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## Divergent effect of Cobalt and Beryllium salts on the fate of peripheral blood monocytes and T lymphocytes.

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5 **Divergent effect of Cobalt and Beryllium salts on the fate of peripheral blood**  
6 **monocytes and T lymphocytes.**  
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## Abstract

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 respond instead producing IFN- $\gamma$ ; conversely CoCl<sub>2</sub> induces apoptosis in T lymphocytes but not in monocytes. Interestingly, both metals induce p53 over-expression, 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 NF- $\kappa$ B and the inflammasome danger signalling pathway leading to the production of pro-inflammatory cytokines. However, CoCl<sub>2</sub>-treated monocytes do not fully differentiate into macrophage or DC, 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, pro-inflammatory state that can contribute to some of the pathologies associated with the exposure to this metal.

**Keywords:** monocytes, lymphocytes, metal salts, lung, inflammation, inflammasome.

## Introduction

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, since 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, while CBD is a multisystem granulomatous disease that primarily affects the lungs by the accumulation of Beryllium-specific CD4<sup>+</sup> major histocompatibility complex (MHC) 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 (Ohori et al., 1989). Moreover, Cobalt is a well known skin sensitizer, causing allergic contact dermatitis, and it can also cause hypersensitivity and

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5 occupational asthma, but there is no convincing evidence for a cell mediated  
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7 immunologic reaction against Cobalt in HMLD (Lison, 1996; Nemery et al.,  
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9 2001). Therefore it appeared interesting to investigate whether the two metals  
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11 have different effects on cells of the immune system such as T lymphocytes and  
12  
13 monocytes. In this paper, we compared the effect of Beryllium and Cobalt on  
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15 peripheral blood mononuclear cells (PBMC) and on purified T lymphocytes or  
16  
17 monocytes subpopulations. The results show that the two metals have strikingly  
18  
19 different effects on monocytes and T cells since Be induces cell death in  
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21 monocytes while leaving T cells alive whereas Cobalt activates monocytes while  
22  
23 causing T cell apoptosis. Interestingly, monocytes, cultured in the presence of  
24  
25 Cobalt, show increased level of p53 and cytoplasmic p21 and produce NF-kB-  
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27 mediated pro-inflammatory cytokines while reducing their capacity to present  
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29 antigens, suggesting that they might contribute to the establishment of a chronic  
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31 inflammation.  
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## Materials and Methods

### Reagents and antibodies

Apoptosis detection Kit and “DuoSet ELISA Development Kit” for cytokines detections in cell culture supernatants were purchased from *R&D Systems (Minneapolis, MN)*. Tetramethylbenzidine and FITC-dextran were purchased from *Sigma Aldrich (St.Louis, MO)*. Monoclonal antibodies 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 peripheral blood mononuclear cells (PBMC) were separated on Lympholyte (*Cedarlane Laboratories*) by density centrifugation of heparinized blood from healthy donors. Monocytes and lymphocytes-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). Cells 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/ml and were suspended in RPMI 1640 supplemented with 10% heat-inactivated foetal calf

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5 serum, 2 mM L-glutamine, 25 U/ml penicillin and 25 U/ml streptomycin (all  
6 purchased from *GIBCO*) in cell culture plates at 37°C in a humidified 7% CO<sub>2</sub>  
7 incubator. 2/3 of the medium were replaced every three days with complete fresh  
8 medium. CoCl<sub>2</sub> and BeSO<sub>4</sub> were adjusted at final concentrations of 84µM CoCl<sub>2</sub>  
9 ·6 H<sub>2</sub>O and 113µM BeSO<sub>4</sub> · 4 H<sub>2</sub>O.

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17 Dendritic Cells (DC) were generated from monocytes isolated using Monocyte  
18 Isolation kit (*Miltenyi Biotec.*). Cells were cultured for 6 days in complete  
19 medium (RPMI 1640 supplemented with 10% heat-inactivated foetal calf serum,  
20 2 mM L-glutamine, 25 U/ml penicillin and 25 U/ml streptomycin) in the presence  
21 of 200U/ml rhGM-CSF and 500 U/ml rhIL-4 (*R&D Systems*).

### 22 23 24 25 26 27 28 29 30 **HLA-DP-Glu69 typing**

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32 Genotyping for the supratypic marker HLA-DPB1<sup>Glu69</sup> of all donors was  
33 performed as previously described (McCanlies et al., 2004) by RFLP (BsrBI)  
34 analysis.  
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### 40 41 **Western blot**

42  
43 For detection of p53, p65 and caspase-1 proteins, western blot analysis was  
44 carried out. After indicated times of culture, monocytes and lymphocytes  
45 untreated or treated with CoCl<sub>2</sub> and BeSO<sub>4</sub> were washed twice with cold PBS and  
46 lysed using RIPA buffer (50 mM Tris-HCl pH: 7.4, 1% Nonidet P40, 0.5%  
47 Sodium deoxycholate, 0.1% Sodium Dodecyl Sulfate [SDS], 1 mM EDTA, 1 mM  
48 EGTA, 0.5 mM NaVO<sub>4</sub>, 50 mM NaF, 1 mM PMSF and protease inhibitor  
49 cocktail, *Roche Hertfordshire, UK*). Cells debris was removed by centrifugation at  
50 20000g for 15 min at 4°C. Protein concentration in cell lysates was determined  
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5 using a Biorad protein assay kit (*Biorad, Hercules, CA*) with bovine serum  
6 albumin used as standard. Cell lysate was mixed with an equal volume of 2x  
7 electrophoresis sample buffer (125mM Tris-HCl, pH 6.8, 4% w/v SDS, 20%  
8 glycerol, 100 mM DTT, 0.02% bromophenol blue) and boiled for 5 minutes.  
9  
10 Twenty-forty  $\mu$ g of total proteins for each sample was separated by 12% SDS-  
11 polyacrilamide gel electrophoresis and subsequently transferred to Hyband  
12 enhanced chemiluminescence (ECL) nitrocellulose membrane (*Amersham*  
13 *Pharmacia Biotech*). After blockade of not-specific binding sites by treatment  
14 with not-fat milk in TBST (Tris buffer saline 0.1% Tween 20) for 1 hour, the  
15 membranes were incubated overnight at 4°C with the primary antibody (anti  
16 human p53, DO-1 *Santa Cruz*;  $\beta$  actin, *Sigma*; anti human caspase-1, anti NF- $\kappa$ B  
17 p65 and phospho-NF- $\kappa$ B p65 (Ser276) antibody; *Cell Signalling*). After three  
18 washes in TBST, membranes were incubated with horseradish peroxidase-  
19 conjugated secondary Ab (*Jackson Immunoresearch Laboratories, Inc. West*  
20 *Grove, PA*), for 1 hour at room temperature and then washed twice in TBST,  
21 followed up by ECL western blotting detection system (*Amersham*) and  
22 exposition to Kodak X-Omat film. The intensity of bands was quantified by  
23 densitometric analysis using the program UN-SCAN-IT gel (*Silk Scientific Inc.,*  
24 *Orem, UT, USA*). Results were evaluated as the ratio of intensity between relevant  
25 protein and  $\beta$  actin from the same sample.  
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28  
29 For detection of activated NF- $\kappa$ B, p21<sup>Cip1/WAF1</sup> and procaspase-3, cell fractionation  
30 was carried out as previously described (Asada et al., 1999). Both the cytoplasmic  
31 and nuclear fractions were subjected to Western blotting. The antibodies used in  
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5 this study were anti-p21 (F-5, *Santa Cruz*) anti-caspase-3 (CPP32, *R&D Systems*)  
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7 and anti NF- $\kappa$ B p65 (*Cell Signalling*). Fractionation efficiency was assessed by  
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9 antibodies against  $\alpha$ -tubulin (B-5-1-2; *Sigma*) and Lamin B1 (ab16048, *Abcam*).  
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### 12 13 **RNA interference treatment**

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15 Monocytes were plated in six-well plates. Cells were stimulated with  $\text{CoCl}_2$  and at  
16  
17 once transfected with p21 small interfering RNA (siRNA) (*SignalSilence; Cell*  
18  
19 *Signalling*) or scrambled control (*Dharmacon, Worcester MA, D-001206-13*).  
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21 siRNAp21 RNA interference was performed at 100 nM. Cells were transfected  
22  
23 using Hiperfect transfection reagent (*Qiagen Inc., Valencia, CA*) according to the  
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25 manufacturer's instructions. The transfection efficiency reached the maximum  
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27 18–24h post transfection as assessed by western blot analysis of p21.  
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### 33 34 **Cell death assay**

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36 Cells were treated with 84  $\mu\text{M}$   $\text{CoCl}_2$  or 113  $\mu\text{M}$   $\text{BeSO}_4$  and the percentage of  
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38 dead, apoptotic and viable cells was then assessed by Annexin-V binding using an  
39  
40 apoptosis detection kit (*R&D Systems*). Expression of phosphatidylserine in the  
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42 external layer of the plasma membrane was determined by studying the fixation  
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44 of fluorescein isothiocyanate (FITC, green fluorescence) Annexin V in the  
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46 presence of  $\text{Ca}^{2+}$  in cells that remain not permeate to Propidium Iodide (PI).  
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48 Briefly, at indicated time points, cells were collected, washed twice with cold PBS  
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50 and then resuspended in binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM  
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52 NaCl, 2.5 mM  $\text{CaCl}_2$ ) at a concentration of  $1 \times 10^6$  cells/ml with saturating  
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54 concentrations of FITC-Annexin V and PI for 15 min at room temperature, in the  
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56 dark, according to the manufacturer's instructions. The labelled cells were  
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5 analysed immediately using a FACScan flow cytometer. Apoptotic cells were  
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7 defined as those with green fluorescence > 98% of unlabeled cells.  
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### 10 **Cytokine assays and statistical analysis**

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12 48 h after stimulation with CoCl<sub>2</sub>, the culture media of monocytes were harvested  
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14 and stored at -80°C until determination. TNF- $\alpha$ , IL1- $\beta$  and MIF were measured  
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16 in supernatants of samples by specific enzyme-linked immunosorbent assays  
17  
18 (sandwich ELISAs) according to manufacturer's instruction (*R&D Systems*). All  
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20 samples were diluted 1:1 in reagent diluent (1% BSA in PBS, pH 7.2, 0.2  $\mu$ m  
21  
22 filtered) and assayed in triplicate. Results were expressed as the means  $\pm$  SD. The  
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24 limits of detection were determined using standard curves with standard cytokine  
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26 preparations (defined concentrations of recombinant cytokine) used as internal  
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28 controls in all test. Optimised standard curve range (pg/ml) was set as  
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30 recommended: for TNF- $\alpha$  , 31.25-1000; for IL1- $\beta$ , 18.75-300 and for MIF, 62.5-  
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32 2000. The comparison among data was achieved by Mann-Whitney U test.  
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### 41 **Intracellular IFN- $\gamma$ staining in lymphocytes**

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43 Intracellular IFN- $\gamma$  staining was performed with FITC labeled monoclonal  
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45 antibody (Clone 25723.11, *BD Biosciences*). After 48h by stimulation,  
46  
47 lymphocytes were washed with 0.1% BSA/PBS and subsequently resuspended for  
48  
49 4h in complete culture medium in the presence of the of the protein transport  
50  
51 inhibitor GolgiPlug containing brefeldin A (*BD*). The cells were washed with  
52  
53 PBS/1% FBS and fixed using Cytofix/Cytoperm Kit (*BD*) according the  
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55 manufacturing datasheet. Cells were washed, resuspended in 1X Perm Wash (*BD*)  
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5 for 15 min at 4°C and stained with FITC-conjugated anti-IFN- $\gamma$  diluted in Perm  
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7 Wash for 30 min at 4°C. The cells were washed twice with Perm Wash and one  
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9 time with PBS and resuspended in PBS for flow cytometry analysis. The mitogen  
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11 phytohemagglutinin (PHA, *Gibco-BRL, MD, USA*) was used as a positive control.  
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### 15 16 **Quantitative IL-1 $\beta$ PCR analysis**

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18 Total RNA was isolated from cell pellets by TRIzol reagent (*Invitrogen,*  
19  
20 *Carlsbad, CA, USA*) as recommended. After denaturation of freshly prepared  
21  
22 RNAs (2  $\mu$ g approximately) at 65 °C for 5 min, SuperScript III Reverse  
23  
24 Transcriptase (*Invitrogen, Paisley, UK*) was used for oligo-dT-primed first-strand  
25  
26 cDNA synthesis. For measurement of the relative level of expression of IL-1 $\beta$  in  
27  
28 untreated and LPS or CoCl<sub>2</sub> stimulated monocytes, real-time RT-PCR was  
29  
30 performed using the 7300 Real-Time PCR System (*Applied Biosystems, ABI*  
31  
32 *Carlsbad, CA*). Reactions were performed in a Micro-Amp Optical 96-well  
33  
34 reaction plates (*ABI*) using 2.5- $\mu$ l cDNA of treated or untreated monocytes, in  
35  
36 triplicate wells, 12.5  $\mu$ l of 2x Master Mix (*ABI*), 1.25  $\mu$ l of validated Assay on  
37  
38 Demand gene expression (*ABI, Hs01555413*). The PCR was performed using the  
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40 following amplification scheme: one cycle of 10 min at 95 °C followed by 60  
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42 cycles of denaturation for 15 s at 95 °C and an annealing/extension step of 1 min  
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44 at 60 °C, in a final volume of 25  $\mu$ l. All reactions were conducted using a 7300  
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46 Sequence Detector thermocycler linked to a PC using the Sequence Detector  
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48 software (*ABI*). Glyceraldehyde-3-phosphate dehydrogenase Assay on Demand  
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50 (*ABI, Hs99999905*) was used to ensure quality of RNA preparation, to account for  
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5 efficiency of the reverse transcription and to check for any loading variation of the  
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7 initial cDNA amount.  
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### 10 **Immunophenotypic characterization of monocytes by flow cytometry**

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12 For immunostaining, at least  $5 \times 10^4$  cells were washed twice in PBS containing  
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14 1% bovine serum albumin (BSA; *Sigma*) and incubated 1h at 4°C with saturating  
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16 concentrations of fluorescein isothiocyanate conjugated (FITC) monoclonal  
17  
18 antibodies (mAb). Afterwards samples were washed, resuspended in 200 µl 1%  
19  
20 BSA/PBS and immediately analysed in a Becton-Dickinson FACScan flow  
21  
22 cytometer for a total of 10000 events. Expression on surface molecules was  
23  
24 reported as percentage and mean fluorescence intensity (MFI). Monocytes were  
25  
26 electronically gated according to light scatter properties to exclude cell debris and  
27  
28 contaminating lymphocytes. Dead cells were gated out after staining with 5 µg/ml  
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30 propidium iodide solution. Flow cytometry data were processed using Cell Quest  
31  
32 software (*Becton-Dickinson*).  
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### 40 **Evaluation of antigen uptake**

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42 The phagocytic activity of monocytes was analysed by flow cytometric analysis.  
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44 Mannose receptor-mediated endocytosis was measured as the cellular uptake of  
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46 FITC-dextran (*Sigma*). Briefly,  $10^5$  cells were resuspended in 200 µl of 10% FCS  
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48 containing RPMI 1640 medium buffered with 25 mM Hepes. Cells were  
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50 incubated with FITC-dextran (0.5 mg/ml) for 2h at 37°C. The incubation was  
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52 stopped by adding 2 ml of cold phosphate-buffered saline. Cells were washed  
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twice with PBS 0.1% sodium azide and analyzed with FACS flow cytometer. 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+ cell isolation kit (*Oxoid*) containing anti-CD14 microbeads. Enriched T cells consisted of more than 95% CD4+ cells were co-cultured at  $4 \times 10^5$  cells/well in round bottomed 96-well culture plates with different amounts of monocytes or DC, 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 pre-treated 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  $\mu$ Ci/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

### 1. Cobalt and Beryllium have opposite effects on monocytes and T lymphocytes viability.

PBMC obtained from blood samples of 18 healthy donors were exposed to escalating doses of either  $\text{CoCl}_2$  or  $\text{BeSO}_4$  and analysed for cell survival at different days of culture (Fig.1). Panel A shows that  $\text{CoCl}_2$ -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  $\text{CoCl}_2$  (336  $\mu\text{M}$ ) was used. Conversely, the exposure to  $\text{BeSO}_4$  (Fig.1B) had a dramatic effect on monocyte survival, already evident after two days of culture at the lowest concentration (56.5  $\mu\text{M}$ ) while not interfering with lymphocytes survival even at four times higher doses (904  $\mu\text{M}$ ). PBMC kept in culture in the absence of metals for the times indicated are plotted in Fig.1C. Fig. 1D reports the histograms plot related to the phenotypic analysis of CD14 expression. On the basis of the dose-response curves the intermediate concentrations of 84  $\mu\text{M}$  for  $\text{CoCl}_2$  highly toxic for T lymphocytes but not affecting monocytes survival and of 113  $\mu\text{M}$  for  $\text{BeSO}_4$  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 behaviour represented in Fig.1 while, in the remaining subjects, monocytes in the presence of Cobalt underwent cell death after two days of culture. We therefore

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5 hypothesized that the presence of Glu69 in the HLA-DP beta chain could make  
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7 the difference. However, this was not the case since the analysis of the allelic  
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9 polymorphism (Glu69) did not reveal any association with the monocyte fate  
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11 (data not shown).  
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## 13 14 15 16 **2. Cobalt induces p53 increase in both monocyte and lymphocyte** 17 18 **populations.** 19

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21 To further investigate the effect of  $\text{CoCl}_2$  and  $\text{BeSO}_4$  on the survival and  
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23 functional status of the monocytes, these cells isolated by negative depletion were  
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25 cultured for 2 days in the presence or absence of metals. Monocytes incubated  
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27 with  $\text{CoCl}_2$  remained alive like the untreated cells, with a viability that was always  
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29 more than 50% (Fig.2A-B). In contrast, Beryllium was toxic for monocytes whose  
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31 number was dramatically reduced already after 2 days of culture. The monocytes  
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33 viability observed in presence of Cobalt was confirmed in 10 subjects and proven  
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35 to be highly reproducible as shown in Fig.2C.  
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41 Since it has been reported that Cobalt may have a genotoxic effect, we addressed  
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43 the question whether cell death seen in lymphocytes was correlated to an  
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45 increased p53 expression. Accordingly, the expression of p53 was investigated in  
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47  $\text{CD3}^+$  T lymphocytes and monocytes after exposure to  $\text{CoCl}_2$  or  $\text{BeSO}_4$ . T cells  
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49 exposed to X-rays and lysed after 24 hours were used as positive controls. After  
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51 16 hours, the expression of p53 was clearly detectable in  $\text{CoCl}_2$ -treated  
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53 lymphocytes but not in those treated with  $\text{BeSO}_4$  (Fig.3A and B). In monocytes  
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55 both  $\text{CoCl}_2$  and  $\text{BeSO}_4$  salts induced p53 up-regulation (Fig.3C and D) with a  
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5 more pronounced effect by the former. After 48h only CoCl<sub>2</sub>-treated monocytes  
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7 were still alive and maintained a high expression of p53. Therefore, monocytes  
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9 survival and death in the presence of Cobalt or Beryllium respectively appear to  
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11 be mediated by different mechanisms both involving p53. This observation is in  
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13 agreement with experimental models showing that wild-type p53 induced cell  
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15 differentiation and, in particular, was found to enhance macrophage  
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17 differentiation (Matas et al., 2004; Navratilova et al., 2007).  
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### 22 23 **3. Cobalt-induced monocytes show cytoplasmic p21<sup>Cip1/WAF1</sup> localization** 24 25 **correlating with the stabilization of procaspase-3.** 26 27

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29 To further investigate the effect of CoCl<sub>2</sub> in monocytes, we examined the  
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31 physiology and morphology of the surviving cells. p21(Waf1/Cip1) is the primary  
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33 mediator of cell cycle arrest in response to DNA damage, to different forms of  
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35 stress and is implicated in senescence and terminal differentiation. The  
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37 differentiation of immature monocytes is associated with a relocalization of  
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39 nuclear p21 to the cytoplasm (Asada et al., 1999). The anti-apoptotic functions of  
40  
41 p21 may be attributed to several of its molecular interactions including the  
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43 binding to procaspase-3 and inhibition of its conversion to mature form (Dotto,  
44  
45 2000). Expression of cytoplasmic p21 concurrent to increased level of procaspase-  
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47 3 in CoCl<sub>2</sub> treated monocytes was demonstrated by Western blot analysis of  
48  
49 cytoplasmic extracts (Fig. 4A). To determine whether p21 expression regulates  
50  
51 procaspase-3 activation in CoCl<sub>2</sub>-treated monocytes, cells were at once transfected  
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53 with p21 small interfering RNA (siRNA) or scrambled control siRNA and  
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55 samples were collected for Western blotting and flow cytometry. The highest  
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5 level of efficiency was reached 16–24 h after transfection. Consistent with a role  
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7 for p21 in regulating procaspase-3 amount, siRNA knockdown of p21 during  
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9 CoCl<sub>2</sub> treatment restored inactive caspase-3 level, whereas scrambled control did  
10  
11 not (Fig. 4B). Quantification of procaspase-3 by western blots obtained from three  
12  
13 separate experiments confirmed this result. In addition, treatment with p21 siRNA  
14  
15 but not control siRNA resulted in increased cell death (data not shown). These  
16  
17 findings support a role for p21-dependent antiapoptotic effect on survival of  
18  
19 monocytes treated with CoCl<sub>2</sub>. Attempts to detect p21 in lymphocytes either in the  
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21 nuclei or in the cytoplasm were unsuccessful (data not shown).  
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#### 27 **4. Cobalt activates NF-κB in monocytes and increases their production of** 28 **pro-inflammatory cytokines.** 29 30 31

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33 Since fully differentiated macrophages, in contrast to monocytes, contain  
34  
35 significant levels of nuclear factor κB (NF-κB) into nuclei and its activation is  
36  
37 required for monocyte differentiation (Pennington et al., 2001), we asked whether  
38  
39 NF-κB was involved in the survival of the Cobalt-treated monocytes. It has been  
40  
41 shown that phosphorylation at Ser276 is critical for p65 NF-κB activation and  
42  
43 regulates the interaction with co-activators p300/CBP (Okazaki et al., 2003).  
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45 Therefore the phosphorylation status of Ser276 in the p65 subunit of NF-κB was  
46  
47 assessed by immunoblotting with a phospho-specific anti-p65 antibody.  
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49 Monocytes treated with Cobalt but not with Beryllium, showed a significant  
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51 increase of phospho-p65 protein (Fig.5).  
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5 Next, we analysed whether the presence of Cobalt had any effect on the  
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7 production of pro-inflammatory cytokines such as TNF- $\alpha$  and IL-1 $\beta$ . The  
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9 supernatants of monocytes were analysed for the presence of these cytokines by  
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11 ELISA (Fig.6) which revealed a clear trend towards an increased production of  
12  
13 TNF- $\alpha$  as well as IL-1 $\beta$  in CoCl<sub>2</sub>-treated compared to untreated monocytes  
14  
15 (Mann-Whitney test:  $p < 0.001$  and  $p < 0.003$  for TNF- $\alpha$  and IL-1 $\beta$ , respectively).  
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17  
18 Since Macrophage Migration Inhibitory factor (MIF) has emerged to be an  
19  
20 important effector molecule of the innate immune system and unlike most  
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22 cytokines is constitutively produced by immune cells (Lue et al., 2002), its  
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24 presence in the supernatants was also analysed. Differently from TNF- $\alpha$  and IL-  
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26 1 $\beta$ , there was no significant correlation between the production of MIF and the  
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28 presence of Cobalt. Notably, some of the donors who produced low amount of  
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30 pro-inflammatory cytokines did secrete MIF.  
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### 38 **5. Cobalt enhances IL-1 $\beta$ expression acting both at transcriptional and at** 39 40 **protein processing level**

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43 The expression of IL-1 $\beta$  is tightly regulated, in that distinct signals are required  
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45 for its transcription and release (Mariathasan and Monack, 2007). The first signal  
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47 can be transmitted via stimulation of Toll-like receptor signalling pathway,  
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49 leading to the activation of NF- $\kappa$ B and the expression of the 35 kDa precursor  
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51 form of IL-1 $\beta$ . A further signal is required to switch on the inflammasome  
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53 complex, leading to the activation of caspase-1 and subsequent processing and  
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55 secretion of the mature cytokine. The amount of IL-1 $\beta$  mRNA in purified  
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5 monocytes from three subjects stimulated with LPS or CoCl<sub>2</sub> for 1, 12 and 24  
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7 hours, was examined by quantitative relative RT-PCR (Fig.7). After CoCl<sub>2</sub>  
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9 exposure, monocytes were still producing a reasonable high level of IL-1β mRNA  
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11 up to 24 hours of culture while the untreated monocytes did not.  
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15 We next tested the caspase-1 activation that is required for IL-1β release. As  
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17 other caspases, caspase-1 undergoes proteolytic activation to produce a tetramer  
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19 of its two active subunits, p20 and p10 (Lamkanfi et al., 2007). To verify the  
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21 status of caspase-1, extracts were prepared from untreated monocytes and those  
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23 stimulated with either CoCl<sub>2</sub> or LPS (0,05μg/ml). The extracts were then  
24  
25 immunoblotted with an antibody that detects pro-caspase-1 (α, β, γ and δ  
26  
27 isoforms) and caspase-1 p20 subunits. Stimulation of monocytes with LPS or  
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29 CoCl<sub>2</sub> induced the processing of pro-caspase-1 into the mature p20 subunit after 4  
30  
31 hours and 12 hours, respectively (Fig.8). Interestingly, however, freshly isolated  
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33 human blood monocytes, kept in culture for the time indicated, also became able  
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35 to produce detectable amount of mature p20 subunit, probably as consequence of  
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37 the differentiation program triggered by plastic adherence (Fig.8, line 5).  
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#### 44 **6. BeSO<sub>4</sub>-treated T lymphocytes produce IFN-γ.**

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47 Untreated, CoCl<sub>2</sub>- or BeSO<sub>4</sub>- stimulated lymphocytes were collected after 48  
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49 hours and stained intracellularly with an antibody specific for IFN-γ. The mitogen  
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51 phytohemagglutinin (PHA) was used as a positive control. The number of IFN-γ  
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53 positive T cells detected by flow cytometry were low in both untreated and CoCl<sub>2</sub>-  
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55 treated lymphocytes; on the contrary, in the presence of BeSO<sub>4</sub>, the number  
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5 increased up close to that of PHA-stimulated cells (Fig.9A). We therefore asked  
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7 whether NF-kB was activated by the two salts. Because the level of phospho-Ser  
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9 276 p65 protein in whole extracts was undetectable in Beryllium-treated  
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11 lymphocytes (not shown), we analyzed directly the nuclear translocation of the  
12  
13 protein. Despite the comparable production of IFN- $\gamma$ , translocation of NF-kB in  
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15 BeSO<sub>4</sub> stimulated lymphocytes appears negligible compared to PHA-treated T  
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17 cells (Fig.9B).  
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### 23 **7. CoCl<sub>2</sub>-treated monocytes show a reduced expression of HLA-class II and** 24 25 **an impaired antigen presentation capability after 6 days of culture.** 26

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28 After 6 days of culture in the presence of Cobalt, more than 60% of cells were still  
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30 alive compared to less than 30% of untreated monocytes (Fig.10A,B) and 0% of  
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32 Beryllium-treated monocytes (data not shown). Fig.10C reports the comparison of  
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34 alive, apoptotic and dead cells performed on monocytes derived from 10  
35  
36 individuals. Lymphocytes treated for six days with the same doses of CoCl<sub>2</sub>  
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38 showed a sharp reduction in survival compared to Beryllium-treated or untreated  
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40 lymphocytes (Fig.10D,F).  
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45 Once established that Cobalt was not toxic for monocytes but rather acting as a  
46  
47 survival factor and a trigger for pro-inflammatory cytokines production, we  
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49 investigated the phenotype of Co-treated monocytes, analysing the expression of  
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51 CD14, CD16, CD11b, CD83, CD86 and HLA-class I and -class II at 6 days of  
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53 culture. The cells expressed CD14, CD11b molecules, but remained negative for  
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55 CD16 and CD83 suggesting that they did not differentiate into either macrophages  
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5 or DC, respectively (Fig.11A). The expression of HLA-class I was slightly  
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7 decreased whereas that of HLA-class II was sharply reduced after 6 days.  
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10 We then asked whether Cobalt-treated monocytes were functionally competent in  
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12 antigen capturing and presentation compared to untreated cells. As shown in  
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14 Fig.11B,  $\text{CoCl}_2$ -treated monocytes have reduced capacity to uptake fluorescent  
15  
16 dextran compared to untreated monocytes. Furthermore, likewise untreated  
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18 monocytes, they were, compared to LPS-matured DC from the same subjects,  
19  
20 poorly efficient in stimulating T cells in a mixed lymphocyte reaction (MLR)  
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22 using allogeneic  $\text{CD4}^+$  T lymphocytes as responder cells, (Fig.11C).  
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## Discussion

Environmental exposure to inhaled dust containing Cobalt particles can cause HMLD that is associated with immunological sensitisation, 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 Reactive Oxygen Species (Kasprzak, 2002) and Reactive Nitrogen Species (RNS) generation (Rengasamy et al., 1999). An important hallmark of HMLD is the giant cell interstitial pneumonitis (GIP) and the presence of multinucleate giant cells in the bronchoalveolar lavage, underlying the occurrence of an inflammatory process 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  $\text{CoCl}_2$ , 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 pro-inflammatory cytokines. This apoptosis-resistant phenotype appears concomitantly with the expression of cytoplasmic  $\text{p21}^{\text{Cip1/WAF1}}$  that induces stabilization of procaspase-3. It is known that p21 can protect against apoptosis in response to growth factor deprivation, p53 over-expression or during the

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5 differentiation of monocytes and it is also been shown to stimulate NF-kB  
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7 mediated transcription. This effect is due to the activation of transcriptional  
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9 cofactors/histone deacetylases p300 and CBP (Abbas and Dutta, 2009). Serine  
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11 276 (Ser276)-phosphorylated RelA binding is seen primarily on a subset of genes  
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13 that are rapidly induced by tumor necrosis factor (TNF), including Gro- $\beta$ ,  
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15 interleukin-8 (IL-8), and I $\kappa$ B $\alpha$ . Recent work has shown that TNF-inducible RelA  
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17 Ser276 phosphorylation is controlled by a reactive oxygen species (ROS)-protein  
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19 kinase A signaling pathway (Novak et al., 2008). Previous investigations  
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21 demonstrated that IKK $\beta$ , the major catalytic subunit of IKK complex, could  
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23 increase p21 expression through upregulation of p21 mRNA level and via  
24  
25 activation of its downstream target Akt which is known to phosphorylate p21 and  
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27 lead to cytoplasmic localization of p21 (Ping et al., 2006). However, although it is  
28  
29 well established that NF-kB is a survival factor activated during monocyte  
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31 differentiation (Pennington et al., 2001), we have not observed a complete  
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33 differentiation of Cobalt-treated monocytes to macrophages or dendritic cells in  
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35 that they remained both CD16 and CD83 negative. These cells did not proliferate  
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37 as shown by the lack of [<sup>3</sup>H]-thymidine incorporation and permanence in the G0  
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39 phase of cell cycle (not shown). Interestingly, T lymphocytes, under the same  
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41 circumstances, underwent a high degree of apoptosis. Strikingly different was the  
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43 fate of cells exposed to Beryllium. This metal is highly toxic for peripheral  
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45 monocytes (this paper) as well as for macrophages (Sawyer et al., 2005), but not  
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47 for T lymphocytes. It has been reported that Cobalt ions are potent inducers of  
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49 hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) (Sandau et al., 2000). HIF-1 $\alpha$  is activated  
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5 during macrophage differentiation and it is an important intracellular regulator of  
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7 numerous genes involved in glucose metabolism, angiogenesis, cell survival,  
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9 proliferation (Oda et al., 2006) and apoptosis inducing accumulation of p53  
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11 through its direct association with HIF-1 $\alpha$  (Won et al., 1998) and consequent cell  
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13 death by apoptosis. This seems to be true for lymphocytes but not for monocytes,  
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15 probably because monocytes are resting cells and therefore less susceptible to the  
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17 effect of genotoxic damage by Cobalt (De Boeck et al., 1998). It is reasonable that  
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19 the high level of p53 in Cobalt-pulsed monocytes plays a role as mediator of NF-  
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21 kB-induced macrophage differentiation (Mudipalli et al., 2001). What is  
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23 intriguing is the opposite effect of Beryllium whose presence is highly toxic for  
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25 monocytes but not for lymphocytes. We do not know what causes this reaction,  
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27 but we observed a detectable increase in p53 expression also in BeSO<sub>4</sub>-treated  
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29 monocytes, suggesting that cell death could be mediated by other mechanisms in  
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31 which there is not the activation of the survival factor NF-kB and cytoplasmic p21  
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33 localization. We show here, in agreement with other reports (Kaufman et al.,  
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35 2008) that, monocytes responding to Cobalt, produce an increased amount of pro-  
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37 inflammatory cytokines such as TNF- $\alpha$  and IL-1 $\beta$ . The observed IL-1 $\beta$   
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39 transcriptional induction could occur via a HIF-1-dependent mechanism during  
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41 hypoxia and be maintained through a NF-kB dependent amplification by autocrine  
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43 IL-1 $\beta$  (Zhang et al., 2006). In this work we have shown that Cobalt induces not  
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45 only the synthesis but also the processing and secretion of IL-1 $\beta$  by activation of  
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47 caspase-1 in human monocytes. Our observation is in agreement with recent  
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49 studies outlining the importance of the inflammasome in activating the danger  
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5 signalling pathway triggered by ROS generated by NADPH oxidase following  
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7 particle phagocytosis (Caicedo et al., 2009; Cassel et al., 2008; Dostert et al.,  
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9 2008; Hewinson et al., 2008; Netea et al., 2009). However, Cobalt-treated  
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11 monocytes appeared to lose their antigen-processing/presenting properties  
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13 displaying a reduced efficiency to capture the antigen as well as to stimulate the  
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15 allo-reactive T cell response. Therefore, Cobalt acts as an activation factor for  
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17 monocytes which can exert their pro-inflammatory effect for long time eventually  
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19 in sites where Cobalt can reach a threshold concentration (i.e. lung in case of  
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21 Cobalt inhalation). Differently from monocytes, peripheral T lymphocytes appear  
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23 to respond to cobalt undergoing apoptosis. Conversely, Beryllium appears to act  
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25 as death-inducer for monocytes while activating T lymphocytes which respond  
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27 secreting IFN- $\gamma$  but not inducing NF $\kappa$ -B translocation (Lombardi et al, 2001 and  
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29 this paper). Interestingly, the lymphocytes derive from healthy donors, yet they  
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31 respond to Beryllium inducing an IFN- $\gamma$ -driven inflammatory response suggesting  
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33 that Beryllium salts might act as mitogenic for at least some populations of T cells  
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35 and, therefore, by-passing the monocyte/DC signals. In conclusion, although the  
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37 two metals induce lung diseases which share some common ground, the molecular  
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39 mechanisms underlying the two pathologies might be mediated by different cell  
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41 types.  
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**Figure Legends.****Fig.1 Effects of Cobalt and Beryllium on human PBMC in cytofluorimetric analysis: kinetics and dose-effect relationships.**

Representative dot plots (SSC vs FSC) of PBMC treated with different concentrations of (A)  $\text{CoCl}_2 \cdot 6 \text{H}_2\text{O}$  (42  $\mu\text{M}$  to 336  $\mu\text{M}$ ) or (B)  $\text{BeSO}_4 \cdot 4 \text{H}_2\text{O}$  (56.5  $\mu\text{M}$  to 904  $\mu\text{M}$ ) or untreated (C) for six days. PBMC from 18 donors were analysed every two days (day 2, day 4 and day 6). Out of 18 donors studied, 14 demonstrated the same behaviour. R1 and R2 indicate monocytes and lymphocytes subpopulations, respectively, as defined by cell size (FSC), granularity/cell density (SSC) and phenotypic analysis of CD14 expression (histograms plot in (D) panel). Debris and dead cells corresponding to R3 gate were excluded in CD14 analysis by FSC/SSC parameters and Propidium Iodide labelling.

**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 hours with 84  $\mu\text{M}$   $\text{CoCl}_2 \cdot 6 \text{H}_2\text{O}$  or 113  $\mu\text{M}$   $\text{BeSO}_4 \cdot 4 \text{H}_2\text{O}$  or untreated (right side) were incubated at 37 °C in 7%  $\text{CO}_2$  humidified atmosphere with RPMI 10 % FCS. Cell viability was determined by Annexin V binding and flow cytofluorimetric analysis. Density plots: (A) SSC vs. FSC; monocytes were

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5 gated on SSC/FSC density plot excluding debris (R1) (B) Propidium Iodide (PI)  
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7 vs. Annexin-V staining: values in quadrants represents the percentage of apoptotic  
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9 (lower right) and dead cells (upper right) from a single experiment representative  
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11 of triplicate experiments with cells from ten different donors. (C) Percentages of  
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13 alive, apoptotic and dead monocytes respectively for each subject analysed.  
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15 Horizontal bars represent median values. In the presence of BeSO<sub>4</sub> monocytes  
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17 viability was < 30 %, while in CoCl<sub>2</sub>-treated monocytes and in control monocytes  
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19 the percentages of viable cells were similar and > 60 %. Reduction of viable cells  
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21 in the presence of BeSO<sub>4</sub> was statistically significant (p <0.0001, not-parametric  
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23 Mann-Whitney U-test).  
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30 **Fig.3 CoCl<sub>2</sub> induces p53 expression in lymphocyte and monocyte populations.**

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32 Untreated, CoCl<sub>2</sub> and BeSO<sub>4</sub>-treated lymphocytes (A) and monocytes (B) were  
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34 cultured for 16 and 48 hours at 37 °C, 7 %CO<sub>2</sub> before lysis and assay by Western  
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36 Blot. An equal amount of proteins for each sample was separated by 12% SDS-  
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38 PAGE and subsequently transferred to a nitrocellulose membrane for ECL  
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40 detection. Reference (Ref) was T cells exposed to X-rays (5Gy) and lysed after  
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42 24h. Anti-β actin was used to ensure equal protein loading. p53 protein levels in  
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44 lymphocytes (C) and monocytes (D) were evaluated by densitometric analysis as  
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46 described in ‘Materials and Methods’. Values are the mean ± s.d. of three  
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48 independent experiments and are reported as percentage of 100% p53 expression  
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50 arbitrarily attributed to control cells.  
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57 **Fig.4 CoCl<sub>2</sub> induces cytoplasmic localization of p21 associated with**  
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59 **procaspase-3 stabilization in monocytes.**  
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5 (A) Untreated, CoCl<sub>2</sub> and BeSO<sub>4</sub> treated monocytes were cultured for 16 hours and  
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7 then were harvested for Western blot analysis. p21 expression was significantly  
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9 increased in the cytoplasmic CoCl<sub>2</sub> treated-monocytes but not in the nuclear  
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11 fractions, where the protein was undetectable (data not shown). Procaspase-3 level  
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13 was also obviously higher in CoCl<sub>2</sub> pulsed-monocytes than in untreated or BeSO<sub>4</sub>  
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15 stimulated cells. Activated caspase-3 was undetectable. (B) Consistent with a role  
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17 of p21 in regulating procaspase-3 amount, siRNA knockdown of p21 during  
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19 CoCl<sub>2</sub> treatment restored the undetectable level of inactive caspase-3 whereas  
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21 scrambled control (Sc) did not. Results are representative of three separated  
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23 experiments.  
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30 **Fig.5 CoCl<sub>2</sub> induces phosphorylation of serine 276 in the p65 NF-κB subunit**  
31  
32 **by monocytes.**

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34 Untreated, CoCl<sub>2</sub> and BeSO<sub>4</sub>-treated monocytes were cultured for 16 hour at 37  
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36 °C, 7 %CO<sub>2</sub>. (A) An equal amount of whole cell lysate for each sample was  
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38 separated by 12% SDS-PAGE, transferred to a nitrocellulose membrane and  
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40 subsequently immunoblotted with anti-phospho-p65 (S276P) (upper panel) and  
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42 anti-p65 (lower panel) antibodies. Monocytes exposed to 0.05 μg/ml of LPS and  
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44 lysed after 1h, were used as positive reference (Ref). (B) Phospho-p65 (S276P)  
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46 protein level in monocytes was evaluated by densitometric analysis as described  
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48 in 'Materials and Methods'. Values are the mean ± s.d. of three independent  
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50 experiments and are reported as percentage of 100% phospho-p65 expression  
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52 arbitrarily attributed to control cells.  
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**Fig.6 Production of TNF- $\alpha$ , IL1- $\beta$  and MIF by monocytes in presence of CoCl<sub>2</sub>.**

Cytokine levels (pg/ml) in harvested media from monocytes incubated in the absence (Unt) or in the presence of CoCl<sub>2</sub> (84  $\mu$ M), was measured by ELISA after 48 hours of culture. Data are mean of triplicate from culture supernatants of ten donors. Standard deviations 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 pro-inflammatory cytokines TNF- $\alpha$  and IL1- $\beta$  induced by CoCl<sub>2</sub> was statistically significant (p<0.001 for TNF- $\alpha$  and p<0.003 for IL1- $\beta$ , respectively, not-parametric 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 hours 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  $\pm$  s.d. of triplicate relative real-time quantification of 3 independent

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5 cultures. AU, Arbitrary Units. Student t-test p-values < 0.05 between the CoCl<sub>2</sub>  
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7 treated monocytes and untreated samples are indicated by \* and < 0.01 by \*\*.  
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18 **Fig.8 Cobalt induced caspase-1 activation by monocytes.**

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20 Untreated, CoCl<sub>2</sub> and LPS-treated monocytes were cultured for indicated times at  
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22 37 °C, 7 %CO<sub>2</sub>. (A) Lysates were separated by 12% SDS-PAGE, transferred to a  
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24 nitrocellulose membrane and subsequently immunoblotted with anti-caspase-1  
25  
26 antibody. The multiple bands are alternative spliced variants of caspase-1  
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28 identified by the antibody used, as reported by the producer. (B) p20 protein  
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30 levels were evaluated by densitometric analysis as described in 'Materials and  
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32 Methods'. Results are representative of two separated experiments.  
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38 **Fig.9 BeSO<sub>4</sub> –treated lymphocytes produce IFN-γ.**

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41 (A) Flow cytometric analysis of IFN-γ production by lymphocytes.  
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44 Alive lymphocytes were gated on FSC/SSC dot plot excluding debris  
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46 and aggregates (not shown). Analysis was then performed on IFN-γ  
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48 FITC vs SSC dot plot. Isotype-matched negative controls and region  
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50 statistics are reported. After 48h, Be-stimulated lymphocytes express  
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52 IFN-γ as much as PHA activated lymphocytes (control). A  
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59 representative of three independent experiments is shown. (B)  
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5 Western Blot analysis of nuclear NF- $\kappa$ B. Untreated, CoCl<sub>2</sub>-, BeSO<sub>4</sub>-  
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7 or PHA-treated lymphocytes were cultured for 16 hour at 37 °C, 7%  
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9 CO<sub>2</sub>. An equal amount of nuclear (N) and cytoplasmic (C) extracts for  
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11 each sample was separated by 12% SDS-PAGE, transferred to a  
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13 nitrocellulose membrane and subsequently immunoblotted with anti-  
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15 p65 antibody. Lymphocytes exposed to 0.05  $\mu$ g/ml of PHA were used  
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17 as positive reference (Ref). (C) Activated NF- $\kappa$ B was evaluated by  
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19 densitometric analysis as ratio between nuclear and cytoplasmic  
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21 fractions. Values are the mean  $\pm$  s.d. of three independent experiments  
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23 and are reported as percentage of PHA-activated control cells  
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25 arbitrarily attributed as 100%.  
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37 **Fig.10 Cell viability of monocytes and lymphocytes after 6 days of culture.**

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39 After 6 days of culture, the viability of CoCl<sub>2</sub>-treated and untreated monocytes  
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41 (A-C) and CoCl<sub>2</sub> or BeSO<sub>4</sub>-treated and untreated Lymphocytes (D-F) was  
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43 compared. Density plots: (A,D) SSC vs. FSC. Monocytes and lymphocytes were  
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45 gated on SSC/FSC density plot excluding debris (R1 and R2 respectively); (B,E)  
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47 PI vs. Annexin V staining. Values within the graphs represent the percentages of  
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49 apoptotic and dead cells from single experiment representative of duplicate  
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51 experiment from ten donors. (C,F) Percentages of alive, apoptotic and dead cells  
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53 from each subject analysed. Horizontal bars represent median values. After 6 days  
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55 monocytes treated with Cobalt are still viable more than 60%. Increasing viable  
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5 cells in the presence of  $\text{CoCl}_2$  were statistically significant ( $p < 0.0001$ , not-  
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7 parametric Mann-Whitney U-test). After 6 days the lymphocytes treated with  
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9 Cobalt are apoptotic ( $> 30\%$ ) unlike cells treated with Beryllium, which are  
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11 substantially similar to not treated ones. The reduction of viable cells in the  
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13 presence of  $\text{CoCl}_2$  was statistically significant ( $p < 0.0001$ , not-parametric Mann-  
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15 Whitney U test)  
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21 **Fig.11 Monocytes kept alive by Cobalt showed reduced expression of HLA-**  
22 **class II and impaired antigen presentation ability.**  
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26 Phenotype monocytes after 6 days of culture: (A) Surface expression of CD14,  
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28 CD16, CD11b, CD86, CD83 and HLA class I and class II in  $\text{CoCl}_2$ -treated (solid  
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30 black line) and in control (solid grey line) monocytes. Dotted-histograms  
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32 represent background staining of isotype-control antibody. The data are  
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34 representative of five different experiments. (B) Antigen uptake capacity. Open  
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36 histograms represent the uptake obtained at  $0^\circ\text{C}$ . Values indicate the percentage  
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38 of cells positive for FITC-dextran. The results from one representative experiment  
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40 out of five independently performed are shown. (C) Allostimulatory capacity.  
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42 After 6 days, cells were collected and their capacity to stimulate T-cell  
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44 proliferation was measured in a MLR assay. Allogenic  $\text{CD4}^+$  T cells were  
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46 cultured for 5 days in 96-well culture microplates ( $4 \times 10^4$ ) as responder cells with  
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48 different numbers of APC. [ $^3\text{H}$ ]-Thymidine incorporation was measured after 16 h  
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50 pulse ( $0,5 \mu\text{Ci/well}$ ). The results are shown as mean counts per minute (axis on  
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52 the left). Dendritic Cells (DC) from the same subjects were used as positive  
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54 control for T cells proliferation (dotted axis on the right of the graph). The data  
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5 are mean values of triplicate samples. One representative experiment out of four is  
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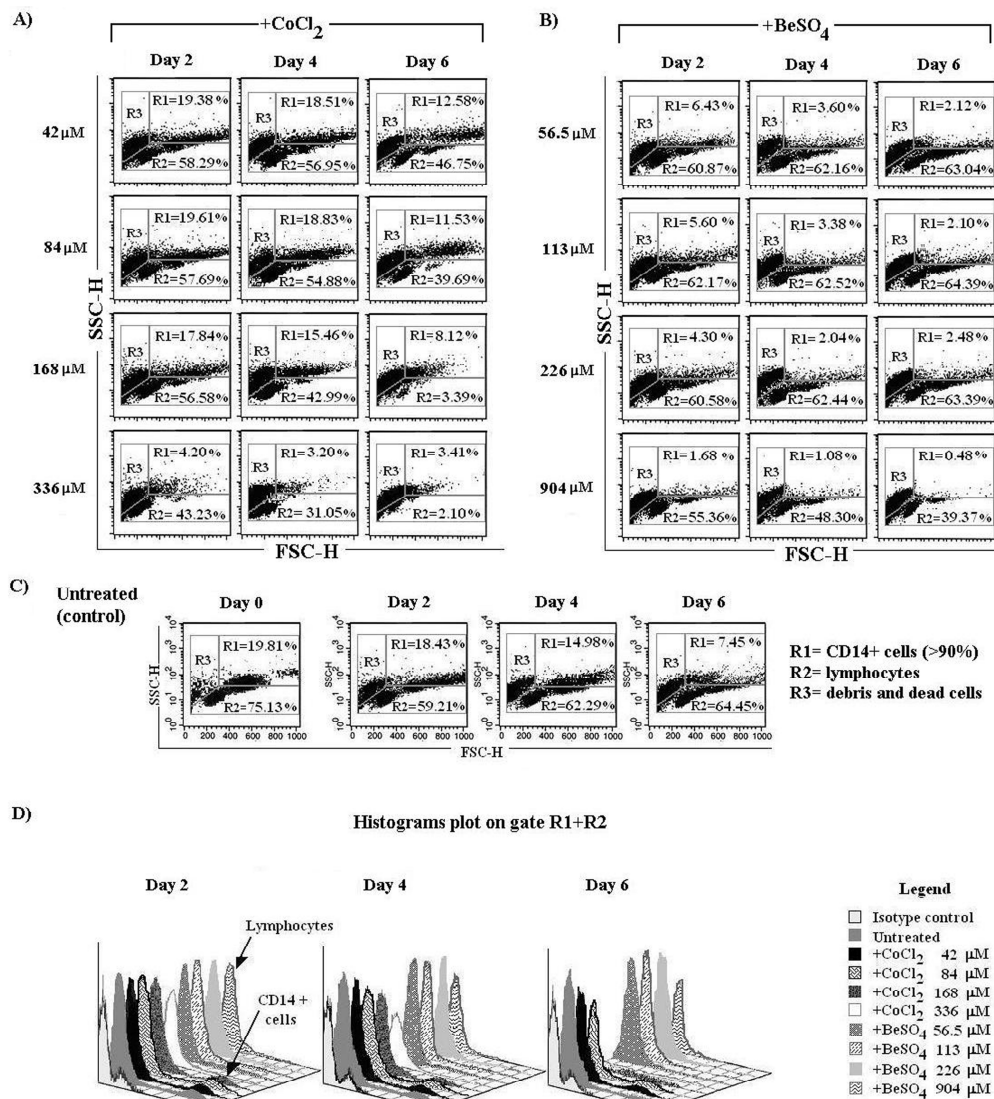


Fig.1 Effects of Cobalt and Beryllium on human PBMC in cytofluorimetric analysis: kinetics and dose-effect relationships.

Representative dot plots (SSC vs FSC) of PBMC treated with different concentrations of (A) CoCl<sub>2</sub> • 6 H<sub>2</sub>O (42 μM to 336 μM) or (B) BeSO<sub>4</sub> • 4 H<sub>2</sub>O (56.5 μM to 904 μM) or untreated (C) for six days. PBMC from 18 donors were analysed every two days (day 2, day 4 and day 6). Out of 18 donors studied, 14 demonstrated the same behaviour. R1 and R2 indicate monocytes and lymphocytes subpopulations, respectively, as defined by cell size (FSC), granularity/cell density (SSC) and phenotypic analysis of CD14 expression (histograms plot in (D) panel). Debris and dead cells corresponding to R3 gate were excluded in CD14 analysis by FSC/SSC parameters and Propidium Iodide labelling.

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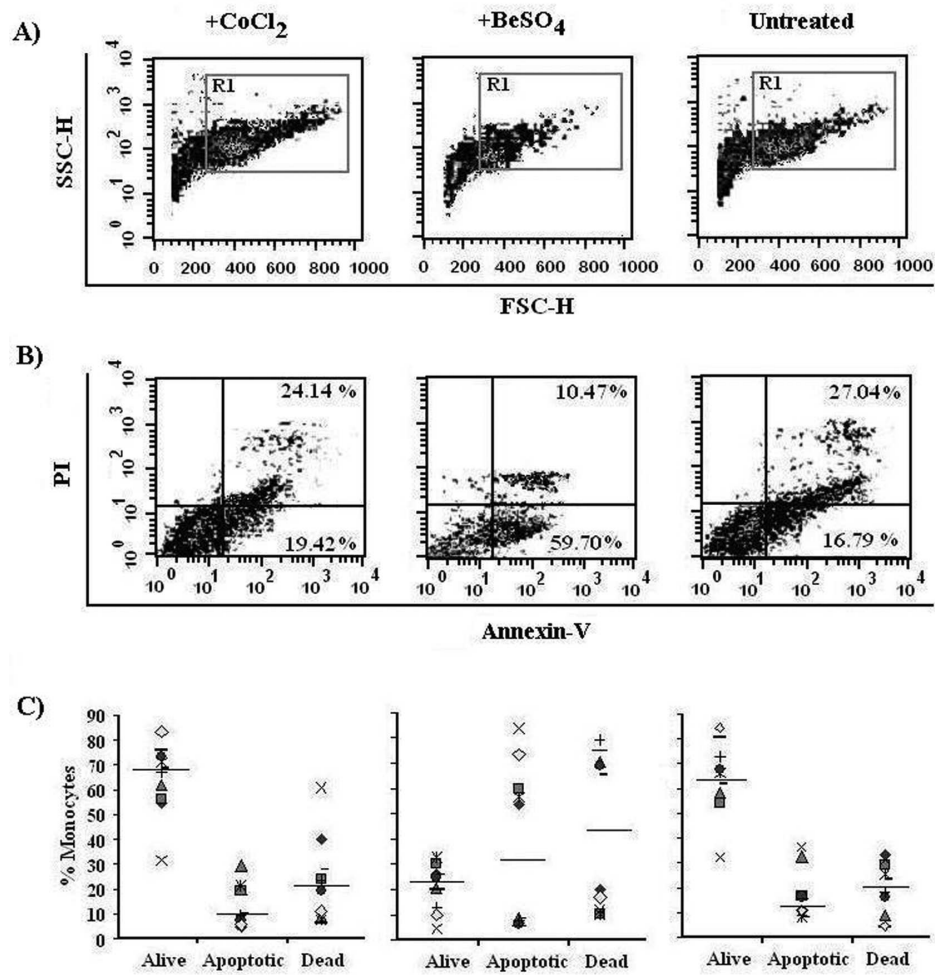


Fig.2 Effects of CoCl<sub>2</sub> and BeSO<sub>4</sub> on human monocytes.

Pure monocytes were isolated by magnetic depletion of lymphocytes. Monocytes, treated for 48 hours with 84  $\mu$ M CoCl<sub>2</sub>• 6 H<sub>2</sub>O or 113  $\mu$ M BeSO<sub>4</sub>• 4 H<sub>2</sub>O or untreated (right side) were incubated at 37 °C in 7% CO<sub>2</sub> humidified atmosphere with RPMI 10 % FCS. Cell viability was determined by Annexin V binding and flow cytofluorimetric analysis. Density plots: (A) SSC/FSC; monocytes were gated on SSC/FSC density plot excluding debris (R1) (B) Propidium Iodide (PI) vs. Annexin-V staining: values in quadrants represents the percentage of apoptotic (lower right) and dead cells (upper right) from a single experiment representative of triplicate experiments with cells from ten different donors. (C) Percentages of alive, apoptotic and dead monocytes respectively for each subject analysed. Horizontal bars represent median values. In the presence of BeSO<sub>4</sub> monocytes viability was < 30 %, while in CoCl<sub>2</sub>-treated monocytes and in control monocytes the percentages of viable cells were similar and > 60 %. Reduction of viable cells in the presence of BeSO<sub>4</sub> was statistically significant ( $p < 0.0001$ , not-parametric Mann-Whitney U-test).

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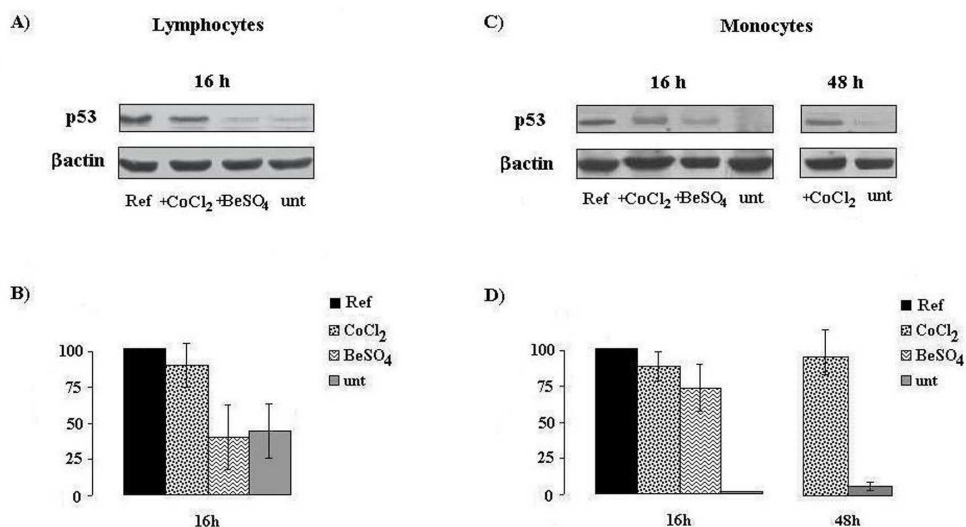


Fig.3 CoCl<sub>2</sub> induces p53 expression in lymphocyte and monocyte populations. Untreated, CoCl<sub>2</sub> and BeSO<sub>4</sub>-treated lymphocytes (A) and monocytes (B) were cultured for 16 and 48 hours at 37 °C, 7 %CO<sub>2</sub> before lysis and assay by Western Blot. An equal amount of proteins for each sample was separated by 12% SDS-PAGE and subsequently transferred to a nitrocellulose membrane for ECL detection. Reference (Ref) was T cells exposed to X-rays (5Gy) and lysed after 24h. Anti-β actin was used to ensure equal protein loading. p53 protein levels in lymphocytes (C) and monocytes (D) were evaluated by densitometric analysis as described in 'Materials and Methods'. Values are the mean ± s.d. of three independent experiments and are reported as percentage of 100% p53 expression arbitrarily attributed to control cells.

152x87mm (600 x 600 DPI)

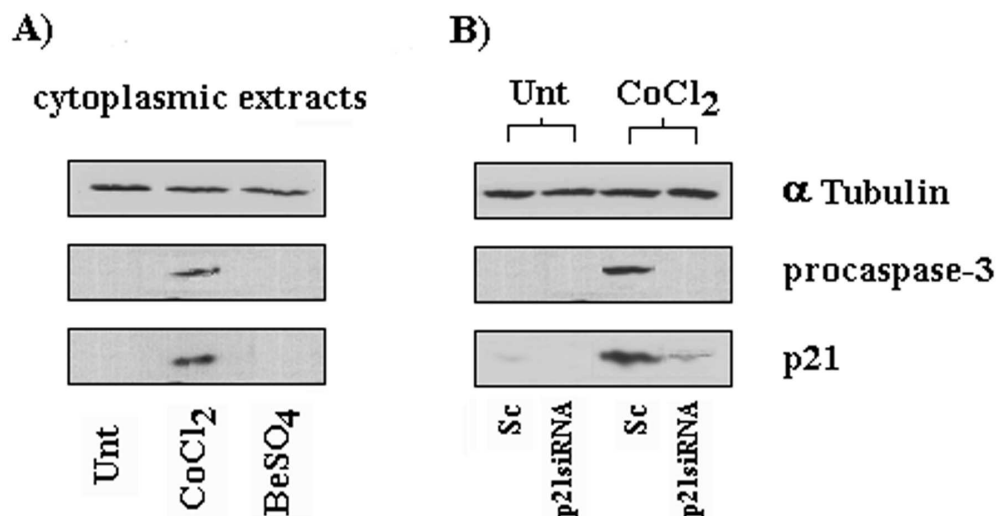


Fig.4 CoCl<sub>2</sub> induces cytoplasmic localization of p21 associated with procaspase-3 stabilization in monocytes.

(A) Untreated, CoCl<sub>2</sub> and BeSO<sub>4</sub> treated monocytes were cultured for 16 hours and then were harvested for Western blot analysis. p21 expression was significantly increased in the cytoplasmic CoCl<sub>2</sub> treated-monocytes but not in the nuclear fractions, where the protein was undetectable (data not shown). Procaspase-3 level was also obviously higher in CoCl<sub>2</sub> pulsed-monocytes than in untreated or BeSO<sub>4</sub> stimulated cells. Activated caspase-3 was undetectable. (B) Consistent with a role of p21 in regulating procaspase-3 amount, siRNA knockdown of p21 during CoCl<sub>2</sub> treatment restored the undetectable level of inactive caspase-3 whereas scrambled control (Sc) did not.

Results are representative of three separated experiments.

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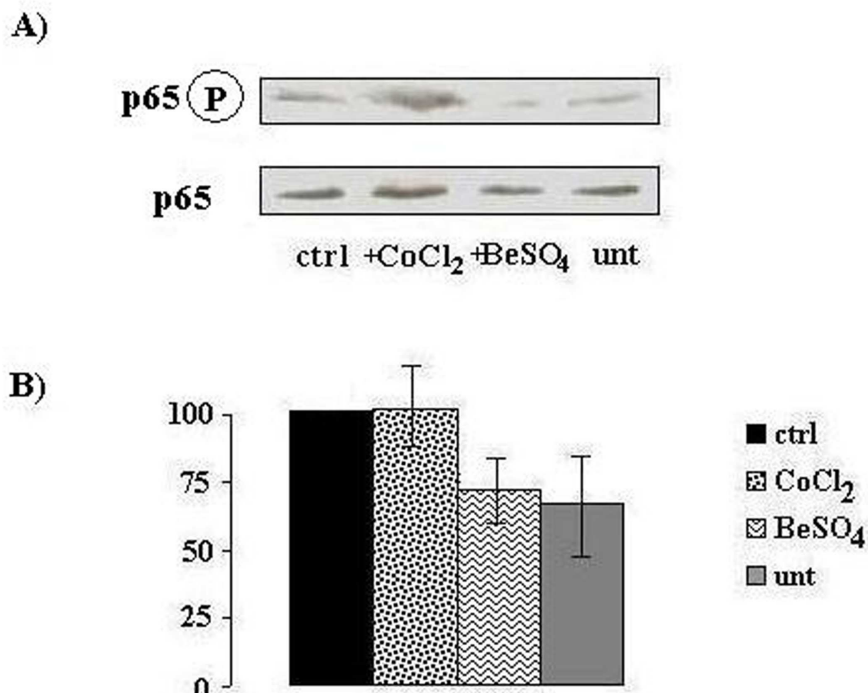


Fig.5 CoCl<sub>2</sub> induces phosphorylation of serine 276 in the p65 NF- $\kappa$ B subunit by monocytes. Untreated, CoCl<sub>2</sub> and BeSO<sub>4</sub>-treated monocytes were cultured for 16 hour at 37 °C, 7 %CO<sub>2</sub>. (A) An equal amount of whole cell lysate for each sample was separated 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 1h, 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'. Values are the mean  $\pm$  s.d. of three independent experiments and are reported as percentage of 100% phospho-p65 expression arbitrarily attributed to control cells.

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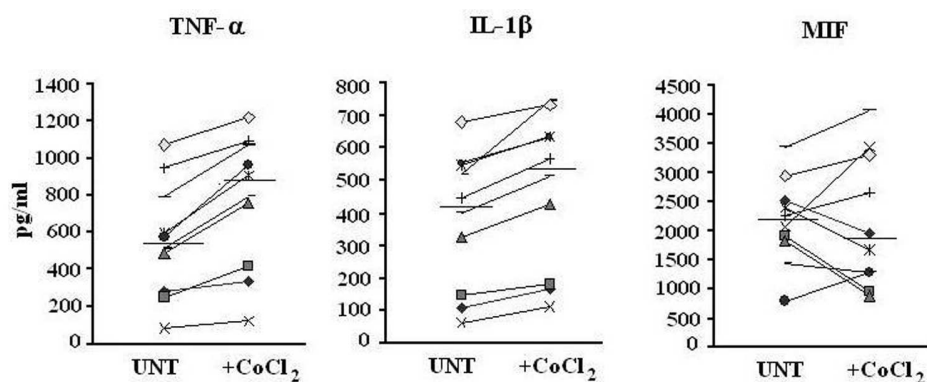


Fig.6 Production of TNF- $\alpha$ , IL1- $\beta$  and MIF by monocytes in presence of CoCl<sub>2</sub>.

Cytokine levels (pg/ml) in harvested media from monocytes incubated in the absence (Unt) or in the presence of CoCl<sub>2</sub> (84  $\mu$ M), was measured by ELISA after 48 hours of culture. Data are mean of triplicate from culture supernatants of ten donors. Standard deviations 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 pro-inflammatory cytokines TNF- $\alpha$  and IL1- $\beta$  induced by CoCl<sub>2</sub> was statistically significant ( $p < 0.001$  for TNF- $\alpha$  and  $p < 0.003$  for IL1- $\beta$ , respectively, not-parametric Mann-Whitney U-test). Differences in MIF production in absence or presence of CoCl<sub>2</sub> were not significant.

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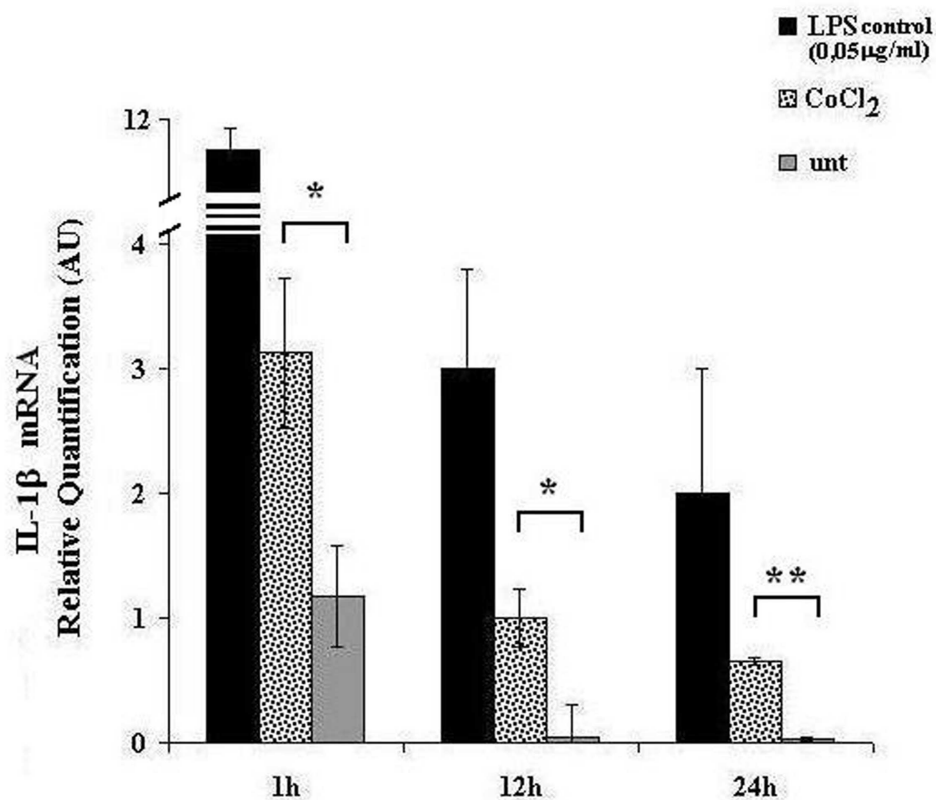


Fig.7 Time-course of the modulation of IL-1 $\beta$  mRNA in CoCl<sub>2</sub> treated- monocytes. CoCl<sub>2</sub> and LPS (control) stimulated or untreated monocytes were cultured for 1, 12 and 24 hours at 37 °C, 7 %CO<sub>2</sub> before to be harvested. Total RNA was isolated by TRIzol reagent and real-time RT-PCR was performed on first-strand cDNAs. Data are mean  $\pm$  s.d. of triplicate relative real-time quantification of 3 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 \* and < 0.01 by \*\*.

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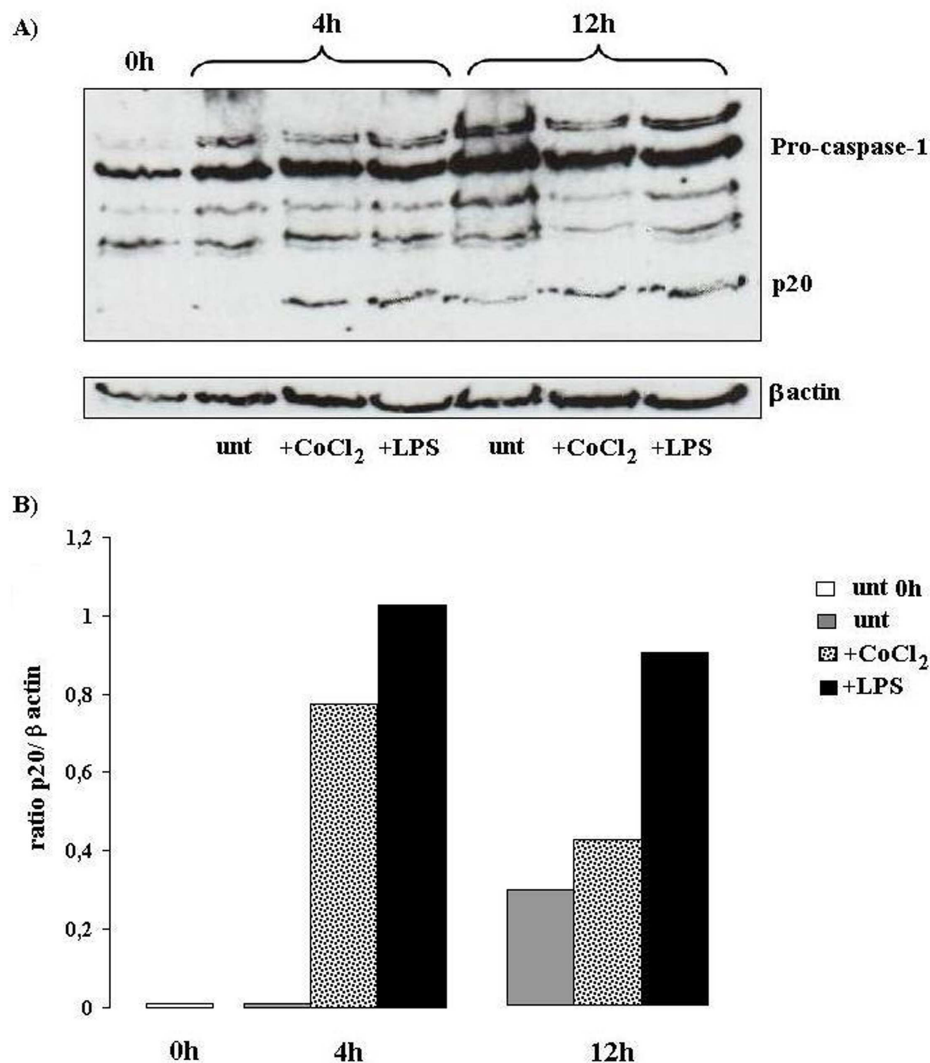


Fig.8 Cobalt induced caspase-1 activation by monocytes.

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 antibody. The multiple bands are alternative spliced variants of caspase-1 identified by the antibody used, as reported by the producer. (B) p20 protein levels were evaluated by densitometric analysis as described in 'Materials and Methods'.

Results are representative of two separated experiments.

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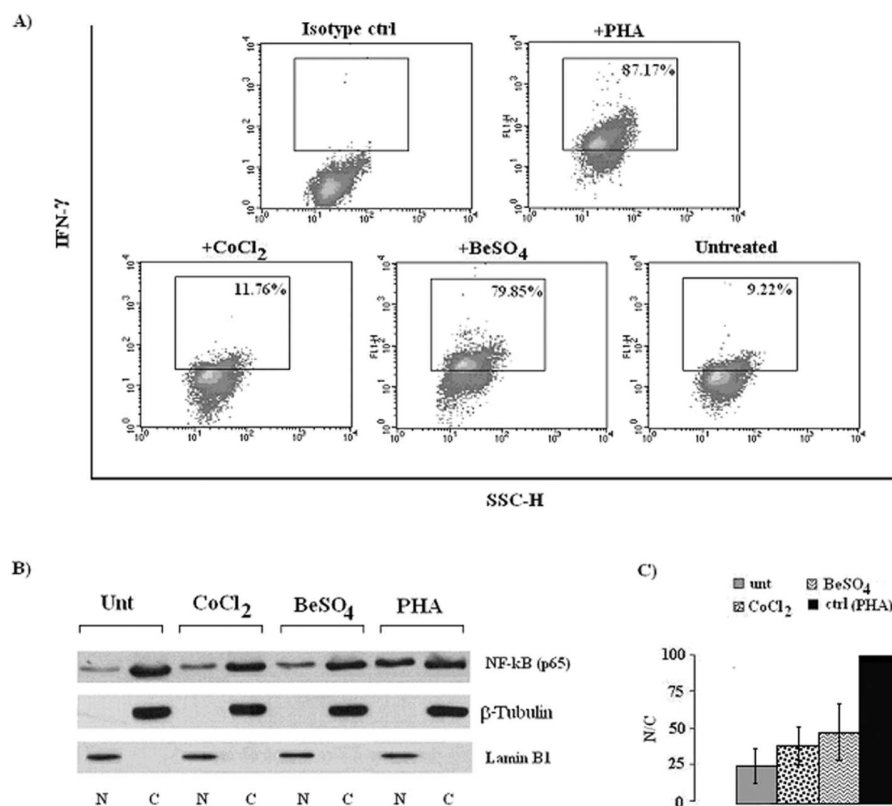


Fig.9 BeSO<sub>4</sub> -treated lymphocytes produce IFN- $\gamma$ .

(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 vs SSC dot plot. Isotype-matched negative controls and region statistics are reported.

After 48h, 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 nuclear NF- $\kappa$ B. Untreated, CoCl<sub>2</sub>-, BeSO<sub>4</sub>- or PHA-treated lymphocytes were cultured for 16 hour 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 antibody. 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  $\pm$  s.d. of three independent experiments and are reported as percentage of PHA-activated control cells arbitrarily attributed as 100%.

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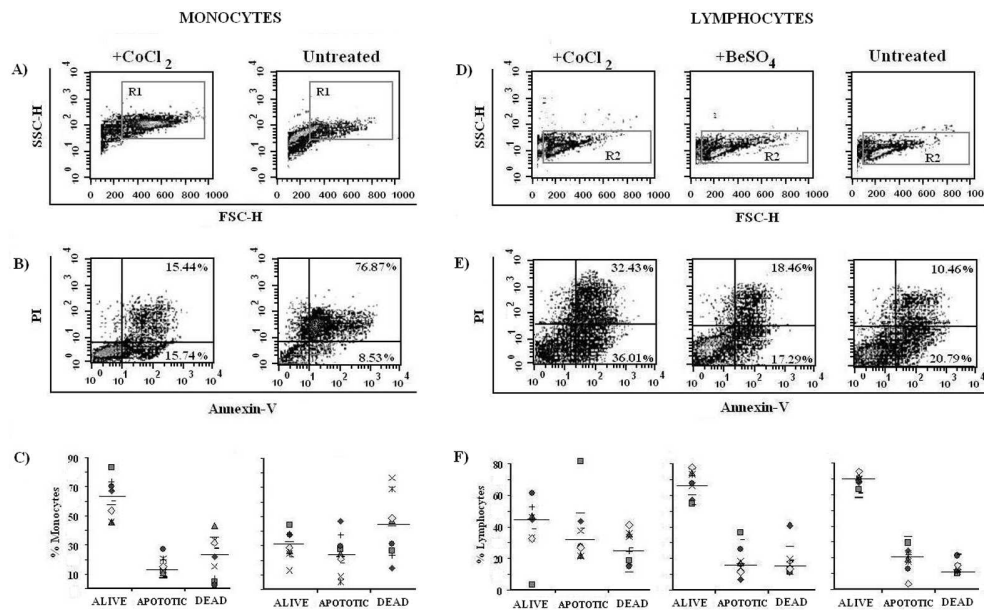


Fig.10 Cell viability of monocytes and lymphocytes after 6 days of culture.

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,D) SSC vs. FSC.

Monocytes and lymphocytes were gated on SSC/FSC density plot excluding debris (R1 and R2 respectively); (B,E) PI vs. Annexin V staining. Values within the graphs represent the percentages of apoptotic and dead cells from single experiment representative of duplicate experiment from ten donors. (C,F) Percentages of alive, apoptotic and dead cells from each subject analysed. 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 ( $p < 0.0001$ , non-parametric 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 ( $p < 0.0001$ , non-parametric Mann-Whitney U test)

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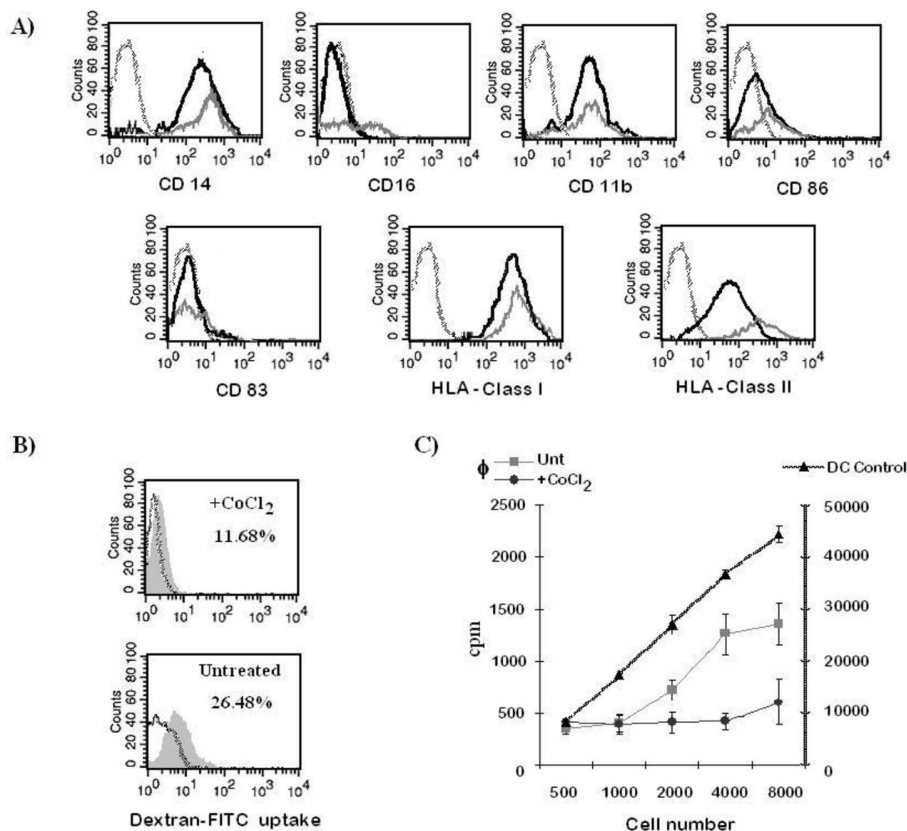


Fig.11 Monocytes kept alive by Cobalt showed reduced expression of HLA-class II and impaired antigen presentation ability.

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 grey line) monocytes. Dotted-histograms represent background staining of isotype-control antibody. 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 a MLR 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 APC. [<sup>3</sup>H]-Thymidine incorporation was measured after 16 h pulse (0,5  $\mu$ Ci/well). The results are shown as mean counts per minute (axis on the left). Dendritic Cells (DC) from the same subjects were used as positive control for T cells 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.

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