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Arsenic Trioxide Induces Apoptosis in Peripheral Blood T Lymphocyte Subsets by Inducing Oxidative Stress: A Role of Bcl-2

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Abstract

Arsenic trioxide (As_2O_3) has been used successfully in the treatment of acute promyelocytic leukemia. However, effects of As_2O_3 in normal peripheral blood T cells have not been studied in detail. The purpose of this study was to investigate whether As_2O_3 would induce apoptosis in normal T cells and therefore may have immunosuppressive side effects. Apoptosis was measured by terminal deoxynucleotidyl transferase-mediated nick end labeling assay, caspase activation by flow cytometry and colorimetric assay, mitochondrial transmembrane potential ($\Delta\psi_m$), intracellular reactive oxygen species (ROS), and intracellular reduced glutathione (GSH) by flow cytometry. The release of cytochrome *c* and apoptosis-inducing factor (AIF) from the mitochondria was measured by confocal microscopy, and the expression of molecules regulating apoptosis was measured by Western blotting. As_2O_3, at clinically achievable therapeutic concentrations, induces apoptosis in peripheral blood T cells. As_2O_3-induced apoptosis was associated with reduced $\Delta\psi_m$, enhanced generation of intracellular ROS, decreased levels of intracellular GSH, release of cytochrome *c* and AIF from the mitochondria, activation of caspases, down-regulation of Bcl-2 and Bcl-x_L, and up-regulation of Bax expression. In addition, exogenous GSH protected lymphocytes from As_2O_3-induced apoptosis. Furthermore, overexpression of Bcl-2 inhibited As_2O_3-induced apoptosis and blocked depolarization of $\Delta\psi_m$, generation of ROS, and release of both cytochrome *c* and AIF. These data indicate that As_2O_3 induces apoptosis in T cells by enhancing oxidative stress and that Bcl-2 appears to play a major role in As_2O_3-induced apoptosis.

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Introduction

Arsenic trioxide (As_2O_3) has been used successfully in the treatment of acute promyelocytic leukemia (1, 2). *In vitro*, arsenic trioxide induces apoptosis in malignant cells from acute promyelocytic leukemia, non-Hodgkin's lymphoma, multiple myeloma, and chronic lymphocytic leukemia (3–5). Arsenic compounds also inhibit certain immune functions (6, 7). However, the mechanism underlying arsenic-induced immune suppression is unclear. Furthermore, the effects of As_2O_3 on apoptosis in normal lymphocytes have not been studied in detail.

Apoptosis is a physiological process that plays an important role in the maintenance of cellular homeostasis and the elimination of self-reactive lymphocytes (8). There are two major pathways of apoptosis, the death receptor pathway (9–12) and the mitochondrial pathway (12, 13). The mitochondrial pathway of apoptosis can be triggered by a variety of stimuli, including chemotherapeutic agents, UV radiation, and oxidative stress. Activation of mitochondria results in dissipation of membrane PTP,[2] resulting in decreased (depolarization) mitochondrial membrane potential ($\Delta\psi_m$) and release of intermembrane contents including cytochrome *c* and AIF from the mitochondria into cytoplasm. Cytochrome *c* binds to an adapter protein, Apaf-1 (apoptotic protease-activating factor), in the presence of ATP or dATP, which then recruits procaspase-9 to form an apoptosome. This results in the autolytic activation of procaspase-9 to active caspase-9, which in turn cleaves and activates effector procaspases to active effector caspases, including caspase-3. These effector caspases cleave a number of substrates, resulting in morphological and biochemical changes of apoptosis (8, 14). The function of PTP and release of intermembrane contents of mitochondria are under regulatory control of a number of proteins of the Bcl-2 family (8, 14).

In this investigation, we show that As_2O_3-induces apoptosis in both CD4+ and CD8+ T cells via the mitochondrial pathway by inducing oxidative stress and that Bcl-2 inhibits As_2O_3-induced apoptosis.

Materials and Methods

Cell Cultures. MNCs were separated from peripheral blood obtained from healthy young volunteers (approved by the

[2] The abbreviations used are: PTP, permeability transition pore; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; ROS, reactive oxygen species; GSH, glutathione; AIF, apoptosis-inducing factor; MNC, mononuclear cell; RT-PCR, reverse transcription-PCR; TMRE, tetramethlyrhodamine ethyl; pNA, *p*-nitinoanalide; VDAC, voltage-dependent anion channel; OM, outer mitochondrial; IAP, inhibitor of apoptosis protein; FMK, fluoromethyl ketone; TBS-T, Tris-buffered saline with Tween 20; FAM, carboxyfluorescein; XIAP, X-chromosome-linked inhibitor of apoptosis protein.

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Institutional Review Board, University of California, Irvine, CA). MNCs were incubated in the presence or absence of various concentrations (1.0–5.0 μM) of As_2O_3 (Sigma, St. Louis, MO) for specified duration and then analyzed.

Apoptosis. Apoptosis was measured by TUNEL assay. Cells (1×10^6 cells/ml) were incubated for 48 h with or without various concentrations of As_2O_3 and stained with PerCp-labeled anti-CD4 and phycoerythrin-labeled anti-CD8 (BD BioSciences, San Jose, CA). Cells were washed with PBS containing 1% BSA and 01% sodium azide and fixed in 2% paraformaldehyde for 30 min at room temperature. Cells were washed with PBS and permeabilized with sodium citrate buffer containing Triton X-100 for 2 min on ice. After washing, cells were incubated with FITC-dUTP in the presence of terminal deoxynucleotidyl transferase enzyme solution containing 1 μM potassium cacodylate and 125 mM Tris-HCl (pH 6.6; In Situ Death Detection Kit; Boehringer Mannheim, Indianapolis, IN) for 1 h at 37°C. After incubation, cells were washed with PBS, and 5000 cells were acquired and analyzed by FACScan.

Determination of Mitochondrial Membrane Potential ($\Delta\psi_m$). MNCs (2×10^6 cells/ml) were incubated for 18 h with or without 5 μM As_2O_3 at 37°C and then incubated for 10 min at 37°C in medium containing 50 nM TMRE (Molecular Probes, Eugene, OR) alone or in the presence of 10 μM carbonyl cyanide *p*-trifluoromethoxy phenyl-hydrazone (Sigma) as an internal control. Cells were stained with PerCp-labeled anti-CD4 and FITC-labeled anti-CD8 monoclonal antibodies, and changes in $\Delta\psi_m$ were measured by FACScan. Data were acquired and analyzed by FACScan using Cell Quest software (Becton Dickinson, Menlo Park, CA).

Measurement of ROS and GSH. Cells (2×10^6 cells/ml) were resuspended in RPMI 1640 containing 5 μM of cell permeable oxidation-dependent fluorescence dye 2′,7′-dichlorofluorescein diacetate (Molecular Probes) for 20 min at 37°C. Then cells were cultured with or without 5 μM As_2O_3 for an additional 3 h at 37°C, stained with PerCp-labeled anti-CD4 and phycoerythrin-labeled anti-CD8 monoclonal antibodies, and analyzed by FACScan.

Intracellular levels of GSH were measured with monobrombiamine (Molecular Probes) by a flow cytometric method described by Hedley and Chow (15).

Analysis of Cytochrome *c* and AIF Release. The release of cytochrome *c* and AIF from the mitochondria was analyzed by confocal microscopy. In brief, MNCs were incubated with 5 μM As_2O_3 for 18 h, washed three times with PBS, and then loaded with 150 nM Mitotracker Orange (Molecular Probes) for 15 min at 37°C. After fixation with 4% (w/v) freshly prepared paraformaldehyde in PBS for 30 min at room temperature and washing three times in PBS, cells were permeabilized with 0.25% (w/v) saponin in PBS for 5 min and washed three times in blocking buffer [0.05% saponin, 3% BSA in PBS (pH 7.4)]. Cells were incubated overnight with a 1:100 dilution of the primary mouse antihuman cytochrome *c* antibody (PharMingen, San Diego, CA) or rabbit antihuman AIF antibody (ProSci Inc., Poway, CA), washed, and then incubated with FITC-labeled goat antimouse IgG antibody (Antibody Inc., Davis, CA) or FITC-labeled goat antirabbit IgG antibody (Oncogene, Boston, MA) for 1 h at room temperature. Cells were washed three times with blocking buffer and cytospun onto slides. Coverslips were mounted with an anti-fade reagent (Bio-Rad, Hercules, CA), slides were imaged using a laser-based confocal microscope, and the percentages of the cells that have released cytochrome *c* or AIF were determined.

Caspase Activity. Cells were incubated with varying concentrations of As_2O_3 at 37°C for 18 h. Caspase-3, caspse-8, and caspase-9 activities were assessed using a colorimetric assay kit (Biovision Research Products, Palo Alto, CA) according to the protocol provided by the manufacturer and by flow cytometry.

The colorimetric assay is based on the spectrophotometric detection of the chromophore pNA after cleavage from the labeled substrate. Briefly, 1×10^6 cells were resuspended in chilled lysis buffer for 10 min, and cytosolic extracts were isolated by centrifugation of lysed cells at $10{,}000 \times g$ for 2 min. Cytosolic extracts were incubated with conjugated caspase-3 substrate DEVD-pNA, caspase-8 substrate IETD-pNA, or caspase-9 substrate LEHD-pNA in the presence of reaction buffer for 1 h at 37°C. The chromophore pNA was detected using a microtiter plate reader at 405 nm. Background readings from cell lysates incubated in the absence of substrates were subtracted from sample readings before determination of fold increase in caspase activity.

The activation of caspases in CD4+ and CD8+ T cells was analyzed with carboxyfluorescein-labeled cell-permeable peptide substrates (Intergen Co., Purchase, NY) that recognize cleaved caspase-9 (FAM-LEHD-FMK), caspase-8 (FAM-IETD-FMK), and caspase 3 (FAM-DEVD-FMK), by triple color analysis using FACScan.

Transfection of Bcl-2 with Expression Plasmid. MNCs were activated with α-CD3 monoclonal antibody for 48 h, cultured in interleukin 2-containing culture medium for an additional 24 h, and transfected with pBcl-2 plasmid (pcDNA3 Bcl-2; Science Reagent, El Cajon, CA) using the Lipofectin reagent (Life Technologies, Inc., Gaithersburg, MD). Briefly, 0.8 ml of the cell suspension (3×10^6 cells/ml) was added to each well in 6-well plates. Two μg of pBcl-2 plasmid DNA were diluted in 98 μl of OPTI-MEM1 medium, and 16 μl of Lipofectin reagent were diluted in 84 μl of OPTI-MEM1 medium. The vector plasmid without *bcl-2* gene was used as a negative control. After a 45-min incubation at room temperature, the DNA and Lipofectin diluents were combined and incubated for 15 min at room temperature. Then, 200 μl of the DNA/Lipofectin mixture were added to each well, and cells were incubated for 12 h at 37°C. Three ml of interleukin 2-containing culture medium were added to each well, and cells were allowed to express Bcl-2 for 2 days. Bcl-2 expression was confirmed by real-time RT-PCR. Total cellular RNA (0.2 μg) was used for reverse transcription, and subsequent PCR amplification was done with specific primers and TaqMan probes for *bcl-2* and glyceraldehyde-3-phosphate dehydrogenase cDNAs.

Western Blotting. Cells treated with or without various concentrations of As_2O_3 were centrifuged, and whole cell extracts were prepared by lysing the cell pellet in 50 μl of cold TGNT buffer with protease and phosphatase inhibitors [100 mM Tris-Cl (pH 7.4), 20% glycerol, 100 mM NaCl, 2%

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Triton X-100, 20 mM EGTA, 100 mM NaF, 4 mM phenylmethylsulfonyl fluoride, 4 mM sodium orthovanadate, and 2 mM *p*-nitrophenol phosphate] and clarified by centrifugation at 4°C for 20 min. Protein concentration of the lysates was determined by Bradford assay (Bio-Rad, Richmond, CA). Aliquots of cell lysates containing 25 μg of total protein were resolved by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes by electroblotting. The membranes were blocked for 2 h at room temperature in TBS-T buffer with 5% nonfat dried milk and sequentially probed by overnight incubation at 4°C with primary antibodies diluted in TBS-T buffer with 5% nonfat dried milk. These antibodies include anti-XIAP, anti-Bcl-x_L, anti-Bax, anti-Bcl-2 (1:2000 dilution; Transduction Laboratory, San Diego, CA), and anti-VDAC (Affinity Bioreagent, Golden, CO). The blots were washed three times for 15 min with TBS-T buffer and then incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody (1:2,000 dilution; Cell Signaling Technology, Beverly, MA) for 1 h at room temperature. After washing three times for 20 min in TBS-T buffer, blots were developed with ECL Plus detection system (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Before each cycle of reprobing, blots were incubated at 50°C for 45 min in stripping buffer [62.5 mM Tris (pH 6.7), 2% SDS, and β-mercaptoethanol]. To normalize protein loading and transfer efficiency, the blots were probed with antiactin antibody (1:20,000 dilution; Chemicon, Temecula, CA).

Statistical analysis was performed by Student's *t* test.

Results

As_2O_3 Induces Apoptosis in Both CD4+ and CD8+ T Cells. *In vitro* As_2O_3 induces apoptosis in malignant lymphocytes (3–5). In this study, we examined the effect of As_2O_3 on apoptosis of peripheral blood T-cell subsets. Peripheral blood MNCs from healthy subjects were incubated with various concentrations of As_2O_3 (1.0–5.0 μM) for 48 h, and apoptosis was measured by triple-color flow cytometry using TUNEL assay and specific monoclonal antibodies against CD4 and CD8 and isotype controls. Fig. 1*A* shows that As_2O_3, in a concentration-dependent manner, induced apoptosis in both CD4+ and CD8+ T cells.

We then examined the effect of As_2O_3 on the activation of caspase-9, caspase-3, and caspase-8 in CD4+ and CD8+ T-cell subsets by intracellular staining. As_2O_3 activated caspase-8 (Fig. 1*B*), caspase-9 (Fig. 1*C*), and caspase-3 (Fig. 1*D*). Fig. 1*E* shows As_2O_3-induced activation of caspases by colorimetric assay. To demonstrate a role of caspases in As_2O_3-induced apoptosis, we examined the effect of caspase inhibitors on As_2O_3-induced apoptosis. The inhibitors of caspase-8 (Z-IETD-FMK), caspase-9 (Z-LEHD-FMK), and caspase-3 (DEVD-FMK) partially but significantly ($P < 0.05$) suppressed As_2O_3-induced apoptosis in both CD4+ and CD8+ T cells (Fig. 1*F*).

As_2O_3 Induces Oxidative Stress and Reduces Intracellular GSH. Arsenic compounds have been shown to induce generation of ROS in tumor cell lines (16, 17), and oxidative stress is one of the mediators of apoptosis (18, 19). Therefore, we examined the effect of As_2O_3 on intracellular levels of ROS. MNCs were incubated with 5 μM As_2O_3 for 3 h, and intracellular ROS was measured with 2′,7′-dichlorofluorescein diacetate by triple-color analysis using FACScan. As_2O_3 significantly enhanced intracellular levels of ROS in both CD4+ and CD8+ T cells (Fig. 2, *A* and *B*).

GSH, an antioxidant, protects cells from undergoing stress-induced apoptosis (20), and reduced intracellular levels of GSH are associated with increased susceptibility to apoptosis (21). Therefore, we analyzed the effect of As_2O_3 on intracellular concentration of GSH. MNCs were incubated with 5 μM As_2O_3, and intracellular GSH contents were measured by flow cytometry using FACS. As_2O_3 reduced intracellular GSH levels in MNCs (Fig. 2*C*).

To determine a role of decreased intracellular level of GSH in As_2O_3-induced apoptosis, MNCs were pre-exposed to 5.0 mM GSH for 2 h and then treated with 5.0 μM As_2O_3 for 48 h, and apoptosis was measured by TUNEL assay. GSH inhibited As_2O_3-induced apoptosis in both CD4+ and CD8+ T cells (Fig. 2*D*), suggesting that As_2O_3 induces apoptosis by altering intracellular redox status.

As_2O_3 Reduces $\Delta\psi_m$ and Induces a Release of Cytochrome *c* and AIF from the Mitochondria into the Cytoplasm. Because apoptosis mediated via the mitochondrial pathway is associated with sequential reduction of $\Delta\psi_m$ and generation of ROS (19), we examined the effect of As_2O_3 on $\Delta\psi_m$. MNCs were incubated with 5.0 μM As_2O_3 for 18 h, and $\Delta\psi_m$ was measured with TMRE dye using FACScan. As_2O_3 induced depolarization of $\Delta\psi_m$ in both CD4+ and CD8+ T cells (Fig. 3).

The depolarization of mitochondrial membrane is associated with release of cytochrome *c* and AIF (13, 22, 23). Therefore, we examined the effect of As_2O_3 on the release of cytochrome *c* and AIF. MNCs were incubated with 5 μM As_2O_3 for 18 h, and release of cytochrome *c* and AIF was analyzed by confocal microscopy using mitochondrial dye Mitotracker Orange and anti-cytochrome *c* and anti-AIF antibodies. Hoechst 33342 dye was also used to examine chromatin fragmentation as an indication of apoptosis. In Fig. 4, *a* and *b, A* is MNCs alone, and *D* is MNCs treated with As_2O_3. As_2O_3 induced the release of cytochrome *c* (Fig. 4*a, D*) in 30% of cells and AIF (Fig. 4*b, D*) in 45% of cells from the mitochondria into the cytoplasm.

As_2O_3 Down-Regulates Bcl-2, Bcl-x_L, and VDAC Expression and Up-Regulates Bax Expression. The release of cytochrome *c* and AIF is under regulatory control of Bcl-2 family proteins; Bcl-2 and Bcl-x_L block the release of cytochrome *c* and AIF, whereas Bax promotes the release of cytochrome *c* and AIF (24, 25). Because As_2O_3 increases intracellular ROS, and Bcl-2 has been shown to serve as an antioxidant (26, 27), we examined the effect of As_2O_3 on the expression of Bcl-2 family proteins. As_2O_3 down-regulated the expression of both Bcl-2 and Bcl-x_L; however, the expression of Bax was up-regulated (Fig. 5). One of the components of the PTP complex is VDAC, which is present in the outer membrane of the mitochondria. It has been suggested that Bcl-2 family proteins bind to VDAC and regulate the release of cytochrome *c* and apoptosis (26). Therefore, we also examined the effect of As_2O_3 on VDAC expression by Western blotting. As_2O_3 down-regulated the expression of VDAC (Fig. 5).

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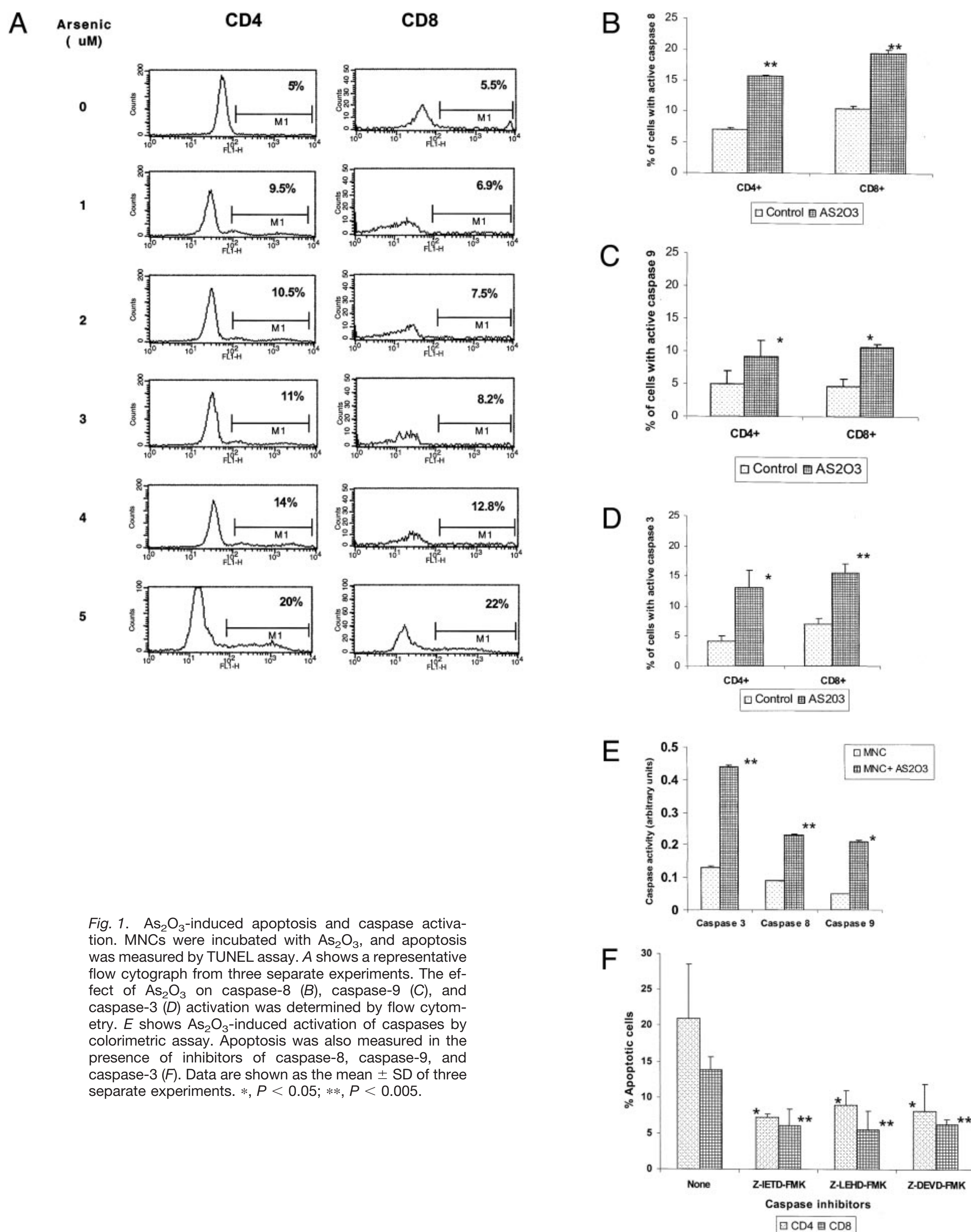

Fig. 1. As_2O_3-induced apoptosis and caspase activation. MNCs were incubated with As_2O_3, and apoptosis was measured by TUNEL assay. *A* shows a representative flow cytograph from three separate experiments. The effect of As_2O_3 on caspase-8 (*B*), caspase-9 (*C*), and caspase-3 (*D*) activation was determined by flow cytometry. *E* shows As_2O_3-induced activation of caspases by colorimetric assay. Apoptosis was also measured in the presence of inhibitors of caspase-8, caspase-9, and caspase-3 (*F*). Data are shown as the mean ± SD of three separate experiments. *, $P < 0.05$; **, $P < 0.005$.

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Bcl-2 Overexpression Protects T Cells from As_2O_3-induced Apoptosis. Bcl-2 regulates $\Delta\psi_m$, generation of ROS, and release of cytochrome *c* and AIF (13, 22, 23) and serves as an antioxidant (27). Because As_2O_3 down-regulated Bcl-2 expression, enhanced generation of ROS, reduced GSH, and induced the release of cytochrome *c* and AIF, we determined whether Bcl-2 plays a major role in As_2O_3-induced apoptosis in T-cell subsets. T cells were

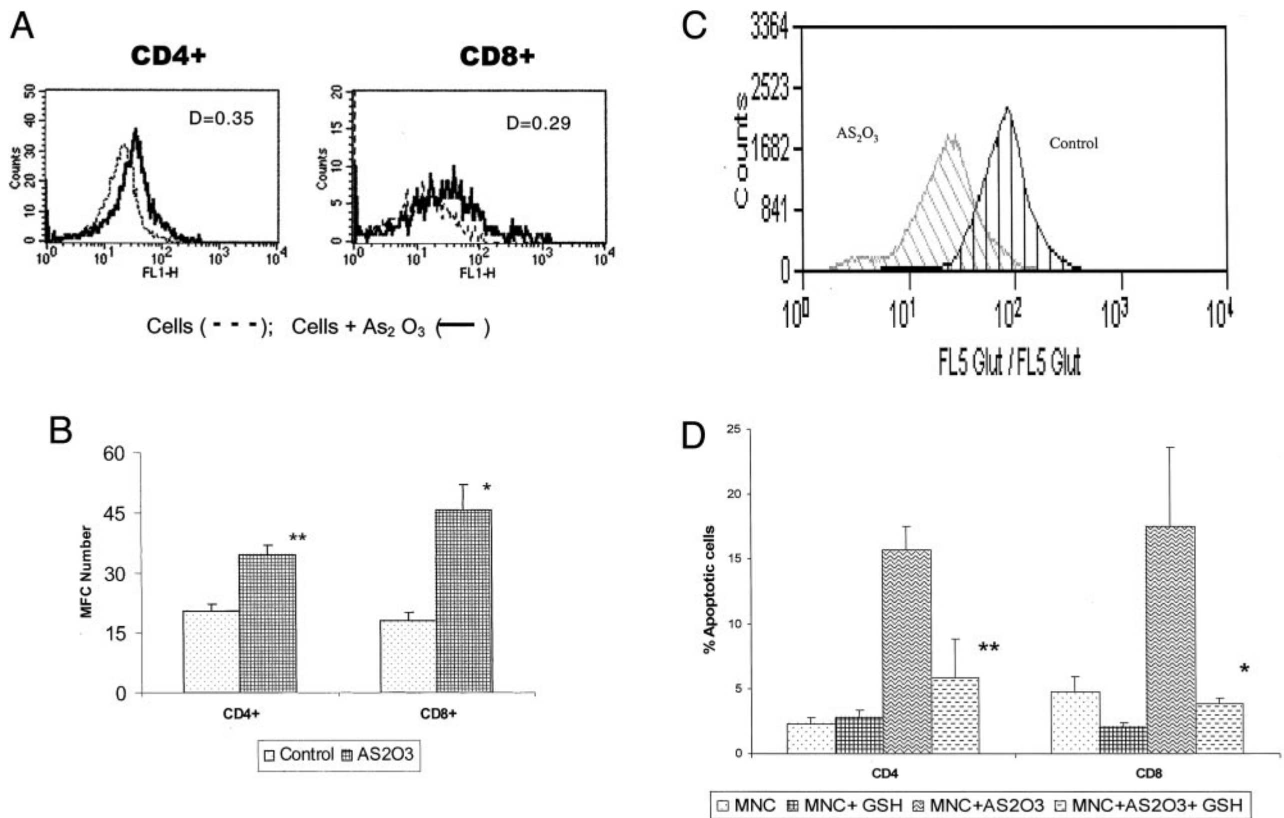

Fig. 2. Effect of As_2O_3 on ROS and GSH levels. *A* is a representative cytofluorograph for the effect of As_2O_3 on ROS. A *D* value of >0.2 is considered significant. *B* represents the mean and SD of three separate experiments. *, $P < 0.05$; **, $P < 0.005$. *C* shows the effect of As_2O_3 on GSH, and *D* shows data for *in vitro* effect of exogenous GSH on As_2O_3-induced apoptosis.

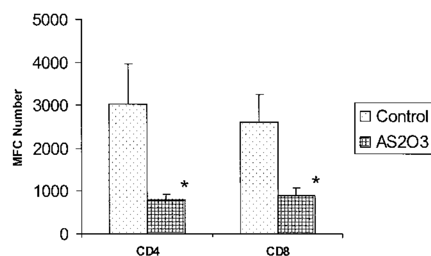

Fig. 3. Effect of As_2O_3 on mitochondrial membrane potential. As_2O_3 reduced $\Delta\psi m$ as determined with TMRE dye using triple-color flow cytometry. Data are shown as the mean ± SD of three separate experiments.

transfected with control plasmid and Bcl-2 expression plasmid. The transfection was confirmed by real-time RT-PCR (Fig. 6*a*). Cells were incubated with 5.0 μM As_2O_3 and examined for apoptosis, $\Delta\psi_m$, ROS, and release of cytochrome *c* and AIF. The transfection of T cells with Bcl-2 resulted in an inhibition of As_2O_3-induced apoptosis (Fig. 6*B*) and an inhibition of activation of caspases (Fig. 6*C*). In addition, overexpression of Bcl-2 resulted in a resistance of T-cell subsets to As_2O_3-induced depolarization of $\Delta\psi_m$ (Fig. 6*D*) and generation of ROS (Fig. 6*E*). Bcl-2 overexpression also inhibited cytochrome *c* and AIF release. Ten percent of Bcl-2-overexpressing cells release cytochrome *c* (Fig. 4*a, F*) and AIF (Fig. 4*b, F*), whereas 30% of control plasmid-transfected cells released cytochrome *c* (Fig. 4*a, E*), and 45% of control plasmid-transfected cells released AIF (Fig. 4*b, E*). These data strongly suggest that Bcl-2 plays a major role in As_2O_3-induced apoptosis in normal T-cell subsets.

Discussion

In this study we have demonstrated that As_2O_3, at a clinically achievable concentration, induces apoptosis in normal peripheral blood CD4+ and CD8+ T cells via mitochondrial pathway by enhancing the generation of oxidative stress and by regulating the expression of Bcl-2 family proteins.

The changes in $\Delta\psi_m$, generation of ROS, and release of cytochrome *c* and AIF are major elements of the mitochondrial pathway of apoptosis (8, 10, 13, 23, 28). Therefore, we examined the effect of As_2O_3 on $\Delta\psi_m$. As_2O_3, in a concentration-dependent manner, decreased $\Delta\psi_m$, suggesting depolarization of the mitochondrial membrane. Similar depolarization of the mitochondrial membrane has been observed with As_2O_3 in various tumor cell lines (17, 29).

Mitochondria are major generators of ROS, which predominantly include superoxide anion, hydrogen peroxide, and the hydroxyl radical (29, 30). The major source of ROS in most cell types is probably the electron leakage from mitochondrial electron transport chain that reduces molecular oxygen

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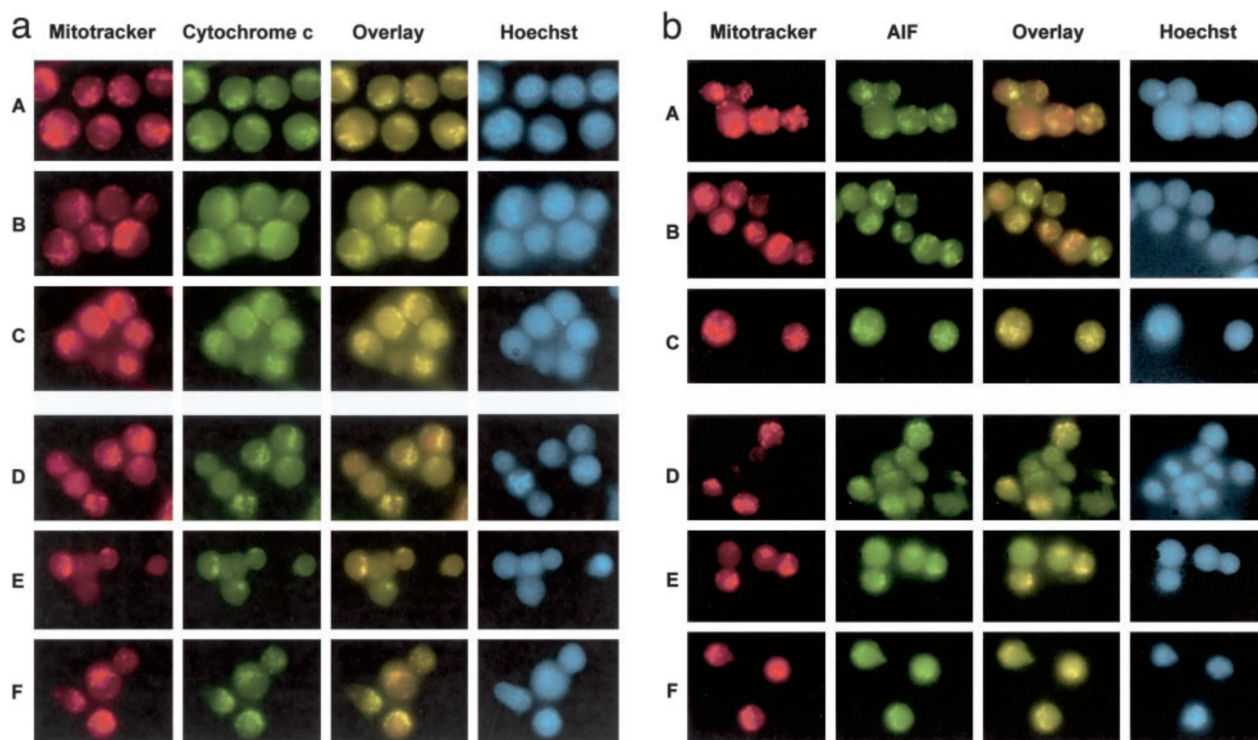

Fig. 4. Effect of As_2O_3 on the release of cytochrome *c* and AIF. The effect of As_2O_3 on release of cytochrome *c* (*a*) and AIF (*b*). In *a* and *b*, *A* shows MNCs alone, *B* shows MNCs transfected with control plasmid, *C* shows MNCs transfected with Bcl-2 expression plasmid, *D* is MNCs stimulated with As_2O_3, *E* shows MNCs transfected with control plasmid and activated with As_2O_3, and *F* is MNCs transfected with Bcl-2 expression plasmid and activated with As_2O_3. *Overlay* is of Mitotracker (*red*) and cytochrome *c* or AIF (*green*). Hoechst 33258 dye is used to examine chromatin fragmentation.

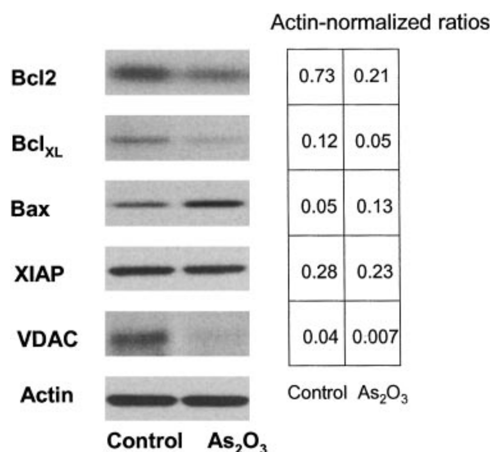

Fig. 5. Effect of As_2O_3 on the expression of proteins regulating apoptosis. Cells treated with As_2O_3 were examined for the expression of molecules regulating apoptosis by Western blotting using specific antibodies. Actin is used as an internal control.

to superoxide ion. Elevated intracellular ROS are sufficient to trigger apoptosis, and it has been suggested that ROS are biochemical mediators of apoptosis (18). In this study, we observed that As_2O_3 increased intracellular levels of ROS in both CD4+ and CD8+ T cells.

Cells contain several antioxidant systems to limit the damage caused by increased ROS, and GSH is an antioxidant that protects cells from oxidative stress-induced apoptosis (20). Glutathione depletion is associated with an increased proportion of cells undergoing apoptosis, and glutathione monoethyl ester decreases apoptosis (21, 31). Furthermore, GSH depletion enforces mitochondrial PTP to induce apoptosis (32), and extrusion of GSH causes cytochrome *c* release (33, 34). In this study, we observed that As_2O_3 reduced intracellular levels of GSH, and *in vitro* glutathione protected T-cell subsets from As_2O_3-induced apoptosis. As_2O_3 has also been shown to reduce GSH levels in tumor cells, and *in vitro* depletion of GSH renders tumor cells more susceptible to As_2O_3-induced apoptosis (21, 29).

The PTP is a channel spanning the inner mitochondrial and OM membrane. The intermembrane space contains a number of molecules including cytochrome *c* and AIF. The permeabilization of the OM, therefore, results in the release of these molecules. Our data show that As_2O_3 induces release of both cytochrome *c* release and AIF from the mitochondria. AIF, once released from mitochondria, is transported into the nucleus, where it stimulates (ATP-independent and caspase-independent) large DNA fragmentation and condensation of chromatin (22). Because As_2O_3 also causes AIF release from the mitochondria, it is possible that, in addition to cytochrome *c*-mediated caspase-dependent apoptosis, As_2O_3 might also induce apoptosis in a caspase-independent manner. This would be consistent with our finding that As_2O_3-induced apoptosis was only partially blocked by caspase inhibitors.

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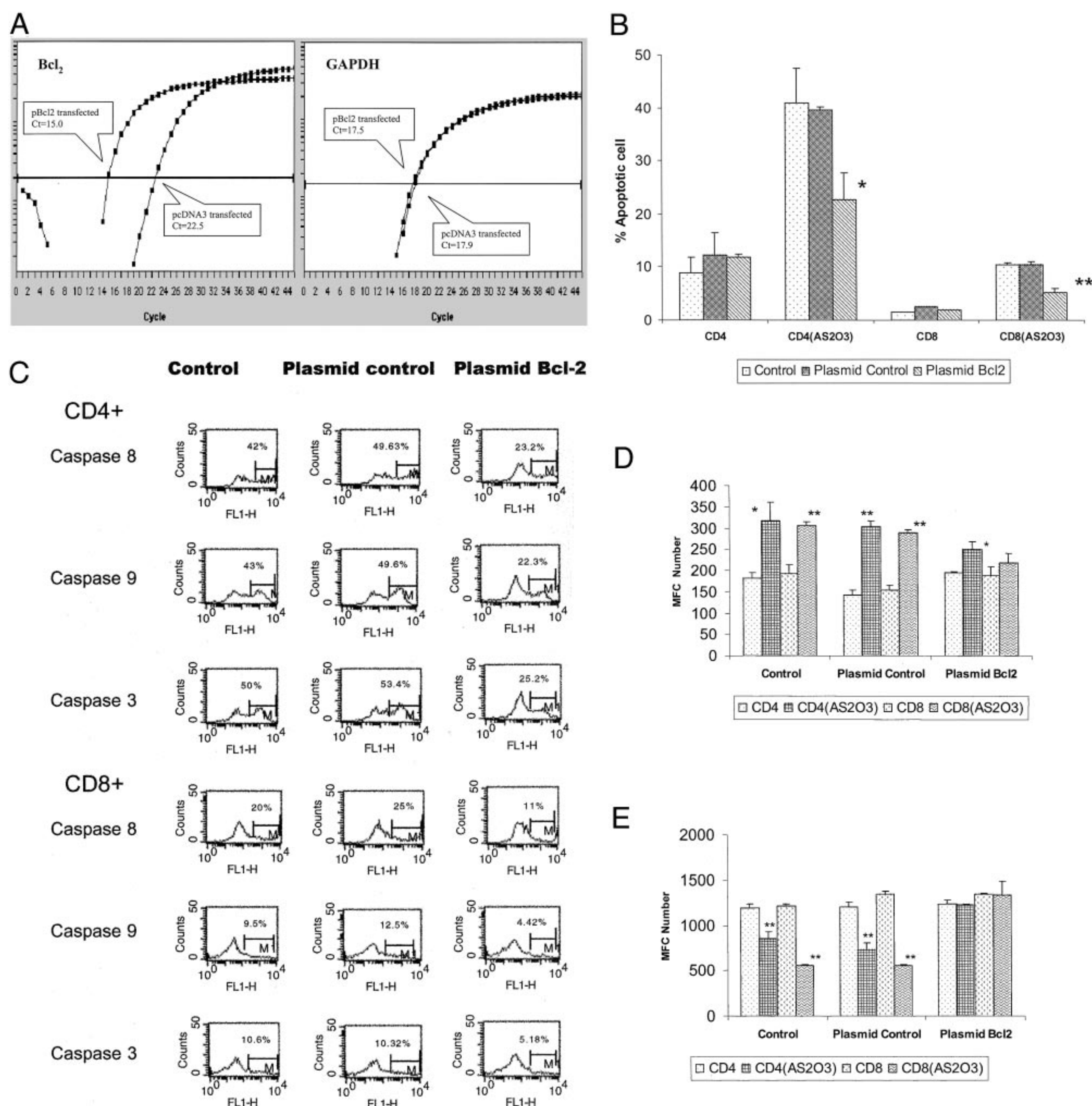

Fig. 6. Effect of overexpression of Bcl-2 on As_2O_3-induced apoptosis, activation of caspases, levels of ROS, and on $\Delta\psi m$. *A* is a representative amplification of real-time RT-PCR for mRNA transcripts of *bcl-2* and glyceraldehyde-3-phosphate dehydrogenase in T cells transfected with *bcl-2* expression plasmid (*pBcl$_2$*) or control plasmid (*pcDNA3*). The cycle numbers in the logarithmic phase of amplification (*Ct values*) were 15.0 or 22.5 for *bcl-2* mRNA in the cells transfected with $pBcl_2$ or pcDNA, indicating that cells transfected with *bcl-2* expression plasmid expressed $2^{7.5}$ times more *bcl-2* mRNA than cells transfected with control plasmid. Decrease of 1 Ct value means a 2-fold increase in mRNA amount. Bcl-2 overexpression inhibited As_2O_3-induced apoptosis (*B*; $*$, $P < 0.05$; $**$, $P < 0.005$), As_2O_3-induced caspase activation (*C*), As_2O_3-induced depolarization of mitochondrial membrane (*D*), and As_2O_3-induced enhancement of ROS (*E*).

Bcl-2 family proteins regulate the release of cytochrome *c* and AIF (35, 36). It has been demonstrated that Bax present in the cytoplasm is translocated to the mitochondrial membrane, where it undergoes conformational changes. The docking of Bax to the OM and its subsequent *in situ* clustering/multimerization cause gating of PTP and release of cytochrome *c* (25). This conformational change and insertion of Bax into mitochondrial membrane are assisted by Bid (37). Bcl-2 and Bcl-x_L inhibit conformational changes in Bax and therefore inhibit release of cytochrome *c* and apoptosis (38). However, a role of Bcl-2 family proteins in the regulation of mitochondrial outer member permeability is controversial.

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Recently Kuwana *et al.* (39) have demonstrated that Bid, Bax, and lipid form supramolecular openings in the OM membrane and that during apoptosis mitochondrial protein release through supramolecular openings in the OM is promoted by Bid/Bax and directly inhibited by Bcl-X_L. In the present study we have demonstrated that As_2O_3 increases the expression of Bax and decreases the expression of Bcl-2 and Bcl-x_L. Chen *et al.* (3) and Akao *et al.* (40) have reported an As_2O_3-induced down-regulation of Bcl-2 in tumor cell lines; however, the effect of As_2O_3 on Bcl-2 in normal lymphocytes and of Bax expression in any cell type has not been documented. Taken together, it appears that As_2O_3 induces apoptosis in T-cell subsets by the release of cytochrome *c* and AIF from the mitochondria by regulating Bcl-2 family proteins.

To determine a role of Bcl-2 in As_2O_3-induced apoptosis, we examined the effect of overexpression of Bcl-2 on various events in As_2O_3-induced apoptosis. An overexpression of Bcl-2 blocked As_2O_3-induced apoptosis, depolarization of $\Delta\psi_m$, generation of ROS, and release of both cytochrome *c* and AIF, demonstrating that Bcl-2 plays a major role in As_2O_3-induced apoptosis in T-cell subsets.

The VDAC, which is present in the outer membrane of mitochondria, has been shown to play a role in apoptosis (26, 28). Furthermore, oxidative stress increases the gating potential of the voltage sensor of PTP (41, 42). Several Bcl-2 family members can bind to VDAC and regulate its channel activity (26). It has been suggested that VDAC undergoes significant conformational changes upon binding to proapoptotic members of Bcl-2 family. In this study, we show that As_2O_3 induces oxidative stress, up-regulates Bax expression, and down-regulates VDAC expression. It is unclear whether down-regulation of VDAC protein represents conformational change or its phosphorylation. The mechanism of down-regulation of VDAC and its role in As_2O_3-induced cytochrome *c* and/or AIF release remains to be investigated.

The release of cytochrome *c* triggers the assembly of Apaf-1 and procaspase-9 to form an apoptosome (8, 13). Procaspase-9 is then autolyticaly cleaved to active caspases-9, which then activates effector caspases including caspase-3, resulting in cleavage of its substrates and apoptosis. In this study, we have demonstrated that As_2O_3 not only activates caspase-9 and caspase-3 but also activates caspase-8. Furthermore, inhibitors of caspase-9, caspase-3, and caspase-8 partially inhibited As_2O_3-induced apoptosis in T-cell subsets. The activation of caspase-8 and partial inhibition of As_2O_3-induced apoptosis by caspase-8 inhibitor suggest that As_2O_3 may also induce apoptosis in T-cell subsets via the death receptor signaling pathway. This observation is in agreement with recent reports that show that As_2O_3-induced tumor necrosis factor α production (43), and tumor necrosis factor α-induced apoptosis is associated with translocation of tBid to the mitochondria (44). Furthermore, Bid/Bax form macro-openings in the OM and induce release of cytochrome *c* and apoptosis (44).

The IAP family of proteins is a novel family of antiapoptotic endogenous proteins (45). IAPs inhibit apoptosis by inhibiting both caspase activation and caspase activity via interaction with caspases-3, -7, and -9. As_2O_3 had no effect on XIAP expression (Fig. 5), demonstrating that XIAP does not play any significant role in As_2O_3-induced apoptosis. This is in contrast to mercury-induced apoptosis in T cells, in which XIAP plays an important role in apoptosis (46).

In summary, As_2O_3 induces apoptosis in human T cells via the mitochondrial pathway by inducing oxidative stress and by regulating Bcl-2 family protein expression. A role of VDAC in As_2O_3-induced apoptosis remains to be investigated. We propose the possibility that long-term treatment of malignant disorders with As_2O_3 may be associated with immune suppression and therefore should be investigated.

References

1. Shen, Z. X., Chen, G. Q., Ni, J. H., Li, X. S., Xiong, S. M., Qiu, Q. Y., Zhu, J., Taang, W., Sun, J. L., Wang, K. Q., Chen, Y., Zhou, L., Fang, Z. W., Wang, Y. T., Ma, J., Zhang, P., Zhang, T. D., Chen, S. J., Chen, Z., and Wang, Z. Y. Use of arsenite trioxide (As_2O_3) in the treatment of acute promyelocytic leukemia. II. Clinical efficacy and pharmacokinetics in relapsed patients. Blood, *89:* 3354–3360, 1997.

2. Soignet, S. L., Maslak, P., Wang, Z. G., Jhanwar, S., Calleja, E., Dardashti, D., Corso, D., DeBlaio, A., Gabrilov, J., Scheinbeg, D. A., Pandolfi, P. P., and Warrell, R. P. Complete remission after treatment of acute promyelocytic leukemia with arsenic trioxide. N. Engl. J. Med., *339:* 1341–1348, 1998.

3. Chen, G. Q., Zhu, J., Shi, X. G., Ni, J. N., Zhong, H. J., Si, G. Y., Jin, X. L., Tang, W., Li, X. S., Xong, S. M., Shen, Z. X., Sun, G. L., Ma, J., Zhang, P., Zhang, T. D., Gazin, C., Naoe, T., Chen, S. J., Wang, Z. Y. and Chen, Z. *In vitro* studies on cellular and molecular mechanisms of arsenic trioxide (As_2O_3) in the treatment of acute promyelocytic leukemia: As_2O_3 induces NB4 cell apoptosis with downregulation of Bcl-2 expression and modulation of PML-RAR α/PML proteins. Blood, *88:* 1052–1061, 1996.

4. Rousselot, P., Labaume, S., Marolleau, J. P., Larghero, J., Noguera, M. H., Brouet, J. C., and Fermand, J. P. Arsenic trioxide and melarsoprol induce apoptosis in plasma cell lines and in plasma cells from myeloma patients. Cancer Res., *59:* 1041–1048, 1999.

5. Shen, L., Chen, T. X., Wang, Y. P., Lin, Z., Zhao, H. J., Zu, Y. Z., Wu, G., and Ying, D. M. As_2O_3 induced apoptosis of the human B lymphoma cell line MBC-1. J. Biol. Regulat. Homeost. Agent, *14:* 116–119, 2000.

6. Vega, L., Ostrosky-Wegman, P., Fortoul, T. I., Diaz, C., Madrid, V., and Saavedra, R. Sodium arsenite reduces proliferation of human activated T-cells by inhibition of the secretion of interleukin-2. Immunopharmacol. Immunotoxicol., *21:* 203–220, 1999.

7. Meng, Z. Q., and Meng, N. Y. Effect of arsenic on blast transformation and DNA synthesis of human blood lymphocytes. Chemosphere, *41:* 115–119, 2000.

8. Hengartner, M. O. The biochemistry of apoptosis. Nature (Lond.), *407:* 770–776, 2000.

9. Ashkenazi, A., and Dixit, V. M. Death receptors: signaling and modulation. Science (Wash. DC), *281:* 1305–1308, 1998.

10. Gupta, S. Molecular signaling in death receptor and mitochondrial pathways of apoptosis. Int. J. Oncol., *22:* 15–20, 2003.

11. Gupta, S. Decision between life and death during TNF-α-induced apoptosis. J. Clin. Immunol., *22:* 175–184, 2002.

12. Krammer, P. H. CD95's deadly mission in the immune system. Nature (Lond.), *407:* 789–795, 2000.

13. Green, D. R., and Reed, J. C. Mitochondria and apoptosis. Science (Wash. DC), *281:* 1309–1312, 1998.

14. Adams, J. M., and Corey, S. L. The Bcl-2 protein family: Arbiters of cell survival. Science (Wash. DC), *281:* 1322–1326, 1998.

15. Hedley, D. W., and Chow, S. Evaluation of methods for measuring cellular glutathione content using flow cytometry. Cytometry, *15:* 349–358, 1994.

16. Lartigue, L., Bauer, M. K. A., Schubert, A., Grimm, S., Hanson, G. T., Remington, S. J., Youle, R. J., Ichas, F., Chen, Y., Shiau, C., Lin, S. Y., and

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Lin, L. K. Involvement of reactive oxygen species and caspase 3 activation in arsenite-induced apoptosis. J. Cell. Physiol., *177:* 324–333, 1998.

17. Woo, S. H., Park, I. C., Park, M. J., Lee, H. C., Chun, Y. J., Lee, S. H., Hong, S. I., and Rhee, C. H. Arsenic trioxide induces apoptosis through a reactive oxygen species-dependent pathway and loss of mitochondrial membrane potential in HeLa cells. Int. J. Oncol., *21:* 57–63, 2002.

18. Buttke, T. M., and Sandstrom, P. A. Oxidative stress as a mediator of apoptosis. Immunol. Today, *15:* 7–10, 1994.

19. Zamzami, N., Marchetti, P., Castedo, M., Decaudin, D., Macho, A., Hirsch, T., Susin, S. A., Petit, P. X., Mignotte, B., and Kroemer, G. Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. J. Exp. Med., *182:* 367–377, 1995.

20. Yu, B. P. Cellular defenses against damage from reactive oxygen species. Physiol. Rev., *74:* 139–162, 1994.

21. Beaver, J. P., and Waring, P. A decrease in intracellular glutathione concentration precedes the onset of apoptosis in murine thymocytes. Eur. J. Biol., *68:* 47–54, 1995.

22. Loeffler, M., Daugas, E., Susin, S. A., Zamzami, N., Metivier, D., Nieminen, A-L., Brothers, G., Penninger, J. M., and Kroemer, G. Dominant cell death induction by extramitochondrially targeted apoptosis-inducing factor. FASEB J., *15:* 758–767, 2001.

23. Jona, N., Susin, S. A., Daugas, E., Stanford, W. L., Cho, S. K., Li, C. Y., Sasaki, T., Elia, A. J., Cheng, H. Y., Ravagnan, L., Ferri, K. F., Zamzami, N., Wakeham, A., Hakem, R., Yoshida, H., Kong, Y. Y., Mak, T. W., Zuniga-Pflucker, J. C., Kroemer, G., and Penninger, J. M. Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. Nature (Lond.), *410:* 549–554, 2001.

24. Marzo, I., Brenner, C., Zamzami, N., Jurgensmeier, J. M., Susin, S. A., Vieira, H. L., Prevost, M. C., Xie, Z., Matsuyama, S., Reed, J. C., and Kroemer, G. The permeability transition pore complex. A target for apoptosis regulation by caspases and Bcl-2-related proteins. J. Exp. Med., *187:* 1261–1271, 1998.

25. De Giorgi, F., Lartigue, L., Bauer, M. K. A., Schubert, A., Grimm, S., Hanson, G. T., Remington, S. J., Youle, R. J., and Ichas, F. The permeability transition pore signals apoptosis by direct Bax translocation and multimerization. FASEB J., *16:* 607–609, 2002.

26. Shimizu, S., Narita, S., and Tsujimoto, Y. Bcl-2 family proteins regulate the release of apoptogenic cytochrome *c* by the mitochondrial channel VDAC. Nature (Lond.), *399:* 483–489, 1999.

27. Hockebery, D. M., Oltvai, Z. N., Yin, X. M., Milliman, C. Y., and Korsmeyer, S. J. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. Cell, *75:* 241–251, 1993.

28. Zamzani, N., and Kroemer, G. The mitochondrion in apoptosis: how Pandora's box opens. Nat. Rev. Mol. Cell. Biol., *2:* 67–71, 2001.

29. Larochette, N., Decaudin, D., Jacotot, E., Brenner, C., Marzo, I., Susin, S. A., Zamzami, N., Xie, Z., Reed, J., and Kroemer, G. Arsenite induces apoptosis via a direct effect on mitochondrial permeability transition pore. Exp. Cell Res., *249:* 413–421, 1999.

30. Brand, M. D., and Murphy, M. P. Control of electron flux through the respiratory chain in mitochondria and cells. Biol. Rev. Camb. Philos. Soc., *62:* 141–193, 1987.

31. Kito, M., Akao, Y., Ohishi, N., Yagi, K., and Nozawa, Y. Arsenic trioxide-induced apoptosis and its enhancement by buthionine sulfoximine in hepatocellular carcinoma cell lines. Biochem. Biophys. Res. Commun., *291:* 861–867, 2002.

32. Armstrong, J. S., and Jones, D. P. Glutathione depletion enforces the mitochondrial permeability transition and causes cell death in Bcl-2 overexpressing HL60 cells. FASEB J., *16:* 1263–1265, 2002.

33. Ghibelli, L., Coppola, S., Fanelli, C., Rotilio, G., Civitareale, P., Scovassi, A. I., and Ciriolo, M. R. Glutathione depletion causes cytochrome *c* release even in the absence of cell commitment to apoptosis. FASEB J., *13:* 2031–2036, 1999.

34. Ghibelli, L., Rescue of cells from apoptosis by inhibition of GSH extrusion. FASEB J., *12:* 479–486, 1998.

35. Finucane, D. M., Bossy-Wetzel, E., Waterhouse, N. J., Cotter, T. G., and Green, D. R. Bax induces caspases activation and apoptosis via cytochrome *c* release from the mitochondria is inhibitable by Bcl-x_L. J. Biol. Chem., *274:* 2225–2233, 1999.

36. Kluck, R. M., Bossy-Wetzel, E., Green, D. R., and Newmeyer, D. D. The release of cytochrome *c* from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science (Wash. DC), *275:* 1132–1136, 1997.

37. Eskes, R., Desagher, S., Antonsson, B., and Matinou, J. C. Bid induces the oligomerization and insertion of bax into the outer mitochondrial membrane. Mol. Cell. Biol., *20:* 929–935, 2000.

38. Cheng, E. H., Wei, M. C., Weiler, S., Flavell, R. A., Mak, T. W., Lindsten, T., and Korsmeyer, S. J. Bcl-2, Bcl-x_L sequester BH3 domain only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. Mol. Cell, *8:* 705–711, 2001.

39. Kuwana, T., Mackey, M. R., Perkins, G., Ellisman, M. H., Latterich, M., Schneiter, R., Green, D. R., and Newmeyer, D. D. Bid, Bax, and lipid cooperate to form supramolecular openings in the outer mitochondrial membrane. Cell, *111:* 331–342, 2002.

40. Akao, Y., Mizoguchi, H., Kojima, S., Naoe, T., Ohishi, N., and Yagi, K. Arsenic induces apoptosis in B cell leukemia cell lines *in vitro*: activation of caspases and downregulation of Bcl-2 protein. Br. J. Haematol., *92:* 1055–1060, 1998.

41. Bernardi, P., Vassanelli, S., Varonese, P., Colonna, R., Szabo, I., and Zoratti, M. Modulation of the mitochondrial permeability transition pore. Effect of protons and divalent cations. Biol. Chem., *268:* 13791–13798, 1992.

42. Petronilli, V., Costantni, P., Scorrano, L., Colonna, R., Passamonti, S., and Bernardi, P. The voltage sensor of mitochondrial permeability transition pore is turned on by the oxidation-reduction state of vicinol thiols. Increase of the gating potential by oxidants and its reversal by reducing agents. J. Biol. Chem., *269:* 16638–16642, 1994.

43. Yu, H. S., Liao, W. T., Chang, K. L., Yu, C. L., and Chen, G. S. Arsenic induces tumor necrosis factor α release and tumor necrosis factor receptor 1 signaling in T helper cell apoptosis. J. Investig. Dermatol., *119:* 812–819, 2002.

44. Perez, D., and White, E. TNF-α signals apoptosis through a Bid-dependent conformational change in Bax that is inhibited by E1B 19K. Mol. Cell, *6:* 53–63, 2000.

45. Deveraux, Q. L., Stennicke, H. R., Salvensen, G. S., and Reed, J. C. Endogenous inhibitor of caspases. J. Clin. Immunol., *19:* 388–399, 1999.

46. Makani, S., Gollapudi, S., Yel, L., Chiplunkar, S., and Gupta, S. Biochemical and molecular basis of thimerosal-induced apoptosis in T cells: a major role of mitochondrial pathway. Genes Immun., *3:* 270–278, 2002.

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