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ORIGINAL ARTICLE

Mycobacterium tuberculosis impairs dendritic cell response by altering CD1b, DC-SIGN and MR profile

Luciana Balboa[1], María Mercedes Romero[1], Noemí Yokobori[1], Pablo Schierloh[1], Laura Geffner[1], Juan I Basile[1], Rosa M Musella[2], Eduardo Abbate[2], Silvia de la Barrera[1], María C Sasiain[1] and Mercedes Alemán[1]

During a chronic infection such as tuberculosis, the pool of tissue dendritic cells (DC) must be renewed by recruitment of both circulating DC progenitors and monocytes (Mo). However, the microenvironment of the inflammatory site affects Mo differentiation. As DC are critical for initiating a *Mycobacterium tuberculosis*-specific T-cell response, we argue that interference of *M. tuberculosis* with a correct DC generation would signify a mechanism of immune evasion. In this study, we showed that early interaction of γ-irradiated *M. tuberculosis* with Mo subverts DC differentiation *in vitro*. We found that irradiated *M. tuberculosis* effect involves (1) the loss of a significant fraction of monocyte population and (2) an altered differentiation process of the surviving monocyte subpopulation. Moreover, in the absence of irradiated *M. tuberculosis,* DC consist in a major DC-specific intercellular adhesion molecule 3-grabbing non-integrin receptor (DC-SIGN^{high})/CD86^{low} and minor DC-SIGN^{low}/CD86^{high} subpopulations, whereas in the presence of bacteria, there is an enrichment of DC-SIGN^{low}/CD86^{high} population. Besides, this population enlarged by irradiated *M. tuberculosis,* which is characterized by a reduced CD1b expression, correlates with a reduced induction of specific T-lymphocyte proliferation. The loss of CD1molecules partially involves toll-like receptors (TLR-2)/p38 MAPK activation. Finally, several features of Mo, which have been differentiated into DC in the presence of irradiated *M. tuberculosis*, resemble the features of DC obtained from patients with active tuberculosis. In conclusion, we suggest that *M. tuberculosis* escapes from acquired immune response in tuberculosis may be caused by an altered differentiation into DC leading to a poor *M. tuberculosis*-specific T-cell response.
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Mycobacterium tuberculosis (Mtb) is a facultative intracellular bacterium responsible for 1.5–2 million deaths annually. *Mtb* has the ability to enter the host through aerosols and parasitize host alveolar macrophages (Mϕ), which are considered the first cells to become infected by *Mtb*.[1] Dendritic cells (DC) are present as a dense network in the airway mucosa[2] and their early interaction with *Mtb* may be critical for mounting a protective immune response particularly in primary tuberculosis. Infected Mϕ and DC enter lung parenchyma where they initiate inflammatory foci to which blood monocytes (Mo) are attracted. Pro-inflammatory, metabolic and immune stimuli all elicit increased recruitment of Mo to peripheral sites, where differentiation into Mϕ and DC occurs, contributing to host defense, and tissue remodeling and repair.[3,4] In chronic infections, Mo are the best candidates for antigen-presenting cell (APC) renewal and they sustain T-lymphocyte activation.[5] Immature DC actively capture and process antigens (Ag) and they enter a terminal activation program in response to maturation-inducing stimuli such as bacterial products or inflammatory cytokines. During this process, DC leave peripheral tissues and migrate to the T-cells-dependent areas of secondary lymphoid organs, where they initiate the immune response.[6]

Culture of Mo with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4)[7] has been very useful for the *in vitro* generation of large quantities of DC, providing a model to investigate the effect of self or environmental agents on the differentiation pathway. The hallmark of *in vitro* differentiation of DC is the acquisition of CD1molecules that present non-peptide lipid Ag to T cells, the expression of which is critical for mounting a protective anti-mycobacterial immune response.[8] Human T cells, which recognize mycobacterial glycolipids in conjunction with CD1, are expanded in *Mtb* infections, produce γ interferon, kill infected target cells and also kill mycobacteria directly.[9] Several hypotheses have been suggested on the capacity of *Mtb* to evade host immune responses during latency and reactivation.[10,11] Subverted DC derived from *Mtb*- or Bacillus Calmette Guérin-infected Mo lost CD1 expression and displayed a reduced upregulation of HLA class II DR and CD80molecules and an impaired capacity to prime IFN-γ producing T lymphocytes.[12,13]

Phagocytic cells express a range of cellular surface receptors that have been implicated in the recognition of *Mtb*. Among these, toll-like receptors (TLR), mannose receptor (MR),[14] DC-specific intercellular adhesion molecule 3-grabbing non-integrin receptor (DC-SIGN)[15]

[1]Instituto de Investigaciones Hematológicas, Academia Nacional de Medicina, Buenos Aires, Argentina and [2]Servicio de Tisioneumonología, Hospital Muñiz, Buenos Aires, Argentina
Correspondence: Dr M Alemán, Instituto de Investigaciones Hematológicas, Academia Nacional de Medicina, Pacheco de Melo 3081, Buenos Aires 1425, Argentina.
E-mail: maleman@hematologia.anm.edu.ar
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and complement receptor 3[16] have been studied in detail. In particular, DC-SIGN is an important *Mtb* receptor on the surface of human Mo-derived DC, dermal DC, lung interstitial DC as well as in lymph nodes.[17] Some lipids are TLR agonists such as phosphatidylinositol mannoside, lipoarabinomannan (LAM), 19 kDa lipoprotein, mycolic acid and glucose monomycolate.[18] Thus, microbes must provide both the TLR agonists to stimulate exogenous lipid processing and the lipid Ag, which are loaded into CD1 proteins.[18]

We have recently demonstrated that γ-irradiated *Mtb (iMtb)* induces maturation of Mo-DC upregulating co-stimulatory molecules together with the downregulation of DC-SIGN in healthy subjects (HS) and in patients with active tuberculosis (TB). However, DC from TB induced lower specific T-cell proliferation in response to *iMtb*.[19] The mechanism responsible for such a lower *iMtb*-specific T-cell proliferation in TB has not been defined yet. One hypothesis to explain this low induction is that *iMtb* exerts a regulatory activity through modulation of APC function.

Although Mϕ and DC are important constituents of normal lungs, it is reasonable to hypothesize that Mo recruitment and differentiation in lungs during persistent *Mtb* infection could modulate immune response and affect the outcome of the disease.[20] Considering that the encounter of Mo with bacteria occurs very early in lungs, in this work we investigated the possible mechanisms by which *iMtb* interferes with Mo differentiation into DC. We evaluated the effect of *iMtb* on *in vitro* DC differentiation in terms of the expression of CD1molecules, CD14 and *Mtb* receptors (DC-SIGN and MR) as well as phagocytosis and *iMtb*-specific T-cell response. Although normally differentiated DC consist in a major DC-SIGN^{high}/CD86^{low} and a minor DC-SIGN^{low}/CD86^{high} subpopulations, the presence of *iMtb* drives the loss of a significant fraction of Mo population and an enrichment of DC-SIGN^{low}/CD86^{high} population. The DC-SIGN^{low}/CD86^{high} population is characterized by a reduced CD1b expression that correlates with a reduced *iMtb*-specific lymphocyte proliferation together with an enhanced mixed leukocyte reaction. We have established that the mechanism by which *iMtb* induced CD1 downregulation partially involves IL-10 secretion and TLR-2 activation in Mo, whereas the exact mechanism by which *iMtb* subverts DC-SIGN and MR expression remains to be established. In conclusion, we suggest that *Mtb* escape from acquired immune response in TB may be caused by an altered differentiation into DC leading to a poor *iMtb*-specific T-cell response.

RESULTS

iMtb impairs Mo differentiation *in vitro*

To determine the effect of *iMtb* on Mo differentiation into DC, peripheral blood Mo from HS were cultured in medium with IL-4 plus GM-CSF for 6 days in absence and presence of *iMtb* (*iMtb*:Mo 2:1 ratio) added at the beginning of the culture, as described in 'Methods.' Notably, *iMtb* exerted a deleterious effect on cell survival at day 6 (cell recovery from Mo differentiated with *iMtb* was 46 ± 9% of the original cells; % mean ± s.e.m., $n=20$, $P<0.002$), which was also evident at 24 h. Thus, for functional analysis the cell number was adjusted to 1×10^6 per ml. Remarkably, these enduring cells from Mo differentiated in the presence of *iMtb* (*iMtb*DC) exhibited a particular forward and scatter pattern that was different from either those Mo differentiated without *iMtb* (DC) or those DC that were matured for additional 48 h with *iMtb* (mDC) (Figure 1a). In addition, *iMtb*DC preserved the monocyte marker CD14 (Table 1) and only a small percentage of cells expressed CD1a and CD1b, which together with the upregulation of HLA-DR, CD54 and CD86 expression tell about a mature phenotype.[21]

To evaluate whether changes in phenotype influence DC capability to present *iMtb* antigens, specific T-cell response (using autologous T cells from PPD+ HS) and mixed lymphocyte reactions (MLR) were assessed. As Figure 1b shows, the presence of *iMtb* during Mo differentiation gave rise to DC with lower capacity to induce *iMtb*-specific T-cell proliferation than mDC, even if *iMtb* was added for an additional 48 h to rule out Ag scarcity (data not shown). This detrimental effect in specific T-cell proliferation exerted by *iMtb* on DC differentiation was dose dependent and detectable at 0.5 *iMtb*:1Mo ratio (Figure 1c). On the contrary, and in agreement with the high expression of co-stimulatory molecules, *iMtb*DC showed an enhanced MLR response that was comparable to mDC (Figure 1d).

iMtb affects DC-SIGN/CD86 profile of DC

DC differentiated from Mo in culture express elevated levels of MR and the main receptor for *Mtb* in DC, namely DC-SIGN.[15] In the context of DC-SIGN and CD86 expression, we observed that DC were mainly DC-SIGN^{high}/CD86^{low} whereas an enrichment in DC-SIGN^{low}/CD86^{high} cells was reached in *iMtb*DC (Figure 2a) (%DC-SIGN^{low}/CD86^{high} *iMtb*DC=44 ± 4; DC=7 ± 1.3; $^*P<0.001$, $n=30$), leading to a loss of just over 40% of DC-SIGN/MR double positive cells (Figure 2b). Furthermore, the percentage of CD1b$^+$ and MR$^+$ cells was also decreased in this DC-SIGN^{low}/CD86^{high} population (Figure 2c). Considering that mDC showed an upregulation in CD86 expression but the pattern of expression of *Mtb* receptors was not changed, we suggest that the maturation itself cannot explain the alterations observed in *Mtb* receptors (Figures 2a and b).

As all these changes in Mo differentiation were observed with irradiated bacteria we wondered whether the same phenotypic profile from *iMtb*DC was induced by viable bacteria. To confirm this possibility, Mo were infected with viable *Mtb* (2:1 MOI Mo:*Mtb*). Viable bacteria did induce a similar profile in the percentage of CD1molecules and DC-SIGN/MR/CD86 expression to *iMtb* (data not shown). Taking into account the phenotypic similarities between the irradiated and the viable *Mtb*, we carried out all the following experiments with *iMtb*.

Having seen the unusual phenotypic profile induced in *iMtb*DC, we decided to study their function in terms of phagocytosis. We found that phagocytosis of *iMtb* was highly reduced in *iMtb*DC compared to DC whereas it was not reduced in Mo differentiated in the presence of latex beads (Figure 2d), suggesting that the loss of phagocytosis might be associated to the low DC-SIGN and MR expression. Furthermore, we found that CD1b$^+$ cells significantly correlated with specific proliferation rates in *iMtb*DC (Figure 2e) leading us to speculate that the reduction in *iMtb*DC function might be associated to the lack of response of CD1-restricted T cells.[22–24] In agreement with this result, the blockage of CD1b indeed inhibited the T-cell proliferation induced by mDC in about 15% whereas the proliferation induced by *iMtb*DC was not inhibited by this blockage (Figure 2f). Overall, these data show that *iMtb* induces a general atypical *in vitro* Mo differentiation into DC involving the downregulation of CD1molecules.

iMtb determines CD1a/CD1b/CD14 levels at early stages of Mo differentiation into DC

Next, the influence of the time of *iMtb* addition to the cultures was analyzed. *iMtb* was added at different time points during Mo differentiation and its effect was evaluated by analyzing the expression of CD1a, CD1b and CD14 at the end of a 6-day culture. As can be seen in Figure 3, DC showed a high percentage of CD1a and CD1b cells and low CD14 levels whereas addition of *iMtb* on day 0 resulted in the differentiation of CD1a^{low} CD1b^{low} CD14^{high} cells and there was no

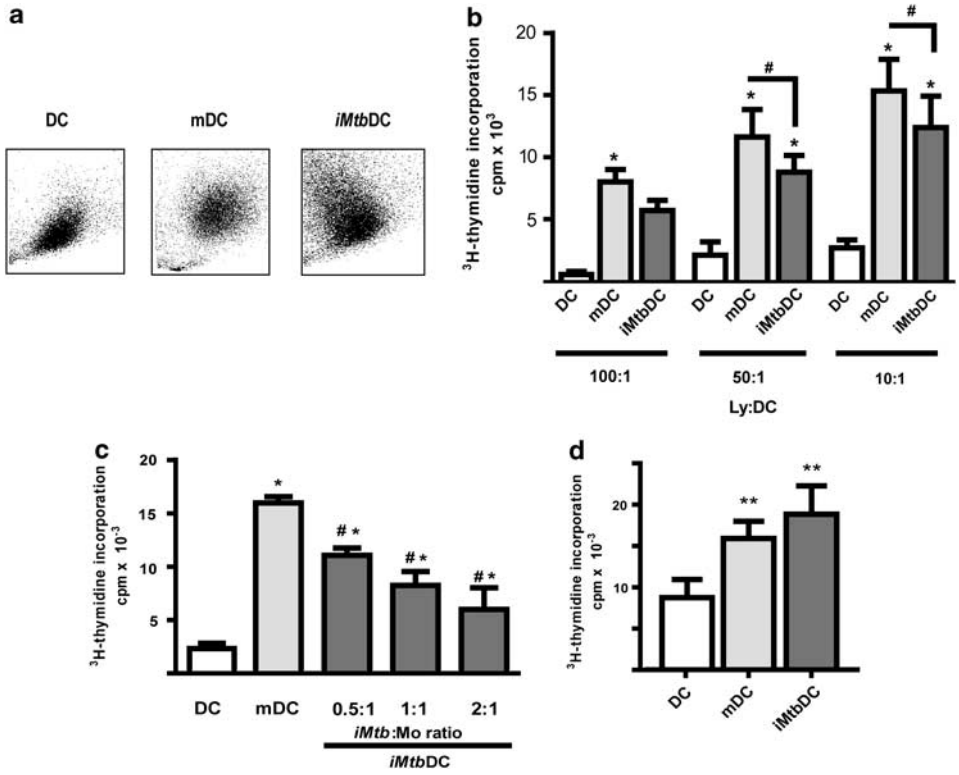

Figure 1 DC differentiated with irradiated *M. tuberculosis (iMtb)* induce a low-specific T-cell response. Mo were incubated for 6 days with IL-4/GM-CSF to obtain immature DC (open bars) or allowed to differentiate for 6 days with IL-4/GM-CSF in the presence of *iMtb* (*iMtb*DC, gray bars); DC were then treated for additional 48 h with *iMtb* to obtain matured dendritic cells (mDC, gray light bars). Isolated lymphocytes were cultured with the different dendritic cell populations at different Ly:DC ratios for 5 days and T-lymphocyte proliferation was determined by [3H]thymidine incorporation. (**a**) Forward and scatter pattern of DC, mDC and *iMtb*DC, a representative experiment is shown. (**b**) *iMtb*-specific T-cell proliferation induced by mDC, *iMtb*DC and DC used as control. Results are expressed as mean ± s.e.m. of c.p.m.$\times 10^{-3}$ n=25. Statistical differences for *iMtb*DC or mDC vs DC: $^*P<0.05$, $^{**}P<0.01$; *iMtb*DC vs mDC: $^{\#}P<0.05$. (**c**) Autologous T-cell proliferation induced by DC, mDC and *iMtb*DC (differentiated at different *iMtb*:Mo ratios) at a 10Ly:1DC ratio. Statistical differences for *iMtb*DC or mDC vs DC: $^*P<0.05$; *iMtb*DC vs mDC: $^{\#}P<0.05$. (**d**) Mixed leukocyte reaction induced by DC, mDC and *iMtb*DC at a 10Ly:1DC ratio. Results are expressed as mean ± s.e.m. of c.p.m.$\times 10^{-3}$, n=10. Statistical differences for *iMtb*DC or mDC vs DC: $^{**}P<0.01$.

Table 1 Characterization of *iMtb*-matured DC and DC differentiated in presence of *iMtb*

Mean % or MFI ± stantard errors[a]	*Treatment*[b]		
	DC	*iMtbDC*	*mDC*
% CD1a$^+$	85 ± 9	10 ± 2*	75 ± 12*
% CD14$^+$	3 ± 1	15 ± 5*	2 ± 1
% CD1b	63 ± 4	24 ± 5*	61 ± 5
MFI DC-SIGN	781 ± 221	310 ± 120*	620 ± 160*
MFI MR	236 ± 28	113 ± 8*	190 ± 32*
MFI CD86	578 ± 110	883 ± 60*	1073 ± 100*
MFI HLA-DR	1300 ± 135	2200 ± 120*	1990 ± 130*
MFI CD54	1197 ± 130	1768 ± 230*	1850 ± 110*

Abbreviations: DC, dendritic cells; DC-SIGN, DC-specific intercellular adhesion molecule 3-grabbing non-integrin receptor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-4, interleukin-4; MFI, median fluorescence intensity; Mo, monocytes; MR, mannose receptor.
[a]Mean percentage or MFI of cell markers measured by flow cytometry. Standard errors for 40 repeated experiments are shown.
[b]Cells differentiated from Mo cultured with IL-4 and GM-CSF (DC) and with *iMtb* from the beginning of the culture (*iMtb*DC) or *iMtb* stimulated at day 6 (mDC). *Statistically significantly different from DC ($^*P<0.05$).

effect when *iMtb* was added later on, suggesting that the impairment of Mo differentiation was established early in culture. Besides, a similar pattern was induced when Mo were treated with supernatants from Mo cultured for 24 h with *iMtb* (Figure 3b), indicating that the impairment was partially mediated by *iMtb*-induced factors. Considering that *iMtb* affects Mo outcome in culture, we wondered whether differentiation into Mϕ could also be altered. To asses this, Mo were differentiated into Mϕ for 6 days with *iMtb* from the beginning of the culture and the resulting cells showed increased CD14 and CD86 as well as a decreased CD1a, CD1b and MR expression. Accordingly, this Mϕ showed increased MLR proliferation and a reduced ability to induce specific cell proliferation (data not shown). All these data confirm that *iMtb* exert an early effect on Mo driving to an abnormal *in vitro* differentiation process that distorts the specific response against *iMtb*.

Kinetics of receptor expression in *iMtb*DC and DC

As the main DC receptors for *Mtb* uptake, namely DC-SIGN and MR, were downregulated in *iMtb*DC,[15], the kinetics of their expression was evaluated during the differentiation process. As Figure 4a shows, the rise of DC-SIGN and MR as well as CD1a started to be significant at 5–12 h and reached a considerable expression after 24 h. The presence of *iMtb* subverted the very early expression of DC receptors, suggesting that *iMtb* probably acted through a Mo receptor. Therefore, the acquisition of *Mtb* receptors in DC is an early event in differentiation that could be impaired by *iMtb* at the Mo level altering the normal DC phenotype development.

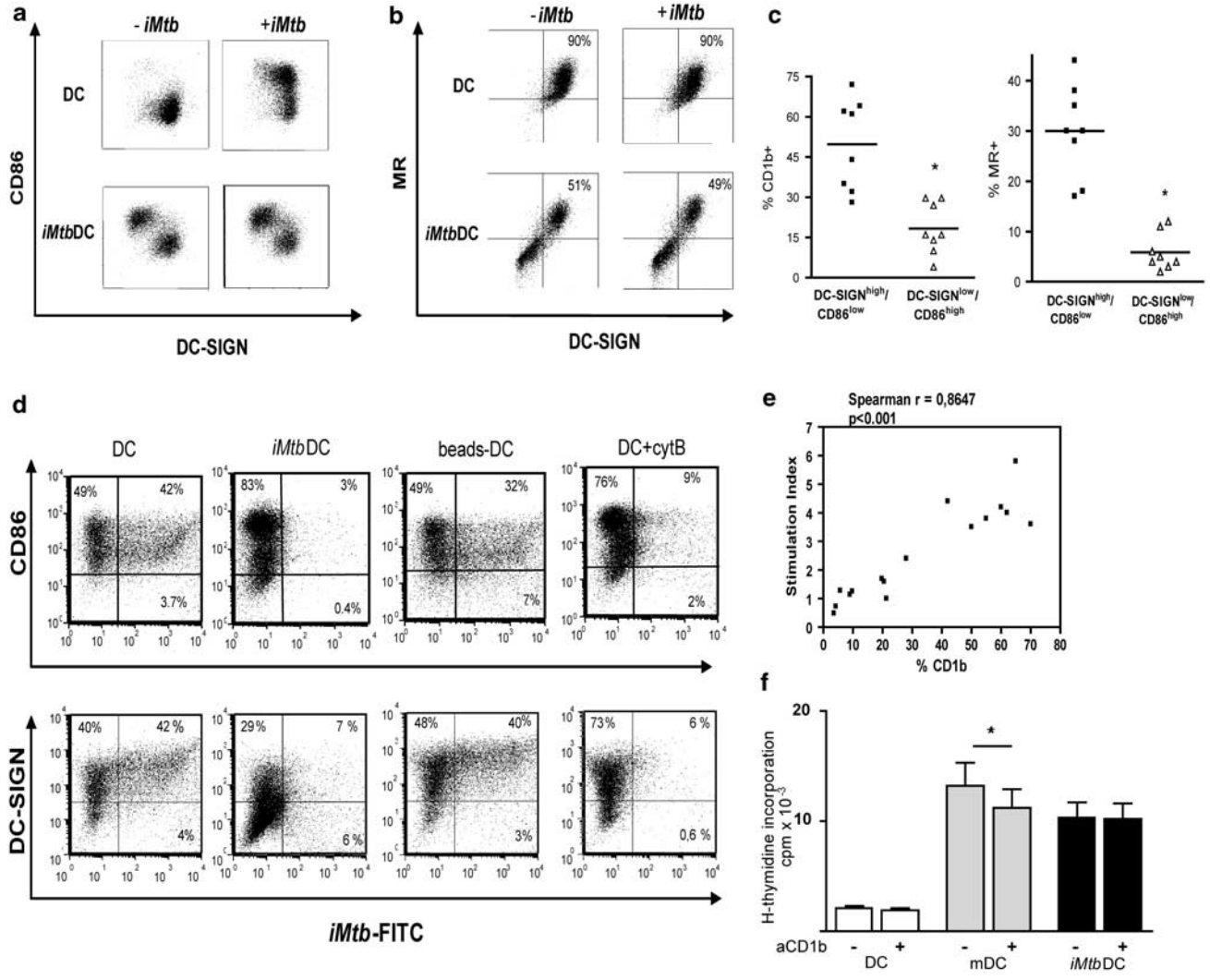

Figure 2 Irradiated *M. tuberculosis (iMtb)* induces a DC population with a DC-SIGN^{low}/CD86^{high}/MR^{low}/CD1b^{low} profile. Mo were differentiated for 6 days with IL-4/GM-CSF (DC) or differentiated with IL-4/GM-CSF in the presence of *iMtb* (*iMtb*DC) and then both populations were matured for additional 48 h with *iMtb* (+*iMtb*). DC-SIGN, CD86, MR and CD1b expression, specific T-cell proliferation and phagocytosis of FITC-labeled *iMtb* were determined. Dot plots of a representative experiment of (**a**) DC-SIGN and CD86 expression, (**b**) DC-SIGN and MR expression. (**c**) Percentage of CD1b and MR-positive cells in DC-SIGN^{low}/CD86^{high} and DC-SIGN^{high}/CD86^{low} populations from *iMtb*DC (n=8). Statitistical differences between populations: $^*P<0.05$. (**d**) *iMtb*-FITC phagocytosis by DC, *iMtb*DC, BeadDC (Mo differentiated with IL-4/GM-CSF in presence of latex beads, 2Beads:1Mo) and DC+cytB (DC preincubated with cytochalasin B). Percentages of DC-SIGN or CD86 and *iMtb*-FITC-positive cells are shown. Dot plots of a representative experiment are shown. (**e**) Percentages of CD1b+ cells from *iMtb*DC correlate with the specific T-cell proliferation induced by *iMtb*DC. Spearman's correlation ($P<0.001$, n=16). (**f**) Effect of CD1b blockage on DC (open bar), mDC (gray light bars) and *iMtb*DC (gray bars) in *iMtb*-specific T-cell proliferation performed at a 10Ly:1DC ratio. Results are expressed as mean ± s.e.m of c.p.m.$\times 10^{-3}$, n=6. Statistical differences for mDC with aCD1b vs mDC without aCD1b: $^*P<0.05$.

Role of IL-10 in *iMtb*-induced DC profile

It has been demonstrated that *Mtb* induces IL-10 secretion by Mo[25] and this cytokine alters phenotype and functional maturity of Mo-derived DC by reducing DC ability to present lipid Ag to CD1-restricted T cells.[26] As mentioned above, *iMtb* impairs differentiation at the Mo level and this is performed in part by soluble factors; thereby, we analyzed whether IL-10 was involved in the development of *iMtb*DC. First, we measured levels of IL-10 from 6-day-cultured *iMtb*DC by ELISA and we observed that IL-10 secretion was dose dependent (Figure 5a). Second, we generated DC in the presence of IL-10 as well as *iMtb*DC in the presence of anti-IL-10 neutralizing Ab. DC plus anti-IL-10 alone were used as control. As it is shown in Figure 5b, when IL-10 was present during Mo differentiation we observed a marked increase in the percentage of CD14 together with a decrease in the levels of CD1a and CD1b whereas CD86 and DC-SIGN expression was not affected (data not shown). The neutralization of IL-10 only counteracted *iMtb*-induced CD14 upregulation but did not restore CD1a or CD1b expression and did not overcome the reduced specific response observed in *iMtb*DC (Figure 5c). Thus, although IL-10 secreted by *iMtb*DC induced the over-expression of CD14 it could not totally explain the pattern induced by *iMtb*.

TLR-2 involvement in *iMtb*-induced DC profile

To identify *Mtb* component/s responsible for the impairment of Mo differentiation into DC, the effect exerted by mannosylated lipoarabinomannan (ManLAM), peptidoglycan (PTG), CWL and culture filtrate protein (CFP) on cellular phenotype was evaluated. On the one hand, ManLAM only decreased CD1b and CFP only decreased CD1a

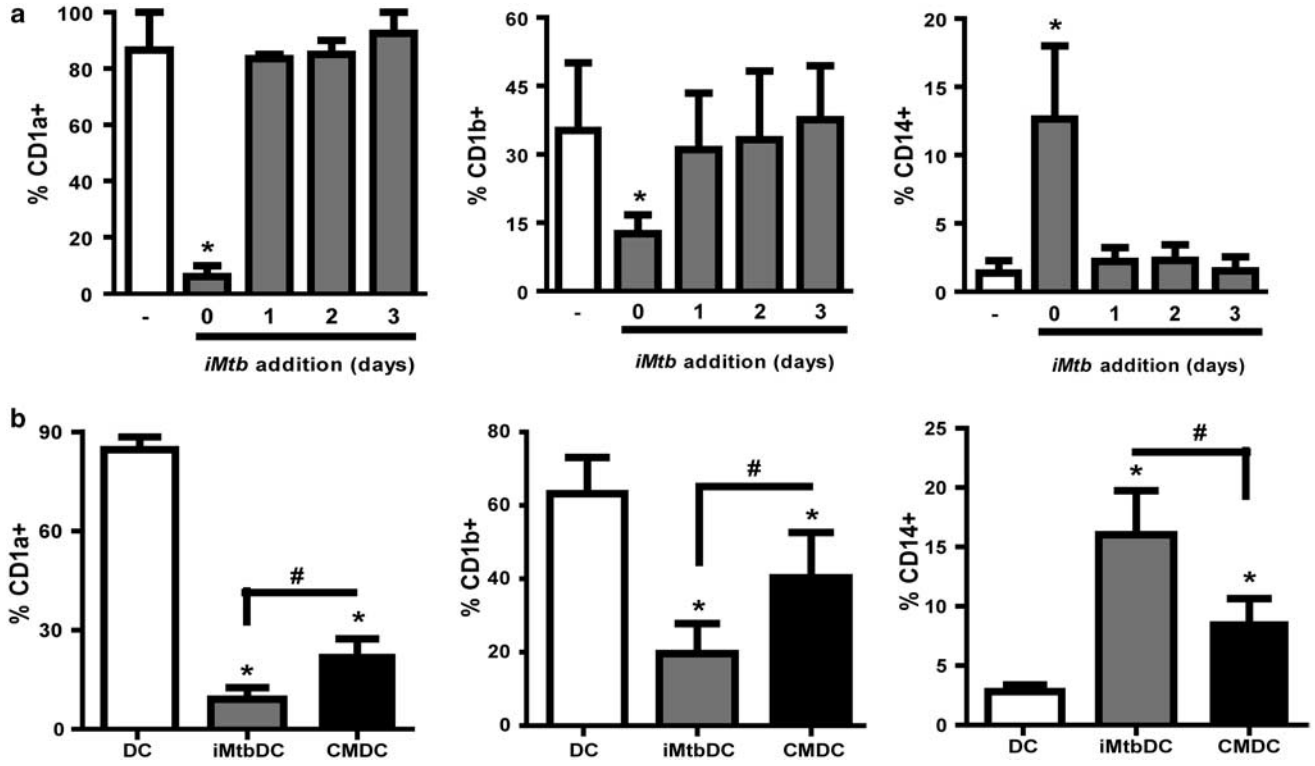

Figure 3 The fate of CD1a/CD1b/CD14 cells profile is determined early during Mo differentiation by irradiated *M. tuberculosis* (*iMtb*) and *iMtb*-induced soluble factors at early stage. Mo were differentiated for 6 days with IL-4/GM-CSF (DC, open bars), and differentiated in the presence of *iMtb* (2*iMtb*:1Mo ratio; *iMtb*DC, gray bars) added at different times of the culture or differentiated with conditioned culture medium (CMDC, black bars) from 24 h *iMtb*-treated Mo. Percentages of CD1a, CD1b and CD14 were determined by flow cytometry on (**a**) DC (open bars) and *iMtb*DC differentiated by *iMtb* added at different time of culture (gray bars) or (**b**) DC (open bars), *iMtb*DC (gray bars) and CMDC (black bars). Results are expressed as mean ± s.e.m (n=6). Statistical differences for *iMtb*DC or CMDC vs DC: $^{*}P<0.05$; CMDC vs *iMtb*DC: $^{\#}P<0.05$.

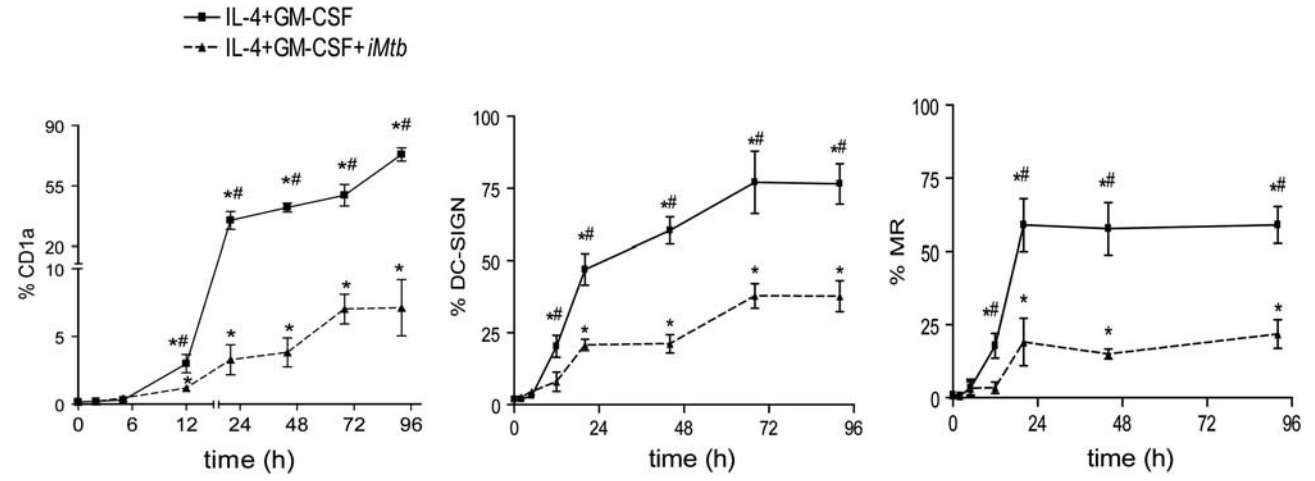

Figure 4 Kinetics of CD1a, MR and DC-SIGN expression along Mo differentiation into DC in presence or not of irradiated *M. tuberculosis* (*iMtb*). Mo were differentiated for 6 days with IL-4/GM-CSF or with IL-4/GM-CSF+ *iMtb* (2*iMtb*:1Mo ratio). CD1a, DC-SIGN and MR percentages were analyzed at the onset of the culture (t=0) or at different time points along the culture by flow cytometry (t=n). Results are expressed as mean ± s.e.m. (n=6). Statistical differences for IL-4+GM-CSF vs IL-4+GM-CSF+*iMtb*: $^{\#}P<0.05$; t=n vs t=0: $^{*}P<0.05$.

expression (Table 2). On the other hand, CWL increased CD86 expression and, as PTG also did, it slightly affected CD1a/CD1b/CD14 levels. Among *Mtb* components, PTG is recognized by TLR-2[27] so we wondered whether *iMtb*DC profile would be triggered by TLR-2-ligation. To confirm this hypothesis, we differentiated Mo in the presence of the synthetic lipopeptide Pam3Cys that binds TLR-2[28] (Pam3DC) and then evaluated DC phenotype and function. As it is shown in Table 2, the phenotype induced in Pam3DC resembled that of PTG in terms of CD86 expression and of CD14/CD1a/CD1b profile. Furthermore, *iMtb*-specific proliferation induced by

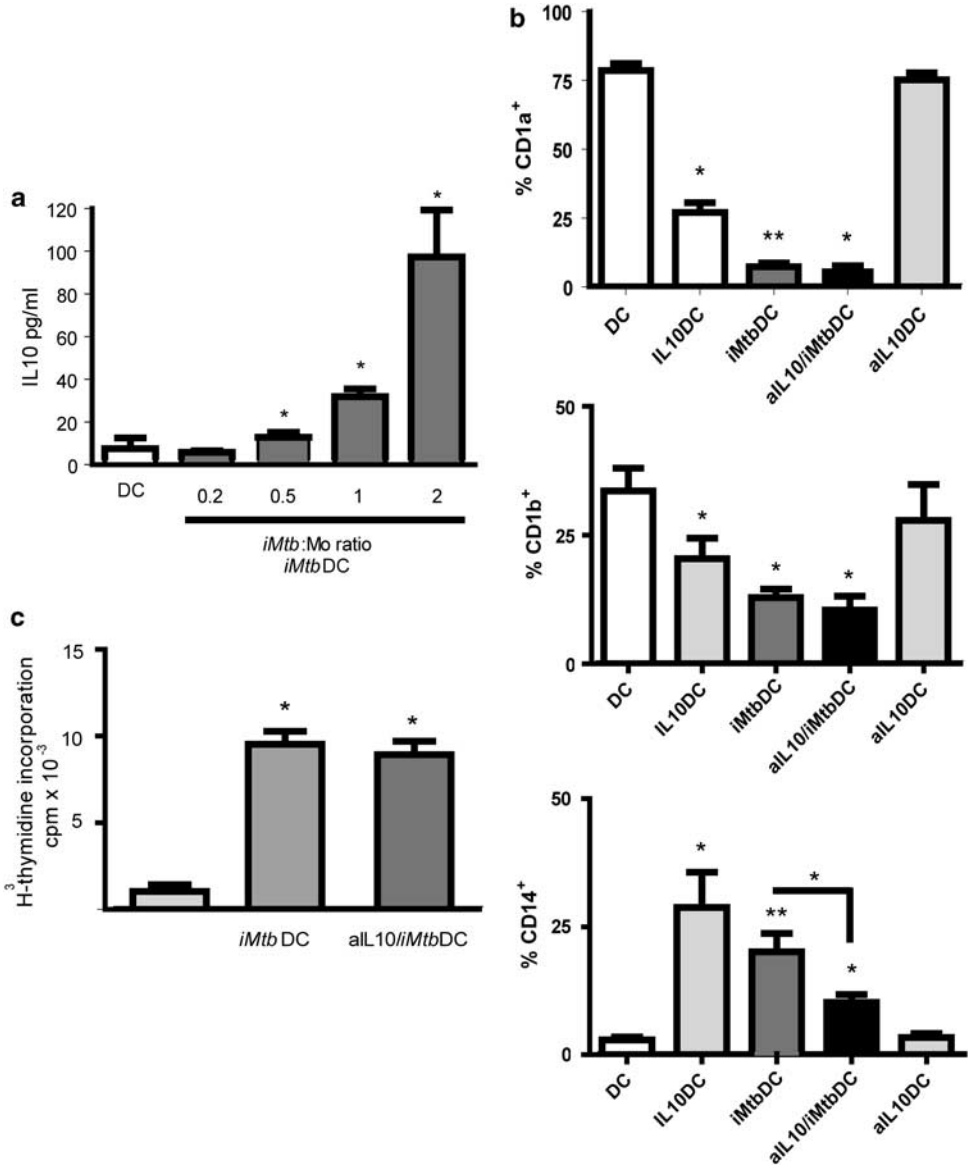

Figure 5 IL-10 secretion induced by irradiated *M. tuberculosis* (*iMtb)* modulates CD14 expression. Mo were cultured with IL-4/GM-CSF (DC) in the presence of *iMtb* (*iMtb*DC), IL-10 (IL10DC), anti-IL-10 plus *iMtb* (aIL10*iMtb*DC) or anti-IL-10 (aIL10DC) for 6 days. Thereafter, phenotype and proliferation assays were performed. (**a**) IL-10 secretion is induced by *iMtb* in a dose-dependent manner. Supernatants were evaluated for the presence of IL-10 by ELISA. Data are expressed as mean ± s.e.m. (n=10). Statistical differences for DC vs *iMtb*DC: $^*P<0.05$. (**b**) CD1a, CD1b and CD14 percentages on DC, *iMtb*DC, IL10DC, aIL10*iMtb*DC and aIL10DC. Results are expressed as mean ± s.e.m. (n=8). Statistical differences for DC vs treatment: $^*P<0.05$; $^{**}P<0.01$; *iMtb*DC vs aIL10*iMtb*DC: $^*P<0.05$. (**c**) Specific T-cell proliferation induced by DC, *iMtb*DC and aIL10*iMtb*DC evaluated at an Ly:DC ratio of 10:1. Results are expressed in c.p.m. as mean ± s.e.m. (n=7). Statistical differences for *iMtb*DC or aIL10*iMtb*DC vs DC: $^*P<0.05$.

Pam3DC (Pam3mDC) was lower than DC, which may be ascribed to the weak levels of CD1b molecules (Figure 6a). Considering that p38 MAPK activation is induced by *Mtb* through TLR-2,[29] we evaluated whether p38 MAPK participates in the generation of *iMtb*DC profile. As it is shown in Figure 6b, chemical inhibition of p38 during culture partially restored the CD14/CD1a/CD1b phenotype whereas inhibition of ERK did not. Surprisingly, neither DC-SIGN nor MR receptors were modulated by any of *Mtb* components, suggesting that though many *Mtb* components appear to be involved in outline CD1a/CD1b/CD14 profile in *iMtb*DC, the whole bacterium structure seems to be required to generate the characteristic DC-SIGN^{low}/MR^{low} pattern.

DC differentiation of Mo from TB

We have shown recently that *iMtb*,[20] as viable bacterium does,[19,21] induces the maturation of DC upregulating the levels of CD83, CD86 and HLA-DR together with a reduction of DC-SIGN expression and, furthermore, IFN-γ and IL-12 production was comparable in cells from HS and TB. Although DC functions were similar in terms of phagocytosis and MLR capability (data not shown), DC from TB induced lower specific T-cell proliferation in response to *iMtb* than HS.[19] Hence, we wondered whether the differences observed in *iMtb*-specific T-cell response in TB could be due to an altered Mo differentiation. For this purpose, Mo obtained from TB were tested

Table 2 Involvement of *Mtb* component/s in the impairment of Mo differentiation to DC

Mean % or MFI ± standard errors[a]	*Treatment*[b]						
	DC	*iMtbDC*	*ManLAM*	*PTG*	*CWL*	*CFP*	*Pam3DC*
% CD1a$^+$	85 ± 9	8 ± 1*	80 ± 9	44 ± 5*	32 ± 4*	43 ± 6*	18 ± 1*
% CD14$^+$	07 ± 0.07	10 ± 2*	1 ± 0.2	4 ± 0.1*	4 ± 0.3*	1 ± 0.2	7 ± 2*
% CD1b$^+$	61 ± 6	15 ± 2*	48 ± 6*	46 ± 5*	33 ± 4*	63 ± 7	21 ± 3*
% DC-SIGN	82 ± 6	41 ± 5*	79 ± 9	75 ± 5	77 ± 5	80 ± 9	81 ± 2
% MR	52 ± 6	33 ± 4*	50 ± 6	45 ± 5	47 ± 5	52 ± 7	54 ± 3
MFI CD86	459 ± 145	872 ± 163*	479 ± 64	407 ± 53	674 ± 101*	484 ± 106	470 ± 101

Abbreviations: DC, dendritic cells; DC-SIGN, DC-specific intercellular adhesion molecule 3-grabbing non-integrin receptor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-4, interleukin-4; MFI, median fluorescence intensity; Mo, monocytes; MR, mannose receptor.
[a]Mean percentage or MFI of cell markers measured by flow cytometry. Six repeated experiments are shown.
[b]Cells differentiated from Mo cultured with IL-4 and GM-CSF (DC) and with *iMtb* from the beginning of the culture (*iMtb*DC) or different *Mtb* antigens: manosilated lipoarabinomannan (ManLAM), peptidoglycan (PTG), cell whole lisate (CWL), cellular filtrated proteins (CFP) and Pam3CysSerLys4 (Pam3DC). *Statistically significantly different from DC (*$P<0.05$).

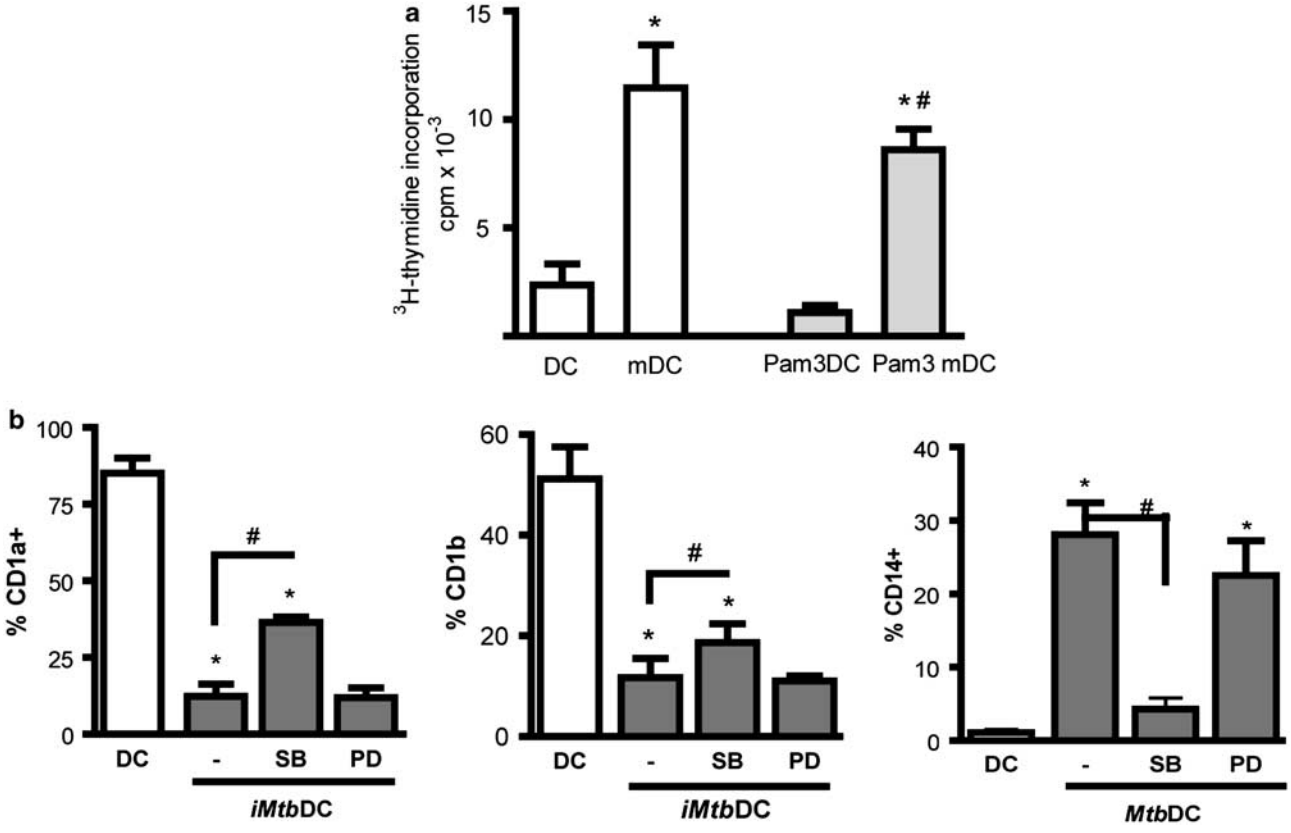

Figure 6 TLR-2 activation impairs CD1a, CD1b and CD14 levels. Mo were differentiated with IL-4/GM-CSF (DC) in the presence of Pam3 (Pam3DC) or *iMtb* (*iMtb*DC) for 6 days. DC and Pam3DC were then matured with *iMtb* for additional 48 h (mDC and Pam3mDC, respectively). When indicated, Mo were pretreated with p-p38 (SB) or ERK (PD) inhibitors and *iMtb* added at the start of differentiation. (**a**) Specific T-cell proliferation from DC, mDC, Pam3DC, Pam3mDC was evaluated, an Ly:DC ratio of 10:1. Results are expressed as mean ± s.e.m. of c.p.m. (n=10). Statistical differences for DC vs mDC or Pam3DC vs Pam3mDC: **$P<0.01$; Pam3mDC vs mDC: $^{\#}P<0.05$. (**b**) CD1a, CD1b and CD14 percentages on DC (open bars) or *iMtb*DC treated or not with SB or PD inhibitors (gray bars). Results are expressed as percentage mean ± s.e.m. (n=7). Statistical differences for inhibitors-treated *iMtb*DC or *iMtb*DC vs DC: *$P<0.05$. *iMtb*DC vs *iMtb*DC plus SB: $^{\#}P<0.05$.

for their ability to differentiate *in vitro* into DC for 6 days and CD1a, CD14, CD86 and DC-SIGN expression were evaluated. As it is shown in Figure 7a, after a 6-day culture, DC from TB showed lower CD1a and higher CD14 levels as well as a larger DC-SIGN^{low}/CD86^{high} population than DC from HS (% TB: 39 ± 4; % HS: 11 ± 2, $P<0.001$; n=30) (Figure 7b), and these DC from TB features resemble those *iMtb*DC features.

DISCUSSION

The immune response to primary TB is most probably initiated after exposure of *Mtb* to immature DC in lungs. Given that in chronic infections Mo are the best candidates for APC renewal, in this study we have analyzed the effect of *iMtb* on *in vitro* Mo differentiation into DC to explain the low specific T-cell proliferation observed in TB. We showed that *iMtb* modified Mo *in vitro* differentiation, giving rise to a

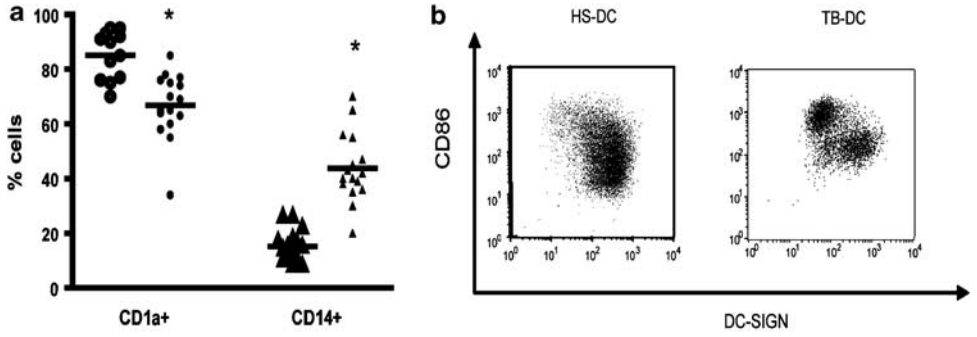

Figure 7 Mo differentiation into DC from patients with TB. Mo from TB and HS were differentiated with IL-4/GM-CSF for 6 days and phenotype was evaluated. (**a**) CD1a and CD14 cell percentages on DC from HS (open symbols) and TB (solid symbols). Lines show means from 10–15 independent experiments. HS-DC vs TB-DC: $*P<0.05$. (**b**) Dot plots of DC-SIGN and CD86 expression of a representative experiment from HS-DC and TB-DC.

subset of DC characterized by an unusual phenotype profile and impaired functions. In accordance with other reports,[30] *iMtb*DC exhibited significant lower CD1 and increased CD14 levels than commonly differentiated DC.

Here, we evaluated for the first time, that though Mo-derived DC are mainly DC-SIGN^{high}/CD86^{low} the presence of *iMtb* induces the generation of a DC-SIGN^{low}/CD86^{high} subpopulation with a reduced CD1b expression (Figure 2a). As shown earlier,[13,31] the more mature phenotype exhibited by *iMtb*DC was in accordance with the high MLR response, whereas lower CD1b expression correlated with their declined ability to present *iMtb* antigens. Our results are in accordance with Gagliardi *et al.*[32] who have identified that DC derived from both α-glucan and mycobacterial cell wall pretreated Mo showed a drastic inhibition of CD1molecule and CD86 increased expression. Another non-proteic compound of cell wall, LAM as well as the cytosol fraction or the pool of secreted proteins of *Mtb* were unable to interfere with CD1molecule expression and only caused a slightly upregulation of CD86 on DC. In fact, heat killed *Mtb* does not induce an altered CD1molecule expression.[13] The lack of CD1molecules is of particular relevance because *Mtb* is one the microorganisms with the highest content of antigenic lipids that are presented by these antigen-presenting molecules to CD1-restricted lipid-specific T lymphocytes. It has been demonstrated that CD1-restricted T cells are expanded in humans as a result of a previous *Mtb* infection, contributing to the initiation of a cell-mediated immune response against the pathogen. In contrast, CD1-restricted T-cell responses are absent or drastically reduced in TB suggesting an effective role during *Mtb* infection.[33]

Our data also showed that the loss of CD14 and the acquisition of CD1molecules as well as DC-SIGN and MR receptors occurred very early in the Mo differentiation process, after which the whole development of DC could not be altered by *iMtb*. Accordingly, *iMtb* was also able to impair Mϕ early differentiation affecting their phenotype and function in a similar way to *iMtb*DC. All these data support the premise that *iMtb* interferes at the monocyte level. Considering that at this point DC-SIGN expression in Mo is incipient, we need to evaluate whether Mo fate impelled by *iMtb* directly involves DC-SIGN receptor and the time point at which Mo begin to express DC-SIGN. In this context, DC have been proposed to be a target of escape for different pathogens and tumors[34–36] and as DC are critical in triggering anti-mycobacterial immune response, they may represent a target of *Mtb* immune evasion.

It has been proposed that IL-10 may provide a mechanism to maintain the balance between a protective immune response and excessive cellular activation in the foci of tuberculous granuloma by converting immature DC into macrophage-like cells that attenuate Th1 response.[37] Local cytokine microenvironment may be dominated by IL-10 in certain stages of the disease,[38] and IL-10 inhibits DC Ag presentation mainly reducing the expression of Ag presentation and co-stimulatory molecules.[39] In this context, we observed that *iMtb* induced the release of IL-10, and that when DC were differentiated in presence of IL-10 to mimic *iMtb*DC effect, only a marked upregulation of CD14 was observed that was reverted by IL-10 neutralization. In line with this, it has been proposed that ManLAM interferes with DC functions through DC-SIGN binding, which induces the release of IL-10 by DC, shifting T cells toward a Th2 profile.[40] As the IL-10 neutralization in *iMtb*DC neither improves Ag-specific T-cell proliferation nor restores DC phenotype, we can conclude that *iMtb*DC profile is not mediated by *iMtb*-induced IL-10 secretion.

Although only whole bacterium was required to further explain the DC-SIGN^{low}/CD86^{high} /MR^{low} pattern, several *Mtb* antigens seem to be involved in the modulation of CD14/CD1a/CD1b expression. On the one hand, ManLAM only decreased CD1b and CFP only decreased CD1a expression. On the other hand, CWL increased CD86 expression and, as PTG also did, it slightly affected CD1a/CD1b/CD14 levels. As among *Mtb* components, PTG is recognized by TLR-2,[27] *iMtb*DC profile would be triggered by TLR-2-ligation. TLR-2 has been identified as a molecular link between *Mtb* and host pro-inflammatory signaling[41] involving NF-κB and p38 MAP kinase activation.[42] Herein, by using the TLR-2 synthetic bacterial lipopeptide Pam3Cys ligand for DC differentiation (Pam3DC), we demonstrated that TLR-2 activation partially mimicked *iMtb*DC in terms of CD1a/CD1b/CD14 profile and in the reduction of CD1molecules. The latter finding was indeed consistent with the reduced Ag-specific T-cell proliferation induced by Pam3DC. However, TLR-2 activation did not trigger the generation of DC-SIGN^{low}/CD86^{high} subpopulation.

DC-SIGN ligands have three possible structure motifs, namely, Lex structure (Gal$^{\beta}$1-4(Fuc$^{\alpha}$1-3) GlcNAc$^{\beta}$), ManGlcNAc and FucGlcNAc.[43] Even though DC-SIGN belongs to the MR family, two of the three structure motifs do not contain mannose epitopes. However, all three do contain *N*-acetylglucosamine (GlcNAc). Similarly, the core lipopolysaccharide (LPS) from *Escherichia coli* K12, *Haemophylus ducreyi*, *Neisseria gonorrhoeae* and *Salmonella typhimurium* do not contain either mannose or fucose, but GlcNAc is part of the core LOS/LPS region in each species.[44,45] In line with this, we observed a similar DC-SIGN^{low}/CD86^{high} pattern expression in Mo differentiated in presence of *E. coli* that did not alter CD1b expression or DC function (data not shown). Therefore, the fact that *Mycobacterium avium* does not bind DC-SIGN and has no effect on Mo differentiation,[46] tempted us to speculate that some bacteria that use DC-SIGN to enter DC share structural components capable of subverting the differentiation process of DC by altering

the expression of their main receptor. However, further studies are required to determine the structures involved and the exact mechanism by which they trigger the generation of this unusual pattern.

Strikingly, other deleterious effect on Mo was exerted by *iMtb* as the recovery of cells in *iMtb*DC cultures was strongly reduced and occurred during the first 24 h of culture. The existence of a subpopulation of Mo more susceptible to die as well as the mechanism involved in *iMtb*-induced Mo death is under study. Interestingly, several features of Mo-derived DC generated *in vitro* in the presence of *iMtb* resemble the features observed in DC obtained from TB characterized by the presence of replicating *Mtb* in lungs. Noticeably, DC from TB showed a reduced recall response,[19] even if they expressed elevated levels of HLA-DR and an improved capability of maturation. Besides, DC from TB expressed low levels of CD1a and high amounts of CD14 together with a larger DC-SIGN^{low}/CD86^{high} population, suggesting that the fate of Mo could be predetermined to induce an altered DC profile.

In summary, we have described a new step in the regulation of the immune response through the interaction of *iMtb* with Mo. *iMtb* effect is defined at an early stage of the differentiation process and involves (1) the loss of an unknown subset of Mo and (2) the altered *in vitro* differentiation of those 'surviving' Mo. These *iMtb*DC lack CD1molecules by a mechanism that partially involves TLR-2 activation and are enriched in the MR^{low}/DC-SIGN^{low} population by a mechanism that remains to be established. In this context, it is important to note that DC-SIGN is also involved in lymphocyte synapsis and migration, and then *Mtb* uptake may not be the only factor that would impact on antigen presentation in tuberculosis. Therefore, this *Mtb* strategy may constitute an effective evasion from the acquired immune response and this process would take place in the infected tissue where the presence of replicating mycobacteria would subvert differentiation of recruited Mo.

METHODS

Subjects

In all, 25 unknown tuberculin (PPD)-test status, 25 PPD+ volunteer blood donors (mean age: 30 years, range: 20–50) and 30 patients with active TB were evaluated (mean age: 43 years, range: 23–50). All patients were diagnosed by the presence of recent clinical respiratory symptoms, abnormal chest radiography and a positive sputum smear test for acid-fast bacilli. Blood samples were collected at 3–10 days after the initiation of antibiotics treatment. Patients were classified according to the extent and type of chest X-ray findings as moderate (n=22) or advanced (n=8). All of them were HIV seronegative and had not other infectious or underlying diseases. Informed consent was obtained from patients and approval for this study was obtained from the Institutional Ethics Review Committee of the Hospital Francisco J Muñiz.

Reagents

Recombinant human IL-10 and affinity purified polyclonal anti-human IL-10-specific antibody were purchased from Peprotech Inc. (Rocky Hill, NJ, USA) and polybead polystyrene microspheres 2 μm from Polysciences (Polysciences Inc, Washington, DC, USA). For inhibition studies, cells were exposed to PD98059 (MEK1 inhibitor) or to SB220025 (MAPK14-α and -β inhibitor) (Calbiochem, San Diego, CA, USA). The cytoskeletal inhibitor Cytochalasin B was purchased from Sigma Chemical Co. (St Louis, MO, USA). Purified monoclonal antibody (mAb) anti-CD1b was used in blocking proliferation assays (BD Pharmingen, San Diego, CA, USA).

Antigens

The *Mtb* γ-irradiated H37Rv strain *(iMtb)* and ManLAM, PTG, whole-cell lysate and CFP from *Mtb* H37Rv were kindly provided by J Belisle (Colorado State University, Fort Collins, CO, USA). *iMtb* was suspended in phosphate-buffered saline (PBS) free of pyrogen, sonicated and adjusted to an OD_{600} of 1, which corresponds to a bacterial suspension of $\sim 1\times10^8$ bacteria per ml. The laboratory strain H37Rv was grown in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA) at 37 °C in 5% CO_2 until log phase. Viable bacteria were harvested, washed three times, suspended in PBS (free of pyrogens) at an optical density at 600 nm of 1 ($\sim 10^8$ bacteria per ml). The synthetic bacterial lipopeptide Pam3CysSerLys4 was purchased from Boehringer Mannheim (Indianapolis, IN, USA).

Generation of Mo-derived DC

Mononuclear cells from peripheral blood samples were isolated by Ficoll-Hypaque gradient. A total of 5×10^6 per ml 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. Non-adherent cells were removed and maintained in culture with suboptimal dose of rIL-2 (1 nM; Peprotech Inc.) for 6 days until their use in autologous proliferation assays. DC were generated from adherent cells by the addition of IL-4 (20 ng ml^{-1}) (R&D Systems, Abingdon, UK) and GM-CSF (50 ng ml^{-1}) (Peprotech Inc.) for 6 days in RPMI-1640 supplemented with penicillin–streptomycin and 10% FCS (complete medium) at 37 °C in 5% CO_2. When indicated *iMtb* (2:1 *iMtb*:Mo ratio) (*iMtb*DC), Pam3Cys 150 ng ml^{-1} (Pam3DC), IL-10 20 ng ml^{-1} (IL10DC), aIL-10 10 ng ml^{-1} (aIL10DC) or beads (2:1 beads:Mo ratio) (BeadDC) were added at the start of the culture. In some experiments, Mo were pre-incubated for 40 min with 30 μM PD98059 or 10 μM SB220025 or with DMSO carrier at the start of the Mo differentiation. Mo were differentiated into Mϕ by the addition of GM-CSF (10 ng ml^{-1}) for 6 days in complete medium. On day 6, DC and Mϕ were analyzed for the expression of surface markers.

DC maturation

The resulting DC were washed and seeded in 24-well plates (Corning) at 1×10^6 DC per ml in RPMI-1640 supplemented with penicillin–streptomycin and 10% FCS, and cellular maturation was achieved by treatment with *iMtb* (2×10^6 bacilli per ml) (mDC) for 48 h at 37 °C. Cells were centrifuged at 800 r.p.m. for 10 min to selectively spin down cells while extra-cellular bacteria remain in the supernatant. Thereafter, cells were washed three times and phenotype and functional assays were evaluated together with survival of activated cells. DC apoptosis was assessed by surface staining with fluorescence-conjugated antibodies against Annexin-V (Sigma) and necrosis was assessed by 7-aminoactinomycin (7-AAD) (BD Bioscience Pharmingen) staining.

Influence of soluble factors on Mo differentiation

Adherent Mo were cultured with IL-4 and GM-CSF in presence or not of *iMtb* (2:1 *iMtb*:Mo) for 24 h; thereafter, supernatants were collected (conditioned culture medium), centrifuged at 6000 *g* three times and passed through a 0.22 μ filter to eliminate bacteria. The ability of culture medium to impair Mo differentiation was tested by culturing fresh Mo with each culture supernatant (supplemented with IL-4, GM-CSF and SFB 10%) and evaluating the DC phenotype at the sixth day of differentiation.

Immunofluorescence analysis

For phenotypic analysis the following FITC, PE or PE-Cy5-labeled mAb against CD1a, CD14, CD86, CD83, HLA-DR, MR, CD54, IL-12, IgG1, IgG2a (eBioscience, San Diego, CA, USA), CD1b and CD1c (Ancell, Bayport, MN, USA) and DC-SIGN (R&D Systems Inc., Minneapolis, MN, USA) were used. Approximately 5×10^5 cells were seeded into tubes and washed once with PBS containing 0.2% FCS and 0.01% azide. Cells were stained for 30 min at 4 °C and washed twice. Stained population was gated according to its forward scatter (FSC) and side scatter (SSC) proprieties analyzed on FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA), 10 000 events were collected in linear mode for FSC and SSC, and log amplification for FL-1 and FL-2. Analysis was performed using the CellQuest software (Becton Dickinson) and isotype matched controls were used to determine autofluorescence and non-specific staining. Results were expressed as median fluorescence intensity or percentage of cells (% cells).

Proliferation assays

Mixed leukocyte reactions were performed by culturing the obtained APC with 1×10^5 allogeneic lymphocytes at an Ly:DC ratio of 10:1 in round bottom 96-well culture plates (Corning) for 5 days. Specific lymphocyte proliferations (recall) were carried out in cells from PPD+ HS by culturing APCs and autologous lymphocytes for 5 days. Blockage of CD1b-dependent T-cell proliferation was carried out by preincubating APC with anti-CD1b blocking mAb ($4\,\mu g\,ml^{-1}$) for 30 min at room temperature and, thereafter, autologous lymphocytes were added for 5 days. After that, 0.5 μCi per well of [*methyl*-^{3}H]thymidine (PerkinElmer, Boston, MA, USA) was added for the last 18 h to the culture and radioactivity was measured in a liquid scintillation counter. The results were expressed as counts of: [^{3}H]thymidine incorporation per minute (c.p.m.).

Phagocytosis assay

The ability of DC to capture Ag was determined by measuring uptake of FITC-labeled *iMtb*. Briefly 10^9 bacteria were labeled by incubation of 0.5 mg FITC per ml (Sigma Chemical Co.) in PBS at room temperature for 1 h, washed four times to remove unbound FITC and suspended in RPMI-1640 containing 10% FCS. DC (5×10^5 per sample) were incubated with FITC-*iMtb* (*iMtb*:DC, 2:1 ratio) for 2 h at 37 or 4 °C. After that, cells were extensively washed to remove extracellular bacteria and the percentage of cells that phagocytosed FITC-*iMtb* was measured by flow cytometry. Control experiments of phagocytosis were performed with cells pretreated for 30 min with Cytochalasin B ($10\,\mu g\,ml^{-1}$, Sigma).

IL-10 determination

Supernatants from DC and *iMtb*DC cultures were harvested at the end of Mo differentiation, filtered (0.2 μm) and stored at −80 °C. IL-10 was measured by ELISA using commercial kits (eBioscience) according to manufacturers' instructions. The limit of detection was $15\,pg\,ml^{-1}$.

Statistical analysis

All values are presented as mean ± s.e.m. of a number of independent experiments. Comparisons between experimental conditions were made using the two-tailed paired Wilcoxon's matched pairs signed-rank test for non-parametric data. Mann–Whitney test was used to compare HS and patients with TB and correlation was evaluated by Spearman's coefficient. A P-value <0.05 was considered significant.

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