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Arsenic trioxide induces apoptosis of human monocytes during macrophagic differentiation through NF-κB-related survival pathway downregulation.

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Running Title Page

A Running title:

As_2O_3 induced monocyte apoptosis by NF-κB inhibition

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D Abbreviations

As_2O_3: arsenic trioxide; X-linked inhibitor of apoptosis protein: XIAP; FLICE-inhibitory protein: FLIP; granulocyte-macrophage colony-stimulating factor: GM-CSF; phorbol 12-myristate 13-acetate: PMA; Annexin-V (A5); Sytox Green (SG); phosphate buffer saline (PBS); FITC: fluorescein isothiocyanate

E Recommended section: toxicology

Abstract

Arsenic trioxide (As_2O_3) is known to be toxic towards leukemia cells. In this study, we determined its effects on survival of human monocytic cells during macrophagic differentiation, an important biological process involved in the immune response. As_2O_3, used at clinically relevant pharmacological concentrations, induced marked apoptosis of human blood monocytes during differentiation with either granulocyte-macrophage colony-stimulating factor (GM-CSF) or M-CSF. Apoptosis of monocytes was associated with increased caspase activities and decreased DNA binding of p65 NF-κB; like As_2O_3, the selective NF-κB inhibitor Bay 11-7082 strongly reduced survival of differentiating monocytes. The role of NF-κB in arsenic toxicity was also studied in promonocytic U937 cells during PMA-induced macrophagic differentiation. In these cells, As_2O_3 first reduced DNA binding of p65 NF-κB and subsequently induced apoptosis. In addition, overexpression of the p65 NF-κB subunit, following stable infection with a p65 retroviral expressing vector, increased survival of As_2O_3-treated U937 cells. As_2O_3 specifically decreased protein levels of X-linked inhibitor of apoptosis protein (XIAP) and FLICE-inhibitory protein (FLIP), two NF-κB-regulated genes in both U937 cells and blood monocytes during their differentiation. Finally, As_2O_3 was found to inhibit macrophagic differentiation of monocytic cells, when used at cytotoxic concentrations; however, overexpression of the p65 NF-κB subunit in U937 cells reduced its effects towards differentiation. In contrast to monocytes, well-differentiated macrophages were resistant to low concentrations of As_2O_3. Altogether, our study demonstrates that clinically relevant concentrations of As_2O_3 induced marked apoptosis of monocytic cells during in vitro macrophagic differentiation likely through inhibition of NF-κB-related survival pathways.

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Introduction

Arsenic trioxide (As_2O_3), an inorganic trivalent salt, is successfully used in the treatment of acute promyelocytic leukemia (Shen et al., 1997). Preliminary clinical data suggest that As_2O_3 has also a potential effectiveness in patients with other malignant hemopathies, including relapsed or refractory multiple myeloma (Munshi et al., 2002). Hematologic remissions are generally reported using As_2O_3 at the doses of 0.15 mg/kg per day for variable durations. Following iv infusions, blood arsenic levels reach to 5-7 µmol/l and then fall approximately to 1 µmol/l and 0.2 µmol/l at 12 h and 24 h after As_2O_3 administration, respectively (Shen et al., 1997). In vitro, low concentrations of As_2O_3 are markedly toxic towards acute promyelocytic leukemia and multiple myeloma cells and to other hematological tumoral cells such as non-Hodgkin lymphoma and chronic lymphocytic leukemia cells (Zhang et al., 1998). Cellular toxicity of As_2O_3 (0.5-2 µM) can first result from oxidative stress-induced mitochondrial damages and subsequent caspase-dependent apoptosis of leukemia cells (Jing et al., 1999; Zhou et al., 2003). On the other hand, arsenic can also induce apoptosis through inhibition of the NF-κB pathway (Mahieux et al., 2001; Mathas et al., 2003; Nasr et al., 2003) which controls expression of various survival proteins, including the caspase inhibitors X-linked inhibitor of apoptosis protein (XIAP) and FLICE-inhibitory protein (FLIP) (Micheau et al., 2001; Zhang et al., 2003). In leukemia cells, arsenic is thought to decrease NF-κB activity by directly inhibiting IκBα kinase (Mathas et al., 2003), thus preventing NF-κB release and translocation to the nucleus.

Besides leukemia cells, As_2O_3 may also be toxic towards normal hematological cells. Indeed, this metalloid was reported to induce neutropenia in 8% and up to 80% of patients suffering from acute promyelocytic leukemia and multiple myeloma, respectively (Soignet et al., 2001; Munshi et al., 2002). In the case of multiple myeloma, severe cytopenia in As_2O_3-treated patients are likely related to myelosuppression due to extensive prior therapy;

nonetheless, it appears that arsenic can be directly toxic, at least in vitro, to blood circulating cells. Notably, it was recently reported that low concentrations of As_2O_3, in the range of clinically effective concentrations (1-5 μM), induce partial apoptosis of T lymphocytes by increasing oxidative stress and caspase activation (Gupta et al., 2003). In addition, sodium arsenite, another trivalent inorganic arsenic salt, was shown to reduce proliferation of normal T lymphocytes at low μM concentrations by delaying production and secretion of interleukin-2 (Galicia et al., 2003). Experimental studies have demonstrated that arsenite also markedly impairs functional integrity of monocytes/macrophages. In vivo, it alters macrophage functions, such as adhesion or phagocytic activity (Sengupta and Bishayi, 2002), and reduces murine responses against experimental bacterial infection (Bishayi and Sengupta, 2003). In vitro, low concentrations of arsenite affect differentiation of human blood monocytes into mature macrophages, in part by reducing cell viability (Sakurai et al., 2005); molecular pathway mediating arsenic-induced monocytic cell death remain however to be determined. In this context, the present study was designed to analyze the effects of As_2O_3 on survival of human monocytic cells, which constitutes a key biological process of macrophagic differentiation (Kiener et al., 1997; Perlman et al., 1999). Indeed, whereas human monocytes rapidly undergo Fas-mediated apoptosis in vitro, growth factor-induced macrophagic differentiation is associated with increased expression of NF-κB-related survival pathways (Perlman et al., 1999; Pennington et al., 2001; Zhang et al., 2003) which could be compromised by As_2O_3, as reported above.

We demonstrate in this work that clinically relevant concentrations of As_2O_3 induced marked apoptosis of human blood monocytes and promonocytic U937 cells during macrophagic differentiation induced by granulocyte-macrophage colony-stimulating factor (GM-CSF) and phorbol 12-myristate 13-acetate (PMA), respectively. Apoptosis of monocytic

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cells likely resulted from decreased NF-κB activity and downregulation of the NF-κB-regulated anti-apoptotic proteins FLIP and XIAP.

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Methods

Chemical reagents and antibodies. As_2O_3, sodium arsenite, cadmium chloride and PMA were purchased from Sigma (Saint Quentin Fallavier, France). Hoechst 33342 and Sytox Green (SG) were from Molecular Probes (Interchim, Montluçon, France). Annexin-V (A5) Alexa™568 was purchased from Roche Diagnostic (Meylan, France). Bay 11-7082, a NF-κB inhibitor, was from Calbiochem (France Biochem, Meudon, France). GM-CSF (sp. act. 1.2 x 10^8 UI/mg) was obtained from Shering Plough (Lyon, France) and macrophage colony-stimulating factor (M-CSF) (sp. act. 1 x 10^5 UI/mg) from Promocell (Heildeberg, Germany). Rabbit polyclonal antibodies against bcl-x_L, caspase-3, IκBα, mcl-1 and p38-kinase were purchased from Santa Cruz Biotechnology (Tebu-bio SA, Le Perray en Yvelynes, France). Rabbit polyclonal anti-FLIP antibody was from Stressgen Biotechnologies (Victoria, Canada), whereas mouse monoclonal anti-bcl-2 and anti-XIAP antibodies were obtained from Pharmingen (San Diego, USA). Mouse monoclonal antibody against caspase-8 was purchased from Alexis Biochemicals (Paris, France). Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody against CD14, CD71, CD11b and CD11c were purchased from Immunotech (Marseille, France).

Cells and treatments. Peripheral blood mononuclear cells were first isolated from bloody buffy coats of healthy donors through Ficoll gradient centrifugation. Human monocytes were then prepared by a 2 h adhesion step, which routinely obtained > 90% of adherent CD14-positive cells as assessed by immunostaining. These monocytic cells were next cultured for 6 days in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 20 UI/mL penicillin and 20 µg/mL streptomycin, in the presence of 800 UI/mL GM-CSF or 50 UI/mL M-CSF to get macrophages as previously reported (Young et al., 1990; van Grevenynghe et al., 2003; Van Grevenynghe et al., 2004). The human promonocytic cell line U937 was grown in RPMI 1640 supplemented with 10 % fetal bovine serum and was induced

to differentiate into macrophage-like cells in the presence of 100 nM PMA during 4 days (Yan et al., 1997). Blood monocytes and U937 cells were induced to differentiate in the absence or presence of As_2O_3. In some experiments, blood monocytes were first differentiated with GM-CSF for 6 days and then treated with As_2O_3.

Measurement of apoptosis and necrosis. A5 and SG staining assays. To analyze alterations of the plasma membrane structure linked to apoptosis, exposition of phosphatidylserine to the extracellular environment was studied. We determined binding of A5, a calcium-dependent protein with high affinity for phosphatidylserine, using A5 conjugated to the fluorescent label Alexa™568. Simultaneously, necrotic cells, which have lost their plasma membrane integrity, were detected with the green fluorescent DNA dye SG. Cells were induced to differentiate in the presence or absence of As_2O_3. Subsequently, cells were collected, washed and incubated with dyes as previously described (Lemarie et al., 2004). Apoptotic ($A5^+/SG^-$) and necrotic ($A5^-/SG^+$ and $A5^+/SG^+$) cells were quantified using a fluorescence Olympus BX60 microscope, in comparison with living cells. At least 200 cells were counted for each cell suspension.

Hoechst 33342 staining assay. In order to look for changes in chromatin structure typical of apoptotic cells, condensed and fragmented nuclei were stained with the Hoechst 33342 fluorescent DNA dye, as previously described (Lemarie et al., 2004). Cells with apoptotic nuclei, i.e. condensed or fragmented, were quantified as described above.

Western-Blot immunoassays. Cells were induced to differentiate into 100 mm dishes in the absence or presence of As_2O_3. Cells were then harvested, centrifuged, washed with PBS and lysed for 20 min on ice in RIPA buffer supplemented with 1 mM phenylmethylsulfonyl fluoride, 5 µg/mL leupeptin, 0.5 µg/mL aprotinin, 0.5 mM dithiothreitol and 1 mM orthovanadate. Cells were then centrifugated at 13 000 rpm for 15 min at 4°C. The resulting supernatants were collected and frozen at -80°C or used immediately. Protein concentration

was quantified using the Bradford's method (Bradford, 1976). 30 µg of each sample or 20 µg of nuclear extract prepared as mentioned below were heated for 5 min at 100°C, then analyzed by 12.5% SDS-polyacrylamide gel electrophoresis and electroblotted overnight onto nitrocellulose membranes (Amersham Biosciences Europe, Orsay, France). After blocking, membranes were hybridized with primary antibody overnight at 4°C and, washed and incubated with appropriate horseradish peroxidase-conjugated secondary antibody. Immunolabelled-proteins were visualized by chemiluminescence.

Caspase activity assay. Caspase activity was assessed as previously described (Huc et al., 2004; Lemarie et al., 2004). 50 µg of crude cell lysate were incubated with 80 µM DEVD-AMC or IETD-AMC, two caspase substrates known to be essentially cleaved by caspase-3 or caspase-8, respectively, for 2 h at 37°C. Caspase-mediated cleavage of substrate-AMC was measured by spectrofluorimetry (SpectraMax Gemini, Molecular Devices) at the excitation/emission wavelength pair of 380/440 nm. Caspase activities were expressed as the ratio of relative activity of treated cells to that of untreated cells.

Measurement of NF-κB DNA Binding. Nuclear proteins were prepared using the Nuclear Extract Kit from Active Motif (Rixensart, Belgium). DNA binding of p65 NF-κB was analyzed using the ELISA-based TransAM™ NF-κB kit (Active Motif) as previously described (Lemarie et al., 2004). In brief, nuclear cell extracts (10 µg) were incubated for 1 h in a 96-well plate to which oligonucleotide containing an NF-κB consensus binding site has been immobilized. After washing, plate was incubated for 1 h with the rabbit anti-NF-κB p65 antibody (1:1000) which specifically detected an epitope accessible only when NF-κB p65 is activated and bound to its cognate oligonucleotide. The plate was then washed and incubated with horseradish peroxidase-conjugated secondary antibody (1:1000) for 1 h at room temperature. After washing, colorimetric readout was quantified by spectrophotometry at 450

nm. In order to monitor the specificity of this assay, wild-type and mutated consensus oligonucleotides were used as competitors for NF-κB binding.

Stable expression of p65 NF-κB in U937 cells. The retroviral vector pMSCV-Puro-p65 was obtained by subcloning the HindIII/HpaI fragment from pEGFP-p65 (kindly provided by Dr J. Schmid (Schmid et al., 2000), Center for Biomolecular Medicine and Pharmacology, Medical University Vienna, Austria) into a modified pMSCV-Puro (Clonetech, Ozyme, Saint Quentin Yvelines, France), containing HindIII and HpaI in its multiple cloning site. Retroviral production and cell transduction were carried out as previously described (Micheau et al., 2001).

Total RNAs isolation and RT-PCR assay. Total RNAs were extracted from monocytes using the TRIzol method (Gibco BRL, USA) and RT-PCR analysis were then performed (Laupeze et al., 2002). The primers used for bcl-2, bcl-$x_{L,}$, XIAP, mcl-1 and the long splice variant of FLIP ($FLIP_L$), have been previously described (Perlman et al., 1999; Cui et al., 2000; Yamaguchi et al., 2002). GAPDH detection was performed as a loading control. PCR products were separated on 1% agarose gel and stained with ethidium bromide.

Flow cytometric immunolabelling assays. After treatment, floating and adherent cells were removed by a 15 min-incubation at 37°C in phosphate buffer saline (PBS) supplemented with 100 µM ethylenediaminetetraacetic acid, collected and centrifuged. Then, phenotypic analysis of monocytic cells was performed using flow cytometric direct immunofluorescence assays (Laupeze et al., 2002). Fluorescence related to immunolabelling was measured using a FACScalibur flow cytometer (Becton Dickinson, San Jose, USA). Each measurement was conducted on 8 000 events and analyzed on Cell Quest software (Becton Dickinson).

Endocytosis and phagocytosis assays. Cells were incubated at 37°C with 1 mg/ml FITC-dextran (Sigma) for 60 min or with 15 µl of fluorescent latex microspheres (Polysciences, Warrington, PA) for 30 min for endocytosis or phagocytosis assays, respectively. Cellular uptake of FITC-dextran and phagocytosis of latex microspheres were then monitored by flow cytometry at 525 nm. Negative controls were performed in parallel by incubating cells with FITC-dextran or latex beads at 4°C instead of 37°C. Each measurement was conducted on 5 000 events and analyzed on Cell Quest software.

Statistical Analysis. The results are presented as means ± S.E.M.. Significant differences were evaluated with the multirange Dunnett's *t* test for experiments in which multiple comparisons were studied. Other differences were evaluated with the Student's *t* test. Criterion of significance of the difference between means was $p<0.05$.

Results

As_2O_3 reduced monocyte survival during differentiation with GM-CSF or M-CSF. After isolation of human peripheral blood mononuclear cells and a 2-h adhesion step, monocytes appeared as firmly adherent cells. When cultured without growth factors, 37 $\pm$ 3.6 % of monocytes died within 24h, as previously described (Kiener et al., 1997). In these experimental conditions, human monocytes were particularly susceptible to Fas/FasL interaction and subsequent caspase-8-dependent apoptosis (Kiener et al., 1997). Growth factors increased monocyte survival and promoted macrophagic differentiation. Indeed, in the presence of GM-CSF or M-CSF for 6 days, monocytes survived and developed into adherent macrophagic cells displaying a "fried-egg"-like morphology (i.e., large round cells with distinct nuclei) or a spindle-shaped/stellate morphology, respectively (Fig. 1A) (Young et al., 1990). Addition of 1 µM As_2O_3 during monocyte differentiation dramatically reduced the number of adherent macrophagic cells and led to an important increase of very small floating cells in culture medium. Using two specific markers of necrosis (SG) and apoptosis (A5), we evaluated global cytotoxicity of As_2O_3 towards differentiating monocytes. As shown on figure 1B, As_2O_3 induced marked dose-dependent apoptosis (A5+/SG-) of monocytes during their differentiation with GM-CSF for 6 days; 1 µM As_2O_3 also significantly altered monocyte survival treated with M-CSF. In contrast, as recently reported (Sakurai et al., 2004), neither arsenite (0.5 µM) nor cadmium (0.5-2 µM) significantly altered monocyte viability during differentiation with GM-CSF (data not shown).

Time-dependent apoptosis was associated with caspase activation and inhibition of NF-κB DNA binding. As_2O_3-induced apoptosis appeared to be time-dependent and detectable after two days of treatment with GM-CSF (Fig. 2A). Apoptosis was confirmed by chromatin and DNA condensation, as assessed by Hoechst 33342 staining, after a three-day treatment (Fig. 2B). Accordingly, at this time point, western blot analysis showed decreased levels of

both procaspase-8 and procaspase-3 and apparition of the caspase-3 active cleaved fragment p17 (Fig. 2C). Involvement of caspases was confirmed by measurements of caspase-8 and -3 activities, using IETD-AMC and DEVD-AMC as specific substrates, respectively. Ratios of relative caspase-8 and-3 activities in cells treated for 6 days with 1 µM As_2O_3 compared to untreated cells were 3.64 ± 0.72 and 3.40 ± 1.25, respectively. Unfortunately, the use of caspase inhibitor to confirm involvement of these cysteine proteases in As_2O_3-induced apoptosis could not be addressed in our cell system. Indeed, as recently reported in M-CSF-treated monocytes (Sordet et al., 2002), we found that, 30 µM of z-VAD-fmk shifted differentiation of GM-CSF-treated monocytes to cell death: 9.03 $\pm$ 1.8 and 40.4 $\pm$ 6 % of A5+/SG- cells were measured in untreated and z-VAD-fmk-treated monocytes after 2 days, respectively.

Different studies report that survival of monocytic cells during growth factor-induced macrophagic differentiation requires NF-κB activity (Pennington et al., 2001; Zhang et al., 2003). We thus determined whether As_2O_3 could alter DNA binding activity of the transcriptionnally active p65 NF-κB subunit which is increased during macrophage differentiation (Conti et al., 1997; Ammon et al., 2000). Figure 3A demonstrates that 1 µM As_2O_3 significantly reduced DNA binding of p65 subunit to κB consensus sites by 20% and 50% in GM-CSF-treated monocytes after 3 and 6 days, respectively. In addition, like arsenic, the specific NF-κB inhibitor inhibitor Bay 11-7082 significantly reduced p65 NF-κB DNA binding (Fig. 3B), prevented cell adhesion (data not shown) and induced potent apoptosis in GM-CSF-treated monocytes after a 3-day treatment (Fig. 3C). Besides NF-κB, we also examined potential roles of p38 kinase and c-Jun N-terminal kinase, two mitogen-activated protein kinases frequently involved in apoptosis induced by inorganic arsenic. Our results demonstrated that neither the p38 kinase inhibitor SB203580 (10 µM) nor the c-Jun N-

terminal kinase inhibitor D-JNKI1 (1 μM) could prevent apoptosis of GM-CSF-treated monocytes exposed to As_2O_3 for 3 days (data not shown).

Arsenic induced apoptosis of U937 cells during macrophagic differentiation through inhibition of NF-κB signals. In order to further study the role of NF-κB in arsenic toxicity towards monocytic cells, we used the promonocytic U937 cell line. Indeed, up-regulation of NF-κB pathway confers survival during macrophagic differentiation of U937 cells with PMA (Pennington et al., 2001). As previously described, PMA-induced U937 cells differentiation was associated with rapid cell adhesion and marked expression of the CD11c β2 integrin after 4 days (data not shown). As_2O_3 was not toxic towards differentiating U937 cells at 1 μM; in contrast, 4 μM As_2O_3 reduced cell adhesion and induced marked apoptosis of differentiating U937 cells (Fig. 4A). Figure 4B indicates that apoptosis was time-dependent and not detectable after a 24h-treatment with As_2O_3.

PMA increased DNA binding activity of the p65 NF-κB subunit during U937 cell differentiation (Fig. 4C); this effect was associated with decrease of IκBα protein levels (Fig. 4D). Addition of As_2O_3 during PMA-induced differentiation reduced p65 NF-κB DNA binding activity in a time dependent manner (Fig. 4C). This effect was significant as early as 24h and was associated with a decrease of nuclear levels of p65 NF-κB (Fig. 4C, insert) and an inhibition of IκBα degradation (Fig. 4D). It thus appears that decrease of NF-κB DNA binding preceded apoptosis and was not a consequent of cell death. In order to determine whether NF-κB inhibition was a causal event mediating As_2O_3-induced apoptosis, a p65 NF-κB subunit expressing retroviral vector was prepared to stably express p65 in U937 cells; human primary monocytes are difficult to transfect and could not be used in this study. As shown in Figure 4E, both p65 DNA binding activity and nuclear level were significantly higher in p65-transduced U937 cells compared to control empty vector, treated or not with

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PMA. Interestingly, As_2O_3 toxicity was significantly reduced in p65 overexpressing U937 cells during differentiation with PMA for 48h (Fig. 4E): percentages of A5+/SG- cells were 28.6 $\pm$ 3.7 and 13.6 $\pm$ 1.3 in control and p65-overexpressing U937 cells, respectively.

As_2O_3 and Bay 11-7082 inhibited FLIP and XIAP expression during differentiation. NF-κB controls expression of various anti-apoptotic proteins, notably FLIP and XIAP (Lin et al., 2001; Micheau et al., 2001; Zhang et al., 2003), two endogenous caspase inhibitors known to increase survival during macrophagic differentiation (Perlman et al., 1999; Lin et al., 2001; Zhang et al., 2003). We analyzed cellular expression of these proteins in response to As_2O_3 treatment. As shown in Figure 5A, As_2O_3 prevented up-regulation of both $FLIP_L$ and XIAP in U937 cells during differentiation with PMA without altering mcl-1 expression. Similarly, As_2O_3 did not significantly impair protein (Fig. 5B) or mRNA (Fig. 5C) levels of the bcl-2, bcl-x_L or mcl-1 anti-apoptotic factors in human blood monocytes during differentiation with GM-CSF. In contrast, it markedly inhibited both mRNA and protein levels of $FLIP_L$ and XIAP in these cells; Bay 11-7082 also markedly reduced mRNAs levels of $FLIP_L$ and XIAP but not those of bcl-2 and bcl-x_L in differentiating monocytes (Fig. 5C).

As_2O_3 inhibited macrophagic differentiation of blood monocytes and U937 cells. Besides its effect on survival, As_2O_3 reduced, in a similar dose-dependent manner, expression of the transferrin receptor CD71, a well-known macrophagic differentiation marker (Fig. 6A) (van Grevenynghe et al., 2003). At 0.125 μM, a dose which did not alter monocyte survival, no effect was observed on CD71 expression. In addition, 1 μM As203 inhibited expressions of the integrins CD11b and CD11c, two other macrophagic markers (Pennington et al., 2001;van Grevenynghe et al., 2003) (Fig. 6B); it also reduced levels of both endocytosis and phagocytosis, two major functions of macrophages (Fig. 6C). As observed with blood monocytes, cytotoxic concentrations of metalloid altered expression of CD11c in U937 cells

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during differentiation with PMA; figure 6D demonstrates that 4 μM As_2O_3 prevented up-regulation of CD11c expression whereas 1 μM As_2O_3 had no effect. Interestingly, Figure 6E shows that overexpression of the p65 NF-κB subunit in U937 cells, not only reduced As_2O_3-induced apoptosis, but also prevented inhibition of CD11c expression.

Mature macrophages are resistant to As_2O_3-induced apoptosis. Once differentiated, mature macrophages display increased NF-κB activity and consequently potently resist to apoptosis (Pagliari et al., 2000). Figure 7A and 7B demonstrate that 1 μM As_2O_3 neither altered viability of human primary macrophages nor decreased levels of $FLIP_L$ or XIAP proteins, respectively; however, a four-fold higher concentration of As_2O_3 induced macrophage apoptosis and reduced protein levels of these caspase inhibitors (Fig. 7A and 7C).

Discussion

Clinical and experimental studies have demonstrated that anticancerous effects of As_2O_3 are mainly due to apoptosis of leukemia cells. In the present study, we demonstrated that low concentrations of As_2O_3 (0.25-1 µM), also markedly decreased survival of human monocytic cells during in vitro macrophagic differentiation.

Our results demonstrate that As_2O_3 induced time-dependent apoptosis of human blood monocytes and promonocytic U937 cells during differentiation with GM-CSF and PMA, respectively. Kinetics of death were slow and similar to those reported for human promelyocytic NB4 cells or human lymphoma cells treated with low concentrations of As_2O_3 (Chen et al., 1997; Mathas et al., 2003). Apoptosis of GM-CSF-treated monocytes was characterized by phosphatidylserine externalization, chromatin condensation, DNA fragmentation and by caspase activation. Involvement of caspases in arsenic-treated monocytes was supported by (i) decreased protein levels of pro-caspase-8 and pro-caspase-3 and increased protein level of the active caspase-3 fragment p17, and (ii) increased activity of both caspase-8 and caspase-3. Caspase-8 is an initiator caspase mainly activated within the death inducing signaling complex by death receptors of the TNF family. It can either directly activate the effector caspase-3 in the cytoplasm and trigger apoptosis, or induce disruption of the outer mitochondrial membrane allowing cytochrome c release and activation of effector caspases, including caspase-3, via the apoptosome. Prevention of arsenic toxicity by caspase inhibition could not be tested; indeed the pan-caspase inhibitor Z-vad-fmk was found, by itself, to shift GM-CSF-induced differentiation to cell death. It thus appears that the level of caspase activity is, in fact, a critical factor since its down-regulation or its excessive up-regulation, like in As_2O_3-treated monocytes, results in failure of macrophagic differentiation and cell death.

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Treatment of monocytic cells with As_2O_3 was associated with marked reduction of DNA binding activity of the transcriptionally active p65 NF-κB subunit. Different arguments support the idea that NF-κB inhibition can be a causal event mediating arsenic toxicity towards monocytic cells. First, NF-κB activity increases survival of monocytic cells during macrophagic differentiation with M-CSF (Zhang et al., 2003), PMA (Pennington et al., 2001) and probably with GM-CSF since we showed that Bay 11-7082, a selective NF-κB inhibitor, induced their apoptosis. Second, our results clearly demonstrate that inhibition of NF-κB DNA binding preceded apoptosis and was not a consequence of U937 cell death. Third, increasing DNA binding activity of the p65 NF-κB subunit in these cells, following stable infection with a p65 expressing vector, significantly reduced metalloid cytotoxicity, during PMA-induced differentiation. The precise mechanism by which As_2O_3 inhibited NF-κB pathway in these cells remains to be further explored. Nevertheless, we show that the metalloid markedly prevented IκBα degradation in PMA-treated U937 cells; this effect might result from inhibition of IκBα kinase activity in As_2O_3-treated U937 cells, as previously described in other cell types (Mathas et al., 2003).

NF-κB regulates expression of some anti-apoptotic proteins, notably FLIP and XIAP in different cell types (Lin et al., 2001; Micheau et al., 2001; Zhang et al., 2003). Our results suggest that NF-κB also controlled expression of these two genes during GM-CSF-induced macrophagic differentiation since Bay 11-7082 specifically reduces their mRNA levels; indeed, this inhibitor had no effect on bcl-2 and bcl-x_L gene expressions which are not regulated by NF-κB pathways in mature macrophages (Pagliari et al., 2000). In addition, our results demonstrated that As_2O_3 selectively reduced mRNA and protein levels of $FLIP_L$ and XIAP in differentiating monocytes and U937 cells. Down-regulation of these NF-κB-regulated-genes did not result from a general toxic effect of As_2O_3 on transcription, since, like Bay 11-7082, it neither alter bcl-2 nor bcl-x_L expressions. $FLIP_L$ has a strong structure

homology to pro-caspase 8 but it lacks catalytic activity; it directly interacts with pro-caspase 8 in the death inducing signaling complex, blocks its cleavage into active fragments and finally prevents caspase-8 dependent apoptosis (Krueger et al., 2001). Down-regulation of $FLIP_L$ expression could thus explain, at least in part, the enhanced caspase-8 activity measured in monocytes exposed to As_2O_3. XIAP is also a potent suppressor of apoptosis and its effects are mainly mediated by direct caspase inhibition. It tightly interacts with caspase-9 and -3 but not with caspase-8 (Salvesen and Duckett, 2002). Consequently, arsenic might impair function of both initiator and effector caspases in monocytic cells. In contrast to monocytes, mature macrophages were lesser sensitive to 1 µM As_2O_3, which is in agreement with their known resistance to apoptosis. At this concentration, metalloid decreased neither cell viability nor $FLIP_L$ protein levels but, unexpectedly, increased those of XIAP. A four-fold higher concentration (4 µM) however allowed both $FLIP_L$ and XIAP down-regulation, and reduced viability of mature macrophages. Altogether, these results suggest that alteration of $FLIP_L$ and XIAP expression, likely due to NF-κB inhibition, is involved in As_2O_3 toxicity towards monocytes/macrophages.

Finally, our study shows that As_2O_3 markedly inhibited macrophagic differentiation of blood monocytes and U937 cells. Different observations strengthen the idea that such an inhibition was mainly related to As_2O_3-induced apoptosis; first, 0.125 µM and 1 µM As_2O_3 modified neither macrophagic marker expressions nor viability in differentiating monocytes and U937 cells, respectively, whereas higher concentrations similarly altered both parameters. Second, blocking of As_2O_3-induced apoptosis in p65-overexpressing U937 cells allowed CD11c upregulation during differentiation of U937 cells exposed to As_2O_3. Differentiation of monocytes into macrophages constitutes a cellular process involved in numerous physiological functions. Indeed, once differentiated, macrophages play a pivotal role in immune defenses by producing cytokines, chemokines, growth factors or eicosanoids. They

also protect against microbial infection and play a role in the tumor cell killing probably via their ability to present antigens to lymphocytes. In addition, they play an important role in inflammation and lipid metabolism and bone-marrow macrophages are notably involved in erythropoiesis. Inhibition of macrophagic differentiation by clinically relevant concentration of As_2O_3 may therefore lead to deleterious adverse effects in As_2O_3-treated patients. On the other hand, macrophages participate to the physiopathology of several diseases, such as hemophagocytic syndrome or rhumatoid arthritis (Koch et al., 1994) . In these circumstances, As_2O_3-induced inhibition of macrophagic differentiation may present a clinical interest.

In conclusion, our study demonstrated that low clinically achievable concentrations of As_2O_3 prevented macrophagic differentiation of human monocytic cells by altering NF-κB-regulated survival pathways.

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Footnotes

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Legends for Figures

Figure 1: As_2O_3 reduces survival of monocytes during macrophagic differentiation with GM-CSF and M-CSF. Blood monocytes were cultured with GM-CSF or M-CSF in the absence or presence of indicated concentrations of As_2O_3, for 6 days (d). **(A)** Photographs of cultured cells were then taken by phase-contrast microscopy (Original magnification, x40). **(B)** Cells were then co-stained with Annexin V-Alexa™568 (A5) and Sytox Green (SG) to detect apoptotic ($A5^+/SG^-$) and necrotic cells ($A5^-/SG^+$ and $A5^+/SG^+$), respectively, and viewed by fluorescence microscopy. Values are means ± SEM of 4 independent experiments. *$p<0.05$, untreated cells versus As_2O_3-treated cells.

Figure 2: Time-dependent apoptosis induced by As_2O_3 is associated with caspase activation. (**A**) GM-CSF-treated monocytes were cultured in the absence or presence of 1 µM As_2O_3 for one to six days (d). Apoptotic cells were analysed by Annexin V-Alexa™568 (A5) and Sytox Green (SG) co-staining. Only apoptotic cells ($A5^+/SG^-$) are represented in the graph. Cells were viewed by fluorescence microscopy. Values are means ± SEM of 4 independent experiments. *$p<0.05$, untreated cells versus As_2O_3-treated cells. (**B**) GM-CSF-treated monocytes were cultured with As_2O_3 at indicated concentrations for 3 days. Subsequently, apoptotic nuclei were analysed by Hoechst 33342 staining and viewed by fluorescence microscopy. Values are means ± SEM of 3 independent experiments. *$p<0.05$, untreated cells versus As_2O_3-treated cells. (**C**) Western-blot analysis of caspase-8 and caspase-3. GM-CSF-treated monocytes were cultured with 1 µM As_2O_3 for 3 days and whole cell lysates were separated by a 12.5% SDS-polyacrylamide gel electrophoresis as described in *Methods*. Equal gel loading and transfer efficiency were checked by protein hybridization with an anti-p38 kinase antibody. These western-blots were repeated 3 times with similar results.

Figure 3: As_2O_3 and Bay 11-7082 reduce DNA binding of NF-κB in monocytes during macrophagic differentiation. GM-CSF-treated monocytes were cultured in the absence or presence of 1 μM As_2O_3 (**A**) or 2.5 μM Bay 11-7082 (**B**) for the indicated times of exposure. Nuclear extracts were prepared and measurement of DNA-binding activity of nuclear p65 NF-κB was performed as described in *Methods*. Wild-type (wt) or mutated (mut) consensus oligonucleotides of p65 NF-κB were used as competitors of nuclear extracts prepared from untreated cells to verify specificity of the assay. Values are means ± SEM of 4 independent experiments. *$p<0.05$. (d = day) (**C**) Blood monocytes were cultured with GM-CSF in the absence or presence of 2.5 μM Bay 11-7082 for 3 days. Then, cells were co-stained with Annexin V-Alexa™568 (A5) and Sytox Green (SG) to detect apoptotic ($A5^+/SG^-$) and necrotic cells ($A5^-/SG^+$ and $A5^+/SG^+$), respectively, and viewed by fluorescence microscopy. Values are means ± SEM of 3 independent experiments. *$p<0.05$, untreated cells versus Bay 11-7082-treated cells.

Figure 4: As_2O_3 induces apoptosis of the human promonocytic U937 cells, during differentiation with PMA, through NF-κB inhibition. Untreated and PMA-treated U937 cells were cultured in the absence or presence of As_2O_3 **(A)** for 4 days (d) at the indicated concentrations or **(B)** at 4 μM for the indicated time intervals. Then, cells were co-stained with Annexin V-Alexa™568 (A5) and Sytox Green (SG) to detect apoptotic ($A5^+/SG^-$) and necrotic cells ($A5^-/SG^+$ and $A5^+/SG^+$), respectively, and viewed by fluorescence microscopy. Only apoptotic cells ($A5^+/SG^-$) are represented in the graph B. Values are means ± SEM of 4 independent experiments. *$p<0.05$, untreated cells versus As_2O_3-treated cells. (**C)** Untreated and PMA-treated cells were cultured in the absence or presence of 4 μM As_2O_3 for the indicated time intervals. Nuclear extracts of untreated and As_2O_3-treated cells were used to measure p65 NF-κB DNA-binding activity and p65 protein levels (insert), as described in *Methods*. Wild-type (wt) or mutated (mut) consensus oligonucleotides of p65 NF-κB were

used as competitors of nuclear extracts prepared from untreated cells to verify specificity of the assay. Values are means ± SEM of 3 independent experiments. (**D**) Western blot of IκBα protein. Untreated and PMA-treated cells were cultured in the absence or presence of 4 µM As_2O_3 for 24 h. Whole cell lysates were prepared and separated by a 12.5% SDS-polyacrylamide gel electrophoresis. Equal gel loading and transfer efficiency were checked by protein hybridization with an anti-p38 kinase antibody. These western-blots were repeated 3 times with similar results. (**E**) U937 cells were stably transduced with empty pMSCV retroviral vector (U937 empty) or a pMSCV-p65 NF-κB construct (U937 p65). Cells were selected by use of puromycine and populations expressing stably p65 were analyzed as follow. Untreated and PMA-treated cell populations were cultured in the absence or presence of 4 µM As_2O_3 for 2 days. (**a**) Nuclear extracts were used to measure DNA-binding activity of p65 NF-κB and p65 protein levels (insert); (**b**) cells were co-stained with Annexin V-Alexa™568 (A5) and Sytox Green (SG) to detect apoptotic ($A5^+/SG^-$) and necrotic cells ($A5^-/SG^+$ and $A5^+/SG^+$), respectively. Only apoptotic cells ($A5^+/SG^-$) are represented in the graph. Cells were viewed by fluorescence microscopy. Values are means ± SEM of 3 independent experiments. *$p<0.05$.

Figure 5: As_2O_3 inhibits $FLIP_L$ and XIAP expression in monocytic cells during macrophagic differentiation. (**A**) Untreated and PMA-treated U937 cells were cultured in the absence or presence of 4 µM As_2O_3 for 4 days and expression of anti-apoptotic proteins was then analyzed by western blot. Equal gel loading and transfer efficiency were checked by protein hybridization with an anti-p38 kinase antibody. Human primary monocytes were cultured with GM-CSF in the absence or presence of 1 µM As_2O_3 (**B, C**) or 2.5 µM Bay 11-7082 (**C**) for 3 days. Expressions of anti-apoptotic genes were then analyzed by western blot (**B**) and RT-PCR (**C**). Experiments in **A**, **B** and **C** were repeated at least 3 times with similar results.

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Figure 6. As_2O_3 inhibited macrophagic differentiation of monocytic cells (A-C) Blood monocytes were cultured with GM-CSF- or M-CSF in the absence or presence of As_2O_3 for six days (d). Parental monocytes and macrophages were stained with monoclonal antibodies directed against the macrophagic differentiation markers CD71 **(A)**, CD11b and CD11c **(B)**. **(C)** GM-CSF-differentiated monocytes were incubated with FITC-dextran (endocytosis) or fluorescent latex microbeads (phagocytosis) at 4°C (negative control) or 37°C. Marker expression and cellular uptakes of FITC-dextran or microbeads were then determined by flow cytometry. U937 cells **(D)** and U937 cells stably transduced with an empty pMSCV retroviral vector (U937 empty) or with a pMSCV-p65 NF-κB construct (U937 p65) **(E)** were treated or not with PMA in the absence or presence of indicated concentrations of As_2O_3 for 4 days **(D)** or 2 days **(E)**. Then, cells were stained with monoclonal antibodies directed against CD11c and analyzed by flow cytometry. Representative histograms of at least 3 individual experiments are shown.

Figure 7: Differentiated macrophages are resistant to low concentrations of As_2O_3. Monocytes were first differentiated in macrophages with GM-CSF for 6 days. **(A)** Macrophages were then treated with As_2O_3 for 3 days (d6-d9) or 6 days (d6-d12). Cells were co-stained with Annexin V-Alexa™568 (A5) and Sytox Green (SG) to detect apoptotic ($A5^+/SG^-$) and necrotic cells ($A5^-/SG^+$ and $A5^+/SG^+$), respectively. Only apoptotic cells ($A5^+/SG^-$) are represented in the graphs. Cells were viewed by fluorescence microscopy. Values are means ± SEM of 3 independent experiments. *$p<0.05$ (d = day). **(B, C)** Western-blot analyses of $FLIP_L$ and XIAP proteins performed with whole cell lysates prepared from macrophages treated for 6 days with 1 µM As_2O_3 **(B)** or for 3 days with 4 µM As_2O_3 **(C)**. Equal gel loading and transfer efficiency were checked by protein hybridization with an anti-p38 kinase antibody. These western-blots were repeated 3 times with similar results.

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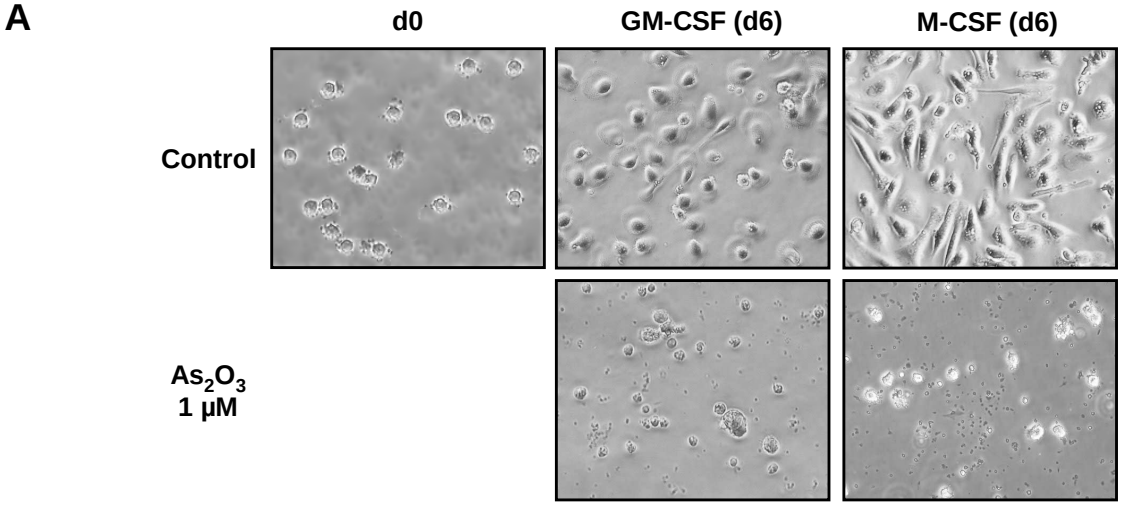

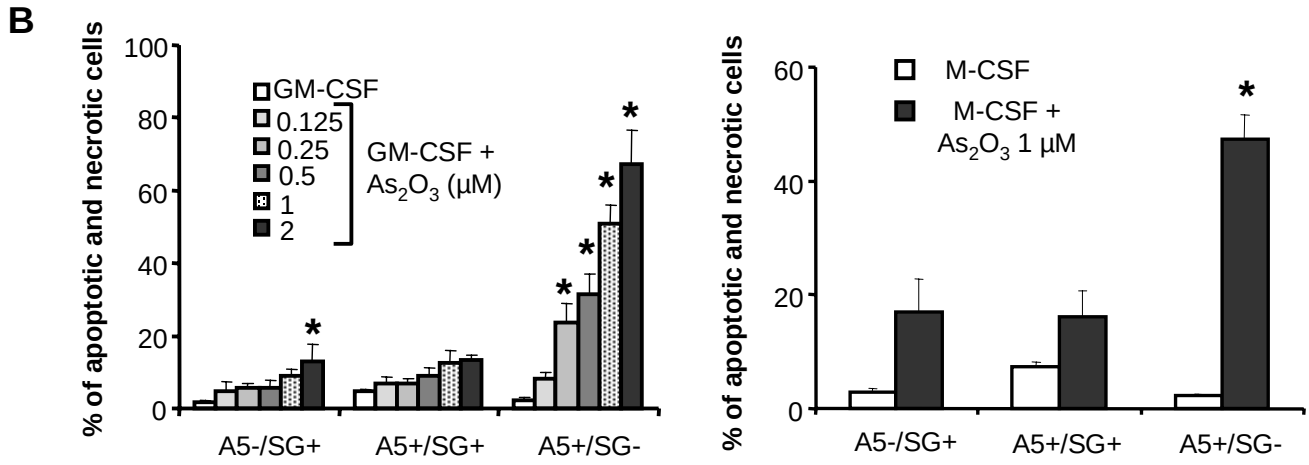

Figure 1

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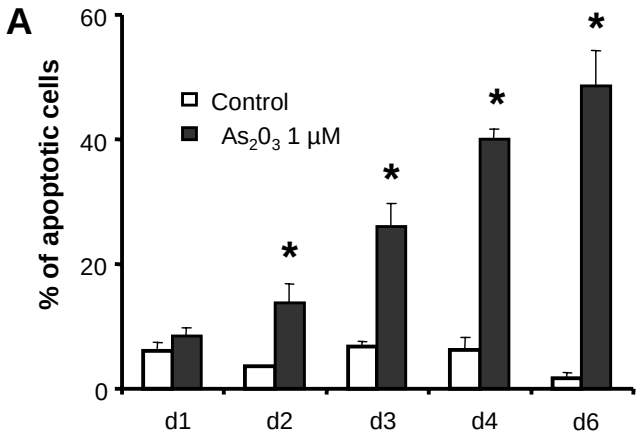

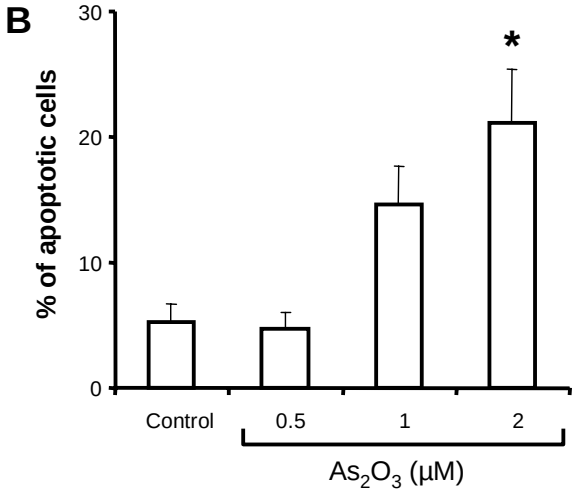

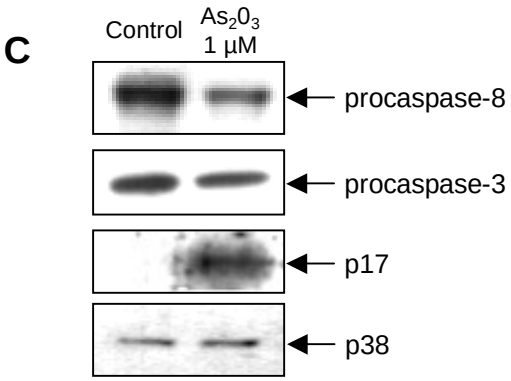

Figure 2

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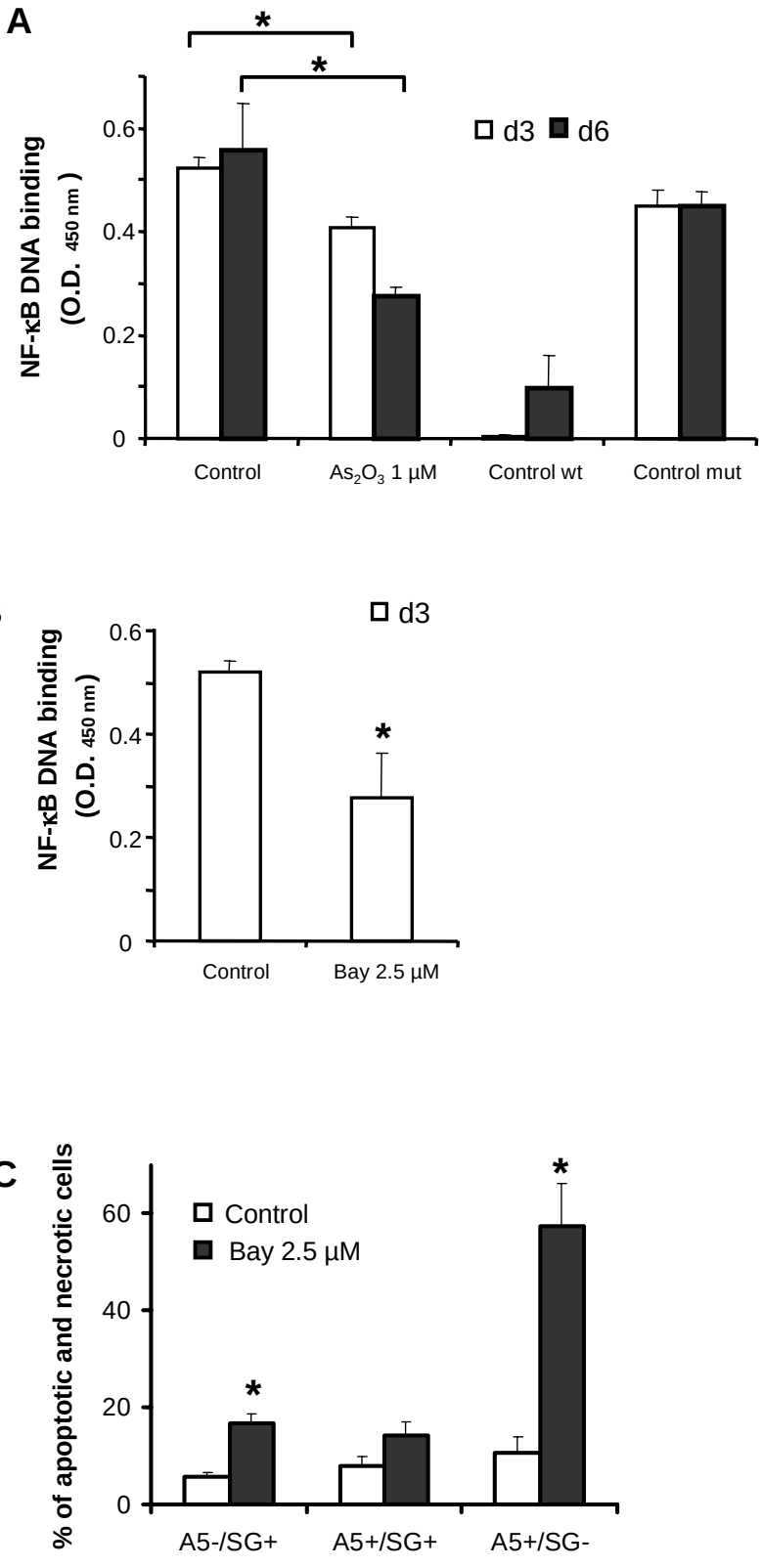

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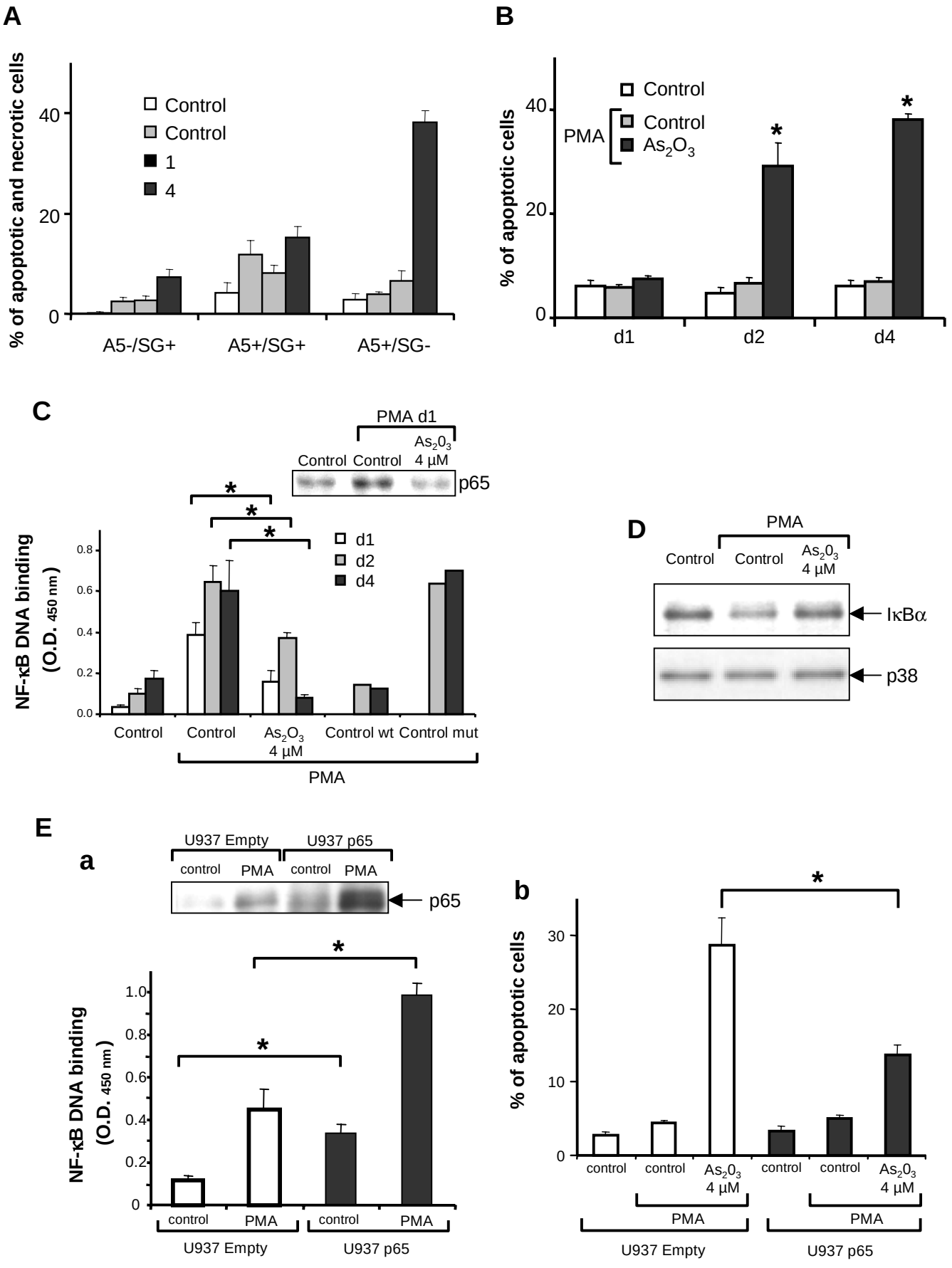

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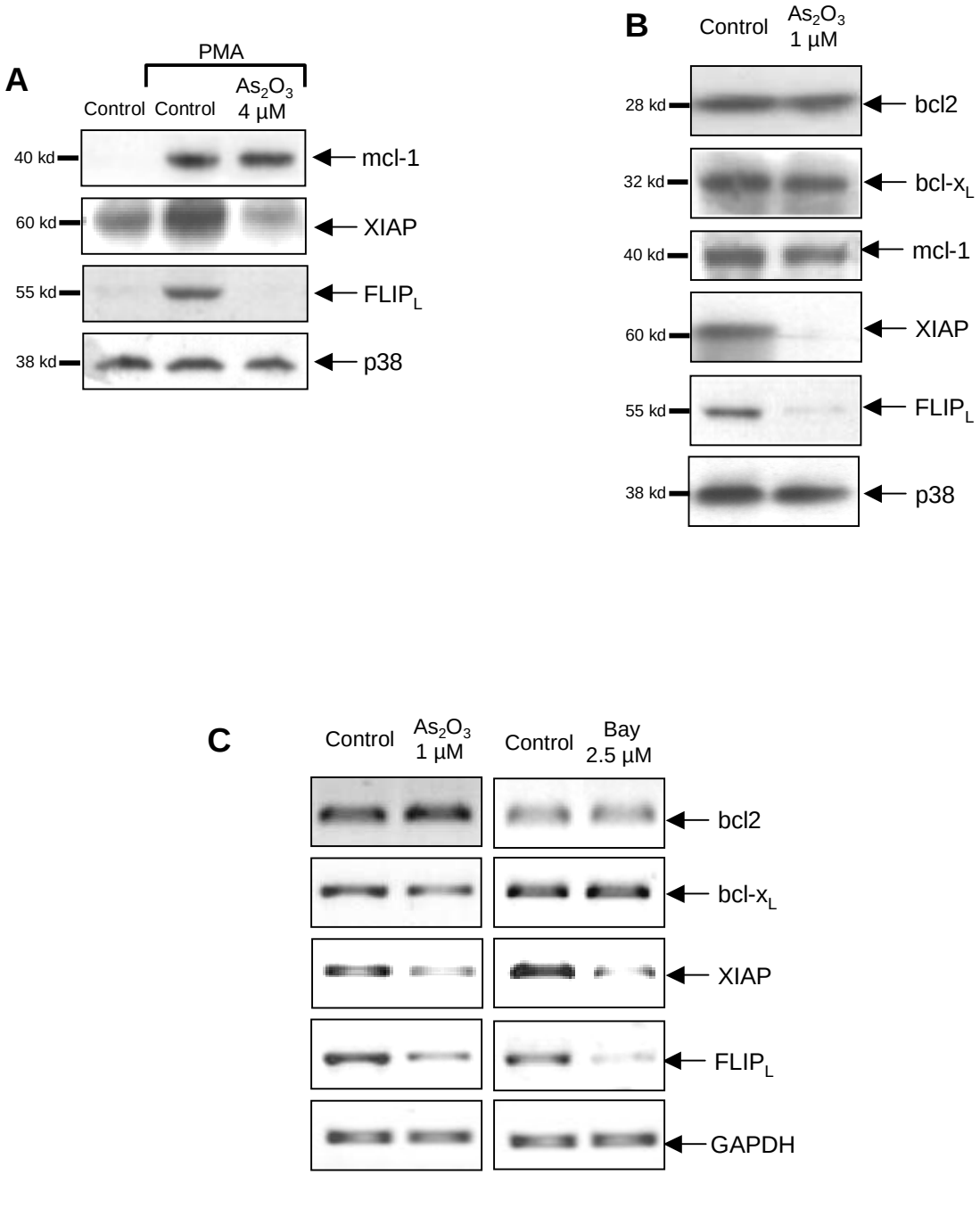

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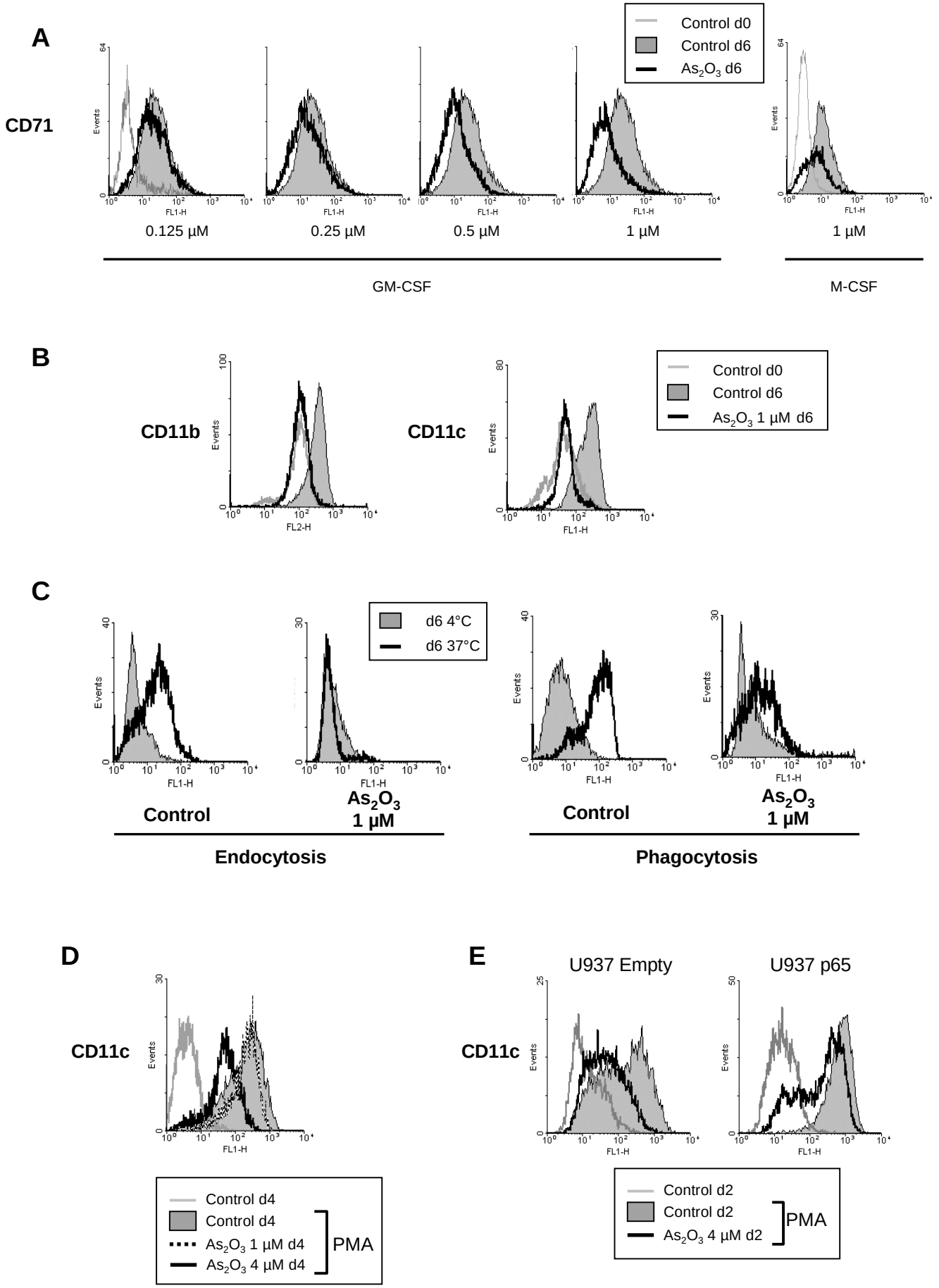

Figure 6

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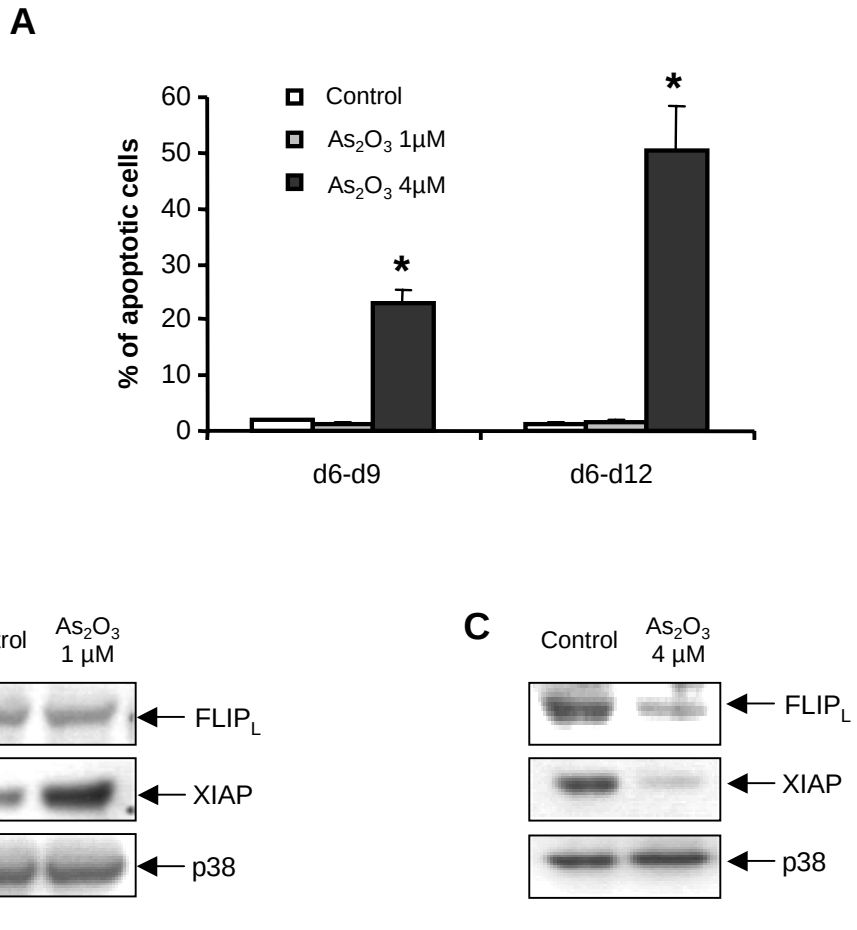

Figure 7