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Role of Monocyte Chemotactic Protein-1/CC Chemokine Ligand 2 on γδ T Lymphocyte Trafficking during Inflammation Induced by Lipopolysaccharide or *Mycobacterium bovis* Bacille Calmette-Guérin[1]

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γδ T lymphocytes are involved in a great variety of inflammatory and infectious responses. However, the mechanisms by which γδ T lymphocytes migrate to inflamed sites are poorly understood. In this study we investigate the role of monocyte chemotactic protein (MCP)-1 in regulating γδ T cell migration after LPS or *Mycobacterium bovis* bacille Calmette-Guérin (BCG) challenge. LPS-induced γδ T cell influx was significantly inhibited by either pretreatment with dexamethasone or vaccinia virus Lister 35-kDa chemokine binding protein, vCKBP, a CC chemokine neutralizing protein, suggesting a role for CC chemokines in this phenomenon. LPS stimulation increased the expression of MCP-1 mRNA and protein at the inflammation site within 6 h. It is noteworthy that LPS was unable to increase MCP-1 production or γδ T cell recruitment in C3H/HeJ, indicative of the involvement of Toll-like receptor 4. γδ T cells express MCP-1 receptor CCR2. Pretreatment with anti-MCP-1 mAb drastically inhibited LPS-induced in vivo γδ T cell mobilization. Indeed, MCP-1 knockout mice were unable to recruit γδ T cells to the pleural cavity after LPS stimulation, effect that could be restored by coadministration of MCP-1. In addition, BCG-induced γδ lymphocyte accumulation was significantly reduced in MCP-1 knockout mice when compared with wild-type mice. In conclusion, our results indicate that LPS-induced γδ T lymphocyte migration is dependent on Toll-like receptor 4 and sensitive to both dexamethasone and CC chemokine-binding protein inhibition. Moreover, by using MCP-1 neutralizing Abs and genetically deficient mice we show that LPS- and BCG-induced γδ T lymphocyte influx to the pleural cavity of mice is mainly orchestrated by the CC chemokine MCP-1. ***The Journal of Immunology*****, 2003, 171: 6788–6794.**

The γδ T lymphocyte constitutes a minor proportion of the T cell subsets (1–5%) that circulate in the blood and peripheral organs of most adult animals. Despite being a minor class, γδ T cells are enriched in the body epithelia, such as skin, lung and intestine, where they can represent up to 50% of T cells. γδ T lymphocytes are able to recognize and respond to several stimuli, such as viral, bacterial, protozoal, and tumoral Ags. A role in host defense has been attributed to γδ T lymphocytes because increased numbers of these cells are found in inflammatory and infectious sites, including Gram-negative bacterial infections (1, 2).

Lymphocyte trafficking is a multistep process controlled by adhesive interactions between lymphocytes and the activated vascular endothelium, as well as by the production of chemoattractants (3). It has been widely demonstrated that chemokines exert an important role in chemotaxis of selective cell populations. Indeed, chemokines are key molecules in the regulation of T lymphocyte homing to secondary lymphoid organs and trafficking to inflamed tissues. The differential expression of chemokines and their receptors dictates the selectivity of T cell subsets recruitment (4, 5). Although an important body of knowledge has been accumulated about chemokines and chemokine receptors in regulating the migration of αβ T lymphocytes, little information is available concerning their roles in γδ T lymphocyte migration and activation. Recently, the patterns of chemokine receptor expression and function in γδ T lymphocytes started to be unveiled. It was demonstrated that blood γδ T lymphocytes express various receptors for chemokines, including higher levels of the CC chemokine receptors CCR1–3 and CCR5 than αβ T lymphocytes (6, 7). Moreover, functional studies demonstrated that purified human γδ T cells migrate in response to CC chemokines in vitro (8).

The chemokine network that contributes to the selective homing and accumulation of γδ T cells into mucosal and lymphoid tissues was recently investigated. Wilson and colleagues (9) provided evidence for functional differences in chemokine sensitivity and chemokine receptor expression among γδ T cell subsets. $CD8^+$ γδ T cells with mucosal tropism, but not $CD8^-$ γδ T cells that accumulate at sites of inflammation, express high levels of CCR7 and migrate toward CC chemokine ligand 21. In addition, CCR9 is highly expressed by small intestine intraepithelial γδ T cells and plays an important role in the homing of these cells (10). However,

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the mechanisms that coordinate γδ T lymphocyte migration to inflamed sites are not fully understood.

We have previously shown that the intrathoracic (i.t.)[4] injection of LPS induces a significant increase of T lymphocyte numbers in the pleural cavity of mice (11), mainly represented by the γδ T lymphocyte subset (12). A putative direct stimulatory effect of LPS on T cells is a matter of controversy. Although it has been demonstrated that γδ T lymphocytes can respond to LPS stimulation directly (13, 14), it is more likely that mediators secreted by macrophages indirectly orchestrate γδ T lymphocyte accumulation.

In the current study, we investigated the mechanisms involved in γδ T cell migration to the inflammatory site induced by LPS. Our results indicate that LPS-induced γδ T lymphocyte migration is dependent on the Toll-like receptor (TLR)*4* and sensitive to both dexamethasone and CC chemokine-binding protein inhibition. Moreover, by using monocyte chemotactic protein (MCP)-1 neutralizing Abs and genetically deficient mice we show that LPS- and BCG-induced γδ T lymphocyte influx to the pleural cavity of mice is mainly orchestrated by the CC chemokine MCP-1.

Materials and Methods

Animals

C57BL/6 and C3H/HeN mice were obtained from the Fundação Oswaldo Cruz Breeding Unit, and C3H/HeJ mice were obtained from Universidade Federal Fluminense (Rio de Janeiro, Brazil). MCP-1 knockout (KO) mice (15) were obtained from Children's Hospital, Harvard Medical School (Boston, MA) and bred at the Laboratory of Applied Pharmacology, Far-Manguinhos (Fundação Oswaldo Cruz, Rio de Janeiro, Brazil) experimental animal facility. Animals were caged with free access to food and fresh water in a room at 22–24°C and a 12 h light-dark cycle in the Department of Physiology and Pharmacodynamic experimental animal facility until used. Animals weighing 20–25 g both male and female were used. All protocols were approved by the Fundação Oswaldo Cruz animal welfare committee.

Abs and reagents

LPS (from *Escherichia coli* serotype 0127:B8), PBS, *o*-phenylenediamine dihydrochloride, and phosphate citrate buffer were purchased from Sigma-Aldrich (St. Louis, MO). Dexamethasone (Decadron) was obtained from Merck (Rio de Janeiro, Brazil). BCG (*M. bovis*) was kindly provided by Fundação Ataulfo de Paiva (Rio de Janeiro, Brazil). Vaccinia virus Lister 35-kDa chemokine binding protein (vCKBP) was generated by Dr. J. A. Symons (Sir William Dunn School of Pathology, University of Oxford, Oxford, U.K.) and kindly provided by Dr. T. J. Williams (Leukocyte Biology Section, Imperial College, London, U.K.) (16). Purified neutralizing anti-MCP-1 mAb and recombinant murine MCP-1 were from R&D Systems (Minneapolis, MN). FITC-conjugated hamster IgG anti-murine CD3 and PE-conjugated hamster IgG anti-murine γδ TCR mAbs were all purchased from BD PharMingen (San Diego, CA). Goat polyclonal IgG anti-murine CCR2 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor 488 protein labeling kit was obtained from Molecular Probes (Eugene, OR).

Pleurisy

Pleurisy was induced under anesthesia by an i.t. injection of LPS (250 ng/cavity) or BCG (100 μg/cavity, ~3.5×10^5 CFU) diluted in sterile PBS to a final volume of 100 μl. Control group received an i.t. injection of 100 μl of sterile PBS. At specific time points after the stimuli, animals were killed by an excess of carbon dioxide and their thoracic cavities were rinsed with 1 ml of saline containing heparin (10 UI/ml).

Treatments

Animals received an i.p. injection of anti-murine MCP-1 mAb (10 μg/cavity) or an i.t. injection of dexamethasone (10 μg/cavity) in a final volume of 100 μl, 30 min or 1 h before stimulation, respectively. In another set of experiments, animals received a 10 pmol dose of vCKBP diluted in the same solution with the stimulus, and incubated for 5 min at 37°C before i.t. injection.

Leukocyte counts

Total leukocyte counts were made in Neubauer chamber, under an optical microscope, after dilution in Türk fluid (2% acetic acid). Differential counts of mononuclear cells, neutrophils, and eosinophils were made using stained cytospins (Cytospin 3; Thermo Shandon, Pittsburgh, PA) by the May-Grünwald-Giemsa method. Counts are reported as numbers of cells per cavity.

Immunofluorescent staining and flow cytometric analysis

Cells recovered from pleural cavities (10^6/ml) were labeled with the appropriate concentration of FITC- or PE-conjugated mAbs to CD3, γδ TCR for 30 min at 4°C after incubation with rat serum to block nonspecific binding. Cells were then washed with PBS/0.1% azide and surface marker analysis was performed using the CellQuest program in a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). Anti-CCR2 was previously stained with Alexa Fluor 488 dye in accordance with instructions provided by the manufacturer. For CCR2 staining, γδ TCR labeled cells were fixed and permeabilized by saponin 0.05%. At least 10^4 lymphocytes were acquired per sample. All data were collected and displayed on a log scale of increasing green and red fluorescence intensity. Data were presented as two-dimensional dot plots. To determine the percentages of the lymphocyte subpopulations, $CD3^+$ T lymphocytes were specifically gated. Counts are reported as numbers of cells per cavity after multiplying the percentage of γδ T lymphocyte by the total number of pleural leukocytes.

ELISA

Levels of MCP-1 in the cell-free pleural fluid were evaluated by ELISA in accordance with the manufacturer's instructions (Quantikine; R&D Systems).

RNase protection assay

Multiple chemokine mRNA analysis was performed in mice leukocytes recovered from pleural cavities 6 h after i.t. injection of LPS or vehicle. Cells were resuspended in the Ultraspec total RNA isolation reagent (Biotecx Laboratories, Houston, TX) at 10^6 cells/ml, and total RNA was purified as recommended by the manufacturer. mRNA expression was evaluated with the multiple chemokine RNase protection assay mCK-5 multiprobe template set, according to BD PharMingen. A total of 10 μg of cellular RNA were applied per lane.

Statistical analysis

Data were reported as the mean ± SEM and were analyzed statistically by means of ANOVA followed by Student-Newman-Keuls test or the Student *t* test with the level of significance set at $p \leq 0.05$.

Results

CC chemokine neutralization by vCKBP inhibits LPS-induced γδ T lymphocyte accumulation in the pleural cavity of mice

The pleural inflammation induced by LPS in mice is characterized by an intense leukocyte accumulation; this augmentation is due to a marked increase in neutrophil numbers within 6 h followed by significant increases in mononuclear cell and eosinophil numbers at later time points (12, 17). LPS i.t. injection (250 ng/cavity) induced a significant rise in γδ T cells in the pleural cavity of C57BL/6 mice within 6 h, and at maximum within 24 h (Fig. 1*A*). The recruitment of monocytes, neutrophils, and eosinophils to the pleural cavity induced by LPS occurs through indirect mechanisms via the release of inflammatory mediators and is sensitive to glucocorticoid treatment (18, 19). Likewise, Fig. 1*B* shows that i.t. pretreatment with dexamethasone (10 μg/cavity) 1 h before stimulation abolished LPS-induced γδ T lymphocyte migration into the pleural cavity of C57BL/6 mice at 24 h (Fig. 1*B*).

Administration of the virally encoded CC chemokine binding protein vCKBP was used to investigate the involvement of CC chemokines in the γδ T cell accumulation induced by LPS in the pleurisy model. It has been described that vCKBP binds with high

[4] Abbreviations used in this paper: i.t., intrathoracic; BCG, bacille Calmette-Guérin; vCKBP, vaccinia virus Lister 35-kDa chemokine binding protein; MCP, monocyte chemotactic protein; WT, wild-type; KO, knockout; TLR, Toll-like receptor.

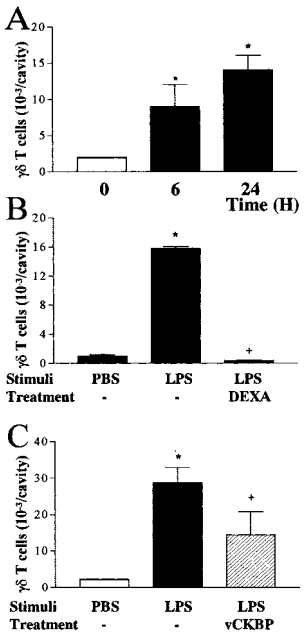

FIGURE 1. *A*, γδ T lymphocyte accumulation in the pleural cavity of C57BL/6 mice 6 h and 24 h after LPS i.t. injection (250 ng/cavity). *B*, Effect of dexamethasone (10 μg/cavity) i.t. pretreatment on the LPS-induced γδ T cell accumulation after 24 h. *C*, Effect of vCKBP (10 pmol/cavity) on the LPS-induced γδ T cell accumulation after 24 h. Animals received a concomitant i.t. injection of vCKBP and the stimulus. Results are expressed as the mean ± SEM from at least six animals. Statistically significant differences between control and agonist stimulated groups are indicated by an asterisk, and + represent differences between treated and untreated groups.

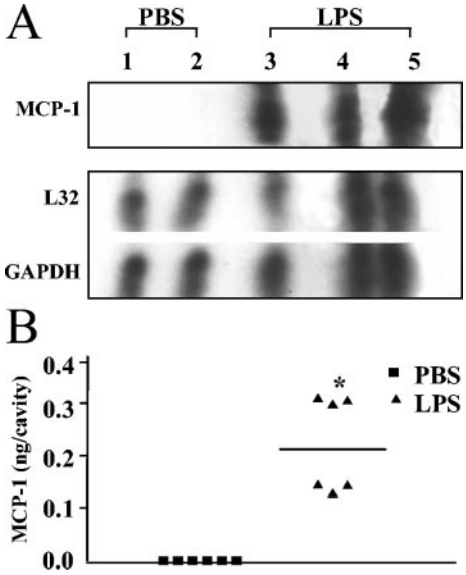

FIGURE 2. LPS-induced MCP-1 expression. *A*, MCP-1 mRNA expression in total leukocytes recovered from mice pleural cavities 6 h after PBS or LPS stimulation. MCP-1 expression was determined by a multiprobe RNase protection assay. RNA from PBS-injected mice (*lanes 1* and *2*) and LPS-injected mice (*lanes 3-5*) are shown. The constitutively expressed genes mL32 (murine ribosomal protein L32) and mGAPDH (murine GAPDH) were used as control. A total of 10 μg of RNA were applied per lane. *B*, MCP-1 protein levels in pleural fluid obtained from PBS- or LPS-injected mice, determined by ELISA in pleural washes recovered at 6 h following stimulation, compared with control groups injected with PBS. Results are expressed as the mean ± SEM from six animals. Statistically significant differences (*, $p \leq 0.05$) are shown.

affinity to virtually all known CC chemokines, but not CXC chemokines, blocking their biological activity by competitive inhibition of chemokine interaction with their respective cellular receptors on target cells in vitro as well as in in vivo inflammatory reactions (16, 17, 20, 21). The concomitant treatment with vCKBP significantly inhibited the LPS-induced γδ T cell influx into mice pleural cavities observed 24 h after stimulation (Fig. 1*C*), suggesting the involvement of CC chemokines in this phenomenon.

LPS induces MCP-1 expression and secretion

CC chemokines, including MCP-1, play important roles in regulating the recruitment of T cell subsets into inflammatory sites (22–26). LPS-induced pulmonary inflammation is a potent stimulus for the production of a wide variety of chemokines (17, 27, 28). The RNase protection assay (mCK-5; BD PharMingen) was performed with RNA recovered from cells obtained from the pleural cavity of C57BL/6 mice 6 h after i.t. LPS stimulation (250 ng/cavity). As previously described (17), animals treated with LPS presented increased expression of mRNA for MCP-1 compared with the control group (Fig. 2*A*). LPS-induced MCP-1 expression and release was confirmed by ELISA. As shown in Fig. 2*B*, LPS induced a significant increase in MCP-1 protein level detected in the 6 h cell-free pleural effluent washes from C57BL/6 mice (Fig. 2*B*). Of note, dexamethasone pretreatment inhibited MCP-1 production induced by LPS stimulation (from 422 pg/ml in nontreated to 142 pg/ml in dexamethasone-treated animals, $p < 0.01$). These results suggest that the inhibition of LPS-induced γδ T cell migration by dexamethasone was probably due to the inhibition of MCP-1 production.

LPS fails to induce γδ T cell migration in C3H/HeJ mice

The LPS receptor complex of leukocytes is composed of TLR4, MD-2, and CD14. LPS signaling through TLR4 was shown to induce or to up-regulate a variety of gene products, including the expression of chemokines and cytokines (29, 30). The involvement of TLR4 in LPS-induced MCP-1 expression and γδ T cell recruitment was analyzed using C3H/HeJ mice. The C3H/HeJ mouse strain is characterized by hyporesponsiveness to LPS (31), due to defective expression of TLR4 (32, 33). The i.t. injection of LPS (250 ng/cavity) failed to induce total leukocyte or γδ T lymphocyte accumulation in the pleural cavity of C3H/HeJ mice 24 h after the stimulation, whereas the same treatment led to cell influx in the control strain C3H/HeN (Fig. 3, *A* and *B*). It is noteworthy that LPS was unable to increase MCP-1 production within 6 h (Fig. 3*C*) or within 24 h (data not shown) in C3H/HeJ. Taken together, these results support the involvement of TLR4 in LPS-induced γδ T cell mobilization and MCP-1 synthesis.

γδ T lymphocytes express CCR2

MCP-1 binds to and activates leukocytes preferentially through the CCR2. To investigate a direct role for MCP-1 in LPS-induced γδ T cell recruitment, we sought first to determine whether γδ T cells express the CCR2. The CCR2 expression in pleural γδ T leukocytes recovered from control or LPS-stimulated animals was determined by two-color flow cytometry. As shown in Fig. 4*A*, CCR2 is expressed on γδ T lymphocytes recovered from the pleural cavity of PBS stimulated C57BL/6 mice. LPS stimulation induced a significant increase in the total number of γδ T lymphocytes expressing CCR2 recovered from the pleural cavity within 6 h (Fig. 4*B*) although CCR2 expression level per cell was unchanged. The

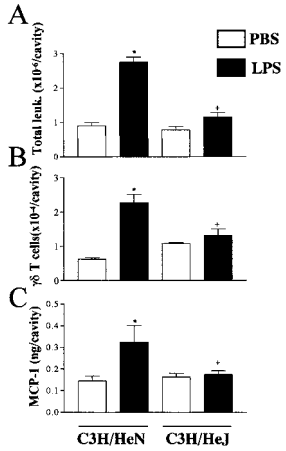

FIGURE 3. Total leukocyte (*A*) and γδ T lymphocyte (*B*) numbers recovered from the pleural cavity of C3H/HeN and C3H/HeJ mice 24 h after LPS stimulation (250 ng/cavity). *C*, MCP-1 levels determined by ELISA in pleural washes recovered at 6 h after LPS stimulation, compared with control groups injected with PBS. Results are expressed as the mean ± SEM from four to six animals. Statistically significant differences (*, $p \leq 0.05$) are shown. +, Differences between C3H/HeN and C3H/HeJ values.

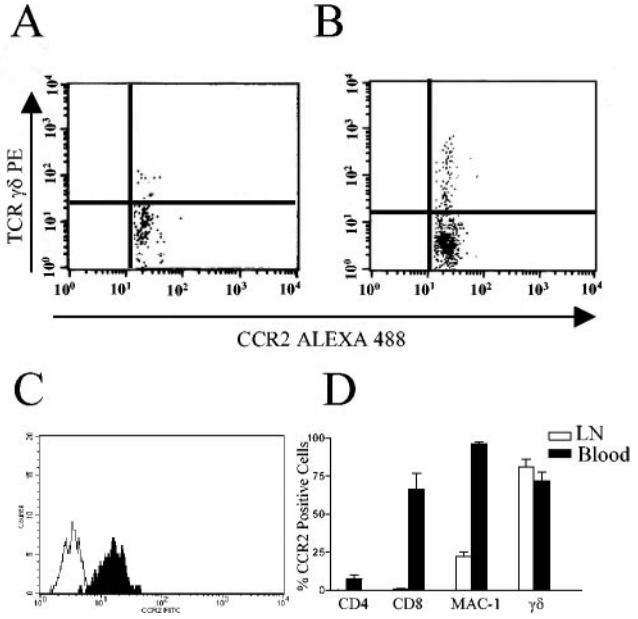

FIGURE 4. Expression of CCR2 on γδ T lymphocytes. Pleural leukocytes were recovered 24 h after PBS (*A*) or LPS (250 ng/cavity) (*B*) i.t. injection. Leukocytes were stained with PE-labeled anti-TCR-γδ plus Alexa 488-labeled anti-CCR2. Results from one representative animal of six mice per group are shown. *C*, Representative histogram shows expression of CCR2 (filled histogram) on peripheral blood $\gamma\delta^+/CD3^+$ T lymphocyte in comparison to the isotype control (open histogram). Results from one representative animal of three mice per group are shown. *D*, Expression of CCR2 on peripheral blood or lymph node in $MAC\text{-}1^+$ leukocytes or $CD4^+$, $CD8^+$, or $\gamma\delta^+/CD3^+$ T lymphocyte. Data represent the mean ± SEM from at least six animals.

expression pattern of CCR2 was also investigated in γδ T lymphocytes recovered from lymph nodes and peripheral blood. As shown in Fig. 4, *C* and *D*, the majority of γδ T lymphocytes obtained from peripheral blood (61–88%) or lymph nodes (71–89%) were $CCR2^+$. Confirming previous findings, only a small population of murine peripheral blood $CD4^+$ T cells (3–11%) or $CD8^+$ T cells (52–78%) was $CCR2^+$-positive, whereas monocytes stained homogeneously positive for CCR2 (94–99%).

MCP-1 accounts for γδ T lymphocyte accumulation induced by LPS

Because we demonstrated the increased expression and secretion of MCP-1 during the inflammatory reaction induced by LPS as well as the expression of the MCP-1 receptor, CCR2, in γδ T cells, we decided to investigate the involvement of MCP-1 in the recruitment of γδ T cells. We pretreated mice with a neutralizing anti-murine MCP-1 mAb (10 μg/cavity), followed by the i.t. administration of LPS (250 ng/cavity). The neutralization of MCP-1 significantly inhibited the γδ T cell accumulation observed 24 h after LPS i.t. injection in C57BL/6 mice (Fig. 5*A*). In accordance, we found that LPS injection failed to induce γδ T lymphocyte accumulation in MCP-1 KO mice (Fig. 5*B*). Moreover, LPS-induced γδ T cell recruitment in MCP-1 KO mice was restored by the addition of exogenous MCP-1, confirming the major role of this chemokine in γδ T cell migration (Fig. 5*B*). Monocyte recruitment after LPS administration was also impaired in MCP-1 KO mice (PBS, 1.75 ± 0.15 vs LPS, 1.98 ± 0.17 mononuclear cells × 10^6/cavity) different to the observed data in wild-type (WT) animals (PBS, 2.21 ± 0.43 vs LPS, 4.12 ± 0.57 mononuclear cells × 10^6/cavity, $p < 0.05$). The neutralization or the absence of MCP-1 did not inhibit the increase in eosinophil numbers triggered by LPS injection (Fig. 5, *C* and *D*, respectively). However, the concomitant administration of MCP-1 lead to a potentiation in LPS-induced pleural eosinophil accumulation (Fig. 5*D*), suggesting that although MCP-1 is not essential to eosinophil recruitment, it may have a modulatory role in this phenomenon.

γδ T cell migration and MCP-1 synthesis induced by BCG

Accumulating evidence points to a role for γδ T lymphocytes in the immunity to intracellular pathogens. In animal models of mycobacterial infection, a drastic expansion of the γδ T cell population is observed in the blood and tissue sites (34–36). In this study we used a model of pleural mycobacteria infection, which allows an evaluation of early inflammatory cell recruitment induced by

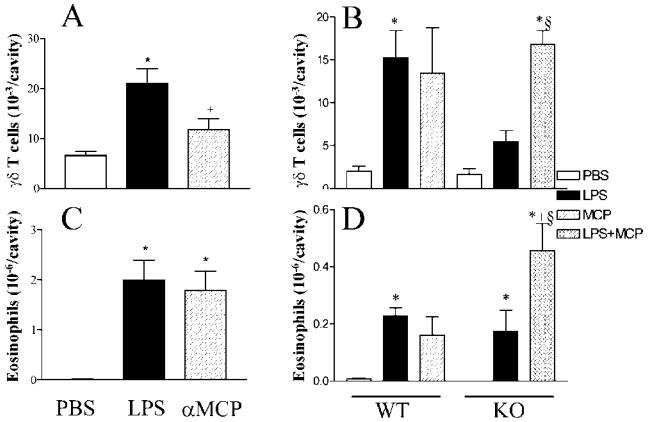

FIGURE 5. Effect of anti-MCP-1 mAb i.p. treatment (10 μg/cavity) on the 24 h γδ T lymphocyte (*A*) and eosinophil (*C*) accumulation induced by LPS stimulation. Effect of LPS (250 ng/cavity) and/or MCP-1 (500 ng/cavity) i.t. injection shown on γδ T lymphocyte (*B*) and eosinophil (*D*) migration in wild-type (WT) or MCP-1 knockout (KO) mice. LPS, MCP-1, or their combination were i.t. injected and analysis was performed within 24 h. Statistically significant differences (*, $p \leq 0.05$) between nonstimulated and stimulated animals, and significant differences (+) between stimulated and treated groups or between WT and KO animals are shown. Differences between LPS and LPS+MCP-1 (§) are also shown. Data represent the mean ± SEM from at least six animals.

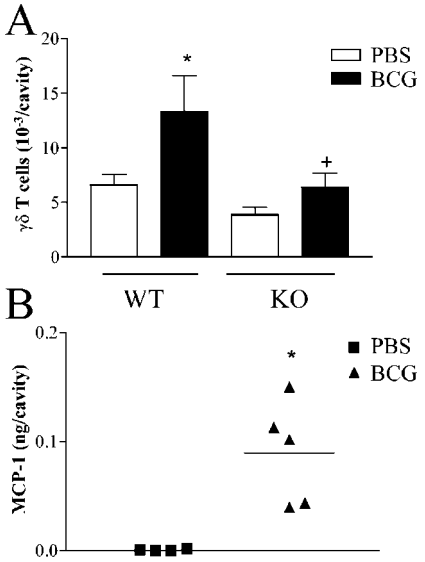

FIGURE 6. *A*, γδ T lymphocyte numbers in wild-type (WT) and MCP-1 knockout (KO) mice after BCG (100 μg/cavity) i.t. injection. Statistically significant differences (*, $p \leq 0.05$) between nonstimulated and stimulated animals, and significant differences (+) between WT and KO mice are shown. *B*, MCP-1 protein levels determined by ELISA in pleural washes recovered at 6 h after BCG stimulation were compared with control groups injected with PBS. Results are expressed as the mean ± SEM from six animals. Statistically significant differences (*, $p \leq 0.05$) are shown.

infection (37, 38). Within 24 h of infection, significantly increased numbers of neutrophils, eosinophils, monocytes, and lymphocytes are recovered from the pleural cavity (37, 38). Accordingly, the i.t. injection of BCG (100 μg/cavity) was also able to induce a significant influx of γδ T lymphocytes to the pleural cavity of WT as compared with a PBS-injected control group within 24 h (Fig. 6*A*). Although MCP-1 KO mice showed increased numbers of γδ T lymphocytes in the pleural cavity after BCG stimulation when compared with nonstimulated MCP-1 KO mice, γδ cell numbers were significantly lower than those found in stimulated WT mice (Fig. 6*A*). In addition, BCG stimulation also induced MCP-1 synthesis within 6 h, as determined by ELISA in WT animals (Fig. 6*B*). These data indicate an important role for MCP-1 in γδ T cell recruitment during mycobacteria infection. It should be noted that BCG-induced recruitment of $CD4^+$ T cells, $CD8^+$ T cells, and monocytes to the pleural cavity was also impaired in MCP-1 KO (data not shown). However, recruitment of eosinophils and neutrophils induced by BCG infection was not different when MCP-1 KO or WT mice were compared (data not shown).

Discussion

Although several studies have described the involvement of γδ T lymphocytes in inflammatory and infectious reactions, the mechanisms responsible for γδ T cell mobilization are still poorly understood. To clarify the mechanisms by which the γδ T cells migrate, we used an in vivo model of pleurisy induced by the i.t. injection of LPS in mice. We and others have previously reported that LPS induced a marked increase in γδ T lymphocytes in celomic cavities of mice (12, 14, 39). Interestingly, we have previously demonstrated that the i.t. administration of LPS in BALB/c *nu/nu* mice also induces an increase in γδ T lymphocyte numbers, suggesting that the γδ T cells accumulating in the pleural cavity in this reaction originate from a population with extrathymic development (12).

Recent observations suggest that LPS-induced γδ T cell influx is not a direct effect of LPS upon this T cell subset (17). Several reports have shown that different cell populations are activated and migrate into inflammatory sites after LPS stimulation via an indirect effect that involves chemoattractant proteins newly synthesized mainly by macrophages (11, 40). Indeed, we have previously demonstrated that in the LPS-induced pleurisy model, the recruitment of neutrophils and eosinophils is not a direct effect of the LPS on these cell populations (41).

Members of the TLR family are the main cellular receptors for LPS, and signaling through TLRs are the prototypical activator of NF-κB leading to generation of cytokines and other proinflammatory responses (29, 30). Macrophages are highly responsive to LPS activation (producing inflammatory mediators) in a TLR4-dependent mechanism (42). Recently, Mokuno and colleagues (14) demonstrate that γδ T cells express mRNA for TLR2, but not TLR4, and γδ T cells respond directly to *E. coli* lipid A through TLR2. To better address whether LPS was acting indirectly to recruit γδ T cells into the pleural cavity we used C3H/HeJ mice that express truncated and functionally deficient TLR4. Here we demonstrate that LPS was unable to attract γδ T cells to the inflammatory site in C3H/HeJ mice. Moreover, the LPS-induced MCP-1 generation was also abolished in these animals. Such observations strongly suggest an involvement of TLR4 in this phenomenon, corroborating previous observations that TLR4-expressing macrophages are the key cells orchestrating leukocyte mobilization in response to LPS (30, 42).

It is well known that glucocorticoids modulate protein synthesis by different cell populations. Dexamethasone treatment exerts a potent inhibitory effect on cytokine/chemokine generation, consequently inhibiting the mobilization of inflammatory cells (44). We have previously demonstrated that in LPS-induced pleurisy, the local pretreatment with dexamethasone inhibits eosinophil mobilization to the inflammatory site, and this in turn seems to be a consequence of the inhibition of a neo-synthesized eosinophilotactic protein (18). Smith and Herschman (20) identified cDNAs for seven LPS-induced genes for chemokines that were attenuated by glucocorticoid, including MCP-1. Several authors have demonstrated that dexamethasone inhibits MCP-1 gene expression by various cell populations; including airway smooth muscle cells (45), eosinophils (46), and PBMC (47). In addition, dexamethasone treatment decreased MCP-1 mRNA expression and protein release in the peritoneal fluid in IL-1-stimulated mice (48). Dexamethasone pretreatment inhibited MCP-1 production and also significantly inhibited LPS-induced γδ T cell migration to the pleural cavity, suggesting that γδ mobilization depends on the neo-synthesis of a chemoattractant factor.

Indeed, LPS is a potent inflammatory stimulus that triggers the production of several inflammatory chemoattractant mediators, including CC chemokines. CC or β chemokines constitute one of the chemokine subfamilies that attracts mainly eosinophils, monocytes and lymphocytes (4). Johnston and collaborators (27) have shown increased pulmonary cytokine and chemokine mRNA levels after inhalation of LPS in C57BL/6 mice. Our group has also observed that the i.t. administration of LPS up-regulates the local production and release of different CC chemokines (17). In this work, we demonstrate an important role for CC chemokines in the LPS-induced γδ T lymphocyte migration, because treatment with vCKBP, a pan CC chemokine neutralizer, significantly inhibited this phenomenon. Of note vCKBP is selective for CC chemokines and does not inhibit CXC chemokines. This selectivity was clearly confirmed because LPS-induced neutrophil influx, which is a CXC-dependent phenomenon, was not inhibited by vCKBP treatment (data not shown).

Among the CC chemokines produced in the mice pleural cavities after LPS challenge (17), we can point RANTES and MCP-1 as potent T lymphocyte chemoattractants (8). Indeed, we confirm in this study that the i.t. injection of LPS induces increased expression of MCP-1 mRNA and protein in the pleural cavities of stimulated mice. The MCP-1 receptor CCR2 has been well characterized in human and murine $\alpha\beta$ T lymphocytes (49, 50). Recently, the expression of CCR2 by human blood $\gamma\delta$ T cells was described (6). However, the expression of CCR2 in murine $\gamma\delta$ T has not been previously examined. Our results demonstrated that resident pleural $\gamma\delta$ T cells in naive animals express CCR2, and the population of $CCR2^+$ $\gamma\delta$ T cells was enhanced after LPS stimulation. Moreover, we demonstrated that a large population of nonstimulated peripheral blood $\gamma\delta$ T lymphocyte, the likely source of recruited cells, is CCR2 positive.

It has been described that MCP-1 is a chemoattractant to human $\gamma\delta$ T lymphocytes (8, 51), thus indicating that CCR2 is functionally active in these cells. However, no report has addressed the relevance of MCP-1 to $\gamma\delta$ T lymphocyte mobilization in in vivo pathophysiological conditions. We demonstrate that the pretreatment with a neutralizing anti-MCP-1 mAb was able to inhibit LPS-induced $\gamma\delta$ T cell accumulation in the mouse pleural cavity, suggesting an important role for MCP-1 in $\gamma\delta$ T cell mobilization. In vivo neutralization of RANTES failed to inhibit the increase in $\gamma\delta$ T lymphocyte numbers observed after LPS i.t. injection (data not shown), discounting a role for this chemokine in $\gamma\delta$ T cell influx after LPS challenge.

More conclusive data concerning the role of MCP-1 in LPS-induced $\gamma\delta$ T cell mobilization was obtained with MCP-1 KO mice. Targeted disruption of MCP-1 has demonstrated that MCP-1 plays an essential and nonredundant role in monocyte recruitment in in vivo models of inflammation (15) including leukocyte recruitment to the pleural cavity (43). Although MCP-1 KO mice have the same basal levels of resident mononuclear cells and T lymphocytes when compared with WT mice, they are unable to mount a cellular response to LPS of the same intensity as WT animals. Our results show that MCP-1 KO mice failed to recruit monocytes and $\gamma\delta$ T lymphocytes into their pleural cavities after LPS administration. Moreover, LPS-induced $\gamma\delta$ T lymphocyte recruitment in MCP-1 KO mice was restored by exogenous administration of MCP-1. Together, these findings indicate that MCP-1 has a requisite role in the recruitment of not only monocytes/macrophages but also of $\gamma\delta$ T lymphocytes to inflamed tissues.

We have previously demonstrated that the LPS-induced eosinophil accumulation in the mice pleural cavity was impaired by previous depletion of $\gamma\delta$ T cells or resident macrophages (12). In this study we show that although MCP-1 neutralization or gene deletion inhibited LPS-induced $\gamma\delta$ T cell accumulation in the mice pleural cavity it did not affect the eosinophil influx induced by LPS. As shown in Figs. 1 and 3–6, there is a population of $\gamma\delta$ T cells (although smaller than in LPS-treated animals) in the pleural cavity of nonstimulated animals. It has been previously shown that $\gamma\delta$ T cells express high levels of TLR2 and can be directly stimulated to proliferate and to produce IFN-γ either by LPS or lipid A (14). These data suggest that LPS may activate resident $\gamma\delta$ T cells to produce cytokines that would regulate macrophages to secrete eosinophil activating factors. Interestingly, we observed a potentiation in LPS-induced pleural eosinophil accumulation by the concomitant administration of MCP-1, suggesting that although MCP-1 is not essential to eosinophil recruitment it may have a modulatory role in this phenomenon.

It is suggested that $\gamma\delta$ T lymphocytes participate in the first line of defense against microorganisms. They increase in numbers during the course of infection with a wild array of pathogens, including bacteria, virus, and parasites (52–54). The reactivity of $\gamma\delta$ T lymphocytes to mycobacteria has attracted considerable attention to the involvement of this T subset in the pathophysiology of tuberculosis. Several reports demonstrated that $\gamma\delta$ T lymphocytes are activated by *M. tuberculosis* and BCG in vitro (34, 55) and also accumulate in the peritoneal cavity and lymph nodes after in vivo stimulation (34, 36). In agreement with these data, we demonstrate in this study that the i.t. administration of BCG induced the accumulation of $\gamma\delta$ T lymphocytes in the pleural cavity of C57BL/6 mice. We next evaluated the role of MCP-1 in $\gamma\delta$ T cell recruitment during BCG infection. Likewise to LPS-induced pleurisy and confirming previous reports of clinical and experimental pleural mycobacterial infection (56–58), we observed a significant increase in pleural MCP-1 following the infection with BCG. Moreover, MCP-1 implication on $\gamma\delta$ T cell migration to the inflammatory site does not seem to be stimulus-specific, as BCG-induced $\gamma\delta$ T cell mobilization was drastically reduced in MCP-1-deficient animals.

In conclusion, we provide evidence that LPS indirectly recruits $\gamma\delta$ T lymphocytes to inflamed sites in a mechanism dependent of TLR4 receptor and sensitive to both dexamethasone and CC chemokine binding protein. Using neutralizing Abs and genetically deficient mice we have implicated MCP-1 as the CC chemokine involved in $\gamma\delta$ T lymphocyte recruitment. Finally, the finding that MCP-1 plays an important role in $\gamma\delta$ T lymphocyte mobilization induced by both LPS and BCG suggests that this chemokine is generally required for the migration of $\gamma\delta$ T cells to inflammatory sites.

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