Mycobacterium tuberculosis Antigens Specifically Modulate CCR2 and MCP-1/CCL2 on Lymphoid Cells from Human Pulmonary Hilar Lymph Nodes

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J Immunol 2007; 179:8381-8391; doi: 10.4049/jimmunol.179.12.8381

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Macrophages and dendritic cells are involved in the immune response to *Mycobacterium tuberculosis* (*Mtb*). Such a response, although extensively studied using animal models and cells from human blood, has not been characterized in cells from pulmonary hilar lymph nodes (PHLN). We characterized populations of myeloid APC from PHLN and determined their expression of CCR2, CCR5, CCR7, CD40, CD54, CD80, and CD86 as well as the cytokine/chemokine microenvironment before and after purified protein derivative (PPD) and mannosilated lipoarabinomannan (ManLAM) stimulation. Results show that there are at least three APC populations in PHLN, defined as CD14$^{\text{high}}$/HLA-DR$^{\text{low}}$/, CD14$^{\text{dim}}$/HLA-DR$^{\text{dim}}$, and CD14$^{\text{dim}}$/HLA-DR$^{\text{high}}$/dendritic cells (DC), with the largest number represented by CD14$^{\text{dim}}$/HLA-DR$^{\text{dim}}$ cells (where dim indicates intermediate levels). CD14$^{\text{dim}}$/HLA-DR$^{\text{high}}$/DC expressed higher levels of costimulatory molecules and lower levels of CCR2 and CCR5, but all cell populations showed similar CCR7 levels. PPD and ManLAM specifically down-regulated CCR2 expression but not that of CCR5 and CCR7, and such down-regulation was observed on all APC populations. *Mtb* Ag did not affect the expression of costimulatory molecules. PPD but not ManLAM specifically induced MCP-1/CCL2 production, which was likely associated with the induction of IFN-γ because this cytokine was highly induced by PPD. We characterized, for the first time, different APC from human PHLN and show that *Mtb* Ag exert fine and specific regulation of molecules closely associated with the immune response to *Mtb* infection. Because knowledge of this response in secondary lymphoid tissues is still poorly understood in humans, such studies are necessary and important for a better understanding of lymphoid cell microenvironment and migrating capacities and their role in the immunopathogenesis of tuberculosis. *The Journal of Immunology*, 2007, 179: 8381–8391.


**Mycobacterium tuberculosis** Antigens Specifically Modulate CCR2 and MCP-1/CCL2 on Lymphoid Cells from Human Pulmonary Hilar Lymph Nodes

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Macrophages and dendritic cells are involved in the immune response to *Mycobacterium tuberculosis* (*Mtb*). Such a response, although extensively studied using animal models and cells from human blood, has not been characterized in cells from pulmonary hilar lymph nodes (PHLN). We characterized populations of myeloid APC from PHLN and determined their expression of CCR2, CCR5, CCR7, CD40, CD54, CD80, and CD86 as well as the cytokine/chemokine microenvironment before and after purified protein derivative (PPD) and mannosilated lipoarabinomannan (ManLAM) stimulation. Results show that there are at least three APC populations in PHLN, defined as CD14$^{\text{high}}$/HLA-DR$^{\text{low}}$/, CD14$^{\text{dim}}$/HLA-DR$^{\text{dim}}$, and CD14$^{\text{dim}}$/HLA-DR$^{\text{high}}$/dendritic cells (DC), with the largest number represented by CD14$^{\text{dim}}$/HLA-DR$^{\text{dim}}$ cells (where dim indicates intermediate levels). CD14$^{\text{dim}}$/HLA-DR$^{\text{high}}$/DC expressed higher levels of costimulatory molecules and lower levels of CCR2 and CCR5, but all cell populations showed similar CCR7 levels. PPD and ManLAM specifically down-regulated CCR2 expression but not that of CCR5 and CCR7, and such down-regulation was observed on all APC populations. *Mtb* Ag did not affect the expression of costimulatory molecules. PPD but not ManLAM specifically induced MCP-1/CCL2 production, which was likely associated with the induction of IFN-γ because this cytokine was highly induced by PPD. We characterized, for the first time, different APC from human PHLN and show that *Mtb* Ag exert fine and specific regulation of molecules closely associated with the immune response to *Mtb* infection. Because knowledge of this response in secondary lymphoid tissues is still poorly understood in humans, such studies are necessary and important for a better understanding of lymphoid cell microenvironment and migrating capacities and their role in the immunopathogenesis of tuberculosis.


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the LN to colonize and home within the extrafollicular T cell areas (16).

CCR2, in particular, has been associated with the immune response to *Mtb* infection, because CCR2-deficient mice had early and persistent defects on macrophage recruitment to the lungs and a reduced number of macrophages and DC in mediastinal LN (17, 18). Indeed, studies of the immune response to *Mtb* and *Leishmania major* infection in mouse models showed that MCP-1/CCL2 and its cognate receptor, CCR2, are involved in controlling migration and localization of DC and macrophages within the LN extrafollicular areas (17, 19, 20). LN cells from CCR2<sup>−/−</sup> mice were unable to increase IFN-γ production after rechallenge with *Mtb* Ag (19). These reports indicate that MCP-1/CCL2 may not only be important in directing the migration of macrophages and DC to the inflammatory site in *Mtb* infection, but also that it may modulate the migration and localization of such cells within peripheral LN.

A role for CCR7 has also been suggested in TB, because the maturation of DC induced in the presence of *Mtb* up-regulated expression of this receptor (21, 22). CCR7 senses gradients of chemokines produced by lymphatic endothelial cells (23) and also resident interdigitating DC cells within the regional LN (24), enabling DC with the capacity to emigrate from the inflammatory environment (25). In contrast to DC, *Mtb* do not up-regulate expression of CCR7 on macrophages (21), which suggests that these cells may be retained at the inflammatory site (21, 26). Up-regulation of CCR7 is likely to be paralleled with enhancement of Ag presentation capacity (27). After arrival in the regional LN, DC display a mature phenotype with high expression of MHC class II molecules and increased levels of costimulatory and adhesion molecules (2, 28), and they readily home within the T cell zones to the extrafollicular areas (16). It is here where DC have intimate interaction with *Mtb* Ag-specific naive T cells, thus facilitating Ag presentation, T cell activation and proliferation, and, through production of the appropriate cytokines, their subsequent differentiation toward Th1 type effector cells (29, 30). Although both macrophages and DC are able to phagocytose/endocytose *Mtb*, up-regulation of costimulatory molecules and Ag presentation capacity are more remarkable in DC (28, 29, 31).

Previous studies show a role for inflammatory chemokines in recruiting lymphoid cells to the site of *Mtb* invasion and also that specific chemokines and their receptors are associated with migration and localization of infected macrophages and DC within LN. Nevertheless, these studies were performed only in animal models (14, 32, 33), and therefore little is known in humans about the role of *Mtb* in modulating chemokine production and migration in peripheral LN.

In this study, we extend these previous studies by using cells from the draining human hilar lung LN, which are the first peripher-
costimulatory molecules, and cytokine/chemokine production and their modulation by Mtb Ag in monocytes/macrophages and DC from these tissues (Table I).

Tissue processing

Following surgical removal, PHLN were quickly chilled to 4°C in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10 mM HEPES, 2 mM l-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% FCS (HyClone), referred hereafter as complete medium (CM), and processed, under sterile conditions, within 10 h of removal from the cadaver. LN were placed on 100 mm petri dishes with 40 ml of cold CM and thoroughly minced using forceps and scalpel. The tissue suspension was transferred to a 50-ml tube and placed vertically for 30 s to allow debris to settle. The supernatant was aspirated, cells were resuspended in CM, and analyzed by flow cytometry.

Freezing procedure and cell storage

Cells were stored in liquid nitrogen until further use, maintained in freezing medium (20% DMSO, 30% FCS, and 50% CM) with no more than 10^9 cells per ampoule. Viability of cells after thawing was consistently >90% as judged by trypan blue exclusion. Cell aliquots below this viability were discarded. Although no experiments were performed to test the effect of freezing conditions on both cytokine/chemokine production and cytokine production and cell surface CCR expression, previous studies showed that cryopreservation of cells from blood and bronchoalveolar lavages does not affect the pattern of expression of these molecules and that such cells possessed an ability in cytokine production and CCR expression as good as that of the fresh cells (34, 35).

Cell culture

Either fresh or thawed MNC from PHLN were cultured in CM supplemented with 10% AB pooled human serum (PHS, BioWhittaker) in U-bottom 96-well plates (Nunc) at 2 × 10^3 cells/well in a final volume of 200 μl in a humidified incubator at 37°C with 5% CO2. The cells were cultured with 10 μg/ml PPD from Mtb (Statens Serum Institute, Copenhagen, Denmark) or 10 μg/ml mannosylated liposomal interferon (ManLAM) from Mtb H37Rv (donated by Prof. P. Brennan, Colorado State University, Fort Collins, CO). The presence of endotoxin in the ManLAM preparation was <37 pg per mg of ManLAM. LPS (from Escherichia coli, serotype O111: B4, Alexis Biochemical) was used as positive control at 0.3 μg/ml, and untreated cells were used as negative controls. After 48 h, the cell supernatants were collected and stored at −70°C for further quantification of chemokines and cytokines. The cells were tested for the expression of CCR and costimulatory molecules by immunofluorescence and flow cytometry.

Immunofluorescence and flow cytometry

Triple staining was performed to determine the expression of CCR and costimulatory molecules on the surface of DC and monocytic/macrophage populations from PHLN before and after culture. Staining was performed in same day cell cultures to compare the modulation of both CCR and costimulatory molecules simultaneously. Either fresh cells or cells under culture were washed twice in cold PBS, resuspended in 100 μl of cold binding buffer (PBS (pH 7.2–7.4) supplemented with 2% PHS plus 0.1% sodium azide), and incubated at 4°C for 30 min with specific mAb. Cells were stained with PE-labeled anti-CCR2, anti-CCR5, and anti-CCR7 mAb or PE- or Cy-Chrome-labeled anti-CD40, anti-CD54, and anti-CD86 mAb combined with FITC-labeled anti-CD19, anti-CD56, and anti-CD14 mAb or PE- or Cy-Chrome-labeled anti-CD80, anti-CD86 mAb combined with FITC-labeled anti-CD19, anti-CD14, anti-CD56, and anti-CD19 mAb, plus Cy-Chrome- or PE-labeled-.direct-DR-PE-DR-Dub. Table II shows a list of the Ab used. After incubation, cells were washed twice with 1 ml of cold PBS and fixed with 400 μl 2% paraformaldehyde (Pischer Scientific) in PBS. One hundred thousand events were acquired in a BD FACSort cytometer (BD Biosciences), and the samples were analyzed using the CellQuest software, version 3.3 (BD Biosciences).

ELISA

Determination of RANTES/CCL5, MIP-1α/CCL3, MIP-1β/CCL4, MCP-1/ CCL2 and MDC/CCL22 CC chemokines and IL-12p70, IL-12p40, IFN-γ, IL-6 (R&D Systems), and IL-10 (Endogen) cytokines released into cell culture supernatants from stimulated and nonstimulated MNC was performed by ELISA protocols recommended by the manufacturers. Coating of ELISA plates (MaxiSorp; Nalgene Nunc International) with 100 μl of a dilution of capture Ab was conducted overnight at room temperature. Then, plates were washed three times with 300 μl of wash buffer (0.05% Tween 20 in PBS (pH 7.4)) and dried by tapping on absorbent paper. Blocking was performed by filling the wells with blocking buffer (PBS containing 1% BSA, 0.1% sucrose, and 0.05% Tween20) followed by the addition of serial dilutions of culture supernatants. Supernatants from cells cultured with Mtb antigen were assayed in triplicate. The absorbance was measured at 450 nm using a microplate reader (ELx800; Bio-Tek Instruments). The data were used to calculate the amount of cytokine or chemokine secreted by stimulated MNC. The standard curve was generated using serial dilutions of recombinant cytokine or chemokine protein.

FIGURE 1. Myeloid APC populations in human pulmonary hilar lymph nodes. Total mononuclear cells were isolated from PHLN as described in Materials and Methods and double stained with FITC-labeled anti-CD3, anti-CD14, anti-CD19, and anti-CD56 mAb plus Cy-Chrome-labeled anti-HLA-DR class II mAb and analyzed by flow cytometry. Granulated and larger cells were gated using forward (FSC) and side scatter (SSC) profiles, and subgating was performed according to double expression of CD14 and HLA-DR class II Ag. Populations of APC are defined according to these two markers as depicted in the figure. Shown is a representative plot of at least six performed from same number of donors. Mean ± SD are as described in Materials and Methods. One-way ANOVA was performed to test for differences in percentages of populations.
and 0.05% NaN₃) and incubating for 2 h. After washing, samples and standards were diluted in diluent buffer (0.1% BSA, 0.05% Tween 20 in Tris-buffered saline (pH 7.3; 20 mM Trizma base, 150 mM NaCl)) and 100 µl of dilutions was added to wells and incubated for 2 h at room temperature. After washing, 100 µl of diluted biotinylated detecting Ab was dispensed into each well, and plates were incubated for 2 h at room temperature. For IFN-γ detection, the anti-IFN-γ biotinylated Ab was diluted 2 h before using in 2% heat inactivated normal goat serum. Plates were then washed and 100 µl of 1/200 dilution of HRP-streptavidin conjugate (R&D Systems) in PBS (pH 7.2–7.4) plus 1% BSA was added into each well and incubated for 20 min. Plates were washed and 100 µl of tetramethylbenzidine (Pierce-Endogen) was dispensed into each well and incubated at room temperature for 12 min in darkness. The reaction was stopped using 50 µl/well stop solution (0.18 M H₂SO₄), and optical densities were read immediately at 450 nm on a plate reader (Bio-Tek Instruments). Results were calculated by interpolating to nonlinear regression standard curves.

Cell proliferation assay
Triplicate cultures of MNC from PHLN (1 x 10⁵ cells/200 µl) were stimulated for 5 days in CM (supplemented with 10% PHS) with 10 µg/ml PPD or with 5 µg/ml PHA (ICN Biomedicals) in round-bottom 96-well plates. Cell proliferation was estimated by incorporation into the DNA of [³H]thymidine (specific activity: 185 GBq/mmol, 5.0 Ci/mmol; Amersham Biosciences). The cells were pulsed with 0.5 µCi of [³H]thymidine per well 18 h before harvesting, and cpm were determined in a liquid scintillation beta counter (Beckman LS 6500). Proliferation response was calculated as the mean ± SD of the cpm from three replicates.

Statistical analyses
Analyses were performed using GraphPad Prism, version 4.00 (GraphPad Software). Descriptive statistics were used to calculate the constitutive expression of CCR and costimulatory molecules. One-way ANOVA was used to test for differences in the numbers of myeloid APC and for differences in the effect of Ag on chemokine and cytokine production with Tukey’s multiple comparison posttest to compare groups by pairs. The nonparametric Kruskal-Wallis one-way ANOVA was performed to compare the expressions of CCR and costimulatory molecules by the different APC populations before and after culture, as well as to compare the effects of Ag stimulation. Dunn’s multiple comparison posttest was performed to analyze for differences between treatments. Differences were considered significant at p < 0.05.

Results
Characterization by flow cytometry of DC and monocyte/macrophage cell populations from human PHLN
We defined the monocyte/macrophage/DC populations in MNC from human PHLN using double immunostaining and flow cytometry. These populations were determined within the gate of large and highly granulated cells usually described as the monocyte gate and according to the expression of HLA-DR Ag and the presence or absence of cell lineage markers. In this way, the DC population was determined by gating cells that, within the monocyte gate, and 0.05% NaN₃) and incubating for 2 h. After washing, samples and standards were diluted in diluent buffer (0.1% BSA, 0.05% Tween 20 in Tris-buffered saline (pH 7.3; 20 mM Trizma base, 150 mM NaCl)) and 100 µl of dilutions was added to wells and incubated for 2 h at room temperature. After washing, 100 µl of diluted biotinylated detecting Ab was dispensed into each well, and plates were incubated for 2 h at room temperature. For IFN-γ detection, the anti-IFN-γ biotinylated Ab was diluted 2 h before using in 2% heat inactivated normal goat serum. Plates were then washed and 100 µl of 1/200 dilution of HRP-streptavidin conjugate (R&D Systems) in PBS (pH 7.2–7.4) plus 1% BSA was added into each well and incubated for 20 min. Plates were washed and 100 µl of tetramethylbenzidine (Pierce-Endogen) was dispensed into each well and incubated at room temperature for 12 min in darkness. The reaction was stopped using 50 µl/well stop solution (0.18 M H₂SO₄), and optical densities were read immediately at 450 nm on a plate reader (Bio-Tek Instruments). Results were calculated by interpolating to nonlinear regression standard curves.

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were negative for CD3, CD19, CD14, and CD56 and that expressed higher levels of HLA-DR Ag. These cells are referred to as CD14^+HLA-DR^{high}/DC. Two populations of monocytes/macrophages were defined using the same gating and staining parameters. One population had higher expression of CD14 and lower or negative expression of HLA-DR Ag, called hereafter CD14^{high}/HLA-DR^{low/-} cells, and a second monocyte/macrophage population with intermediate levels of both HLA-DR and CD14, defined onwards as CD14^{dim}/HLA-DR^{dim} cells. The latter was consistently the largest population (CD14^{high}/HLA-DR^{high}/DC, 29.5 ± 16.0%; CD14^{dim}/HLA-DR^{dim}, 57.8 ± 16.8%; CD14^{high}/HLA-DR^{low/-}, 12.6 ± 2.9%; p < 0.0001). These percentages were similar after 48 h of in vitro culture (data not shown). There was a small cluster of cells at approximate coordinates (101.75, 101.5) that did not bind any of the 11 cell surface markers used in this study, including cell lineage markers and costimulatory and CCR molecules. We speculate that this group of cells could represent dead cells or cells in the process of dying. The likelihood that these cells are stromal cells that do not make up part of the lymphoid parenchyma has also to be considered. Fig. 1 shows a representative plot describing these cell populations.

Expression of chemokine receptors by monocytes/macrophages/DC populations from PHLN

After defining the different monocytes/macrophages/DC populations in the PHLN, we first wanted to determine the expression of CCR2, CCR5, and CCR7 by these cell populations and define whether there was differential expression of these receptors according to cell phenotype. To this purpose, triple immunostaining was performed using mAbs against the aforementioned receptors. Table III shows the percentage of positive cells and the mean fluorescence intensity (MFI) for CCR2, CCR5, and CCR7 of monocyte/macrophage/DC populations. There was a high donor-to-donor variability in expression of these receptors, as illustrated in the table. Approximately one-third of CD14^{high}/HLA-DR^{low/-} and CD14^{dim}/HLA-DR^{dim} cells expressed CCR2, CCR5, and CCR7. In contrast, a small percentage of CD14^{high}/HLA-DR^{high}/DC expressed CCR2 and CCR5, also with low MFI. These low levels were only statistically significant for CCR2. Indeed, CCR2 expression was significantly lower in the CD14^{high}/HLA-DR^{high}/DC population, both in the percentage of positive cells (p < 0.03) and the MFI (p < 0.04) when compared with CD14^{high}/HLA-DR^{low/-} cells (Table III). CCR7 was expressed evenly in the three cell populations.

Expression of costimulatory molecules by monocyte/macrophage/DC populations from PHLN

It is established that the process of differentiation of CD14^+ monocytes toward either macrophages or DC is accompanied by a switch in the expression of costimulatory molecules (36). Therefore, we sought to determine whether expression of costimulatory molecules varied according to the cell phenotype described above. Expression of CD40, CD54, CD80, and CD86 was determined, as for CCR, by triple immunostaining and flow cytometry on the three cell populations. Table IV depicts the percentage and MFI values for these molecules. As for CCR, there was very high variability between donors in the expression of these molecules. Nonetheless, as illustrated in Fig. 2, although statistic analysis only showed differences in MFI expression of CD54 and CD86, CD14^+HLA-DR^{high}/DC cells expressed much higher levels of costimulatory molecules than CD14^{high}/HLA-DR^{low/-} cells, indicating that the former population in fact corresponds to DC. Similar results were obtained for the percentage of positive cells (Table IV). This DC population was, however, highly immature because approximately one-third of the cells expressed the maturation markers CD40 and CD86 (Table IV).
Effect of Mtb Ag on expression of chemokine receptors and costimulatory molecules by monocyte/macrophage/DC populations from PHLN

Once the expression of CCR and costimulatory molecules by monocyte/macrophage/DC populations was defined in cells before culture, we wondered whether such expression would be modulated under culture conditions and by stimulation with Mtb Ag. For this purpose, total MNC from PHLN were cultured for 48 h in the presence of PPD and ManLAM, both from Mtb H37Rv, and expression of CCR and costimulatory molecules was tested by flow cytometry in the cell populations described above. LPS was used as a positive control of stimulation, and untreated cells were used as a negative control. It was observed that placing the cells in culture did not affect the expression of CCR2 and CCR5 but provided a trend for an increase in the number of CCR7 molecules per cell in CD14<sup>dim</sup>HLA-DR<sup>dim</sup> and CD14<sup>+</sup>HLA-DR<sup>high</sup>/DC (data not shown). However, the values were not statistically significant, probably due to the low number of donors and the high variability between them. Stimulation with Mtb Ag, PPD, and ManLAM, significantly decreased the number of CCR2<sup>+</sup> cells (Fig. 3A) but not that of CCR5<sup>+</sup> and CCR7<sup>+</sup> cells in all three populations (data not shown). CCR2 MFI was inhibited by Mtb Ag only on CD14<sup>+</sup>HLA-DR<sup>high</sup>/DC cells (Fig. 3B). Overall, the inhibitory effect on CCR2 expression by Mtb Ag was stronger on the CD14<sup>+</sup>HLA-DR<sup>high</sup>/DC cell population (Fig. 3, A and B). LPS did not significantly affect the expression of CCR2. However, LPS significantly increased the expression of CCR7 at the MFI level only on CD14<sup>+</sup>HLA-DR<sup>high</sup>/DC cells (Fig. 3C).

Mtb Ag did not affect the expression of costimulatory molecules by any of the cell populations analyzed (data not shown). Importantly, the number of APC populations observed before culture (see above) was similar after 48 h regardless of the presence of Mtb Ag.

Modulation of chemokine production by Mtb Ag on MNC from PHLN

Because both PPD and ManLAM specifically inhibited the expression of CCR2, we wanted to determine whether such inhibition was associated with the induction of its ligand, MCP-1/CCL2. It has been suggested that regulation of CCR2, as well as CCR5, is at least in part associated with receptor-ligand binding-induced internalization. For this purpose, the presence of the CC-chemokines MCP-1/CCL2, MIP1α/CCL3, MIP-1β/CCL4, RANTES/CCL5, and MDC/CCL22 were tested by ELISA in the culture supernatants obtained from the same Mtb-stimulated cell cultures where CCR and costimulatory molecule expression was tested previously. Fig. 4A shows that the production of chemokines was highly variable between donors. There was constitutive production of all chemokines, and MDC/CCL22 was produced at nearly 1000 pg/ml in unstimulated conditions. RANTES/CCL5 was produced in very small amounts with values hardly reaching 200 pg/ml, even after Ag stimulation (Fig. 4A). To test for differences in chemokine production after Mtb Ag stimulation, values were normalized and expressed as the fold increase of chemokines produced under unstimulated conditions. Results show that the induction of chemokines by Mtb Ag was specific for both Ag and chemokine. Indeed, PPD, but not ManLAM, specifically induced MCP-1/CCL2 release (p < 0.01), and although PPD increased levels of MIP-1α/CCL3 and MIP-1β/CCL4, these levels were not significantly different from those released by unstimulated cells (Fig. 4B). However, induction of these two chemokines by PPD was significantly higher than that by ManLAM (p < 0.05; Fig. 4B). RANTES/CCL5 and MDC/CCL22 were not induced by Mtb Ag. LPS significantly induced higher levels of MIP-1α/CCL3, MIP-1β/CCL4, and RANTES/CCL5 but not MCP-1/CCL2 and MDC/CCL22 (Fig. 4B). These data suggest that induction of CC-chemokines by Mtb Ag on MNC from PHLN is specific for MCP-1/CCL2 and that such induction depends on PPD in contrast to ManLAM.

Cytokine microenvironment and its modulation by Mtb Ag on MNC from PHLN

It has been established that protection against Mtb is associated with a Th1 immune response characterized by the production of Th1 cytokines, most notably IFN-γ (2). More recently, chemokines and their receptors have been also associated with Th response patterns (37, 38), and MCP-1/CCL2 has been described...
within a Th1-type chemokine response (17, 19, 39). Because PPD was specific in inducing MCP-1/CCL2 by MNC from human PHLN, we wanted to test whether Mtb Ag were inducing a predominant Th1-type cytokine microenvironment in cultures of these cells, which could explain the MCP-1/CCL2-specific induction. For this purpose, the cytokines IFN-γ, IL-12p70, IL-12p40, IL-10, and IL-6 were tested by ELISA after stimulation with PPD and ManLAM. The cytokines IL-12p40, IL-12p70, and IL-10 were not detected under any type of antigenic stimulation, including LPS. IFN-γ and IL-6 were detected, but at low concentrations (Fig. 5). PPD, but...
not ManLAM, induced significant amounts of IFN-γ, and neither Ag induced significant levels of IL-6. LPS had an opposite effect, because it only induced significant amounts of IL-6. These data suggest that IFN-γ may be involved in the induction of MCP-1/ CCL2 by PPD.

Discussion

We characterized different types of APC in MNC from human PHLN and determined the modulatory capacity of Ag from Mtb H37Rv on molecules critically involved in cell activation and cell migration/homing in the immune response to Mtb.

We found that there are three myeloid APC populations in the PHLN based on the expression of HLA-DR and CD14. These populations were named CD14+ HLA-DR+/−/DC, CD14dimHLA-DR+/−, and CD14dimHLA-DR−/−. The first may correspond to DC, the intermediate to macrophages or cells in the process of differentiation toward DC or macrophages, and the last population may correspond to monocytes. There appears to be a continuum between these populations with a tendency of cells to down-regulate the expression of CD14 and acquire HLA-DR.

Because there is little knowledge of how monocytes are recruited to peripheral lymphoid tissues (40), the finding of bright CD14+ cells with very low expression of HLA-DR within these tissues is remarkable. These cells likely correspond to monocytes and their role may be that of precursors of macrophages and DC. Noteworthy, these monocytes express high levels of CCR2 (see Table III), and CCR2+ monocytes have been recognized as inflammatory monocytes that are recruited to the inflammation site to become macrophages (40). The process of monocyte differentiation was studied in a model of subendothelial transmigration and suggested that the majority of migrating DC derive from CD14+ monocytes (36). In that report, the differentiation of CD14+ cells into DC was characterized by enhanced expression of costimulatory molecules (36). Other studies suggested that the activation of inflammatory monocytes by phagocytosis stimulus in the periphery induces migration of these cells to regional LN, where they become potent Ag-presenting DC (41). In line with these observations, we show that expression of CD40, CD54, CD80, and CD86 was higher on CD14+ HLA-DR+/−/DC than on monocytes and macrophages. In addition, when cells were cultured for up to 48 h, the number of monocytes positive for costimulatory molecules decreased (data not shown), suggesting that the fate of monocytes within PHLN is to terminally differentiate toward DC. Thus, this is the first evidence of a role for monocytes as precursors of DC within peripheral human lymphoid tissues.

It has been suggested that the up-regulation of costimulatory molecules on maturing DC associates with CCR7 up-regulation (22), and in this study we confirmed that observation. However, although monocytes and macrophages expressed low levels of costimulatory molecules, CCR7 was still observed in high levels on these cells, indicating that parallel up-regulation of CCR7 and costimulatory molecules is more specific for the DC population, at least in cells from human PHLN. Monocytes and macrophages in these tissues may express high levels of CCR7 to remain within the PHLN.

Contrary to the even expression of CCR7 on APC, CCR2 and CCR5 expression was lower on CD14+ HLA-DR+/−/DC than CD14dimHLA-DR−/− monocytes. High expression of CCR2 on monocytes and macrophages has been described in mice (19) and humans (42), and its expression on such cells from mediastinal human LN has been described previously only by our group (43). CCR2 presence on these cells and also its low levels on DC suggest a role for this receptor in APC migration, localization, and interaction with T cells within the LN, as suggested previously (20), and strengthen the concept of Randolph et al. (41) that peripheral tissue monocytes that become DC readily migrate to the LN. Of note, the low CCR2 levels observed on PHLN DC indicate that down-regulation of this receptor in maturing DC cells is not an all-or-none phenomenon (20). However, persistence of this receptor on these cells may be due to the presence of DC that are not fully mature, because only about one-third of DC from PHLN expressed molecules associated with DC maturation.

Little is known in humans of the immune events occurring in the regional LN after Mtb infection. Therefore, we studied the effect of Mtb Ag (PPD and ManLAM) on the expression of costimulatory and CCR molecules by the monocyte/macrophage/DC populations. Mtb Ag did not affect expression of costimulatory molecules or CCR by any of the cell populations studied. These results contrast with previous reports that show up-regulation of these molecules by Mtb on monocyte-derived DC (28, 31) and monocyte-derived macrophages (31). Discrepancy between these results may be due to the different origin of the cells, i.e., blood monocyte-derived macrophages/DC vs PHLN, or simply that APC from PHLN respond differently to Mtb than blood-derived cells. In this regard, we reported previously that human peripheral blood MNC are more likely to be affected by the modulatory effects of Mtb Ag than cells from spleen and PHLN (43, 44).

An important finding of this work was that PPD and ManLAM down-regulated CCR2 expression on the three APC populations, suggesting that CCR2 may be important in affecting migration of these APC in response to Mtb not only at the inflammatory foci but also within secondary lymphoid tissues. Inducing down-regulation of CCR2 on APC, especially monocytes and DC, could be a mechanism used by Mtb to hamper the emigration of DC-maturing monocytes to and within LN, thus inhibiting APC-T cell interactions and therefore the development of an effective immune response. An important role for CCR2 in response to Mtb has been suggested previously (17–19, 45). Interestingly, LPS did not significantly affect expression of CCR2, even though CCR2 down-regulation on human blood monocytes by LPS has been reported previously (46, 47).

Mtb Ag did not affect CCR7 expression. This contrasts with a previous report showing that live virulent Mtb up-regulated CCR7 expression on murine bone marrow-derived DC but not macrophages (21). Differences in the models used, the source of cells, and the type of Ag stimulation may account for the contradictory
findings. However, it is important to highlight that CCR7 expression was already high on all APC from PHLN before Ag stimulation. LPS increased CCR7 expression on all APC from these tissues, although such an increase was statistically significant only for DC. CCR7 up-regulation by LPS on peripheral blood monocyte-derived DC has been also observed by others (48).

The capacity of PPD and ManLAM to modulate chemokine production was also studied. We found that PPD Ag induced chemokine production, whereas ManLAM was completely incapable of doing so. Importantly, chemokine induction by PPD was specific, because only MCP-1/CCL2 was produced in significant amounts when compared with unstimulated or ManLAM-treated cells. Although PPD also induced levels of MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5, and MDC/CCL22, the differences were not significant.

Induction of MCP-1/CCL2 by Mtb or its products on cells from both humans and mice has been described before (49, 50). More recently, MCP-1/CCL2 production by human monocytes and monocyte-derived macrophages in response to Mtb was reported (51), and high levels of MCP-1/CCL2 were detected in bronchoalveolar lavage fluid from TB patients (9). In addition, monocytes from TB patients produced higher levels of MCP-1/CCL2 in response to heat-killed Mtb than healthy PPD controls (3). The previous reports clearly establish a role for Mtb in inducing MCP-1/ CCL2 production by lymphoid cells. Our data clearly confirm the previous findings but, importantly, MCP-1/CCL2 induction was analyzed on cells from previously unexplored tissues. The specific modulatory effect of Mtb Ag on MCP-1/CCL2/CCR2 molecules constitutes a new research target for the study of the immune response to Mtb infection. These findings are supported by recent work in mice that define a role for CCR2 in macrophage and DC migration and localization within the LN in a protective immune response against Mtb (17) and L. major (20). Of note, although PPD and ManLAM both down-regulated CCR2, only PPD enhanced MCP-1/CCL2. Because the binding of MCP-1/CCL2 to CCR2 has been implicated in its down-regulation (42), these results suggest that receptor-ligand binding is not the only CCR2 regulatory mechanism or that other ligand(s) different from MCP-1/CCL2 are induced by ManLAM.

Noteworthy, ManLAM failed to induce CC chemokine production, which contrasts with previous reports that associate ManLAM with chemotactic activity on human peripheral lymphoid cells (12, 52). A previous study showed inhibition of IL-12 by ManLAM on blood-derived DC (53) and, because IL-12 has been associated with CC-chemokine production (54), it is possible that down-regulation of IL-12 by ManLAM accounts for its lack of capacity to induce CC-chemokines on MNC from PHLN. However, neither IL-12p40 nor IL-12p70 were detected under any type of stimulation. Indeed, neither form of IL-12 was detected even after stimulation with LPS, which induced significant amounts of MIP-1α/CCL3, MIP-1β/CCL4, and RANTES/CCL5. This lack of production of IL-12 was not due to a lack of sensitivity in the detection of this cytokine, because the assay used detected IL-12p70 and also IL-12p40 after the stimulation of peripheral blood-monocyte-derived dendritic cells with LPS plus CD40L/CD154 and IFN-γ (our unpublished data). However, we could not completely exclude the possibility that levels of IL-12 undetectable by our ELISA (i.e., <7 pg/ml) could induce production of these CC chemokines.

IL-10 is produced mainly by macrophages, and it down-regulates Th1 responses through the inhibition of IL-12 production (31). Although IL-10 could explain the undetectable IL-12 and subsequent lack of CC chemokine production in response to ManLAM, this appears unlikely because MNC from PHLN did not produce detectable levels of IL-10 after Ag stimulation. Therefore, extrapolation of the immune response to Mtb by cells from blood to that of PHLN may not be straightforward. Other negative regulatory molecules such as TGF-β induced on monocytes by ManLAM could explain the incapacity of ManLAM to enhance CC chemokine production (55).

Contrary to ManLAM, LPS significantly enhanced production of MIP-1α/CCL3, MIP-1β/CCL4, and RANTES/CCL5, although not that of MCP-1/CCL2. Apart from a previous study in blood-derived monocytes (49) that also showed the incapability of LPS to induce MCP-1/CCL2, most of the studies published to date, both in vitro and in vivo, showed induction of MCP-1/CCL2 by LPS (56–58). One study showed that human blood-derived DC respond differently to TLR2 and TLR4 in terms of chemokine and cytokine production, with TLR4 preferentially inducing IP-10 and IL-12p35 and TLR2 preferentially inducing IL-12p19 (59). However, mechanisms of MCP-1/CCL2 induction in PHLN may be independent of TLR. In line with this hypothesis, MCP-1/CCL2 release by mouse splenocytes induced by invasive Listeria monocytogenes was MyD88 independent (60).

To obtain insight into the complex MCP-1/CCL2 regulatory mechanisms, we analyzed the predominant Mtb Ag-induced cytokine microenvironment in cultured MNC from PHLN. PPD induced IFN-γ whereas LPS induced IL-6. Because MCP-1/CCL2/CCR2 interaction has been associated with Th1-type immune responses (17, 19), induction of IFN-γ by PPD suggests that this cytokine may play an important role, at least in PHLN, for MCP-1/CCL2 production. It is intriguing that PPD failed to induce significant levels of MIP-1α/CCL3, MIP-1β/CCL4, and RANTES/CCL5, which contrasts with previous publications that associate the synthesis of these chemokines with Th1-type immune responses (61–63). Still more intriguing was the finding that LPS induced both of these CC chemokines and IL-6, raising the possibility of a role for IL-6 in modulating MIP-1α/CCL3, MIP-1β/CCL4, and RANTES/CCL5 in PHLN.

In summary, we characterized, for the first time, different APC from human LN, and their response to Mtb Ag in terms of chemokine receptors, costimulatory molecule expression, and chemokine/cytokine production. Results indicate that Mtb Ag exert fine and specific modulation of MCP-1/CCL2 and its receptor, CCR2, on cells from LN of the hilar lung, which are the peripheral lymphoid tissues most likely involved in Mtb Ag presentation and the initiation of an effective immune response in TB.

Acknowledgments
We acknowledge the kind contribution of surgeons Dr. Alvaro Velásquez and Dr. Jorge Gutierrez from the Transplant Unit at the University Hospital San Vicente de Paúl, Medellín, Colombia, and the families of cadaveric donors for providing access to the lymphoid tissues. We are grateful to Catherine Cifuentes from the unit of flow cytometry at the Facultad de Medicina, Universidad de Antioquia, and Liliana Sauvedra and Dr. Iairo Tovar at Universidad Javeriana, Bogotá, Colombia for technical assistance, and to Rina Patel and Piedad Cardona for administrative assistance. We also thank Natalia Rendón for her invaluable help in finding clinical and demographic information of donors, and Dr. Rajko Reljic for helpful discussions and critical reading of this manuscript.

Disclosures
The authors have no financial conflict of interest.

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