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Monocyte-derived dendritic cells early exposed to *Mycobacterium tuberculosis* induce an enhanced T helper 17 response and transfer mycobacterial antigens

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ABSTRACT

Tuberculosis (TB) is a complex disease, and the success of the bacterium depends on its ability to evade the immune response. Previously, we determined that *Mycobacterium tuberculosis* (*Mtb*) impairs the function of dendritic cells (DC), promoting the generation of cells that are poor stimulators of mycobacterial antigen-specific CD4T cells, which are required to control this persistent infection. In this study, we aimed to determine the mechanisms by which monocyte-derived DCs differentiated in the presence of *Mtb* (*Mtb*DC) may impact on the proliferation of specific anti-mycobacterial T cells. We found that the presence of *Mtb* during monocyte-derived DC differentiation favours T helper (Th) 2 and Th17 polarization, in detriment of a Th1 response, compared to DC mature with *Mtb*. The bias on T cell polarization was associated to the profile of C-type lectin receptors expression found in *Mtb*DC (DC-SIGN^{low}/MR^{low}/Dectin-1^{high}). Alternatively, *Mtb*DC release *Mtb* antigens (Ag) that can be taken up and presented by bystander DC, promoting the proliferation of CD4T cells, but to a lesser extent than direct presentation by *Mtb*-matured DC. In summary, we have further characterized the generation of *Mtb*DC as an effective evasion strategy driven by the pathogen, leading to the inhibition of Ag-presentation and bias of T cell polarization towards Th2 and Th17 profiles, features which partially explain the persistence of *Mtb* in the host.

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1. Introduction

Tuberculosis (TB) remains a major health problem, with million deaths and 9 million new cases annually (WHO, 2014). At the same time, one third of the world's total population is predicted to be infected with the etiological agent, *Mycobacterium tuberculosis (Mtb).* Fewer than 10% of the infected individuals will eventually develop disease. The persistence of *Mtb* in discrete lesions in healthy individuals indicates that although the immune system is highly effective in containing the pathogen, it fails to eradicate *Mtb* (Barry et al., 2009; Kaufmann, 2002). The chronic nature of this infection implies that the bacilli have developed strategies to avoid both the innate and adaptive immune responses (Baena and Porcelli, 2009; Bhatt and Salgame, 2007).

The recognition of *Mtb* is mediated by variety of receptors expressed on innate immune cells, including the toll-like receptors, complement receptors, nucleotide oligomerization domain like receptors, scavenger receptors, Fc receptors (*e.g.* CD14), and C-type lectin receptors (CLR), such as dendritic cell-specific intercellular adhesion molecule grabbing nonintegrin (DC-SIGN), mannose receptors (MR) (Hossain and Norazmi, 2013). Interaction of mycobacterial ligands with pattern recognition receptors (PRR) provokes macrophages and dendritic cells (DC) to secrete cytokines, which in turn modulate the activation of T cells (Geijtenbeek and Gringhuis, 2009; van Crevel et al., 2002). Since immune control of persistent infection with *Mtb* requires a sustained pathogen-specific CD4T cell response, alterations in the generation and maintenance of *Mtb* protective CD4T cells are central for determining the outcome of the infection. Moreover, the development of T helper 1 cells (Th1) cells is crucial for mounting an effective acquired immune response given that Interferon (IFN)-γ activates the killing mechanisms of infected macrophages. In fact, IFN-γ derived from CD4T cells is essential for host survival and enhances CD8T cell function during the infection (Green et al., 2013). In this context, DCs play a decisive role in orchestrating the

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course of the immune response by regulating the differentiation of CD4T cells into Th effectors, such as Th1, Th2, and Th17 cells, among others (Harrington et al., 2005; Park et al., 2005; Tato and O'Shea, 2006; Wynn, 2005). DCs display an extensive capacity for antigen (Ag) uptake in the periphery. After Ag-internalization, DCs migrate to the draining lymph node where they present antigenic peptides, generated by intracellular Ag-processing, to orchestrate naïve T cells activation (Amigorena and Savina, 2010; Buckwalter and Albert, 2009). The processes of Ag-collection and -presentation to T cells are not necessarily carried on by the same single cell, but rather can be performed through collaboration between various DC subsets (Wakim and Bevan, 2011). As DCs dictate T cells polarization, it is reasonable to consider that *Mtb* may interfere with DC generation leading to immune evasion. In a previous study, we demonstrated that the contact between viable (or irradiated-*Mtb)* and monocytes leads to the impairment of monocyte-derived DC differentiation, generating a cell population that is characterized by a poor capacity to induce specific anti-mycobacterial T clones proliferation (Balboa et al., 2010). This escape mechanism becomes relevant given that the major DC subset involved in mycobacterial infections are monocyte-derived DC, otherwise known as 'inflammatory DC' (Humphreys et al., 2006; Reljic et al., 2005). In the present study, we determined the mechanisms by which DC differentiated in the presence of *Mtb* may impact on the proliferation of specific anti-mycobacterial T cells, and consequently dictate the outcome of the infection.

2. Methods

2.1. Subjects

Blood samples from twenty healthy subjects were provided by the Blood Transfusion Service, Hospital Fernandez, Buenos Aires (agreement number CEIANM-52-5-2012). Also, ten healthy tuberculin (PPD)-test status positive volunteer blood donors (average age: 43 years, range: 28–62) were evaluated. As BCG vaccination is compulsory in Argentina, PPD is considered positive if the induration is bigger than 15 mm. The research was carried out in accordance with the Declaration of Helsinki (2013) of the World Medical Association.

2.2. Antigens

The *Mtb* γ-irradiated H37Rv strain was obtained from BEI Resource, USA.

2.3. Generation of monocyte-derived DC

Mononuclear cells from peripheral blood samples were isolated by Ficoll-Hypaque gradient. A total of 5×10^6 per ml mononuclear cells were seeded in six-well plates (Corning, NY, USA) for 2 h in RPMI-1640 (Gibco Lab., NY, USA) supplemented with 2% fetal calf serum (FCS) (Gibco) for adherence. The mean purity of adherent monocytes was 85% (range: 80–92%). Immature DCs (iDC) were generated from monocytes by the addition of recombinant IL-4 (20 ng/ml) (R&D Systems, Minneapolis, MN, USA) and granulocyte-macrophage colony-stimulating factor (GM-CSF, 50 ng/ml) (Peprotech Inc., NJ, USA) for 6 days in RPMI-1640 supplemented with penicillin–streptomycin and 10% FCS (complete medium) at 37 °C in 5% CO_2. To generate *Mtb*DC, *Mtb* was added to monocytes at different ratios (bacilli:cell) at the start of the differentation towards DC as previously described (Balboa et al., 2010).

2.4. DC maturation

iDC were washed and seeded in 24-well plates (Corning) at 1×10^6 cells per ml in complete medium, and their maturation was achieved by treatment with either *Mtb* (2×10^6 bacilli per ml, mDC-*Mtb*) or LPS (10 ng/ml, mDC-LPS) for 24 h at 37 °C. Cells were centrifuged at 800 rpm for 10 min to selectively spin down cells while extra-cellular bacteria remained in the supernatant. When indicated, conditioned media derived from iDC, mDC-*Mtb* or *Mtb*DC were used at different doses to induce DC maturation. These cells were then washed three times, and their phenotype and functionality were evaluated together with survival of activated cells; cell number and viability was determined by trypan blue exclusion assays.

2.5. Purification of autologous CD4T cells

Autologous non-adherent cells from the PBMC fraction were maintained in culture with suboptimal dose of recombinant interleukin (IL)-2 (1 nM; Peprotech Inc.) for 6 days until their use in proliferation assays. The purity of CD4T cells in the non-adherent fraction was about 51% (range: 40–65%). After 6 days of culture, CD4T cells were isolated from the non-adherent fraction using the column-based untouched MACS separation CD4T Cell Isolation Kit from Miltenyi Biotec (Auburn, CA, USA), obtaining a purity average of CD4T cells of 92% (range: 87–95%). The phenotype and functionality of CD4T cells was evaluated by determining the activation markers CD25 and CD69 before and after stimulation with 20 ng/ml phorbol myristate acetate (PMA, SIGMA, Chem. Co, St. Louis Mo, USA) and 1 μM Ionomycin (Io, SIGMA) for 4 and 24 h. Cells were counted and viability was determined by trypan blue exclusion assays.

2.6. Proliferation assays

Specific lymphocyte proliferation (recall) assays were carried out in cells from healthy PPD^+ donors by culturing DC populations and autologous carboxyfluorescein succinimidyl ester (CFSE)-labelled CD4T cells at a ratio of 10 to 1 DC in round bottom 96-well culture plates (Corning) for 5 days as detailed previously (Balboa et al., 2013). The numbers of DCs were adjusted to live cells before the start of the co-cultures. When indicated, monoclonal antibodies (mAb) were added to inhibit human IL-1β (1 μg/ml, Sigma) or human Dectin-1/CLEC7A (3 μg/ml, R&D) and their corresponding isotype antibodies as mock controls (mouse IgG1, clone MOPC 21 from SIGMA; and mouse IgG2B, clone 20116 from R&D). In order to gate out dead lymphocytes, the gate of CD4T cells with increased SSC and low FSC was excluded (Swat et al., 1991). Of note, this exclusion gate represented less than 15% of total CD4T cells for all evaluated conditions.

2.7. Immunofluorescence analysis

The FITC-, PE- or PE-Cy5-labelled mAbs were used for the phenotypic analysis of the following cell-surface receptor repertoires: i) CD1a, CD14, CD80, CD86, HLA-DR, and CCR7 from eBioscience (San Diego, CA, USA), ii) DC-SIGN (CD209) and Dectin-1 (CLEC7A) from R&D, iii) MR (CD206) and CD25 and CD69 from BD Pharmingen (San Diego, CA, USA), and iv) CD4 from Biolegend (San Diego, CA, USA). Approximately 5×10^5 cells were seeded into tubes and washed once with PBS containing 0.2% FCS. Cells were stained for 30 min at 4 °C and washed twice. In the case of CCR7 staining, cells were washed at room temperature and stained at 37 °C for 30 min. Stained populations were gated according to its forward scatter (FSC) and side scatter (SSC) properties analyzed on FACScan (Becton Dickinson). Isotype matched controls were used

to determine auto-fluorescence and non-specific staining. Analysis was performed using the FCS Express (De Novo Software) and results were expressed as mean fluorescence intensity (MFI) or percentage of positive cells.

To determine intracellular cytokines in CD4T cells, brefeldin A (5 μg/ml; Sigma Chemical Co.) was added for the last 4 h of culture to block cytokine secretion. As positive control of cytokine production by CD4T cells, PMA (20 ng/ml) and ionomycin (Io, 1 μM) were added together with brefeldin A for the last 4 h. Thereafter, CFSE-labelled lymphocytes were stained with anti-CD4-PerCP-Cy5.5 mAbs and fixed with 0,5% paraformaldehyde for 15 min. Cells were then permeabilized with 500 μl Perm2 (Becton Dickinson, Cockysville, MD, USA) for 10 min and were incubated with PE-labelled anti-IFN-γ (Invitrogen, California, USA), -IL-4 (BD Pharmingen), -IL-10 (BD Pharmingen), and -IL-17A (R&D) mAbs. For the assessment of intracellular DC cytokine content, cells were harvested after a challenge with *Mtb* or LPS either for 6 h to measure tumor necrosis factors (TNF)-α and IL-1β, or 24 h to measure IL-10 and IL-12p70. Cells were then fixed and permeabilized (as mentioned above) and incubated with anti-IL-10, -IL-12p70 (BD Pharmingen), -TNF-α (eBioscience) or -IL-1β (eBioscience) mAbs.

2.8. Soluble cytokines determinations

Supernatants from DC populations were harvested after a 24-h challenge with *Mtb* or LPS, and the assessment for TNF-α, IL-1β, IL-10, IL-12p70, IL-6, and transforming growth factor (TGF)-β production was measured by ELISA, according to manufacturers' instructions (IL-10, IL-12p70, TNF-α, and TGF-β from eBioscience; IL-1β and IL-6 from Peprotech). The detection limit was 3 pg/ml for TNF-α; 8 pg/ml for IL-10, IL-12p70 and IL-6; and 15.6 pg/ml for IL-1β and TGF-β. Also, supernatants were harvested at day 5 from co-cultures between autologous CD4T cells and DC populations, or from PMA/Io stimulated T cells, and the IFN-γ, IL-4 and IL-17A production was measured by ELISA, according to manufacturer's instructions (eBioscience). The detection limit was 3 pg/ml for IL-17A, and 6.25 pg/ml for IFN-γ and IL-4.

2.9. Chemotactic activity of DC

Each DC population (4×10^5 cells in 75 μl) was placed on the upper chamber of a Transwell insert (5 μm pore size, 96-well plate; Corning), and 230 μl of media (RPMI with 0.5% FCS) with different amounts of human recombinant CCL21 (Peprotech) were placed in the lower chamber. After 3 h, cells that had migrated to the lower chamber were removed and analyzed. The relative number of cells migrating was determined on a flow cytometer using Calibrite beads (BD Biosciences, San Jose, CA, USA), where a fixed number of beads was included in each sample and the number of cells per 1000 beads was evaluated. Data were normalized to the number of initial cells.

2.10. Apoptosis determination assay

The binding of FITC-Annexin V was measured in *Mtb*DC, iDC and mDC-*Mtb*. For this, 100 μl of 1× annexin-binding buffer (25 mM HEPES, 140 mMNaCl, 1 mM EDTA, pH 7.4, 0.1% bovine serum albumin) containing 5 μl of FITC-Annexin V and 1 μl of 100 μg/ml propidium iodide (PI) was added to the cells for 15 min at room temperature according to the manufacturer's instructions (Molecular Probes, Oregon, USA). Thereafter, cells were immediately analyzed by flow cytometry.

2.11. Generation of conditioned media and fractions

For the generation of the exosome-fractions, DCs were cultured in medium with FBS-exosome-free; serum-derived exosomes were depleted by ultracentrifugation of RPMI FBS (20%) overnight at 100,000*g*, 4 °C, and filtered through 0.22 μm pore-size membrane. Monocytes were cultured with RPMI exosome-free-FBS (10%) in the presence of IL-4 and GM-CSF, under *Mtb* challenge (or not), in order to obtain the different DC populations. Thereafter, the conditioned media (CM) was collected from iDC, mDC-*Mtb*, *Mtb*DC, monocytes and *Mtb*-stimulated monocytes. These CM were then centrifuged sequentially at 300*g* for 10 min, 2000 *g* for 10 min, and 10,000 *g* for 30 min; for each centrifugation the pellet was removed and the supernatants were filtered through 0.22 μm pore-size membrane to ensure the removal of any remaining intact bacteria. When indicated, CM was further processed in order to obtain two fractions by ultracentrifugation at 100,000*g* for 70 min to separate the exosome-containing pellets and the supernatants termed "SUP". Finally, pellets were washed in PBS by an extra ultracentrifugation at 100,000*g* for additional 70 min to yield the pellet fraction called "Ex" (Théry et al., 2006).

2.12. Mycobacterial Ags determination

To determine the presence of Ags in the CM from the different DC populations, we adapted a home-made ELISA protocol based on our previous work (Schierloh et al., 2014). Briefly, serum from six TB patients with high antibodies titer against mycobacterial Ags (S27, S36, MDR9, MDR10, MDR18 and MDR19, see Schierloh et al., 2014) were pooled and used as "primary antibody" in the ELISA assay (Schierloh et al., 2014). ELISA plates were coated with the CM from the different DC populations, or from cells challenged with (5×10^5) heat-killed *Mtb* (1 h at 95 °C) or one tuberculin unit of PPD (from the Instituto Nacional de Producción de Biológicos, Malbran, Buenos Aires, Argentina) for 24 h. After coating, the wells were washed extensively, blocked by adding PBS 1X with 5% BSA (for 1 h at room temperature) and incubated overnight in the presence of the pool of TB sera (1/200 dilution). After washing extensively, anti-human polyvalent immunoglobulin conjugated to peroxidase (1/2000, SIGMA) was added for 1 h at room temperature, wash again, and the substrate (TMB from eBioscience) was added. Reaction was stopped by adding sulphuric acid and absorbance was detected at 450 nm.

2.13. Transwell coculture

iDC (1×10^6/ml each well) were plated in 24-well dishes. Autologous iDC, *Mtb*DC or mDC-*Mtb* were plated (2×10^5/200 μl each well) in transwell chambers (Greiner bio-one, Kremsmünster, Austria) and placed above the wells containing iDC. The transwell allows exchange of the medium, but prevents cell–cell interactions through a physical barrier in the form of PET membranes with 0.4 μm size-pore. After 48 h, the cells from the lower compartment were recovered to evaluate their Ag-presenting capacity in a proliferation assay, as described above.

2.14. Statistical analysis

All values are presented as median ± range of a number of independent experiments. Each independent experiment corresponds to 1 donor. Comparisons between unpaired experimental conditions were made using the Friedman test followed by Dunn's Multiple Comparison Test for non-parametric data. Comparisons between paired experimental conditions were made using the

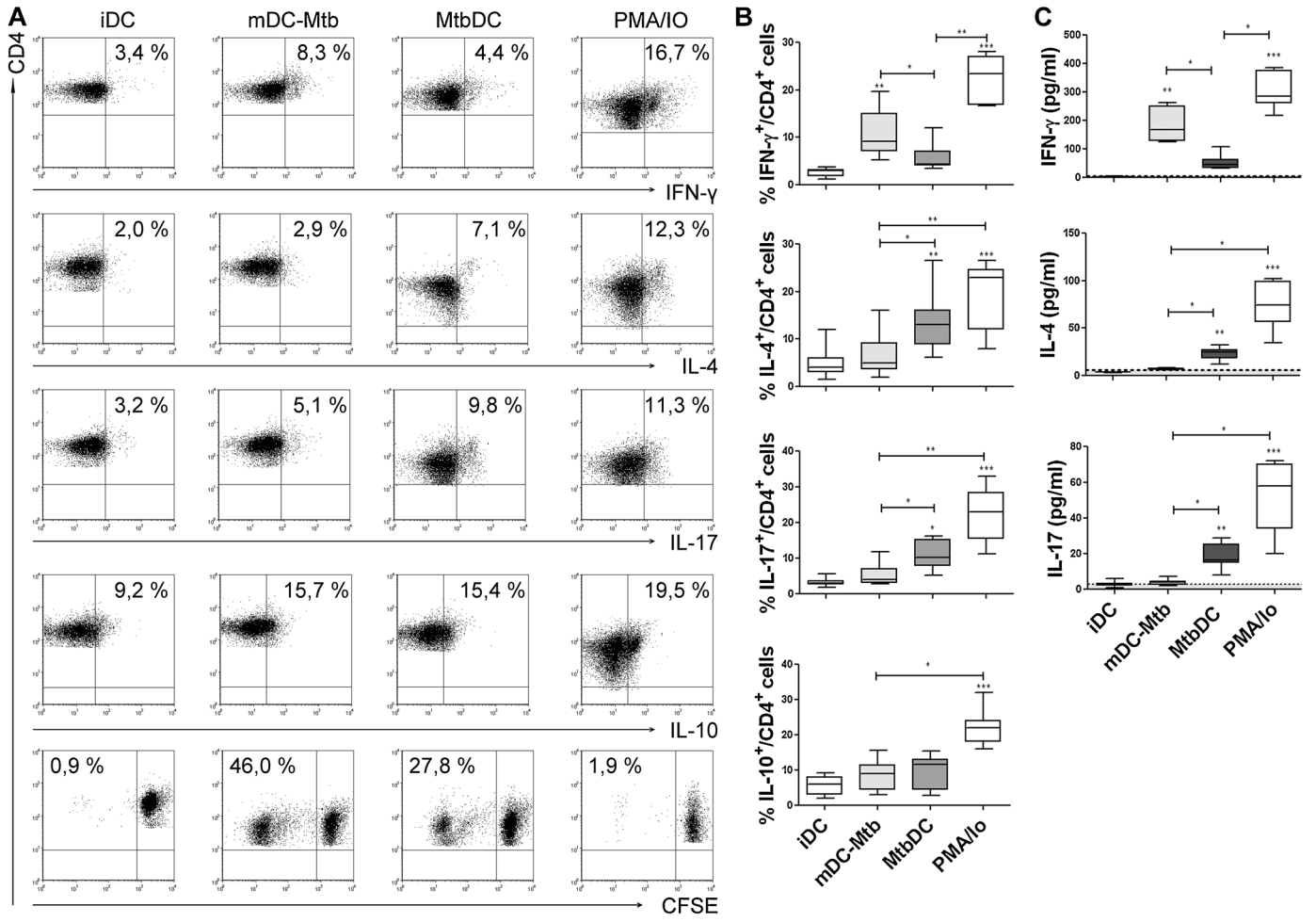

Fig. 1. DCs differentiated in the presence of *Mtb* promote the preferential activation of Th2 and Th17 cells.
Monocytes from PPD⁺ healthy donors were differentiated towards DCs in the presence of *Mtb* either at the beginning of the culture (*Mtb*DC), or at fully differentiated stage day 5 (iDC) for a 24-h challenge (mDC-*Mtb*). All DC populations were co-cultured with CFSE-labelled autologous CD4T cells additional for 5 days. (A) Representative dot plots from 1 independent experiment showing the percentages of CD4T cells positive for cytokine production (IFN-γ, IL-4, IL-17, or IL-10), or CFSE dye, as a result of the co-cultures with different DC populations (iDC, mDC-*Mtb* or *Mtb*DC), or after PMA/Io treatment as positive control. (B) Median percentages of CD4T cells positive for cytokine production induced by iDC, mDC-*Mtb*, *Mtb*DC or PMA/Io. Data were obtained from 9 independent experiments. (C) Extracellular cytokine production (IFN-γ, IL-4, IL-17) measured by ELISA. Data obtained from 7 independent experiments. Friedman test followed by Dunn's Multiple Comparison Test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, condition vs iDC, or as indicated in the graph.

two-tailed Wilcoxon Signed Rank Test for non-parametric data. A p-value of 0.05 was considered significant.

3. Results

3.1. *The presence of* Mtb *during monocyte differentiation towards a DC program determines their ability to polarize anti-mycobacterial Th clones*

In previous studies, we have demonstrated that monocyte-derived DC differentiated in the presence of *Mtb* (2 bacilli per cell) from the beginning of the culture display poor capacity to induce anti-mycobacterial T cell proliferation, including CD1-restricted T-cell responses (Balboa et al., 2010). Regardless whether these cells promise an already characterized subset of DCs or of macrophages, we will called the cells resulting from the culture of monocytes in the presence of *Mtb* as *Mtb*DC because they were generated in the presence of IL-4/GM-CSF. Alternatively, those monocyte-derived DC generated in the absence of *Mtb*, but further exposed to the bacilli once fully differentiated will be called as mDC-*Mtb*. In order to shed light on the capacity of *Mtb*DC to dictate Th cell polarization, we compared the cytokine expression profile displayed by CFSE-labelled T cells from PPD⁺ healthy subjects activated by autologous immature DC (iDC), *Mtb*-mature DC (mDC-*Mtb*) and *Mtb*DC. To accomplish this, DC populations were generated by culturing freshly isolated human monocytes with recombinant IL-4 and GM-CSF for 6 days under the presence (or not) of *Mtb*. Thereafter, the iDC population was challenged for an additional 24 h with *Mtb* (mDC-*Mtb*) or with LPS (mDC-LPS) when indicated (Supplementary Fig. 1A). In agreement with our previous work (Balboa et al., 2010), we confirmed the "monocyte-like" phenotype displayed by *Mtb*DC, characterized by the reduction of the FSC/SSC profile, the preserved monocyte marker CD14, and the lack of the IL-4 driven-DC markers, such as CD1a and DC-SIGN (Supplementary Fig. 1B). Moreover, *Mtb*DC exhibit a mature phenotype in terms of the CD86 expression as observed in mature normally differentiated DC (mDC-*Mtb* and mDC-LPS). In parallel, the non-adherent T cells were maintained in culture for 7 days in the presence of a suboptimal dose of IL-2 as we have previously established (Balboa et al., 2010). In order to verify the functional integrity of 7-days cultured lymphocytes, the levels of the activation markers CD25 and CD69 were measured at day 0 and day 7 of culture with or without PMA/Io stimulation for 4 or 24 h. We also verified that lymphocytes remain inactivated throughout the culture, as judged by the

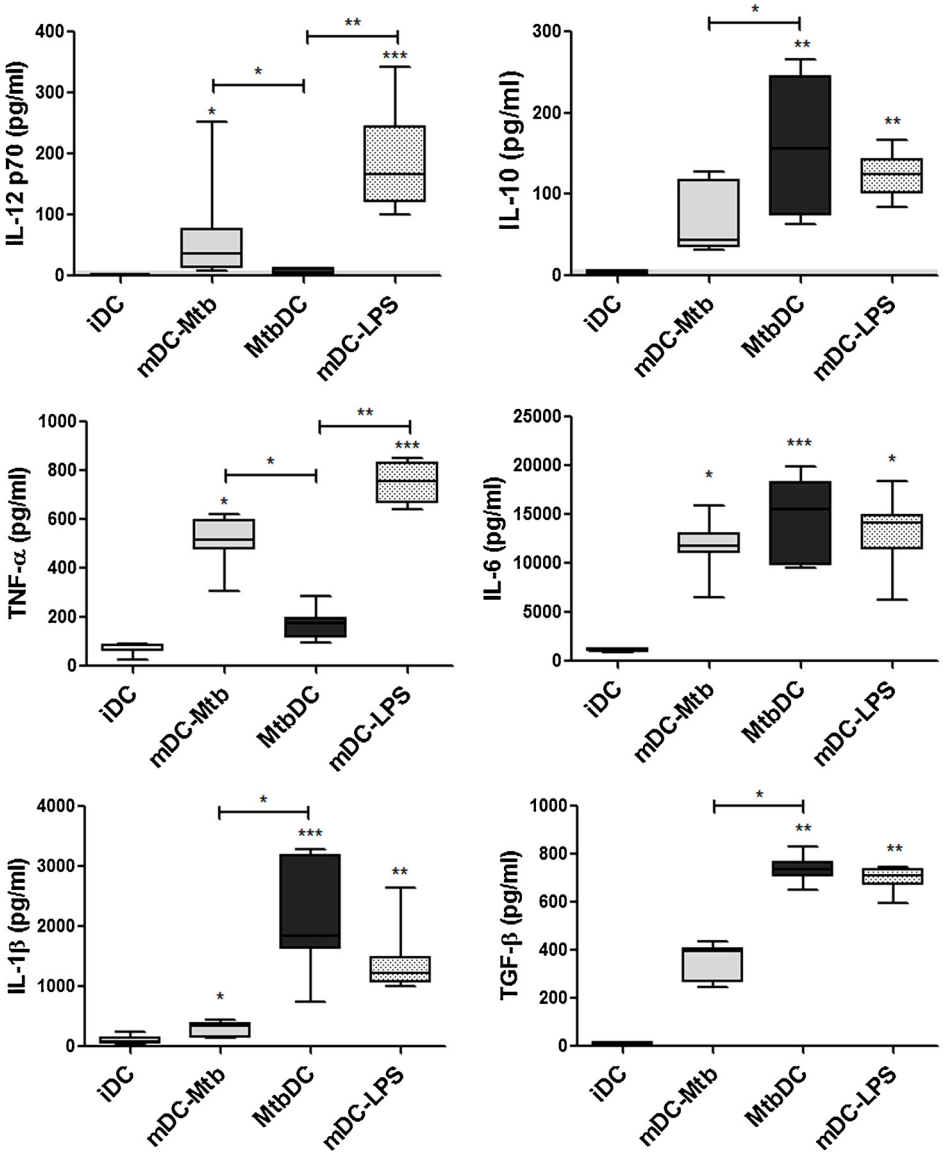

Fig. 2. DCs differentiated in the presence of *Mtb* display an altered production of cytokines.
Monocytes from PPD$^+$ healthy donors were differentiated towards DCs in the presence of *Mtb* either at the beginning of the culture (*Mtb*DC), or at fully differentiated stage day 5 (iDC) for a 24-h challenge (mDC-*Mtb*). Cytokine production by all DC populations was determined by ELISA. A positive control was included in the form of LPS-treated iDC (mDC-LPS). Data were obtained from 7 independent experiments. Friedman test followed by Dunn's Multiple Comparison Test: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$, condition vs iDC, or as indicated in the graph.

lack of CD25 and CD69 acquisition and by the conserved cell viability (Supplementary Fig. 2A-C); these were indeed able to respond to PMA/Io activation at day 7 (Supplementary Fig. 2B). The autologous CD4T cells were then isolated from the cultured non-adherent fraction by immunomagnetic separation (Supplementary Fig. 2D). Thereafter, these lymphocytes were cultured with the different monocyte-derived DC populations for 5 days, and their cytokine expression profile and proliferation was compared to T cells stimulated with PMA/Io (positive control). As it is shown in Fig. 1 and Supplementary Fig. 3, *Mtb*DC induced preferentially the proliferation of IL-4$^+$ and IL-17$^+$ CD4T cells compared to mDC-*Mtb*, which favoured instead the proliferation of IFN-γ$^+$ T cells. In addition, no differences in the percentages of IL-10$^+$ CD4T cells were observed between *Mtb*DC and mDC-*Mtb* (Fig. 1A and B and Supplementary Fig. 3). Therefore, the presence of *Mtb* during the monocytes differentiation results in the generation of monocyte-derived DCs that preferentially induce Th2/Th17 polarization in detriment of a Th1 response.

3.2. Mtb*DC display an altered profile of cytokines expression in response to Mtb*

Considering that the production of different cytokine profiles by DCs can orchestrate Th differentiation, we aimed to determine the cytokine profile expression (*i.e.* IL-12p70, IL-10, TNF-α, IL-6, IL-1β and TGF-β) displayed by *Mtb*DC compared to that in mDC-*Mtb* and mDC-LPS (positive control). As illustrated in Fig. 2, *Mtb*DC produced higher amounts of IL-10, IL-1β, and TGF-β, and lower amounts of IL-12 and TNF-α, in comparison to mDC-*Mtb* and mDC-LPS. Since the CLR family is among the most prominent receptors expressed in DCs that are known to actively engage mycobacterial ligands and modulate cytokine production (Geijtenbeek and Gringhuis, 2009; Hoving et al., 2014), we inferred that *Mtb*DC might display a different CLR repertoire compared to the other monocyte-derived DC populations. For this reason, we studied the expression profile of three relevant CLR that can mediate mycobacterial interactions, such as MR, DC-SIGN and Dectin-1. Unlike Dectin-1, we

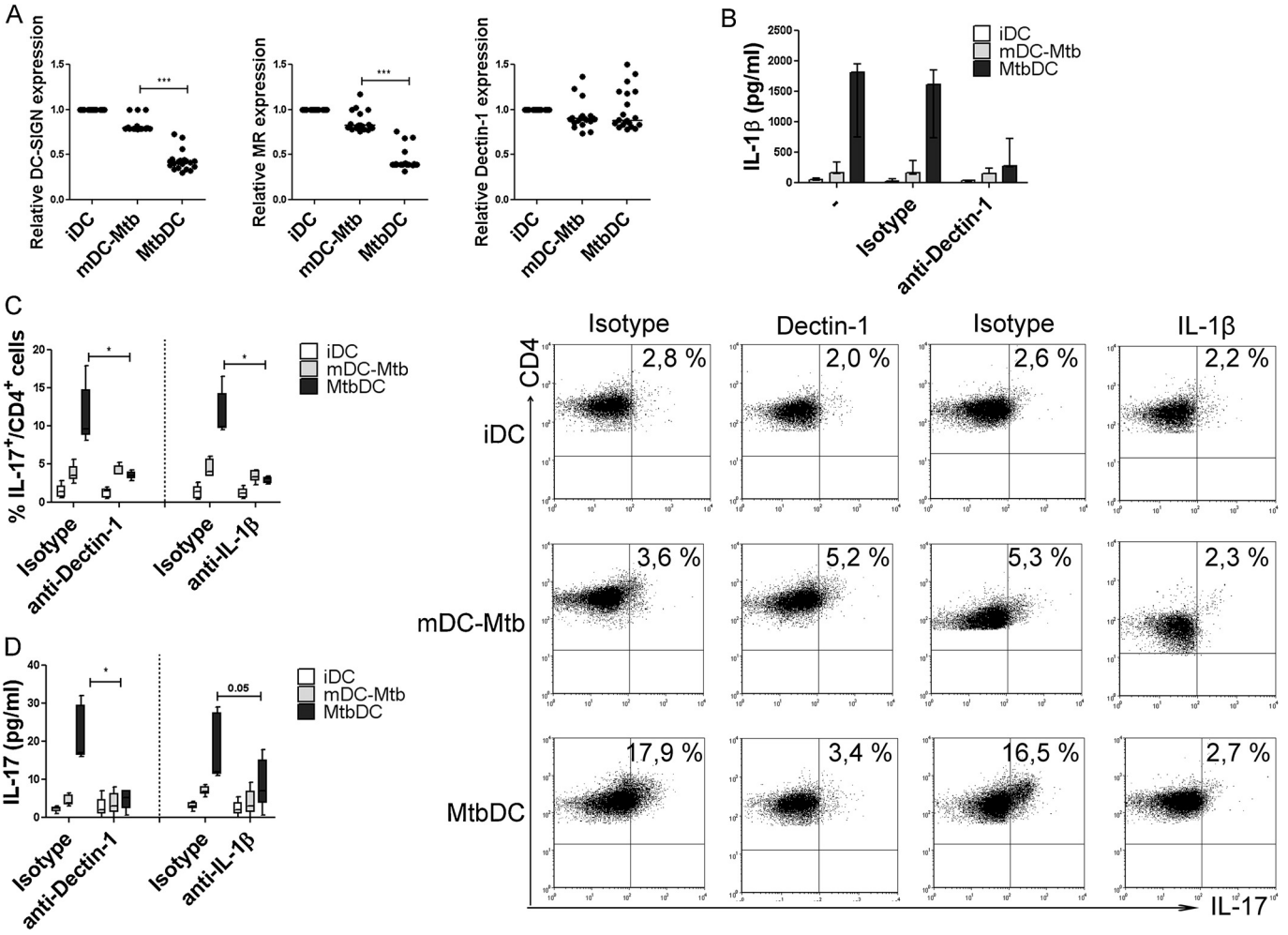

Fig. 3. Dectin-1-driven production of IL-1β and differentiation of Th17 cells by *Mtb*DC.
(A) Relative expression of DC-SIGN, MR and Dectin-1 in iDC, mDC-*Mtb* or *Mtb*DC, as measured by FACS. MFI were normalized to iDC expression. Data represent 20 independent determinations. Wilcoxon signed rank test: ***, $p < 0.001$, for mDC-*Mtb* vs *Mtb*DC. (B) Extracellular IL-1β production by iDC, mDC-*Mtb* or *Mtb*DC, in the presence (or not) of neutralizing antibodies against Dectin-1 or its isotype control. Data represent the median of 3 independent experiments ± interquartile range. (C) Left: Representative dot blots from 1 independent experiment showing the percentages of CD4T cells positive for IL-17 production activated by iDC, mDC-*Mtb* or *Mtb*DC, in the presence of neutralizing antibodies anti-Dectin-1, anti-IL-1β, or their respective isotype controls. Right: Box and whiskers graphs obtained from 5 independent experiments. (D) Extracellular IL-17A production by CD4T cells activated by iDC, mDC-*Mtb* or *Mtb*DC, in the presence of neutralizing antibodies anti-Dectin-1, or anti-IL-1β, or their respective isotype controls. Data are obtained from 5 independent experiments. Wilcoxon signed rank test: *, $p < 0.05$, for isotype vs neutralizing antibodies.

found that MR and DC-SIGN were downregulated in *Mtb*DC compared to mDC-*Mtb* (Fig. 3A). Taking into account that a high ratio of Dectin-1 to DC-SIGN/MR results in higher IL-1β production and increased capacity to generate Th17 by monocyte-derived DCs in response to *Mtb* (Zenaro et al., 2009), we evaluated the effect of inhibiting Dectin-1 in all monocyte-derived DC populations. As shown in Fig. 3B, Dectin-1 inhibition reduced the IL-1β secretion by *Mtb*DC, but not mDC-*Mtb,* in response to challenge. Moreover, either the inhibition of Dectin-1, or the neutralization of IL-1β, resulted in the specific reduction of the proliferation of IL-17$^+$ CD4T cells induced by *Mtb*DC (Fig. 3C), as well as in the overall secretion of IL-17 (Fig. 3D). Altogether, these results argue that a higher ratio of Dectin-1 to DC-SIGN/MR expression in *Mtb*DC leads to an altered cytokine profile characterized by enhanced IL-1β production, and thus a higher capacity by *Mtb*DC to differentiate Th17 cells.

3.3. Mtb*DC can transfer mycobacterial Ags to bystander monocyte-derived DCs*

Beyond the effects of cytokine secretion, we wondered whether T cell polarization could also be altered by soluble factors released by monocyte-derived DCs. For this purpose, we first determined whether the conditioned medium from *Mtb*DC (CM_*Mtb*DC) could modulate the maturation of monocyte-derived DC. Hence, the expression of CD83, CD86 and HLA-DR was measured on iDC exposed for 24 h to CM_*Mtb*DC and compare with those exposed to CM from iDC (CM_iDC), or from mDC-*Mtb* (CM_mDC-*Mtb*). As positive control of maturation, we evaluated the expression of these receptors under the direct challenge with *Mtb* or LPS (Balboa et al., 2010). The addition of CM_iDC did not induce the expression of CD83, CD86 or HLA-DR (Fig. 4). By contrast, their expression level was significantly increased upon exposure to CM_*Mtb*DC or CM_mDC-*Mtb* in a dose-dependent manner (Fig. 4).

Next, we wondered whether iDC exposed to CM_*Mtb*DC and CM_mDC-*Mtb* could also trigger the proliferation of mycobacterial-specific T clones. To accomplish this, we assessed the capacity of these conditioned media from monocyte-derived DCs to promote the proliferation of CFSE-labelled autologous T cells from healthy PPD$^+$ donors. As previously demonstrated (Balboa et al., 2010), *Mtb*DC displayed a reduced ability to induce the proliferation of mycobacterial-specific CD4T cells in comparison to mDC-Mtb (Fig. 5A). Interestingly, iDC exposed to CM_*Mtb*DC induced a

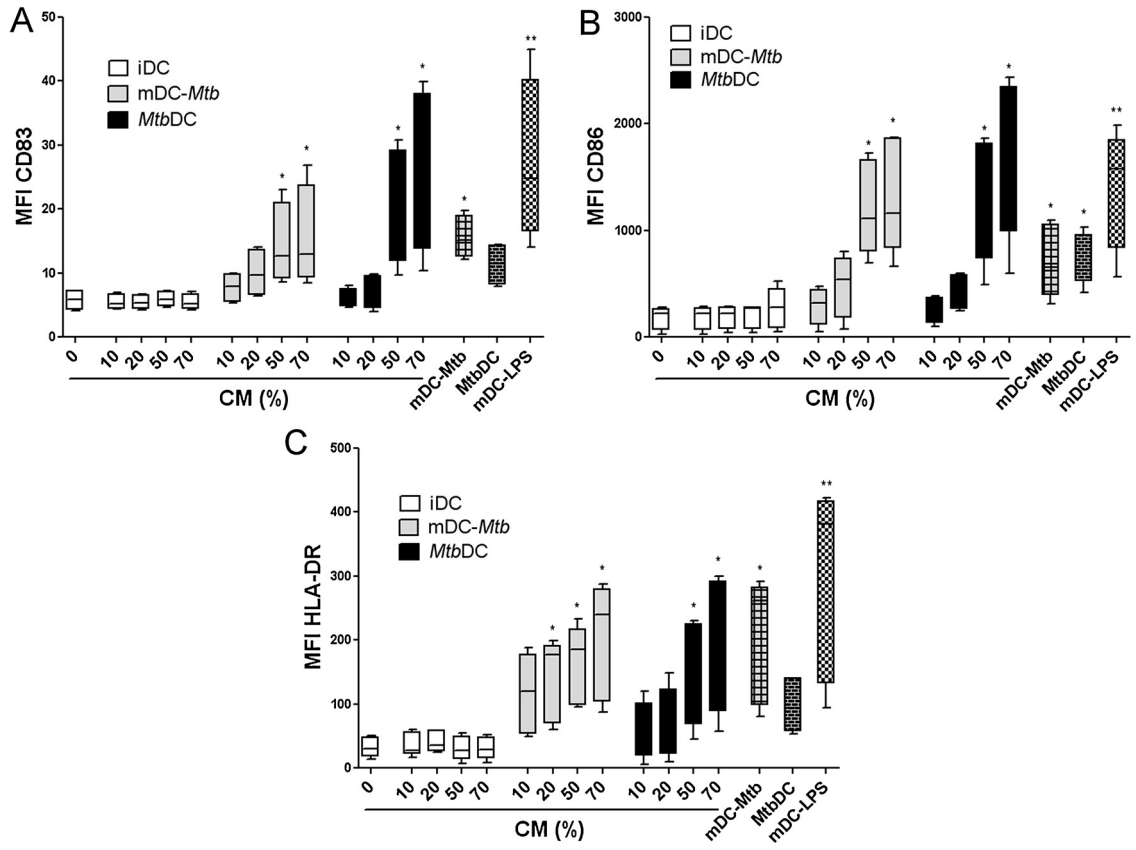

Fig. 4. Conditioned media from monocytes differentiated in the presence of *Mtb* towards DCs can promote DC maturation.
Fully differentiated immature DCs (iDC) were incubated for 24 h in the presence of different percentages of the conditioned medium derived from either iDC (CM-iDC), mDC-*Mtb* (CM-mDC-*Mtb*) or *Mtb*DC (CM-*Mtb*DC). Thereafter the maturation state was evaluated by the mean fluorescence intensity of CD83 (A), CD86 (B), and HLA-DR (C), as determined by flow cytometry. DC matured with *Mtb* (mDC-*Mtb*) or LPS (mDC-LPS) are also shown as positive controls. Data were obtained from 5 independent experiments. Friedman test followed by Dunn's Multiple Comparison Test: *, $p < 0.05$; **, $p < 0.01$; only the differences vs untreated iDC are shown.

significant increase in the levels of autologous T cell proliferation; while iDC exposed to CM_mDC-*Mtb* or media from *Mtb*-challenged monocytes (CM_Mo*Mtb*) did not promote T cell proliferation (Fig. 5A). Moreover, only iDC exposed to CM_*Mtb*DC were able to promote the production of IFN-γ and IL-4 by co-cultured T cells (Fig. 5B).

Based on these results, we inferred that CM_*Mtb*DC can not only promote monocyte-derived DC maturation but also contain mycobacterial Ags. In order to test this hypothesis, we analysed the content of mycobacterial Ags found in the supernatants from iDC, *Mtb*DC, mDC-*Mtb* or mDC-LPS, by ELISA using PPD and heat-killed *Mtb* as positive controls. As it is shown in Fig. 5C, mycobacterial Ags were specifically enriched in the supernatant from *Mtb*DC but not from other monocyte-derived DC population. Another way to measure indirectly the release mycobacterial Ags by monocyte-derived DCs is to characterize the Ag-presenting capacity by bystander DCs, which may efficiently take up and present soluble Ags to T cells, by using a transwell system. To test this, iDC were cultured within the lower compartment of a transwell system containing iDC, *Mtb*DC or mDC-*Mtb* in the upper compartment, and allow the exchange of soluble factors between populations without cell–cell contact. After 48 h, we recovered the cells from the lower compartment and evaluated their Ag presenting capacity. As demonstrated in Fig. 6, proliferation of autologous T cells and IFN-γ release were observed only by iDC exposed to factors released by *Mtb*DC and no other CM. Collectively, these results suggest that soluble Ags could be released from *Mtb*DC and efficiently taken up by bystander monocyte-derived DCs, which in turn could be presented to T cells.

3.4. *Mycobacterial Ags released by* Mtb*DC can occur in the absence of apoptosis and/or exosome release*

It has been proposed that exosomes released by DC could serve as Ag spreaders (Thery et al., 2002). However, it has also been demonstrated that viral Ag transfer from infected ephitelium of the Peyer's patches to $CD8\alpha^{-}/CD11b^{-}$ DCs can happen during the generation of apoptotic bodies (Fleeton et al., 2004). In addition, it has been recently shown that *M. tuberculosis*-infected bone marrow-derived DC can release Ags as soluble, unprocessed proteins (Srivastava and Ernst, 2014). To discriminate among these scenarios, we have addressed whether the Ags released by *Mtb*DC were contained preferentially inside exosome vesicles and/or apoptotic bodies. For this reason, we first determined cell death induction in *Mtb*DC, iDC and mDC-*Mtb*. In agreement with our previous study (Balboa et al., 2010), *Mtb* has a detrimental effect on cell viability when added to monocytes during the differentiation process towards the DC program (*Mtb*DC), or to fully differentiated DCs (mDC-*Mtb*) (Fig. 7A). Moreover, *Mtb* induces cell apoptosis in *Mtb*DC and mDC-*Mtb*, and an incidence of necrosis in *Mtb*DC only (Fig. 7B and C). In order to elucidate whether cell death participates in the release of Ags, *Mtb*DC were generated by exposing monocytes to different amounts of *Mtb* and the recovery of cells was measured at the end of the differentiation process. As illustrated in Fig. 7D, a lower challenge with *Mtb* (1 bacillus to 5 cells, and 1 bacillus to 2 cells) did not result in cell death in monocyte-derived DCs. However, the CM derived from *Mtb*DC (generated from a ratio of 1 bacilli to 5 cells) still activates CD4T cell proliferation and IFN-γ release (Fig. 7E–G), suggesting that cell death is not the main mechanism

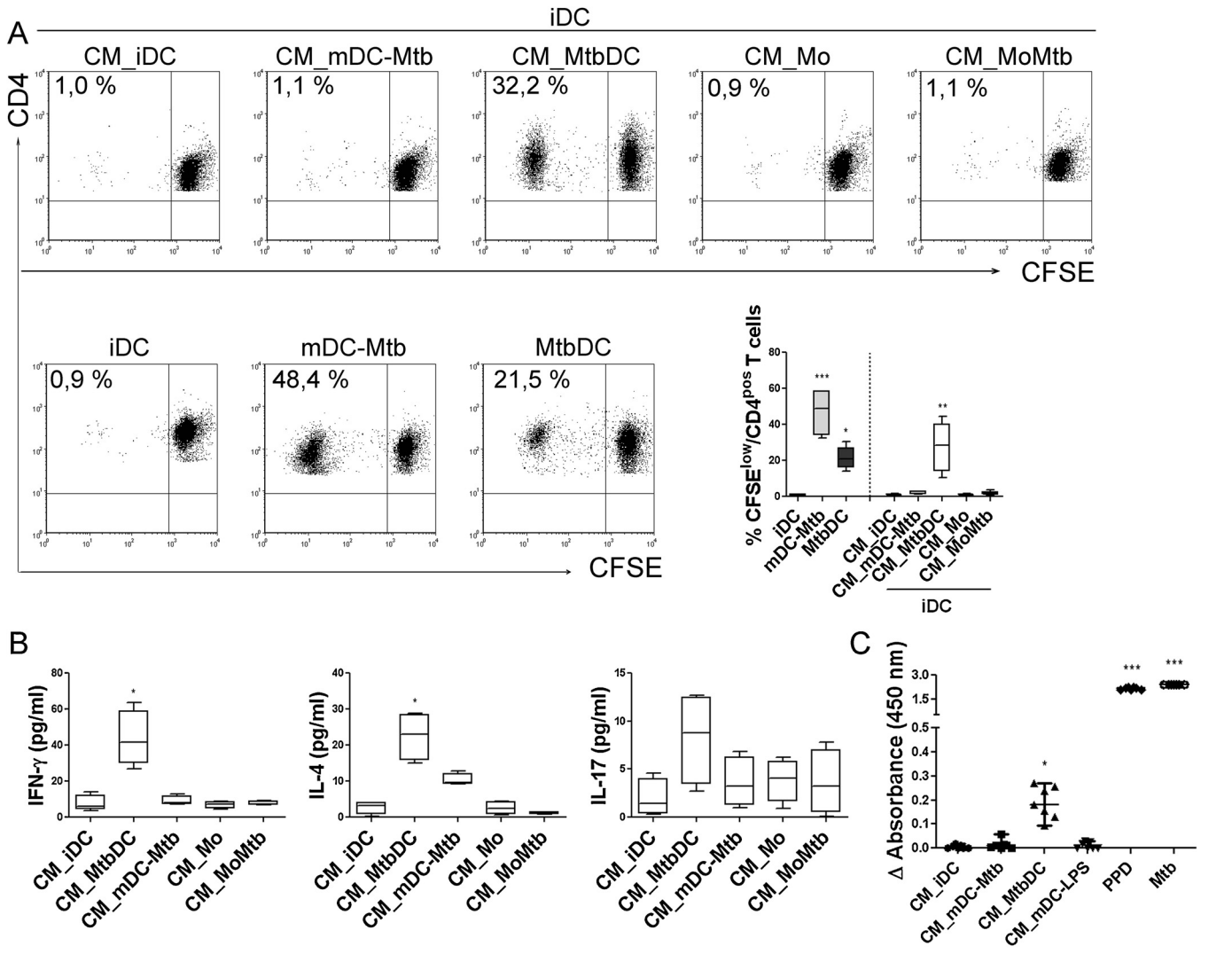

Fig. 5. Monocytes differentiated in the presence of *Mtb* towards DCs can release mycobacterial Ags.
Fully differentiated immature DCs (iDC) from PPD^{pos} healthy donors were exposed for 24 h to 50% of the conditioned medium (CM) from iDC (CM-iDC), mDC-*Mtb* (CM-mDC-Mtb), *Mtb*DC (CM-*Mtb*DC), monocytes (CM-Mo) or *Mtb*-stimulated monocytes (CM-Mo*Mtb*), and then placed in co-culture with CFSE-labelled autologous CD4T cells for 5 days. Alternatively, mDC-*Mtb* or *Mtb*DC were used as antigen-presenting cells. The percentages of specific proliferating CD4 lymphocytes were determined by flow cytometry. (A) Representative dot plots from 1 independent experiment and box and whisker graph showing the percentages of CFSE^{low} CD4T cells obtained from 6 independent experiments. Friedman test followed by Dunn's Multiple Comparison Test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; only the differences vs untreated iDC are shown. (B) Extracellular cytokine production (IFN-γ, IL-4, IL-17) measured by ELISA. Data obtained from 4 independent experiments. Friedman test followed by Dunn's Multiple Comparison Test: *, $p < 0.05$; only the differences vs untreated iDC are shown. (C) Determination of mycobacterial Ags in the CM from iDC, *Mtb*DC, mDC-*Mtb* or mDC-LPS, by ELISA. PPD and heat-killed *Mtb* were included as positive controls. Δ absorbance was calculated as the optical density at 450 nm minus the values at 570 nm. Data were obtained from 7 independent experiments. Friedman test followed by Dunn's Multiple Comparison Test: *, $p < 0.05$; ***, $p < 0.001$; only the differences vs CM-iDC are shown.

responsible for Ag transfer in *Mtb*DC. Furthermore, we evaluated the role of exosome release in the Ag transfer by *Mtb*DC. For that, CM from iDC, mDC-*Mtb* and *Mtb*DC were ultracentrifuged and the antigenic activity of the supernatants and pellets (containing apoptotic vesicles and exosomes) were examined on iDC. Higher antigenic activity was found only after the treatment of iDC with the supernatant fraction from *Mtb*DC, correlating with the release of IFN-γ by CD4T cells (Fig. 7H and I). Taken together, these results show that under these specific experimental conditions, *Mtb*DC may release mycobacterial Ags preferentially as a soluble form instead of being confined inside apoptotic bodies or exosome vesicles.

3.5. *Chemotactic activity of* Mtb*DC*

In order to evaluate the capacity of *Mtb*DC to migrate and transfer mycobacterial Ags to the lymph nodes, we compared the expression of the chemokine receptor CCR7 between all monocyte-derived DC populations. As shown in Fig. 8A and B, *Mtb*DC and mDC-*Mtb* display intermediate levels of CCR7 expression compared to iDC and mDC-LPS, suggesting that these cell populations might be prone to migrate. To test this, the chemotactic activity was compared among iDC, *Mtb*DC, mDC-*Mtb* and mDC-LPS, in response to a gradient containing the CCR7-ligand, CCL21. As expected, mDC-LPS and *Mtb*DC displayed high chemotactic activities in comparison to mDC-*Mtb* and iDC, which displayed a poor capacity to migrate. All things considered, we infer that *Mtb*DC may be able to migrate to lymph nodes and transfer mycobacterial Ags to resident bystander DCs.

4. Discussion

During *Mtb* infection, monocytes are highly recruited to the lungs (Balboa et al., 2015), where they can differentiate into DC or macrophages. Several studies have identified mycobacterial

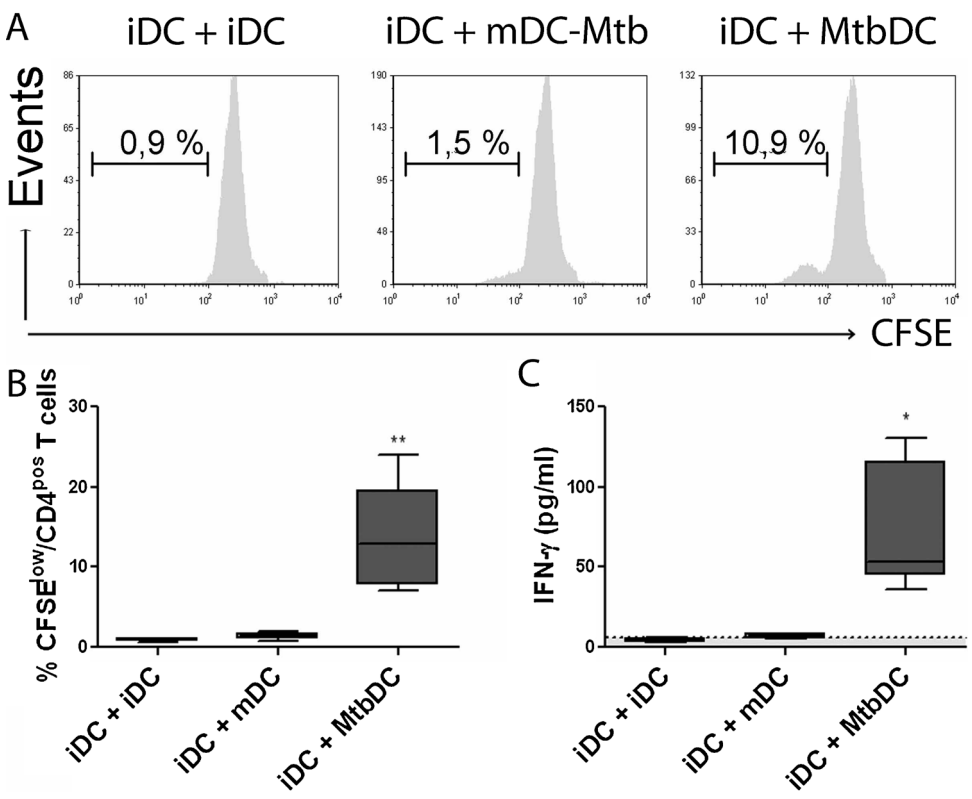

Fig. 6. Mycobacterial Ags released by *Mtb*DC can be further taken up and presented by bystander monocytes-derived DCs.
iDC from PPD$^+$ healthy donors were plated in the lower compartment of a transwell system, and a 0.4 μm pore-size insert was added containing autologous iDC, *Mtb*DC or mDC-*Mtb* (upper compartment). After 48 h, cells were recovered from the lower compartment and co-cultured with CFSE-labelled CD4T cells for 5 days, and the percentages of specific proliferating CD4 lymphocytes were determined by flow cytometry. (A) Representative histograms from 1 independent experiment showing the percentages of CFSE^{low} CD4T cells induced in co-cultured with iDC, mDC-*Mtb* or *Mtb*DC, using the transwell system. (B-C) Box and whisker graphs showing the percentages of CFSE^{low} CD4T cells (B), and the extracellular production of IFN-γ (C), obtained from 6 independent experiments. Friedman test followed by Dunn's Multiple Comparison Test: *, $p < 0.05$; **, $p < 0.01$; only the differences vs iDC + iDC are shown.

pathways capable of limiting Ag-presentation by infected cells, especially via the MHC class II pathway (Harding and Boom, 2010). Clearly, the activation of CD4T cells is critical for generating protective responses against mycobacterial infections (Caruso et al., 1999; Ladel et al., 1995; Mogues et al., 2001). In this context, the early interaction of *Mtb* with monocytes drives an altered differentiation program that promotes the generation of cells (*Mtb*DC) with a poorer Ag-presenting capacity (Balboa et al., 2010; Gagliardi et al., 2009). In the present study, we looked deeper into the potential mechanisms that inhibit the Ag-presentation process during the *in vitro* differentiation of human monocytes into DC in the presence of *Mtb*.

We showed that the interaction of γ-irradiated *Mtb* with monocytes along their differentiation into DCs impairs IL-12 and TNF-α, but favours IL-10, IL-1β, and TGF-β secretion, instructing CD4T cells towards Th2/Th17 differentiation in detriment of the Th1 program. Our data argues that the lower production of IL-12 by *Mtb*DC may be responsible for their poor capacity to induce Th1 polarization. In the case of the activation of Th2 cells, we did not detect the secretion of the master regulator cytokines, IL-4 or IL-13, which could clearly explain the better capacity of *Mtb*DC to activate the Th2 program. While we cannot rule out the possibility that the diminution of Th1 cells may lead by default to induce Th2 polarization, given the mutual antagonism between Th1 and Th2 responses, it could be possible that the increase secretion of IL-6 by *Mtb*DC may indeed shift Th0 towards Th2 cells (Rincon et al., 1997). In the case of the observed Th17 program, our results point out IL-1β as the principal cytokine derived from *Mtb*DC to drive the polarization of Th17 cells. Indeed, *Mtb*DC displayed a 8.5 fold increase for IL-1β in comparison to the 2 fold increase of TGF-β production, relative to the cytokine content in mDC-*Mtb*. Of note, IL-1β plays an important role in Th17 differentiation (Acosta-Rodriguez et al., 2007; Cosmi et al., 2008). Interestingly, the balance between Th1 and Th17 responses has been previously associated to the CLR expression pattern in DCs within the TB context; for example, Dectin-1 expression favours the generation of both Th1 and Th17 cells, whereas that of DC-SIGN or MR actually inhibits Th17 differentiation while increasing Th1 cells (Zenaro et al., 2009). In fact, a cross-talk occurs between the signalling pathways triggered by these CLRs to control the production of IL-1β, where both DC-SIGN and MR inhibit the Dectin-1-driven expression of this cytokine (Zenaro et al., 2009). In our hands, the blockage of Dectin-1 signalling in *Mtb*DC reduced the production of IL-1β, which in turn resulted in lower induction of Th17 polarization, and thus alluding to the enhanced production of IL-1β by *Mtb*DC as the principal culprit for the enhanced capacity to differentiate Th17 cells. Our finding demonstrates that, while Dectin-1 expression remains similar among all tested DC populations, the level of DC-SIGN and MR expression is particularly low in *Mtb*DC, suggesting a lack of inhibition of Dectin-1-dependent signalling and IL-1β production in these cells. This is supported by our observations where the inhibition of Dectin-1 that resulted in the expected reduction of IL-1β secretion by *Mtb*DC. Likewise, direct inhibition of IL-1β reduced the capacity by *Mtb*DC to drive Th17 differentiation. This is important given that, despite the fact that IL-17 drives pro-inflammatory immune responses, it has been also associated with tissue-damaging inflammation. Given that Th17 cells can mediate both antibacterial and pro-inflammatory responses, data on the role for Th17 during primary *Mtb* infection are

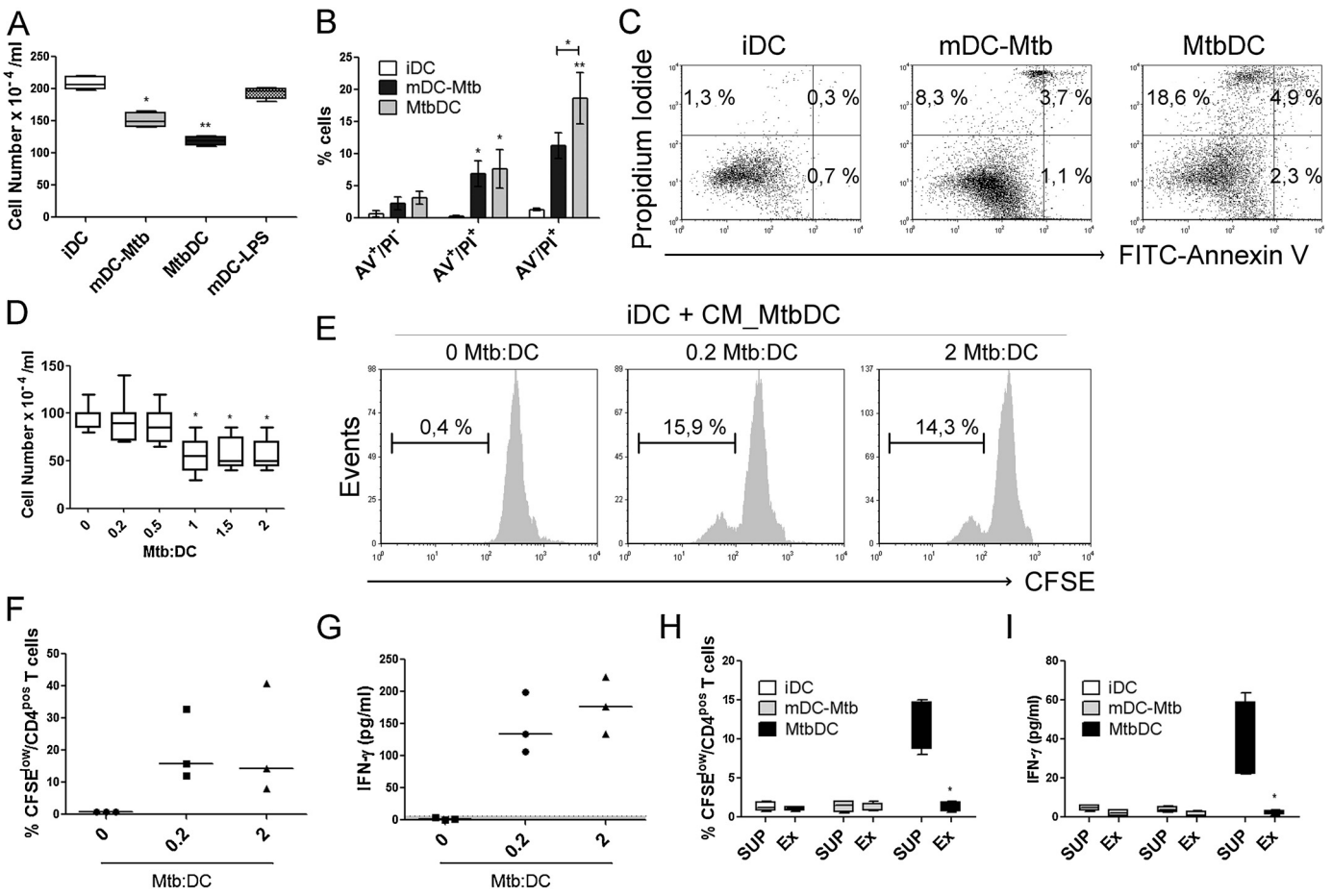

Fig. 7. Mycobacterial antigens transfer can occur in the absence of apoptosis or exosome release.
(A) Percentages of viable cells in iDC, mDC-*Mtb*, *Mtb*DC or mDC-LPS, were determined by trypan blue exclusion. (B) Percentages of early apoptotic (AV^+/PI^-), late apoptotic (AV^+/PI^+) or necrotic (AV^-/PI^+) cells, was determined by FITC-Annexin V (AV) and propidium iodide (PI) staining. Data were obtained from 7 independent experiments. Friedman test followed by Dunn's Multiple Comparison Test: *, $p < 0.05$; **, $p < 0.01$; only the differences vs iDC are shown. (C) Representative dot plot from 1 independent experiment of AV and PI staining in iDC, mDC-*Mtb* or *Mtb*DC. (D) Percentages of viable cells in *Mtb*DC differentiated from monocytes exposed to different amounts of *Mtb* (ratio, *Mtb*:DC) in the presence of IL-4 and GM-CSF for 6 days. Data were obtained from 7 independent experiments. Friedman test followed by Dunn's Multiple Comparison Test: *, $p < 0.05$; only the differences vs 0 *Mtb*:DC are shown. (E) Representative histograms from 1 independent experiment showing the percentages of autologous $CFSE^{low}$ CD4T cells induced by iDC exposed for 24 h to the conditioned medium derived from *Mtb*DC cultures generated in the presence of 0, 0.2 or 2 *Mtb*:DC ratio. (F-G) Box and whisker graphs showing the percentages of $CFSE^{low}$ CD4T cells (F), and the extracellular production of IFN-γ (G), obtained from 3 independent experiments. (H-I) Box and whisker graphs showing the percentages of autologous $CFSE^{low}$ CD4T cells induced by iDC exposed for 24 h to the supernatants (SUP) or the exosomes (Ex) fractions derived from the sequential centrifugation of the conditioned medium from iDC, mDC-*Mtb* or *Mtb*DC (H), and determination of the extracellular production of IFN-γ (I), obtained from 4 independent experiments. Friedman test followed by Dunn's Multiple Comparison Test: *, $p < 0.05$; comparisons between SUP and Ex for each condition are shown.

conflicting and quite likely depend on the model and the degree of inflammation (Lyadova and Panteleev, 2015). Although evidences coming from the mice model thus far suggest that IL-17 is important during the first steps of infection (Gopal et al., 2014), excessive IL-17 responses may be detrimental, resulting in a destructive influx of granulocytes as well as increased amounts of MIP-2, TNF-α and IL-6 within affected lungs (Cruz et al., 2010). Likewise, contradictory results have been obtained in humans. While some authors reported reduced frequencies of blood IL-17 producing cells in TB patients compared to healthy donors suggesting that Th17 contribute to the protection (Scriba et al., 2008), others show that the proportion of IL-17 CD4T cells correlates with the severity of the disease (Jurado et al., 2012), and that patients infected with multidrug resistant clinical *Mtb* isolates display an augmented Th17 response associated with persistent and high antigen load (Basile et al., 2011). Therefore, we consider that the outcome will highly depend on the chronicity and dysregulation of the production of IL-17. In the context of our findings, we speculate that at advance stages of the infection, sustained bacillary loads may favour the generation of *Mtb*DC from recruited monocytes, and these cells may further promote Th17 polarization, in detriment of Th1, contributing to tissue damage.

Beside the effect observed on Th polarization by *Mtb*DC, this study provides strong evidence in favour of the Ag-transfer between *Mtb*DC and bystander monocyte-derived DCs. We demonstrate that i) the detection of mycobacterial Ags is higher in conditioned media from *Mtb*DC in comparison to that of other monocyte-derived DC populations, ii) conditioned media from *Mtb*DC has a higher capacity to activate the recall response of bystander monocyte-derived DCs, and iii) this capacity in *Mtb*DC is mediated through the exchange of soluble factors without cell–cell contact. Based on our previous study showing that *Mtb*DC lose their phagocytic capacity (Balboa et al., 2010), we predict that the Ags that are released at the stage of *Mtb*DC are produced after the Ag-processing taking place at the monocyte stage. These findings are in agreement with current concepts promoting that the transfer of Ags between DCs stimulated in tissues and resident DCs in lymph nodes is a key mechanism for the induction of immunity or the establishment of T cell memory response (Wakim and Bevan, 2011). In the TB context, the involvement of Ag-transfer

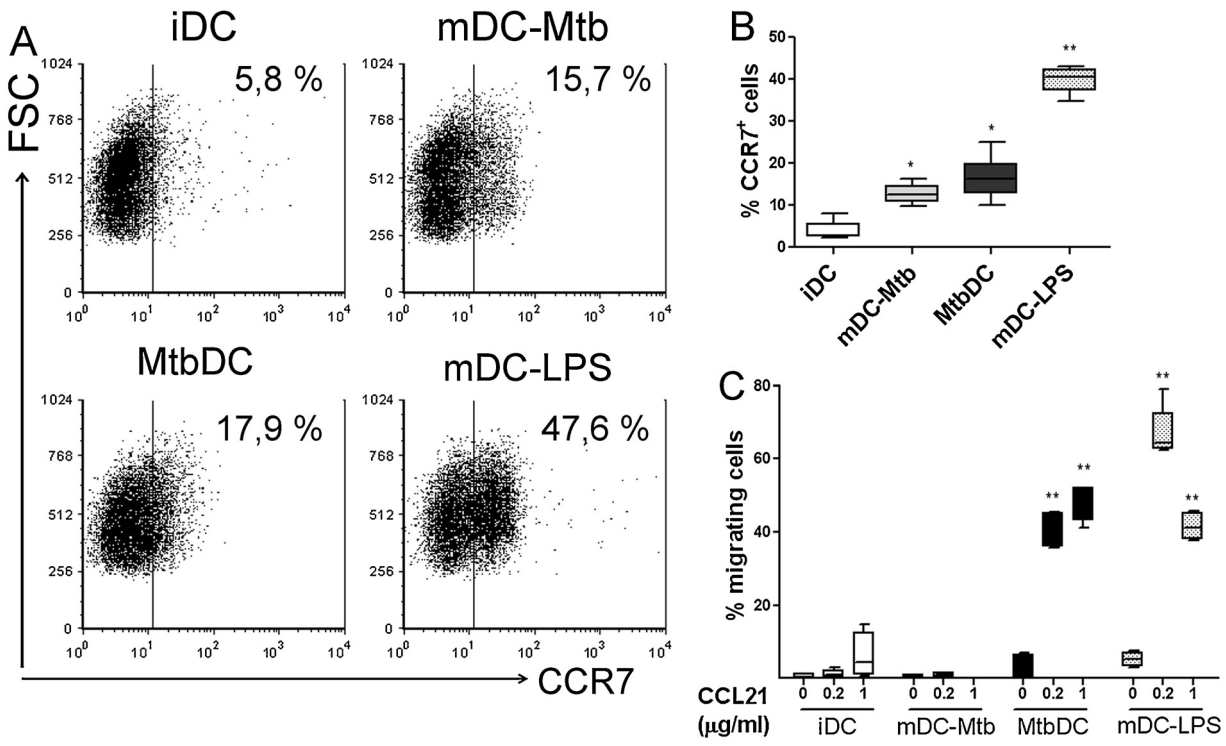

Fig. 8. *Mtb*DC show a high chemotactic activity towards the lymphatic homing chemokine CCL21.
(A) Representative dot plots from 1 independent experiment showing the expression of CCR7 displayed by iDC, mDC-*Mtb*, *Mtb*DC or mDC-LPS. (B) Expression of CCR7 determined in 6 independent experiments. Friedman test followed by Dunn's Multiple Comparison Test: *, $p < 0.05$; **, $p < 0.01$; only the differences vs iDC are shown. (C) iDC, mDC-*Mtb*, *Mtb*DC and mDC-LPS were seeded on the upper compartment of chemotaxis chambers against the different amounts of CCL21. After 4 h of incubation at 37 °C, cells were recovered from the lower compartment and were quantified by flow cytometry. Percentages were calculated relative to the control medium. Data were obtained from 6 independent experiments. Friedman test followed by Dunn's Multiple Comparison Test: **, $p < 0.01$; only the differences vs iDC are shown.

and its impact on the induction of T cell immunity to *Mtb* has been recently demonstrated (Samstein et al., 2013; Srivastava and Ernst, 2014; Srivastava et al., 2016). In fact, after the Ag-capture by macropinocytosis, $CD103^+$ DCs have a unique capacity to store and regurgitate unprocessed Ags that can be then taken up by B cells (Le Roux et al., 2012). It remains to be determined if the release of unprocessed Ags by infected DC also activates B cells and whether this event contributes to the host immune response against *Mtb*. Whatever the case may be, Ag-export is proposed as an effective mechanism of immune evasion that promotes persistence of *Mtb*. For instance, it has been recently demonstrated that the blocking of Ag-export can improve CD4T cell activation and the control of the infection (Srivastava et al., 2016). More specifically, it has been shown that *Mtb* uses a vesicular transport pathway to divert bacterial Ags from the MHC class II pathway, and out of infected cells, to ultimately minimize CD4T cell activation; the Ag-uptake and subsequent presentation of bystander cells can only compensate partially for the otherwise large and diverse T cell repertoire obtained through a normal and uninhibited Ag-processing and -presentation (Srivastava et al., 2016). In our study, the activation of CD4T cells by bystander monocyte-derived DCs exposed to conditioned media containing mycobacterial Ags did not reach the same proliferation levels observed from fully mature monocyte-derived DCs challenged with *Mtb* (mDC-*Mtb*). Taken together, Ag-export may provide a novel explanation for the lack of antigenic variation by reducing the selection pressure from T cell recognition during *Mtb* infection, as recently proposed by others (Comas et al., 2010; Copin et al., 2014; Coscolla et al., 2015).

Another important finding in this study is that Ag-exchange between the *Mtb*DC and bystander monocyte-derived DCs can occur in the absence of apoptosis. Given that microvesicle shedding is a major secretory pathway for rapid IL-1β release from activated monocytes (MacKenzie et al., 2001), we had expected that *Mtb*DC could release Ags inside exosomes. Nonetheless, our results showed that exosome-enriched fractions from *Mtb*DC did not provide Ags for presentation to CD4T cells. Another mechanism largely accepted in the TB context is the Ag-transfer through the process of apoptosis or apoptotic bodies (Schaible et al., 2003; Winau et al., 2006). Nonetheless, we discarded this scenario since i) conditioned media derived from *Mtb*DC (under a low multiplicity of infection causing no cell death) was still able to activate T cell proliferation and IFN-γ release, and ii) fully mature DCs challenge with *Mtb* (mDC-*Mtb*) did not release detectable Ags despite showing significant levels of apoptosis. The notion that *Mtb*DC may actively release soluble mycobacterial Ags is in line with novel studies in the murine model in which infected bone marrow-derived DCs can release *Mtb* Ags as soluble, unprocessed proteins, for uptake and presentation by uninfected resident lymph node DCs (Srivastava and Ernst, 2014). Likewise, Ag-export and −transfer to uninfected cells has been shown to occur in lungs during an *in vivo* infection (Srivastava et al., 2016). The recent demonstration of the occurrence of Ag-transfer between cells in lungs of *Mtb*-infected mice constitutes an important support for our *in vitro* findings. On the other side, given that we have evaluated the response of the whole repertoire of specific CD4T cells against *Mtb*, including several antigens, we consider that our findings can complement the results obtained in the P25 TCR-Tg transgenic mouse limited to the study of the peptide Ag–85B of *Mtb* (Srivastava et al., 2016). Despite this, it is still important to consider that our study is limited by the use of irradiated bacteria; thereafter we have dismissed the contribution of those antigens that can be produced *de novo* by *Mtb* throughout the course of the infection.

Finally, our study also evidences that *Mtb*DC displays a higher capacity to migrate efficiently towards a gradient containing the

lymphatic chemokine, CCL21 (Fig. 8). We infer that *Mtb*DC may be able to readily transport Ags to the lymph nodes and then transfer them to bystander resident DCs. Interestingly; other monocyte-derived DC populations like mDC*Mtb* were not able to respond to CCL21 despite expressing CCR7. This result suggests that *Mtb* has a differential effect on the migration depending on whether the bacilli interact with monocytes at early stages of after cells are fully differentiated, independently on the acquisition of CCR7. This is in agreement with a recent study reporting a significant reduction in the ability of *Mtb*-infected monocyte-derived DCs to migrate toward the lymphatic chemokines CCL19/CCL21, despite of a similar CCR7 expression level in comparison to LPS-treated DCs, which migrate normally under these conditions (Roberts and Robinson, 2014). The impairment of DC migration within the TB context has been correlated to the delay in the onset of an adaptive immune response against *Mtb*, which is thought to allow subsequently the bacterial growth and dissemination. This is supported by the fact that *Mtb*-infected monocyte-derived DCs exhibit a significant reduction in the migration towards lymphatic chemokines (Roberts and Robinson, 2014), and that variants in the *ASAP1* gene, a regulator of DC migration, for example, are associated to the susceptibility to tuberculosis (Curtis et al., 2015).

All things considered, we propose that the generation of *Mtb*DC could be negative consequence of the *Mtb* infection by hampering the host immune response due to the concerted i) bacterial inhibition of Ag presentation of the infected DC, ii) driving Th cells towards Th2 and Th17 profiles, and iii) releasing mycobacterial Ags to other bystander DCs that can activate specific T cell clones but at a lesser extent than fully matured DCs challenged with *Mtb*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijmm.2016.06.004.

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