IFN-$\alpha\beta$ Released by *Mycobacterium tuberculosis*-Infected Human Dendritic Cells Induces the Expression of CXCL10: Selective Recruitment of NK and Activated T Cells[1]

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We recently reported that dendritic cells (DC) infected with *Mycobacterium tuberculosis* (Mtb) produce Th1/IFN-γ-inducing cytokines, IFN-$\alpha\beta$ and IL-12. In the present article, we show that maturing Mtb-infected DC express high levels of CCR7 and they become responsive to its ligand CCL21. Conversely, CCR5 expression was rapidly lost from the cell surface following Mtb infection. High levels of CCL3 and CCL4 were produced within 8 h after infection, which is likely to account for the observed CCR5 down-modulation on Mtb-infected DC. In addition, Mtb infection stimulated the secretion of CXCL9 and CXCL10. Interestingly, the synthesis of CXCL10 was mainly dependent on the Mtb-induced production of IFN-$\alpha\beta$. Indeed, IFN-$\alpha\beta$ neutralization down-regulated *CXCL10* expression, whereas the expression of *CXCL9* appeared to be unaffected. The chemotactic activity of the Mtb-infected DC supernatants was evaluated by migration assays using activated NK, $CD4^+$, and $CD8^+$ cells that expressed both CCR5 and CXCR3. Mtb-induced expression of *CCL3*, *CCL4*, *CXCL9*, and *CXCL10* was involved in the stimulation of NK and T cell migration. In accordance with the data on the IFN-$\alpha\beta$-induced expression of *CXCL10*, neutralization of IFN-$\alpha\beta$ significantly reduced the chemotactic activity of the supernatant from Mtb-infected DC. This indicates that IFN-$\alpha\beta$ may modulate the immune response through the expression of *CXCL10*, which along with CXCL9, CCL3, and CCL4 participates in the recruitment and selective homing of activated/effector cells, which are known to accumulate at the site of Mtb infection and take part in the formation of the granulomas. ***The Journal of Immunology*, 2003, 170: 1174–1182.**

Tuberculosis (TB)[4] is one of the major causes of death throughout the world, since approximately one-third of the world's population is infected with *Mycobacterium tuberculosis* (Mtb). Most of the infected persons never develop an active disease, indicating that in most cases the host immune response keeps the infection under control. However, the increasing incidence of TB over the last decade has made the need to delineate host factors that control the individual susceptibility to TB more urgent (1).

Although innate immunity initially predominates in anti-Mtb responses, the subsequent recruitment of T lymphocytes to the lung is necessary for the containment of Mtb within granulomas, which consist of activated macrophages surrounded by T lymphocytes, fibroblasts, and epitheloid cells (2). A complex series of interactions between different cell populations controls Mtb infection and prevents the disease from being reactivated. NK cells, $\gamma\delta$ T lymphocytes, and $\alpha\beta$ T lymphocytes of $CD4^+$ and $CD8^+$ phenotypes are recruited in sequential order into the site of Mtb infection through a gradient of specific chemokines (1, 3).

The human chemokine system comprises more than 50 chemokines and 18 chemokine receptors (4). Chemokines are divided into four families according to the N-terminal cysteine positioning in their amino acid sequence (C, CC, CXC, CX_3C subtypes) or into two categories on the basis of their physiological features (inducible and constitutive). Chemokines induce changes in target cell membrane protein composition and cell architecture, thus enhancing chemotaxis and penetration of leukocytes through the vascular endothelium into the inflammed tissues. On the target cells, chemokines bind to seven transmembrane domain receptors coupled to G protein and downstream of effector molecules (5). Chemokine receptor expression varies among different leukocyte populations; therefore, different leukocyte types can be specifically recruited to the site of inflammation in response to local chemokine milieu (5). It has been shown that CCL3, CCL4, CCL5, and CXCL8 are released by human alveolar macrophages upon infection with Mtb in vitro (6), whereas CXCL8 and CXCL1 are produced by Mtb-infected polymorphonuclear granulocytes (7). Moreover, increased chemokine production has been observed in monocytes, lymph node-derived cells, mesothelial cells, and bronchoalveolar lavage fluid from pulmonary TB patients compared to the production in healthy individuals (6, 8–10). It has been reported that Mtb-induced chemokine production from macrophages is regulated by a complex interplay between host cell surface molecules (CD14, mannose receptor, and Toll-like receptors) and mycobacterial membrane or cell wall components (1, 11). Conversely, chemokines released from Mtb-infected dendritic cells (DC) have been investigated to a limited extent. DC are highly represented in sites of Mtb infection at the onset of the inflammatory response (12–14). After interacting with the bacteria, DC mature in the lung mucosa

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[4] Abbreviations used in this paper: TB, tuberculosis; Mtb, *Mycobacterium tuberculosis*; DC, dendritic cell; RPA, RNase protection assay; MOI, multiplicity of infection.

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and, before their migration out of the inflamed tissues, they produce CCL3, CCL4, and CCL5, which further recruit immature DC to the site of infection and replace the mobilized population.

IFN were originally identified on the basis of their ability to induce cellular resistance to viral infections (15). IFN have been divided into two classes (types I and II) on the basis of their structure, function, and cellular origin. Although type I IFN (IFN-$\alpha\beta$) can be secreted by virtually all virus-infected cells, type II IFN (IFN-γ) is mainly produced as a result of stimulation of T lymphocytes and NK cells (15). Recent studies have highlighted the production of type I IFN following bacterial infections (16, 17). THP-1 cells or human DC have been reported to be able to produce type I IFN in response to Mtb infection (17–19). The production of type I IFN in infected cells is a central event in innate immunity, since these cytokines control the proliferation, differentiation, activation, and maturation of different leukocytic populations, such as DC, NK, Th1, and memory $CD8^+$ lymphocytes (20–24). Thus, one important effect of type I IFN is its ability to stimulate NK and T cell IFN-γ production (25, 26), which then activates the development of cell-mediated immune response (21). Interestingly, it has been shown that both IFN-$\alpha\beta$ and -γ may induce the expression of certain chemokine genes, such as those of *CXCL9*, *CXCL10*, and *CXCL11* (27, 28). These chemokines can specifically attract $CXCR3^+$ activated T cells into the inflamed tissues (27).

Given the role played by chemokines in the recruitment and selective homing of activated/effector cells into the site of Mtb infection to form the granuloma, we sought to characterize the profile of chemokines produced by human DC following Mtb infection. In this study, we have examined the expression of chemokines and chemokine receptors during the infection of DC and analyzed the role of Mtb-induced chemokines in their ability to attract activated NK, $CD4^+$, and $CD8^+$ cells. Interestingly, we observed that the expression of *CXCL10* was mainly dependent on the Mtb-induced type I IFN, as neutralization of IFN-$\alpha\beta$ downregulated the expression of this chemokine gene. Thus, our results indicate that IFN-$\alpha\beta$ may modulate, in an autocrine and paracrine fashion, the expression of *CXCL10* and, in turn, influence the chemotactic activity of Mtb-infected DC at the sites of Th1-type inflammation.

Materials and Methods

Abs and other reagents

mAbs specific for CD1a, CD3, CD4, CD8, CD14, CD16, CD56, CD83, CCR3, CCR5, CCR7, and CXCR3 as well as IgG1, IgG2a, and IgG2b (BD PharMingen, San Diego, CA) 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. Where indicated, IFN-α2 (a generous gift from R. Pine, Public Health Research Institute, Newark, NJ) was used at a concentration of 1000 U/ml. Rabbit polyclonal antiserum raised against IFN-α and -β was used at 20 μg/ml (PBL Biomedical Laboratories, New Brunswick, NJ). LPS from *Escherichia coli* 0111:B4 (Sigma-Aldrich, St. Louis, MO) was used at a concentration of 1 μg/ml to induce DC maturation.

DC and Mtb infection

DC were prepared as previously described (18). Briefly, PBMC were isolated from freshly collected buffy coats obtained from healthy voluntary blood donors (Blood Bank of the University "La Sapienza", Rome, Italy) by density gradient centrifugation using Lympholyte-H (Cedarlane Laboratories, Hornby, Ontario, Canada). Monocytes were purified by positive sorting using anti-CD14-conjugated magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The recovered cells were >99% $CD14^+$ as determined by flow cytometry with anti-CD14 Ab. DC were generated by culturing monocytes in six-well tissue culture plates (Costar, Cambridge, MA) with 25 ng/ml GM-CSF and 1000 U/ml IL-4 (R&D Systems, Abingdom, U.K.) 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). No antibiotics were added to the cultures. At day 5, the cells were 70–80% $CD1a^+$ and 95% $CD14^-$. DC were infected with Mtb at a multiplicity of infection (MOI) of 5 Mtb/cell as previously described (18).

Mtb H37Rv (ATCC 27294) was grown with gentle agitation (80 rpm) in Middlebrook 7H9 broth (Difco, Detroit, MI) supplemented with 0.05% Tween 80 (Sigma-Aldrich) and 10% Middlebrook oleic acid albumin dextrose catalase enrichment (BD Biosciences, 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) and contained <10 pg/ml LPS.

NK, $CD4^+$, and $CD8^+$ T cells

NK cells were obtained by coculturing non-nylon adherent human PBMC from buffy coats (4×10^5 cells/ml) with irradiated (4000 rad) Epstein-Barr virus-transformed B cell line RPMI 8866 (1×10^5 cells/ml) for 10 days, as previously described (29). On day 10, NK cells were purified by depletion with anti-CD3, anti-CD4-, anti-CD8-, anti-CD20-, and anti-CD14-conjugated magnetic microbeads (Dynal Biotech, Oslo, Norway). The resulting cell population was >95% pure, as assessed by flow cytometric analysis using anti-CD56 and anti-CD16 Abs.

Human neonatal leukocytes were isolated from freshly collected, heparinized, neonatal blood by Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation. T cells were purified by negative sorting using pan T magnetic microbeads (Miltenyi Biotec). The recovered cells were >96% $CD3^+$ as determined by flow cytometry with anti-CD3 Ab. Cells were stimulated with 2 μg/ml PHA (Wellcome, Beckenham, U.K.) in the presence of 20 ng/ml IL-12 (Hoffmann-LaRoche, Nutley, NJ) and 200 ng/ml neutralizing anti-IL-4 Abs (18500D; BD PharMingen) for Th1/Tc1 cultures. Cells were washed on day 3 and expanded in complete RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 U/ml IL-2 (Hoffmann-LaRoche). $CD4^+$ and $CD8^+$ cells were purified by negative selection using anti-CD8 and anti-CD4 microbeads, respectively, according to a protocol supplied by the manufacturer (Miltenyi Biotec). $CD4^+$ and $CD8^+$ cells were analyzed for chemokine receptor expression and chemotaxis between days 12 and 16.

FACS analysis and intracellular cytokine staining

Cells (1×10^5) 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 (BD Biosciences).

T cells were stimulated with 10^{-7} PMA plus 1 μg/ml ionomycin (Sigma-Aldrich) for 4 h. Brefeldin A (10 μg/ml; Sigma-Aldrich) was added during the last 2 h. Then the cells were fixed with 4% paraformaldehyde and permeabilized with saponin (Cytofix/Cytoperm kit; BD PharMingen). Fixed cells were stained with anti-human IFN-γ-FITC (BD PharMingen) and anti-human IL-4-PE (BD PharMingen) as recommended by the manufacturer and analyzed on a FACSCalibur using CellQuest software (BD Biosciences).

Chemokine detection

Supernatants from control and Mtb-infected DC cultures were harvested at different times after infection, filtered (0.2-μm filters), and stored at −80°C. Ab pairs used in ELISA for CCL4, CCL3, and CXCL10 were obtained from R&D Systems. CXCL9 was analyzed with Ab pairs and standards obtained from BD PharMingen. Supernatants from 6 to 10 separate experiments were analyzed. All ELISA assays were carried out according to the manufacturers' instructions.

Chemotaxis assay

NK and T cell chemotaxis assays were performed using 5-μm pore polycarbonate filters of a 24-well Transwell chamber (Costar). NK and T cells were washed with RPMI 1640 containing 0.5% BSA (Sigma-Aldrich) and resuspended at 2×10^6/ml. A dilution of 1/10 of the culture supernatants (final volume, 600 μl) from control and Mtb-infected DC cultures was added to the bottom chamber of the Transwell while 2×10^5 cells were added to the top chamber (input cells). The plates were then incubated for 3 h at 37°C. To block CXCL10 and CXCL9 binding to CXCR3 receptor, the cells were preincubated with a saturating dose of CXCL10 (10 μg/ml; PeproTech, London, U.K.) for 1 h at 37°C before transmigration assays.

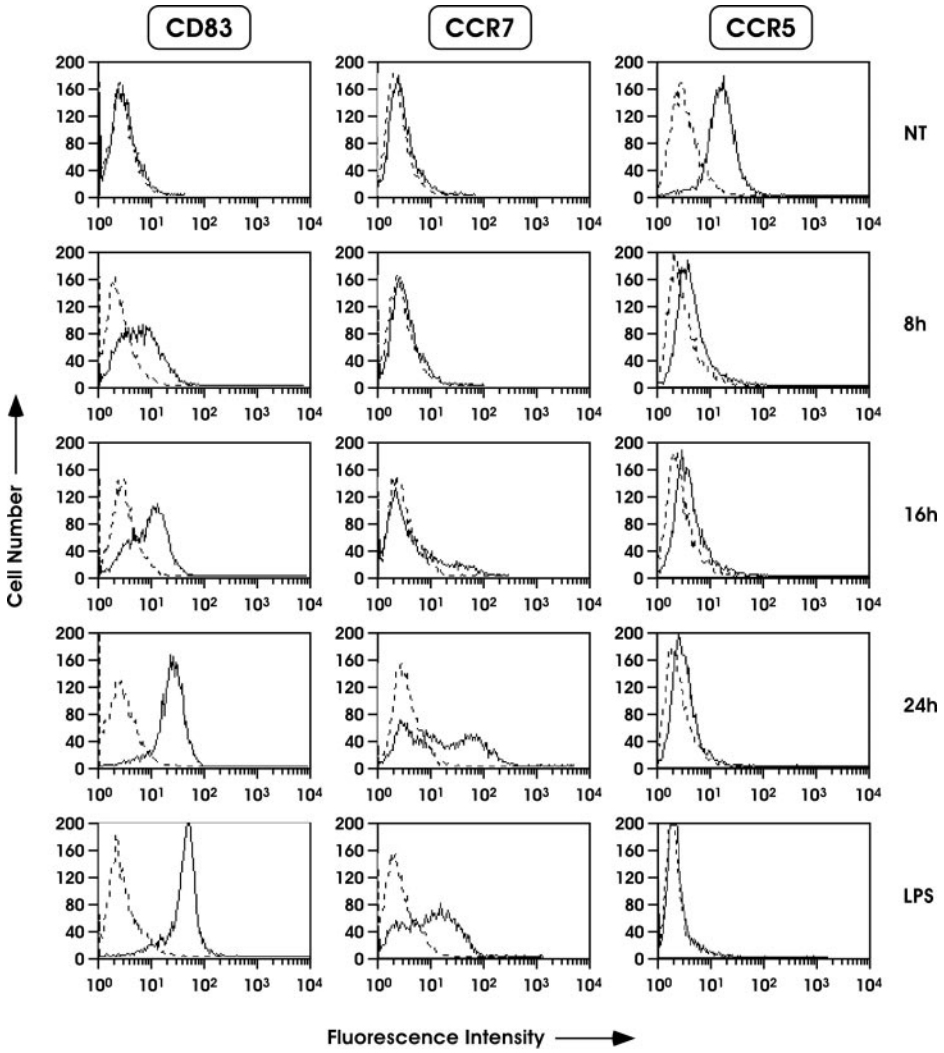


FIGURE 1. Analysis of chemokine receptor expression on DC infected with Mtb. Cells were infected with Mtb at a MOI of 5 and the expression of CCR5, CCR7, and CD83 was analyzed by FACS at different time points after infection. DC treated with LPS for 24 h were used as a control for DC maturation. A total of 5000 cells were analyzed per sample. Unstimulated and stimulated cells stained with a control Ab are represented by the dashed line. Representative FACS profiles of one experiment, which was repeated an additional four times using DC from a total of five different blood donors, are shown. NT, Not treated.

The migrated cells were counted with a flow cytometry by 1-min acquisition at a flow rate of 100 μl/min with a continuous mixing. Results in the migration assay are presented as a percentage of the cells migrated from the input. All experimental points were performed in duplicate. Results are means ($\pm$SE) of three chemotaxis assays done with supernatants from three representative experiments.

For the transmigration assay of DC, the cells were collected after Mtb infection, washed once with RPMI 1640 containing 0.5% BSA, and then resuspended at 2×10^6/ml. Chemotaxis assay was performed using 8-μm pore polycarbonate filters of a 24-well Transwell chamber (Costar). To test the migratory properties of DC through the chemokine receptor CCR7, 2×10^5 DC were seeded in the top chamber, whereas different doses of CCL21 (PeproTech) were added to the bottom chamber. The plates were incubated for 4 h at 37°C. Before counting, the migrated cells were fixed overnight with 2% paraformaldehyde to inactivate mycobacterial particles.

RNase protection assay (RPA)

RNA was extracted from DC with an RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. A phenol/chloroform extraction was performed to inactivate residual mycobacterial particles. RPA was performed as previously described (18). The hCK-5 multiprobe template set (RiboQuant; BD PharMingen) was used to analyze the chemokine expression.

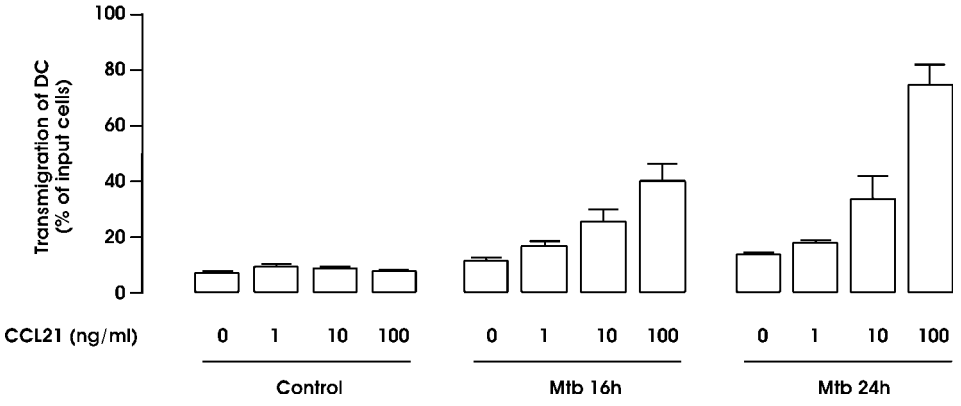


FIGURE 2. Migratory response of Mtb-infected DC. Migration assay was performed using immature DC (Control) and mature DC infected with Mtb for 16 or 24 h in response to increasing concentrations of CCL21. Results in the migration assay are shown as percentage of migrated cells from the input. All experimental points were performed in duplicate. Results are means $\pm$ SE of three chemotaxis assays.

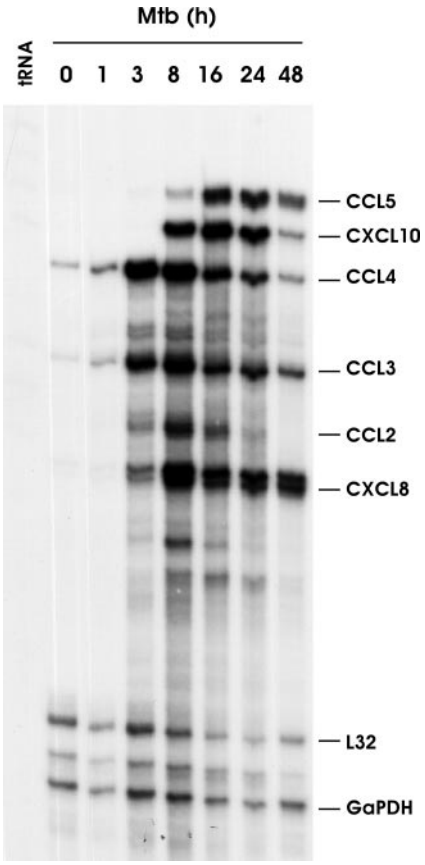


FIGURE 3. Kinetics of chemokine mRNA expression in DC infected with Mtb. DC were collected at different times after Mtb infection. Total cellular RNA (5 μg) was isolated and analyzed by RPA. This is a representative RPA experiment of three independent experiments with RNA extracted from different Mtb-infected DC cultures.

Statistical analysis

Data are expressed as means ± SE. Statistical significance of differences was determined by the paired two-tailed Student's *t* test ($p < 0.05$ was considered significant).

Results

Modulation of chemokine receptor expression in maturing Mtb-infected DC

It has been described that following maturation DC change their migratory behavior in relation to the differential expression of chemokine receptors (30). Maturing DC down-regulate the expression of receptors specific to different inflammatory chemokines, while inducing the expression of CCR7, which mediates the migration of DC into secondary lymphoid organs. To analyze whether Mtb infection modulates cell surface expression of chemokine receptors, DC were infected with Mtb and CCR7 and CCR5 expression was analyzed by flow cytometric analysis at different time points (Fig. 1). CCR7 was strongly up-regulated at 24 h after Mtb infection. Conversely, the expression of CCR5 was rapidly lost in maturing DC starting 8 h after Mtb infection. As previously described (18), the expression of CD83, a marker for mature DC, was induced 8 h after exposure to Mtb and increased for up to 24 h. DC treated with LPS for 24 h were used as a control for DC maturation.

To evaluate whether the induced expression of CCR7 correlates with the acquired responsiveness to secondary lymphoid tissue chemokines, Mtb-infected DC were tested for their capacity to respond to different doses of CCL21, a CCR7 ligand. The migration activity of DC was evaluated 16 and 24 h after Mtb infection by chemotaxis assay (Fig. 2). In accordance with CCR7 expression (Fig. 1), CCL21 induced the migration of mature DC. The higher expression of CCR7 observed at 24 h after infection was associated with a stronger chemotactic response to CCL21.

Chemokine expression in Mtb-infected DC

Next, we analyzed the kinetics and the profile of chemokine expression in DC during Mtb infection. DC were collected at different times after Mtb infection. Total cellular RNA was isolated and chemokine gene expression was analyzed by RPA (Fig. 3). After 3 h of stimulation, Mtb up-regulated mRNA expression of CCL3, CCL4, CXCL8, and, to a lesser extent, CCL2. The expression of *CCL5* and *CXCL10* was induced at 8 h after Mtb infection. Interestingly, although CXCL8 and CCL5 mRNA levels remained elevated at 48 h after infection, a reduced expression of transcripts coding for CCL2, CCL3, CCL4, and CXCL10 was observed.

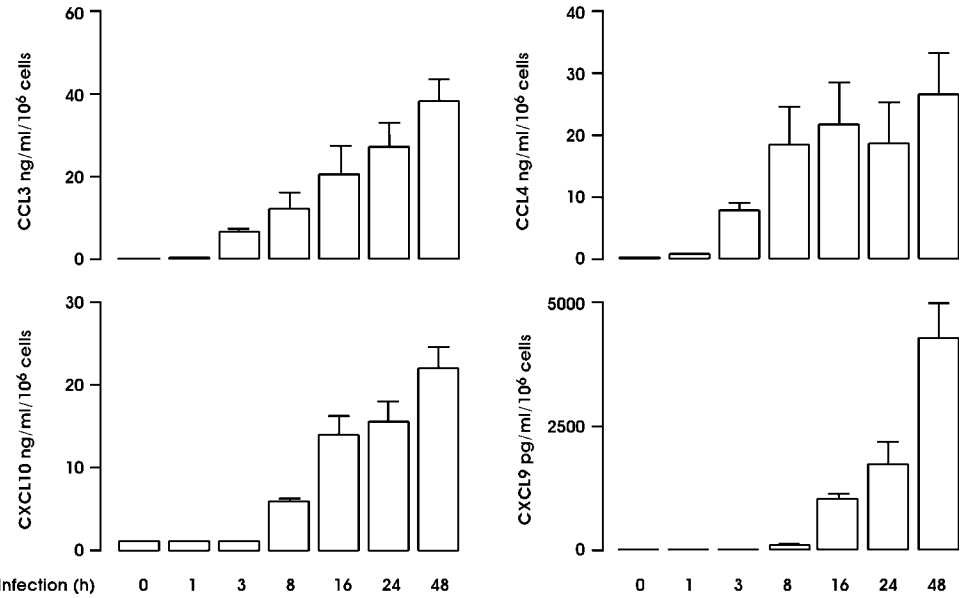
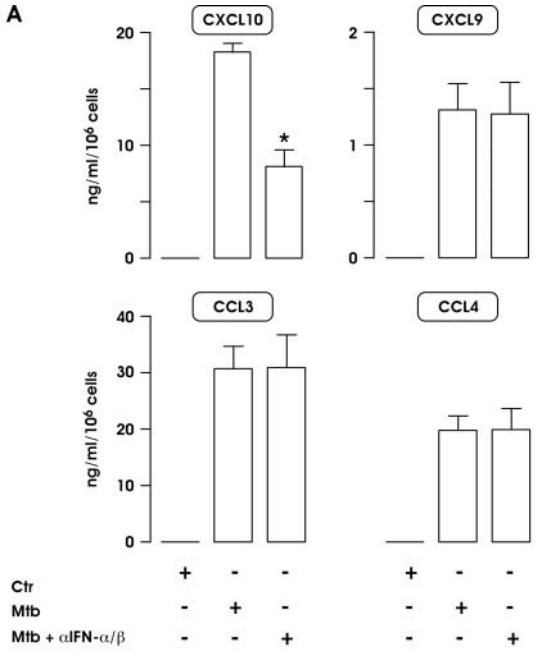
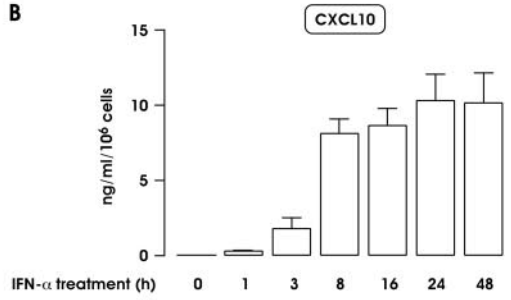
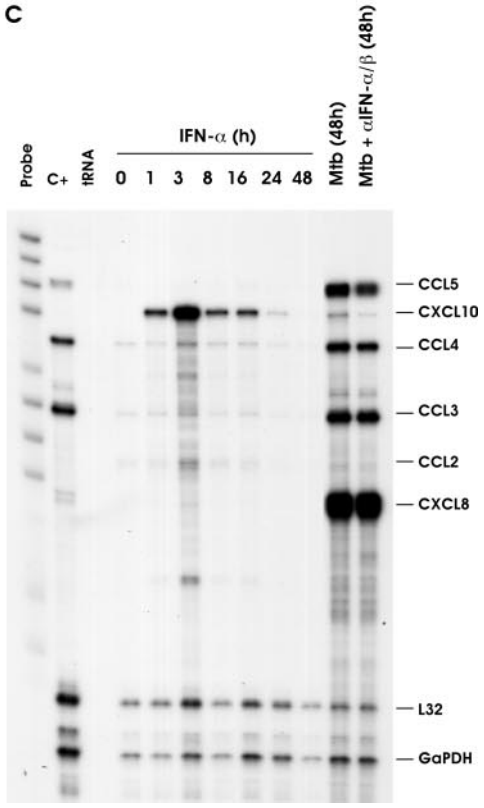


FIGURE 4. Kinetics of chemokine production following Mtb infection of DC. Cells were infected with Mtb at a MOI of 5 and cell culture supernatants were collected at different time points after infection and analyzed for chemokine production with specific ELISAs. The results represent the means ± SE of 10 separate experiments. Note a difference in scales.







To determine whether the up-regulation of chemokine mRNA expression correlated with the secretion of these chemokines, we measured the concentration of CCL3, CCL4, CXCL9, and CXCL10 in Mtb-infected DC supernatants. Cell culture supernatants were collected at different time points after the infection and the levels of CCL3, CCL4, CXCL9, and CXCL10 were determined by ELISA (Fig. 4). DC infected with Mtb showed enhanced production of CCL3, CCL4, CXCL9, and CXCL10. Some differences in the kinetics were seen. CCL3 and CCL4 production was slightly faster and evident already at 3 h after infection, while CXCL9 and CXCL10 steadily increased for up to 8 or 16 h. Along with the results obtained by the flow cytometric analysis of chemokine receptors, these data indicate that CCL3 and CCL4 production by Mtb-infected DC results in a rapid autodesensitization of CCR5.

IFN-αβ mediates induction of CXCL10 gene expression in Mtb-infected DC

Infection of DC with Mtb leads to the production of different cytokines including IFN-$\alpha\beta$ (18). Since IFN-$\alpha\beta$ has already been shown to be able to induce *CXCL10* gene expression (28, 31, 32), we analyzed whether IFN-$\alpha\beta$ regulates the chemokine gene expression in Mtb-infected DC. Thus, we analyzed the production of CCL3, CCL4, CXCL9, and CXCL10 in the supernatants of Mtb-infected DC cultures in the presence or absence of neutralizing anti-IFN-$\alpha\beta$ Abs (Fig. 5*A*). *CXCL10* expression was significantly reduced ($p < 0.05$) at 24 h in response to the IFN-$\alpha\beta$ neutralization, whereas no effect was detected in the other chemokines analyzed (Fig. 5*A*).

Since IFN-$\alpha\beta$ was likely to regulate CXCL10 production in Mtb-infected DC, we analyzed whether exogenous IFN-α was able to stimulate CXCL10 production. In accordance with IFN-$\alpha\beta$ neutralization experiments, DC treated with 1000 U/ml IFN-α rapidly produced CXCL10 as analyzed by ELISA (Fig. 5*B*). This indicates that in Mtb-infected DC the expression of CXCL10 was in part dependent on type I IFN production. To further study the role of type I IFN in CXCL10 gene expression, total cellular RNA was isolated from DC treated for different times with 1000 U/ml IFN-α, and the CXCL10 steady-state mRNA level was analyzed by RPA (Fig. 5*C*). An induction of CXCL10 mRNA was observed as early as 1 h after IFN-α treatment. A sustained expression of CXCL10 mRNA was still present at 16 h followed by its complete disappearance at 24–48 h. Moreover, to study the role of type I IFN in Mtb-induced CXCL10 production, the cells were infected in the presence and absence of neutralizing anti-IFN-$\alpha\beta$ Ab. Neutralization of type I IFN reduced CXCL10 mRNA expression at 24 h after Mtb infection, whereas no major changes in the mRNA levels in other Mtb-induced chemokines were detected (Fig. 5*C*).

FIGURE 5. Effects of type I IFN on chemokine production in Mtb-infected DC. *A*, Cell culture supernatants were collected 24 h after Mtb infection and analyzed with chemokine-specific ELISAs. Where indicated, neutralizing anti-IFN-$\alpha\beta$ Abs were used to treat the DC cultures during Mtb infection. The results represent the means ± SE of six independent experiments. *B*, DC were treated with exogenous IFN-α2 (1000 U/ml) for different periods of times and the production of CXCL10 was analyzed in cell culture supernatants. *C*, DC were collected at different times after IFN-α treatment as indicated. Total cellular RNA (5 μg) was isolated and analyzed by RPA. This is a representative RPA experiment, which was repeated for an additional two times with RNA extracted from different DC cultures. Ctr, Control.

Recruitment of NK, CD4$^+$, and CD8$^+$ cells by chemokines secreted from Mtb-infected DC

CCL3, CCL4, and CXCL10 are known to function as chemotactic factors for NK cells and activated CD4$^+$ and CD8$^+$ cells, which represent the major IFN-γ-producing leukocytes in granulomas.

NK cells represent a cell type of innate immunity that could be rapidly recruited at the site of infection. Thus, the expression of CXCR3 and CCR5 was analyzed on human NK cells (Fig. 6*A*). Flow cytometric analysis revealed a high expression of CXCR3, while CCR5 was expressed at lower levels. To evaluate the functional significance of this chemokine receptor expression, supernatants from Mtb-infected DC were used to stimulate NK cell migration. Figure 6*B* shows the percentage of NK cells responding to the Mtb-induced chemokines present in DC supernatants. The saturation of CXCR3 receptor with CXCL10 reduced NK migration almost to the level seen in the control supernatant, indicating that the chemotactic activity in Mtb-infected DC supernatants could mainly be attributed to CXCL10.

Next, we analyzed the ability of Mtb-induced chemokines to stimulate the chemotaxis of CD4$^+$ (Fig. 7) and CD8$^+$ (Fig. 8) T cells. Th1 and Tc1 cells were prepared by stimulating human cord blood lymphocytes with the appropriate cytokines. As was demonstrated at the single-cell level by measuring intracellular cytokine production, neonatal T cells differentiated into IFN-γ-producing cells (Figs. 7*A* and 8*A*). The expression of the CCR3, CCR5, and CXCR3 was analyzed in activated T cells using specific mAbs. CXCR3 and CCR5 were expressed at high levels on both CD4$^+$ and CD8$^+$ T cells (Figs. 7*B* and 8*B*). The expression of CCR3, generally acquired after Th2 polarization, was absent (data not shown). Next, we wanted to investigate the capacity of CD4$^+$ and CD8$^+$ T cells to respond to Mtb-induced chemokines via CXCR3 and CCR5. Supernatants from Mtb-infected DC were added to migration chambers containing Th1 or Tc1 cells, and the number of migrated cells was counted (Figs. 7*C* and 8*C*, respectively). The chemokines released from DC stimulated with Mtb for 48 h significantly enhanced ($p < 0.001$) the migration of both Th1 and Tc1 cells (Figs. 7*C* and 8*C*). Neutralization of CXCR3 by preincubating Th1 and Tc1 cells with CXCL10 prior to the migration assay resulted in markedly reduced migration, by a reduction of approximately 50% (Figs. 7*C* and 8*C*, $p < 0.001$). Similar results were obtained when the chemotactic activity of CXCL10 present in the supernatants from Mtb-infected DC was blocked by the addition of Abs raised against CXCR3 (data not shown). Interestingly, supernatants obtained from DC infected with Mtb in the presence of neutralizing IFN-αβ Abs exhibited similarly reduced chemotactic activity for Th1 or Tc1 cells (Figs. 7*C* and 8*C*, **, $p < 0.003$). The migration experiments were also performed using Th1 and Tc1 cells prepared by stimulating human PBL. The results were comparable to those obtained with Th1 and Tc1 cells prepared by stimulating neonatal T cells (data not shown). Overall, these results indicate that IFN-α-mediated production of CXCL10 is critical for the selective recruitment of activated T cells. However, the residual

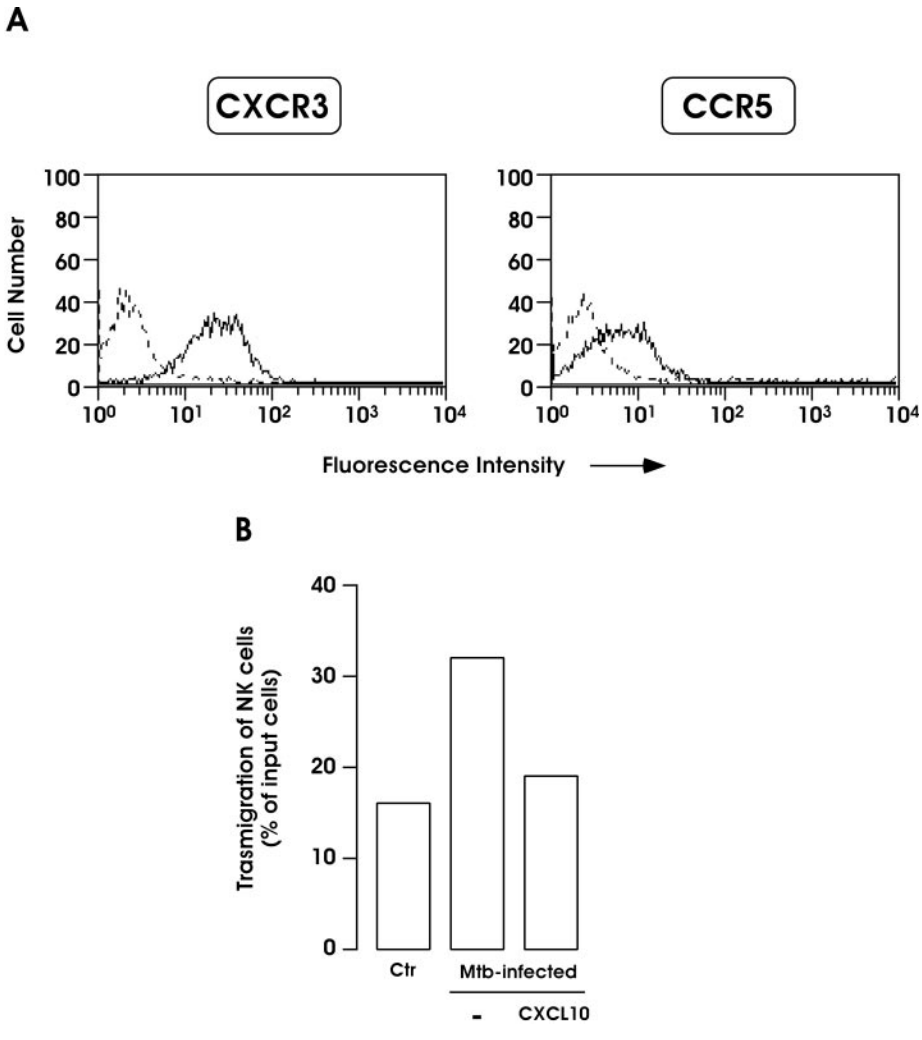


FIGURE 6. Chemotactic activity of NK cells for Mtb-infected DC cell culture supernatants. *A*, NK cells cultivated for 10 days were characterized for the expression of CXCR3 and CCR5. *B*, The migration assay of NK cells stimulated with supernatants from cultures of control DC or from cultures of DC infected with Mtb for 48 h is shown. Where indicated, the cells were preincubated with a saturating dose of CXCL10 (10 μg/ml) for 1 h at 37°C. Values of the migration assay were expressed as percentage of migrated cells from the input. All experimental points were performed in duplicate. A representative experiment of two chemotaxis assays performed with supernatants from two different DC cultures is shown. Ctr, Control.

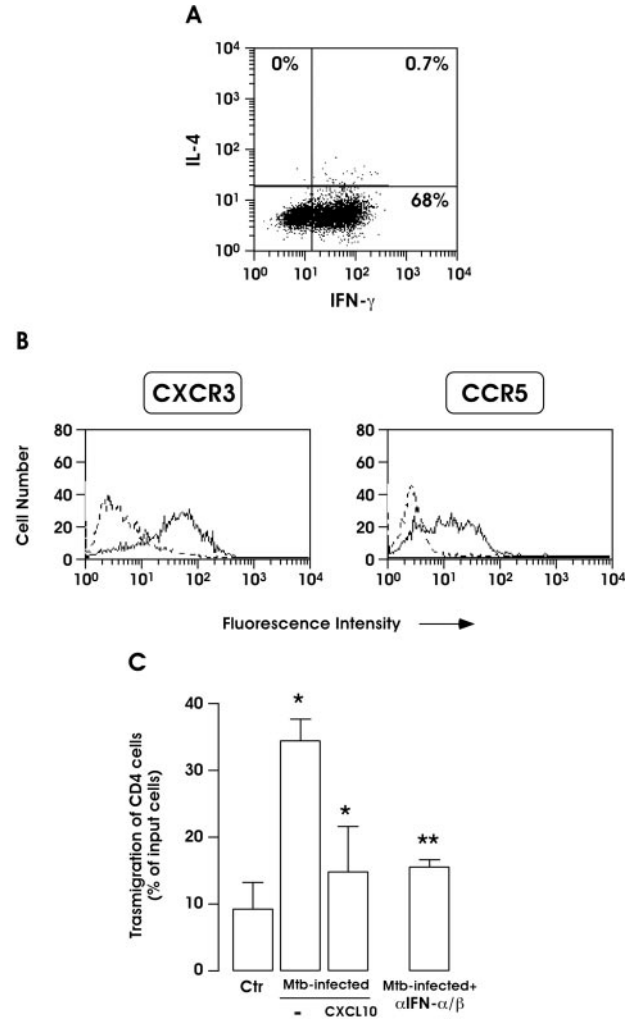


FIGURE 7. Chemotactic activity in supernatants of Mtb-infected DC for $CD4^+$ cells. $CD4^+$ T cells were analyzed for IFN-γ and IL-4 production by intracellular staining (*A*) and for the expression of CXCR3 and CCR5 (*B*). Comparable results were obtained in three additional experiments. *C*, Migration assay was performed using Th1 cells in response to supernatants from cultures of control DC or from cultures of DC infected with Mtb for 48 h in the presence or absence of neutralizing IFN-$\alpha\beta$ Abs. Where indicated, the cells were preincubated for 1 h at 37°C with a saturating dose of CXCL10 (10 μg/ml). Values of the migration assay are expressed as percentage of migrated cells from the input. All assays were performed in duplicate. Results are means ± SE of four chemotaxis assays done with supernatants from three independent experiments. Ctr, Control.

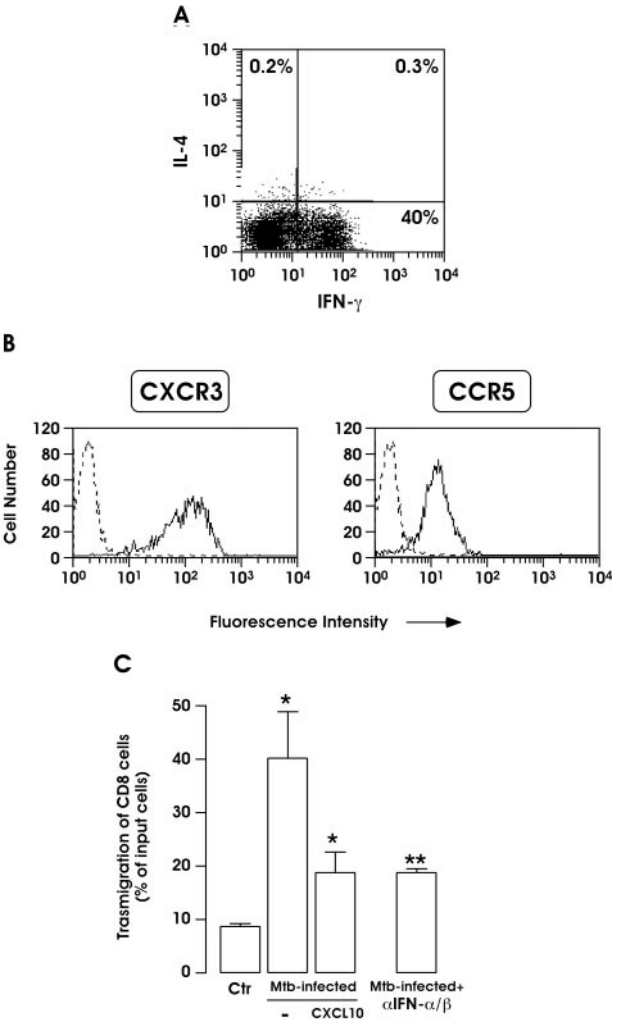


FIGURE 8. Chemotactic activity in supernatants of Mtb-infected DC for $CD8^+$ cells. Cord $CD8^+$ T cells were analyzed for IFN-γ and IL-4 production by intracellular staining (*A*) and for the expression of CXCR3 and CCR5 (*B*). Comparable results were obtained in three additional experiments. *C*, Migration assay was performed using Tc1 cells in response to supernatants from cultures of control DC or from cultures of DC infected with Mtb for 48 h in the presence or absence of neutralizing IFN-$\alpha\beta$ Abs. Where indicated, the cells were preincubated for 1 h at 37°C with a saturating dose of CXCL10 (10 μg/ml). Values of the migration assay are expressed as percentage of migrated cells from the input. All experimental points were performed in duplicate. Results are means ± SE of three chemotaxis assays done with supernatants from three independent experiments. Ctr, Control.

chemotactic activity in the supernatants of Mtb-infected DC cultures treated with neutralizing IFN-$\alpha\beta$ Abs could be ascribed to CCL3, CCL4, CXCL9, and also to the portion of CXCL10 whose expression was not affected by IFN-$\alpha\beta$ (Fig. 5*A*).

Discussion

In this study, we have shown that DC respond to Mtb infection by secreting different inflammatory chemokines and by switching their migratory capacity through the expression of various chemokine receptors. For the first time, we demonstrated that the Mtb-induced production of IFN-$\alpha\beta$ modulated the DC chemoattractive properties regulating the expression of *CXCL10* in an autocrine and paracrine fashion.

Following interaction with Mtb, the DC showed a rapid and robust production of CCL3 and CCL4 (Fig. 4) that may down-modulate CCR5 expression in maturing DC. Moreover, the production of CCL3 and CCL4 may regulate the recruitment of different leukocyte populations expressing CCR5, such as immature DC, monocytes, macrophages, activated T lymphocytes, and NK cells to infected lung tissue. In addition, Mtb infection stimulated the secretion of CXCL9 and CXCL10 that preferentially attracted Th1- and Tc1-activated lymphocytes and NK cells through CXCR3. Mtb-induced production of CCL2, CCL3, CCL4, CCL5, and CXCL8 has been previously observed in human alveolar macrophages, in monocytic THP1 cells, and in bronchoalveolar lavage or pleural fluid from pulmonary TB patients (6, 9, 10). However, the induction of CXCL9 and CXCL10 following Mtb infection is a novel finding. A particular feature shared by these two chemokines is their inducibility by IFN (32), therefore we investigated whether type I IFN released from Mtb-infected DC (18, 19) could modulate the expression of *CXCL9* and *CXCL10*. We found that

although the production of CXCL9 was not affected by the addition of Abs to IFN-$\alpha\beta$, Mtb-induced *CXCL10* expression was significantly dependent on IFN-$\alpha\beta$ since its neutralization reduced both the mRNA steady-state level and the protein content secreted into the supernatants of Mtb-infected DC cultures (Fig. 5). The remaining expression of CXCL10 observed in the presence of neutralizing IFN-$\alpha\beta$ Abs could be ascribed to the activation of the NF-κB pathway induced by the direct interaction of Mtb with the DC. Indeed, the infection of the DC by Mtb stimulated a rapid binding of NF-κB to the κB site within the *CXCL10* gene promoter (data not shown) that may cooperate with other transcription factors in the regulation of *CXCL10* gene transcription (33). In addition, we also observed that IFN-$\alpha\beta$ directly caused a significant increase in CXCL10 mRNA expression and protein production in treated cells (Fig. 5). Altogether these results suggest that the expression of *CXCL10* is regulated in Mtb-infected DC in two ways: first, by direct interactions between mycobacterial components and DC leading to the activation of the NF-κB complex (data not shown) and, second, in a paracrine and autocrine fashion by the release of type I IFN from Mtb-infected cells. Interestingly, the kinetics of CXCL10 release suggested that DC began to produce this chemokine 8 h after Mtb infection (Fig. 4), just a few hours before their migration into secondary lymphoid organs due to the acquisition of CCR7 and the loss of CCR5 expression (Figs. 1 and 2).

The functional impact of chemokines produced by infected DC on the recruitment of NK cells and $\alpha\beta$ T lymphocytes of $CD4^+$ and $CD8^+$ phenotypes was investigated since these cells would be recruited to the site of Mtb infection due to the presence of CCR5 and CXCR3 (Figs. 6–8). Interestingly, these cells show cytolytic activity and are able to produce IFN-γ, which plays a central role in the development of cell-mediated immunity and host immune response against the Mtb (1). The effect of Mtb-induced chemokines on NK cells is consistent with a recent report on the role of NK cell-mediated innate immune response against Mtb (34). Moreover, the chemotaxis of NK cells induced by chemokines released from Mtb-infected DC is of particular interest in light of a new observation suggesting significant cross-talk between NK cells and DC that was required for their reciprocal activation and maturation (35).

Supernatants from Mtb-infected DC clearly displayed a chemotactic activity for activated $CD4^+$ and $CD8^+$ cells expressing CXCR3 and CCR5 receptors (Figs. 7*C* and 8*C*). The blocking of CXCR3 dramatically reduced the ability of Mtb-stimulated DC supernatants to stimulate chemotaxis in Th1 and Tc1 cells, indicating that CXCR3 ligands, CXCL9 and CXCL10, also have a major role in Mtb-induced Th1 and Tc1 cell chemotaxis. Accordingly, the neutralization of IFN-$\alpha\beta$ in Mtb-infected DC cultures significantly reduced Th1 and Tc1 migration, further pointing to the importance of CXCL10 expression in the selective recruitment of activated $CD4^+$ and $CD8^+$ cells.

In summary, our results suggest the following sequence of events that may regulate some aspects of the immune response against Mtb. At early phases of Mtb infection, before the generation of Ag-specific T cells, DC-derived CXCL10 may recruit NK cells to the site of infection where they may directly destroy Mtb-infected cells and produce IFN-γ, which stimulates macrophage activation. At the later stages of infection when Mtb-specific Th1 and Tc1 are expanded, their recruitment is mediated by the expression of inflammatory chemokines from the Mtb-infected DC. The release of IFN-γ sustains the activation of macrophages required to maintain the inflammatory response within granuloma through interactions of macrophages with $CD4^+$ and $CD8^+$ T cells. The production of IFN-γ may also reinforce the production of CXCL9 and CXCL10 from macrophages, DC, and pulmonary epithelial cells to sustain the lymphocyte traffic into the alveolar compartment. Moreover, the maturing DC, that migrate into the secondary lymphoid organs, continue CXCL10 secretion that might regulate DC-Th1 cluster formation and the retention of CD4-positive T cells, as was recently demonstrated in a murine granulomatous liver disease model (36). Thus, the mature DC may direct the complete polarization process of T lymphocytes within T cell areas of regional draining lymph node through different mechanisms comprising CXCL10 production, the release of Th1/IFN-γ-inducing cytokines, IFN-$\alpha\beta$ and IL-12, and the expression of costimulatory and Ag-presenting molecules.

The importance of the type I IFN/CXCL10 axis in the regulation of the immune response against Mtb has to be confirmed in vivo to define a possible protective role of IFN-$\alpha\beta$. Indeed, although recent studies demonstrated a clinical improvement in drug-sensitive or multidrug-resistant TB patients treated with IFN-α (37–39), opposite results were obtained in mice where the induction of type I IFN by Mtb infection may be pathogenic (40). A better understanding of these events may improve and reveal new targets for TB treatment and prevention.

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