

A Human Dendritic Cell-Based *In Vitro* Model to Assess *Mycobacterium Tuberculosis* SO2 Vaccine Immunogenicity

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Summary

Among the tuberculosis (TB) vaccine candidates, SO2 is the prototype of the first live-attenuated vaccine that recently entered into clinical trials. To investigate the capacity of SO2 to stimulate an appropriate immune response *in vitro* within a human immunological context, a comparative analysis of the effects promoted by SO2, the current *Bacille Calmette-Guerin* (BCG) vaccine and *Mycobacterium tuberculosis* (Mtb) was conducted in human primary dendritic cells (DC), which are critical modulators of vaccine-induced immunity.

In particular, we found that SO2 promotes the expression of maturation markers similarly to BCG but to a lower extent than Mtb. Moreover, SO2-infected DC released higher levels of interleukin (IL)-23 than BCG-infected cells, which accounts for the expansion of interferon (IFN)- γ -producing T cells in an IL-12-independent manner. In the autologous mixed leukocyte reaction setting, the expansion of IL-17-producing T cells was also observed in response to SO2 infection. Interestingly, apoptosis and autophagic flux, events required for antigen presentation within MHC class II complex, were not affected in DC infected with SO2, converse to what we observed upon Mtb stimulation.

Collectively, our results indicate that SO2 represents a promising TB vaccine candidate, which displays an attenuated phenotype and promotes in DC a stronger capacity to stimulate the Th response than BCG vaccine. Interestingly, the data obtained by using the human DC-based experimental setting mirrored the results derived from studies in animal models, suggesting that this system could be used for an efficient and rapid down-selection of new TB vaccine candidates, contributing to achieving the 3Rs objective.

Keywords: human, DC, IL-23, tuberculosis, vaccine

1 Introduction

Tuberculosis (TB) remains an urgent global health problem with about 9 million new cases and 1.4 million deaths each year (WHO, 2012). An estimated one third of the world population is latently infected with *Mycobacterium tuberculosis* (Mtb) and at risk of developing TB. The dual pandemic of TB and HIV/AIDS and the increasing emergence of (multi) drug-resistant strains severely aggravate the issue and hamper current control strategies (WHO, 2012). Thus, to achieve effective and sustainable control of TB, there is a compelling need for vaccines that

can reduce the development of disease both in adolescents and adults following exposure to new infection, and in the 2 billion individuals who have already been exposed to Mtb.

The current TB vaccine, the live-attenuated *Bacille Calmette-Guerin* (BCG) derived from *Mycobacterium bovis*, is effective in preventing severe disseminated forms of TB, including meningitis and miliary TB, in infants. However, BCG presents variable efficacy against pulmonary TB in adults and is poorly effective in preventing development of disease in latently infected individuals. In addition, BCG is unsafe in HIV positive infants and is not recommended for use in this population (WHO, 2012).

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Abbreviations: Abs, antibodies; Ag, antigen; AMLR, autologous mixed lymphocyte reaction; APC, Ag presenting cells; BCG, *Bacille Calmette-Guerin*; CBA, cytometric bead array; CFU, colony forming units; DC, dendritic cells; ESAT-6, 6 kDa early secretory antigenic target; ESX, ESAT-6 secretory system; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; IFN, interferon; IL, interleukin; MFI, median fluorescence intensity; MHC, major histocompatibility complex; MOI, multiplicity of infection; Mtb, *Mycobacterium tuberculosis*; PBMC, peripheral blood mononuclear cells; SEM, standard error of the mean; TB, tuberculosis; Th, T helper; TNF, tumor necrosis factor



Among the most promising TB live vaccine candidates (Otenhoff and Kaufmann, 2012; Kaufmann and Gengenbacher, 2012), the prototype vaccine SO2 was attenuated by insertion of a kanamycin-resistance cassette in the *phoP* gene of a Mtb clinical isolate (Perez et al., 2001). The transcriptional regulator PhoP controls approximately 2% of Mtb coding capacity, including the synthesis of the trehalose-derived lipids, diacyl- and polyacyl-trehaloses, and the secretion of the virulence factor ESAT-6 (Frigui et al., 2008; Walters et al., 2006; Gonzalo Asensio et al., 2006, 2008). In addition, SO2 contains also a deletion of *fadD26* gene, which is essential for the synthesis of the complex lipid phthioceroldimycocerosates, one of the major mycobacterial virulence factors (Camacho et al., 1999). Accordingly, preclinical testing of SO2 has provided robust data for its high degree of safety and improved immunogenicity and protective efficacy compared to BCG in relevant animal models of TB, from mice to non-human primates (Nambiar et al., 2012; Martin et al., 2006; Verreck et al., 2009; Cardona et al., 2009). To fulfil the Geneva consensus requirements to enter into clinical trials (Walker et al., 2010), the first Mtb vaccine candidate MTBVAC was constructed from SO2 by removing antibiotic-resistance markers (Arbues et al., 2013). MTBVAC is currently in clinical trials (NCT02013245) as the first live attenuated Mtb-based vaccine.

In this study we sought to investigate how SO2 modulates the functions of human DC, which are key regulators and players of the immune defense against pathogens, including Mtb. The rationale of this study relies on previous findings showing that an experimental setting based on human primary DC could be used to compare the immunogenicity of BCG and Mtb (Giacomini et al., 2001, 2006). In particular, we observed that BCG was less efficient in inducing DC maturation and, in turn, in promoting a T helper (Th) 1-oriented T cell response compared to Mtb (Giacomini et al., 2006). By using this DC-based setting, we found that Mtb H37Rv inhibits the fusion of autophagosome to lysosome, whereas no inhibition was observed in DC infected with either live or heat-inactivated Mtb H37Ra or BCG (Romagnoli et al., 2012; Martin et al., 2006; Cardona et al., 2009; Williams et al., 2005).

Here, we compared the effects induced by BCG vaccine, SO2 and the parental strain by studying DC phenotype, release of regulatory and pro-inflammatory cytokines, and capacity to either promote T cell response or modulate apoptosis and autophagy. In line with the results obtained in animal models (Aguilar et al., 2007), we found that SO2 displays interesting features as a vaccine candidate since it possesses a stronger capacity to expand the Th1 and Th17 response than the BCG Danish vaccine.

Having found that data on SO2 immunogenicity, achieved by using the *in vitro* human DC setting, mirrored the results obtained in animal models, we seek to propose this experimental system as a preclinical evaluation of the capacity of vaccine candidates to stimulate an appropriate immune response within a human immunological context, achieving in this way the 3Rs (Replacement, Refinement, Reduction) objective for reduction of animal use in research.

2 Materials and methods

Antibodies (Abs) and other reagents

Monoclonal Abs specific for CD1a, CD14, CD38, CD86, CD83, HLA-DR, IgG1, IgG2a (BD Bioscience, San Jose, CA), Annexin V (Abcam, Cambridge, UK) and Fixable Viability Dye eFluor®780 (FvDye, eBioscience, San Diego, CA) were used as direct conjugates to fluorescein isothiocyanate (FITC), phycoerythrin or allophycocyanin 7 as needed. For immunoblotting analysis, rabbit anti-MAP1LC3 (Cell Signaling, Danvers, MA), mouse anti-actin (Sigma-Aldrich, St. Louis, MO), mouse anti-SQSTM1 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-caspase-3 (Santa Cruz Biotechnology) and horseradish peroxidase-conjugate secondary Abs anti-mouse and anti-rabbit (Santa Cruz Biotechnology) were used. Rapamycin (0.2 μ M; Sigma-Aldrich) or staurosporin (1 μ M; Sigma-Aldrich) were used as inducers of autophagy and apoptosis, respectively.

DC preparation and viability

Istituto Superiore di Sanità Review Board approved the research project (CE/13/387). DC were prepared as previously described (Giacomini et al., 2001). Briefly, peripheral blood mononuclear cells (PBMC) were isolated from freshly collected buffy coats obtained from healthy, voluntary blood donors (Blood Bank of University “La Sapienza”, Rome, Italy) by density gradient centrifugation using Lympholyte-H (Cedarlane, Hornby, Ontario, Canada). Monocytes were purified by positive sorting using anti-CD14-conjugated magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The recovered cells were >99% CD14⁺, as determined by flow cytometry with anti-CD14 Ab. DC were generated by culturing monocytes in six-well tissue-culture plates (Costar Corporation, Cambridge, MA) with 25 ng/ml GM-CSF and 1000 U/ml IL-4 (R&D Systems, Abingdon, GB) for 5 days at 0.5 $\times 10^6$ cells/ml in RPMI 1640 (BioWhittaker Europe, Verviers, Belgium), supplemented with 2 mM L-glutamine and 15% fetal bovine serum (FBS) (Lonza, Verviers, Belgium). At day 5, the cells were tested for their differentiation status by evaluating CD1a expression (>90% CD1a⁺) and lack of CD14 (>95% CD14⁻). Before infection, the medium was replaced with RPMI without cytokines and supplemented only with 2 mM L-glutamine and 15% FBS. Cytokine deprivation did not affect DC survival rate, which was >90%. Cellular vitality was evaluated with FvDye staining according to the manufacturer’s protocol.

Bacterium preparation and DC infection

Mtb H37Rv (ATCC 27294; American Type Culture Collection), BCG Danish (1331) and SO2 prototype vaccine strains were grown as previously described (Giacomini et al., 2001; Aporta et al., 2012). Logarithmically growing cultures were centrifuged at 800 rpm for 10 min to eliminate clumped mycobacteria and then washed three times in RPMI 1640. Mycobacteria were resuspended in RPMI 1640 containing 10% FBS and then stored at -80°C. Bacterial frozen vials were thawed and bacterial viability was determined by counting the number of colony forming units (CFU). All bacterium preparations were tested for endotoxin contamination (<1 endotoxin unit/ml) by the Limu-

lus lysate assay (Lonza). DC cultures were then infected with a multiplicity of infection (MOI) of 1 bacterium/cell as previously described (Giacomini et al., 2006).

CFU assay

DC were infected with Mtb H37Rv, SO2 and BCG Danish at MOI 1. Cells were lysed immediately or 6 h after infection with distilled water containing 0.1% saponin for 5 min at RT. Six h after infection, cell cultures were gently washed three times with RPMI 1640. DC were then centrifuged at 150 x g for 10 min to selectively spin down cells while extracellular bacteria remained in the supernatants. Samples were assayed in triplicate for CFU counting. Cell lysates were briefly sonicated and 10-fold dilutions were performed using 0.05% Tween 80 in distilled water before plating in duplicate on Middlebrook 7H10 agar (Difco, BD Bioscience). After 21 days of incubation at 37°C in 5% CO₂ atmosphere, CFU of each plate were enumerated.

Acid-fast staining

DC monolayers were stained with the Kinyoun method as previously described (Giacomini et al., 2001). Bacteria were identified by light microscopy on Nikon Eclipse Ni-U microscope (Nikon corporation, Tokyo, Japan) using a 60x magnification. Images were captured with a high-definition color camera head DS-Fi2 (Nikon). Duplicate monolayers were prepared for each experimental condition.

Flow cytometry analysis

Cells (10⁵) were washed once in PBS containing 2% FBS and incubated with mAbs at 4°C for 30 min. After washing the cells were fixed in 4% formaldehyde (PanreacQuimica, Castellar del Valles, Spain) before analysis on a FACSCan using CellQuest software (Becton Dickinson, Mountain View, CA). A total of 5000 cells were analyzed per sample. The viable DC were gated by forward and side scatter and then the expression of cell surface molecules was evaluated using the median fluorescence intensity (MFI) after subtraction of the values of the isotype Ab controls. Only cells comprised in the viable cell gate were considered for the analysis.

Cytokine determination

Supernatants of DC cultures were filtered and stored at -80°C. The production of IL-12, TNF- α , IL-1 β and IL-6 was then measured by human inflammation Cytometric Bead Array (CBA) (BD Bioscience). The production of IL-23, IFN- γ and IL-17 was instead measured with ELISA (R&D Systems).

Apoptosis detection

Phosphatidylserine exposure and membrane integrity were analyzed by using Annexin V-FITC and FvDye according to manufacturing protocols. Twenty-four h post infection, DC were stained with FvDye and then labelled for 15 min with 5X Annexin V-FITC. Finally, the cells were fixed with 4% formaldehyde for 30 min before analysis on a FACSCanto (BD Bioscience). Data were analyzed by FlowJo software (TreeStar Inc., Ashland, OR).

Immunoblotting analysis

Whole cell extracts (30 μ g) were used for immunoblotting analysis as previously described (Romagnoli et al., 2012). Briefly, proteins were separated on 12% PAGE Bis-Tris gel and electroblotted onto polyvinylidene fluoride membranes (Millipore, Billerica, MA). Blots were incubated with primary Ab in 5% non-fat dry milk in TBS plus 0.1% Tween20 (Sigma-Aldrich) overnight at 4°C. Detection was achieved using horseradish peroxidase-conjugate anti-mouse or -rabbit secondary Abs (Santa Cruz Biotechnology) and visualized with the Enhanced Chemiluminescence plus kit (GE Healthcare, Freiburg, Germany). B-actin levels were analyzed by mouse anti- β -actin Ab (Sigma-Aldrich) to verify the loaded protein amount.

AMLR experiment

Autologous PBMC were frozen in RPMI supplemented with 20% FBS and 10% dimethyl sulfoxide soon after Lympholyte-H separation. After DC differentiation and infection, PBMC were thawed in order to isolate autologous naïve T cells by indirect magnetic sorting with naïve CD45RA⁺ T cell Isolation Kit II (Miltenyi Biotec). Immature DC and DC infected for 16 h with the indicated mycobacterium strains were resuspended with X-VIVO 15 medium at 0.4x10⁵ cells/ml and gently added to naïve CD45RA⁺ T cells (Ratio DC:T=1:10) in 96-well U-bottomed tissue culture plates. After 5 days recovered T cells were resuspended at 1x10⁶ cells/ml and stimulated with anti-CD3/CD28 Dynabeads (Invitrogen Life Technology, Carlsbad, CA) (ratio 1:1) for 24 h, then supernatants were harvested for IFN- γ and IL-17 detection.

T-cell activation and expansion

To investigate the effects induced by cytokines on Th cell differentiation, purified naïve CD45RA⁺ T cells were plated in 96-well U-bottomed tissue culture plates at 0.4x10⁶ cells/ml with supernatants obtained from control or 24 h infected DC. Where indicated, 1 μ g/ml of anti-human IL-23 p19 neutralizing or mouse IgG1 κ isotype control Abs (eBioscience) were used. Conditioned naïve T cells were stimulated with anti-CD3/CD28 Dynabeads (ratio 1:1) for 5 days. Supernatants were harvested for IFN- γ detection following the stimulation of 1x10⁶ T cells with anti-CD3/CD28 beads for an additional 24 h.

Statistical analysis

Statistical analysis was calculated using a two-tailed Student's t-test for paired data. A *P* value \leq 0.05 was considered statistically significant.

3 Results

3.1 The attenuated Mtb strain SO2 neither affects viability nor promotes apoptotic-like features in human DC

For an optimal induction of host immune response, an attenuated live vaccine should not affect the viability of antigen presenting cells (APC) (Palucka et al., 2010). To this aim, the impact of the prototype vaccine SO2 stimulation was firstly

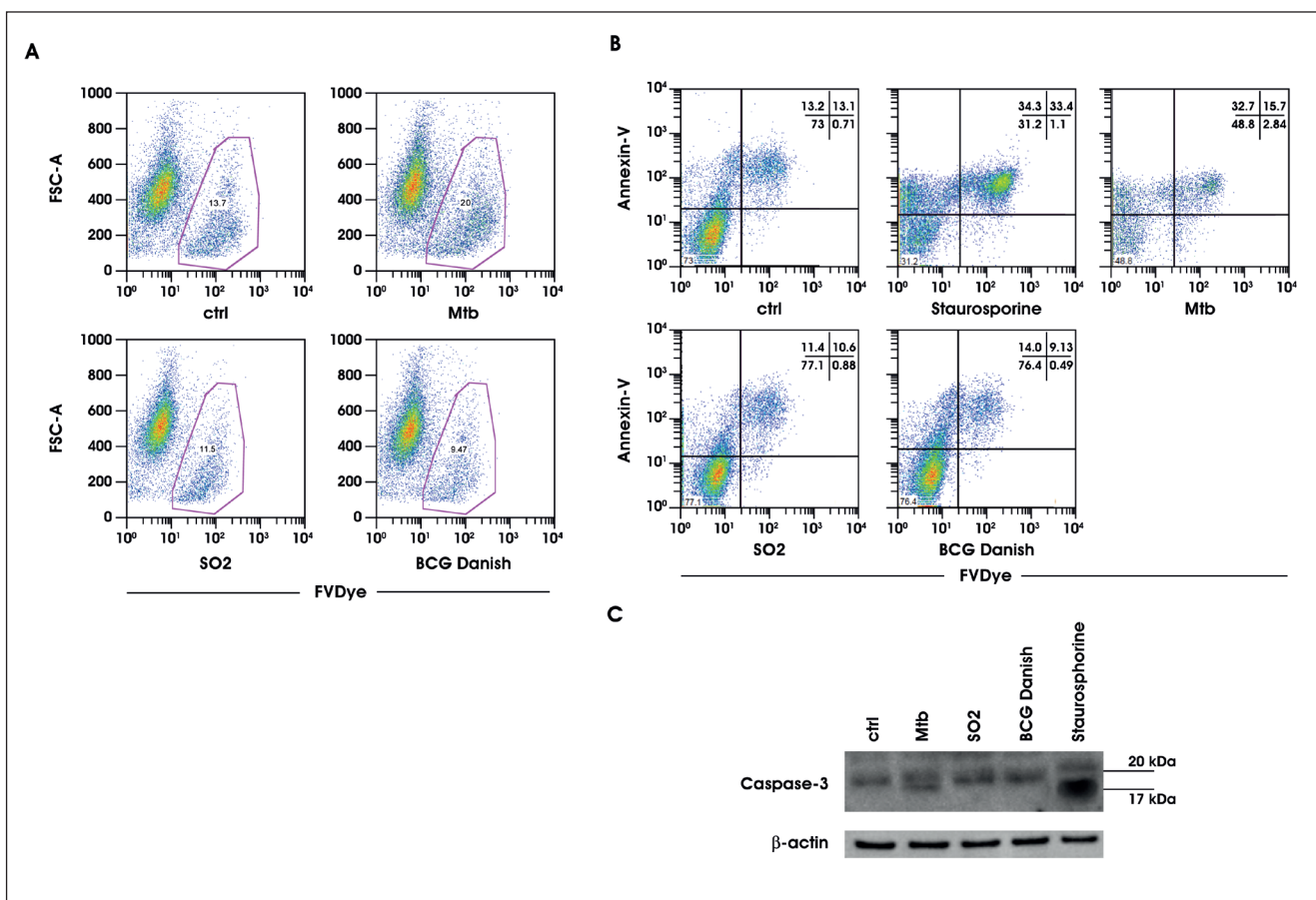


Fig. 1: The attenuated SO2 strain does not affect either DC viability or apoptosis

DC were left untreated (ctrl), or infected with Mtb, SO2 or BCG Danish (MOI 1:1) for 24 h. Cell viability was assessed by staining DC with FvDye (A). A representative experiment, out of 5 independent experiments performed, is shown. Numbers in dot plot are the percentage of positive cells for FvDye. (B) Apoptosis was investigated by staining DC with annexin V and FvDye. As internal control, DC were treated with 1 μ M staurosporine for 16 h. A representative experiment, out of 5 independent experiments performed, is shown. Numbers in the dot plots correspond to the percentage of cells in each quadrant. (C) Caspase-3 activation was investigated by immunoblotting. B-actin levels were analyzed to verify loaded protein amount. The result shown is from one out of 3 independent experiments that yielded similar results.

evaluated on DC viability. The percentage of live versus dead cells was assessed by FACS analysis on DC stained with FvDye, a compound that irreversibly labels dead cells prior to the fixation procedure necessary to neutralize Mtb infectivity (Fig. 1A). Keeping with the data obtained with murine macrophages (Aporta et al., 2012; Aguilo et al., 2013), the live attenuated SO2 vaccine does not affect DC viability, a result similar to that observed in uninfected or in BCG-infected DC cultures. On the contrary, the virulent strain Mtb H37Rv significantly reduces the number of viable DC. Dead cells stained by FvDye, indeed, were doubled when DC were treated with Mtb H37Rv (Fig. 1A).

Apoptosis of Mtb-infected cells was studied with a novel approach set up in our laboratory, based on double staining with annexin V together with FvDye. Unlike 7-AAD and propidium iodide, cells labeled with FvDye can be fixed to neutralize Mtb infectivity and, then, permeabilized and stained for surface or

intracellular antigens (Ag) without any loss of fluorescence intensity of the dead cells. By using this procedure, we found that the basal level of early apoptotic (13.2% annexin⁺/FvDye⁻ DC) and late apoptotic cells (13.1% annexin⁺/FvDye⁺ DC) observed in uninfected DC cultures was strongly upregulated only in response to Mtb infection (32.7% annexin⁺/FvDye⁻ and 15.7% annexin⁺/FvDye⁺ DC), while it was slightly reduced in response to SO2 and BCG stimulation (Fig. 1B). In line with these data, we observed by immunoblotting analysis caspase-3 cleavage only in Mtb infected DC as a result of self proteolysis of the inactive pro-enzyme occurring in cells undergoing apoptosis (Fig. 1C) (Boatright and Salvesen, 2003). As expected, no caspase-3 activation was observed in control or BCG- and SO2-infected DC cultures, while the treatment with staurosporine, a classical inducer of the apoptotic process, strongly promoted the cleavage of caspase-3 to its active form. These data correlate with what has been observed in mouse macrophages (Aporta et al., 2012).

3.2 The constitutive autophagy of DC is not affected by SO2

Another important event controlling host immune response via DC activation is autophagy (Kuballa et al., 2012). In this regard, DC display peculiar features showing in a constitutive manner high levels of autophagy (Schmid et al., 2007), a cellular process that continuously provides peptidic antigens for MHC class II presentation in order to induce immunity or tolerance by immature and mature DC, respectively. In particular, autophagy controls mycobacterial Ag processing and degradation, both processes required for induction of T cell responses by DC (Deretic et al., 2009). In line with these findings, Jagannath and collaborators showed, in a mouse model, that the stimulation of autophagy by Rapamycin enhanced the capacity of DC to present mycobacterial antigens into the MHC class II complex (Jagannath et al., 2009).

Thus, to assess whether the attenuated SO2 vaccine might perturb autophagy, we analyzed by immunoblotting two autophagosome markers, namely MAP1LC3 and SQSTM1 (Fig. 2). We found that SO2, like BCG, does not promote the accumulation of both MAP1LC3-II and SQSTM1, indicating that the prototype vaccine does not alter the constitutive autophagic process present in DC. As previously observed (Romagnoli et al., 2012), Mtb promotes, instead, a significant increase of both MAP1LC3-II and SQSTM1 level due to an impaired ability of autophagosomes to fuse with lysosomes, where the degradation of these molecules occurs in association with Mtb destruction. As expected, treatment with the autophagy inducer Rapamycin enhanced the basal autophagic flux, which correlates with the

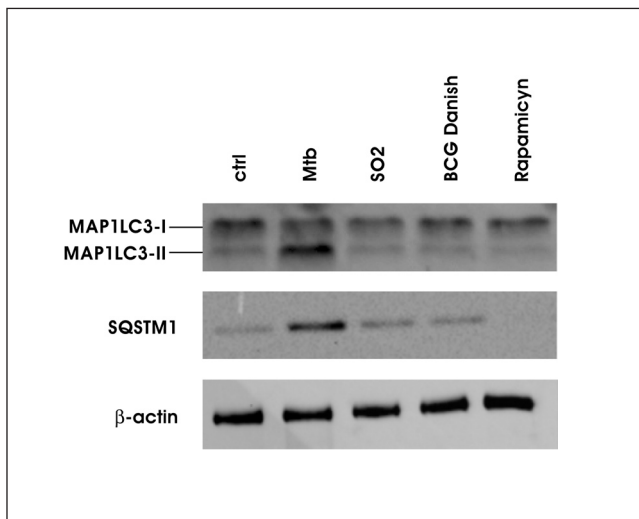


Fig. 2: The basal autophagic process of DC is not affected by SO2 infection

The autophagy levels were analyzed by means of MAP1LC3 and SQSTM1 expression by using immunoblotting. DC were left untreated or infected for 24 h with Mtb, SO2 or BCG Danish. As control, DC were treated with Rapamycin (Rapa, 0.2 μ M; 20 h). B-actin levels were analyzed to verify loaded protein amount. The results shown are from one out of 3 independent experiments that yielded similar results.

degradation of both SQSTM1 and MAP1LC3-II (Romagnoli et al., 2012). Through this comparative analysis, we also confirmed previous data showing that the impairment of the autophagic flux only occurred if the engulfed live mycobacteria had the capacity to secrete intact ESAT-6/CFP-10 via the ESX-1/type VII secretion system (Romagnoli et al., 2012), given that both SO2 and BCG are unable to release ESAT-6.

3.3 The live attenuated vaccine SO2 promotes DC maturation

To compare the impact of either SO2 or BCG stimulation on DC maturation, we evaluated the immunophenotype of these cells, focusing on the molecules involved in Ag presentation and T cell interaction (Tab. 1). DC were challenged with SO2, BCG or Mtb for 24 h and cell surface expression of CD86, CD83, HLA-DR and CD38 was examined by FACS analysis. Both SO2- and BCG-infected DC showed an enhanced expression of the analyzed molecules compared to the uninfected counter-

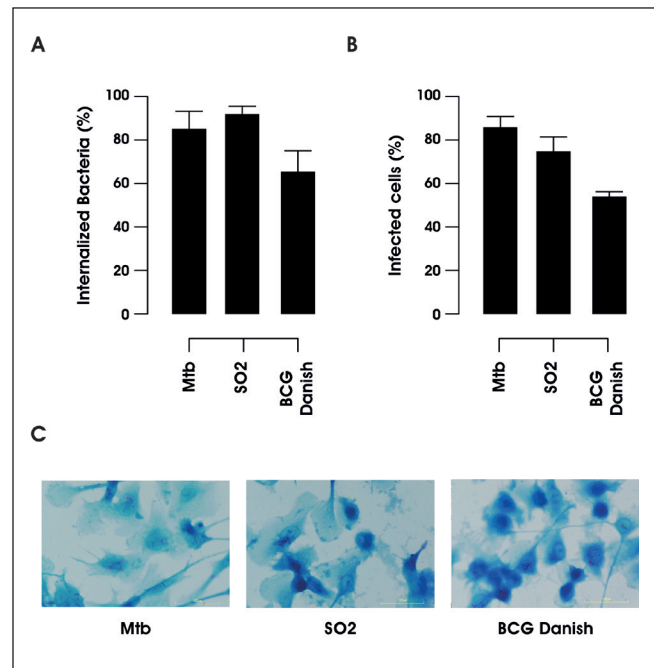


Fig. 3: DC internalize SO2 and BCG Danish to a similar extent

DC were infected with Mtb, SO2 or BCG Danish at MOI 1 for 6 h. (A) DC were lysed to determine the number of internalized bacteria by CFU counting upon 21 days. The results are presented as a percentage of internalized bacteria 6 h after the infection with respect to the mycobacterial input used to stimulate DC cultures measured soon after the infection. The values are the means \pm standard deviation of 5 independent experiments. (B) Percentage of DC infected with the different bacterial strains was estimated following acid-fast staining by calculating the ratio of infected versus uninfected cells per 20 different fields in each experiment out of 2 separately performed. Results are expressed as mean percentage \pm standard error of the mean (SEM). (C) Representative pictures of DC infected with Mtb, SO2 or BCG Danish obtained by acid-fast staining are shown. Bars: 100 μ m.

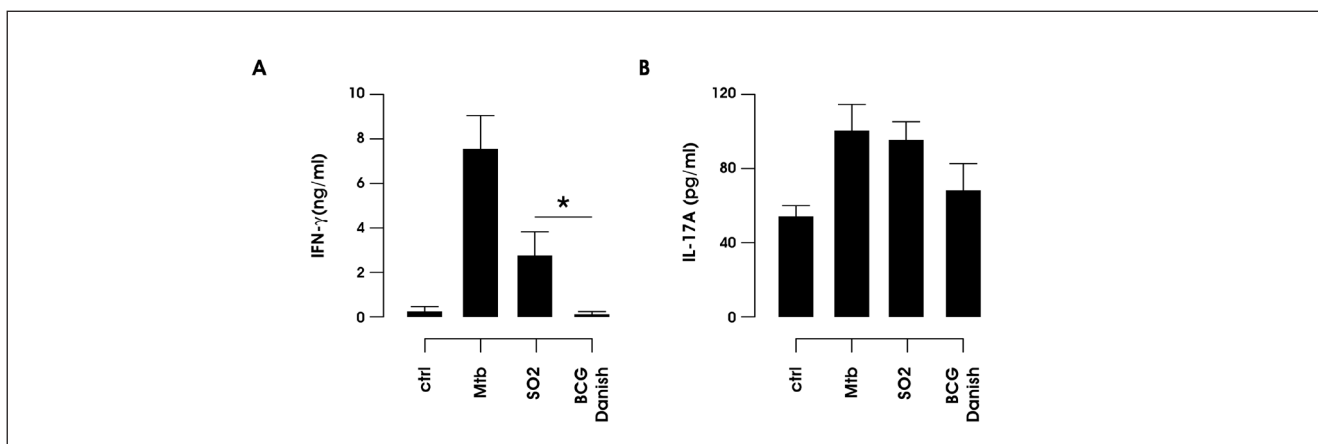


Fig. 4: SO2 equips DC with the capacity to expand Th response in AMLR experiments

The response of naïve CD4⁺ T cells was evaluated upon co-culture with autologous DC infected with Mtb, SO2 or BCG Danish by AMLR. The level of IFN-γ (A) and IL-17 (B) was measured with ELISA. The results shown represent means \pm SEM of 5 independent experiments. Significance was calculated by using Student's t-test; p-value: * = 0.04.

Tab. 1: Expression of maturation markers in human primary DC stimulated with SO2, BCG and Mtb

	CD86	CD38	HLA-DR	CD83
ctrl	14 \pm 3	3 \pm 0.3	52 \pm 10	3 \pm 1
Mtb	163 \pm 19	11 \pm 2	95 \pm 9	12 \pm 0.6
SO2	44 \pm 13	5 \pm 0.6	60 \pm 9	7 \pm 0.8
BCG Danish	47 \pm 13	5 \pm 0.6	65 \pm 10	7 \pm 0.9

DC were left untreated (ctrl) or infected with Mtb, SO2 and BCG Danish for 24 h. Expression of indicated molecules was calculated by subtracting MFI values obtained from isotype control Abs to those derived from each different experimental condition. Data are reported as MFI mean \pm SEM of 5 independent experiments.

part, while Mtb-infected DC displayed a robust induction of all maturation markers (Tab. 1).

The stronger capacity of Mtb in respect to SO2 and BCG Danish to promote DC maturation was not related to differences in the infection rate, which resulted to be similar between Mtb and SO2 and slightly (not statistically significant) lower for BCG as evaluated 6 h after the infection by CFU counting and acid-fast staining of intracellular bacteria (Fig. 3A,B). The results of the internalization rate related to each strain (Fig. 3A) are comparable to the number of infected cells observed upon acid-fast staining (Fig. 3B).

To investigate whether the SO2-stimulated DC maturation resulted in an enhanced capacity to promote the expansion of Th1-oriented CD4⁺ T cells, we studied naïve T-cell polarization in an AMLR setting (Volpe et al., 2008; Torrado and Cooper, 2010). As shown in Figure 4A, the production of IFN-γ released by naïve T cells stimulated with autologous SO2-infected DC was significantly higher than the level found in T cells co-cultured with BCG-infected DC. As expected, a robust expansion of IFN-

γ-producing naïve T cells was stimulated by Mtb-infected DC. Interestingly, IL-17 levels measured in co-cultures of T cells with either SO2- or Mtb-infected DC doubled those observed when T cells were cultured with uninfected or BCG-infected DC (Fig. 4B). Nevertheless, the differences in IL-17 production were not statistically significant.

3.4 IL-23 released by SO2-stimulated DC is required for the expansion of IFN-γ producing T cells

In an attempt to identify how SO2 enables DC with Th1 polarizing properties, the profile of cytokines involved in Th polarization was investigated. Cell culture supernatants were collected 24 h after infection and the production of IL-12, IL-1β, IL-6, TNF-α and IL-23 was analyzed (Fig. 5). According to what we previously published (Giacomini et al., 2001), Mtb infection induced a robust production of IL-12 by DC, whereas SO2- and BCG-stimulated cells failed to do so. Conversely, TNF-α, IL-1β and IL-23 secretion was significantly higher in DC infected with SO2 than the levels found in BCG-stimulated DC cultures. In addition, IL-6 production was similar in response to both BCG and SO2 challenge, while it was strongly induced upon Mtb infection (Fig. 5). However, as expected, a robust expression of TNF-α, IL-1β and IL-23 occurred in DC infected with Mtb.

To investigate whether SO2-induced IL-23 could account for the expansion of a Th1-cell response in the absence of IL-12, naïve CD4⁺ T cells were stimulated with anti-CD3/CD28 beads in combination with supernatants obtained from autologous or heterologous DC cultures infected with BCG, SO2 or Mtb for 24 h (Fig. 6), as previously described by Gerosa and collaborators (Gerosa et al., 2008). This experimental setting allows evaluating exclusively the contribution of soluble factors, released from infected DC, on T cell polarization without bias due to differences in DC viability or maturation. The addition of supernatants derived from SO2-stimulated DC resulted in an im-

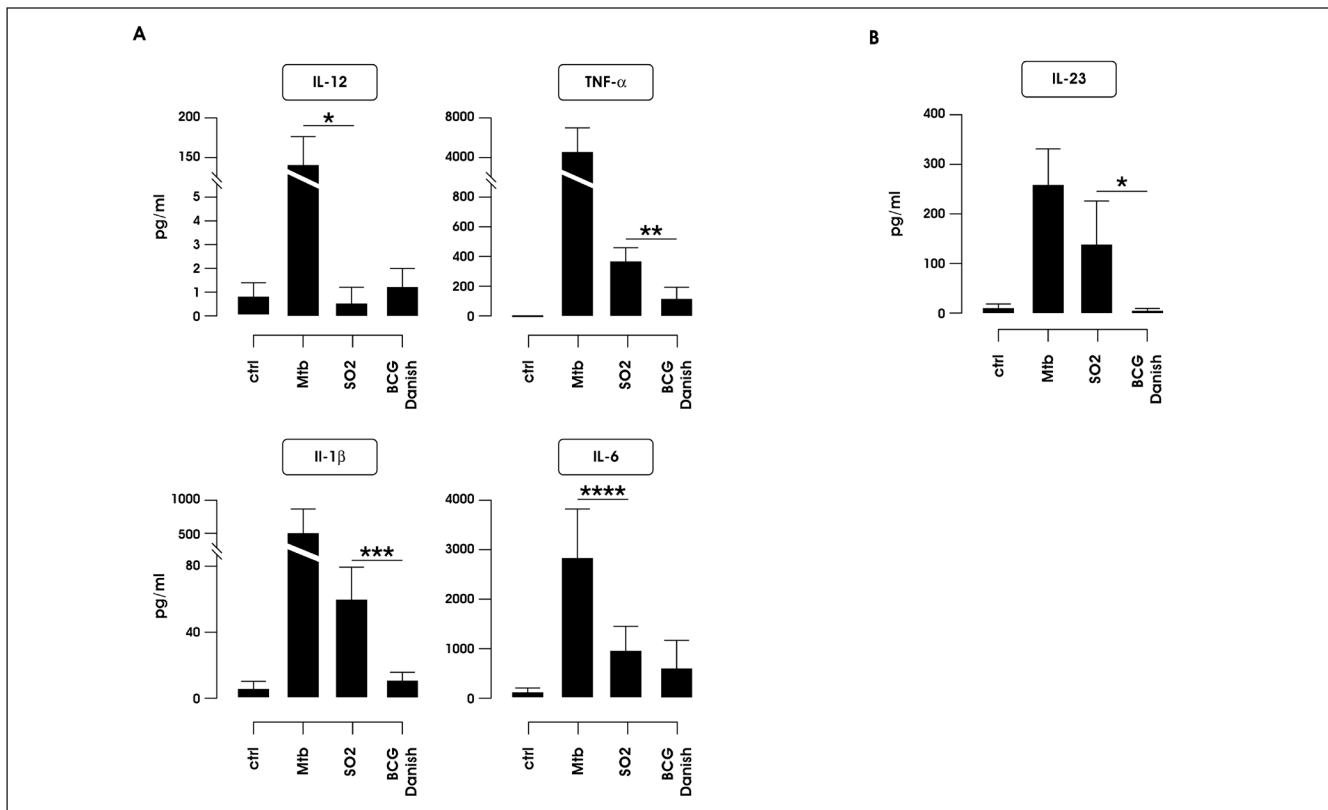


Fig. 5: SO2 stimulates a specific cytokine profile in DC

DC were left untreated or infected with Mtb, SO2 and BCG Danish for 24 h. (A) The production of IL-12, TNF- α , IL-1 β and IL-6 was evaluated by cytometric bead array inflammation kit. (B) IL-23 production was measured with ELISA. The results represent means \pm SEM of 7 independent experiments. Significance was calculated by Student's t-test; p-values: * \leq 0.003; ** = 0.02; *** = 0.03; **** = 0.01.

proved expansion of IFN- γ producing T cells, which, however, was lower than that induced by Mtb-conditioned supernatants (Fig. 6). Interestingly, we observed that the neutralization of IL-23 activity by the addition of an anti-IL-23 p19 Ab significantly reduced the expansion of IFN- γ producing CD4⁺ T cells, while the isotype control Ab failed to do so. Collectively, these results suggest a role for the IL-23 pathway in driving Th1 differentiation in response to SO2 challenge in the absence of IL-12, as recently suggested by Gopal and colleagues in a model of BCG vaccination in mice (Gopal et al., 2012).

4 Discussion

A good vaccine candidate needs to mimic as close as possible the natural infection without causing disease and, at the same time, should elicit an immune response more efficient and incisive than those induced by vaccines currently in use. Advances in the fields of immunology and Mtb pathogenesis, vaccine studies and technological developments make possible the construction of new, effective and safe vaccines against TB (Marinova et al., 2013). The current global TB preventive vaccine portfolio includes two main strategies: the subunit vaccines, which deliver immunodominant mycobacterial Ags and aim at increasing pro-

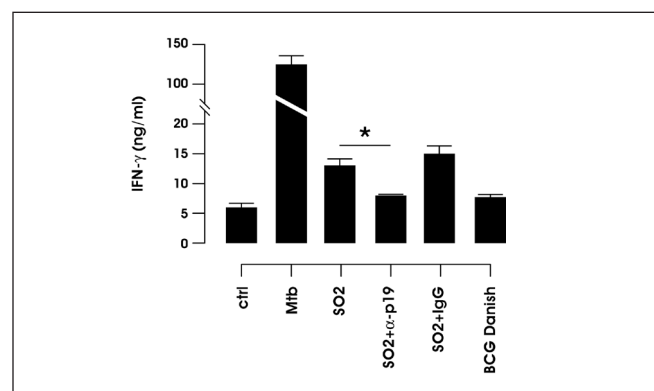


Fig. 6: SO2-induced IL-23 production contributes to the expansion of IFN- γ producing cells

Naïve CD4⁺ T cells were stimulated with anti-CD3 / CD28 magnetic beads in the presence of supernatants derived from DC cultures untreated or infected for 24 h with Mtb, SO2 and BCG Danish. Where indicated, 1 μ g/ml of anti-human IL-23 p19 or IgG κ isotype Abs were added to supernatants of DC cultures infected with SO2. IFN- γ production was evaluated on harvested supernatants by ELISA. The results shown represent means \pm SEM of 6 independent experiments. Significance was calculated by Student's t-test, p-value: * = 0.008.



tection over that of the present BCG; and priming live vaccines based either on genetically manipulated BCG strains or on *Mtb* attenuated strains (Rowland and McShane, 2011). On the other hand, given current understanding of immunology and protective immunity in TB, it is difficult to apply meaningful criteria to the selection of the better or best candidate from a plethora of likely possible novel vaccines (Barker et al., 2012). In this context, it must be kept in mind that the information obtained from animal models of disease and vaccination may not necessarily reflect the outcome in humans (Ernst, 2012). For instance, although mice, guinea pigs and rabbits all provide models for certain stages of TB infection and immunity, none of these animals can efficiently transmit TB. In the case of mice, the commonly used strains (C57/BL6 and BALB/c) are highly resistant to *Mtb* infection and do not form caseous granuloma in lungs, therefore the different lung architecture and the absence of cavitation in infected lungs make difficult the establishment of a murine TB transmission model.

Based on these assumptions, the aim of our study was to compare, in the context of a human-based preclinical model, the capacity of SO2 prototype vaccine to the current vaccine BCG to stimulate the immunomodulatory properties of human DC, which are critical regulators of vaccine-induced immunity. Firstly, we evaluated SO2 virulence by studying the apoptotic process in DC. Apoptosis is a mechanism widely used by intracellular pathogens to favor infection outcome (Aguilo et al., 2013). In the case of *Mtb*, the role of apoptosis induced in macrophages by mycobacteria has been amply discussed, generating controversial data (Briken and Miller, 2008). Nevertheless, apoptosis induced by mycobacteria in human DC is poorly studied. Our results indicated that the virulent *Mtb* strain H37Rv induces a classical apoptotic phenotype in human DC, which correlates with phosphatidylserine exposure, and caspase-3 activation. Conversely, SO2 and BCG attenuated strains do not promote apoptosis in maturing DC, which, on the contrary, remain alive over the time required for T cell stimulation. As DC are one of the most important APC of the immune system, *Mtb*-induced cell death could represent a mechanism of virulence used by *Mtb* to impair adaptive response establishment. Therefore, the obtainment of an attenuated phenotype in SO2 is crucial to elicit a protective T cell response via a proper DC activation, which is one of the main features for a promising vaccine. The absence of apoptosis in human DC infected with SO2 correlates well

with animal model data (see Tab. 2). Indeed, C57BL/6 mice, intranasally challenged with SO2, showed neither active caspase-3 activation nor positive TUNEL staining in the lung, as observed in granulomatous lung lesions of *Mtb* infected mice (Aguilo et al., 2013; Seimon et al., 2010). Accordingly, safety studies on SO2 conducted in mice, guinea pigs and non-human primates showed that the first attenuated live *Mtb* vaccine possesses a safety profile comparable to BCG vaccine (Cardona et al., 2009; Martin et al., 2006; Verreck et al., 2009).

The attenuated phenotype, however, does not alter the immunogenic properties of SO2. In particular, SO2 did not inhibit the constitutive autophagic process of human DC. This result is interesting, taking into account that autophagy plays an important role in the regulation of the immune response against *Mtb* (Bradford et al., 2013; Romagnoli et al., 2012). Having found that only *Mtb* blocked autophagy, we confirmed previous data showing that a functional ESX-1 secretion system is required for this inhibition (Romagnoli et al., 2012), since neither SO2 nor BCG alter this process in the infected DC. Another positive feature of SO2 candidate relies in its capacity to induce the expression of maturation markers involved in T cell stimulation as well as the release of inflammatory and regulatory cytokines, such as IL-23, IL-1 β , TNF- α and IL-6, whose crucial role in tuning the fate of the Th-mediated immune response has been previously described (Giacomini et al., 2001, 2009; Cooper et al., 2011; Cooper and Torrado, 2012). The cytokine cocktail released by SO2-infected DC contributes to the expansion of both Th1 and Th17 cells. In this context, we were able to confirm the key role of IL-23 in promoting the expansion of Th1 cells and, in turn, in driving the immune response against *Mtb* infections, as previously described in a mouse model (Khader et al., 2007; Desel et al., 2011).

Collectively, our results indicate that the experimental setting based on human primary DC represents a robust biological platform to predict the immunological potential of novel vaccine strains within a human *in vitro* system and, therefore, could be considered as a new tool for the preclinical assessment of immunogenicity of TB vaccine candidates in the context of an animal-free model. In addition, the use of human DC will facilitate the preclinical down-selection process of different vaccine candidates in line with the principle of the 3Rs, which promotes the development of alternative test methods for reduction of animal use in research.

Tab. 2: Comparison of *in vitro* results from human DC with *in vivo* data in animal models

	Human DC	Animal model
Immunogenicity	SO2-infected DC stimulated a robust production of IFN- γ from naïve T cells	Immunization of mice and rhesus macaques with SO2 resulted in enhanced expansion of <i>M. tuberculosis</i> -specific CD4 ⁺ T cells compared with BCG vaccine (Nambiar et al., 2012; Verreck et al., 2009)
Virulence	SO2 does not induce apoptosis in DC	SO2 does not induce apoptotic events in mouse lung infection (Aporta et al., 2012)
Protection	Not applicable	SO2 protects against <i>Mtb</i> infection in mice, guinea pigs and non human primates (Cardona et al., 2009; Martin et al., 2006; Verreck et al., 2009)

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Conflict of interest

Carlos Martin is co-inventor on a composition of matter patent "tuberculosis vaccine" filed by the University of Zaragoza. There are no other conflicts of interest.

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