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Infection of Human Macrophages and Dendritic Cells with *Mycobacterium tuberculosis* Induces a Differential Cytokine Gene Expression That Modulates T Cell Response¹

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Macrophages and dendritic cells (DC) play an essential role in the initiation and maintenance of immune response to pathogens. To analyze early interactions between *Mycobacterium tuberculosis* (Mtb) and immune cells, human peripheral blood monocyte-derived macrophages (MDM) and monocyte-derived dendritic cells (MDDC) were infected with Mtb. Both cells were found to internalize the mycobacteria, resulting in the activation of MDM and maturation of MDDC as reflected by enhanced expression of several surface Ags. After Mtb infection, the proinflammatory cytokines TNF- α , IL-1, and IL-6 were secreted mainly by MDM. As regards the production of IFN- γ -inducing cytokines, IL-12 and IFN- α , was seen almost exclusively from infected MDDC, while IL-18 was secreted preferentially by macrophages. Moreover, Mtb-infected MDM also produce the immunosuppressive cytokine IL-10. Because IL-10 is a potent inhibitor of IL-12 synthesis from activated human mononuclear cells, we assessed the inhibitory potential of this cytokine using soluble IL-10R. Neutralization of IL-10 restored IL-12 secretion from Mtb-infected MDM. In line with these findings, supernatants from Mtb-infected MDDC induced IFN- γ production by T cells and enhanced IL-18R expression, whereas supernatants from MDM failed to do that. Neutralization of IFN- α , IL-12, and IL-18 activity in Mtb-infected MDDC supernatants by specific Abs suggested that IL-12 and, to a lesser extent, IFN- α and IL-18 play a significant role in enhancing IFN- γ synthesis by T cells. During Mtb infection, macrophages and DC may have different roles: macrophages secrete proinflammatory cytokines and induce granulomatous inflammatory response, whereas DC are primarily involved in inducing anti-mycobacterial T cell immune response. *The Journal of Immunology*, 2001, 166: 7033–7041.

One-third of the world's population is infected with *Mycobacterium tuberculosis* (*M. tuberculosis* or Mtb).³ Most of the infected persons never develop active disease, indicating that generally the immune response keeps the infection under control. However, the increased incidence of tuberculosis over the last decade has made more urgent the need to delineate host factors that control susceptibility to tuberculosis.

Once inhaled, Mtb particles are readily phagocytosed, processed, and presented by alveolar macrophages (1). Initially, the establishment of a productive infection depends on the ability of the mycobacteria to invade the alveolar space and to survive within the macrophages. In contrast, infection of the macrophages by Mtb leads to the activation of multiple microbicidal mechanisms, including phagolysosome fusion and respiratory burst, and the production of proinflammatory cytokines, which limit the growth of ingested organisms and the recruitment and activation of additional leukocytes (2, 3).

While innate immune responses initially predominate, the subsequent recruitment of T lymphocytes to the lung is necessary to the containment of Mtb within granulomas, which consist of activated macrophages surrounded by T lymphocytes, fibroblasts, and epitheloid cells (4). The kinetics of production and the balance between proinflammatory (IL-1, IL-6, IL-12, and TNF- α) and inhibitory (IL-10 and TGF- β) cytokines secreted by mononuclear phagocytes after the exposure to microbial Ags regulate subsequent T cell responses and are also critical for the formation and maintenance of the granuloma. In turn, cytokines produced by T cells, such as IFN- γ , can activate monocyte and macrophages to become microbicidal. Therefore, the cytokine cross-talk between T cells and mononuclear phagocytes is essential for the final result of Mtb infection. Four potential outcomes of Mtb infection can occur according to the fate of the microorganism inside the macrophages. In fact, Mtb can be immediately eliminated, becomes dormant indefinitely inside the host, causes a primary tuberculosis, or reactivates many years after the primary infection.

Recent studies support the hypothesis that dendritic cells (DC) also strengthen the cellular immune response against mycobacterium infection (5–9). Even if the critical role of DC in the initiation of immune response has been established (10), their involvement in Mtb infection is poorly defined. DC are highly represented in sites of Mtb infection at the onset of the inflammatory response (11–13). Immature DC present in the lung mucosa are specialized for Ag up-take and processing. After interacting with pathogens, they mature and migrate in lymphoid organs where they prime T cells through the cell surface expression of MHC and costimulatory molecules and the secretion of immunoregulatory cytokines such as IL-12 (7, 10).

In this study, we have investigated the interactions of virulent Mtb H37Rv strain with human peripheral blood monocyte-derived macrophages (MDM) and monocyte-derived dendritic cells

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³ Abbreviations used in this paper: Mtb, *Mycobacterium tuberculosis*; DC, dendritic cells; MDM, monocyte-derived macrophages; MDDC, monocyte-derived dendritic cells; MOI, multiplicity of infection; RPA, RNase protection assay.

(MDDC). We have examined the modulation of host cell activation markers during the infection and analyzed the kinetics of cytokine gene expression from Mtb-infected cells focusing on their ability to stimulate IFN- γ production or enhance IL-18R expression on T cells.

Materials and Methods

Abs and other reagents

mAbs specific for CD1a, CD1b, CD14, CD11b, CD64, CD86, CD83, CD40, CD54, HLA-DR, HLA-DQ, CD58, CD80, mannose receptor, IgG1, IgG2a (BD PharMingen, San Diego, CA), and IL-18R (R&D Systems, Abingdom, U.K) were used as pure Abs or as direct conjugates to FITC or PE. Goat anti-mouse IgG F(ab')₂ FITC was used as secondary Ab where necessary. Anti-CD3 Ab (OKT3, 1 mg/ml; Ortho Diagnostics, Raritan, NJ) was used for precoating the plate wells for 1 h at 37°C. Following removal of unbound Ab, T cells were added. Neutralizing anti-IL-12 Ab (R&D Systems) and control IgG (BD PharMingen) were used at 20 μ g/ml after preincubation for 1 h at 37°C with supernatants to neutralize the IL-12 production. Neutralizing mouse monoclonal anti-IL-18 Ab was used at the concentration of 40 μ g/ml (Euroclone, Devon, U.K.) and rabbit polyclonal anti-IFN- α was used at 20 μ g/ml (PBL Biomedical Laboratories, New Brunswick, NJ). Recombinant human soluble IL-10R was purchased from R&D Systems and used at 5 μ g/ml after preincubation for 1 h at 37°C with the supernatants. IL-12 was obtained from R&D Systems. A concentration of 1 μ g/ml LPS from *Escherichia coli* 0111:B4 (Sigma, St. Louis, MO) was used to induce cytokine gene expression.

Monocytes, macrophages, DC, and T cells

PBMCs were isolated from freshly collected buffy coats obtained from healthy voluntary blood donors (Blood Bank of University "La Sapienza", Rome, Italy) by density gradient centrifugation using Lympholyte-H (Cederlane, Hornby, Ontario, Canada). Monocytes were purified by positive sorting using anti-CD14-conjugated magnetic microbeads (Miltenyi Biotec, Bergisch Gladsbach, Germany). The recovered cells were >99% CD14⁺ as determined by flow cytometry with anti-CD14 Ab. Macrophages were obtained by culturing adherent monocytes in six-well tissue culture plates (Costar, Cambridge, MA) with 0.1 ng/ml GM-CSF (Schering-Plough, Innishannon, Ireland) for 5 days at 0.5×10^6 cells/ml in RPMI 1640 (BioWhittaker Europe, Verviers, Belgium) supplemented with 2 mM L-glutamine and 15% FCS (BioWhittaker Europe). DC were generated by culturing adherent monocytes in six-well tissue culture plates (Costar) with 25 ng/ml GM-CSF and 1000 U/ml IL-4 (R&D Systems) for 5 days at 0.5×10^6 cells/ml in RPMI 1640 with supplements as above. No antibiotics were ever added to the cultures. After day 5 of culture, the cells were analyzed for the expression of surface markers associated with DC as well as macrophage differentiation. The resulting DC were 70–80% CD1a⁺ and 95% CD14⁺, while the macrophages were 80–90% CD14⁺.

T cells were purified by negative sorting using magnetic microbeads (Miltenyi Biotec). The recovered cells were >96% CD3⁺ as determined by cytometry with anti-CD3 Ab. Purified T cells were primarily stimulated with plate-bound anti-CD3 mAb and cultured for 5 days in the presence of 100 U/ml IL-2 (BD PharMingen) in RPMI 1640 supplemented with 10% FCS. IL-2-containing medium was removed from T cells 16 h before stimulation with MDM or MDDC supernatants.

Mtb and infection of MDM and MDDC

Mtb H37Rv (ATCC 27294; American Type Culture Collection, Manassas, VA) was grown with gentle agitation (80 rpm) in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI) supplemented with 0.05% Tween 80 (Sigma) and 10% Middlebrook oleic acid albumine dextrose catalase enrichment (Becton Dickinson, Sparks, MD). Logarithmically growing cultures were centrifuged at 800 rpm for 10 min to eliminate clumped mycobacteria and then washed three times in RPMI 1640. Mycobacteria were resuspended in RPMI 1640 containing 10% FCS and 10% glycerol and then stored at -80°C. Vials were thawed and bacterial viability was 90% as enumerated by CFU on Middlebrook 7H10 agar plates. All Mtb preparations were analyzed for LPS contamination by the *Limulus* lysate assay (BioWhittaker Europe) and contained <10 pg/ml LPS.

Bacterial suspensions, at a multiplicity of infection (MOI) from 0.1 to 10 Mtb/cell, were added on macrophages and DC, and, after 16 h of infection at 37°C, the cultures were gently washed (three times) with medium. Macrophages and DC were centrifuged at 800 rpm for 10 min to selectively spin down cells while extracellular bacteria remain in the supernatants. Cells were resuspended in RPMI 1640 supplemented with 2% FCS and cultured for the times indicated in each experiment.

CFU assay

Triplicate samples were assayed for CFU. Culture medium was removed and cells were lysed with water containing 0.06% SDS. Serial dilutions of the bacterial suspensions were plated (six replicates for each dilution) on Middlebrook 7H10 agar plates.

Acid-fast staining

The medium overlying the infected cells attached on coverslips (Nunc, Roskilde, Denmark) was gently aspirated. The monolayers were fixed in 2% formalin for 10 min, dried, and stained with the Kinyoun method (14). After drying and mounting, bacteria were observed by light microscopy. Duplicate monolayers were prepared for each experimental condition.

FACS analysis

Approximately $1-2 \times 10^5$ cells were aliquoted into tubes and washed once in PBS containing 2% FCS. The cells were incubated with purified mAbs at 4°C for 45 min. The cells were then washed and fixed overnight with 2% paraformaldehyde before analysis on a FACSCalibur using CellQuest software (Becton Dickinson, Mountain View, CA).

Cytokine determinations

Supernatants from control and Mtb-infected macrophage and DC cultures were harvested at different times after infection, filtered (0.2- μ m filters) and stored at -80°C. Ab pairs used in ELISA for IL-1 β , IL-6, IL-10, and TNF- α cytokine levels were obtained from R&D Systems. IL-12- and IFN- γ -specific ELISA kits were obtained from R&D Systems. IL-18 ELISA was obtained from Hayashibara Biochemical Laboratories (Fujisaki Institute, Okayama, Japan), and IFN- α ELISA was obtained from PBL Biomedical Laboratories. Supernatants from 6 to 10 separate experiments were considered. All ELISA were conducted according to manufacturers' instructions.

RNase protection assay (RPA)

RNA was extracted from MDM and MDDC with RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. A phenol/chloroform extraction was performed to inactivate residual mycobacterial particles. Then 5 μ g of each target RNA was analyzed by RPA using the hCK-2 multiprobe template set (Riboquant; BD PharMingen). Linearized DNA templates were used for T7-directed synthesis of ³²P-labeled riboprobes using [α -³²P]UTP (3000 Ci/mmol, 10 mCi/ml; Amersham Life Science, Amersham, U.K.). The probes were hybridized overnight and then digested with RNase T1 and RNase A to remove unhybridized probes and mRNAs. The protected probes were purified and electrophoresed on a 6% denaturing polyacrylamide gels. Bands were visualized by autoradiography (XAR film; Eastman Kodak, Rochester, NY).

Results

Infection of human macrophages and DC with Mtb

Initial studies were designed to determine the infectivity of Mtb in macrophages and DC. MDM and immature MDDC were generated from the same blood donors and were allowed to differentiate in culture for 5 days. Once their differentiated phenotypes were acquired, the cells were infected with increasing MOI, starting at 0.1, 1, and 10 per cell (Fig. 1).

The percentages of infected cells were measured 16 h after infection by acid-fast staining; they were $11\% \pm 2$, $45\% \pm 3$, and $69\% \pm 7$ for macrophages and $15\% \pm 3.5$, $38\% \pm 6.5$ and $85\% \pm 8$ for DC at MOI values of 0.1, 1, and 10, respectively, as measured by acid-fast staining (Fig. 1A). A clear difference was observed in the number of internalized bacteria, which was 2-fold higher in DC compared with macrophages (at MOI = 1 the DC harbored 3.1 ± 0.4 bacteria vs 1.4 ± 0.3 bacteria present in macrophages Fig. 1B).

Cell viability was evaluated both by phase-contrast light microscopic examination and trypan blue dye exclusion method. Infections of MDM and MDDC with a MOI of 0.1 and 1 apparently had no effect on cell viability during a 6-day follow-up period, whereas high cell mortality was seen in MDDC cultures infected with a MOI of 10 after 3 days. Moreover, the morphology attained by infected vs uninfected cultures was dependent on the bacterial

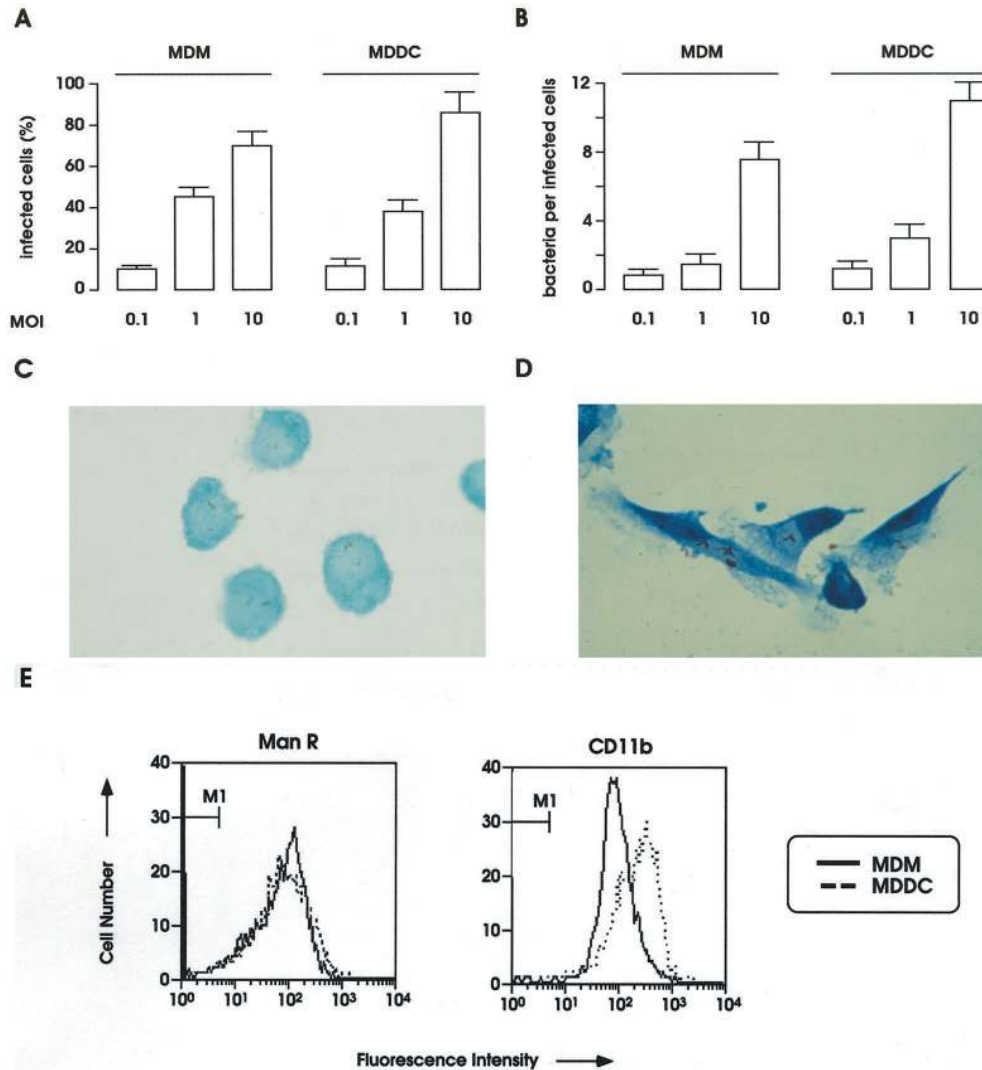


FIGURE 1. Infection of MDM and MDDC with Mtb. *A*, Human MDM and MDDC were infected with increasing MOI. After 16 h of infection at 37°C, cells were fixed and stained with the Kinyoun method. The percentage of infected cells (*A*) and the number of internalized bacteria (*B*) were determined. The results, obtained from 200 cells, are expressed as mean \pm SE of four independent experiments. Representative pictures of infected MDM (*C*) and MDDC (*D*) obtained by acid-fast staining are shown. Analysis of mannose receptor and CD11b cell surface expression is shown in *E*. Unstimulated and stimulated cells stained with a control Ab were contained in the M1 bar. Data are representative FACS profiles of one experiment, which was repeated three additional times, using macrophages and DC from a total of four different blood donors.

doses used. In fact, the morphology acquired by the infected macrophages with MOIs of 1 or 10 clearly showed cells firmly attached to the plastic surface (Fig. 1*C*). Likewise, the percentage of DC showing the typical morphology with extended fine dendrites increases when MOIs of 1 or 10 were used to infect the cells (Fig. 1*D*). We choose the infectious dose of MOI = 1 to perform the experiments, because it resulted in cellular activation and maturation without considerably inducing cell death.

To investigate the differential ability of DC vs macrophages to internalize mycobacteria, the cell surface expression of two markers involved in the receptor-mediated uptake (15), i.e., mannose receptor and CD11b, was analyzed (Fig. 1*E*). No differences were detected in the expression of mannose receptor, while the levels CD11b were higher in DC compared those present on the surface of macrophages.

Up-regulation of markers peculiar of activated macrophages and mature DC by Mtb infection

To analyze whether the effect of Mtb infection alters cell surface expression of markers involved in Ag presentation and T cell in-

teraction, MDM and MDDC were infected with Mtb and the cell surface expression of MHC class II DR and DQ, ICAM-1 (CD54), B7.1 (CD80) and B7.2 (CD86), CD40, Fc γ RI (CD64), LFA-3 (CD58), and CD83 was examined (Fig. 2).

Mtb-infected MDM showed enhanced expression of costimulatory and adhesion molecules CD40 and CD54, whereas a slight down-regulation of MHC class II DQ expression was seen (Fig. 2*A*). No changes in CD80, CD86, CD64, and MHC class II DR were observed. Conversely, a strong increase in CD83 expression as well as in costimulatory molecules CD40, CD80, CD86 and adhesion molecules CD58 and CD54 was observed in Mtb-infected MDDC (Fig. 2*B*). Contrary to MDM, MDDC showed a significant up-regulation of class II DR and DQ molecules, suggesting that Mtb-infected DC are likely to function as efficient APCs compared with Mtb-infected macrophages. These observations are in line with previous reports describing a diminished capacity for MHC class II-restricted Ag presentation of macrophages following Mtb infection (16, 17). Also, the low expression of CD1a and CD1b molecules on macrophages, as compared with

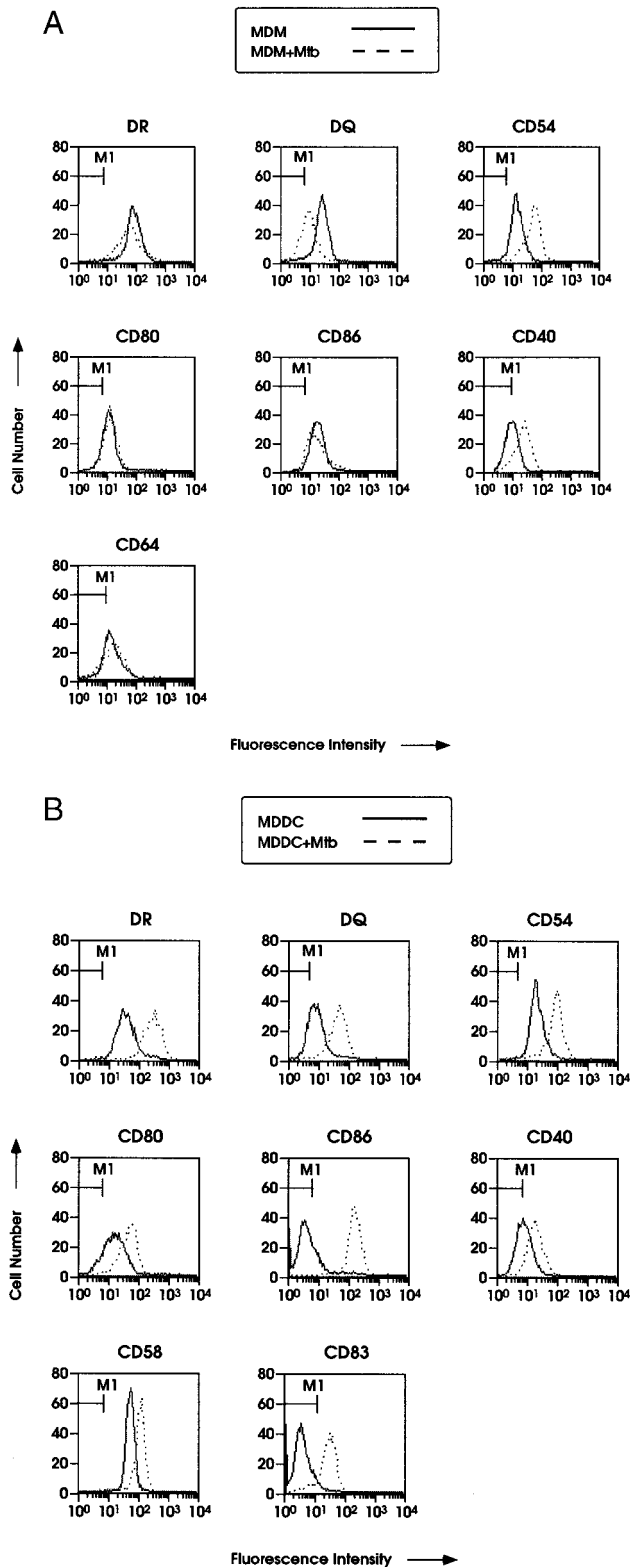


FIGURE 2. Analysis of cell surface phenotypes of MDM (A) and MDDC (B) infected with Mtb. Cells were infected with a MOI of 1 for 48 h, and the cell surface phenotypes were analyzed by FACS. A total of 5000 cells were analyzed per sample. Unstimulated and stimulated cells stained with a control Ab were contained in the M1 bar. These are representative FACS profiles of one experiment, which was repeated four additional times, using macrophages and DC from a total of five different blood donors.

the level present on DC, did not comply with CD1-restricted Mtb Ag presentation to T cells (data not shown).

Cytokine secretion from Mtb-infected macrophages and DC

Next we analyzed the kinetics and the profile of cytokine secretion from MDM and MDDC during Mtb infection. Cell culture supernatants were collected at different time points after the infection and cytokine levels were determined by ELISA (Fig. 3, A and B). MDM infected with Mtb showed enhanced production of IL-1 β , IL-6, IL-10, TNF- α , and IL-18. Some differences in the kinetics were seen. In fact, IL-1 β and IL-18 production was fast and evident already at 6–16 h after infection, while IL-6, IL-10 and TNF- α steadily increased up to 24 or 48 h after infection. A clearly different situation was observed in Mtb-infected MDDC, which produced low, but reproducible, levels of IL-12 p70 and significant amount of IFN- α , up to 400 pg/ml (equivalent to ~40 IU/ml) (Fig. 3B). In MOI = 1-infected MDDC, some IL-1 β , TNF- α , and IL-18 production was seen, whereas IL-6 and IL-10 secretion was not clearly detectable.

To investigate whether higher MOI would have an effect on the cytokine expression profile, different infectious doses were used to infect MDM and MDDC cultures. DC infected with a MOI of 10 produced slightly more cytokines (Fig. 4), but the relative cytokine expression profile remained similar to the one seen at a lower MOI value. Similarly, in macrophages the cytokine expression pattern was not markedly altered by a higher infectious dose of Mtb (MOI of 10) and no production of IL-12 and IFN- α was detected (Fig. 4). Thus, the inhibitory role of IL-10 on IL-12 production was evaluated. IL-12 production was restored in Mtb-infected macrophages when the effect of IL-10 was blocked by the addition of soluble IL-10R (Fig. 5). A 1.5-fold increase in IL-12 was also found in Mtb-infected MDDC, pointing to a low production of IL-10, probably undetectable by conventional ELISA (Fig. 5).

To further study whether the kinetics and the profile of cytokine secretion correlated with gene transcription, MDM and MDDC were collected at different times after Mtb infection or LPS treatment. Total cellular RNA was isolated, and cytokine gene expression was analyzed by RPA. LPS treatment was used as a positive control for cytokine gene induction. As expected, the different cytokine production patterns between MDM and MDDC were also detected at the mRNA level (Fig. 6). In particular, there was a clear correlation between the IL-12 p70 secretion from DC and the up-regulation of IL-12 p35 at the mRNA level, which was observed at 16 h after infection. IL-12 p40 mRNA expression, instead, was strongly induced as early as 8 h after exposure of DC to Mtb. Conversely, LPS-stimulated IL-12 p35 gene transcription both in MDM and MDDC. High-level expression of IL-1 β mRNA was detected in DC and especially in macrophages infected with Mtb, whereas IL-10 gene expression starting at 3 h after infection was only seen in macrophages. The expression of IL-1 α , IL-1Ra (receptor antagonist) and IL-6 was observed in higher levels in Mtb-infected MDM compared with MDDC (Fig. 6).

Stimulation of T cell IFN- γ production and IL-18R expression by cytokines secreted from macrophage and DC infected with Mtb

Next we investigated whether the cytokines produced by Mtb-infected macrophages and DC were able to induce IFN- γ production from T cells. For these experiments, MDM, MDDC, and T cells were obtained from the same blood donor. Purified T cells were initially stimulated with plate-bound anti-CD3 mAbs and cultured in presence of 100 IU/ml IL-2 for 5 days. IL-2 was removed from T cells 16 h before stimulation with supernatants from infected MDM and MDDC cultures collected at 24 h after infection. T cells were incubated for 16, 24, or 48 h and secreted IFN- γ levels were

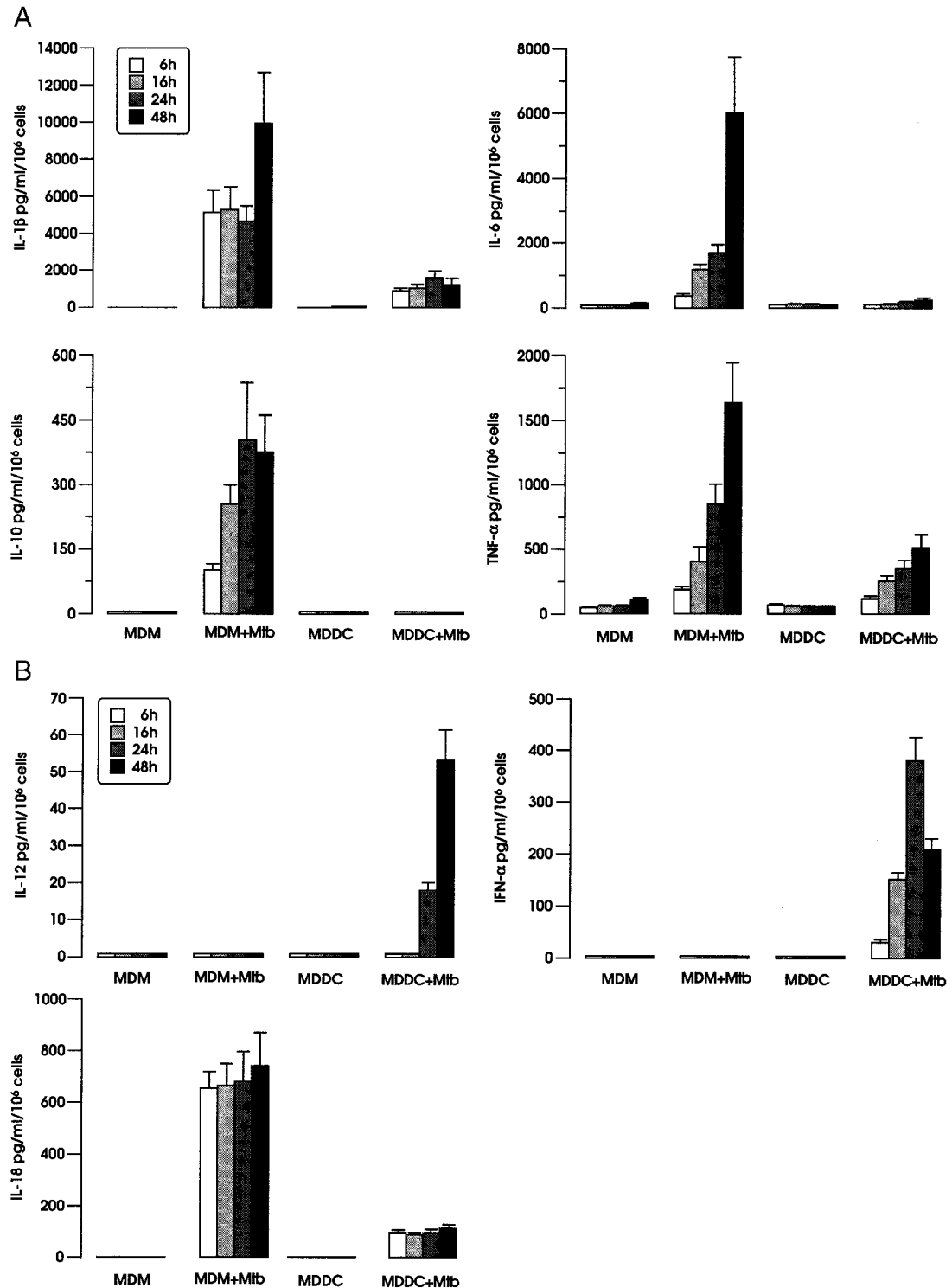


FIGURE 3. Kinetics of cytokine production following Mtb-infected MDM and MDCC. Cells were infected with Mtb at a MOI of 1, and supernatants were collected at different time points after infection and analyzed with specific ELISA for proinflammatory cytokines (A) and Th1/IFN- γ -inducing cytokines (B). The results represent the means \pm SE of 10 separate experiments.

analyzed by ELISA (Fig. 7A). As a control, T cells were also stimulated with IL-12 (20 ng/ml). Supernatants obtained from MDM culture were unable to stimulate IFN- γ production, whereas MDCC supernatants readily induced IFN- γ synthesis in T cells (Fig. 7A).

To characterize in more detail the role of Mtb-induced cytokines on IFN- γ gene expression, neutralization and immunodepletion experiments were conducted. Because IL-10 may down-regulate IFN- γ production (18), we tested whether soluble IL-10R affected

the capacity of Mtb-infected MDM supernatants to stimulate IFN- γ production (Fig. 7B). Pretreatment of MDM supernatants with soluble IL-10R leads to a clearly detectable increase in T cell IFN- γ secretion (Fig. 7B). To determine the role of IL-12, IFN- α , and IL-18 in the induction of IFN- γ gene expression, neutralizing anti-IFN- α , anti-IL-12, or anti-IL-18 Abs were used. Pretreatment of infected MDCC supernatants with anti-IL-12 Abs significantly down-regulated T cell IFN- γ production, while anti-IFN- α and

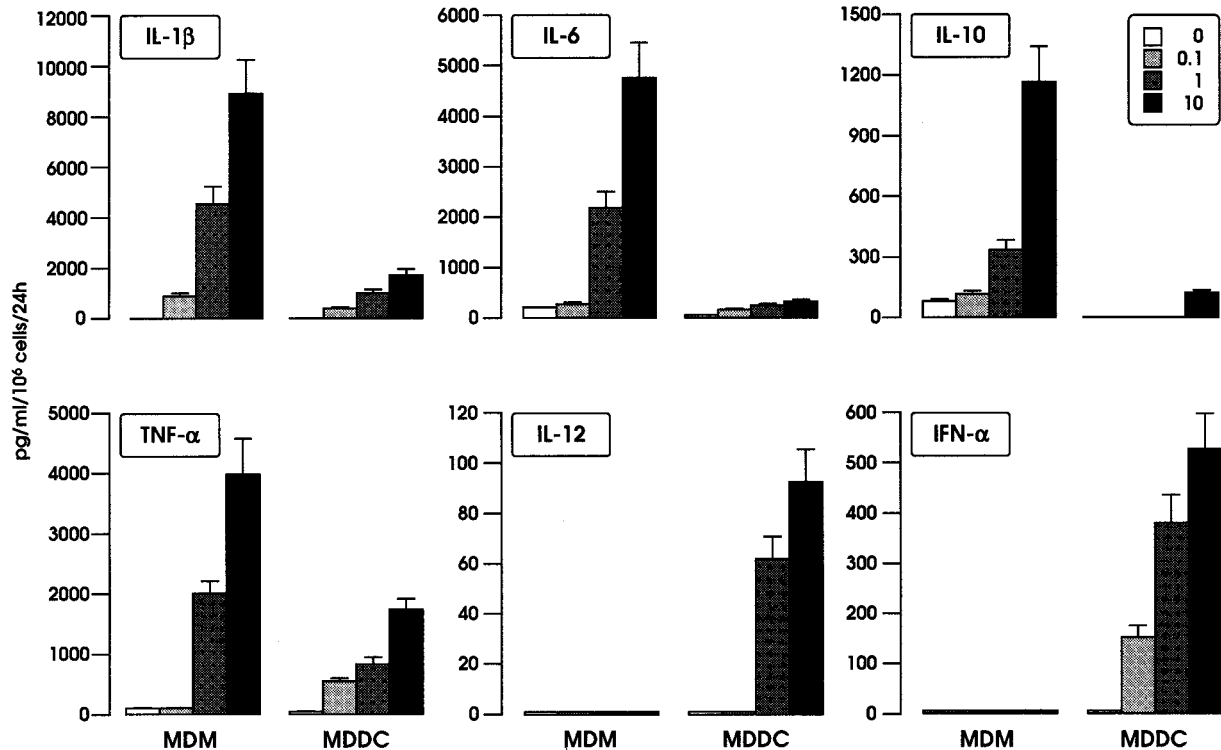


FIGURE 4. Effects of increasing MOI on cytokine production from MDM and MDDC. Cells were infected with different MOI of *Mtb* and supernatants were collected after 24 h of infection and analyzed with specific ELISA. The results represent the means \pm SE of six independent experiments.

anti-IL-18 Abs exerted a less pronounced, but clearly detectable reduction of IFN- γ secretion (Fig. 7C).

In addition of enhancing IFN- γ production, IFN- α and IL-12 may stimulate a Th1-type response by inducing the expression of IL-18R (19). Therefore, we tested whether supernatants from *Mtb*-infected MDM and MDDC would also stimulate T cell IL-18R expression. Infected DC supernatants as well as IL-12 were able to enhance the expression IL-18R on the T cell surface as examined by FACS analysis (Fig. 8). Treatment of *Mtb*-infected MDDC supernatant with anti-IL-12 and anti-IFN- α Abs reduced, at different extent, the number of T cells expressing IL-18R. Supernatant from infected MDM was a poor inducer of IL-18R, but after pretreatment with soluble IL-10R the supernatant gained some ability to stimulate expression of IL-18R on the surface of T cells.

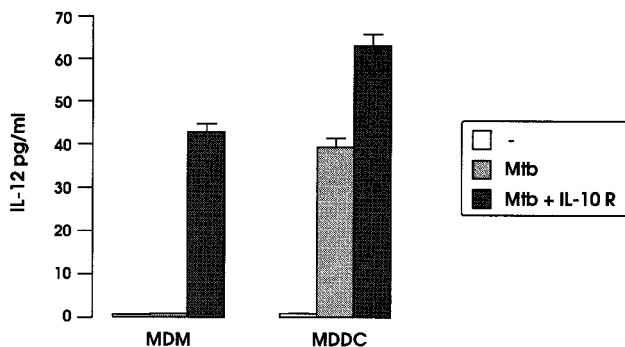


FIGURE 5. Effect of IL-10 on IL-12 production from *Mtb*-infected MDM. MDDC and MDM cultures were treated for 4 h with soluble IL-10R before infection with *Mtb*. Supernatants were collected 24 h after infection, and IL-12 levels were determined by ELISA. The results represent the means \pm SE of six independent experiments.

Discussion

Clinical and experimental data demonstrate that both innate and acquired immunity are involved in the protection to *Mtb* infection

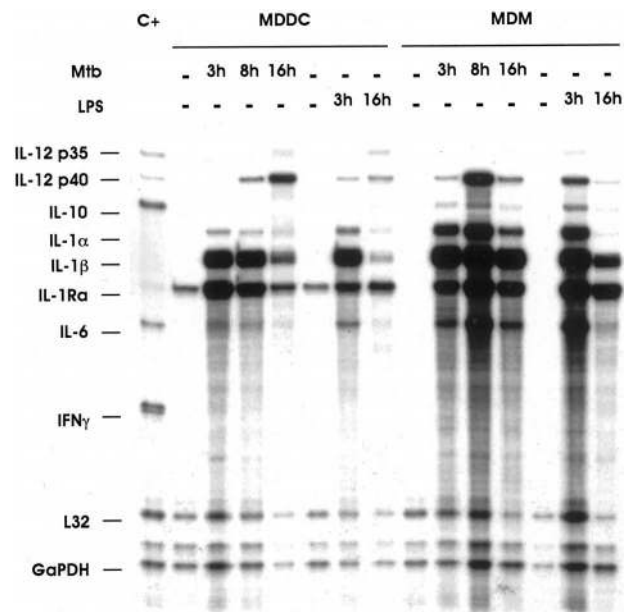


FIGURE 6. Kinetics of cytokine mRNA expression in macrophages and DC infected with *Mtb*. MDDC and MDM were collected at different times after the infection. LPS treatment (1 μ g/ml) was used as inducer for cytokine expression. Total cellular RNA (5 μ g) was isolated and analyzed by RPA. C+ is an internal positive control of the RPA kit. Data are representative of one RPA experiment, which was repeated an additional three times with RNA extracted from different MDM and MDDC cultures infected with *Mtb* or treated with LPS.

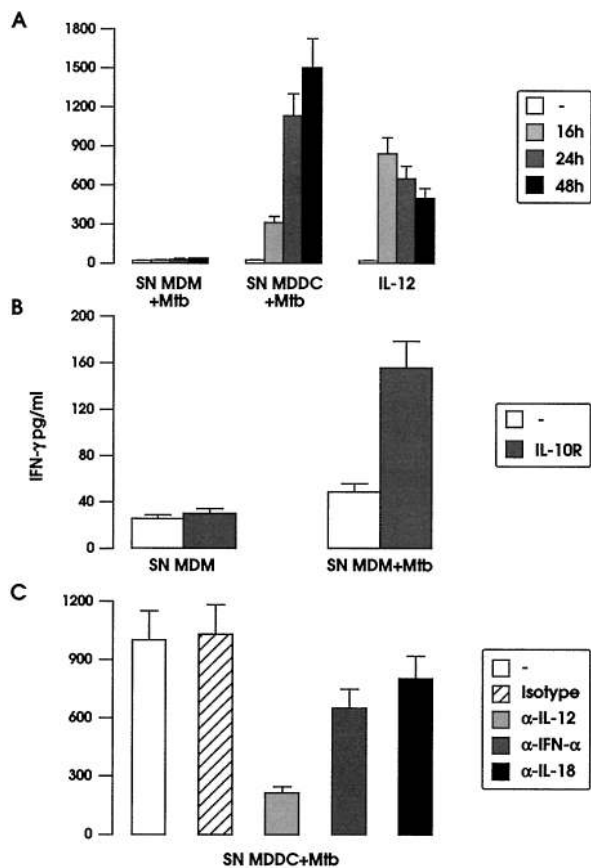


FIGURE 7. Regulation of IFN- γ production by cytokines secreted by Mtb-infected MDM and MDDC. *A*, IFN- γ production in human T cells stimulated for 24 h with supernatants harvested from MDM and MDDC infected with Mtb at different time points. T cells stimulated with human IL-12 (20 ng/ml) were used as a control. *B*, Neutralization of IL-10 secreted from infected macrophages on IFN- γ production from T cells treated for 24 h with the indicated supernatants. *C*, The effect of neutralizing anti-IFN- α , anti-IL-12, and anti-IL-18 Abs on IFN- γ production from T cells treated for 24 h with the indicated supernatants. The results represent the means \pm SE of six independent experiments.

(20). A complex series of interactions between various cell populations controls and contains the Mtb infection as well as prevents from reactivation (21). NK cells, $\gamma\delta$ T lymphocytes, and $\alpha\beta$ T lymphocytes of CD4 and CD8 phenotype are recruited in a sequential order after Mtb infection. All these cells share potential

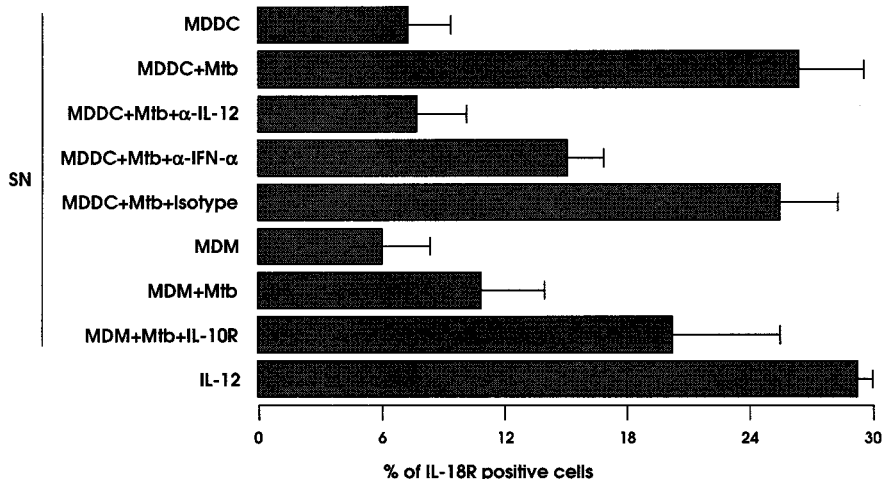
cytolytic activity and are able to produce IFN- γ , which plays a central role in the host defense against the Mtb. Activation of these cell subsets is primarily regulated by cytokine production and presentation of Mtb Ags by infected macrophages (3, 21). Characterization of some human severe immunodeficiencies has highlighted an essential role of IL-12 and IFN- γ in the control of Mtb (22–26). Therefore, studies committed to investigate the balance existing in the granulomatous response between mononuclear phagocytes and T cells are essential to understand the changes leading to the dissemination of mycobacteria and disease or to the reactivation of latent infection.

To investigate the effects of the initial interactions between Mtb and macrophages or DC on the profile of secreted cytokines, we used in vitro-cultured human immature MDDC and MDM. Both cell types took up the Mtb although MDDC appeared to be more active than MDM to internalize bacteria probably through CD11b-mediated uptake (Fig. 1). The invasion of DC may be advantageous for intracellular mycobacteria because it may allow their multiplication and spreading into draining lymph nodes and lungs.

Moreover, we extended our analysis on the expression of cell surface markers (Fig. 2). DC infected with Mtb expressed high levels of costimulatory and adhesion molecules, while macrophages exhibited only a considerable induction of CD40 and CD54 following Mtb infection. Moreover, a significant increase of MHC class II DR and DQ was observed in MDDC while the constitutive expression of MHC class II molecules was slightly down-modulated in infected MDM as previously reported (16). Conversely, the up-regulation of these surface markers in infected DC underlines the capacity of DC to mature following Mtb infection, which correlates with the acquired ability to present Ag to T lymphocytes. Thus, our results suggest that while Mtb infection results in the direct activation and maturation of DC followed by enhanced presentation of Ag and capacity to stimulate T cells (7), it impairs the ability of macrophages to process and/or present soluble Ag and in turn, to serve as accessory cells in T cell activation.

The production of proinflammatory cytokines is essential for host resistance against Mtb infection. TNF- α production is an important early event that leads to granuloma formation and a protective host immune response (27, 28). Macrophage-derived IL-1 enhances IL-2 production, IL-2R expression, and subsequent clonal expansion of the CD4⁺ T cells (3). IL-6 has also been suggested to be a pivotal proinflammatory cytokine during acute infection (29). It has been recently found that IL-18, another proinflammatory cytokine that enhances innate and specific Th1 immune response (30), is important for the generation of protective

FIGURE 8. Induction of IL-18R on T cells. Human T cells were stimulated for 24 h with supernatants obtained from MDM and MDDC infected with Mtb or with IL-12 (20 ng/ml). The neutralization of IL-10, IFN- α , and IL-12 present in the supernatants of Mtb-infected cultures was performed, and the effect on IL-18R expression on T cells was analyzed. The percentage of cells expressing IL-18R was evaluated by FACS analysis, which was repeated four additional times, using T cells from a total of five different blood donors.



immunity to mycobacteria (31, 32). The differentiation process of T cells is generally initiated by triggering the Ag receptor and is directed by cytokines present at the time of priming (33). The expression of inflammatory and immunoregulatory cytokines was therefore analyzed in supernatants obtained from MDM and MDDC infected with Mtb. Proinflammatory cytokines TNF- α , IL-1, IL-6, and IL-18 were secreted rapidly at high levels and in a sustained fashion, preferentially by Mtb-infected MDM, whereas MDDC produced low or undetectable levels of these cytokines (Fig. 3). However, when MOIs of 10 were used to infect MDDC, low, but reproducible, levels of TNF- α , IL-1, IL-6 were secreted, suggesting that a stronger stimulus is required to induce the expression of these inflammatory cytokines (Fig. 4). This suggests that macrophages and DC respond to Mtb in a different fashion. A different mechanism is instead responsible for the absence of IL-18 production from infected DC. It is likely that caspase activation, which is a prerequisite for the processing and secretion of IL-18 (30), was not taking place in DC. In fact, it has been recently described that the precursor form of IL-18 is constitutively produced by DC although the secretion of the biologically active form requires CD40 engagement of DC (34). Similarly, we observed a clear induction of IL-1 β mRNA in Mtb-infected MDDC at all examined time points, whereas a modest secretion of mature IL-1 β protein was seen. Whether this is due to differential ability of Mtb to induce caspase activation in macrophages and DC is presently not known.

Mtb-infected macrophages produce the immunosuppressive cytokine IL-10. IL-10 has been shown to inhibit the activation of macrophages (18, 35, 36) and more recently the differentiation of DC (37, 38). It is thus likely that high IL-10 production levels in Mtb-infected macrophages plays an antiinflammatory role through the inhibition of IL-12 expression (39–41) as well as the inhibition of the MHC class II transport to the cell membrane (42). In line with this concept is that the neutralization of IL-10 rescued IL-12 production from Mtb-infected macrophages (Fig. 6); however, we cannot exclude that other mechanisms could suppress IL-12 synthesis in Mtb-infected macrophages. Some increase of IL-12 synthesis was also observed in Mtb-infected MDDC (Fig. 6), suggesting a low level of IL-10 synthesis in DC as well. However, despite low IL-10 production, MDDC were able to produce high levels of IL-12 compared with MDM.

IL-10 also inhibits IFN- γ production and Ag-specific proliferation of Th1 (43). The hypothesis that IL-10 secretion from infected macrophages may down-modulate the T cell responses was confirmed by adding soluble IL-10R to supernatants obtained from Mtb-infected-MDM (Fig. 8). The presence of IL-18 in the supernatants of infected macrophages was not sufficient to stimulate IFN- γ production from T cells despite the neutralization of IL-10. In fact, it has been described that IL-18 is a weak inducer of IFN- γ synthesis from T cells without the cooperation of IL-12 and IFN- α (44). Therefore, the present findings of impaired ability of Mtb-infected MDM to stimulate T cells suggests a possible mechanism by which mycobacteria may evade immune recognition through the reduced expression of MHC class II molecules and the increased IL-10 production by infected macrophages.

A typical pattern of Th1/IFN- γ -inducing cytokine production was produced by DC after Mtb infection. The contact between Mtb and MDDC resulted in an elevated expression of IL-12 and IFN- α . This observation is in line with the results obtained in vivo with human subjects demonstrating enhanced IL-12 expression in skin lesions of patients with tuberculoid leprosy and in tuberculous pleuritis (45). Moreover, our findings in DC are in agreement with the recent observations by Mohagheghpour et al. who showed that *Mycobacterium avium*-infected DC secreted larger amounts of

IL-12 than infected macrophages (9). Few in vitro studies have examined the ability and mechanisms of Mtb to directly stimulate the production of the bioactive p70 IL-12 in human monocytes (46, 47) or in DC (7). In fact, the presence of activated T lymphocytes that produce IFN- γ or express CD40 ligand is generally required to obtain the expression of p70 IL-12 from macrophages or DC.

IFN- α production following Mtb infection of DC is a novel finding. In fact, IFNs were originally identified as cytokines that mediate antiviral immunity, but were also found to mediate a protective role against bacterial infections (48, 49). Our results about IFN- α production from Mtb-infected MDDC are consistent with the data of Cella et al., who found plasmacytoid DC produce type I IFN in mycobacteria-infected lymph nodes (50). It has been also shown that Mtb infection leads to secretion of type I IFN from THP-1 cells (48). All together these observations indicate that the production of IFN type I could play a dual role in Mtb infection by promoting both Th1 and DC differentiation (51–54).

Next, we examined the effects of the cytokines produced by infected MDM and MDDC on T cell stimulation measured by IFN- γ release, a parameter that is indicative of a favorable outcome of tuberculosis (Fig. 7). Supernatants obtained from MDM culture were unable to stimulate IFN- γ production, whereas MDDC supernatants readily induced IFN- γ synthesis in T cells (Fig. 7A). Similar effects were observed when MDM and MDDC supernatants were used to study the expression of IL-18R (Fig. 8). Immunodepletion of IL-12 produced in infected DC significantly down-regulated IFN- γ synthesis and IL-18R expression in T cells, whereas the anti-IFN- α and anti-IL-18 Abs exerted less pronounced effects, but consistent reduction of both IFN- γ secretion and IL-18R expression, indicating that both cytokines are inducers of Th1 cell response. These observations are in line with recent data indicating that IL-12, IL-18, and IFN- α have a significant role in enhancing Th1 immune response by inducing T cell IFN- γ production and the expression of Th1-type cytokine receptors (19, 44, 51, 55).

In the present study, we have demonstrated that human macrophages and DC are infected by Mtb and these cell types have a unique way to respond to the infection. Both cell types showed a differential expression of some cellular adhesion molecules and activation markers following Mtb infection, in particular MHC class II gene expression that resulted up-regulated in DC but down-modulated in macrophages. Moreover, macrophages readily produced proinflammatory cytokines and IL-10 in response to mycobacteria infection, whereas DC failed to produce these cytokines in significant amounts and instead released Th1/IFN- γ -inducing cytokines IL-12 and IFN- α . These features also correlate with the different localizations of activated cells, in particular the infected DC migrate to lymphoid organs where they liaise with and activate Ag-specific T cells while macrophages are inside the granuloma and are involved in the establishment of inflammation. Thus, the results suggest that macrophages and DC clearly have a different role in Mtb infection. DC are engaged in inducing T cells in virtue of their production of Th1/IFN- γ -inducing cytokines and expression of costimulatory molecules while macrophages are primarily involved in the formation of the granuloma where tissue macrophages harboring tubercle bacilli are surrounded by and interact with effector T lymphocytes. Thus, the development of a new generation of vaccine against tuberculosis has to elicit a strong activation of DC to stimulate the maximal Ag presentation, the production of IFN- α and IL-12 cytokines, and consequently a protective T cell response.

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