

Mycobacterium tuberculosis Inhibits Maturation of Human Monocyte-Derived Dendritic Cells In Vitro

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To induce effector immunity, dendritic cells (DCs) must differentiate into fully mature cells. We show that, after human monocyte-derived DCs were infected with virulent *Mycobacterium tuberculosis*, up-regulation of cellular-surface maturation markers was minimal and reversible. In the presence of a potent stimulus for maturation (tumor necrosis factor [TNF]- α , interleukin [IL]- 1β , and prostaglandin E₂ [PGE₂]), *M. tuberculosis* inhibited phenotypic DC maturation. *M. tuberculosis*-infected DCs had an impaired ability to induce allogeneic lymphoproliferation and activated autologous memory CD4⁺ and CD8⁺ T cells optimally only in the presence of TNF- α , IL- 1β , and PGE₂. Thus, virulent *M. tuberculosis* inhibits phenotypic and functional maturation of human monocyte-derived DCs. This mechanism, which has been described elsewhere for various viruses and for the virulent mycobacterium *M. leprae*, may be a novel mechanism that this pathogen uses to evade the host's immune response.

Worldwide, 8.4 million new cases of tuberculosis occurred in 1999 alone; in 2005, this figure will rise to a staggering 10.2 million new cases [1]. Although effective antituberculosis drug therapy is available, control of the epidemic is hampered by poor adherence to the long duration of therapy, emergence of multidrug resistance, human immunodeficiency virus-induced immune compromise, and social and economic public-health constraints. An effective new vaccine may present the only sustainable option for achieving control of tuberculosis;

however, vaccine development has been slow because of an incomplete understanding of the host immune response to the organism [2, 3]. In particular, the role of antigen-presenting cell function in the induction of specific T cell immunity to *Mycobacterium tuberculosis* has not been fully elucidated.

Dendritic cells (DCs), the most potent antigen-presenting cells, play a central role in induction and regulation of protective immunity against microorganisms [4–6]. In the lungs, immature DCs line alveolar spaces and readily take up foreign material [7], presumably including inhaled *M. tuberculosis*. To present antigen efficiently, these immature DCs must differentiate into activated, mature DCs. During this process, DC phagocytic capacity is down-regulated and antigen-presenting capacity is up-regulated. Mature DCs express high levels of the antigen-presenting molecules major-histocompatibility-complex (MHC) class I, MHC class II, and CD1 and of T cell costimulatory molecules—such as CD40, CD80, and CD86—on their surfaces. These cells can migrate to regional lymph nodes by means of up-regulated surface expression of the chemokine receptor

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CCR7. Within lymph nodes, mature DCs interact with the abundant T cells that traffic through this site, to activate both naive (primary) and memory (secondary) T cell immunity.

How *M. tuberculosis* infection affects this process is not clear. Henderson et al. [8] found that DCs phagocytose *M. tuberculosis* efficiently, a process resulting in up-regulation of MHC class I and MHC class II. CD40, CD54, CD58, and CD80 were also up-regulated in *M. tuberculosis*-infected DCs. In contrast to these findings suggesting that DC maturation follows infection, Stenger et al. [9] demonstrated that infection of antigen-presenting cells, with *M. tuberculosis*, resulted in down-regulation of the CD1 molecules. The antigen-presenting cells used in that study were generated in vitro by incubation of monocytes, with interleukin (IL)-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF), for 3 days, compared with incubation for 5–7 days that is normally required for differentiation of monocytes into immature DCs. Giacomini et al. [10] showed that DCs in cultures exposed to *M. tuberculosis* up-regulated surface MHC class II, the DC maturation marker CD83, and CD40, CD54, CD58, CD80, and CD86. Together, these studies demonstrated that *M. tuberculosis* infection may have diverse phenotypic effects on immature human DCs in vitro.

In the present study, we introduced a unique model to elucidate the effects that virulent *M. tuberculosis* has on phenotypic DC maturation. By using *M. tuberculosis* expressing green fluorescent protein (*gfp*), we could describe differential effects on infected DCs and on uninfected, bystander cells from the same cultures. We compared the effects that *M. tuberculosis* has on DCs with the effects that an optimal positive control of DC maturation has, effects induced by the “maturation cocktail” of tumor necrosis factor- α (TNF- α), IL-1 β , and prostaglandin E₂ (PGE₂) [11]. We also determined the effect that *M. tuberculosis* has on full and irreversible DC maturation induced by this cocktail. To assess functional consequences of the effects that *M. tuberculosis* has on DC phenotype, we determined the ability of these cells to activate both naive and memory T cells.

MATERIALS AND METHODS

Generation of immature human DCs in vitro. Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation, from sodium-heparinized blood obtained from healthy adult volunteers. Written-informed consent was obtained from healthy blood donors, and experimentation guidelines of the Department of Health and Human Services and of respective institutional review boards were followed. Monocytes were purified by positive selection of CD14⁺ cells, by use of magnetic beads (Miltenyi Biotec), and were shown to be >95% pure, by flow cytometry. Immature DCs were generated by culturing of monocytes, in 5% pooled human AB serum (Gemcell)

in RPMI (Gibco), with IL-4 (1 ng/mL) (Endogen) and GM-CSF (0.2 ng/mL) (Endogen), in Falcon 6-well plates (BD Biosciences), at 37°C in 5% CO₂. Antibiotics were not used in any cultures, in any experiment described. Cytokines were replenished at 2, 4, and 6 days. On either day 6 or day 7, nonadherent immature DCs were harvested and were placed in Falcon 24-well plates (BD Biosciences), in 10% pooled human AB serum in RPMI.

M. tuberculosis infection of DCs. Virulent *M. tuberculosis*, strain H37Rv, expressing *gfp* (H37Rv-*gfp*) (made by C.B.) when viable, was grown in 7H9 medium (Difco). Stocks were stored at -70°C, until use, when bacteria were rapidly thawed and sonicated to create single-cell suspensions. Immature DCs were plated in Falcon 24-well plates, at 2×10^5 cells in 1.5 mL of 10% pooled human AB serum in RPMI per well, without antibiotics, and then were infected with H37Rv-*gfp*. IL-4 and GM-CSF were added to these cultures and were replenished 2 days after infection. To validate the flow-cytometric differentiation of infected and uninfected DCs, by *gfp*, DCs expressing *gfp* were sorted from *gfp*⁻ DCs 2 days after infection, by flow cytometry (FACSCalibur; BD Biosciences). The DCs were disrupted and were cultured on Middlebrook 7H11 agar (Difco), and colony-forming units were enumerated. At an MOI of 3, mycobacterial culture demonstrated means of 4.4 organisms/cell in *gfp*⁺ DCs and 0.4 organisms/cell in *gfp*⁻ DCs.

Phenotypic DC maturation. DC phenotype was assessed by flow-cytometric analysis of surface-marker expression. Infected DCs were incubated in 0.02% EDTA (Sigma) for 15 min at room temperature, before harvest, to obtain the total cellular content of wells. Harvest was followed by a single wash in PBS (Gibco). Harvested cells were then incubated in 2% fetal calf serum (Gemcell) in 0.09% sodium azide (Sigma) in PBS, with combinations of fluorescence-conjugated antibodies to the following surface markers: CD14, CD25, CD40, CD80, CD83, CD86, MHC class I, MHC class II, and CCR7 (all from BD Biosciences). Flow-cytometric acquisition was performed on unfixed DCs, within 1 h of surface staining, in a biosafety level 3 facility. DC apoptosis was assessed by surface staining with fluorescence-conjugated antibodies against Annexin-V (BD Biosciences), and necrosis was assessed by 7-aminoactinomycin (7-AAD) (BD Biosciences) staining. In all experiments evaluating DC phenotypic maturation, dead cells (7-AAD⁺) were excluded from analysis. *gfp*-expressing (i.e., H37Rv-*gfp*-infected) DCs and uninfected DCs were analyzed either in combination or separately. Preliminary kinetic experiments demonstrated peak expression of surface markers 2 days after infection; this incubation period was therefore chosen for evaluation of DC maturation.

As a positive control for DC maturation, DCs were incubated with a combination of the following proinflammatory molecules: TNF- α (10 ng/mL) (Alexis), IL-1 β (10 ng/mL) (Pharmingen), and PGE₂ (1 μ g/mL) (Sigma). This maturation cock-

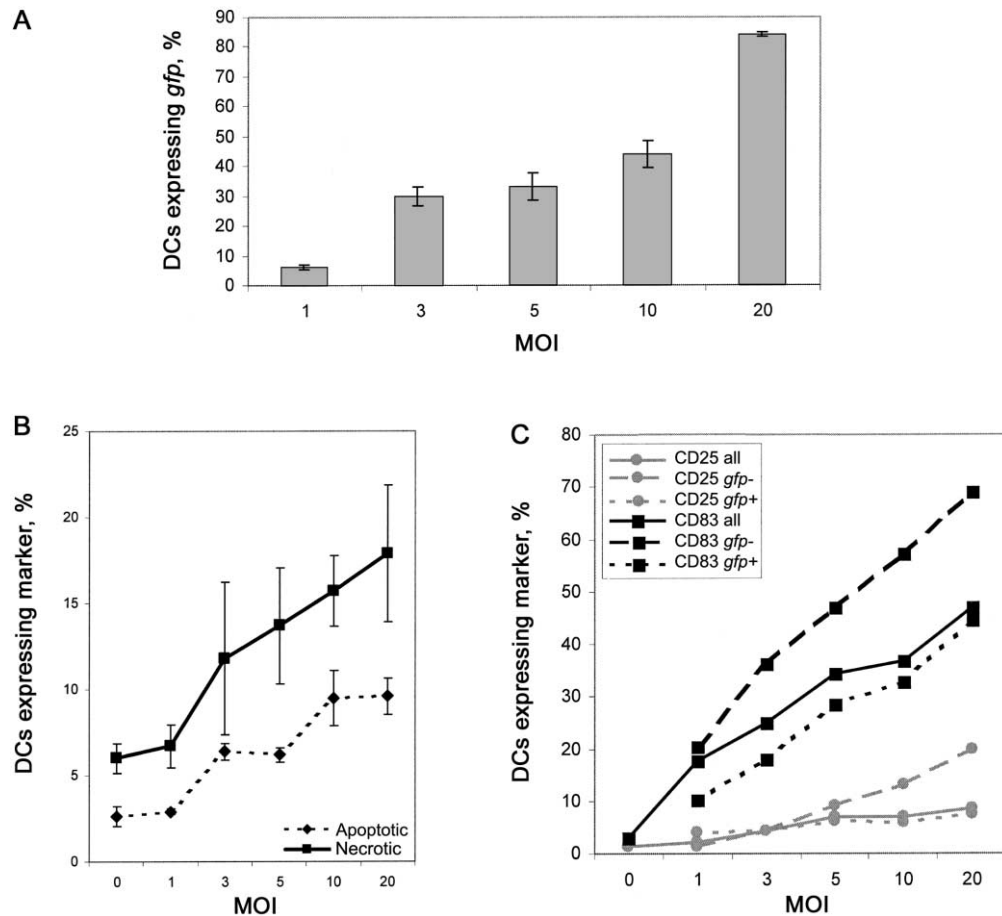


Figure 1. A, Effect of MOI with *Mycobacterium tuberculosis* H37Rv–green fluorescence protein (*gfp*), on percentage of dendritic cells (DCs) expressing *gfp*, by flow cytometry. B, Effect of MOI on rate of both apoptotic and necrotic cells, in cultures. Apoptotic cells were detected by surface expression of Annexin-V and necrotic cells by uptake of 7-AAD, by flow cytometry. C, Effect of MOI on surface expression of CD83 and CD25, by flow cytometry. Only viable cells (7-AAD⁻) were evaluated. Surface-marker expression was measured in total cells from cultures exposed to mycobacteria (all, solid line) and in uninfected (*gfp*⁻, dashed line) and infected (*gfp*⁺, dotted line) DCs, within these cultures. Results are representative of 3 experiments.

tail lacks IL-6, which was used in a previously described combination of proinflammatory molecules (to induce DC maturation) [11], because our preliminary experiments demonstrated that IL-6 did not affect, to a detectable level, DC maturation.

In some experiments, infected DCs were removed from IL-4 and GM-CSF 2 days after infection and were reincubated in 10% pooled human serum in RPMI. DC surface phenotype was again evaluated 2 days later. As a control, DCs matured with TNF- α , IL-1 β , and PGE₂ were also removed from these cytokines 2 days after infection and were reincubated without cytokines, and surface phenotype was evaluated 2 days later.

DC-induced naive T cell activation. The ability of infected DCs to activate naive T cells was assessed by allogeneic lymphoproliferation. DCs were harvested 2 days after infection with H37Rv-*gfp*, were washed once in PBS, and were suspended in 10% pooled human AB serum in RPMI, without antibiotics or

cytokines. Infected DCs were used to stimulate allogeneic CD14⁻ PBMCs (purified by magnetic beads), at ratios of 1:40–1:3000. CD14⁻ PBMCs, rather than naive CD4⁺ T cells, were used to assess allogeneic lymphoproliferation, because preliminary experiments demonstrated identical results when infected DCs were incubated with allogeneic CD4⁺CD45RA⁺ PBMCs (purified by magnetic beads). After DCs were cocultured with CD14⁻ PBMCs for 5 days, [³H]thymidine incorporation was used to assess lymphoproliferation.

DC-induced memory T cell activation. The ability of infected DCs to induce memory T cell activation was assessed by flow cytometry–based assay of T cell–associated interferon- γ (IFN- γ) expression after incubation of infected DCs with autologous CD14⁻ PBMCs. Blood from tuberculin skin test–positive, healthy adult donors was used for these experiments. DCs were derived from their monocytes and were infected with H37Rv-*gfp*, and, 2 days after infection, the cells were harvested,

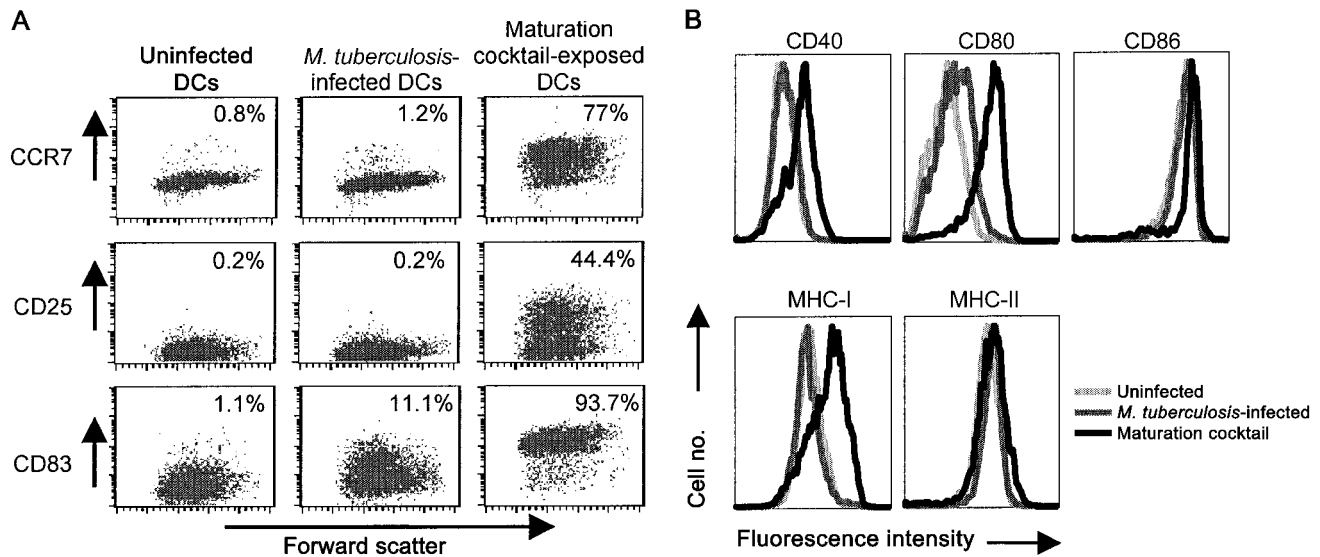


Figure 2. Effect of *Mycobacterium tuberculosis* infection on phenotypic maturation of human monocyte-derived dendritic cells (DCs). *A*, DC surface expression of CCR7, CD25, and CD83 2 days after infection or exposure to maturation cocktail of tumor necrosis factor- α , interleukin-1 β , and prostaglandin E $_2$, by flow cytometry. *B*, DC surface expression of costimulatory and antigen-presenting molecules (MHC [major histocompatibility complex]) 2 days after infection or exposure to maturation cocktail, by flow cytometry. Results are representative of 6 experiments.

were washed once with PBS, and then were plated in 10% human AB serum in RPMI, without cytokines or antibiotics, in Falcon 24-well plates. Autologous CD14⁻ PBMCs (isolated by magnetic bead separation) were added at a CD14⁻ PBMC:DC ratio of 40:1. Cultures were incubated for 13 h at 37°C with 5% CO $_2$, and then brefeldin-A (10 μ g/mL) (Sigma) was added, to capture intracellular cytokines. After an additional 5 h of incubation, EDTA in PBS was added for 15 min at room temperature, for a final concentration of EDTA of 0.02%, to disaggregate DCs and lymphocytes. The well contents were then transferred to 4-mL flow-cytometry tubes (Falcon 2054; Becton Dickinson). Becton Dickinson FACS lysing solution (2 mL) was added for 10 min to fix the cells, and then the cells were pelleted, and supernatants were removed. Permeabilization solution (2 mL) consisting of 0.1% saponin (Sigma) plus 0.1% bovine serum albumin (Sigma) in PBS was added to each tube, which was vortexed and was incubated for 10 min at room temperature. Cells were again pelleted, and supernatants were removed. Approximately 50 μ L of permeabilization solution with cells remained in the tube, to which the following antibodies were added: anti-CD4–allophycocyanin, anti-CD8–peridinin chlorophyll protein, and anti-IFN- γ –phycoerythrin (all from Becton Dickinson). After 30 min of incubation, the cells were washed once in permeabilization solution and were pelleted, before being fixed in 250 μ L of 1% paraformaldehyde (Fisher). Four-color flow cytometry was performed. Principles of rare-event detection were used to optimize flow-cytometric analysis; these included use of an initial fluorescence threshold to gate on CD4⁺ or CD8⁺ cells and exclusion of autofluorescent

cells by use of a fluorescence channel for which cells were not specifically stained.

CD14⁻ PBMCs incubated with uninfected DCs served as negative controls; CD14⁻ PBMCs not incubated with DCs but stimulated with 10 ng/mL staphylococcal enterotoxin B (Sigma) served as positive controls. In some experiments, DCs were infected in the presence of the maturation cocktail of TNF- α , IL-1 β , and PGE $_2$, before incubation with CD14⁻ PBMCs. In other experiments, autologous CD14⁻ PBMCs were incubated with monocytes that had been infected with H37Rv-*gfp* (MOI of 3:1) 2 days before, at a CD14⁻ PBMC:monocyte ratio of 40:1.

Statistical considerations. Differences in expression of DC maturation markers were evaluated by nonparametric Wilcoxon rank sum test. $P < .05$ was chosen to indicate statistical significance.

RESULTS

Effect of *M. tuberculosis* infection on DC viability and maturation. Immature human DCs were infected with viable *M. tuberculosis* H37Rv-*gfp*, at MOIs of 1–20. Increasing MOIs were associated with increasing numbers of DCs expressing *gfp*, as evaluated by flow cytometry, 2 days after infection (figure 1*A*). However, at high MOIs, H37Rv-*gfp* was toxic to the cells, because progressively increasing numbers of apoptotic (Annexin-V⁺) and necrotic (7-AAD⁺) DCs were demonstrated by flow cytometry (figure 1*B*). High MOIs were also associated with a reduction in the number of cells detected by flow cytometry

Table 1. Effect of *Mycobacterium tuberculosis* infection on phenotypic maturation of human monocyte-derived dendritic cells (DCs).

Maturation marker	Uninfected DCs	DCs exposed to maturation cocktail ^a	Cultures exposed to <i>M. tuberculosis</i> H37Rv- <i>gfp</i>	
			<i>M. tuberculosis</i> -infected DCs (<i>gfp</i> ⁺) ^b	Uninfected DCs (<i>gfp</i> ⁻) ^c
CD25	57	184	59	83
CD40	92	198	128	159
CD80	156	1473	573	585
CD83	37	693	84	95
CD86	161	284	214	152
CCR7	36	592	60	79
MHC class I	96	283	149	159
MHC class II	111	188	162	163

NOTE. DC surface expression of multiple maturation markers was measured by flow cytometry 2 days after infection. Data are percentage increase in geometric mean fluorescence intensity of maturation-marker expression over this period (median of 6 experiments). MHC, major histocompatibility complex; maturation cocktail: tumor necrosis factor- α , interleukin-1 β , and prostaglandin E₂.

^a $P < .05$ vs. uninfected DCs.

^b $P < .05$ vs. uninfected DCs (except for CD25 [$P > .05$]) and vs. DCs exposed to maturation cocktail.

^c $P > .05$ vs. *M. tuberculosis*-infected DCs.

(data not shown), a finding indicating that some of the cells had died, had disintegrated, or may have been phagocytosed by other DCs in the culture.

When DC surface expression of the maturation markers CD25 and CD83 was evaluated in viable (7-AAD⁻) cells 2 days after infection, increasing MOIs were associated with increasing numbers of cells expressing the maturation markers (figure 1C). Interestingly, as the MOIs increased, larger numbers of DCs that had remained uninfected (i.e., *gfp*⁻) demonstrated maturation-marker expression, a finding indicating preferential maturation of uninfected, bystander cells (figure 1C). To further evaluate the effect that H37Rv-*gfp* has on DC maturation, we assessed surface expression of other surface markers of maturation, including the chemokine receptor CCR7, the costimulatory molecules CD40, CD80, and CD86, and the antigen-presenting molecules MHC class I and MHC class II (figure 2). To avoid DC death, a single MOI of 3 was used for these experiments. Infected DCs were compared with DCs both not exposed to *M. tuberculosis* and exposed to the maturation cocktail of TNF- α , IL-1 β , and PGE₂ (figure 2). Only viable cells (7-AAD⁻) were evaluated. Two days after infection, some up-regulation of DC surface expression of these maturation markers was noted. However, infection-induced maturation was minimal, compared with that induced by the maturation cocktail of TNF- α , IL-1 β , and PGE₂. Similar results were obtained when *M. tuberculosis* H37Rv not expressing *gfp* was used in the experiments (data not shown). Further, as demonstrated above, uninfected, bystander (*gfp*⁻) DCs from cultures exposed to H37Rv-*gfp* demonstrated slightly higher expression of these maturation markers, compared with that expressed by infected

DCs (*gfp*⁺), although the expression was not statistically significant (table 1). We concluded that virulent *M. tuberculosis* infection of DCs, at the low, physiological MOI of 3, induced minimal phenotypic maturation.

Effect of removal of cytokines from cultures, after *M. tuberculosis* infection, on DC phenotype. One of the hallmarks of mature DCs is that they retain their mature phenotype after removal of the maturation stimulus. Thus, when IL-4 and GM-CSF are removed from cultures of mature DCs, the cells will not revert to a macrophage phenotype, whereas immature DCs will revert to this phenotype. Therefore, H37Rv-*gfp*-infected DCs were removed from cultures containing IL-4 and GM-CSF 2 days after infection, were washed, and were reincubated in medium without cytokines. Two days after reincubation, surface phenotype was evaluated in viable (7-AAD⁻) cells and was compared with the phenotype before removal of cytokines. Removal of IL-4 and GM-CSF caused down-regulation of the low levels of expression that CD25 and CD83 demonstrated 2 days earlier (figure 3). Furthermore, surface expression of CD14 was dramatically up-regulated. Light microscopy demonstrated the morphological appearance of adherent macrophages (data not shown). In contrast, maturation cocktail-exposed DCs retained their mature DC phenotype 2 days after all cytokines in the medium had been removed (figure 3) and had the morphological appearance of mature, nonadherent, highly veiled DCs (not shown). We concluded that minimal maturation induced by *M. tuberculosis* infection of DCs was reversible and that infected DCs changed back into macrophages after removal of the DC differentiation cytokines IL-4 and GM-CSF.

Effect of *M. tuberculosis* infection of DCs, on complete mat-

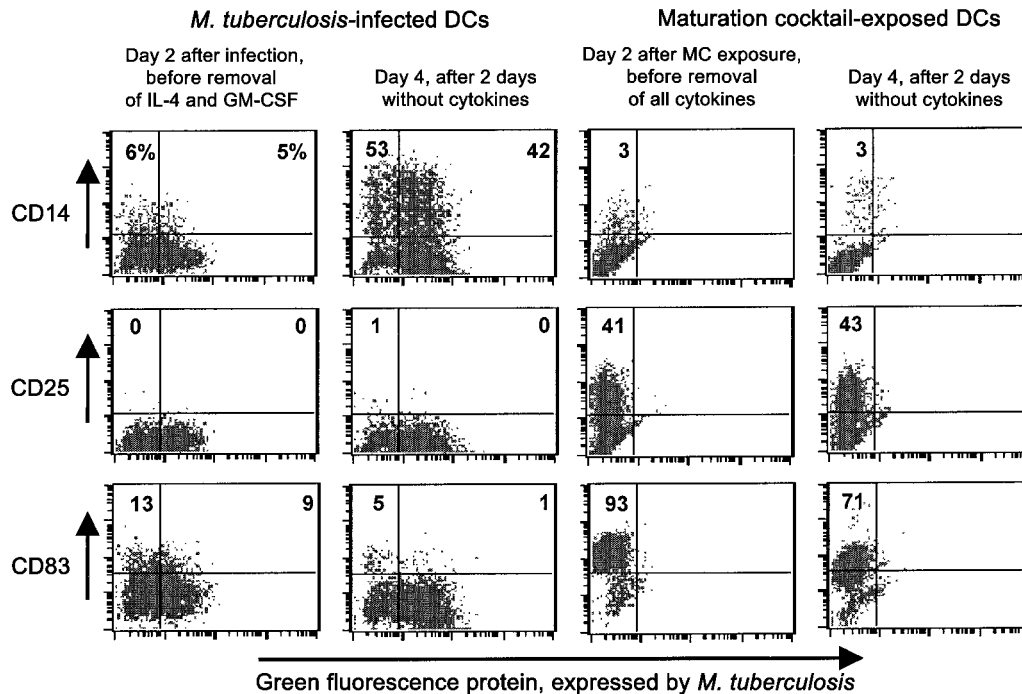


Figure 3. Effect of removal of *Mycobacterium tuberculosis*-infected or maturation cocktail (MC) (tumor necrosis factor- α , interleukin-1 β , and prostaglandin E₂)-exposed dendritic cells (DCs), from cytokine-containing medium (interleukin-4 [IL-4] and granulocyte-macrophage colony-stimulating factor [GM-CSF]), on DC phenotype. DCs were removed from cytokine-containing medium 2 days after infection or exposure to MC and were reincubated in media without cytokines. Surface phenotype was assessed by flow cytometry, both at the time of removal from cytokine-containing media and 2 days later. Macrophage marker CD14 and DC maturation markers CD25 and CD83 were measured. Results are representative of 2 experiments.

uration induced by the maturation cocktail of TNF- α , IL-1 β , and PGE₂. Both limited phenotypic maturation induced by *M. tuberculosis* and possible interference with DC maturation by the pathogen raised the question whether the bacillus would inhibit maturation induced by the maturation cocktail of TNF- α , IL-1 β , and PGE₂. These proinflammatory molecules are likely to be present at the disease site. DCs were infected with H37Rv-*gfp* (MOI of 3), in the presence of the maturation cocktail. Among viable (7-AAD⁻) cells, infected DCs (*gfp*⁺) showed down-regulation of the maturation markers CCR7, CD25, and CD83, compared with expression by *gfp*⁻ (uninfected) cells (figure 4). This effect became even more striking when progressive dilutions (0.5 \times and 0.05 \times) of the maturation cocktail were used in the cultures (figure 4). The inhibitory effect that H37Rv-*gfp* has on phenotypic maturation, in the presence of a maturation stimulus, was also demonstrated when other surface markers characteristic of maturation—including CD40, CD80, MHC class I, and MHC class II—were assessed (not shown). The striking differential maturation of infected and uninfected DCs from the same cultures also remained constant both whether infection occurred before or after exposure to the maturation cocktail and when higher MOIs of H37Rv-*gfp* were used (data not shown). We concluded that virulent *M. tuberculosis* inhibited phenotypic maturation of DCs induced by proinflammatory stimuli.

Effect of *M. tuberculosis* infection on the ability of DCs to activate naive T cells. Next, the antigen-presenting capacity of DCs was evaluated. The ability of the cells to induce naive (primary) T cell activation was assessed by quantitating the proliferation of allogeneic T cells exposed to DCs. Mature DCs normally induce significant allogeneic lymphoproliferation at DC:naive T cell ratios of $\leq 1:300$, as demonstrated by DCs fully matured with TNF- α , IL-1 β , and PGE₂ (figure 5). In contrast, lymphoproliferation induced by DCs exposed to H37Rv-*gfp* (including both infected DCs and uninfected, bystander cells from the same cultures) more closely resembled lymphoproliferation induced by DCs from cultures not exposed to the bacilli (figure 5). Remarkably, DCs infected with H37Rv-*gfp*, in the presence of the maturation cocktail, also induced significantly lower allogeneic proliferation than did uninfected DCs, in the presence of the maturation cocktail (figure 5).

These results suggested that “incompletely” matured H37Rv-*gfp*-infected DCs might induce some naive T cell activation but that this capacity was relatively impaired, compared with DCs exposed to the maturation cocktail. Also, H37Rv-*gfp* infection inhibited the ability of DCs to completely mature functionally—that is, to induce naive T cell activation in the presence of the maturation cocktail of TNF- α , IL-1 β , and PGE₂.

Effect of the maturation cocktail of TNF- α , IL-1 β , and PGE₂ on optimal memory T cell activation by *M. tubercu-*

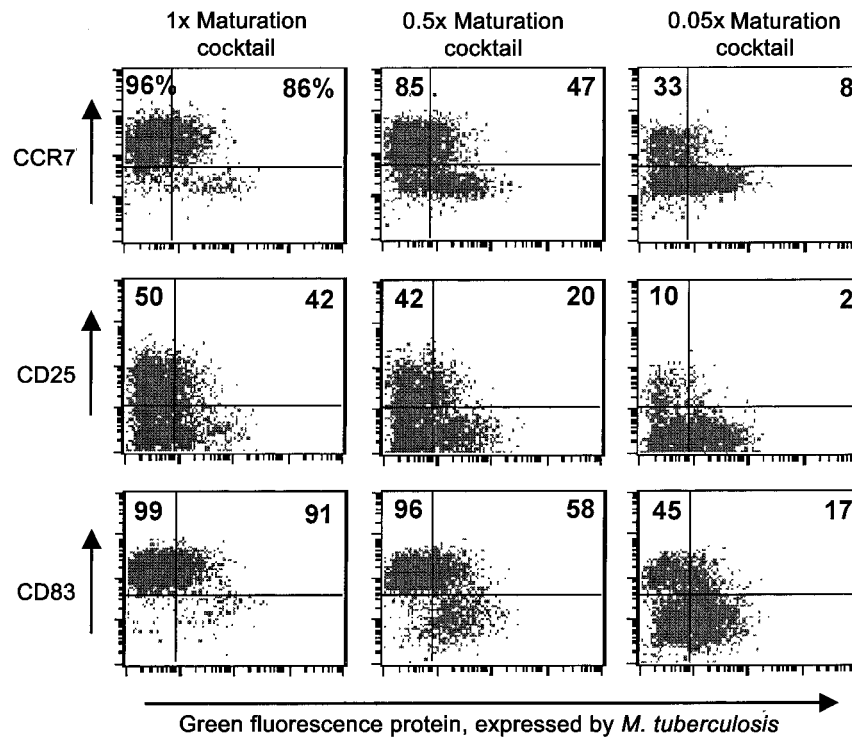


Figure 4. Effect of dendritic cell (DC) infection with *Mycobacterium tuberculosis*, on maturation induced by maturation cocktail of tumor necrosis factor- α , interleukin-1 β , and prostaglandin E₂. Data are percentage of DCs that express CCR7, CD25, and CD83, by flow cytometry, in both uninfected (*gfp*⁻) and infected (*gfp*⁺) DCs. This effect was examined at serially lower concentrations of maturation cocktail. Results are representative of 3 experiments.

lois-infected DCs. Next, the functional ability of incompletely matured *M. tuberculosis*-infected DCs to induce memory (secondary) T cell activation was evaluated. Autologous PBMCs from persons who were tuberculin skin test-positive, which is a clear indication of presence of a memory mycobacteria-specific immune response, were used as effector cells. A flow-cytometric assay of CD4⁺ and CD8⁺ lymphocyte-associated IFN- γ production after exposure of PBMCs to DCs was used. H37Rv-*gfp*-infected DCs were able to induce robust IFN- γ production in both CD4⁺ and CD8⁺ T cells after 18 h of incubation (figure 6). This response was double that induced by infected monocytes, a finding indicating the superior antigen-presenting capacity of even relatively immature DCs (figure 6). The frequency of IFN- γ -producing lymphocytes increased when infected DCs were cultured in the presence of the maturation cocktail (figure 6). This assay was mycobacteria-specific, because uninfected DCs or DCs exposed to the maturation cocktail in the absence of H37Rv-*gfp* did not induce significant IFN- γ production in CD4⁺ and CD8⁺ lymphocytes (figure 6).

These results suggest that DCs infected with H37Rv-*gfp*, although incompletely matured, are still markedly superior activators of memory T cell responses than are monocytes. However, optimal induction of memory T cell responses by DCs in

vitro required the presence of the maturation cocktail of TNF- α , IL-1 β , and PGE₂.

DISCUSSION

We have shown that in vitro infection of immature human monocyte-derived DCs, with virulent *M. tuberculosis*, results in only limited phenotypic maturation of these cells. Infected DCs demonstrated lower maturation-marker expression than did uninfected, bystander DCs from the same cultures, a finding suggesting that *M. tuberculosis*-infected DCs may have produced soluble factors that affected maturation of bystander DCs from the same cultures or that this bystander effect was cell contact dependent. Furthermore, the limited maturation induced by *M. tuberculosis* infection was reversible when IL-4 and GM-CSF were removed: the cells reacquired the phenotypic and morphological appearance of macrophages. Inhibition of DC maturation, by intracellular *M. tuberculosis*, was confirmed by demonstrating significantly depressed phenotypic maturation of the infected cells, in the presence of an optimal maturation cocktail (TNF- α , IL-1 β , and PGE₂).

Inhibition of maturation of human DCs has been reported after in vitro infection with various viruses [12–15] and with the parasite *Trypanosoma cruzi* [16]. With regard to obligate

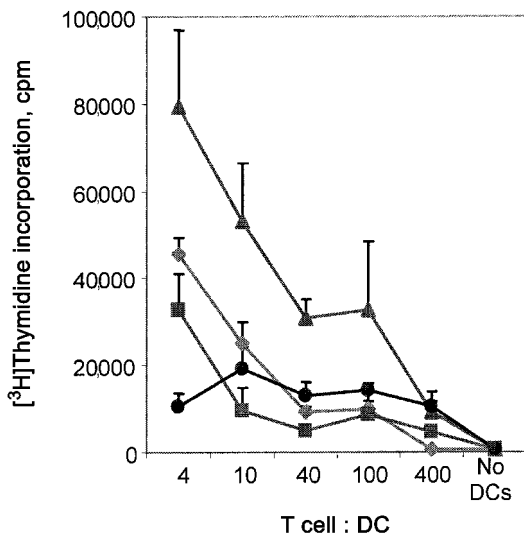


Figure 5. Ability of *Mycobacterium tuberculosis*-infected dendritic cells (DCs) to induce allogeneic lymphoproliferation in CD14⁺ peripheral blood mononuclear cells (PBMCs), at different PBMC:DC ratios, by [³H]thymidine incorporation in PBMCs (mean + SE). *M. tuberculosis*-infected DCs (squares), uninfected DCs (diamonds), DCs matured with cocktail of tumor necrosis factor- α , interleukin-1 β , and prostaglandin E₂ (triangles), and DCs infected with *M. tuberculosis*, in presence of maturation cocktail (circles), are shown. Results are representative of 5 experiments.

and facultative intracellular bacteria, various microorganisms have been shown to be potent inducers of DC maturation in vitro (summarized in [17]). For example, up-regulated surface expression of phenotypic maturation markers after in vitro infection of human monocyte-derived DCs, with the intracellular organism *Listeria monocytogenes*, has been reported [18]. Among mycobacteria, *M. bovis* [19–21] has been reported to induce maturation. However, it was recently reported that virulent *M. leprae*, which causes leprosy, induced minimal maturation of monocyte-derived DCs in vitro [21]. *M. leprae*-infected DCs also inhibited cellular activation induced by CD40L, a potent stimulant of maturation. This pattern was remarkably similar to our findings of DC interactions with virulent *M. tuberculosis*. Although this pathogen has been described elsewhere to induce DC activation, which was termed “maturation” [8–10, 22], our experimental approach differed significantly from that of these reports and enabled us to examine this phenomenon in more detail. First, detection of green fluorescence expressed only by viable H37Rv-*gfp* allowed us to sort infected cells from uninfected, bystander cells. The importance of this procedure is underscored by our observation that the phenotypes of total DCs exposed to virulent *M. tuberculosis* often differed from the phenotypes seen after sorting of infected and uninfected DCs from the same cultures. Second, by using an optimal positive control of maturation (that is, the DC phenotype induced by the maturation cocktail of TNF- α ,

IL-1 β , and PGE₂), we could distinguish minimal or partial DC maturation from complete maturation.

We have investigated the functional implications of limited phenotypic DC maturation or inhibition of maturation caused by *M. tuberculosis* infection in greater detail than has been reported elsewhere [8–10, 22]. Our results strongly suggest that *M. tuberculosis*-infected DCs are compromised in their ability to activate naive T cells, as determined by induced allogeneic lymphoproliferation. Moreover, the ability of maturation cocktail-matured DCs to activate naive T cells was inhibited by simultaneous *M. tuberculosis* infection. For these experiments, the total cellular content of cultures exposed to *M. tuberculosis* was used to induce naive T cell activation. Future experiments should focus on sorting this culture content for infected (*gfp*⁺) and uninfected (*gfp*⁻) DCs, to differentially assess the ability of these cells to activate naive T cells. It also remains to be

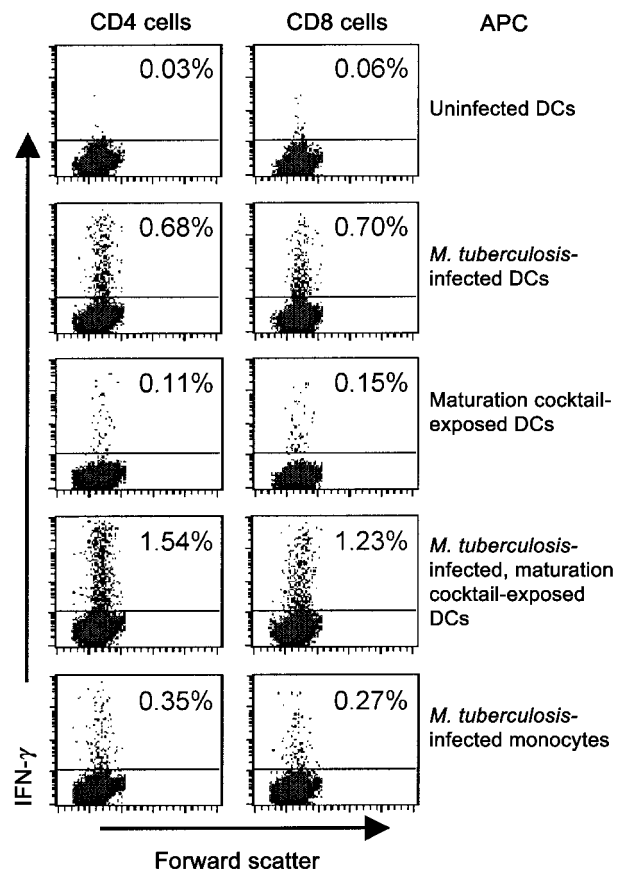


Figure 6. Effect of dendritic cell (DC) maturation on interferon- γ (IFN- γ) production by CD4⁺ or CD8⁺ lymphocytes, after 18 h of incubation with antigen-presenting cells (APC)—DCs or monocytes infected with *Mycobacterium tuberculosis* H37Rv-*gfp* and infected and uninfected DCs exposed to maturation cocktail of tumor necrosis factor- α , interleukin-1 β , and prostaglandin E₂—with autologous peripheral blood mononuclear cells, at a ratio of 1:40. Flow cytometric analysis of ~40,000 gated events, for either CD4⁺ lymphocytes or CD8⁺ lymphocytes, is shown. Results are representative of 5 experiments.

demonstrated whether infected DCs are compromised in their ability to activate autologous naive T cells, compared with that of DCs exposed to antigens known to activate naive T cells, in this setting.

We also examined the memory T cell response and observed that, at inducing autologous memory CD4⁺ and CD8⁺ T cell IFN- γ production, *M. tuberculosis*-infected DCs were more efficient than were *M. tuberculosis*-infected monocytes. This is not surprising, because antigen-pulsed DCs may even induce autologous memory T cell activation after fixation of the antigen-presenting cells (data not shown). Nevertheless, optimal activation of mycobacterial memory T cells occurred only when DCs were infected in the presence of the maturation cocktail. Together, these results suggest that the major functional consequence of limited phenotypic maturation of *M. tuberculosis*-infected DCs may be reduced naive T cell activation, whereas capacity to activate memory T cells is relatively preserved.

Our findings of the inhibition of functional antigen-presenting capacity of *M. tuberculosis*-infected DCs were again similar to the findings of the effects that *M. leprae*-infected DCs have on T cell activation [21]. *M. tuberculosis*-infected DCs were poor inducers of both autologous T cell lymphoproliferation and cytokine production, in blood from donors not previously exposed to mycobacteria. In contrast, Hashimoto et al. showed that DCs infected with avirulent mycobacteria (that is, *M. bovis* bacille Calmette-Guérin and *M. avium*) could efficiently induce T cell activation. Together, these findings suggest that virulent mycobacteria may inhibit DC function, whereas avirulent mycobacteria do not.

Inhibition of DC maturation, by virulent mycobacteria, such as *M. tuberculosis*, may therefore be a novel immune-evasion mechanism that is used by the pathogen to evade host immune responses. Incompletely matured DCs may not be able to activate T cell effector immunity efficiently in vivo. Furthermore, antigen-pulsed DCs that have remained immature or that are not in a fully activated mature state may have a tolerogenic effect in vivo, an effect resulting in suppression of preexisting T cell immunity [23–25]. This effect may be mediated through preferential activation of regulatory CD4⁺ or CD8⁺ T cells, which suppress activity of memory CD4⁺ and CD8⁺ T cells [26–29]. It remains to be demonstrated whether incompletely matured *M. tuberculosis*-infected DCs preferentially activate regulatory T cells and possibly suppress specific immunity in this manner.

Our findings have important implications for potential novel DC-based vaccination strategies. Delivering antigen-pulsed DCs to hosts or specifically targeting DCs in vivo are attractive vaccination strategies, because optimal immunity in a broad range of T cell subsets may be induced. In tuberculosis, multiple T cell subsets have been shown to be important in protective

immunity (reviewed in [3]). In mice, administration of mycobacteria-pulsed DCs has induced antimycobacterial immunity [30], which has resulted in protection against *M. tuberculosis* challenge similar to that afforded by subcutaneous bacille Calmette-Guérin vaccination [30]. If *M. tuberculosis* prevents optimal DC maturation, as we have shown, or if antigens of *M. tuberculosis* prevent maturation, as has been shown for lipoarabinomannan [31], this vaccination approach may not induce optimal T cell immunity. Therefore, further studies should focus on the mechanisms of inhibition of DC maturation, by *M. tuberculosis*, which may have important implications for these immunotherapeutic approaches. In a recent study, Geijtenbeek et al. [32] did suggest one such mechanism: Lipoarabinomannan from *M. bovis* bacille Calmette-Guérin specifically prevented DC maturation by targeting the surface C-type lectin DC-SIGN. As *M. tuberculosis* enters DCs by binding DC-SIGN [33], this pathogen may also inhibit DC maturation through this receptor.

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