Tuberculosis 99 (2016) 31–40

Contents lists available at ScienceDirect

Tuberculosis

journal homepage: http://intl.elsevierhealth.com/journals/tube

IMMUNOLOGICAL ASPECTS

TLR4 and DC-SIGN receptors recognized *Mycobacterium scrofulaceum* promoting semi-activated phenotype on bone marrow dendritic cells

Marisa Cruz-Aguilar [a], Antonia I. Castillo-Rodal [a], Alejandro Schcolnik-Cabrera [a], Laura C. Bonifaz [b], Gabriela Molina [a], Yolanda López-Vidal [a, *]

[a] *Programa de Inmunología Molecular Microbiana, Departamento de Microbiología y Parasitología, Facultad de Medicina, Universidad Nacional Autónoma de México, Ciudad de Mexico, Mexico*
[b] *Unidad de Investigación Médica en Inmunoquímica, Instituto Mexicano del Seguro Social, México, DF, Mexico*

ARTICLE INFO

Article history:
Received 31 October 2015
Received in revised form
18 February 2016
Accepted 5 April 2016

Keywords:
Dendritic cells
DC maturation
Innate immunology
M. scrofulaceum

SUMMARY

Nontuberculous mycobacteria (NTM) are recognized as emerging pathogens and their immune regulatory mechanisms are not well described yet. From them, *Mycobacterium avium* is known to be a weak activator of dendritic cells (DCs) that impairs the response induced by BCG vaccine. However, whether other NTM such as *Mycobacterium scrofulaceum* may modulate the activation of DCs, has not been extensively studied. Here, we exposed bone marrow-derived DCs (BMDCs) to *M. scrofulaceum* and we analyzed the effect on the activation of DCs. We found that *M. scrofulaceum* has a comparable ability to induce a semi-mature DC phenotype, which was produced by its interaction with DC-SIGN and TLR4 receptors in a synergic effect. BMDCs exposed to *M. scrofulaceum* showed high expression of PD-L2 and production of IL-10, as well as low levels of co-stimulatory molecules and pro-inflammatory cytokines. In addition to immunophenotype induced on DCs, changes in morphology, re-organization of cytoskeleton and decreased migratory capacity are consistent with a semi-mature phenotype. However, unlike other pathogenic mycobacteria, the DC-semi-mature phenotype induced by *M. scrofulaceum* was reversed after re-exposure to BCG, suggesting that modulation mechanisms of DC-activation used by *M. scrofulaceum* are different to other known pathogenic mycobacteria. This is the first report about the immunophenotypic characterization of DC stimulated by *M. scrofulaceum*.

1. Introduction

Nontuberculous mycobacteria (NTM) are ubiquitous microorganisms [1,2] and are considered opportunistic pathogens in both humans and animals. NTM can be isolated from various water sources, including drinking water distribution systems. Additionally, the exposure to certain species of NTM could affect the efficacy of the bacillus Calmette-Guerin BCG vaccination against pulmonary tuberculosis [3,4].

Mycobacterium scrofulaceum is a kind of pathogenic NTM frequently isolated from different sources of water from various regions of Mexico and South America, and is responsible for the development of cervical lymphadenitis and lung infections in children as well as disseminated disease in immunodepressed individuals [5,6].

Biochemically, it is characterized by its resistance to chlorine and testing positive to catalase and superoxide dismutase (SOD) activity, which provides the advantage to colonize drinking water distribution systems as well as surviving the oxidative burst of activated macrophages [5,7,8]

To date, the mechanisms by which *M. scrofulaceum* can evade the immune system to persist within the host and generate disease is completely unknown. However, since it shares several biochemical characteristics with other pathogenic mycobacteria it is possible that it could modulate the immune response from innate immunity like *Mycobacterium avium*, with dendritic cells (DCs) being key elements in this regulation.

DCs are antigen-presenting cells (APCs) that integrate a variety of incoming signals and orchestrate the immune response [9]. DC

* Corresponding author. Programa de Inmunología Molecular Microbiana, Departamento de Microbiología y Parasitología, Facultad de Medicina, Universidad Nacional Autonoma de Mexico, Mexico.
E-mail addresses: maricruzmca1804@yahoo.com (M. Cruz-Aguilar), castillorodal@gmail.com (A.I. Castillo-Rodal), a.schcolnik@garvan.org.au (A. Schcolnik-Cabrera), labonifaz@yahoo.com (L.C. Bonifaz), gabriela.molina86@yahoo.com.mx (G. Molina), lvidal@unam.mx (Y. López-Vidal).

http://dx.doi.org/10.1016/j.tube.2016.04.003
1472-9792/

activation is crucial for the effective stimulation of antigen-specific effector T cell responses. This activation is characterized by the up-regulation of MHC class II (MHC II), of co-stimulatory molecules (i.e., CD40, CD80, CD86), and cytokine secretion, such as IL-12, IFN-γ and TNF [10]. Partially mature DCs express low to intermediate levels of co-stimulatory molecules and pro-inflammatory cytokines, but high levels of PD-L2, a potent co-inhibitor for T cell responses [11], and of the anti-inflammatory cytokine IL-10.

The precise mechanism underlining the effects of NTM on the immune response has not been completely clarified. The host response to *M. avium* is perhaps the best understood. Previous studies have demonstrated that *M. avium* (strain WAg206) induced down-regulation of activation markers on bone marrow-derived dendritic cells (BMDCs), and the release of anti-inflammatory cytokines in murine DC-macrophage co-cultures compared with lipopolysaccharide (LPS). This report also showed that after pre-immunization with *M. avium*, mice do not to respond efficiently to BCG vaccine [12]. Recently, our group showed that the interaction of mouse DC with *M. avium* results in partial cell maturation and tolerogenic responses [13]. These findings open the possibility that exposure to some pathogenic NTM such as *M. scrofulaceum* also have the ability to modulate the activation of DC and, subsequently, the adaptive immune response. In this study, we characterized the relationship between *M. scrofulaceum* and the DC phenotype, as well as its mechanism of interaction.

2. Materials and methods

2.1. Mycobacterial strains

Mycobacterium bovis strain BCG Phipps. *Mycobacterium arupense* (JN049511), *M. avium* (JN049509), *Mycobacterium fortuitum* (JN049501), *Mycobacterium gordonae* (JN049504), *Mycobacterium nonchromogenicum* (JN049498), *Mycobacterium peregrinum* (JN049500), *M. scrofulaceum* (JN049503), and *Mycobacterium smegmatis* (JN049499) isolated from drinking water and reuse water of México city were cultured in Sauton broth until the logarithmic growth phase was reached. Prior to use, the CFU and viability of the BCG Phipps and NTMs cultures were determined by serial plating in supplemented Middlebrook 7H10 (BD. Munich, Germany) and ATP SL Luminescent Assay Kit (Biotherma AB) respectively.

2.2. Study animals

Male Balb/c mice of 6–8 weeks of age were sacrificed, and DC precursors were isolated. All experiments with animals were performed according to the guidelines of the ethics and institutional biosafety committees CIEFM-055-11.

2.3. DC generation from bone marrow

DCs were obtained from the femurs and tibias of the Balb/c mice by successive irrigations with RPMI 1640 medium (GIBCO, BRL) as described previously [14]. The obtained cells were cultivated in RPMI medium supplemented with 10% fetal bovine serum (GIBCO, BRL), 2 mM-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin (all from GIBCO BRL) and 10% culture supernatant of a cell line producer of granulocyte and monocyte colony stimulating factor (GM-CSF). At day 3 post-culture, 10 mL of RPMI 1640 medium supplemented with FBS and GM-CSF was added. At day 5, the expression of $CD11b^+$ and $CD11c^+$ in BMDC was analyzed by flow cytometry.

2.4. DC stimulation with BCG or NTM

At day 5 post-culture, DC were harvested and quantified at a density of 5×10^6 cells and cultured for 24 h in the absence of stimulation or with any of the following stimuli: live cultures of *M. bovis* BCG Phipps, NTM at a 1:1 ratio (DC:Mycobacteria) or with 0.1 μg/mL PPD (Sanofi-Aventis Pasteur, Toronto, Canada) as a positive control for activation. Assays were performed in 24-well plates with 1 mL fresh RPMI. After 24 h, DCs were harvested and the expression of surface markers and cytokine production in the culture supernatant was analyzed by flow cytometry.

For re-stimulation assays, at day 5 post-culture DCs were cultured at a density of 5×10^6 cells with live cultures of *M. bovis* BCG Phipps, *M. avium* or *M. scrofulaceum* at a 1:1 ratio (DC:Mycobacteria) for 3 h. Harvested cells were positively selected with CD11c anti-mouse MACS MicroBeads (Miltenyi, Biotec, Auburn, CA, USA) and plated in 24-well plates to re-stimulation with *M. bovis* BCG Phipps, *M. avium* or *M. scrofulaceum* at a 1:1 ratio for 18 h. The surface molecule expression was analyzed by flow cytometry and the supernatant was collected for cytokine detection (CBA, Mouse Inflammation Kit BD).

In some assays, DCs were incubated for 1 h with 10 μg/mL of individual polyclonal antibodies against TLR2 (Santa Cruz Biotechnology, Inc.), TLR4 (Santa Cruz Biotechnology, Inc.), DC-SIGN (BD Biosciences), or with an isotype control. After receptors blockade, the BMDC were stimulated with *M. scrofulaceum* for 24 h and harvested cells were analyzed for expression of cell surface molecules and cytokines released into the supernatant by flow cytometry.

2.5. Flow cytometry

Stimulated or re-stimulated DCs with *M. bovis* BCG Phipps, NTM or PPD were treated with 2% fetal bovine serum in PBS 1× for 1 h at room temperature to block FCγ receptors. Surface molecules on DCs were analyzed with APC-CD11c anti-mouse antibody, anti-CD11b FITC, anti-mouse PD-L1 (B7-H1), PD-L2 (B7-DC), CD80 and CD86 coupled to PE or isotype control (all from Pharmingen-BD Biosciences) for 20 min at 4 °C, according to the manufacturer's recommendations.

The cells were washed with 1× PBS and fixed with 4% paraformaldehyde (Sigma–Aldrich, Inc.) for 20 min at room temperature; they were then washed and processed with a FACS Calibur flow cytometer (BD Biosciences) and the results were analyzed using the FlowJo software (Tree Star Inc., Ashland, Oregon, USA).

The identification of DCs population was performed by three-color flow cytometry and sequential gating analysis. After the exclusion of doublets, debris and dead cells, BMDCs were identified based on the expression of the markers CD11b and CD11c. The expression of activation markers was presented as median fluorescence intensity of Phycoerythrin (PE). Data were normalized to un-stimulated cells and the fold change was obtained.

2.6. Cytokine secretion

The presence of IFN-γ, TNF, IL-10 and IL-12p70 in the supernatant of stimulated cells was determined using a Cytometric Bead Array Mouse Inflammation Kit (Pharmingen-BD Biosciences) according to the manufacturer's instructions. The concentration of the standards was shown in a range of 20–5000 pg/mL. The results were evaluated by flow cytometry and analyzed using the FlowJo software.

2.7. Immunofluorescence and confocal microscopy

DC without stimulation or stimulated by BCG, NTMs or PPD were grown on 12 mm, round coverslips covered with poly-L-lysine (BD BioCoat TM, BD Biosciences). After 24 h of stimulation, DCs were washed with PBS and fixed with 4% paraformaldehyde (ultrapure grade, Tousimis Research Co., Rockville, MD) at room temperature for 20 min. Cells were permeabilized with acetone (Sigma–Aldrich, Inc.) for 5 min at −20 °C, and actin microfilaments were stained with 0.01 U/mL rhodaminated phalloidin (Molecular Probes, Invitrogen) at room temperature for 20 min. Cells were stained with 5 μg/mL of 4′,6-diamidino-2-phenylindole dilactate (DAPI, Molecular Probes, Invitrogen). After washing with PBS at 1×, the coverslips were placed on a slide using a mounting solution from Immuno Mount Mounting Medium (GeneTex) and analyzed with confocal microscope (Leica SP5, DM16000, Mo) with the Image Pro Plus V 6.0 software from Media Cybernetics.

2.8. Statistical analysis

Data are presented as the mean of two or three individual experiments. The data was compared using a one-way analysis of variance (ANOVA), followed by a post hoc Turkey's test to determine significance. A p value less than 0.01 was considered significant.

3. Results

3.1. M. scrofulaceum induces an immature phenotype and overexpression of PD-L2 in BMDCS

NTM such as *M. arupense*, *M. fortuitum*, *M. gordonae*, *M. nonchromogenicum*, *M. peregrinum*, *M. smegmatis*, *M. avium* or *M. scrofulaceum* were previously isolated from samples of different water sources of Mexico City [5].

With the aim of determining the phenotype induced in the DCs after stimulation with different NTMs, DCs were differentiated from bone marrow precursors of Balb/c mice obtaining 60% double positive cells for $CD11c^{+}$, $CD11b^{+}$ (Figure S1).

Initially, we evaluated the ability of different NTM strains to induce the maturation of DCs. NTMs co-cultured with immature murine BMDC for 24 h without exogenous cytokines were analyzed by flow cytometry by the expression of surface markers (Figure 1A). We used BCG Phipps and *M. avium* as positive and negative controls of BMDC activation, respectively, because previous works have shown that BCG Phipps stimulated the DCs towards a mature phenotype while *M. avium* was a weak inducer of mature DC phenotype [13]. Additionally, the mature DC phenotype was verified on BMDC stimulated by protein-purified derivative (PPD) and comparing with non-stimulated control cells. Isotype control was used as negative control to measure the level of non-specific background signal caused by primary antibodies.

Because the un-stimulated cells express basal levels of co-stimulatory and inhibitory molecules, the median fluorescence intensity of these was greater than that of isotype control (Figure 1A). Figure 1B shows the fold induction of stimulatory and inhibitory molecules of activation, positive and negative controls, as well as BMDCs stimulated with *M. scrofulaceum*, which showed significantly different phenotype compared with the other NTM tested.

The exposure to NTM such as *M. arupense*, *M. fortuitum*, *M. gordonae*, *M. nonchromogenicum*, *M. peregrinum* or *M. smegmatis* induced a mature phenotype on DCs similar to that induced by BCG Phipps or PPD. The high level expression of co-stimulatory molecules such as CD40 or CD86 on cell surface suggested the DC activation (Figure S2).

By contrast, *M. scrofulaceum* failed to induce a mature phenotype and the BMDCs showed a phenotype characterized by high expression level of the co-inhibitory molecule PD-L2 (5.3-fold expression), similar to that observed with the exposure to *M. avium* (Figure 1B). Compared with BCG-stimulated DC, lower levels of co-stimulatory molecules (CD40 and CD86) where detected on *M. scrofulaceum*-stimulated DC.

Because the mature DC phenotype is defined also by the production of pro-inflammatory cytokines, the IFN-γ, TNF and IL-10 cytokine levels were measured in the culture supernatants of non-stimulated BMDCs and in those exposed to different stimuli (Figure 1C). Compared with PPD or BCG controls, DCs stimulated with *M. scrofulaceum* showed a two-fold increase in IL-10 production, which was similar to the levels induced by *M. avium* (Figure 1C). The increased expression of IL-10 induced in *M. scrofulaceum*-stimulated BMDCs correlated with the high levels of PD-L2 expression. In contrast, a significant decreased level of pro-inflammatory cytokines production such as INF-γ and TNF was obtained (60% and 75%, respectively).

These results showed that *M. scrofulaceum* avoided the activation of BMDCs, which was characterized by increased levels of the co-inhibitory molecule PD-L2 and of IL-10 synthesis. This behavior of *M. scrofulaceum* on DC phenotype increased our interest to investigate the mechanisms used by *M. scrofulaceum* to modulate the phenotype and activation of the BMDCs.

3.2. M. scrofulaceum is recognized by BMDC through TLR4 and DC-SIGN receptors

Previous reports have shown that mycobacteria target DC-SIGN receptor to affect TLR-mediated immune responses by impairing DC-maturation and enhancing IL-10 production [15]. Because our results showed that *M. scrofulaceum* induced a semi-mature DC phenotype and IL-10 increased levels, our next goal was to determine which receptors on the cell surface are involved in the *M. scrofulaceum* recognition.

BMDCs were treated with a blocking antibody against TLR2, TLR4 or DC-SIGN before the stimulation with *M. scrofulaceum* and DC phenotype was determined. Co-stimulatory molecules expression was not affected by blocking TLR2 compared with BMDCs exposed to *M. scrofulaceum* without previous TLR2 blockade (Figure 2A and B). However, an increase in the expression of CD40 and CD80 was detected when TLR4 (2.0 and 1.5 fold change respectively) or DC-SIGN (2.5 and 1.2 fold change) were blocked.

In addition, under this condition PD-L2 (2.5 fold change) and IL-10 production was significantly decreased (approximately 40% less), and the pro-inflammatory cytokines TNF and IFN-γ were considerably increased, although they did not reach similar levels to those induced by BCG (Figure S3). These results suggest that *M. scrofulaceum* uses the TLR4 and DC-SIGN receptor on DC for its recognition.

3.3. The recognition of M. scrofulaceum through TLR4 and DC-SIGN receptors impairs the mature phenotype of DC in a synergic manner and dependent of signaling pathway kinase Raf-1

It has been showed on previous reports that mycobacteria exploit the convergence of DC-SIGN with TLR4 to affect DC maturation and to enhance IL-10 production [15]. In this work we have analyzed the effect induced over the DC maturation when DC-SIGN and TLR4 receptors were simultaneously blocked prior to DC stimulation with *M. scrofulaceum*.

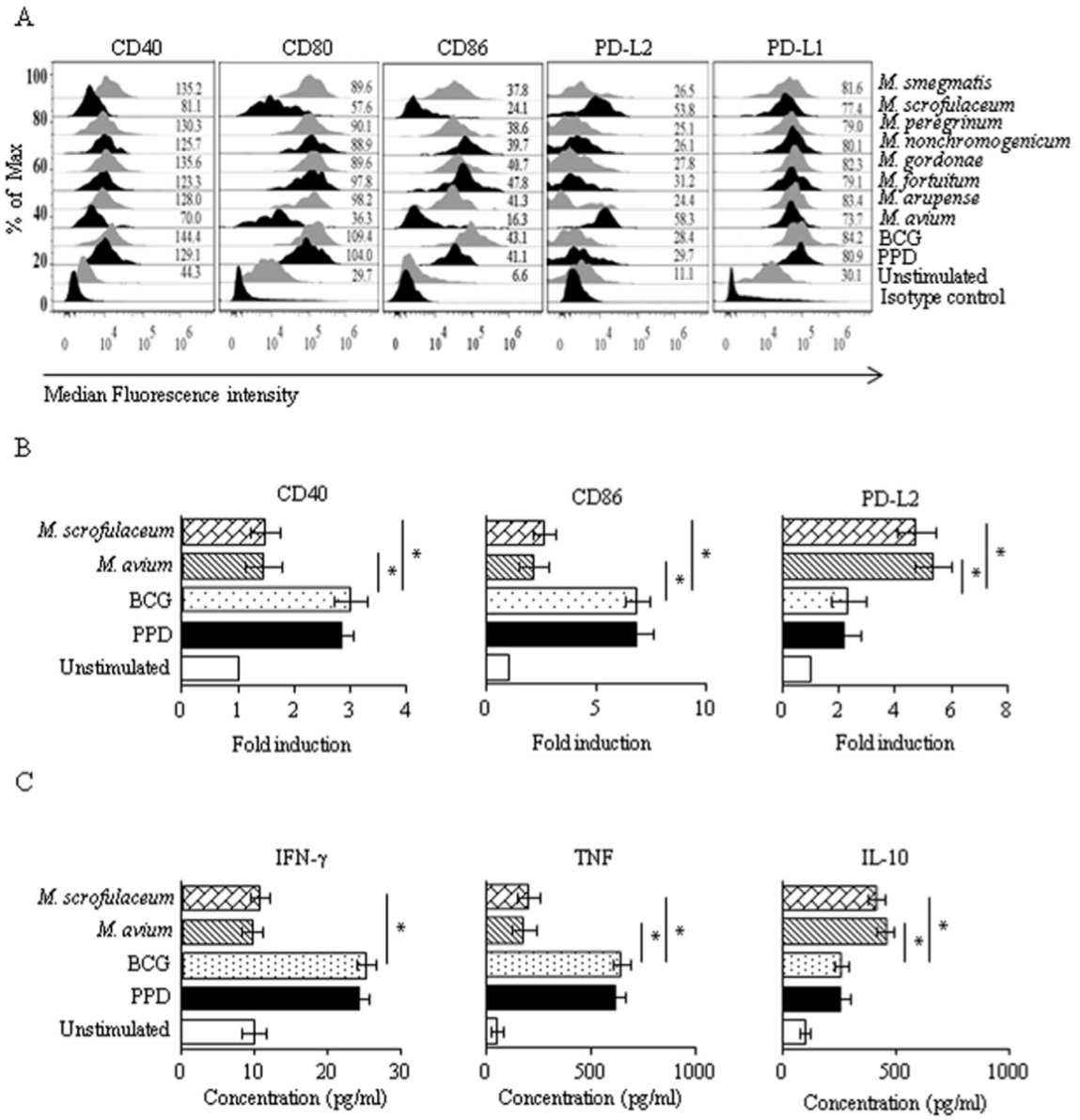

Figure 1. DC exposure to *M. scrofulaceum* induced a semi-mature phenotype with high expression of PD-L2 and IL-10. Bone marrow-derived DC (BMDC) were stimulated with different NTM for 24 h at ratio of 1:1 (DC:Mycobacteria), and the expression of cell surface markers were analyzed by flow cytometry. (A) Representative histogram of three experiments. BMDC stimulated by *M. bovis* (BCG) or 0.1 μg/mL Purified Protein Derivative (PPD) were used as DC activation-positive controls and by *M. avium* as activation-negative control. The median fluorescence intensity to each histogram of co-stimulatory or inhibitory molecule on $CD11b^{+}$ $CD11c^{+}$ gated cells is indicated. (B) Fold induction of the expression of CD40, CD86 and PD-L2 following stimulation with *M. scrofulaceum*, *M. avium*, BCG or PPD. The results were normalized to un-stimulated BMDC levels. (C) Levels of different pro- or anti-inflammatory cytokines (pg/mL) in culture supernatants of BMDC with different stimuli were determined using a Cytometric bead array and analyzed using flow cytometry. *$p < 0.01$ by one-way analysis of variance (ANOVA).

As seen in Figure 2, the simultaneous blocking of TLR2 with TLR4 or DC-SIGN receptors showed a similar effect in respect to individual-receptor blocking, suggesting that TLR2 is not relevant in *M. scrofulaceum* recognition by DCs. However, at simultaneous blockade of TLR4/DC-SIGN (Figure 2B and C), a synergistic effect was observed. This effect was characterized by an increase in the level of co-stimulatory molecules ($p < 0.01$) and a decrease in the PD-L2 level respect to unblocked DCs stimulated with *M. scrofulaceum* (approximately 50% less). Figure 2C shows that the level of IFN-γ and TNF cytokine are increased (30% and 17% respectively) in comparison with receptors blocked individually ($p < 0.01$). In contrast, the synthesis of IL-10 showed an additional reduction of 15% when TLR4 and DC-SIGN receptors were blocked simultaneously. These results show that TLR4 and DC-SIGN receptors participate synergistically in the recognition of *M. scrofulaceum* and therefore, in the modulation of DC phenotype.

Different studies have reported that upon pathogen binding, DC-SIGN induces an intracellular signaling pathway with a central role for the serine/threonine kinase Raf-1 [16]. Then in order to confirm the activation of DC-SIGN receptor by *M. scrofulaceum*, we treated BMDCs with a specific inhibitor of Raf-1 (GW5074) prior to their stimulation with *M. scrofulaceum* and we analyzed the effect on BMDC phenotype (Figure 3). Figure 3B shows that PD-L2 expression levels and synthesis of IL-10 were negatively affect by the inhibitor of Raf-1 protein (Figure 3C).

In contrast to the expression of PD-L2, the levels of co-stimulatory molecules and pro-inflammatory cytokines (IFN-γ or TNF) were increased, indicating that DC-SIGN receptor is important

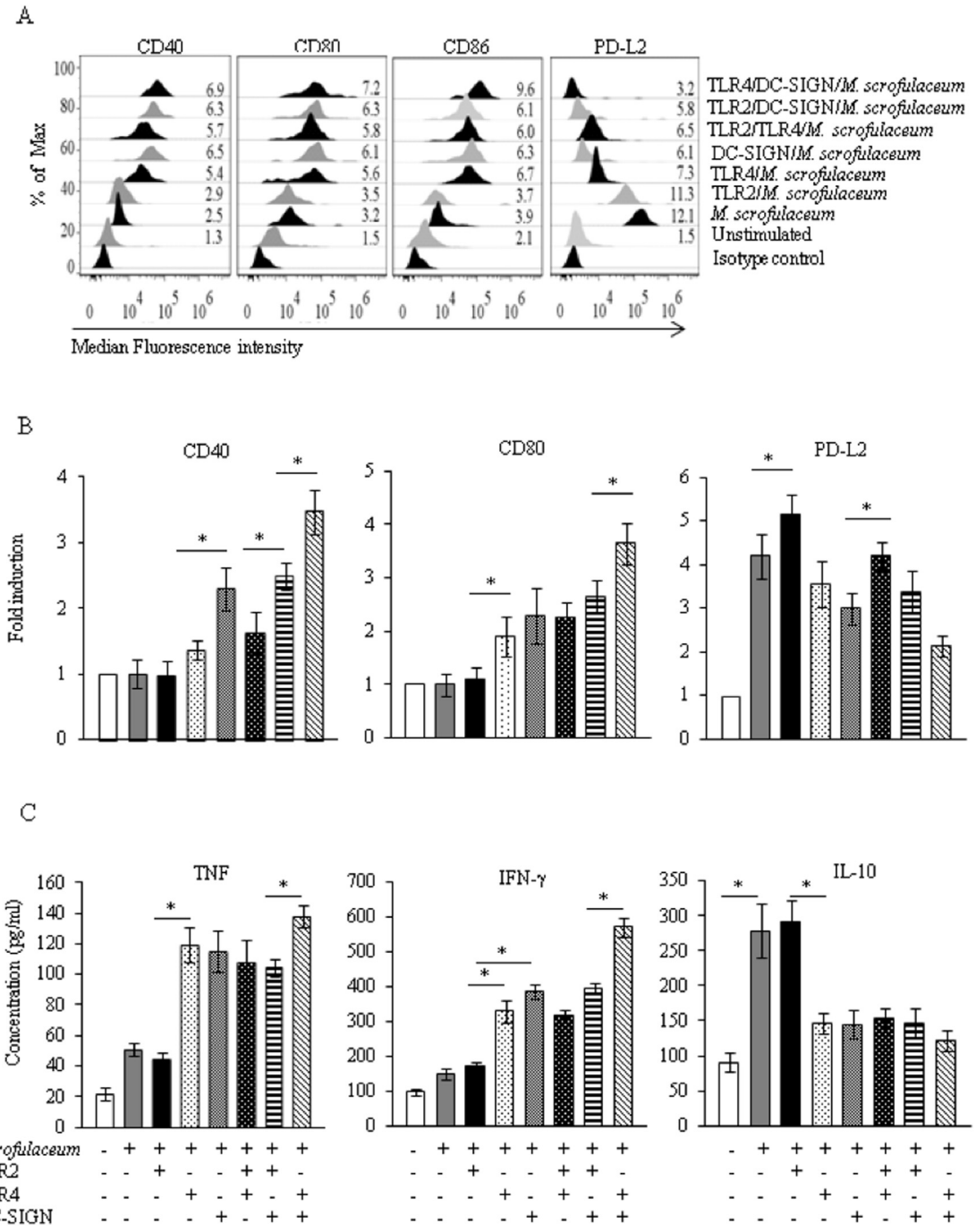

Figure 2. The recognition of *M. scrofulaceum* by BMDCs through TLR4 and DC-SIGN induces the expression of PD-L2 and IL-10. BMDCs were incubated for 1 h with or without blocking antibody anti-TLR2 (α-TLR2), anti-TLR4 (α-TLR4), anti-DC-SIGN (α-DC-SIGN) or a combination of these antibodies. Enriched $CD11c^{+}/CD11b^{+}$ cells were then exposed to *M. scrofulaceum* for 24 h. (A) Representative histogram of three experiments. (B) Expression of CD40, CD80 and PD-L2 on BMDCs exposed to *M. scrofulaceum* after receptors blockade. (C) Concentrations of pro- and anti-inflammatory cytokines in the culture supernatants were measured using a cytometric bead array. The IL-10 levels decreased on BMDCs treated simultaneously with blocking antibodies against TLR4 and DC-SIGN. The mean of three experiments is shown. *$p < 0.01$. Un-stimulated DCs.

in *M. scrofulaceum* recognition, and together with TLR4, both are involved in promoting the immature DC phenotype induced by *M. scrofulaceum*.

3.4. BMDCs exposed to M. scrofulaceum reorganize the cytoskeleton and reduce the cellular migration

Because mature DCs are characterized by the induction of cytoskeletal rearrangements that favor their migration to lymphoid organs, mainly in response to chemokines such as CCL21, we analyzed the correlation between the DC immature phenotype and architectural changes of the actin cytoskeleton as part of the phenotypic characterization of the DC exposed to *M. scrofulaceum*.

BMDCs exposed to PPD, BCG, *M. avium* or *M. scrofulaceum* were fixed on coverslips coated with poly-L-lysine, stained actin filaments and microtubules with phalloidin-rhodamine and anti-β-tubulin antibody respectively, and were analyzed using confocal microscopy. BMDCs stimulated with BCG or PPD expressed co-

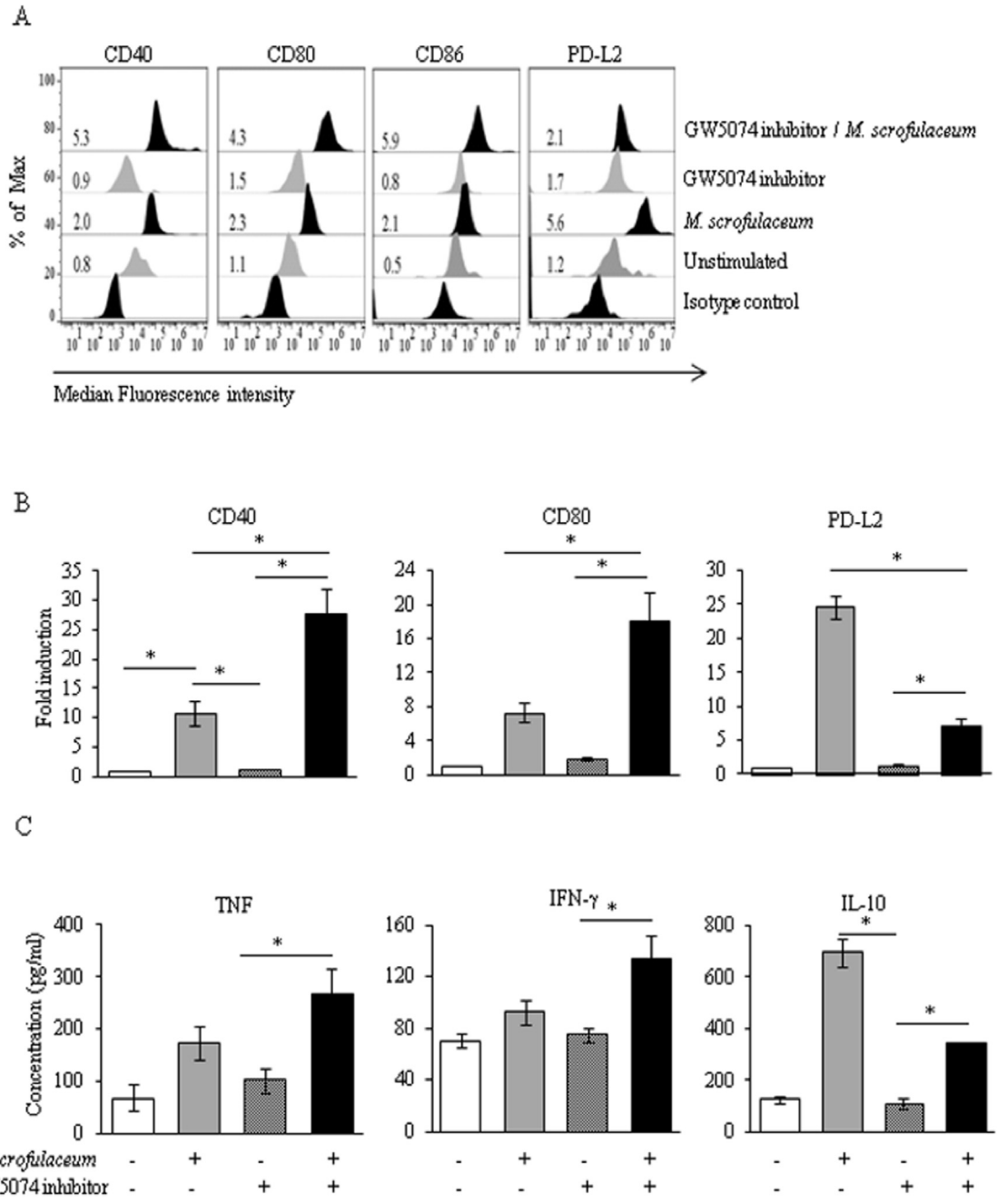

Figure 3. The blocking of Raf-1 signaling pathway by GW5074 inhibitor increased co-stimulatory molecules and inflammatory cytokines levels in BMDCs exposed to *M. scrofulaceum*. The GW5074 inhibitor was used to blockade downstream signaling pathway of DC-SIGN previous the stimuli of BMDCs with *M. scrofulaceum*. (A) Representative histogram of three experiments. (B) Fold induction of the expression of co-stimulatory and PD-L2 molecules following different stimuli. (C) Cytokines concentration (pg/mL) in culture supernatants of BMDC was measured. The data is presented as the mean of three independent experiments. $^{*}p < 0.01$.

stimulatory molecules, produced pro-inflammatory cytokines in line with a mature phenotype and showed an extended morphology (Supplemental Figure S4). The actin microfilament network was uniformly distributed throughout the cytoplasm and the spike-like protrusions were clearly distinguished. By contrast, *M. avium* and *M. scrofulaceum* stimulation showed obvious changes with slightly extended morphology. Unlike BMDCs exposed to BCG or PPD in which actin is localized in a rearrangement in the subcortical membrane, BMDCs stimulated by *M. avium* or *M. scrofulaceum* formed short filament aggregates, which were stained with phalloidin-rhodamine and scattered in both the cell periphery and cytoplasm. The formation of actin-rich structures, such as spikes or pseudopodia, was limited and apparently smaller than BCG-exposed BMDCs, suggesting possible defects in DC migration.

Thus, in order to evaluate the migratory capacity of BMDCs stimulated by different NTMs or BCG, an *in vitro* migration assay in response to chemokine CCL21 in transwell-type plates was established. As shown in Supplemental Figure S5, BMDCs stimulated by LPS, PPD or BCG exhibited migratory capacity 25-fold higher than non-stimulated cells (Nil) or those cultured in the absence of CCL21 (medium). However, the chemotactic ability of BMDCs to CCL21 was severely affected by *M. scrofulaceum* or *M. avium* stimulation (52% and 38% fewer migrating cells, respectively), compared to cells

stimulated by BCG. Interestingly, the number of DCs that migrated in response to chemoattractant was significantly higher in those stimulated by *M. scrofulaceum* than those by *M. avium* ($p < 0.01$). Taken together, these data showed that *M. scrofulaceum* affected the organization of actin cytoskeletal and actin-dependent BMDC migration suggesting a correlation with semi-mature phenotype.

3.5. The mature phenotype of BMDCs induced by BCG Phipps is dominant over the phenotype induced by M. scrofulaceum

Because previous studies have shown that the high expression of PD-L2 and the synthesis of IL-10 in DCs exposed to *M. avium* are maintained even in the presence of BCG, interfering with acquired protection induced by BCG vaccination [13], we investigated whether the *M. scrofulaceum*-induced DC phenotype like *M. avium*, could be predominant to that induced by BCG.

DCs pre-cultured with BCG, *M. avium* or *M. scrofulaceum* and later re-stimulated with any of these organisms were evaluated to determine the expression of DC-activation molecules by flow cytometer. Low expression levels of CD40 and CD80, and a high PD-L2 expression levels were observed when DCs were exposed to *M. avium* as first or second stimulus (Figure 4A). Furthermore, the increased synthesis of IL-10 (Figure 4B) correlated with the increased expression level of PD-L2 (Figure 4A).

In contrast with the dominant phenotype of BMDCs induced by *M. avium*, evident changes were observed when BMDCs were exposed to *M. scrofulaceum* prior or subsequent to BCG. Under these conditions, co-stimulatory molecules levels and inflammatory cytokines production were significantly higher than DC exposed to *M. scrofulaceum* as single stimulus (Figure 4A and B). This suggests that there was a shift toward mature phenotype induced by BCG, which was predominant over that induced by *M. scrofulaceum*.

4. Discussion

We evaluated the effect of the interaction of BMDCs with *M. scrofulaceum*, focusing on the Analysis of the induced phenotype on BMDCs, which is determinant on the T cell activation. In this paper, we report for the first time that *M. scrofulaceum* modulates the activation of DCs, preventing their maturation.

BMDCs exposed to *M. scrofulaceum* have a distinct phenotype from that exposed to BCG or PPD (Figure 1). While BCG and PPD induce the expression of co-stimulatory molecules and pro-inflammatory cytokines, *M. scrofulaceum* not only represses the production of those molecules, but also induces the expression of the inhibitory molecule PD-L2 (Figure 1B) and the production of IL-10 (Figure 1C), generating a semi-mature DC phenotype. A similar effect was observed previously with *M. avium*, which has been determined as a weak inducer of mice and human's DCs [13]. Although this semi-mature DC phenotype was induced by both *M. scrofulaceum* and *M. avium*, we identified that each of them was the result of the interaction with specific innate receptors on the cell surface of the DCs analyzed.

Previous studies of the role of TLRs in BCG recognition have demonstrated that TLR2, TLR4, TLR9 and probably TLR10 are important in the induction of TNF and IL-12 in murine macrophages and DCs [17–19]. However, in this study we observed that *M. scrofulaceum* recognition was mediated through TLR4 and Raf-1 signal pathway-dependent DC-SIGN. This was suggested by the blockade of DC-SIGN and Raf-1, which produced decreased levels of PD-L2 and IL-10, as well as up-regulation of CD40 and CD86 (Figures 2 and 3 and S3). A similar behavior was observed when TLR4 receptor was blocked prior to exposition of DC to *M. scrofulaceum*, but not with the blockade of TLR2 or by the use of Raf-1 inhibitor alone, which demonstrates the relevance of TLR4 and DC-SIGN in recognition of *M. scrofulaceum*. In contrast, *M. avium* recognition is exclusively through TLR2 and the IL-10 production in macrophages and DCs induced by *M. avium* depends of the activation of the p38 MAPK signaling pathway [13,20].

Interestingly, when the two receptors (TLR4 and DC-SIGN) were blocked simultaneously in DCs (Figure 2) prior to its approach with *M. scrofulaceum*, the PD-L2 levels were drastically reduced relative to the blocked with either receptor individually. This result indicates that both receptors act synergistically to regulate expression of co-stimulatory and co-inhibitory molecules and therefore modulating DC activation. However, because PD-L2 expression was not observed completely depressed, it suggests that another pathway in addition to the TLR4 and Raf-1 signaling pathway-dependent DC-SIGN could be involved.

It is also possible that the DC-SIGN receptor acts as a modulator of TLR4. Various studies have reported that mycobacteria targets DC-SIGN to affect TLR4-mediated immune responses by impairing DC maturation and enhancing IL-10 production [15]. The interaction of DC-SIGN with TLR4 could result in enhanced TLR4 signaling, as has been described previously for SIGNR1, a murine homolog of DC-SIGN [21].

DC-SIGN, a carbohydrate receptor that belongs to the C-type lectin family and which is expressed mainly on DCs, serves as a pattern recognition receptor as well as an adhesion receptor [22,23]. One of the main ligands responsible for mycobacterial binding to DC-SIGN is Man-LAM [15], a cell wall component abundantly expressed by pathogenic mycobacterial species. DC-SIGN can recognize Man-LAM and lipomannans expressed on the cell surface of mycobacteria, and it has been seen previously that DC-SIGN on DC interacts with Man-LAM to internalize BCG, but interestingly by blocking DC-SIGN there is a restriction on the infection of the DC [22]. Man-LAM induces the production of the immunosuppressive cytokine IL-10 through Raf-1 and the phosphorylation of NF-kB p65 at serine 276 (Ser276) [16]. Also, signaling downstream of Raf-1 appears to be dependent on the specific DC-SIGN ligands involved. For most pathogens such as *Mycobacterium tuberculosis*, interaction with DC-SIGN modulates immune response by targeting NF-κB, which can form homodimers and heterodimers such as p65-p50 [24]. DC-SIGN-signaling controls p65 activity by phosphorylation of p65 at Ser276, and acetylation is dependent on Raf-1-activation. DC-SIGN-mediated acetylation of p65 leads to increased synthesis of IL-10 by DC [25].

It is known that DC-SIGN cannot activate p65 by itself, but can modulate p65-activity when activation of p65 has been induced by another receptor. Therefore, with our results we can suggest that upon stimulation, immune modulation of NF-kB activation occurs when mycobacteria trigger DC-SIGN simultaneously with another receptor that activates NF-kB, such as TLR4 triggering. However, the modulation of immune responses by DC-SIGN is probably not restricted to TLR activation, but instead might also include other NF-kB-dependent responses, so additional studies should be conducted to determine this point.

Furthermore, our results suggest that the effect observed with blocking receptors prior to exposure to *M. scrofulaceum* depends on the extracellular interaction of bacteria with DC-SIGN and TLR4 receptors, and it was independent on the uptake of the bacteria by DC. This is possible considering that the phenotype of *M. scrofulaceum*-exposed DCs without blocking was different. Conversely, if the effect depends on uptake of bacteria, it would be expected that DCs phenotype induced by *M. scrofulaceum* with or without receptor blockade were similar.

In conjunction with PD-L2 expression and the synthesis of IL-10, semi-mature phenotypes induced by *M. scrofulaceum* were characterized by changes in the organization of the actin cytoskeleton, which had a negative effect on DC morphology and migration

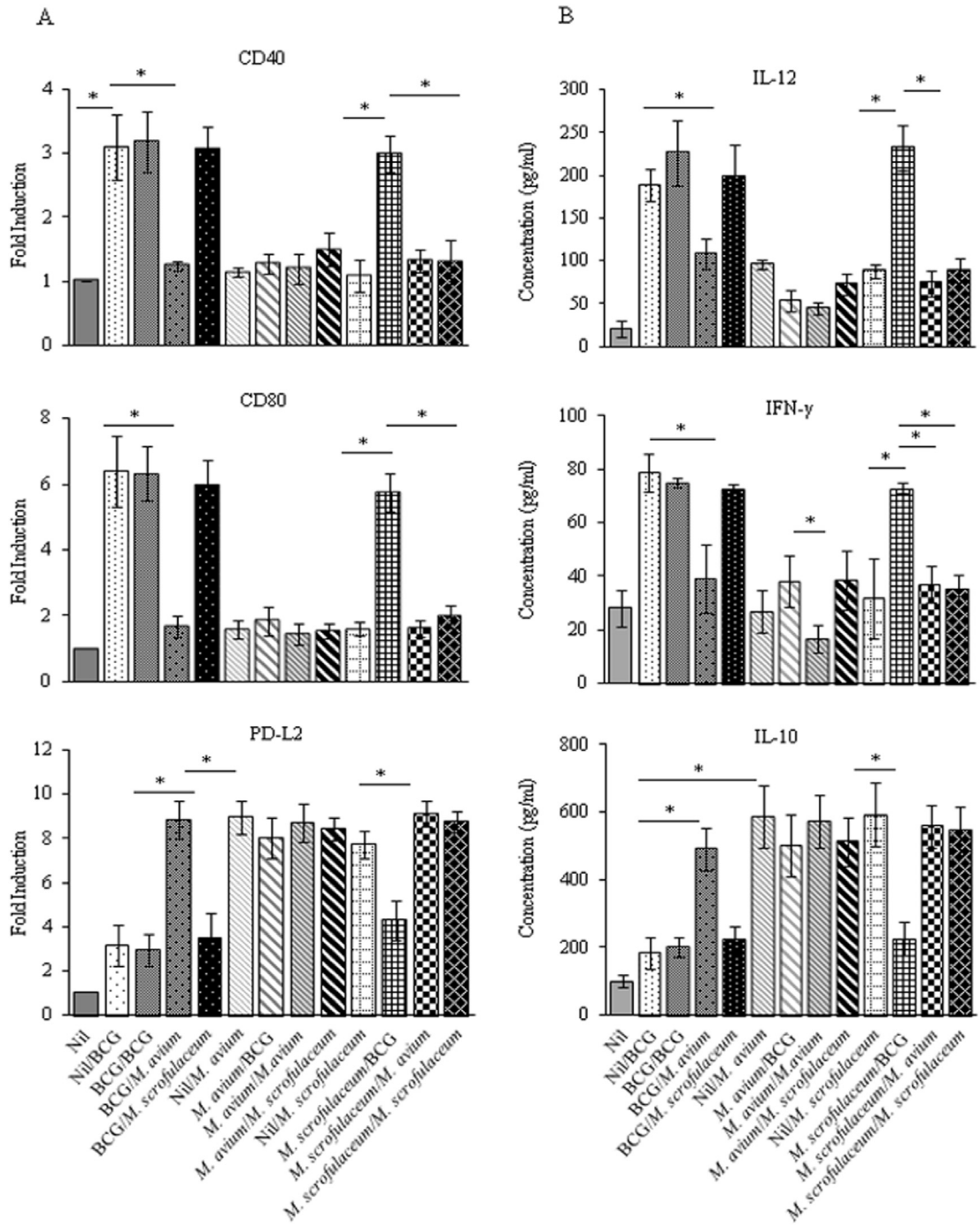

Figure 4. DC semi-mature phenotype induced by *M. scrofulaceum* was reverted by the stimulation with BCG. Enriched $CD11c^+$, $CD11b^+$ cells were exposed to *M. bovis* (BCG), *M. avium* or *M. scrofulaceum* for 3 h and were then subjected to a second exposure to any of the above stimuli for 18 h. The expression level of maturity molecules was determined by flow cytometry. (A) Fold induction in the expression of surface markers on BMDCs is shown. (B) Concentration of cytokines (pg/mL) in culture supernatants of BMDC doubly stimulated was determined by cytometric bead array. The data is the mean of three independent experiments. *$p < 0.01$ tested by one-way ANOVA. Un-stimulated DCs (Nil).

(Figures S4 and S5). Previous reports on different pathogens have shown that the modulation of cytoskeleton dynamics is a way to facilitate different functions. The successful microbial pathogens drive different effectors proteins to modulate the actin cytoskeleton and microtubules [26]. Whether NTM induces a similar behavior on DC cytoskeleton has been poorly studied. This study showed that the modest formation of actin-dependent structures such as filopodia after the recognition of *M. scrofulaceum*, correlated with a marked reduction in the motile capacity of DCs. Actin remodeling mediated by TLRS signaling also enhances antigen capture by DCs through the formation of filopodia and lamellipodia induced by strong actin polymerization on the cell periphery. On macrophage, cytoskeletal rearrangements dependent of TLR2 signaling induced by BCG challenge has been observed [26].

In vivo, DC migration to lymphoid nodules and the induction of Th1 response requires maturation stimuli [27]. We observed that reduction on DC migration was dependent on changes in the cytoskeletal organization. The negative effect observed on cell

migratory capacity, the high expression of PD-L2 and the elevated synthesis of IL-10 induced in DCs exposed to *M. scrofulaceum* were consistent with a semi-mature phenotype. However, despite knowing that in some pathogenic microorganisms this modulation in cytoskeleton dynamics facilitates many functions to prevent intracellular killing by alterations in different molecules, the actual mechanism after recognition of NTMs has not been determined.

In a previous work it was determined that the semi-mature DC phenotype induced by *M. avium* is dominant over the mature DC phenotype induced by BCG, although the DC exposition to BCG occurred before or after stimulation with *M. avium* [13]. We investigated whether the semi-mature DC phenotype induced by *M. scrofulaceum* could be maintained even after exposure to a second stimulus given by other mycobacteria such as *M. avium* or BCG (Figure 4).

In this work we evidenced that DC exposed to *M. scrofulaceum* after challenge with BCG Phipps showed a mature phenotype with high expression of CD40 and CD80, in addition to elevated synthesis of IFN-γ and IL-12, similar to that observed in DC only exposed to BCG (Figure 4A and B). This result indicated that the phenotype induced in *M. scrofulaceum*-stimulated DCs is not dominant over the mature phenotype induced by BCG. The fact that the phenotype of DCs induced by *M. scrofulaceum* was reverted in the presence of BCG (Figure 4) may be explained as a competition between various TLR signals during the initiation of the immune response and thus, the immune response could depend on the predominant TLR signal. It is possible that the set of signaling pathways activated by BCG are more intense than those induced by *M. scrofulaceum* and because of this, the mature DC phenotype is favored.

This could be feasible considering that BCG-cell wall is composed of mycolic acid, arabinogalactan, and peptidoglycan that are strong ligands to various TLRs-dependent signaling pathways that induce DC activation. Therefore, the strong recognition of several components of cell wall of BCG by DCs receptors confers to the mycobacteria adjuvant activity with a large immunostimulatory capacity [24].

Meanwhile, *M. scrofulaceum* was recognized through TLR4 and DC-SIGN but not by TLR2 as *M. avium* or BCG (Figure 2). This indicates that *M. scrofulaceum* ligands are different to either BCG or *M. avium* and consequently, its recognition by DC. In fact, some reports have been shown that resistance to chlorine in *M. scrofulaceum* is lower than *M. avium*, possibly devoid a differences in membrane fluidity, which is related to their lipid composition [28]. It is possible that signals downstream provided by *M. scrofulaceum*-ligands are weak and short-lived regard to that induced by BCG.

It has been reported that DCs require various signals before they can be activated completely. The signals for DC may be originated through TLRs and the pattern of recognition would be considered as the first signal. The co-activating signal provided by other TLR ligands could be provided as a second signal. The receipt of the first and second signal in DC produces a strong stimulus for maturation, together with a third signal such as cytokines [29].

When the first signal is limited (i.e. insufficient crosslink of TLRs), a second (co-activating) signal for immature DC would be required for the expression of co-stimulatory molecules and to acquire a mature phenotype. Maturation would require additionally a third signal [30]. Then, if any of these signs is insufficient, the DC will remain in a state of immaturity. Thus, the outcome of immune response would depend on competition between various TLR signals during the initiation of the immune response. This is, the acquired DC phenotype can generate two kinds of responses: one that activates T cells specific for a particular antigen (mature phenotype), or another that generates regulatory T cells [31].

According to the above, we propose that *M. scrofulaceum*-exposed DC stimulates signaling insufficiently and then, they remained in a state of immaturity that was reverted when a strong activator like BCG or PPD favored their maturation. However, further studies are needed to clarify the signaling pathways activated in DC through *M. scrofulaceum* recognition.

In summary, the results presented in this study show that *M. scrofulaceum* induces a semimature DC phenotype that is characterized by PD-L2 over-expression and elevated synthesis of IL-10, most likely through TLR4 and Raf-1 signaling pathway-dependent DC-SIGN stimulation. The consequences of the semi-mature phenotype induced by *M. scrofulaceum* can be change to mature phenotype before or after BCG stimulation.

Acknowledgments

Marisa Cruz-Aguilar is the recipient of a DGAPA-UNAM Postdoctoral fellowship. Alejandro Schcolnik-Cabrera is a student from the Plan de Estudios Combinados en Medicina (PECEM), UNAM.

Funding: CONACYT SALUD2010-C01-140998 and DGAPA IN227611 and IN214713 financially supported this study.

Competing interests: None declared.

Ethical approval: All experiments with animals were performed according to the guidelines of the ethics and institutional biosafety committees CIEFM-055-11.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tube.2016.04.003.

References

[1] Falkinham 3rd JO. Surrounded by mycobacteria: nontuberculous mycobacteria in the human environment. J Appl Microbiol 2009;107:356–67.
[2] Howard ST, Byrd TF. The rapidly growing mycobacteria: saprophytes and parasites. Microbes Infect Inst Pasteur 2000;2:1845–53.
[3] de Lisle GW, Wards BJ, Buddle BM, Collins DM. The efficacy of live tuberculosis vaccines after presensitization with *Mycobacterium avium*. Tuberculosis 2005;85:73–9.
[4] Flaherty DK, Vesosky B, Beamer GL, Stromberg P, Turner J. Exposure to *Mycobacterium avium* can modulate established immunity against *Mycobacterium tuberculosis* infection generated by *Mycobacterium bovis* BCG vaccination. J Leukoc Biol 2006;80:1262–71.
[5] Castillo-Rodal AI, Mazari-Hiriart M, Lloret-Sanchez LT, Sachman-Ruiz B, Vinuesa P, Lopez-Vidal Y. Potentially pathogenic nontuberculous mycobacteria found in aquatic systems. Analysis from a reclaimed water and water distribution system in Mexico City. Eur J Clin Microbiol Infect Dis 2012;31:683–94.
[6] Falkinham JO. The changing pattern of nontuberculous mycobacterial disease. Can J Infect Dis 2003;14:281–6.
[7] Gangadharam PR, Pratt PF. In vitro response of murine alveolar and peritoneal macrophages to *Mycobacterium intracellulare*. Am Rev Respir Dis 1983;128:1044–7.
[8] Hilborn ED, Covert TC, Yakrus MA, Harris SI, Donnelly SF, Rice EW, et al. Persistence of nontuberculous mycobacteria in a drinking water system after addition of filtration treatment. Appl Environ Microbiol 2006;72:5864–9.
[9] Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998;392:245–52.
[10] Labeur MS, Roters B, Pers B, Mehling A, Luger TA, Schwarz T, et al. Generation of tumor immunity by bone marrow-derived dendritic cells correlates with dendritic cell maturation stage. J Immunol 1999;162:168–75.
[11] Lutz MB, Schuler G. Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? Trends Immunol 2002;23:445–9.
[12] Young SL, Slobbe L, Wilson R, Buddle BM, de Lisle GW, Buchan GS. Environmental strains of *Mycobacterium avium* interfere with immune responses associated with *Mycobacterium bovis* BCG vaccination. Infect Immun 2007;75:2833–40.

[13] Mendoza-Coronel E, Camacho-Sandoval R, Bonifaz LC, Lopez-Vidal Y. PD-L2 induction on dendritic cells exposed to *Mycobacterium avium* downregulates BCG-specific T cell response. Tuberculosis 2011;91:36–46.
[14] Lutz MB, Kukutsch N, Ogilvie AL, Rossner S, Koch F, Romani N, et al. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J Immunol methods 1999;223:77–92.
[15] Geijtenbeek TB, Van Vliet SJ, Koppel EA, Sanchez-Hernandez M, Vandenbroucke-Grauls CM, Appelmelk B, et al. Mycobacteria target DC-SIGN to suppress dendritic cell function. J Exp Med 2003;197:7–17.
[16] Hodges A, Sharrocks K, Edelmann M, Baban D, Moris A, Schwartz O, et al. Activation of the lectin DC-SIGN induces an immature dendritic cell phenotype triggering Rho-GTPase activity required for HIV-1 replication. Nat Immunol 2007;8:569–77.
[17] Tsuji S, Matsumoto M, Takeuchi O, Akira S, Azuma I, Hayashi A, et al. Maturation of human dendritic cells by cell wall skeleton of *Mycobacterium bovis* bacillus Calmette-Guerin: involvement of toll-like receptors. Infect Immun 2000;68:6883–90.
[18] Uehori J, Matsumoto M, Tsuji S, Akazawa T, Takeuchi O, Akira S, et al. Simultaneous blocking of human Toll-like receptors 2 and 4 suppresses myeloid dendritic cell activation induced by *Mycobacterium bovis* bacillus Calmette-Guerin peptidoglycan. Infect Immun 2003;71:4238–49.
[19] von Meyenn F, Schaefer M, Weighardt H, Bauer S, Kirschning CJ, Wagner H, et al. Toll-like receptor 9 contributes to recognition of *Mycobacterium bovis* Bacillus Calmette-Guerin by Flt3-ligand generated dendritic cells. Immunobiology 2006;211:557–65.
[20] Sweet L, Schorey JS. Glycopeptidolipids from *Mycobacterium avium* promote macrophage activation in a TLR2- and MyD88-dependent manner. J Leukoc Biol 2006;80:415–23.
[21] Nagaoka K, Takahara K, Tanaka K, Yoshida H, Steinman RM, Saitoh S, et al. Association of SIGNR1 with TLR4-MD-2 enhances signal transduction by recognition of LPS in gram-negative bacteria. Int Immunol 2005;17:827–36.
[22] Hossain MM, Norazmi MN. Pattern recognition receptors and cytokines in *Mycobacterium tuberculosis* infection – the double-edged sword? BioMed Res Int 2013;2013:179174.
[23] Gagliardi MC, Teloni R, Giannoni F, Pardini M, Sargentini V, Brunori L, et al. *Mycobacterium bovis* Bacillus Calmette-Guerin infects DC-SIGN- dendritic cell and causes the inhibition of IL-12 and the enhancement of IL-10 production. J Leukoc Biol 2005;78:106–13.
[24] Mitchell DA, Fadden AJ, Drickamer K. A novel mechanism of carbohydrate recognition by the C-type lectins DC-SIGN and DC-SIGNR. Subunit organization and binding to multivalent ligands. J Biol Chem 2001;276:28939–45.
[25] Gringhuis SI, den Dunnen J, Litjens M, van Het Hof B, van Kooyk Y, Geijtenbeek TB. C-type lectin DC-SIGN modulates Toll-like receptor signaling via Raf-1 kinase-dependent acetylation of transcription factor NF-kappaB. Immunity 2007;26:605–16.
[26] Lasunskaia EB, Campos MN, de Andrade MR, Damatta RA, Kipnis TL, Einicker-Lamas M, et al. Mycobacteria directly induce cytoskeletal rearrangements for macrophage spreading and polarization through TLR2-dependent PI3K signaling. J Leukoc Biol 2006;80:1480–90.
[27] Bertho N, Adamski H, Toujas L, Debove M, Davoust J, Quillien V. Efficient migration of dendritic cells toward lymph node chemokines and induction of T(H)1 responses require maturation stimulus and apoptotic cell interaction. Blood 2005;106:1734–41.
[28] Falkinham JO. The changing pattern of nontuberculous mycobacterial disease. Can J Infect Dis J Can Malad Infect 2003;14:281–6.
[29] Steinman RM, Hawiger D, Nussenzweig MC. Tolerogenic dendritic cells. Annu Rev Immunol 2003;21:685–711.
[30] Blander JM, Medzhitov R. Regulation of phagosome maturation by signals from toll-like receptors. Science 2004;304:1014–8.
[31] Mihret A, Mamo G, Tafesse M, Hailu A, Parida S. Dendritic cells activate and mature after infection with *Mycobacterium tuberculosis*. BMC Res Notes 2011;4:247.