

## Divergent Effect of Cobalt and Beryllium Salts on the Fate of Peripheral Blood Monocytes and T Lymphocytes

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Occupational exposure to metals such as cobalt and beryllium represents a risk factor for respiratory health and can cause immune-mediated diseases. However, the way they act may be different. We show here that the two metals have a divergent effect on peripheral T lymphocytes and monocytes: BeSO<sub>4</sub> induces cell death in monocytes but not in T lymphocytes, which instead respond by producing Interferon gamma (IFN- $\gamma$ ); conversely, CoCl<sub>2</sub> induces apoptosis in T lymphocytes but not in monocytes. Interestingly, both metals induce p53 overexpression but with a dramatic different outcome. This is because the effect of p53 in CoCl<sub>2</sub>-treated monocytes is counteracted by the antiapoptotic activity of cytoplasmic p21<sup>Cip1/WAF1</sup>, the activation of nuclear factor  $\kappa$ B, and the inflammasome danger signaling pathway leading to the production of proinflammatory cytokines. However, CoCl<sub>2</sub>-treated monocytes do not fully differentiate into macrophage or dendritic cells, as inferred by the lack of expression of CD16 and CD83, respectively. Furthermore, the expression of HLA-class II molecules, as well as the capability of capturing and presenting the antigens, decreased with time. In conclusion, cobalt keeps monocytes in a partially activated, proinflammatory state that can contribute to some of the pathologies associated with the exposure to this metal.

**Key Words:** monocytes; lymphocytes; metal salts; lung; inflammation; inflammasome.

Cobalt and beryllium are among those metals for which occupational exposure is associated with hypersensitivity reactions (Chiappino, 1994; Freiman and Hardy, 1970). Inhalation of beryllium can lead to a chronic granulomatous lung reaction (chronic beryllium disease, CBD), and some aspects of this pathology are similar to the “hard metal lung disease” (HMLD) caused by inhalation of metal dusts containing tungsten carbide (WC) and cobalt powdered into a polycrystalline material. The discovery that the use of diamond tools, which do not contain WC but contain cobalt, could lead

to HMLD confirmed that cobalt plays a relevant role in this pathology (Demedts *et al.*, 1984). Apparently, CBD and HMLD share a common genetic ground because both show a positive association with the presence of an allelic polymorphism (Glu69) of the HLA-DP beta chain (McCanlies *et al.*, 2004; Potolicchio *et al.*, 1997; Richeldi *et al.*, 1993). However, whereas CBD is a multisystem granulomatous disease that primarily affects the lungs by the accumulation of beryllium-specific CD4<sup>+</sup> major histocompatibility complex class II-restricted T lymphocytes (Fontenot *et al.*, 2000; Lombardi *et al.*, 2001; Saltini *et al.*, 1989), several attempts to obtain cobalt-specific T cell clones from patients with HMLD in our hands have not been successful. The pathology of HMLD is unique as it is characterized, at least in typical cases, by the presence of “cannibalistic” multinucleated giant cells in airspaces and bronchoalveolar lavage (Ohuri *et al.*, 1989). Moreover, cobalt is a well-known skin sensitizer, causing allergic contact dermatitis, and it can also cause hypersensitivity and occupational asthma, but there is no convincing evidence for a cell-mediated immunologic reaction against cobalt in HMLD (Lison, 1996; Nemery *et al.*, 2001a). Therefore, it appeared interesting to investigate whether the two metals have different effects on cells of the immune system such as T lymphocytes and monocytes. In this paper, we compared the effect of beryllium and cobalt on peripheral blood mononuclear cells (PBMC) and on purified T lymphocyte or monocyte subpopulations. The results show that the two metals have strikingly different effects on monocytes and T cells because Be induces cell death in monocytes while leaving T cells alive, whereas cobalt activates monocytes while causing T cell apoptosis. Interestingly, monocytes, cultured in the presence of cobalt, show increased level of p53 and cytoplasmic p21 and produce nuclear factor  $\kappa$ B (NF- $\kappa$ B)-mediated proinflammatory cytokines while reducing their capacity to present antigens,

suggesting that they might contribute to the establishment of a chronic inflammation.

## MATERIALS AND METHODS

**Reagents and antibodies.** Apoptosis Detection Kit and “DuoSet ELISA Development Kit” for cytokine detections in cell culture supernatants were purchased from R&D Systems (Minneapolis, MN). Tetramethylbenzidine and fluorescein isothiocyanate (FITC)-dextran were purchased from Sigma Aldrich (St Louis, MO). Monoclonal antibodies (mAb) for immunostaining were purchased from Becton-Dickinson (St Jose, CA), Cell Signalling Technology Inc. (Beverly, MA), Santa Cruz Biotechnology Inc. (Santa Cruz, CA), and Sigma Aldrich.  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  and  $\text{BeSO}_4 \cdot 4\text{H}_2\text{O}$  ( $\text{CoCl}_2$  and  $\text{BeSO}_4$  in the text) with purity of 99% were purchased from Sigma Aldrich; their stock solutions were freshly prepared in distilled water and then were diluted into complete medium.

**Isolation of peripheral human monocytes and lymphocytes and cell culture.** Human PBMC were separated on Lympholyte (Cedarlane Laboratories) by density centrifugation of heparinized blood from healthy donors. Monocyte- and lymphocyte-enriched PBMC suspensions were sorted using Isolation kits and Depletion Column Type LS (Miltenyi Biotec Inc., Auburn, CA) according to the supplier’s instructions (monocytes were isolated from PBMC fraction by negative immunoselection). Cell viability as measured by Trypan blue exclusion always exceeded 95%. Monocytes and lymphocytes isolated by this procedure were, respectively, > 80 and 98% pure by FACScan flow cytometer (FACSort, Becton-Dickinson) analysis. Cells were adjusted to a concentration of  $10^6$  cells per milliliter and were suspended in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 2mM L-glutamine, 25 U/ml penicillin, and 25 U/ml streptomycin (all purchased from GIBCO) in cell culture plates at 37°C in a humidified 7%  $\text{CO}_2$  incubator. Two out of three of the medium were replaced every 3 days with complete fresh medium.  $\text{CoCl}_2$  and  $\text{BeSO}_4$  were adjusted at final concentrations of 84 $\mu\text{M}$   $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  and 113 $\mu\text{M}$   $\text{BeSO}_4 \cdot 4\text{H}_2\text{O}$ .

Dendritic cells (DCs) were generated from monocytes isolated using Monocyte Isolation kit (Miltenyi Biotec). Cells were cultured for 6 days in complete medium (RPMI 1640 supplemented with 10% heat-inactivated FCS, 2mM L-glutamine, 25 U/ml penicillin, and 25 U/ml streptomycin) in the presence of 200 U/ml recombinant human granulocyte-macrophage colony stimulating factor and 500 U/ml recombinant human Interleukin-4 (R&D Systems).

**HLA-DP-Glu69 typing.** Genotyping for the supratypic marker HLA-DPB1<sup>Glu69</sup> of all donors was performed as previously described (McCanlies *et al.*, 2004) by restriction fragment length polymorphism (BsrBI) analysis.

**Western blot.** For detection of p53, p65, and caspase-1 proteins, Western blot analysis was carried out. After indicated times of culture, monocytes and lymphocytes untreated or treated with  $\text{CoCl}_2$  and  $\text{BeSO}_4$  were washed twice with cold PBS and lysed using RIPA buffer (50mM Tris-HCl, pH 7.4, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, 1mM EDTA, 1mM EGTA, 0.5mM  $\text{NaVO}_4$ , 50mM NaF, 1mM PMSF, and protease inhibitor cocktail, Roche Hertfordshire, UK). Cell debris was removed by centrifugation at  $20,000 \times g$  for 15 min at 4°C. Protein concentration in cell lysates was determined using a Biorad protein assay kit (Biorad, Hercules, CA) with bovine serum albumin (BSA) used as standard. Cell lysate was mixed with an equal volume of 2 $\times$  electrophoresis sample buffer (125mM Tris-HCl, pH 6.8, 4% wt/vol SDS, 20% glycerol, 100mM dithiothreitol, 0.02% bromophenol blue) and boiled for 5 min. Twenty-forty micrograms of total proteins for each sample was separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) and subsequently transferred to Hyband-enhanced chemiluminescence (ECL) nitrocellulose membrane (Amersham Pharmacia Biotech). After blockade of nonspecific binding sites by treatment with nonfat milk in Tris buffer saline 0.1% Tween 20 (TBST) for 1 h, the membranes were incubated overnight at 4°C with the primary antibody (Ab) (anti-human p53, DO-1 Santa Cruz;  $\beta$  actin, Sigma; anti-human caspase-1, anti-NF- $\kappa$ B p65, and phospho-NF- $\kappa$ B

p65 [Serine 276 {Ser276}] Ab. Cell Signalling). After three washes in TBST, membranes were incubated with horseradish peroxidase-conjugated secondary Ab (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA) for 1 h at room temperature and then washed twice in TBST followed up by ECL Western blotting detection system (Amersham) and exposition to Kodak X-Omat film. The intensity of bands was quantified by densitometric analysis using the program UN-SCAN-IT gel (Silk Scientific Inc., Orem, UT). Results were evaluated as the ratio of intensity between relevant protein and  $\beta$  actin from the same sample.

For detection of activated NF- $\kappa$ B, p21<sup>Cip1/WAF1</sup>, and procaspase-3, cell fractionation was carried out as previously described (Asada *et al.*, 1999). Both the cytoplasmic and nuclear fractions were subjected to Western blotting. The antibodies used in this study were anti-p21 (F-5, Santa Cruz) anti-caspase-3 (CPP32, R&D Systems), and anti-NF- $\kappa$ B p65 (Cell Signalling). Fractionation efficiency was assessed by antibodies against  $\alpha$ -tubulin (B-5-1-2; Sigma) and Lamin B1 (ab16048, Abcam).

**RNA interference treatment.** Monocytes were plated in six-well plates. Cells were stimulated with  $\text{CoCl}_2$  and at once transfected with p21 small interfering RNA (siRNA) (SignalSilence; Cell Signalling) or scrambled control (Dharmacon, Worcester, MA, D-001206-13). siRNAp21 RNA interference was performed at 100nM. Cells were transfected using Hiperfect transfection reagent (Qiagen Inc., Valencia, CA) according to the manufacturer’s instructions. The transfection efficiency reached the maximum 18–24 h posttransfection as assessed by Western blot analysis of p21.

**Cell death assay.** Cells were treated with 84 $\mu\text{M}$   $\text{CoCl}_2$  or 113 $\mu\text{M}$   $\text{BeSO}_4$ , and the percentage of dead, apoptotic, and viable cells was then assessed by Annexin-V binding using an Apoptosis Detection Kit (R&D Systems). Expression of phosphatidylserine in the external layer of the plasma membrane was determined by studying the fixation of FITC (green fluorescence) Annexin-V in the presence of  $\text{Ca}^{2+}$  in cells that remain not permeate to propidium iodide (PI). Briefly, at indicated time points, cells were collected, washed twice with cold PBS, and then resuspended in binding buffer (10mM HEPES/NaOH, pH 7.4, 140mM NaCl, and 2.5mM  $\text{CaCl}_2$ ) at a concentration of  $1 \times 10^6$  cells per milliliter with saturating concentrations of FITC-Annexin-V and PI for 15 min at room temperature, in the dark, according to the manufacturer’s instructions. The labeled cells were analyzed immediately using a FACScan flow cytometer. Apoptotic cells were defined as those with green fluorescence > 98% of unlabeled cells.

**Cytokine assays and statistical analysis.** After 48 h of stimulation with  $\text{CoCl}_2$ , the culture media of monocytes were harvested and stored at  $-80^\circ\text{C}$  until determination. Tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL) 1 $\beta$  and migration inhibitory factor (MIF) were measured in supernatants of samples by specific ELISAs (sandwich ELISAs) according to manufacturer’s instruction (R&D Systems). All samples were diluted 1:1 in reagent diluent (1% BSA in PBS, pH 7.2, 0.2  $\mu\text{m}$  filtered) and assayed in triplicate. Results were expressed as the means  $\pm$  SDs. The limits of detection were determined using standard curves with standard cytokine preparations (defined concentrations of recombinant cytokine) used as internal controls in all tests. Optimized standard curve range (picograms per milliliter) was set as recommended: for TNF- $\alpha$ , 31.25–1000; for IL-1 $\beta$ , 18.75–300; and for MIF, 62.5–2000. The comparison among data was achieved by Mann-Whitney *U*-test.

**Intracellular IFN- $\gamma$  staining in lymphocytes.** Intracellular IFN- $\gamma$  staining was performed with FITC-labeled mAb (Clone 25723.11, BD Biosciences). After 48 h by stimulation, lymphocytes were washed with 0.1% BSA/PBS and subsequently resuspended for 4 h in complete culture medium in the presence of the protein transport inhibitor GolgiPlug containing brefeldin A (BD). The cells were washed with PBS/1% fetal bovine serum and fixed using Cytofix/Cytoperm Kit (BD) according the manufacturing datasheet. Cells were washed, resuspended in  $1 \times$  Perm Wash (BD) for 15 min at 4°C, and stained with FITC-conjugated anti-IFN- $\gamma$  diluted in Perm Wash for 30 min at 4°C. The cells were washed twice with Perm Wash and one time with PBS and resuspended in PBS for flow cytometry analysis. The mitogen phytohemagglutinin (PHA, GIBCO-BRL, MD) was used as a positive control.

**Quantitative IL-1 $\beta$  PCR analysis.** Total RNA was isolated from cell pellets by TRIzol reagent (Invitrogen, Carlsbad, CA) as recommended. After denaturation of freshly prepared RNAs (approximately 2  $\mu$ g) at 65°C for 5 min, SuperScript III Reverse Transcriptase (Invitrogen, Paisley, UK) was used for oligo-dT-primed first-strand complementary DNA (cDNA) synthesis. For measurement of the relative level of expression of IL-1 $\beta$  in untreated and lipopolysaccharide (LPS) or CoCl<sub>2</sub>-stimulated monocytes, real-time RT-PCR was performed using the 7300 Real-Time PCR System (Applied Biosystems, ABI Carlsbad, CA). Reactions were performed in a Micro-Amp Optical 96-well reaction plates (ABI) using 2.5- $\mu$ l cDNA of treated or untreated monocytes, in triplicate wells, 12.5  $\mu$ l of 2 $\times$  Master Mix (ABI), and 1.25  $\mu$ l of validated Assay on Demand gene expression (ABI, *Hs01555413*). The PCR was performed using the following amplification scheme: one cycle of 10 min at 95°C followed by 60 cycles of denaturation for 15 s at 95°C and an annealing/extension step of 1 min at 60°C in a final volume of 25  $\mu$ l. All reactions were conducted using a 7300 Sequence Detector thermocycler linked to a PC using the Sequence Detector software (ABI). Glyceraldehyde-3-phosphate dehydrogenase Assay on Demand (ABI, *Hs9999905*) was used to ensure quality of RNA preparation, to account for efficiency of the reverse transcription, and to check for any loading variation of the initial cDNA amount.

**Immunophenotypic characterization of monocytes by flow cytometry.** For immunostaining, at least  $5 \times 10^4$  cells were washed twice in PBS containing 1% BSA (; Sigma) and incubated 1 h at 4°C with saturating concentrations of FITC-conjugated mAb. Afterward, samples were washed, resuspended in 200  $\mu$ l 1% BSA/PBS, and immediately analyzed in a Becton-Dickinson FACScan flow cytometer for a total of 10,000 events. Expression on surface molecules was reported as percentage and mean fluorescence intensity. Monocytes were electronically gated according to light scatter properties to exclude cell debris and contaminating lymphocytes. Dead cells were gated out after staining with 5  $\mu$ g/ml PI solution. Flow cytometry data were processed using Cell Quest software (Becton-Dickinson).

**Evaluation of antigen uptake.** The phagocytic activity of monocytes was analyzed by flow cytometric analysis. Mannose receptor-mediated endocytosis was measured as the cellular uptake of FITC-dextran (Sigma). Briefly,  $10^5$  cells were resuspended in 200  $\mu$ l of 10% FCS containing RPMI 1640 medium buffered with 25mM HEPES. Cells were incubated with FITC-dextran (0.5 mg/ml) for 2 h at 37°C. The incubation was stopped by adding 2 ml of cold PBS. Cells were washed twice with PBS 0.1% sodium azide and analyzed with fluorescence activated cell sorter. Staining of cells incubated at 0°C was taken as background value reference.

**Primary mixed cultures.** PBMC were obtained by Lymphoprep gradient centrifugation of heparinized blood. PBMC were treated with CD4<sup>+</sup> cell isolation kit (Oxoid) containing anti-CD14 microbeads. Enriched T cells consisting of more than 95% CD4<sup>+</sup> cells were cocultured at  $4 \times 10^5$  cells per well in round-bottomed 96-well culture plates with different amounts of monocytes or DCs, in a total volume of 200  $\mu$ l of complete medium per well, for 5 days at 37°C in a 7% CO<sub>2</sub>-humidified atmosphere. Monocytes were pretreated or not with CoCl<sub>2</sub> for 6 days and washed with culture medium before to be used in the mixed lymphocyte culture. During the last 16 h of culture, the cells were pulsed with 0.5 microcurie per well of [<sup>3</sup>H]thymidine (specific activity 5 Ci/mM, PerkinElmer). Cultures were harvested onto glass fiber filters (Skatron). [<sup>3</sup>H]Thymidine incorporation was measured using a beta scintillation counter (Beckman). [<sup>3</sup>H]Thymidine incorporations of T cells or DCs only were taken as background and subtracted. Mean values from three replicates were taken from each sample culture and expressed as mean counts per minute.

## RESULTS

### *Cobalt and Beryllium Have Opposite Effects on Monocyte and T Lymphocyte Viability*

PBMC obtained from blood samples of 18 healthy donors were exposed to escalating doses of either CoCl<sub>2</sub> or BeSO<sub>4</sub> and

analyzed for cell survival at different days of culture (Fig. 1). Panel A shows that CoCl<sub>2</sub> treatment caused a dose- and time-dependent reduction of lymphocytes (R2) but not of monocytes (R1), which did not significantly decrease in number unless a very high dose of CoCl<sub>2</sub> (336 $\mu$ M) was used. Conversely, the exposure to BeSO<sub>4</sub> (Fig. 1B) had a dramatic effect on monocyte survival, already evident after 2 days of culture at the lowest concentration (56.5 $\mu$ M) while not interfering with lymphocyte survival even at four times higher doses (904 $\mu$ M). PBMC kept in culture in the absence of metals for the times indicated are plotted in Figure 1C. Figure 1D reports the histogram plots related to the phenotypic analysis of CD14 expression. On the basis of the dose-response curves, the intermediate concentrations of 84 $\mu$ M for CoCl<sub>2</sub> highly toxic for T lymphocytes but not affecting monocyte survival and of 113 $\mu$ M for BeSO<sub>4</sub> to which, on the opposite, monocytes promptly respond by undergoing apoptosis, whereas lymphocytes are not affected, were chosen for subsequent experiments.

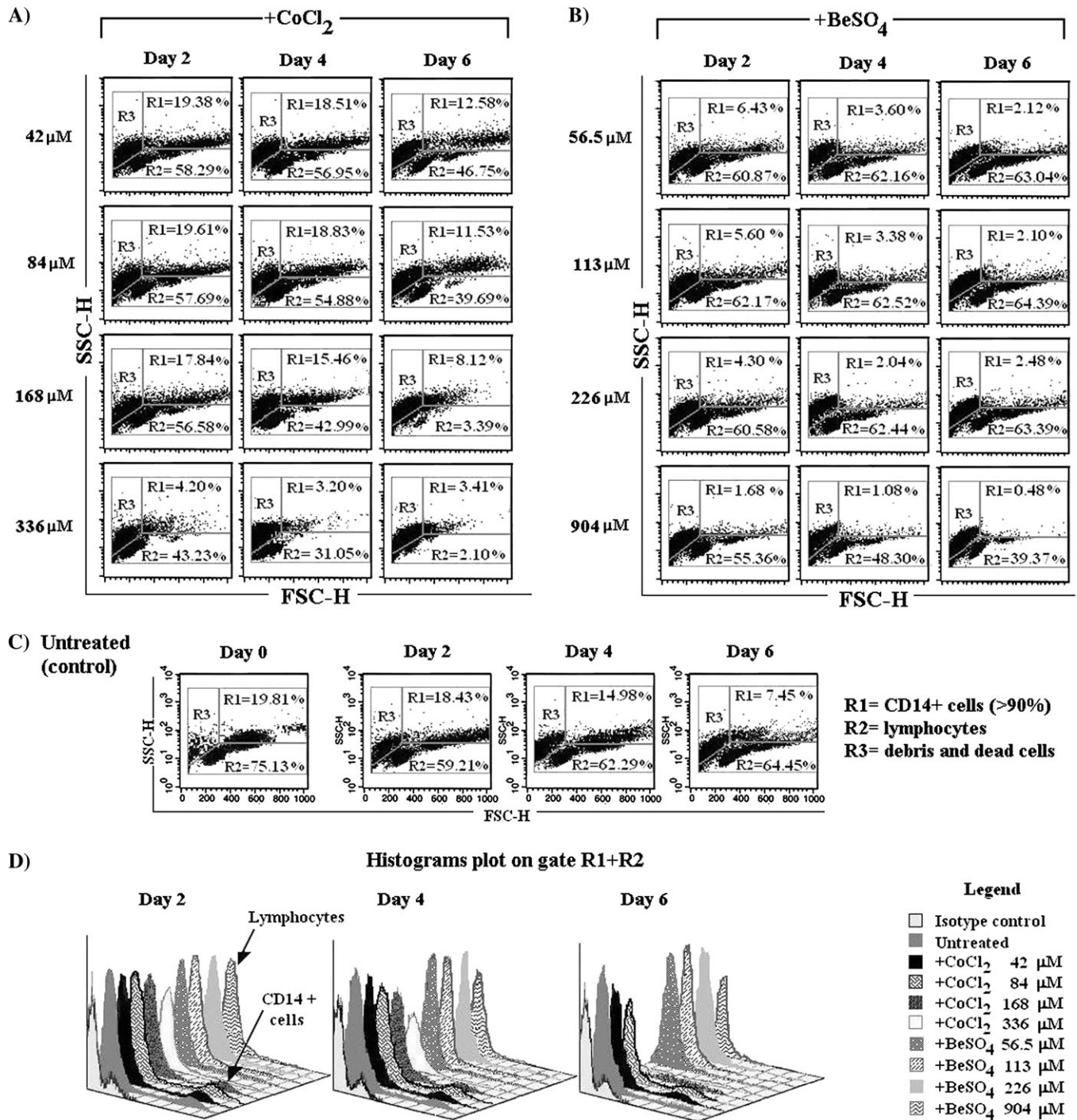
Interestingly, out of 18 donors studied, 14 demonstrated the same behavior represented in Figure 1, whereas in the remaining subjects, monocytes in the presence of cobalt underwent cell death after 2 days of culture. We therefore hypothesized that the presence of Glu69 in the HLA-DP beta chain could make the difference. However, this was not the case because the analysis of the allelic polymorphism (Glu69) did not reveal any association with the monocyte fate (data not shown).

### *Cobalt Induces p53 Increase in both Monocyte and Lymphocyte Populations*

To further investigate the effect of CoCl<sub>2</sub> and BeSO<sub>4</sub> on the survival and functional status of the monocytes, these cells isolated by negative depletion were cultured for 2 days in the presence or absence of metals. Monocytes incubated with CoCl<sub>2</sub> remained alive like the untreated cells, with a viability that was always more than 50% (Figs. 2A and 2B). In contrast, beryllium was toxic for monocytes whose number was dramatically reduced already after 2 days of culture. The monocyte viability observed in presence of cobalt was confirmed in 10 subjects and proven to be highly reproducible as shown in Figure 2C.

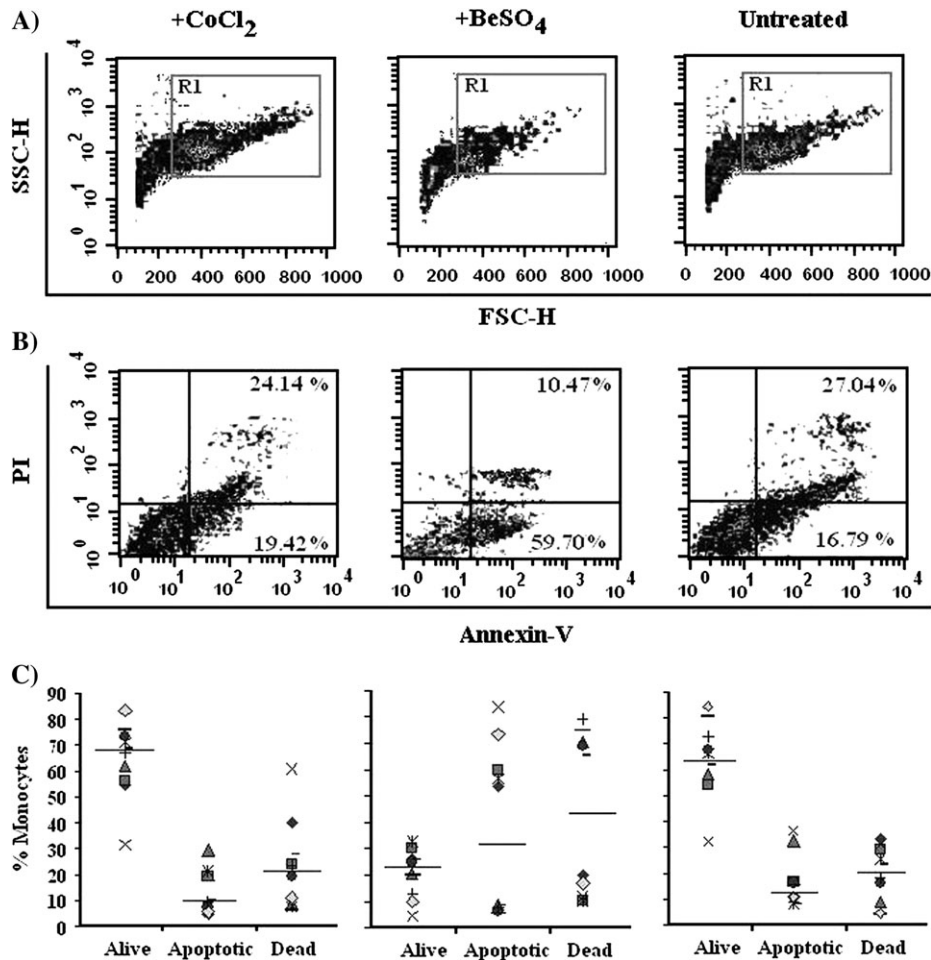
Because it has been reported that cobalt may have a genotoxic effect, we addressed the question whether cell death seen in lymphocytes was correlated to an increased p53 expression. Accordingly, the expression of p53 was investigated in CD3<sup>+</sup> T lymphocytes and monocytes after exposure to CoCl<sub>2</sub> or BeSO<sub>4</sub>. T cells exposed to x-rays and lysed after 24 h were used as positive controls. After 16 h, the expression of p53 was clearly detectable in CoCl<sub>2</sub>-treated lymphocytes but not in those treated with BeSO<sub>4</sub> (Figs. 3A and 3B). In monocytes, both CoCl<sub>2</sub> and BeSO<sub>4</sub> salts induced p53 upregulation (Figs. 3C and 3D) with a more pronounced effect by the former. After 48 h, only CoCl<sub>2</sub>-treated monocytes were





still alive and maintained a high expression of p53. Therefore, monocyte survival and death in the presence of cobalt or beryllium, respectively, appear to be mediated by different mechanisms both involving p53. This observation is in

agreement with experimental models showing that wild-type p53 induced cell differentiation and, in particular, was found to enhance macrophage differentiation (Matas *et al.*, 2004; Navrátilová *et al.*, 2007).

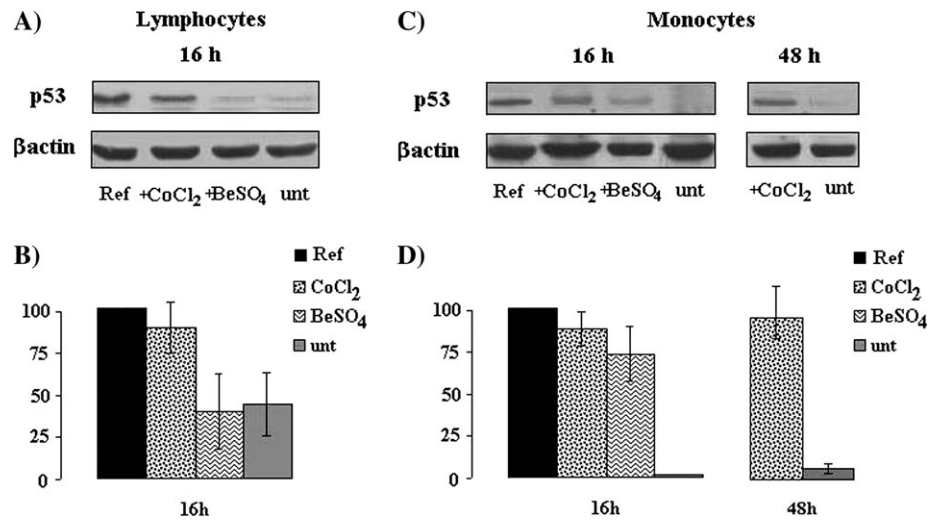


**FIG. 2.** Effects of  $\text{CoCl}_2$  and  $\text{BeSO}_4$  on human monocytes. Pure monocytes were isolated by magnetic depletion of lymphocytes. Monocytes, treated for 48 h with  $84\mu\text{M CoCl}_2 \cdot 6\text{H}_2\text{O}$  or  $113\mu\text{M BeSO}_4 \cdot 4\text{H}_2\text{O}$  or untreated (right side), were incubated at  $37^\circ\text{C}$  in  $7\% \text{CO}_2$ -humidified atmosphere with RPMI 10% FCS. Cell viability was determined by Annexin-V binding and flow cytometric analysis. Density plots: (A) SSC versus FSC; monocytes were gated on SSC/FSC density plot excluding debris (R1). (B) PI versus Annexin-V staining: values in quadrants represent the percentage of apoptotic (lower right) and dead cells (upper right) from a single experiment representative of triplicate experiments with cells from 10 different donors. (C) Percentages of alive, apoptotic, and dead monocytes, respectively, for each subject analyzed. Horizontal bars represent median values. In the presence of  $\text{BeSO}_4$ , monocyte viability was  $< 30\%$ , whereas in  $\text{CoCl}_2$ -treated monocytes and in control monocytes, the percentages of viable cells were similar and  $> 60\%$ . Reduction of viable cells in the presence of  $\text{BeSO}_4$  was statistically significant ( $p < 0.0001$ , nonparametric Mann-Whitney  $U$ -test).

### *Cobalt-Induced Monocytes Show Cytoplasmic p21<sup>Cip1/WAF1</sup> Localization Correlating with the Stabilization of Procaspase-3*

To further investigate the effect of  $\text{CoCl}_2$  in monocytes, we examined the physiology and morphology of the surviving cells. p21(CIP/WAF1) is the primary mediator of cell cycle arrest in response to DNA damage to different forms of stress and is implicated in senescence and terminal differentiation. The differentiation of immature monocytes is associated with a relocalization of nuclear p21 to the cytoplasm (Asada *et al.*, 1999). The antiapoptotic functions of p21 may be attributed to several of its molecular interactions including the binding to procaspase-3 and inhibition of its conversion to mature form (Dotto, 2000). Expression of cytoplasmic p21 concurrent to

increased level of procaspase-3 in  $\text{CoCl}_2$ -treated monocytes was demonstrated by Western blot analysis of cytoplasmic extracts (Fig. 4A). To determine whether p21 expression regulates procaspase-3 activation in  $\text{CoCl}_2$ -treated monocytes, cells were at once transfected with p21 siRNA or scrambled control siRNA, and samples were collected for Western blotting and flow cytometry. The highest level of efficiency was reached 16–24 h after transfection. Consistent with a role for p21 in regulating procaspase-3 amount, siRNA knockdown of p21 during  $\text{CoCl}_2$  treatment restored inactive caspase-3 level, whereas scrambled control did not (Fig. 4B). Quantification of procaspase-3 by Western blots obtained from three separate experiments confirmed this result. In addition, treatment with p21 siRNA but not control siRNA resulted in increased cell death (data not shown). These findings support



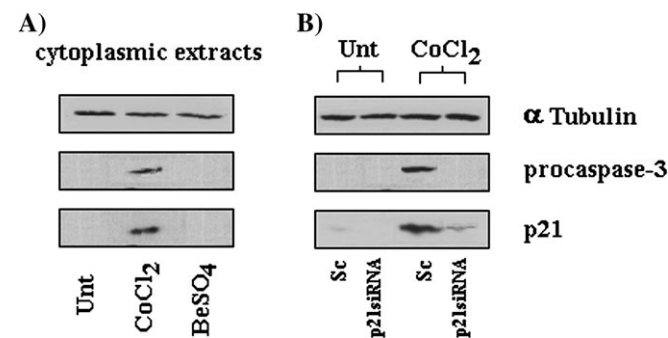
a role for p21-dependent antiapoptotic effect on survival of monocytes treated with CoCl<sub>2</sub>. Attempts to detect p21 in lymphocytes either in the nuclei or in the cytoplasm were unsuccessful (data not shown).

#### Cobalt Activates NF-κB in Monocytes and Increases Their Production of Proinflammatory Cytokines

Because fully differentiated macrophages, in contrast to monocytes, contain significant levels of NF-κB into nuclei

and its activation is required for monocyte differentiation (Pennington *et al.*, 2001), we asked whether NF-κB was involved in the survival of the cobalt-treated monocytes. It has been shown that phosphorylation at Ser276 is critical for p65 NF-κB activation and regulates the interaction with coactivators p300/CBP (Okazaki *et al.*, 2003). Therefore, the phosphorylation status of Ser276 in the p65 subunit of NF-κB was assessed by immunoblotting with a phospho-specific anti-p65 Ab. Monocytes treated with cobalt but not with beryllium showed a significant increase of phospho-p65 protein (Fig. 5).

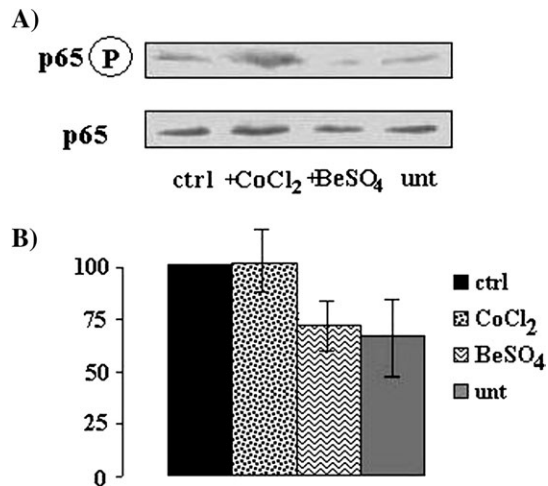
Next, we analyzed whether the presence of cobalt had any effect on the production of proinflammatory cytokines such as TNF-α and IL-1β. The supernatants of monocytes were analyzed for the presence of these cytokines by ELISA (Fig. 6), which revealed a clear trend toward an increased production of TNF-α as well as IL-1β in CoCl<sub>2</sub>-treated compared with untreated monocytes (Mann-Whitney test:  $p < 0.001$  and  $p < 0.003$  for TNF-α and IL-1β, respectively). Because macrophage MIF has emerged to be an important effector molecule of the innate immune system and unlike most cytokines is constitutively produced by immune cells (Lue *et al.*, 2002), its presence in the supernatants was also analyzed. Differently from TNF-α and IL-1β, there was no significant correlation between the production of MIF and the presence of cobalt. Notably, some of the donors who produced low amount of proinflammatory cytokines did secrete MIF.



**Cobalt Enhances IL-1β Expression Acting both at Transcriptional and at Protein Processing Level**

The expression of IL-1β is tightly regulated in that distinct signals are required for its transcription and release (Mariathasan





**FIG. 5.** CoCl<sub>2</sub> induces phosphorylation of serine 276 in the p65 NF- $\kappa$ B subunit by monocytes. Untreated, CoCl<sub>2</sub>-, and BeSO<sub>4</sub>-treated monocytes were cultured for 16 h at 37°C, 7% CO<sub>2</sub>. (A) An equal amount of whole-cell lysate for each sample was separated by 12% SDS-PAGE, transferred to a nitrocellulose membrane, and subsequently immunoblotted with anti-phospho-p65 (S276P) (upper panel) and anti-p65 (lower panel) antibodies. Monocytes exposed to 0.05  $\mu$ g/ml of LPS and lysed after 1 h were used as positive reference (Ref). (B) Phospho-p65 (S276P) protein level in monocytes was evaluated by densitometric analysis as described in “Materials and Methods” section. Values are the mean  $\pm$  SD of three independent experiments and are reported as percentage of 100% phospho-p65 expression arbitrarily attributed to control cells.

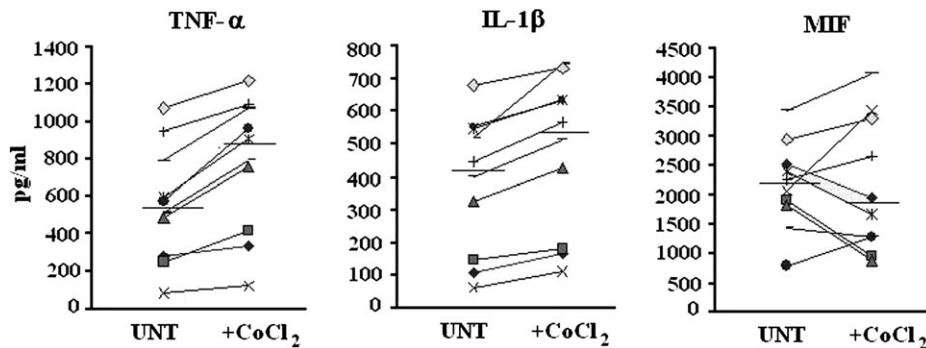
and Monack, 2007). The first signal can be transmitted via stimulation of Toll-like receptor signaling pathway, leading to the activation of NF- $\kappa$ B and the expression of the 35-kDa precursor form of IL-1 $\beta$ . A further signal is required to switch on the inflammasome complex, leading to the activation of caspase-1 and subsequent processing and secretion of the mature cytokine. The amount of IL-1 $\beta$  messenger RNA (mRNA) in purified monocytes from three subjects stimulated with LPS or CoCl<sub>2</sub> for 1, 12, and 24 h was examined by quantitative relative

RT-PCR (Fig. 7). After CoCl<sub>2</sub> exposure, monocytes were still producing a reasonable high level of IL-1 $\beta$  mRNA up to 24 h of culture, whereas the untreated monocytes did not.

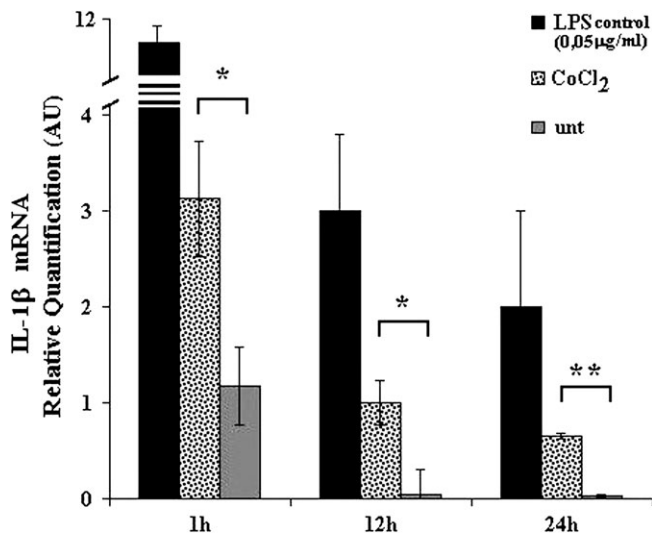
We next tested the caspase-1 activation that is required for IL-1 $\beta$  release. As other caspases, caspase-1 undergoes proteolytic activation to produce a tetramer of its two active subunits, p20 and p10 (Lamkanfi *et al.*, 2007). To verify the status of caspase-1, extracts were prepared from untreated monocytes and those stimulated with either CoCl<sub>2</sub> or LPS (0.05  $\mu$ g/ml). The extracts were then immunoblotted with an Ab that detects procaspase-1 ( $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  isoforms) and caspase-1 p20 subunits. Stimulation of monocytes with LPS or CoCl<sub>2</sub> induced the processing of procaspase-1 into the mature p20 subunit after 4 and 12 h, respectively (Fig. 8). Interestingly, however, freshly isolated human blood monocytes, kept in culture for the time indicated, also became able to produce detectable amount of mature p20 subunit, probably as consequence of the differentiation program triggered by plastic adherence (Fig. 8, line 5).

#### BeSO<sub>4</sub>-Treated T Lymphocytes Produce IFN- $\gamma$

Untreated, CoCl<sub>2</sub>-, or BeSO<sub>4</sub>-stimulated lymphocytes were collected after 48 h and stained intracellularly with an Ab specific for IFN- $\gamma$ . The mitogen PHA was used as a positive control. The number of IFN- $\gamma$ -positive T cells detected by flow cytometry were low in both untreated and CoCl<sub>2</sub>-treated lymphocytes; on the contrary, in the presence of BeSO<sub>4</sub>, the number increased up close to that of PHA-stimulated cells (Fig. 9A). We therefore asked whether NF- $\kappa$ B was activated by the two salts. Because the level of phospho-Ser276 p65 protein in whole extracts was undetectable in beryllium-treated lymphocytes (not shown), we analyzed directly the nuclear translocation of the protein. Despite the comparable production of IFN- $\gamma$ , translocation of NF- $\kappa$ B in BeSO<sub>4</sub>-stimulated lymphocytes appears negligible compared with PHA-treated T cells (Fig. 9B).



**FIG. 6.** Production of TNF- $\alpha$ , IL-1 $\beta$ , and MIF by monocytes in presence of CoCl<sub>2</sub>. Cytokine levels (picograms per milliliter) in harvested media from monocytes incubated in the absence (Unt) or in the presence of CoCl<sub>2</sub> (84 $\mu$ M) were measured by ELISA after 48 h of culture. Data are mean of triplicate from culture supernatants of 10 donors. SDs were always < 5%. Numbers of viable monocytes did not differ between control and treated cells for each donor. Horizontal bars represent median values. The increase in the production of proinflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$  induced by CoCl<sub>2</sub> was statistically significant ( $p < 0.001$  for TNF- $\alpha$  and  $p < 0.003$  for IL-1 $\beta$ , respectively, nonparametric Mann-Whitney *U*-test). Differences in MIF production in absence or presence of CoCl<sub>2</sub> were not significant.



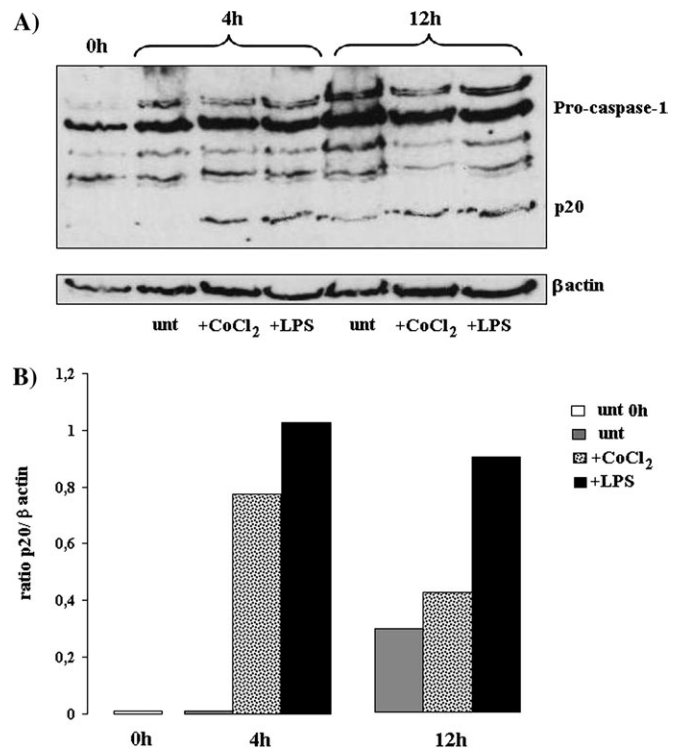
**FIG. 7.** Time course of the modulation of IL-1 $\beta$  mRNA in CoCl<sub>2</sub>-treated monocytes. CoCl<sub>2</sub>- and LPS (control)-stimulated or untreated monocytes were cultured for 1, 12, and 24 h at 37°C, 7% CO<sub>2</sub> before to be harvested. Total RNA was isolated by TRIzol reagent, and real-time RT-PCR was performed on first-strand cDNAs. Data are mean  $\pm$  SD of triplicate relative real-time quantification of three independent cultures. AU, arbitrary units. Student *t*-test *p* values < 0.05 between the CoCl<sub>2</sub>-treated monocytes and untreated samples are indicated by asterisks and < 0.01 by double asterisks.

#### *CoCl<sub>2</sub>-Treated Monocytes Show a Reduced Expression of HLA-Class II and an Impaired Antigen Presentation Capability after 6 Days of Culture*

After 6 days of culture in the presence of cobalt, more than 60% of cells were still alive compared with less than 30% of untreated monocytes (Figs. 10A and 10B) and 0% of beryllium-treated monocytes (data not shown). Figure 10C reports the comparison of alive, apoptotic, and dead cells performed on monocytes derived from 10 individuals. Lymphocytes treated for 6 days with the same doses of CoCl<sub>2</sub> showed a sharp reduction in survival compared with beryllium-treated or untreated lymphocytes (Figs. 10D and 10F).

Once established that cobalt was not toxic for monocytes but rather acting as a survival factor and a trigger for proinflammatory cytokine production, we investigated the phenotype of cotreated monocytes, analyzing the expression of CD14, CD16, CD11b, CD83, CD86, and HLA-class I and class II at 6 days of culture. The cells expressed CD14 and CD11b molecules but remained negative for CD16 and CD83, suggesting that they did not differentiate into either macrophages or DCs, respectively (Fig. 11A). The expression of HLA-class I was slightly decreased, whereas that of HLA-class II was sharply reduced after 6 days.

We then asked whether cobalt-treated monocytes were functionally competent in antigen capturing and presentation compared with untreated cells. As shown in Figure 11B, CoCl<sub>2</sub>-treated monocytes have reduced capacity to uptake fluorescent dextran compared with untreated monocytes. Furthermore, like untreated monocytes, they were, compared



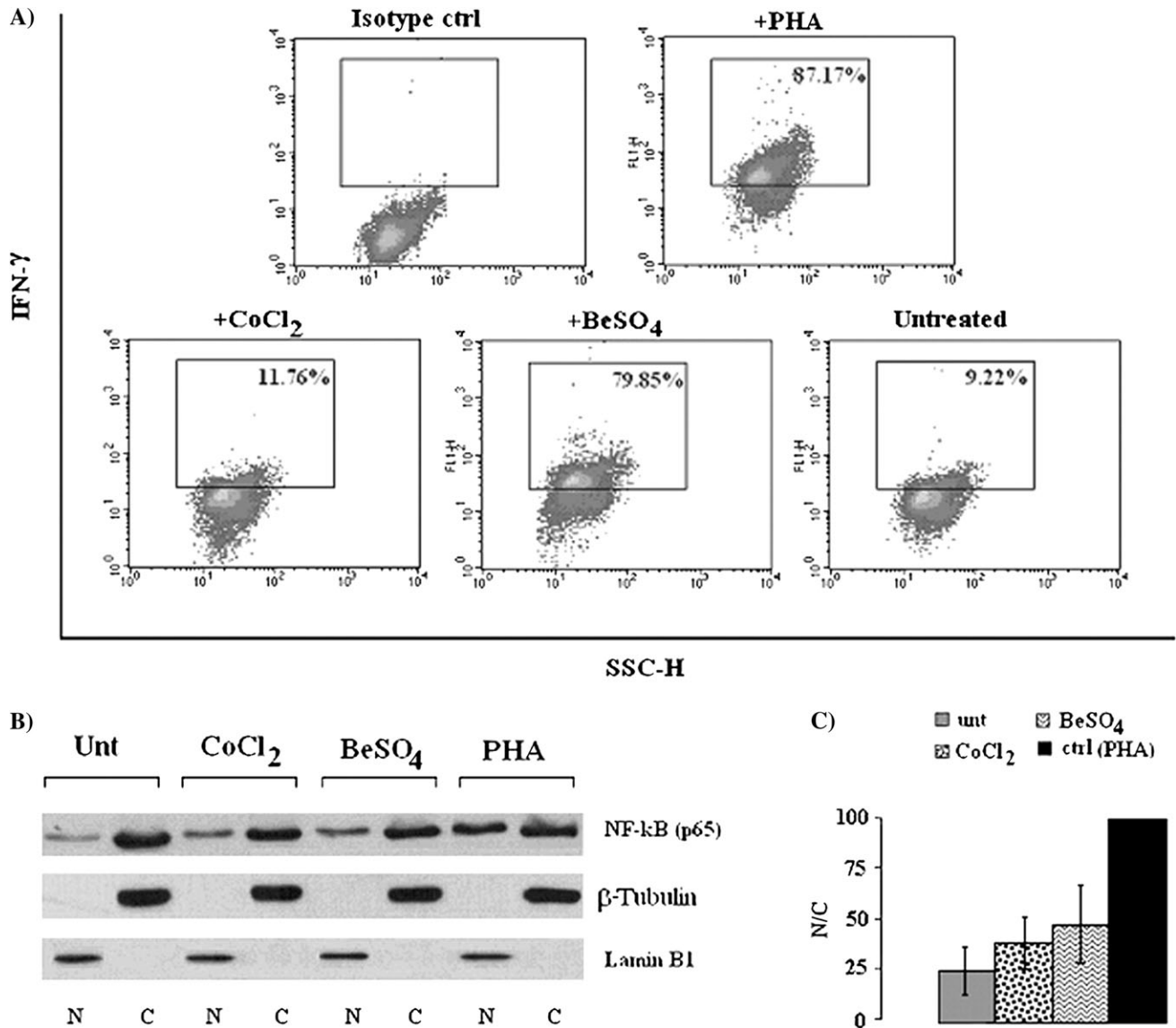
**FIG. 8.** Cobalt-induced caspase-1 activation by monocytes. Untreated, CoCl<sub>2</sub>-, and LPS-treated monocytes were cultured for indicated times at 37°C, 7% CO<sub>2</sub>. (A) Lysates were separated by 12% SDS-PAGE, transferred to a nitrocellulose membrane, and subsequently immunoblotted with anti-caspase-1 Ab. The multiple bands are alternative spliced variants of caspase-1 identified by the Ab used, as reported by the producer. (B) p20 protein levels were evaluated by densitometric analysis as described in "Materials and Methods" section. Results are representative of two separated experiments.

with LPS-matured DCs from the same subjects, poorly efficient in stimulating T cells in a mixed lymphocyte reaction using allogeneic CD4<sup>+</sup> T lymphocytes as responder cells (Fig. 11C).

## DISCUSSION

Environmental exposure to inhaled dust containing cobalt particles can cause HMLD that is associated with immunological sensitization, respiratory tract irritation, asthma, and fibrosis. An important aspect of HMLD is that the disease may occur after a short duration of exposure, suggesting that the individual susceptibility rather than cumulative exposure plays a major role (Nemery *et al.*, 2001). Previous evidence indicates that many of these responses are similar to those elicited by reactive oxygen species (ROS), and the cellular and molecular effects of metal exposure in the lung can be the result of ROS (Kasprzak, 2002) and reactive nitrogen species generation (Rengasamy *et al.*, 1999). An important hallmark of HMLD is the giant cell interstitial pneumonitis and the presence of multinucleate giant cells in the bronchoalveolar lavage, underlying the occurrence of an inflammatory process

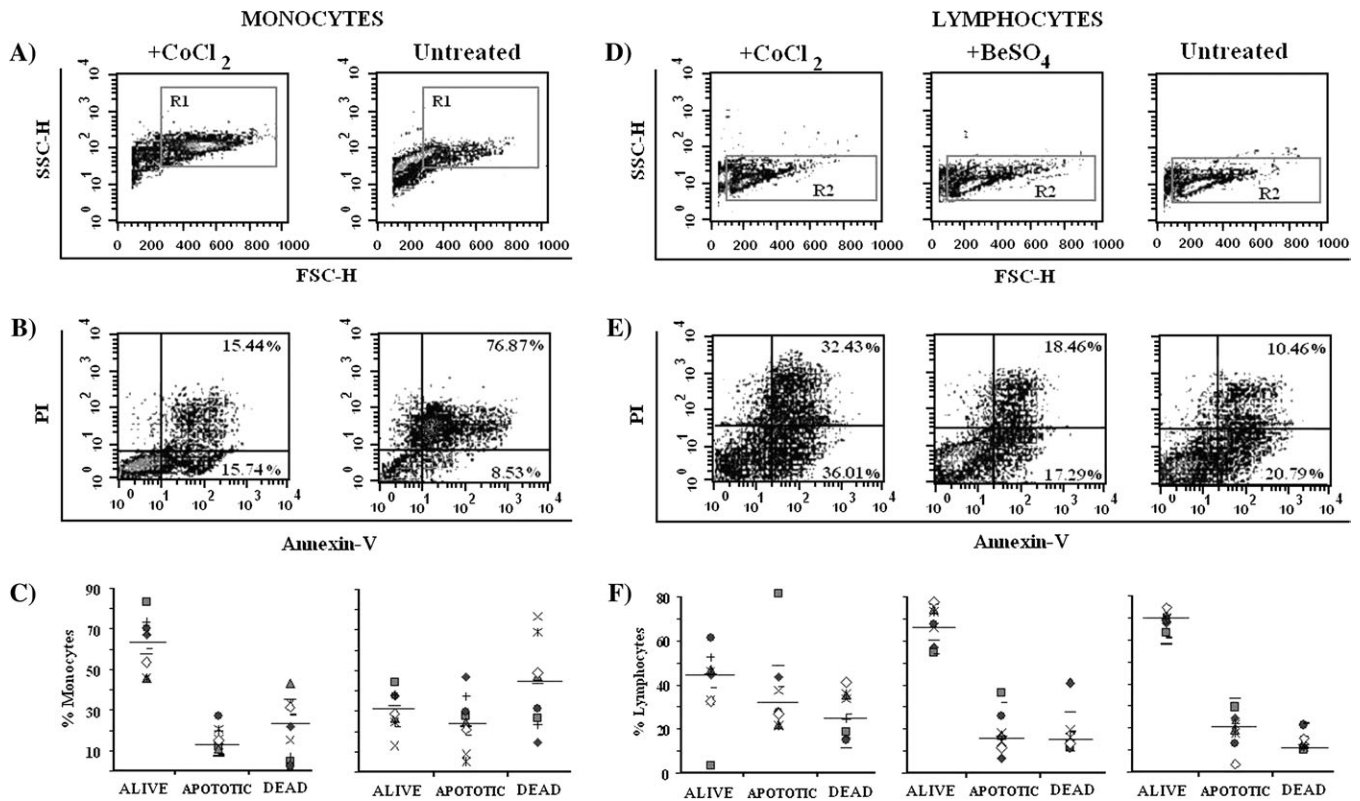




**FIG. 9.** BeSO<sub>4</sub>-treated lymphocytes produce IFN- $\gamma$ . (A) Flow cytometric analysis of IFN- $\gamma$  production by lymphocytes. Alive lymphocytes were gated on FSC/SSC dot plot excluding debris and aggregates (not shown). Analysis was then performed on IFN- $\gamma$  FITC versus SSC dot plot. Isotype-matched negative controls and region statistics are reported. After 48 h, Be-stimulated lymphocytes express IFN- $\gamma$  as much as PHA-activated lymphocytes (control). A representative of three independent experiments is shown. (B) Western blot analysis of NF- $\kappa$ B. Untreated, CoCl<sub>2</sub>-, BeSO<sub>4</sub>-, or PHA-treated lymphocytes were cultured for 16 h at 37°C, 7% CO<sub>2</sub>. An equal amount of nuclear (N) and cytoplasmic (C) extracts for each sample was separated by 12% SDS-PAGE, transferred to a nitrocellulose membrane, and subsequently immunoblotted with anti-p65 Ab. Lymphocytes exposed to 0.05  $\mu$ g/ml of PHA were used as positive reference (Ref). (C) Activated NF- $\kappa$ B was evaluated by densitometric analysis as ratio between nuclear and cytoplasmic fractions. Values are the mean  $\pm$  SD of three independent experiments and are reported as percentage of PHA-activated control cells arbitrarily attributed as 100%.

in the lung tissue (Rivolta *et al.*, 1994). In this paper, we investigated the effect of cobalt on monocytes and showed that these cells, once exposed to CoCl<sub>2</sub>, undergo metabolic and morphological changes. Indeed, the transcriptional activity of NF- $\kappa$ B is early stimulated, and monocytes survive in culture for several days and secrete proinflammatory cytokines. This apoptosis-resistant phenotype appears concomitantly with the expression of cytoplasmic p21<sup>Cip1/WAF1</sup> that induces stabilization of procaspase-3. It is known that p21 can protect against

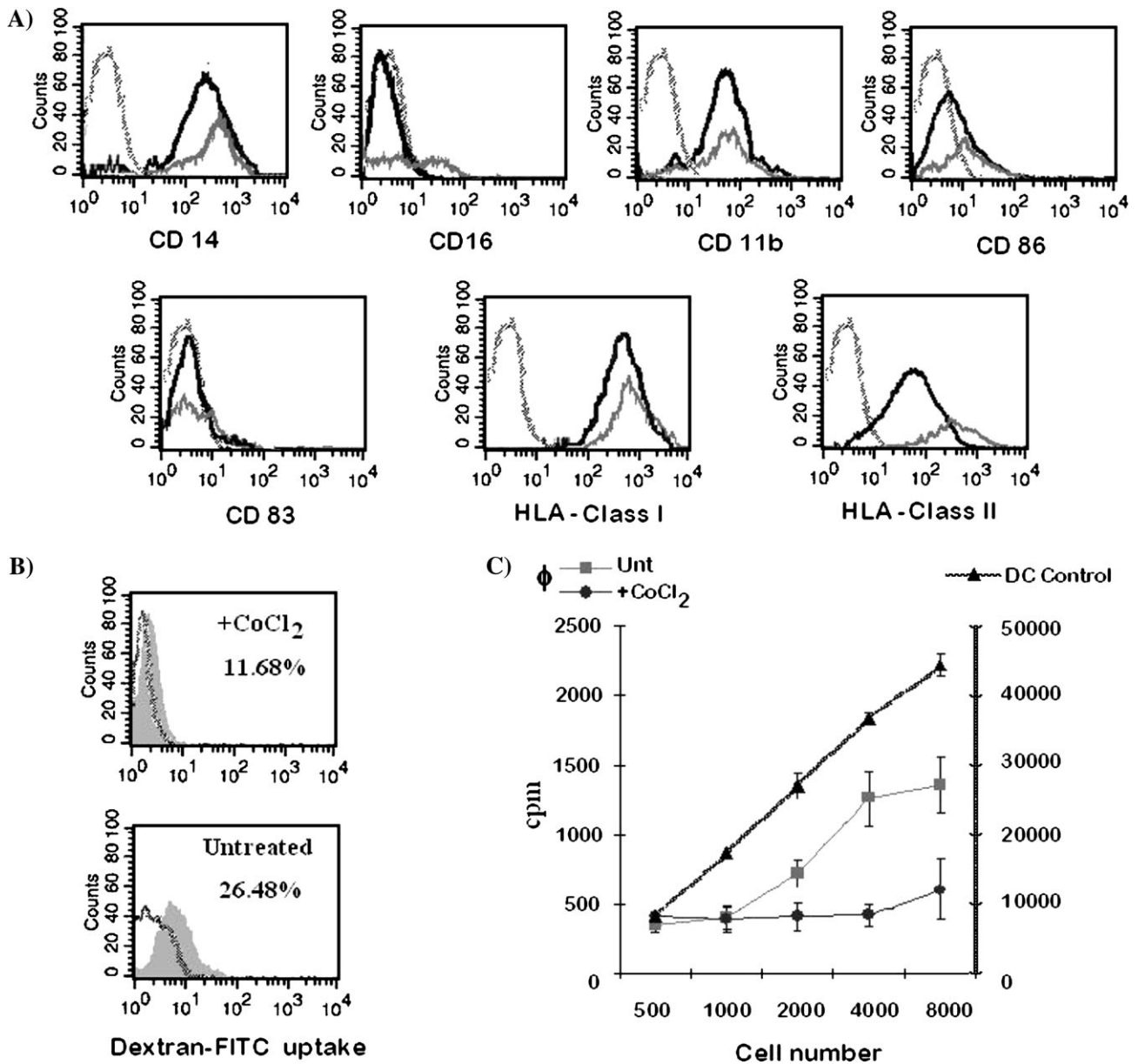
apoptosis in response to growth factor deprivation, p53 overexpression, or during the differentiation of monocytes, and it is also been shown to stimulate NF- $\kappa$ B-mediated transcription. This effect is because of the activation of transcriptional cofactors/histone deacetylases p300 and CBP (Abbas and Dutta, 2009). Ser276-phosphorylated RelA binding is seen primarily on a subset of genes that are rapidly induced by TNF, including Gro- $\beta$ , IL-8, and I $\kappa$ B $\alpha$ . Recent work has shown that TNF-inducible RelA Ser276 phosphorylation is



**FIG. 10.** Cell viability of monocytes and lymphocytes after 6 days of culture. After 6 days of culture, the viability of CoCl<sub>2</sub>-treated and untreated monocytes (A–C) and CoCl<sub>2</sub>- or BeSO<sub>4</sub>-treated and untreated lymphocytes (D–F) was compared. Density plots: (A and D) SSC versus FSC. Monocytes and lymphocytes were gated on SSC/FSC density plot excluding debris (R1 and R2, respectively); (B and E) PI versus Annexin-V staining. Values within the graphs represent the percentages of apoptotic and dead cells from single experiment representative of duplicate experiment from 10 donors. (C and F) Percentages of alive, apoptotic, and dead cells from each subject analyzed. Horizontal bars represent median values. After 6 days, monocytes treated with cobalt are still viable more than 60%. Increasing viable cells in the presence of CoCl<sub>2</sub> were statistically significant ( $p < 0.0001$ , nonparametric Mann-Whitney  $U$ -test). After 6 days, the lymphocytes treated with cobalt are apoptotic ( $> 30\%$ ) unlike cells treated with beryllium, which are substantially similar to not treated ones. The reduction of viable cells in the presence of CoCl<sub>2</sub> was statistically significant ( $p < 0.0001$ , nonparametric Mann-Whitney  $U$ -test).

controlled by an ROS-protein kinase A signaling pathway (Novak *et al.*, 2008). Previous investigations demonstrated that IKK $\beta$ , the major catalytic subunit of IKK complex, could increase p21 expression through upregulation of p21 mRNA level and via activation of its downstream target Akt, which is known to phosphorylate p21 and lead to cytoplasmic localization of p21 (Ping *et al.*, 2006). However, although it is well established that NF- $\kappa$ B is a survival factor activated during monocyte differentiation (Pennington *et al.*, 2001), we have not observed a complete differentiation of cobalt-treated monocytes to macrophages or DCs in that they remained both CD16 and CD83 negative. These cells did not proliferate as shown by the lack of [<sup>3</sup>H]thymidine incorporation and permanence in the G0 phase of cell cycle (not shown). Interestingly, T lymphocytes, under the same circumstances, underwent a high degree of apoptosis. Strikingly different was the fate of cells exposed to beryllium. This metal is highly toxic for peripheral monocytes (this paper) as well as for macrophages (Sawyer *et al.*, 2005), but not for T lymphocytes. It has been reported that cobalt ions are potent inducers of hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) (Sandau *et al.*, 2000). HIF-1 $\alpha$  is

activated during macrophage differentiation, and it is an important intracellular regulator of numerous genes involved in glucose metabolism, angiogenesis, cell survival, proliferation (Oda *et al.*, 2006), and apoptosis-inducing accumulation of p53 through its direct association with HIF-1 $\alpha$  (Won *et al.*, 1998) and consequent cell death by apoptosis. This seems to be true for lymphocytes but not for monocytes, probably because monocytes are resting cells and therefore less susceptible to the effect of genotoxic damage by cobalt (De Boeck *et al.*, 1998). It is reasonable that the high level of p53 in cobalt-pulsed monocytes plays a role as mediator of NF- $\kappa$ B-induced macrophage differentiation (Mudipalli *et al.*, 2001). What is intriguing is the opposite effect of beryllium whose presence is highly toxic for monocytes but not for lymphocytes. We do not know what causes this reaction, but we observed a detectable increase in p53 expression also in BeSO<sub>4</sub>-treated monocytes, suggesting that cell death could be mediated by other mechanisms in which there is noactivation of the survival factor NF- $\kappa$ B and cytoplasmic p21 localization. We show here, in agreement with other reports (Kaufman *et al.*, 2008), that monocytes responding to cobalt produce an increased amount of



**FIG. 11.** Monocytes kept alive by cobalt showed reduced expression of HLA-class II and impaired antigen presentation ability. Phenotype monocytes after 6 days of culture: (A) surface expression of CD14, CD16, CD11b, CD86, CD83, and HLA-class I and class II in CoCl<sub>2</sub>-treated (solid black line) and in control (solid gray line) monocytes. Dotted histograms represent background staining of isotype control Ab. The data are representative of five different experiments. (B) Antigen uptake capacity. Open histograms represent the uptake obtained at 0°C. Values indicate the percentage of cells positive for FITC-dextran. The results from one representative experiment out of five independently performed are shown. (C) Allostimulatory capacity. After 6 days, cells were collected and their capacity to stimulate T cell proliferation was measured in an mixed lymphocyte reaction assay. Allogenic CD4<sup>+</sup> T cells were cultured for 5 days in 96-well culture microplates ( $4 \times 10^4$ ) as responder cells with different numbers of antigen presenting cells. [<sup>3</sup>H]thymidine incorporation was measured after 16-h pulse (0.5 microcurie per well). The results are shown as mean counts per minute (axis on the left). DCs from the same subjects were used as positive control for T cell proliferation (dotted axis on the right of the graph). The data are mean values of triplicate samples. One representative experiment out of four is shown.

proinflammatory cytokines such as TNF- $\alpha$  and IL-1 $\beta$ . The observed IL-1 $\beta$  transcriptional induction could occur via a HIF-1-dependent mechanism during hypoxia and be maintained through a NF- $\kappa$ B-dependent amplification by autocrine IL-1 $\beta$  (Zhang *et al.*, 2006). In this work, we have shown that cobalt induces not only the synthesis but also the processing and

secretion of IL-1 $\beta$  by activation of caspase-1 in human monocytes. Our observation is in agreement with recent studies outlining the importance of the inflammasome in activating the danger signaling pathway triggered by ROS generated by NADPH oxidase following particle phagocytosis (Caicedo *et al.*, 2009; Cassel *et al.*, 2008; Dostert *et al.*, 2008; Netea *et al.*,



2009). However, cobalt-treated monocytes appeared to lose their antigen-processing/presenting properties displaying a reduced efficiency to capture the antigen as well as to stimulate the alloreactive T cell response. Therefore, cobalt acts as an activation factor for monocytes, which can exert their proinflammatory effect for long time eventually in sites where cobalt can reach a threshold concentration (i.e., lung in case of cobalt inhalation). Differently from monocytes, peripheral T lymphocytes appear to respond to cobalt undergoing apoptosis. Conversely, beryllium appears to act as death inducer for monocytes while activating T lymphocytes, which respond secreting IFN- $\gamma$  but not inducing NF- $\kappa$ B translocation (Lombardi *et al.*, 2001 and this paper). Interestingly, the lymphocytes derive from healthy donors; yet, they respond to beryllium inducing an IFN- $\gamma$ -driven inflammatory response, suggesting that beryllium salts might act as mitogenic for at least some populations of T cells and, therefore, bypassing the monocyte/DC signals. In conclusion, although the two metals induce lung diseases, which share some common ground, the molecular mechanisms underlying the two pathologies might be mediated by different cell types.

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