sun Z, Chen W, Eblin KE, Gandolfi JA, Zhang DD. Nrf2 protects human bladder urothelial cells from arsenite and monomethylarsonous acid toxicity. Toxicol Appl Pharmacol. 2007; 225: 206–213. https://doi.org/10.1016/j.taap.2007.07.016 PMID: 17765279

13. Chan K, Lu R, Chang JC, Kan YW. NFE2, a member of the NFE2 family of transcription factors, is not essential for murine erythropoiesis, growth, and development. Proc Natl Acad Sci U S A. National Academy of Sciences; 1996; 93: 13943–8. Available: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1943040

14. Dinkova-Kostova AT, Holtclaw WD, Cole RN, Itoh K, Wakabayashi N, Katoh Y, et al. Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. Proc Natl Acad Sci U S A. National Academy of Sciences; 2002; 99: 11908–13. https://doi.org/10.1073/pnas.172398899 PMID: 12193649

15. Itoh K, Wakabayashi N, Katoh Y, Igarashi K, Engel JD, et al. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. Genes Dev. Cold Spring Harbor Laboratory Press; 1999; 13: 76–86. https://doi.org/10.1101/gad.13.1.76

16. McMahon M, Itoh K, Yamamoto M, Hayes JD. Keap1-dependent proteasomal degradation of transcription factor Nrf2 contributes to the negative regulation of antioxidant response element-driven gene expression. J Biol Chem. American Society for Biochemistry and Molecular Biology; 2003; 278: 21592–600. https://doi.org/10.1074/jbc.M300931200 PMID: 12682069

17. Venugopal R, Jaiswal AK, Kan YW. Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the expression. J Biol Chem. American Society for Biochemistry and Molecular Biology; 2003; 278: 21592–600. https://doi.org/10.1074/jbc.M300931200 PMID: 12682069

18. Kim H-J, Nel AE. The role of phase II antioxidant enzymes in protecting memory T cells from spontaneous apoptosis in young and old mice. J Immunol. American Association of Immunologists; 2005; 175: 2948–59. https://doi.org/10.4049/JIMMUNOL.175.5.2948

19. Thimmulappa RK, Scollick C, Traore K, Yates M, Trush MA, Liby KT, et al. Nrf2-dependent protection from LPS-induced inflammatory response and mortality by CDDO-Imidazolide. Biochem Biophys Res Commun. 2006; 351: 883–889. https://doi.org/10.1016/j.bbrc.2006.10.102 PMID: 17097057

20. Johnson DA, Amirahmadi S, Ward C, Fabry Z, Johnson JA. The absence of the pro-antioxidant transcription factor Nrf2 exacerbates experimental autoimmune encephalomyelitis. Toxicol Sci. Oxford University Press; 2010; 114: 237–46. https://doi.org/10.1093/toxsci/kfp274 PMID: 19910389

21. Osburn WO, Yates MS, Dolan PD, Chen S, Liby KT, Sporn MB, et al. Genetic or pharmacologic amplification of nrf signaling inhibits acute inflammatory liver injury in mice. Toxicol Sci. NIH Public Access; 2008; 104: 218–27. https://doi.org/10.1093/toxsci/kfn079 PMID: 18417483

22. Rangasamy T, Guo J, Mitzner WA, Roman J, Singh A, Fryer AD, et al. Disruption of Nrf2 enhances susceptibility to severe airway inflammation and asthma in mice. J Exp Med. 2005;202.
28. Thimmulappa RK, Lee H, Rangasamy T, Reddy SP, Yamamoto M, Kensler TW, et al. Nrf2 is a critical regulator of the innate immune response and survival during experimental sepsis. J Clin Invest. American Society for Clinical Investigation; 2006; 116: 984–95. https://doi.org/10.1172/JCI25790 PMID: 16585964
29. Li J, Stein TD, Johnson JA. Genetic dissection of systemic autoimmune disease in Nrf2-deficient mice. Physiol Genomics. 2004; 18: 261–272. https://doi.org/10.1152/physiolgenomics.00209.2003 PMID: 15173550
30. Yoh K, Itoh K, Enomoto A, Hirayama A, Yamaguchi N, Kobayashi M, et al. Nrf2-deficient female mice develop lupus-like autoimmune nephritis. Kidney Int. 2001; 60: 1343–1353. https://doi.org/10.1046/j.1523-1755.2001.00939.x PMID: 11576348
31. Rockwell CE, Zhang M, Fields PE, Klaassen CD. Th2 skewing by activation of Nrf2 in CD4(+) T cells. J Immunol. American Association of Immunologists; 2012; 188: 1630–7. https://doi.org/10.4049/jimmunol.1101712 PMID: 22250088
32. Turley AE, Zagorski JW, Rockwell CE. The Nrf2 activator tBHQ inhibits T cell activation of primary human CD4 T cells. Cytokine. Elsevier Ltd; 2015; 71: 289–295. https://doi.org/10.1016/j.cyto.2014.11.006 PMID: 25484350
33. Castellanos MC, Munoz C, Montoya MC, Lara-Pezzi E, Lopez-Cabrera M, de Landazuri MO. Expression of the leukocyte early activation antigen CD69 is regulated by the transcription factor AP-1. Eur J Immunol. 2002; 32: 3108–17. https://doi.org/10.1002/1521-4141(200211)32:11<3108::AID-IMMU3108>3.0.CO;2-D PMID: 12385031
34. El Gazzar M, Church A, Liu T, McCall CE. MicroRNA-146a regulates both transcription silencing and translation disruption of TNF-α during TLR4-induced gene reprogramming. J Leukoc Biol. 2011; 89: 2094–2095. https://doi.org/10.1172/jlb.0211074 PMID: 21562054
35. Lai WS, Carballo E, Strum JR, Kennington EA, Phillips RS, Blackshear PJ. Evidence that tristetraprolin binds to AU-rich elements and promotes the deadenylation and destabilization of tumor necrosis factor alpha mRNA. Mol Cell Biol. 1999; 19: 4311–23. 0270-7306/99 PMID: 10330172
36. Conde P, Acosta-Saavedra LC, Goytia-Acevedo RC, Calderon-Aranda ES. Sodium arsenite-induced inhibition of cell proliferation is related to inhibition of IL-2 mRNA expression in mouse activated T cells.
46. Tenorio EP, Saavedra R. Differential effect of sodium arsenite during the activation of human CD4+ and CD8+ T lymphocytes. Int Immunopharmacol. 2005; 5: 1853–1869. https://doi.org/10.1016/j.intimp.2005.06.006 PMID: 16275621

47. Das S, Pan D, Bera AK, Rana T, Bhattacharya D, Bandyapadhyay S, et al. Sodium arsenite mediated immuno-disruption through alteration of transcription profile of cytokines in chicken splenocytes under in vitro system. Mol Biol Rep. Springer Netherlands; 2011; 38: 171–176. https://doi.org/10.1007/s11033-010-0091-5 PMID: 2039924

48. Soto-Peña GA, Vega L. Arsenic interferes with the signaling transduction pathway of T cell receptor activation by increasing basal and induced phosphorylation of Lck and Fyn in spleen cells. Toxicol Appl Pharmacol. 2008; 230: 216–26. https://doi.org/10.1016/j.taap.2008.02.029 PMID: 18407307

49. Morzadec C, Macoche M, Kerdine-Römer S, Fardel O, Vernhet L. Nrf2 expression and activity in human T lymphocytes: stimulation by T cell receptor activation and priming by inorganic arsenic and tert-butylhydroquinone. Free Radic Biol Med. 2014; 71: 133–145. https://doi.org/10.1016/j.freeradbiomed.2014.03.006 PMID: 24632831

50. Karin M. The regulation of AP-1 activity by mitogen-activated protein kinases. J Biol Chem. American Society for Biochemistry and Molecular Biology; 1995; 270: 16483–6. https://doi.org/10.1074/JBC.270.28.16483

51. Kim HP, Imbert J, Leonard WJ. Both integrated and differential regulation of components of the IL-2/IL-2 receptor system. Cytokine Growth Factor Rev. 2006; 17: 349–366. https://doi.org/10.1016/j.cytogfr.2006.07.003 PMID: 16911870

52. Barbulescu K, Becker C, Schlaak JF, Schmitt E, Meyer zum Buschenfelde K-H, Neurath MF. Cutting Edge: IL-12 and IL-18 Differentially Regulate the Transcriptional Activity of the Human IFN-gamma Promoter in Primary CD4. J Immunol. 1998; 160: 3642–3647. Available: http://www.jimmunol.org/content/160/8/3642 PMID: 9558063

53. Jain J, Valge-Archer VE, Rao A, Jain J, Vilia +, Valge-Archer E, et al. Analysis of the AP-1 sites in the IL-2 promoter. J Immunol. 1992; 148: 1240–1250. Available: http://www.jimmunol.org/content/148/4/1240 PMID: 1737937

54. Rhoades KL, Golub SH, Economou JS. The Regulation of the Human Tumor Necrosis Factor a Promoter Region in Macrophage, T Cell, and B Cell Lines. J Biol Chem. 1992; 267: 22102–22107. PMID: 1429562

55. Thomas RS, Tymms MJ, Mckinlay LH, Shannon MF, Seth A, Kola I. ETS1, NFkB and AP1 synergistically transactivate the human GM—CSF promoter. Oncogene. 1997; 14: 2855–2855. https://doi.org/10.1038/sj.ong.1201795 PMID: 19990901

56. Wang C-Y, Bassuk AG, Boise LH, Thompson CB, Bravo R, Leiden JM. Activation of the Granulocyte-Macrophage Colony-Stimulating Factor Promoter in T Cells Requires Cooperative Binding of Elf-i and AP-1 Transcription Factors. Mol Cell Biol. 1994; 1153–1159. PMID: 8269796

57. Yedjou CG, Tchounwou PB. Modulation of p53, c-fos, RARE, cyclin A, and cyclin D1 expression in human leukemia (HL-60) cells exposed to arsenic trioxide. Mol Cell Biochem. Springer US; 2009; 331: 207–214. https://doi.org/10.1007/s11010-009-0160-z PMID: 19444595

58. Yang H, Maglinick N, Lee C, Kalmaz D, Ou X, Chan JY, et al. Nrfl and Nrfl2 regulate rat glutamate-cysteine ligase catalytic subunit transcription indirectly via NF-kappaB and AP-1. Mol Cell Biol. American Society for Microbiology; 2005; 25: 5933–46. https://doi.org/10.1128/MCB.25.14.5933-5946.2005