Cellular Microbiology (2016) doi:10.1111/cmi.12562

Reactive oxygen species production by human dendritic cells involves TLR2 and dectin-1 and is essential for efficient immune response against Mycobacteria

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Summary

Tuberculosis remains the single largest infectious disease with 10 million new cases and two million deaths that are estimated to occur yearly, more than any time in history. The intracellular replication of *Mycobacterium tuberculosis* (*Mtb*) and its spread from the lungs to other sites occur before the development of adaptive immune responses. Dendritic cells (DC) are professional antigen-presenting cells whose maturation is critical for the onset of the protective immune response against tuberculosis disease and may vary depending on the nature of the cell wall of *Mtb* strain. Here, we describe the role of the endogenous production of reactive oxygen species (ROS) on DC maturation and expansion of *Mtb*-specific lymphocytes. Here, we show that *Mtb* induces DC maturation through TLR2/dectin-1 by generating of ROS and through Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) in a ROS independently manner. Based on the differences observed in the ability to induce DC maturation, ROS production and lymphocyte proliferation by those *Mtb* families widespread in South America, i.e., Haarlem and Latin American Mediterranean and the reference strain H37Rv, we propose that variance in ROS production might contribute to immune evasion affecting DC maturation and antigen presentation.

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Introduction

Mycobacterium tuberculosis (*Mtb*) infects approximately one third of the world's population (WHO, 2013). *Mtb* is able to target endothelial cells, alveolar macrophages and dendritic cells (DCs), a first line of defense within the lungs. DCs are professional antigen-presenting cells that exhibit the ability to activate naïve lymphocytes against microbial antigens, resulting in the initiation of protective immune response, so that activated $CD4^+$ and $CD8^+$ T-cells migrate back to the lungs to further alveolar macrophages activation (Banchereau and Steinman, 1998). DCs may be differentiated *in vitro* from $CD14^+$ monocytes (Mo) cultured in the presence of granulocyte-macrophage colony-stimulating factor and interleukin-4 (IL-4). These DCs are immature, but after activation with lipopolysaccharide (LPS), cytokines (Sallusto *et al.,* 2000) or *Mtb* (Alemán *et al.,* 2007) expression of co-stimulatory molecules (CD86) and maturation markers (CD83, major histocompatibility complex class II) as well as the production of tumor necrosis factor alpha and interferon gamma (IFNγ), IL-12, IL-1β e IL-6 are increased acquiring T-cell stimulatory capacity (Hickman *et al.,* 2002; Fricke *et al.,* 2006).

It has been reported that TLR2 mediates DC maturation induced by microbial lipopeptides conferring them a potent capacity to stimulate T cells in a mixed leukocyte reaction (MLR) (Hertz *et al.,* 2001). TLRs are a key step in initiating innate immune responses upon mycobacterial infection, (van Crevel *et al.,* 2002) and in this context, it has been demonstrated that TLR2 stimulation enhances the production of reactive oxygen species (ROS) that is accompanied by a sustained phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK), suggesting an essential role of ROS in TLR2-signaling pathways (Yang *et al.,* 2008). The very low levels of ROS production in DCs suggest that it would not be directly involved in microbe killing (DCs express but around 5% of the levels found in neutrophils) (Kantegawa *et al.,* 2003) but would be important in TLR-mediated maturation of DC to control dissemination of the pathogen (Elsen *et al.,* 2004). In this way, it has been demonstrated that DC maturation could also be affected by oxidative stress (Vulcano *et al.,* 2004).

It is now known that relatively low concentrations of ROS serve as second messengers during cellular responses to a variety of physiological stimuli (Reth, 2002). In this regard, in chronic granulomatous disease,

patients who are incapable of producing ROS, tuberculosis disease progression is much more severe (Lee *et al.,* 2008). Moreover, it has recently been reported that ROS generated by DCs during antigen presentation, enhances the antigen-specific T-cell proliferation (Matsue *et al.,* 2003), suggesting that endogenous production of ROS may act as an important mediator during antigen presentation.

Many pathogens have evolved mechanisms to avoid being eliminated, and such mechanisms have allowed *Mtb* to become a very successful pathogen (Fratti *et al.*, 2003; Chua *et al.,* 2004). Within the Haarlem lineage, the multidrug-resistant M strain is highly prosperous in Argentina and is able to build up further drug resistance without impairing its ability to spread. In a previous work, we showed that this clinical strain fails in their ability to induce ROS and apoptosis in neutrophils, a mechanism possibly governed by carbohydrate composition of mycobacteria cell wall (Romero *et al.,* 2014). Here, we describe the *Mtb*-induced ROS production in DCs and the impact on immune response against Mycobacterium tuberculosis, directing the discussion to how this phenomenon could contribute to immune evasion.

Results

Mycobacterium tuberculosis induce dendritic cell phenotypic maturation and T-cell proliferation

Human Mo-derived DCs were exposed to γ-irradiated H37Rv for 24 h, at a ratio of 2 bacilli to 1 DC to minimize cell death, and thereafter, the expression of maturation markers was determined by flow cytometry on the basis of CD86 and DC-specific intercellular adhesion molecule-3 grabbing non-integrin (SIGN) expression. The results show that *Mtb* induced increase of CD86, CD83 and Human Leukocyte Antigen – antigen D Related (HLA-DR), as well as the production of IL-12, similar to that obtained with LPS (100 ng/ml), compared with non-stimulated cells. Until now, it was believed that IFNγ was produced only by lymphocytes; however, it was lately shown that Bacillus Calmette–Guérin (BCG) vaccine or lipopeptide-stimulated DCs produce IFNγ (Fricke *et al.,* 2006; Hertz *et al.,* 2001). Here, we observed that in contrast to LPS stimulation, γ-irradiated H37Rv induced a significant production of IFNγ in DCs (Figure 1A).

Mature DCs are potent stimulators of T-cell proliferation either in association with peptide antigen or in

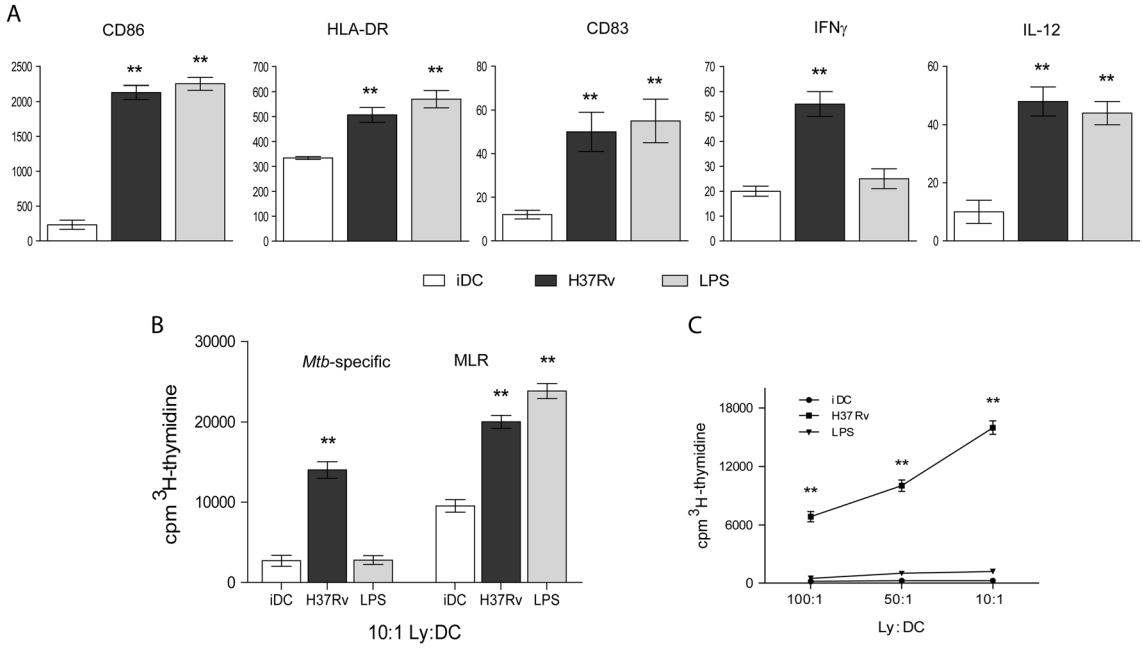

Fig. 1. *Mycobacterium tuberculosis*-matured DCs are potent stimulators of T-cell proliferation.
A. Human Mo-derived dendritic cells (DCs) (white bars) were exposed to H37Rv (dark grey bars) or lipopolysaccharide (LPS) (light gray bars) for 24 h, and the expression of CD86, HLA-DR, CD83, INFγ and IL-12 was determined by flow cytometry on the basis of CD86 and DC-specific intercellular adhesion molecule-3 grabbing non-integrin expression. Results are expressed as mean ± standard error (SE) ($n = 25$). H37Rv or LPS versus non stimulated DC (iDC) $^{**}p < 0.001$.
B. Immature and H37Rv-matured or LPS-matured DC were conducted in proliferation assays either by using autologous lymphocytes from healthy donors with positive response to the antigen-purified protein derivative (PPD^{+}) as a specific response, or by using human lymphocytes from non related donor (MLR) for 5 days at 1:10 ratios of DCs to T-cells. The results show the lymphocyte proliferative response and are expressed as mean ± SE ($n = 9$) of the counts of ^{3}H-thymidine incorporation per minute (cpm). Specific response: H37Rv versus iDC, $^{**}p < 0.001$; MLR: H37Rv or LPS versus iDC, $^{**}p < 0.001$.
C. Immature or H37Rv- and LPS-matured DCs were conducted in specific proliferation assays for 5 days with different ratios of DCs to T-cells (10:1; 50:1; 100:1). Results are expressed mean ± SE ($n = 7$). H37Rv versus iDC, $^{**}p < 0.001$ for all DC:Ly ratios. HLA-DR, Human Leukocyte Antigen – antigen D Related; IFNγ, interferon gamma.

alloreaction (MLR). Thus, in order to evaluate DC functionality, *Mtb*-matured DCs were co-cultured with autologous lymphocytes from healthy donors with positive response to the antigen purified protein derivative (PPD) (PPD^+) or human lymphocytes from non related donor (MLR) in order to evaluate lymphocyte proliferation. As shown in Figure 1B, *Mtb* as well as LPS improved MLR, reflecting a functional maturation due to MLR is known to be highly dependent on CD80/CD86-mediated co-stimulation (Tzachanis *et al.,* 2002). Besides, *Mtb*-matured DCs were able to induce antigen presentation (Figure 1B and C).

Toll-like receptor 2 mediates Mycobacterium tuberculosis-induced dendritic cell maturation

It has been suggested that TLR2 and TLR4 are receptors for mycobacteria on human DCs (Means *et al.,* 1999) and TLR2 mediates DC maturation (Alemán *et al.,* 2007; Fricke *et al.,* 2006; Hertz *et al.,* 2001). Therefore, we evaluated the role of TLR2 in *Mtb*-induced expression of maturation markers following CD86+/DC-SIGN+ gating strategy (Figure 2A). Then IFNγ measured by flow cytometry (Figure 2A and B) and by Enzyme-Linked Immuno Sorbent Assay (ELISA) (Figure 2C) as well as HLA-DR expression (Figure 2D) were abrogated, whereas the CD86 expression was partially reduced by the blockage of TLR2 (Figure 2E). Kinetic studies of MAPK phosphorylation showed that *Mtb* induces both Extracellular signal-regulated kinases (ERK) and p38 activation in human Mo (Reiling *et al.,* 2001; Schorey and Cooper, 2003). To assess the role of MAPK in DC maturation, cells were incubated with specific inhibitors for p38, ERK and PI3K, before bacteria challenge. The viability of DCs was not affected in the presence of specific inhibitors (data not shown). Noticeably, pretreatment of DCs with the p38 inhibitor SB203580 abrogated the *Mtb*-induced IFNγ production and HLA-DR expression, whereas the *Mtb*-induced CD86 expression was strongly reduced but remained detectable in the DC surface. On the other hand, ERK or PI3K inhibitors (PD98059 and wortmannin respectively) had no effect (Figure 2B–E). Consequently, DC functionality was also affected (Figure 2F), even for the specific T-cell proliferation, which was also reduced in both DCs and Mo (Figure 2F and G), which could be attributed to a lack of DC maturation and/or antigen presentation.

Reactive oxygen species play an essential role in Mycobacterium tuberculosis-induced dendritic cell maturation and antigen presentation

It has been described that intracellular ROS operates as a signaling molecule (Finkel, 2003) inducing DC maturation (Kantegawa *et al.,* 2003) and besides, ROS are also necessary for TLR2-induced p38 MAPK activation in macrophages (Yang *et al.,* 2008). To determine whether ROS are involved in *Mtb*-induced DC maturation and antigen presentation, oxidases inhibitor (DPI), a selective inhibitor of xanthine oxidase (allopurinol) and a selective inhibitor of NADPH oxidase (apocynin), were employed to inhibit ROS production before *Mtb* challenge. As can be observed in Table 1, HLA-DR and IFNγ expression induced by *Mtb* was abrogated by DPI treatment, which was in coherence with the impaired T-cell proliferation. In addition, treatment with apocynin or allopurinol significantly reduced DC maturation and lymphocyte proliferation. In contrast, CD86 expression was partially reduced, prompting us to suppose that other mechanisms participate in inducing CD86 expression, cooperating with ROS in DC maturation triggered by *Mtb*.

Role of Toll-like receptor 2 in Mycobacterium tuberculosis-induced reactive oxygen species production in DCs

Considering that ROS affect DC maturation, we sought to determine whether receptors associate with *Mtb* recognition are involved in ROS production. Thus, ROS and CD86 expression were assessed in the presence of blocking antibodies against TLR2, mannose receptor (MR) (Stahl and Ezekowitz, 1998), DC-SIGN (Tailleux *et al.,* 2003), the fungal β-glucan receptor dectin-1 (Brown *et al.,* 2003) and CD11b (Velasco-Velázquez *et al.,* 2003). As shown in Figure 3A, ROS production was abrogated by the blockage of TLR2 or dectin-1 but not by anti-DC-SIGN, anti-CD11b nor anti-MR. On the other hand, the increase in CD86 expression was affected but not completely inhibited by the blockage of TLR2, dectin-1 or DC-SIGN blockage. However, when all these receptors were blocked simultaneously, the increase in CD86 expression was totally abrogated supporting the idea that DC maturation is mediated by ROS, involving TLR2 and dectin-1, and by other mechanism, which up-regulate CD86 expression through DC-SIGN (Figure 3B). The intracellular expression of the activated form of the spleen tyrosine kinase (Syk), which is associated to dectin-1, was significantly induced under *Mtb* challenge confirming the participation of dectin-1 in *Mtb* recognition. In turn, the *Mtb*-induced expression of Spleen tyrosine kinase (Syk) was abrogated by blocking dectin-1 (Figure 3C). Moreover, ROS was significantly reduced by Syk inhibition, suggesting that those pathways downstream of TLR2 and dectin-1 are both involved in the generation of ROS (Figure 3D).

Mycobacterium tuberculosis-induced reactive oxygen species production affects lymphocyte proliferation

Thereafter, we evaluated whether *Mtb*-induced ROS production affects antigen presentation directly or by inducing maturation of the cell. To that, DCs were first treated with LPS for 24 h to induce maturation of DCs, and we observed that even re-stimulating the last 2 h, ROS production was not induced compared with *Mtb* stimulation (Figure 4A and B). It

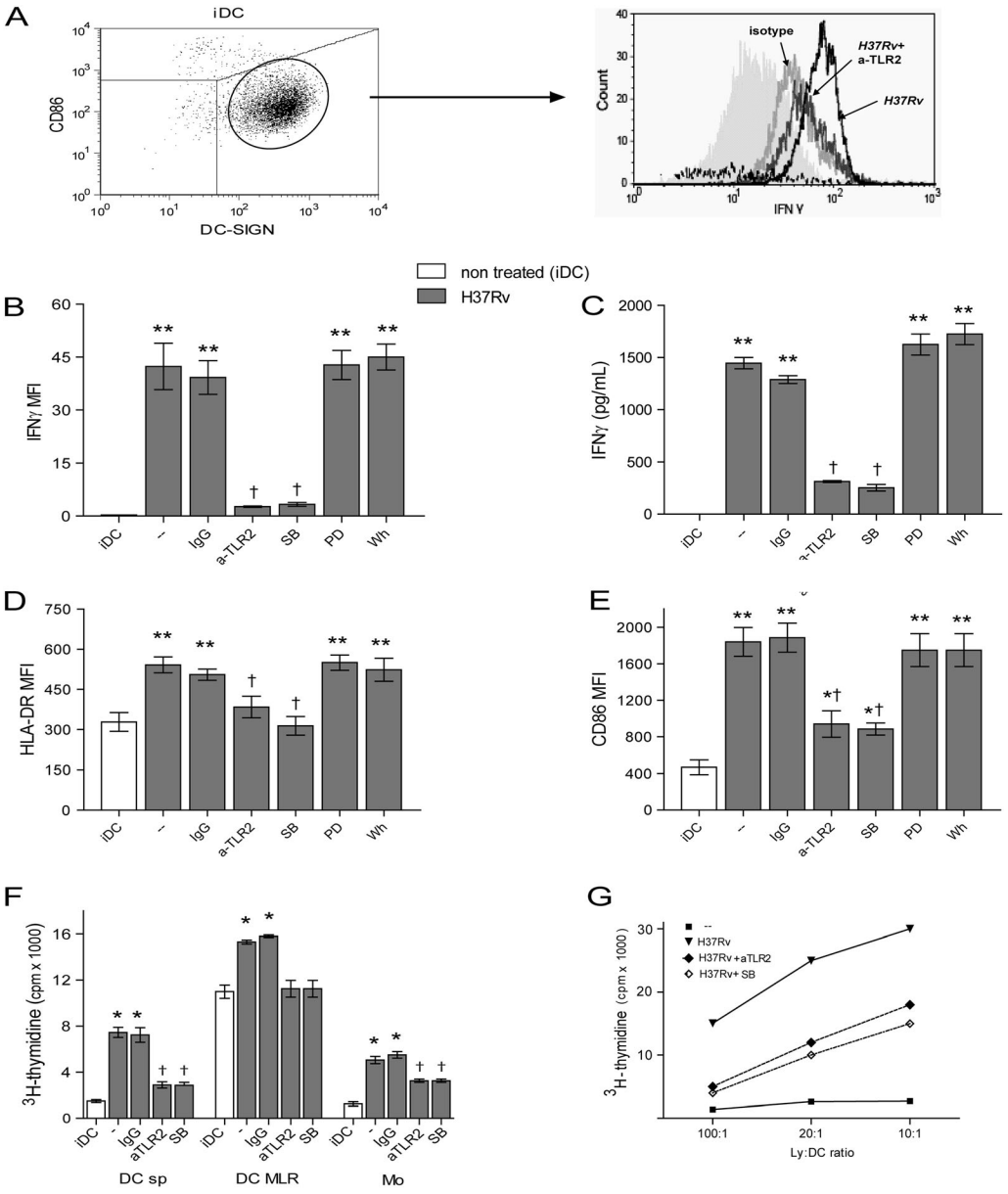

Fig. 2. Toll-like receptor 2 mediates *Mycobacterium tuberculosis* (*Mtb*)-induced dendritic cell (DC) maturation.
A. DC population was gated on the basis of CD86 and DC-specific intercellular adhesion molecule-3 grabbing non-integrin (SIGN) expression. Intracytoplasmic expression of IFNγ was evaluated in this gate, and histogram shows a representative experiment of 20 carried out. Immature DC (iDC) (white bars), were stimulated with *Mtb* H37Rv (grey bars) in the presence or not of specific inhibitors for p38 (SB203580), ERK (PD98059) and PI3K (wortmannin), or blocking mAbs against TLR2, before *Mtb* treatment and maturation markers were evaluated. The results are expressed as mean ± standard error ($n = 25$).
B. IFNγ was measured by flow cytometry or
C. quantified by ELISA; H37Rv, irrelevant IgG Ab + H37Rv, H37Rv + PD98059 and H37Rv + wortmannin versus iDC, $^{**}p < 0.001$; H37Rv + anti-TLR2 or H37Rv + SB203580 versus H37Rv, $\dagger p < 0.0001$
D. HLA-DR expression: H37Rv, irrelevant IgG antibody + H37Rv, H37Rv + PD98059 and H37Rv + wortmannin versus iDC, $^{**}p < 0.001$; H37Rv + anti-TLR2 or H37Rv + SB203580 versus H37Rv, $\dagger p < 0.0001$
E. CD86 expression; H37Rv, irrelevant IgG antibody + H37Rv, H37Rv + PD98059 and H37Rv + wortmannin versus iDC, $^{**}p < 0.001$; H37Rv + anti-TLR2 or H37Rv + SB203580 versus H37Rv, $\dagger p < 0.0001$; H37Rv + anti-TLR2 or H37Rv + SB203580 versus iDC $^{*}p < 0.01$.
F. *Mtb*-induced mixed leukocyte reaction (MLR) in DCs and in specific T-cell proliferation in both DCs and Mo at 1:10 ratios of DCs or Mo to T cells. H37Rv or irrelevant IgG Ab + H37Rv antibody versus iDC, $^{*}p < 0.005$; H37Rv + anti-TLR2 or H37Rv + SB203580 versus H37Rv, $\dagger p < 0.0001$
G. Representative experiment of specific T-cell proliferation at 1:10; 1:50 and 1:100 DC to T-cells ratio is shown.

Table 1. Role of ROS in *Mtb*-induced DC maturation and function

	CD86 (MFI)	HLA-DR (MFI)	IFNγ (MFI)	MLR (3H-thymidine)
iDC	546 ± 153	369 ± 21	20 ± 2	8386 ± 100
+H37Rv	1984 ± 190[a]	623 ± 51[a]	65 ± 5[a]	13450 ± 420[a]
+H37Rv + DPI	997 ± 140[b]	388 ± 40	25 ± 4	7550 ± 230
+H37Rv + allop	1200 ± 100[a]	550 ± 51[b]	37 ± 4[b]	8500 ± 230[c]
+H37Rv + apocyn	1011 ± 101[a]	475 ± 51[b]	43 ± 6[b]	8100 ± 230[c]

a. Expression of CD86, HLA-DR and INFγ in dendritic cells exposed to H37Rv for 24 h, at a ratio of 2 bacilli to 1 DC in the presence or not of different oxidases inhibitors before *Mtb* treatment, was determined by flow cytometry.
b. Results are expressed as mean ± standard deviation ($n = 11$) of mean fluorescence intensity (MFI).
c. Mixed leukocyte reaction (MLR) at 10:1 lymphocyte/dendritic cell are expressed as mean ± standard deviation ($n = 11$) of counts per minute (cpm). H37Rv versus iDC.
[a] **d.** $p < 0.001$; H37Rv + DPI, H37Rv + allopurinol or H37Rv + apocynin versus iDC.
[b] **e.** $p < 0.02$; H37Rv + allopurinol or H37Rv + apocynin versus H37Rv.
[c] **f.** $p < 0.005$.
g. iDC, Immature dendritic cells; HLA-DR, Human Leukocyte Antigen – antigen D Related; IFNγ, interferon gamma.

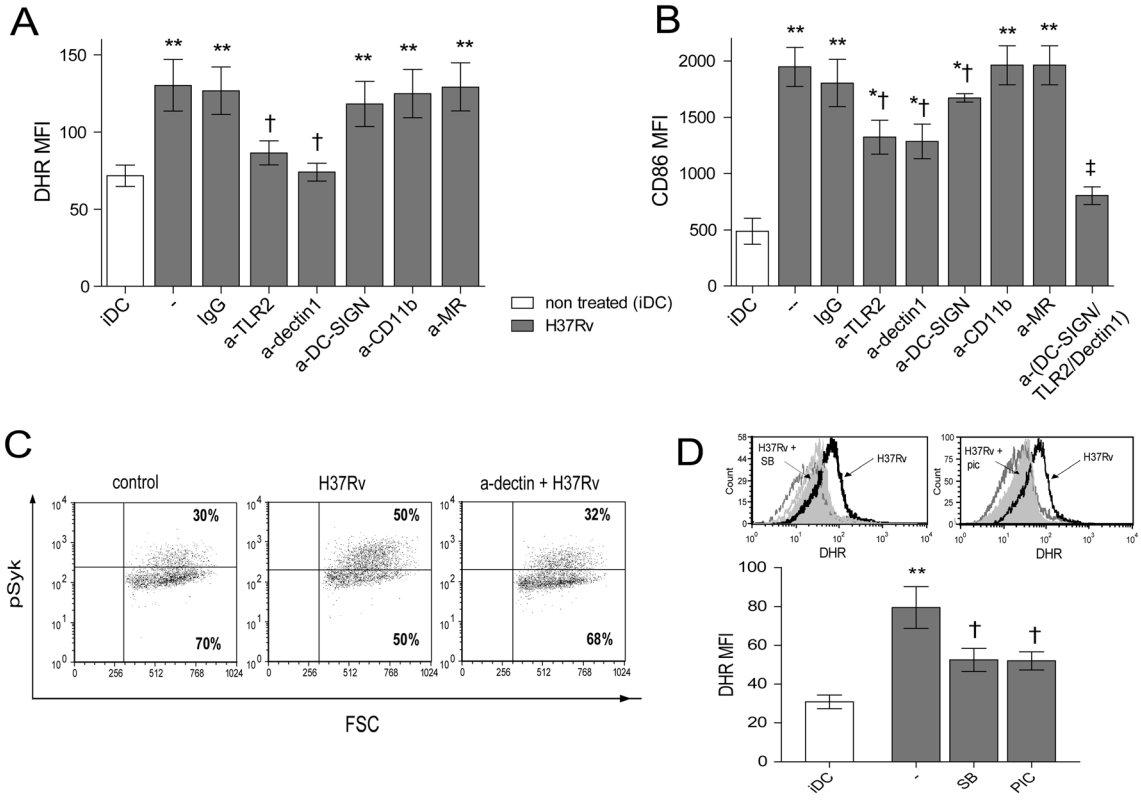

Fig. 3. Toll-like receptor 2 participates in *Mycobacterium tuberculosis* (*Mtb*)-induced reactive oxygen species production in dendritic cells (DCs). DCs were incubated in the presence of blocking antibodies against TLR2, dectin-1, DC-SIGN, CD11b and MR, and then stimulated (grey bars) or not (white bars) with a 2:1 *Mtb* : DC ratio (H37Rv).
A. DCs were incubated 15 min with DHR before the addition of blocking mAbs or irrelevant antibodies and then incubated with *Mtb* for 2 h. The emission of oxidized DHR was evaluated by flow cytometry. Results are expressed as a mean ± standard error (SE) ($n = 20$): H37Rv, irrelevant IgG + H37Rv, H37Rv + a-DC-SIGN, H37Rv + a-CD11b and H37Rv + a-MR versus iDC $^{**}p < 0.001$; H37Rv + a-TLR2, H37Rv + a-dectin-1 versus H37Rv †$p < 0.001$
B. CD86 expression was evaluated by flow cytometry and results are expressed as a mean ± SE ($n = 20$): H37Rv, irrelevant IgG + H37Rv, H37Rv + a-DC-SIGN, H37Rv + a-CD11b and H37Rv + a-MR versus iDC $^{**}p < 0.001$; H37Rv + a-TLR2, H37Rv + a-dectin versus control $^{*}p < 0.005$; H37Rv + a-TLR2, H37Rv + a-dectin and H37Rv + a-DC-SIGN versus H37Rv †$p < 0.001$; H37Rv + a-DC-SIGN + a-TLR2 versus H37Rv ‡$p < 0.0001$.
C. Percentage of cells expressing the activated form of Syk in *Mtb*-stimulated DCs. A representative experiment of 7 done is depicted.
D. DCs incubated with DHR were treated or not with inhibitors specific for p38 (SB203580) or Syk (piceatannol, pic). Thereafter, cells were incubated with *Mtb* H37Rv (grey bars) or media alone (white bars) as in (A). The results are expressed as mean ± SE ($n = 12$): H37Rv versus control $^{**}p < 0.001$; H37Rv + SB and H37Rv + pic versus H37Rv †$p < 0.001$. Representative histograms are embedded.

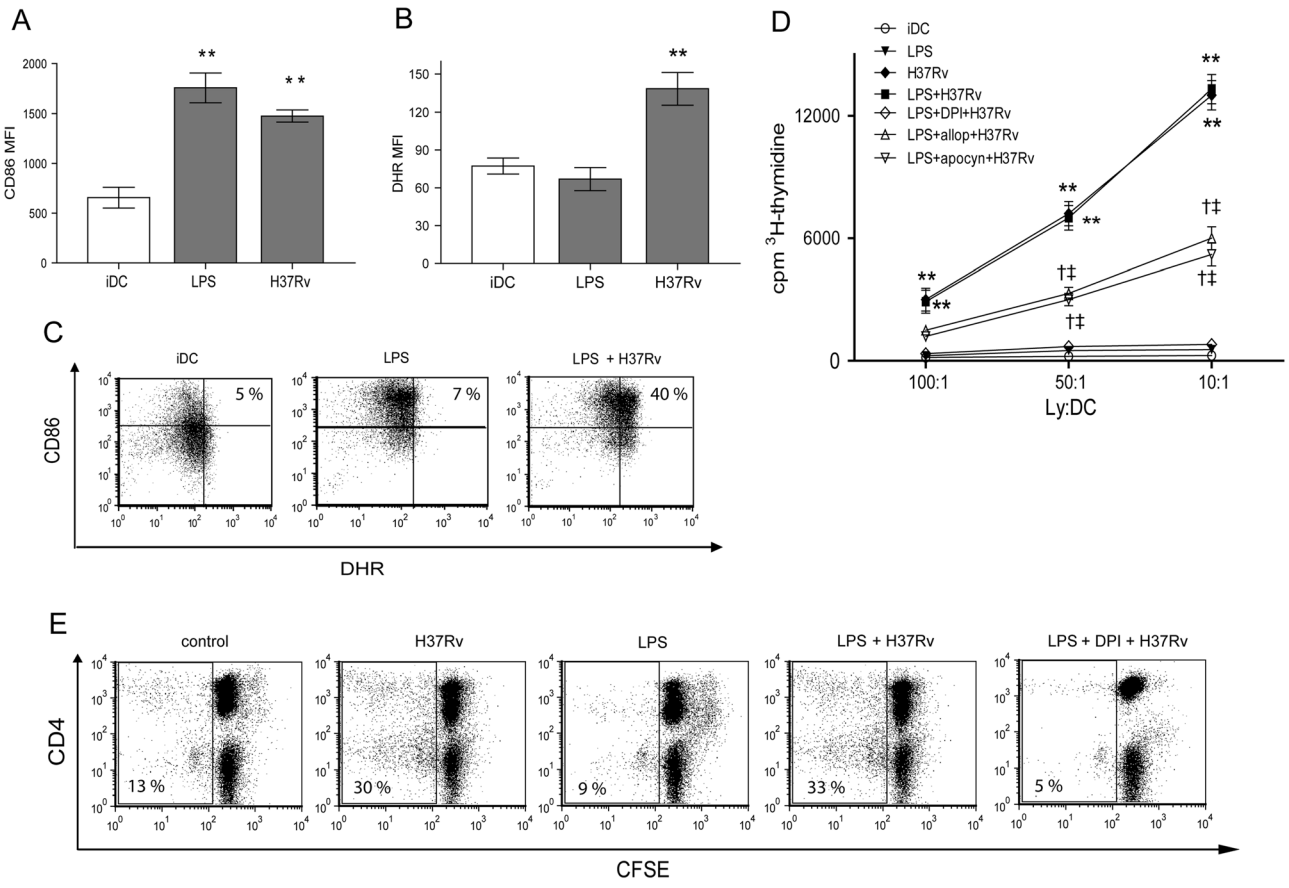

Fig. 4. *Mycobacterium tuberculosis* (*Mtb*)-induced reactive oxygen species production affects lymphocyte proliferation. Dendritic cells (DCs) were cultured for 24 h in the presence of lipopolysaccharide (LPS) or *Mtb* (H37Rv)

A. CD86 expression: H37Rv and LPS versus immature DC (iDC) $*p < 0.001$. And

B. reactive oxygen species production was evaluated 2 h after re-stimulation; DHR: H37Rv versus iDC $*p < 0.001$. Results are expressed as a mean ± standard error ($n = 20$).

C. Percentage of cells that express oxidized DHR and/or CD86 in DCs that were first matured with LPS for 24 h and thereafter stimulated or not with *Mtb* for 2 more hours. A representative experiment of seven carried out is depicted.

D. Specific lymphocyte proliferation in response to different ratios of DC treated as in (C) with or without oxidase inhibition before *Mtb* treatment. Results are expressed as mean ± standard error ($n = 9$) of the counts of ^{3}H-thymidine incorporation per minute. LPS + H37Rv and H37Rv versus all conditions $**p < 0.001$; LPS + allopurinol + H37Rv and LPS + apocynin + H37Rv versus control $\dagger p < 0.005$; LPS + allopurinol + H37Rv and LPS + apocynin + H37Rv versus LPS + H37Rv $\ddagger p < 0.005$

E. CFSE and CD4 expression on lymphocyte gated on $CD3^{+}$ cells and cultured for 5 days at 1:10 of DC to T-cell ratio. A representative experiment of four carried out is depicted.

is known that LPS induces DC maturation through TLR4 and could priming ROS production in DC (Kaisho and Akira, 2001); thus, we matured DC with LPS, and thereafter, LPS-matured DC were incubated for 2 h with *Mtb* to generate ROS. The resulting DCs showed a mature phenotype and *Mtb*-induced ROS production (Figure 4C). So, in these conditions, we were able to evaluate the role of ROS in antigen presentation beyond DC maturation. As shown in Figure 4D and E, when oxidase inhibitors (DPI) were supplied after LPS treatment and before the addition of *Mtb,* the specific lymphocyte proliferation was totally abrogated, suggesting that *Mtb*-induced ROS formation of LPS-matured DCs affects lymphocyte proliferation albeit. In addition, *Mtb*-induced lymphocyte proliferation was partially reduced by allopurinol or apocynin, suggesting the involvement of both oxidase pathways.

Mycobacterium tuberculosis-strains differ in their ability to induce reactive oxygen species, dendritic cell maturation and lymphocyte proliferation

Two *Mtb* multi-drug resistant clinical strains, M and Ra, were evaluated in their ability to induce DCs maturation. These strains are representative of widespread *Mtb* families in South America, i.e., Haarlem and Latin-American Mediterranean respectively, being strain M able to generate further drug resistance and to disseminate aggressively. In a previous work, we demonstrated that Ra

Table 2. *Mycobacterium tuberculosis* strains differentially induce DC maturation.

(MFI)	CD86	HLA-DR	IFNγ	IL-12
iDC	502 ± 95	334 ± 6	18 ± 2	10 ± 4
+H37Rv	1861 ± 154[b]	507 ± 30[a]	50 ± 8[b]	48 ± 8[b]
+Ra	1928 ± 204[b]	580 ± 35[a]	49 ± 6[b]	52 ± 4[a]
+M	1131 ± 100[b]	350 ± 15	25 ± 5	14 ± 3

a. Dendritic cells were exposed to H37Rv, Ra or M strains for 24 h at a ratio of 2 bacilli to 1 DC.
b. The expression of maturation markers was determined by flow cytometry.
c. The results show the increased expression of CD86 and HLA-DR in membrane and IFNγ and IL-12 in cytoplasm after *Mycobacterium tuberculosis* strains treatment and are expressed as mean ± standard deviation ($n = 16$); H37Rv and Ra versus iDC.
[a] **d.** $p < 0.001$; M versus iDC.
[b] **e.** $p < 0.02$; M versus H37Rv and Ra.
[c] **f.** $p < 0.005$.
g. iDC, Immature dendritic cells; HLA-DR, Human Leukocyte Antigen – antigen D Related; IFNγ, interferon gamma; MFI, mean fluorescence intensity.

strongly induces ROS in neutrophils, whereas M lacks the ability to induce ROS, as well as activation of p38 and Syk, and consequently, it fails at induce neutrophil apoptosis (Romero *et al.,* 2014). Here, we show that DC maturation (Table 2), ROS production (Figure 5A), Syk activation (Figure 5B) and T-cell proliferation (Figure 5C–E) induced by Ra were comparable with H37Rv strain (Table 2). However, strain M triggered a poor response, which coincides with a low production of ROS, being partially restored by the exogenous addition of H_2O_2. As it has been previously shown, exogenous addition of H_2O_2 fails at inducing LPS-induced DC maturation (Kantegawa *et al.,* 2003); thus, our results reinforce the notion that ROS makes the difference at processing *Mtb* antigens.

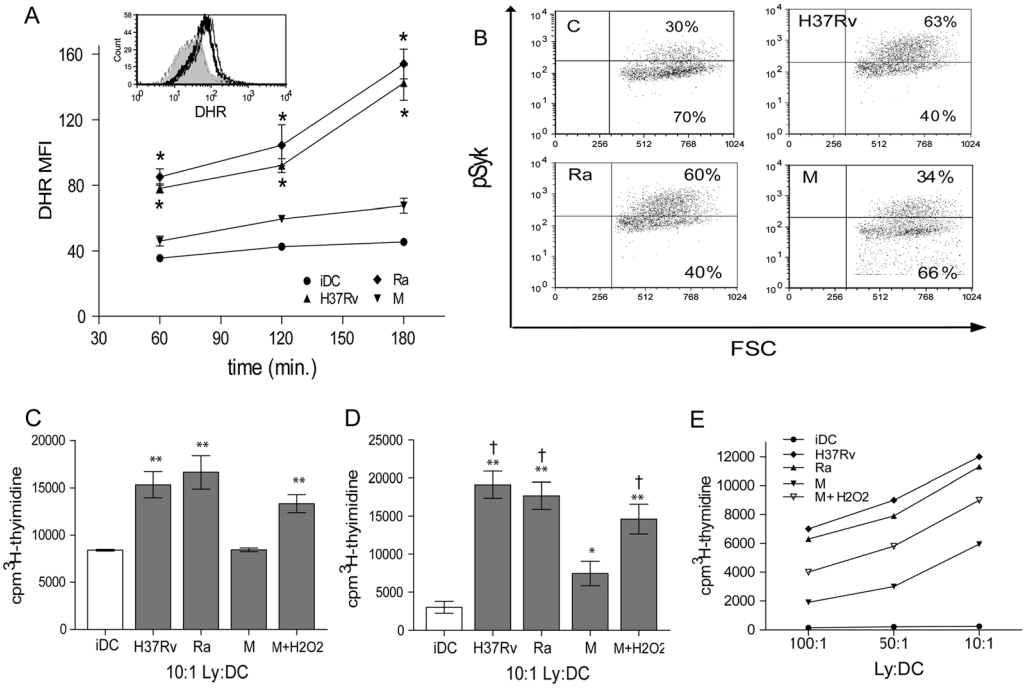

Fig. 5. *Mycobacterium tuberculosis* (*Mtb*) strains differentially induce reactive oxygen species, dendritic cells (DC) maturation and lymphocyte proliferation. Two *Mtb* multi-drug resistant clinical strains, M and Ra, were evaluated in their ability to induce DC functionality.
A. DCs were cultured for 24 h in the presence *Mtb* (H37Rv) or clinical isolates and reactive oxygen species production was evaluated 2 h after re-stimulation. Results were expressed as the mean ± standard error ($n = 20$) of emission of oxidized DHR and evaluated by flow cytometry: H37Rv and Ra versus immature DC (iDC) $*p < 0.001$. A representative experiment is embedded.
B. Percentage of cells that express the activated form of Syk in DC stimulated with H37Rv, Ra and M strain. A representative experiment of seven carried out is depicted
C. Mixed leukocyte reaction assay at 1:10 ratios of DC to T cells. DCs were cultured as in (A). Results are expressed as mean ± standard error ($n = 9$) of the counts of ^{3}H-thymidine incorporation per minute, H37Rv, Ra or M + H_2O_2 versus iDC $**p < 0.001$
D. lipopolysaccharide-matured DC, and were loaded with *Mtb* strains and *Mtb*-specific T-cell proliferation at 1:10 ratios of DCs to T-cells were evaluated. H37Rv and Ra versus iDC $**p < 0.001$; M versus iDC $*p < 0.05$; M versus H37Rv, Ra or M + H_2O_2 $\dagger p < 0.002$,
E. representative experiment of specific T-cell proliferation at 1:10; 1:50 and 1:100 DC : T ratio.

Discussion

Dendritic cells are present as a dense network in the airway mucosa (Holt, 2005) and phagocyte viable *Mtb* (Henderson *et al.,* 1997), which is critical for mounting a protective immune response (Jiao *et al.,* 2002; Flynn and Chan, 2001). *Mtb*-induced DC maturation is a prerequisite for an efficient T-cell activation (Hertz *et al.,* 2001; Tsuji *et al.,* 2000) and involves the up-regulation of several molecules (Giacomini *et al.,* 2001; Tailleux *et al.,* 2005; Mellman and Steinman, 2001). Here, we observed that *Mtb*-induced DC maturation comprised the up-regulation of CD86, HLA-DR and IL-12 similarly to LPS stimulation and was reflected in DC functionality, whereas IFNγ production was induced by *Mtb* and not by LPS. In this context, it has been described that DCs produce IFNγ in response to *Mycobacterium bovis* but not LPS (Fricke *et al.,* 2006), suggesting an autocrine DC activation upon bacterial encounter.

Some *Mtb* components are able to stimulate DC maturation through TLR2 and TLR4 (Tsuji *et al.,* 2000; Quesniaux *et al.,* 2004) involving activation of MAPK (Re and Strominger, 2001). In line with this, our group has shown that p38 MAPK can be activated by *Mtb* in DCs and neutrophils (Alemán *et al.,* 2007). Here, we observed that IFNγ production as well as HLA-DR up-regulation induced by *Mtb* totally involves TLR2/p38 but not ERK or PI3K pathways. On the other side, the blockage of TLR2/p38 MAPK partially inhibited the up-regulation of CD86 that was consequently reflected on DC function. In addition to the cytotoxic potential, ROS can be produced within the cell and served as signaling molecules (Finkel, 2003), regulating various intracellular cascades (Forman and Torres, 2002). In this respect, ROS could regulate gene expression by their action on redox-regulated transcription factors, like NF-κB and AP-1 (Schreck *et al.,* 1992). In this context, it has been proposed a role of ROS in TLR2 signaling together with a sustained phosphorylation of p38 MAPK (Yang *et al.,* 2008) and also being involved in DC maturation and antigen presentation (Matsue *et al.,* 2003; Kantegawa *et al.,* 2003). Moreover, LPS-induced DC maturation involves NADPH oxidase without participation of xanthine oxidase system (Vulcano *et al.,* 2004). The release of oxygen radicals may involve multiple enzymatic pathways (Droge, 2002), and in this way here, we showed that *Mtb*-induced DC maturation involves both NADPH oxidase and xanthine oxidase system without the requirement of being pre-treated with a priming agent. Furthermore, NADPH oxidase could be associated with TLR2 in response to *Mtb* initiating the inflammatory response in macrophages (Yang *et al.,* 2008), leading us to speculate that it could probably exists an early linkage between TLR2 and ROS production within *Mtb* recognition and subsequent DC maturation. Moreover, here, we observed that while ROS was strictly TLR2 and dectin-1 dependent, CD86 expression was also dependent on DC-SIGN by mechanisms that dot not involves ROS. Dectin-1 is a C-type lectin receptor for fungal wall-derived β-glucans, which also participate in the recognition of mycobacteria. This receptor is expressed on Mo, macrophages, neutrophils, DCs and Langerhans cells (Velasco-Velázquez *et al.,* 2003) and was described to promote *Mtb*-induced IL-12p40 in DC (Rothfuchs *et al.,* 2007) involving Syk activation (Underhill *et al.,* 2005). Here, we show that the increase in the activated form of Syk was coincident with the participation of dectin-1 in *Mtb* recognition, and this was also in accordance with a previous work describing a TLR2/dectin-1 contribution in *Mtb*-induced ROS production in neutrophils (Romero *et al.,* 2014).

The role of ROS in antigen presentation has been described in Kupffer cells (Maemura *et al.,* 2005). Moreover, it has been reported that oxidation of protein antigens improves both processing and immunogenic presentation to specific T cells (Carrasco-Marín *et al.,* 1998) and furthermore, NADPH oxidase can also participate in the regulation of phagosome pH, which may promote peptide loading of major histocompatibility complex molecules class II (Henderson *et al.,* 1987). In line with this, we show the effect of ROS in DC functionality, which encompasses optimization of antigen presentation either by inducing DC maturation as well as promoting the processing and presentation of *Mtb* antigens. Nonspecific *Mtb*-induced proliferation was not surprising because MLR is well known to be highly dependent upon CD80/CD86 co-stimulation. However, the role of ROS in specific T-cell proliferation may also be important in antigen processing and presentation because despite DC is matured, ROS was necessary for inducing lymphocyte proliferation. Moreover, strain M that was unable to induce ROS resulting in a poor DC activation restored DC functionality in presence of H_2O_2. This result is consistent with that described in a previous work showing that M strain lacks the ability to induce ROS in neutrophils delaying apoptosis (Romero *et al.,* 2014).

The ability of a pathogen to avoid inducing inflammatory cytokine release, as well as to limit the expression of those molecules implicated in antigen presentation, may allow certain strains to remain undetected by DCs evading an effective immune response (Urban *et al.,* 1999). In this context, certain intracellular pathogens have evolved avoiding immune reactivity in the host, and in this manner, some other *Mtb* strains are able to manipulate DCs as a means of immune evasion (Buchan *et al.,* 2009). For instance, it was suggested that certain strains of *Mtb* can spread more effectively and cause disease more frequently than others, resulting in different profiles in the immune response (Velasco-Velázquez *et al.,* 2003; Portevin *et al.,* 2011). In this way, *Mtb* and BCG differ in

their capacity to induce DC maturation *in vitro* (Mazurek *et al.,* 2012), which might be dependent on the different nature of cell wall associated molecules produced by the divergent mycobacterial strains.

In conclusion, our study describes and unveils a possible role played for endogenous ROS produced in DC maturation and antigen presentation demonstrating that *Mtb*-specific lymphocyte proliferation is dependent on *Mtb*-induced DC maturation through the interaction with DC-SIGN and by the generation of ROS. Besides, *Mtb*-induced ROS production is dependent on TLR2 and dectin-1, which in turn affects antigen processing and a proper initiation of an adaptive immune response. This work also provides experimental evidence of a different capability of *Mtb* strains in modulating the phenotypic and functional maturation of DCs based on their differences in inducing ROS production, inviting us to hypothesize that *Mtb*-induced ROS production may contribute to immune evasion by affecting DC maturation and function. Our present studies are focused on revealing which of those bacterial components interacting with target immune cell may have significant impact on DC-based vaccine for tuberculosis.

Antigens

The γ-irradiated *Mtb* H37Rv strain was kindly provided by J. Belisle (Colorado State University, Fort Collins, CO, USA). *Mtb* clinical strains were obtained from sputum culture positive patients. Two multidrug-resistant strains were employed in this study: Ra 11608 of the Latin-American Mediterranean family and M 6548, of the Haarlem family. The isolates belonged to the collection kept at the Reference Laboratory for Mycobacteria, Instituto Nacional de Enfermedades Infecciosas ANLIS 'Carlos G. Malbran' in Buenos Aires, Argentina. Bacteria were inactivated by gamma irradiation (2.4 MRad), suspended in phosphate-buffered saline (PBS) at an OD_{600} nm of 1 (~ 10^8 bacteria/ml) and stored at −20°C until their use. The isolates had been previously submitted to drug susceptibility testing and genotyping by IS*6110* DNA fingerprinting and spoligotyping using standardized protocols.

Dendritic cell differentiation and maturation

Mononuclear cells from peripheral blood samples were isolated by Ficoll-Hypaque gradient and seeded in plates (Corning, NY, USA) for 2 h in RPMI-1640 and 2% fetal calf serum (FCS) (Gibco Lab., NY, USA) for adherence. Non-adherent cells were removed and maintained in culture with suboptimal dose of rIL-2 (1 nM; Peprotech Inc.) for 6 days. Adherent monocytes were cultured with of IL-4 (20 ng/ml) (R&D Systems, Abingdon, UK) and granulocyte-macrophage colony-stimulating factor (50 ng/ml) (Peprotech Inc.) for 6 days in RPMI-1640 supplemented with penicillin–streptomycin and 10% FCS (complete medium, CM) at 37 °C in 5% CO_2. DC maturation was achieved by treatment with LPS (100 ng/ml), H37Rv or *Mtb* clinical strains (2:1 *Mtb*:DC ratio) for 24 h at 37 °C. When indicated, DCs were incubated in the presence 10 μg/ml of blocking antibodies against TLR2, CD11b (BioLegend, San Diego, California, USA), dectin-1, DC-SIGN, MR, or IgG1 isotype control Ab (R&D Systems Inc. Minneapolis, MN, USA), or the oxidase inhibitors DPI (10 μM) (Cayman Chemical, Michigan, USA), allopurinol (50 μM) and apocynin (10 μM), or the MAPK inhibitors SB203580 (20 μM), PD98059 (50 μM) and wortmannin (2 μM) or inhibitor of Syk piceatannol (15 mM) (Calbiochem-Behring, La Jolla, CA, USA) 30 min before maturation. Cytokine secretion was evaluated after 24 h in supernatants and determined employing an ELISA kit (BioLegend). DC apoptosis and necrosis were assessed by Annexin-V (Sigma) and 7-aminoactinomycin (7-AAD) (BD Bioscience Pharmingen TM, USA) staining.

Immunofluorescence analysis

Cell surface molecules were analyzed by staining with anti-CD86-PeCy5 (clone IT2.2), anti-DC-SIGN-PE, anti-DC-SIGN-FITC (R&D Systems Inc., Minneapolis, MN, USA), anti-CD1b-PE (clone SN13-(K5-1B8), anti-CD1a-FITC (clone HI149) and anti-CD14-PerCP/Cy5.5 (clone M5E2), IFNγ-FITC (clone 4S.B3), IL-12-PE (clone C11.5) (BioLegend, San Diego, California, U.S.), anti-HLA-DR-FITC (BD Pharmigen, San Diego, CA) and CD83-FITC (eBioscience, San Diego, CA, USA). Thereafter, cells were washed and fixed in 1% paraformaldehyde, and later, 10 000 events were collected in linear mode for forward scatter and side scatter, and log amplification for FL-1, FL-2 and FL-3 using a FACScan (Becton-Dickinson Immunocytometry Systems, San Jose, CA). Analysis was performed using the CellQuest software (Becton-Dickinson).

For intracellular staining of the phosphorylated form of Syk, DCs were incubated with different *Mtb* strains for 30 min, in the presence of ortovanadate in order to avoid phosphatases activation. Thereafter, cells were permeated by using a Fix and Perm kit (Caltag, Burlingame, CA, USA) and washed with PBS containing 1% Na azide and 5% fetal calf serum. Afterward, cells were stained with mouse antihuman p-(Tyr525/526)-Syk (Cell Signaling Technology, Inc., Danvers, MA) and revealed with donkey anti-mouse-IgG-FITC antibodies (Cell Signaling Technology, Inc.).

Proliferation assays

Mixed leukocyte reactions were performed by culturing DCs with 1×10^5 allogeneic lymphocytes (Ly) at 10Ly : 1DC ratio for 5 days. Specific lymphocyte proliferations (recall) were carried out in cells from PPD^+ healthy subjects by culturing DC and autologous lymphocytes for 5 days. When indicated, 10 mM of H_2O_2 was added 15 min after *Mtb* treatment. Afterward, 0.5 μCi per well of

(methyl-^{3}H)thymidine (PerkinElmer, Boston, MA, USA) was added the last 18 h to the culture. Lymphocyte proliferation was also evaluated by Carboxyfluorescein succinimidyl ester (CFSE) staining. Briefly, cell suspension was incubated with PBS/2% FCS and CFSE (0.5 μM) (Invitrogen, Massachusetts, USA) at 37°C for 10 min. After several washings, cells were added to *Mtb*-matured DCs (10:1 ratio) and cultured for 5 days. Afterward, cells were stained with mAbs directed to CD3-PE and CD4-PerCP/Cy5.5 in the cell surface, and later, 10 000 events were collected and CFSE low (proliferating lymphocytes, FL-1) and analyzed as described earlier.

Oxidative burst assay

Intracellular ROS were measured by Dihydrorhodamine123 assay (DHR). Briefly, 5×10^5 of 24 h *Mtb* or LPS-matured DC was incubated with 100 μl DHR (5 μg/ml) for 15 min at 37°C. Afterwards, DCs were re-stimulated with *Mtb* (or LPS when indicated) for 2 more hours to induce oxidative stress with or without oxidase inhibitors. Thereafter, DCs were washed and collected in a flow cytometer as mentioned earlier.

Statistics

The statistical analysis of the data was performed using one-way ANOVA followed by Tukey's multiple comparison tests to compare more than two groups followed by Wilcoxon test to compare two groups. The significance adopted was $p < 0.05$. The graphical representation of the values is given by mean ± standard error of the mean.

Acknowledgments

This work was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica, (ANPCyT PAE-PICT 2007–2329 and PAE-PICT 2007–2328); Consejo Nacional de Investigaciones Científicas y Técnicas, CONICET (PIP 112-200801-01476) and Fundación Alberto J. Roemmers. We thank National Commission of Atomic Energy (CONEA) for *Mtb* gamma-irradiation.

Author contribution: M. M. R. conceived the study and carried out experimental procedures. J. I. B. and L. C. F. participated in performing assays and in revising the manuscript. B. L. and V. R. tested drug susceptibility of *Mtb* strains and genotyped by IS6110 DNA fingerprinting and spoligotyping. M. A. conceived the study, revised experimental procedures and wrote the manuscript.

Ethic statement

This research was carried out in accordance with the Declaration of Helsinki (2008) of the World Medical Association and has been approved by the Ethics Committee of IMEX-CONICET-ANM and Hemocentro Buenos Aires; all subjects provided informed written consent.

Conflict of Interest

Authors must declare whether or not there are any competing financial interests in relation to the work described.

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