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Nrf2-dependent repression of interleukin-12 expression in human dendritic cells exposed to inorganic arsenic

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ABSTRACT

Inorganic arsenic, a well-known Nrf2 inducer, exerts immunosuppressive properties. In this context, we recently reported that the differentiation of human blood monocytes into immature dendritic cells (DCs), in the presence of low and noncytotoxic concentrations of arsenic, represses the ability of DCs to release key cytokines in response to different stimulating agents. Particularly, arsenic inhibits the expression of human interleukin-12 (IL-12, also named IL-12p70), a major proinflammatory cytokine that controls the differentiation of Th1 lymphocytes. In the present study, we determined if Nrf2 could contribute to these arsenic immunotoxic effects. To this goal, human monocyte-derived DCs were first differentiated in the absence of metalloid and then pretreated with arsenic just before DC stimulation with lipopolysaccharide (LPS). Under these experimental conditions, arsenic rapidly and stably activates Nrf2 and increases the expression of Nrf2 target genes. It also significantly inhibits IL-12 expression in activated DCs, at both mRNA and protein levels. Particularly, arsenic reduces mRNA levels of IL12A and IL12B genes which encodes the p35 and p40 subunits of IL-12p70, respectively. *tert*-Butylhydroquinone (tBHQ), a reference Nrf2 inducer, mimics arsenic effects and potently inhibits IL-12 expression. Genetic inhibition of Nrf2 expression markedly prevents the repression of both IL12 mRNA and IL-12 protein levels triggered by arsenic and tBHQ in human LPS-stimulated DCs. In addition, arsenic significantly reduces IL-12 mRNA levels in LPS-activated bone marrow-derived DCs from $Nrf2^{+/+}$ mice but not in DCs from $Nrf2^{-/-}$ mice. Finally, we show that, besides IL-12, arsenic significantly reduces the expression of IL-23, another heterodimer containing the p40 subunit. In conclusion, our study demonstrated that arsenic represses IL-12 expression in human-activated DCs by specifically stimulating Nrf2 activity.

Introduction

Arsenic is a major environmental pollutant to which millions of individuals are chronically exposed over the world, mainly through consumption of contaminated drinking water [1]. This metalloid promotes or initiates the development of nonmalignant and cancerous diseases by altering physiologic functions of several organs including those of the immune system [2]. Indeed, arsenic induces toxic effects on key immune cells that may contribute to the development of lung diseases, infections, and cancers [2]. At noncytotoxic concentrations, arsenic impairs the proliferation and activation of T cells and suppresses humoral immunity [3,4]. In addition, we previously demonstrated that arsenic markedly alters human innate responses by repressing the differentiation of macrophages [5] and dendritic cells (DCs) [6]. Notably, we recently reported that differentiation of human peripheral blood monocytes into immature DCs, in the presence of nanomolar concentrations (100 to 500 nM) of sodium arsenite, reduces their ability to secrete major cytokines when DCs are subsequently activated in an arsenic-free medium [6]. Particularly, arsenic inhibits the expression and release of the human IL-12p70. This cytokine is well known to stimulate the polarization of T lymphocytes into T helper 1 cells and the production of cytotoxic T lymphocytes, which play a central role in immunosurveillance against infections and

Abbreviations: Nrf2, nuclear factor-erythroid 2-related-2; As, arsenite; NQO1, NAD(P)H quinone oxidoreductase 1; DC, dendritic cells; BMDC, bone marrow-derived DC; Keap1, Kelch-like ECH-associated protein 1; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-a; Ab, antibody; IL, interleukin; SDS-PAGE, sodium dodecyl sulfate polymerase gene electrophoresis; TBS, Tris-buffered saline; tBHQ, *tert*-butylhydroquinone; RT-PCR, reverse-transcription polymerase chain reaction; IRF3, interferon regulated factor 3; SP1, specific protein 1

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cancers [7]. IL-12p70 consists of the p35 and p40 subunits encoded by the IL12A and IL12B genes, respectively. The p40 subunit also forms a homodimer, named IL-12p40, that is less active than IL-12p70 [7]. Our previous results showed that differentiation of monocytes in the presence of arsenic repressed the transcription of IL12A and IL12B genes during DC activation [6]. However, the molecular mechanisms mediating this arsenic-dependent repression of IL-12p70 expression have not been determined.

Arsenic is a prooxidant metalloid well known to induce expression and activity of the nuclear factor-erythroid 2-related-2 (Nrf2) in several cell types including human T lymphocytes and macrophages [8,9]. Nrf2 levels are generally low in normal cells, not exposed to an environmental stress. Indeed, newly synthesized Nrf2 is rapidly and tightly bound to Kelch-like ECH-associated protein 1 (Keap1), an E3 ubiquitin ligase complex, in a way that allows Nrf2 degradation through the 26S proteasome [10]. Reactive oxygen species or prooxidant chemicals such as *tert*-butylhydroquinone (tBHQ) can oxidize thiol groups in specific cysteine residues of Keap1 which modifies its affinity for Nrf2 and leads to a new conformation of the Keap1/Nrf2 complex blocking Nrf2 degradation [11]. Different studies have clearly showed that arsenic can increase Nrf2 expression by reducing activity of the Keap1-ubiquitine ligase complex [12,13]. Furthermore, besides this canonical pathway, arsenic can also stimulate Nrf2 expression and activity by impairing the basal autophagic pathway in a p62-dependent manner [14]. Upregulation of Nrf2 levels in cells exposed to arsenic is generally considered as a protective stress response which stimulates the expression of several antioxidant enzymes and prevents the cells from deleterious intracellular damage [11]. In contrast, the possibility that Nrf2 expression could also directly contribute to specific toxic effects of the metalloid has never been reported.

Several studies using murine Nrf2$^{-/-}$ models have demonstrated that genetic invalidation of Nrf2 expression not only reduces antioxidant defenses but also promotes the development of major inflammatory-related diseases such as atherosclerosis, chemical-induced allergy, and chronic obstructive pulmonary diseases [15–17]. Interestingly, Nrf2 inhibition markedly alters the phenotype of murine DCs. Indeed, Nrf2$^{-/-}$ DCs have reduced glutathione levels, impaired phagocytic functions, and enhanced T cell stimulatory capacity [18]. Moreover, these cells secrete higher levels of cytokines such as TNF-α, IL-6, and IL-12 [19], suggesting that constitutive or induced Nrf2 levels may limit their expression.

The present study was thus designed to determine if arsenic can repress IL-12p70 expression in human DCs by stimulating Nrf2 activation. Our results demonstrate that the pretreatment of human immature DCs with noncytotoxic concentrations of inorganic arsenic markedly increases the expression and activity of Nrf2 and inhibits the production of IL-12p70 in DCs activated with lipopolysaccharide (LPS). Genetic invalidation of Nrf2 prevents this repression of IL-12 expression in human and murine LPS-stimulated DCs pretreated with arsenic, thus demonstrating that Nrf2 activation contributes to immunotoxic effects of this metalloid in DCs.

Materials and methods

Chemical reagents and antibodies (Abs)

Trivalent inorganic arsenic (arsenite), tBHQ, MG132, actinomycin D, propidium iodide (PI), and LPS (*Escherichia coli* 055:B5) were from Sigma-Aldrich. Granulocyte–macrophage colony stimulating factor (GM-CSF) was provided by Shering Plough. IL-4 was from Miltenyi Biotec. Primary antibodies (Abs) directed against Nrf2 (H-300, sc-13032), Hsc70, NQO1, p300, ARNT, p65 NF-κB subunit, and p38-kinase were from Santa Cruz. Abs against phospho-Jun kinase (JNK), phospho-extracellular regulated kinase (ERK), phospho-p38 kinase, and phospho-IκBα were purchased from Cell Signaling Technology.

Cell culture

Peripheral blood mononuclear cells were obtained from blood buffy coats of healthy donors (provided by Etablissement Francais du Sang, Rennes, France) through Ficoll gradient centrifugation. Human monocytes were isolated by positive selection using a magnetic separator QuadroMACS (Miltenyi Biotec) and micromagnetic beads coated with a monoclonal Ab directed against CD14 (Miltenyi Biotec). After washings, monocytes were seeded in 6-well plates at a concentration of 500,000 cells/mL in complete RPMI-1640 (Invitrogen) supplemented with 10% fetal calf serum (Gibco), 500 IU/mL GM-CSF, and 50 ng/mL IL-4 at 37 °C in a 5% CO_2 atmosphere. After 6 days, the phenotype of these cells was concordant with an immature DC population: DC-SIGN^{high}, CD1a^{high}, CD14$^{very\ low}$, HLA-DR^{low}, CD80^{low}, CD83$^{very\ low}$, CD86^{low}, and CD40^{low} [6]. DCs were then washed with complete RPMI-1640, incubated with arsenic or tBHQ for 2 h, and then nonstimulated or stimulated for the indicated times with LPS (25 ng/mL), in the absence or presence of IFN-γ (50 ng/mL), to obtain mature DCs.

Cell number and viability

Viability was determined by the analysis of intracellular PI staining using a FC500 flow cytometer and a CXP Analysis software (Beckman Coulter).

Immunolabeling by flow cytometry.

Phenotypic analysis of DCs was performed by direct immunofluorescence using flow cytometry. Cells were first blocked in phosphate-buffered saline (PBS) supplemented with 5% fetal calf serum for 20 min to avoid nonspecific binding, resuspended in 30 μl PBS, and then incubated with specific Ab or appropriate isoptypic controls. After 30 min, cells were washed and analyzed by flow cytometry (10,000 events per sample), using a FC500 flow cytometer. The following Abs were used: phycoerythrin (PE)-conjugated mouse monoclonal Ab against CD14 (Miltenyi Biotech) or fluorescein isothiocyanate-conjugated mouse monoclonal Abs against CD1a (Miltenyi Biotech), CD80, CD83, or CD86 (BD Bioscience). Corrected mean fluorescence intensities (cMFI) were calculated by subtracting values of isotypic control MFI from those of specific Ab MFI.

Quantification of cytokine levels

Levels of IL-12p40, IL-12p70, TNF-α, IL-6, IL-8, and IL-23 secreted in culture medium were quantified by ELISA, using specific Duoset ELISA development system kits (R&D Systems) following the manufacturer's instructions.

RNA isolation and reverse transcriptase–polymerase chain reaction (RT-PCR) assays

Total RNAs were extracted by the TRIzol method (Invitrogen) and then analyzed by RT-PCR using the fluorescent dye SYBR Green methodology and an ABI Prism 7300 detector (Applied Biosystem), as previously described [6]. Specific gene primer sets were from Qiagen. Specificity of gene amplification was checked up at the end of PCR through analysis of dissociation curves of the PCR products. Amplification curves were analyzed by the comparative cycle threshold method. Relative quantification of the steady-state target mRNA

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