# *Leishmania*-Induced Cellular Recruitment during the Early Inflammatory Response: Modulation of Proinflammatory Mediators

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**This study investigated whether *Leishmania* species, the etiologic agent of cutaneous *(Leishmania major)* and visceral *(Leishmania donovani)* leishmaniasis, could differentially elicit early inflammatory events in vivo correlating with the subsequent development of their reciprocal pathogenesis. By use of the murine air pouch system, injection of *Leishmania* led to a rapid and transient accumulation of a mixed population of leukocytes, and *L. major* recruited 31-fold more leukocytes than did controls, compared with 7-fold more leukocytes for *L. donovani*. *L. major* promastigotes were better than *L. donovani* promastigotes at inducing proinflammatory cytokine secretion and chemokine gene expression in pouch exudates. *L. major* infection elicited significantly increased chemokine receptor gene expression, compared with *L. donovani* infection. Collectively, the data reveal that *L. major* is a strong inducer of the early inflammatory response, compared with *L. donovani,* and suggest that such an immunologic event potentially could restrain this parasite to the inoculation site, favoring the development of local swelling and cutaneous lesions.**

The intracellular protozoan parasites *Leishmania* species are transmitted to humans and various mammals through the bites of sand flies. Fifteen million people worldwide are infected with this parasite, and >400,000 new cases are reported annually [1]. *Leishmania* infections are the cause of several pathologic processes, ranging from cutaneous and mucocutaneous lesions to deadly visceral disease. *Leishmania donovani* usually causes redness at inoculation sites before migrating via the lymphatic and blood systems toward the liver and spleen. On the other hand, *Leishmania major*–infected persons develop skin lesions and ulcerations due to chronic inflammation and cell infiltration into the dermal layers. This inflammatory reaction usually limits the pathogen to the inoculation site. Modulation of proinflammatory cytokines by *Leishmania* species in vitro and in vivo has been reported elsewhere [2–7]. *Leishmania* infections induce tumor necrosis factor (TNF)–$\alpha$ production [5, 6], and interleukin (IL)–1$\beta$ generation is abolished by *L. donovani* infection in vitro and in vivo [2, 3, 7], whereas it is induced by *L. major* infection [4]. Together, these observations establish that various species of *Leishmania* can differentially modulate important mediators of the inflammatory response, the proinflammatory cytokines. In addition, it is now well documented that these cytokines play an important role in the modulation of chemokines, which are recognized for their pivotal role in cell recruitment and the promotion of the inflammatory reaction [8].

Chemokines are polypeptides of low molecular mass (8–11 kDa) that have been grouped, according to the position of the cysteine residues in the N-terminus portions, into 4 subfamilies: CXC, CC, C, and $CX_3C$ [9–12]. Although each type attracts competent cells to the inflammatory site, their actions are specific to particular cellular groups [13]. For example, members of the CXC class, such as IL-8, growth-related oncogene (GRO)–$\alpha$ (KC, murine homologue), and platelet factor–4, act mainly on neutrophils, whereas members of the CC class, such as monocyte chemotactic protein (MCP)–1 (JE, murine homologue), monocyte inflammatory protein (MIP)–1$\alpha$, MIP-1$\beta$, and RANTES (regulated on activation normal T cell–expressed and –secreted protein), act on a larger group of cells, including monocytes, basophils, eosinophils, and lymphocytes, but not neutrophils. Lymphotactin, the only C chemokine, acts solely on specific subgroups of B and T lymphocytes [14]. Recently, a $CX_3C$ branch member termed fractalkine was discovered and was reported to attract monocytes, neutrophils, and T lymphocytes [11, 12], although this remains controversial. Chemokines are secreted in a stimulus-specific manner from a variety of cell types, including leukocytes, fibroblasts, epithelial cells, and endothelial cells [15–18]. As mentioned above, chemokines represent some of the most important mediators of inflammation and have been reported to operate in inflammatory responses toward numerous infectious agents [19–22].

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Some studies have reported that, in leishmaniasis, chemokine expression follows infection; indeed, in vitro infection of murine macrophages with *L. major* led to a rapid and transient expression of MCP-1 and IL-8 [23, 24]. Biopsy samples from patients with localized cutaneous leishmaniasis contained important amounts of MCP-1, compared with samples from diffuse cutaneous leishmaniasis, in which expression of MIP-1$\alpha$ was predominant [25]. A recent report demonstrated the induction of some chemokine genes (e.g., MCP-1, MIP-1$\alpha$, and interferon-inducible protein [IP]–10) in livers of BALB/c and SCID mice infected with *L. donovani* [26]. Nevertheless, in vivo comparisons of the pattern of chemokine expression during cutaneous and visceral leishmaniasis have yet to be done. Because *Leishmania* infections can progress toward the development of species-specific pathologic disorders, we postulate that the early inflammatory events occurring at the site of inoculation may reflect and direct the type of species-specific pathogenesis that will develop at a later time. Thus, in the present study, we were interested to determine, by use of a murine air pouch system, whether *Leishmania* could induce chemokine production by macrophages in vitro and, more important, in vivo. Of utmost interest, we want to establish whether the development of cutaneous leishmaniasis can be associated with a species-specific chemokine profile and inflammatory response.

## Materials and Methods

*Materials.* Isotopes were obtained from Mandel Scientific. Six- to eight-week-old male BALB/c mice, 20–30 *g* body weight, were purchased from Charles River. Lipopolysaccharide (LPS; *Escherichia coli,* serotype O111:B4) was purchased from Sigma. Endotoxin-free PBS was purchased from Gibco BRL.

*Macrophage cell line.* B10R macrophages [27] were grown in Dulbecco's MEM (Gibco BRL) supplemented with 10% fetal bovine serum (Hyclone Laboratories), 5% L-glutamine (Gibco BRL), and 5% penicillin-streptomycin (Gibco BRL). Cells were maintained at 37°C in a humidified 5% $CO_2$ atmosphere and transferred biweekly.

*Parasite cultures.* Promastigotes of *L. donovani* 1S2D and *L. major* Friedlin were grown at room temperature and transferred weekly in SDM-79 culture medium (Gibco BRL), as described elsewhere [28, 29]. B10R macrophages were infected in vitro at a parasite:cell ratio of 20:1.

*Air pouch and leukocyte migration.* Air pouches were raised on the dorsum of BALB/c mice, as described elsewhere [30, 31]. All stimulations were done by injection of *L. major* and *L. donovani* stationary-phase promastigotes ($5 \times 10^6$ parasites in 1 mL of endotoxin-free PBS) into the air pouches. Control mice were injected with endotoxin-free PBS (negative control) and LPS (100 ng/mL; positive control). At various times after intrapouch inoculation (0, 3, 6, 12, 24, and 48 h), 5–10 animals per experimental group were lethally anesthetized, and the pouch contents were washed with a total of 5 mL of endotoxin-free PBS to collect leukocytes of the exudates. Recruited cells were counted directly with a hemocytometer. Differential cell counts were done microscopically on cytospin preparations stained with Diff-Quik (Baxter Healthcare). Collected supernatants, recruited leukocytes, and dissected air pouch linings were kept at −20°C for further evaluation.

*RNase protection assay.* Chemokine gene expression induced by *Leishmania* parasites in vitro and in vivo was monitored by an RNase protection assay (mCK-5 Riboquant kit; Pharmingen) [31], allowing the simultaneous determination of a large number of these proinflammatory molecules (i.e., lymphotactin, RANTES, eotaxin, MIP-1$\alpha$, MIP-1$\beta$, MIP-2, IP-10, MCP-1, and T cell activation protein [TCA]–3). In brief, the commercial probe was labeled with [$^{32}$P] UTP, as specified by the manufacturer. Next, 10 $\mu$g of total RNA was prepared as described elsewhere [32] and was solubilized in 8 $\mu$L of hybridization buffer, and the labeled probe ($3 \times 10^5$ cpm/$\mu$L) was added to each RNA sample. Finally, the incubated samples were subjected to RNase treatment. Next, a proteinase K cocktail was mixed with RNase digests, followed by a phenol/chloroform extraction before RNA precipitation. The air-dried samples were heated, cooled on ice, and loaded onto a 5% acrylamide gel. The gel was dried and exposed to a BIOMAX-MR film at −70°C. A sample of the probe (1000–2000 cpm/lane) was diluted in loading buffer to serve as a size standard.

*ELISA.* Previously collected in vivo air pouch exudate samples were centrifuged at 250 *g* for 10 min to remove cells. ELISA was used to detect TNF-$\alpha$ and IL-1$\beta$ in the specific supernatants, as specified by the manufacturer (Endogen).

*Statistical analysis.* Statistically significant differences between groups were determined by analysis of variance followed by Fisher's least significant difference test with use of SAS software (version 6.07; SAS Institute). $P < .05$ was considered to be statistically significant. All in vitro and in vivo experiments were done at least 2–3 times if not stated otherwise.

## Results

*Ability of* Leishmania *species to elicit leukocyte recruitment in vivo.* Leukocyte recruitment was shown to be induced rapidly after parasite inoculation within the air pouch (figure 1), reaching a maximal peak at 6 h after infection and then declining over a 48-h period. Although inoculation of PBS did not induce leukocyte accumulation, inoculation of *L. major* led to an important transient accumulation of leukocytes (31-fold increase over PBS). Similarly, LPS inoculation led to an important influx of leukocytes (20-fold increase over PBS) into the air pouches, but less than in *L. major* infection at 6 h after inoculation. Of interest, *L. donovani* proved to be a poor inducer of leukocyte accumulation (7-fold increase over PBS). Neutrophils, monocytes, eosinophils, and lymphocytes were enumerated at different time points after parasite inoculation. As shown in figure 2, after stimulation with LPS, a great number of neutrophils (figure 2*A*) and smaller numbers of macrophages (figure 2*B*), eosinophils (figure 2*C*), and lymphocytes (figure 2*D*) were recruited. More than 87% of cells recruited in response to LPS were neutrophils (figure 3). However, injection of *Leishmania* led to the recruitment of mixed cell populations (figure 2), comprising ~53% neutrophils, 25% monocytes/macrophages, and

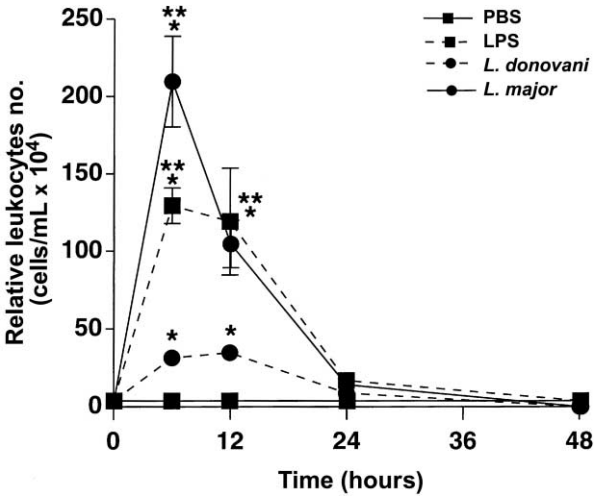



**Figure 1.** Number of leukocytes accumulating in air pouch exudate in response to *Leishmania* inoculation. Air pouches were raised on the backs of 6–8-week-old male BALB/c mice. One milliliter of endotoxin-free PBS with or without lipopolysaccharide (LPS; 20 $\mu$g), *Leishmania major,* or *Leishmania donovani* ($10^7$ promastigotes) was injected into pouches, and exudate was collected at 6, 12, 24, and 48 h after inoculation. Leukocytes were enumerated microscopically. Data are mean ± SD of ⩾5 mice. $*P < .01$, experimental mice vs. PBS control mice ($n = 5$). $**P < .01$, *L. major*– or LPS-inoculated mice vs. *L. donovani*–inoculated mice ($n = 5$).

22% eosinophils with few lymphocytes 6 h after infection (figure 3). As shown in figure 2, *L. major* was a more potent recruiter of all cell types than was *L. donovani* injection at 6–12 h after infection. Analysis of cell populations recruited in response to both species of *Leishmania* demonstrated that neutrophils, although lower in proportion than in PBS-injected controls, made up the main population (figure 3) in the early period after injection and declined thereafter (data not shown). The important population of eosinophils noted at 6 h after infection also declined thereafter. Conversely, the already important proportion of macrophages (figure 3) and, to a lesser extent, lymphocytes (figure 3) increased with time to reach 50% and 15%, respectively, by 48 h after infection (data not shown). Thus, although stimulation by both LPS and *L. major* are recognized to result in the onset of inflammation, *L. major* induced a more intense accumulation of leukocytes than did LPS. These in vivo experiments establish that *L. major* could differentially modulate cell recruitment during early inflammatory responses, compared with *L. donovani.*

*Accumulation of TNF-$\alpha$ and IL-1$\beta$ proteins in the air pouch after* Leishmania *inoculation.* To examine whether differential *Leishmania*-induced cell recruitment could be paralleled by the accumulation of the proinflammatory cytokines TNF-$\alpha$ and IL-1$\beta$ in the air pouches, we used ELISA to evaluate the extracellular production of these cytokines in the exudate supernatants. As reported in figure 4, the supernatants collected 6 h after inoculation from air pouches revealed that *L. major* was the more potent inducer of TNF-$\alpha$ (5-fold more) and IL-1$\beta$ (21-fold more) than was *L. donovani.* These observations digress from in vitro experiments showing that IL-1$\beta$ was not inducible by *L. donovani,* although *L. donovani*– and *L. major*–induced expression of TNF-$\alpha$ has been reported elsewhere [2–7].

Leishmania-*induced chemokine gene expression in vitro and in vivo.* Modulation of CXC and CC chemokine mRNA expression in response to *Leishmania* infection was tracked by use of an RNase protection assay. As shown in figure 5*A,* chemokine gene expression of RANTES, MIP-1$\alpha$, MIP-1$\beta$, MIP-2, IP-10, MCP-1, and TCA-3 in the exudate cells was increased by *L. major* or *L. donovani* after their injection within air pouches. There was no induction of lymphotactin gene expression at either site, nor was there a difference in TCA-3 and MIP-2 expression in exudate cells following infection by either species. A different pattern of induction of other chemokine gene expression was observed depending on the strain of *Leishmania.* Indeed, *L. major* injection led to a greater up-regulation of RANTES, MIP-1$\alpha$, MIP-1$\beta$, IP-10, and MCP-1 in exudate cells than did *L. donovani* infection. The expression of MIP-1$\alpha$, MIP-1$\beta$, and MCP-1 was amplified slightly in response to *L. major,* compared with *L. donovani,* whereas the greatest difference was observed for RANTES and IP-10. In contrast, there was a modest enhanced induction of eotaxin, MIP-1$\alpha$, MIP-1$\beta$, and MIP-2 by *L. donovani* in the pouch lining tissue, compared with *L. major* infection (figure 5*A*). This greater accumulation of chemokine transcripts in the air pouch lining of *L. donovani*–infected mice suggests, in part, that inflammatory leukocytes have been attracted to the inoculation site but were unable to transmigrate through the epithelium of the pouch. MCP-1 and TCA-3 gene expression was similarly induced by both species of *Leishmania.* Thus, this last set of data establishes that *Leishmania* species were able to induce a broad range of chemokine gene expression in vivo and that *L. major* can induce several of these chemokines to a greater extent than can *L. donovani* infection. In contrast, experiments performed in vitro (figure 5*B*) revealed that stationary-phase promastigotes of *L. major* and *L. donovani* can transiently and similarly induce macrophage chemokine gene expression (i.e., MIP-1$\alpha$, MIP-1$\beta$, MIP-2, and MCP-1). Maximal chemokine gene expression was detected 2 h after stimulation and rapidly declined thereafter. These data indicate that *Leishmania* species can selectively induce a subset of chemokine genes, but a strong capacity to differentially induce them was not observed in vitro, with the exception of the MIP-2 chemokine gene, which seems to be more expressed in *L. major*–infected macrophages than in *L. donovani*–infected cells.

*Effect of* L. major *and* L. donovani *infection on specific CC chemokine receptor (CCR) gene expression in air pouch exudate cells.* Because we obtained evidence that *L. major* is a better inducer of proinflammatory molecules than is *L. donovani,* we became interested in evaluating whether *L. major* could

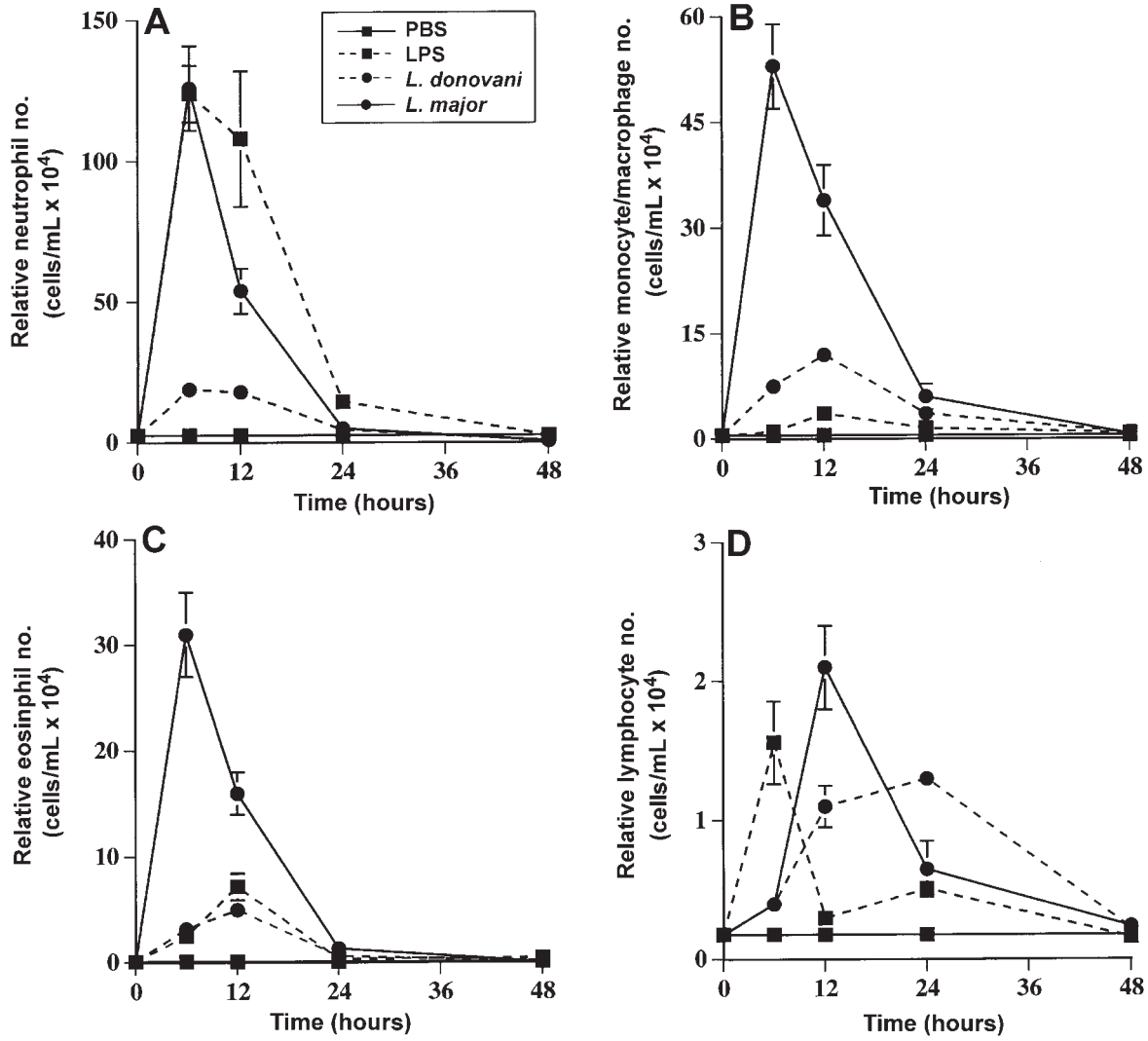



**Figure 2.** Total numbers of neutrophils *(A),* monocytes/macrophages *(B),* eosinophils *(C),* and lymphocytes *(D)* recruited in pouch exudate in response to lipopolysaccharide (LPS), *Leishmania major,* or *Leishmania donovani* inoculation. Stimulations were done as described in the legend to figure 1. Exudate cells were placed onto microscope slides by use of cytospin and stained with Diff-Quik solution (Dade Behring); proportions of neutrophils, monocytes/macrophages, eosinophils, and lymphocytes/300 cells were enumerated; and relative cell numbers were calculated from total exudate leukocytes. Data are mean ± SD of ⩾5 mice. Differences in experimental cell counts over PBS control were all significant ($P < .01$; $n = 5$) at time point 6–12 h. The capacity of *L. major* to recruit all leukocyte types was significantly greater ($P < .01$; $n = 5$) than that of *L. donovani.*

also modulate CC chemokine receptor gene expression differently. As shown in figure 6, expression of CCR3, CCR5, and CCR2 was induced to a greater magnitude by *L. major* than by *L. donovani.* There was ~2 times more induction of these transcripts after injection of *L. major,* as shown by densitometric analysis. This observation is of importance and suggests that the greater chemokine receptor gene expression induced by *L. major* infection could explain, in part, why leukocytes were recruited to the inoculation site more readily than they were by *L. donovani* infection.

## Discussion

Leukocytes play an important role in host defense, and their recruitment into infected tissue might be a crucial event in *Leishmania*-induced inflammatory reactions leading to skin lesion development observed in cutaneous leishmaniasis. To better understand the physiologic mechanisms responsible for the development of this type of pathologic disorder, we evaluated the modulation of diverse inflammatory events and leukocyte recruitment during the early phase of 2 different *Leishmania* in-

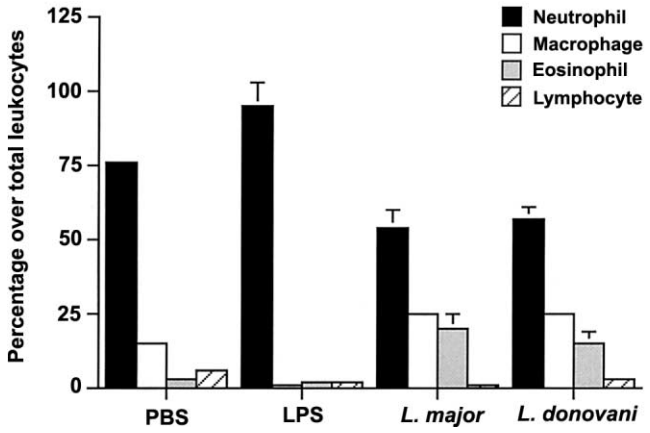



**Figure 3.** Percentages of neutrophils, monocytes/macrophages, eosinophils, and lymphocytes recruited in pouch exudate in response to lipopolysaccharide (LPS), *Leishmania major,* or *Leishmania donovani* 6 h after inoculation. Stimulation and analysis were done as described in the legends to figures 1 and 2. Data are mean ± SD of ⩾5 mice. Differences observed for neutrophils, monocytes/macrophages, and eosinophils in LPS-, *L. donovani*–, and *L. major*–inoculated mice were significant ($P < .01$; $n = 5$), compared with PBS control.

fections leading to the development of cutaneous or visceral leishmaniasis.

In vivo studies making use of a murine air pouch system enabled us to simulate the inoculation site of the sand fly in a closed environment, provided a suitable space for the induction of inflammation by a variety of agents, and allowed for a subsequent analysis of the inflammatory parameters and mediators [33]. Injection of *L. major* and *L. donovani* into air pouches caused a rapid and transient increase in exudate cellular content during the first 48 h. The kinetic of leukocyte accumulation confirmed and extended previous data obtained from other studies [34–36] with respect to a rapid and transient increase of neutrophils and eosinophils, which peaked in the early phase of cutaneous leishmaniasis, and an increasing macrophage response predominating thereafter. Some morphologic aspects of the parasite once inside the granulocytes suggested that intracellular killing may have been occurring (data not shown). Thus, neutrophils and eosinophils may possess leishmanicidal activity that restrains parasite progression at the initial step of the infection, as has been demonstrated elsewhere [36]. The monocyte/macrophage population, which is recognized to regulate the infection by its ability to potentially phagocytose and kill the parasite if it can avoid *Leishmania*-mediated functional inhibition [37], mainly served as a reservoir for parasite replication. It is interesting to note that *L. major* was the more potent recruiter of leukocytes into air pouches, which suggests that this important leukocyte accumulation could help reduce parasitic load and restrict spreading of *Leishmania* infection, although permitting the development of a characteristic skin lesion. On the other hand, because *L. donovani* infection did not strongly induce leukocyte recruitment into the pouch, this possibly could indicate that this parasite has developed a strategy that minimizes early inflammatory responses, permitting unrestrained progression within the host.

In fact, lipophosphoglycan, the most abundant glycolipid on the cell surface of *Leishmania* promastigotes [38], possesses potent cell inhibitory activity [39]. Results reported by Lo et al. [40], who found that lipophosphoglycan of *L. donovani* blocked LPS-mediated expression of E-selectin, intercellular adhesion molecule–1, and vascular cell adhesion molecule–1 on endothelial cells, suggest that the discrepancy we found between the capability of *L. donovani* and *L. major* parasites to induce transendothelial migration of monocytes potentially could be explained by the inhibitory action of *L. donovani* lipophosphoglycan. Nevertheless, lipophosphoglycan injection per se (10–25 $\mu M$) of both *Leishmania* species (provided by S. Turco, University of Kentucky, Lexington) did not show a significant difference in their capacity to recruit leukocytes (authors' unpublished data). Nonetheless, the total number of leukocytes recruited in response to lipophosphoglycans reached rates was comparable ($20 \times 10^4$, 6 h after inoculation) to those in response to *L. donovani* injection, as we report here. Therefore, although *L. donovani* lipophosphoglycan has been reported to affect in vitro molecules involved in transendothelial migration, we excluded their involvement in the triggering of cellular recruitment.

In keeping with their central role as regulators of the proinflammatory response, TNF-$\alpha$ and IL-1$\beta$ are released from activated neutrophils and monocytes/macrophages for direct stimulation of the expression of genes in these cells [8]. In the present study, we show that injection of *Leishmania* leads to an early production of these proinflammatory cytokines; however, *L. major* infection induces a greater release in the pouch exudate.

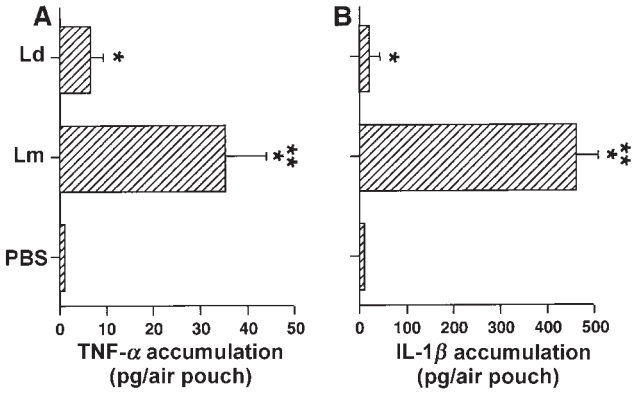
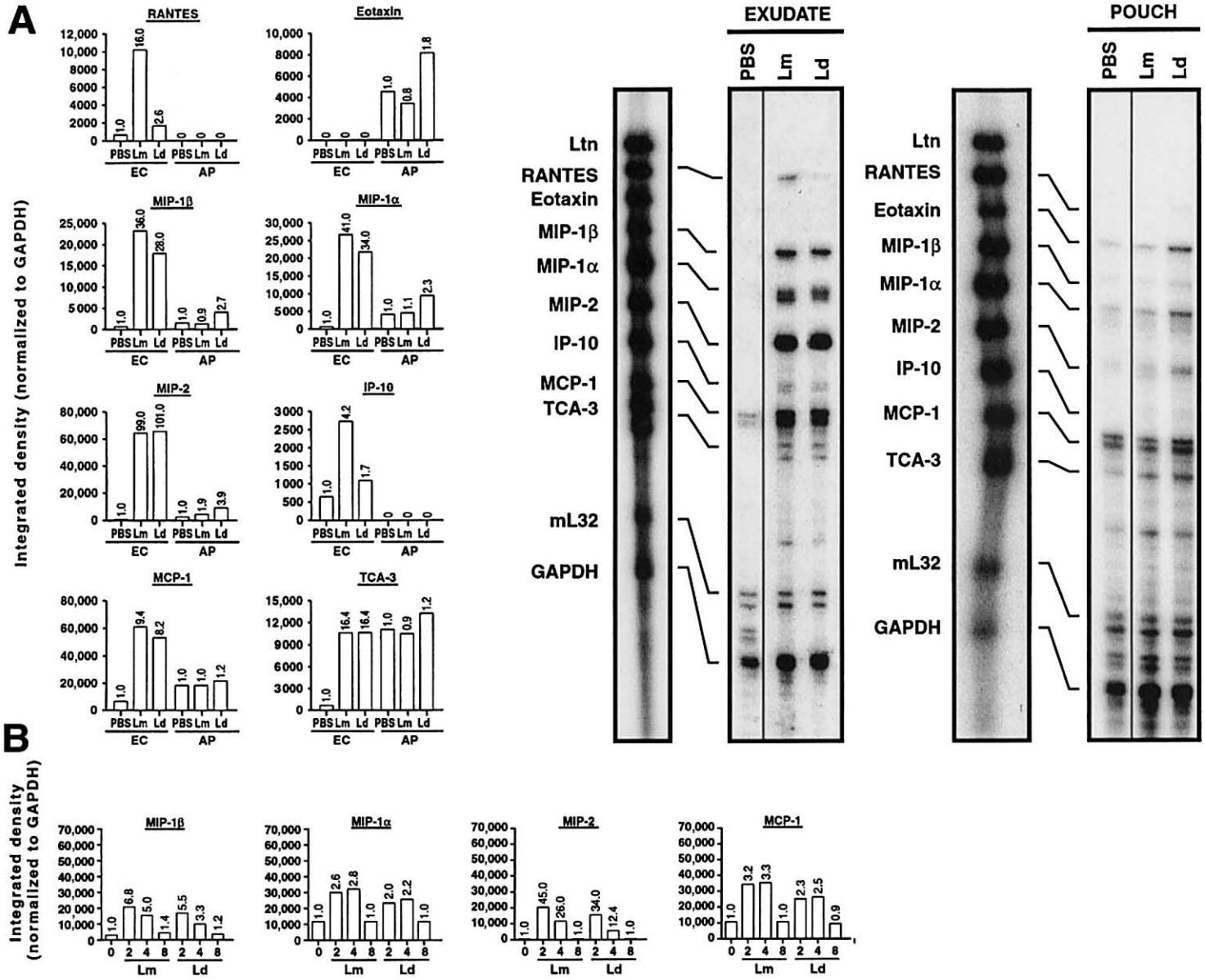



**Figure 4.** Production of cytokines tumor necrosis factor (TNF)–$\alpha$ *(A)* and interleukin (IL)–1$\beta$ *(B)* in air pouch exudates in response to *Leishmania donovani* (Ld) or *Leishmania major* (Lm) infections. Animals were injected into their air pouches as described in the legend to figure 1. Exudates were collected 6 h after inoculation and centrifuged, and resulting supernatants were subjected to ELISA. Data are mean ± SE of 4 animals. *$P < .01$, experimental mice vs. PBS control mice ($n = 5$). **$P < .01$, *L. major*– vs. *L. donovani*–inoculated mice ($n = 5$).


A
RANTES
Eotaxin
MIP-1β
MIP-1α
MIP-2
IP-10
MCP-1
TCA-3
Integrated density (normalized to GAPDH)
PBS Lm Ld PBS Lm Ld
EC AP
EXUDATE
POUCH
PBS
Lm
Ld
Ltn
RANTES
Eotaxin
MIP-1β
MIP-1α
MIP-2
IP-10
MCP-1
TCA-3
mL32
GAPDH
B
Integrated density (normalized to GAPDH)
MIP-1β
MIP-1α
MIP-2
MCP-1
0 2 4 8 2 4 8
Lm Ld

The smaller amount of this cytokine measured in pouches of mice infected with *L. donovani* is consistent with the results reported by Hatzigeorgiou et al. [7]. Another study has demonstrated a decrease in production of both cytokines in vitro by mononuclear cells of patients with visceral leishmaniasis during the acute disease [41]. Previous reports that IL-1$\beta$ production is increased in murine peritoneal macrophages infected with *L. major* [4] support our observation that *L. major* was a better inducer of both cytokines in pouch exudates. Because proinflammatory cytokines (TNF-$\alpha$ and IL-1$\beta$) lack chemotactic properties, they cannot directly cause leukocyte infiltration after *Leishmania* infection. On the other hand, previous studies [42, 43] have reported their implication in the induction of expression of several chemokine genes.

A diversified cellular population (neutrophils, monocytes/macrophages, and eosinophils) was recruited into the pouch exudate in response to *Leishmania;* this cellular accumulation may be associated with the pattern of chemokine gene expression. This study demonstrated that the cells of the pouch exudate recruited in response to *Leishmania* infection express RANTES (*L. major* only), MIP-1$\alpha$, MIP-1$\beta$, MIP-2, IP-10, MCP-1, and TCA-3. In addition, evaluation of the expression of these chemokines in the cells composing air pouch lining tissue demonstrated, although less impressively than cell exudates, an induction of the MIP-1$\alpha$, MIP-1$\beta$, MIP-2, MCP-1, and TCA-3 genes in response to both parasites. Of interest, the fact that chemokine gene expression was stronger in air pouch lining of *L. donovani*–infected animals suggests that transendothelial migration could have been blocked by *L. donovani* lipophosphoglycan, as suggested by Lo et al. [40]. The results regarding the chemokine profiles detected in mouse pouches might explain, at least in part, the mixed population recruited in response to *Leishmania.* Indeed, chemokines induced in response to *Leishmania* are known to recruit monocytes/macrophages (RANTES, MIP-1$\alpha$, MIP-1$\beta$, IP-10, and MCP-1), neutrophils (MIP-2 and TCA-3), and eosinophils (RANTES, eotaxin, and MIP-1$\alpha$). Nevertheless, the stronger expression of RANTES and, to a lesser extent, IP-10, MIP-1$\alpha$, and MIP-1$\beta$, induced by *L. major,* compared with *L. donovani,* in pouch exudates could play an important role in the greater leukocyte accumulation in pouch exudates, resulting in the amplification of inflammation at extravascular sites. Studies have reported a potential importance in cutaneous inflammation for RANTES. Both TNF-$\alpha$ and IL-1$\beta$ proinflammatory cytokines are known to stimulate dermal fibroblasts to express RANTES [44]. Moreover, this chemokine seems to play a key role in delayed-type hypersensitivity reactions, in which the monocyte/macrophage lineage was found to represent the major contribution to RANTES gene expression [45]. This up-regulation of RANTES expression by *L. major* may be impli-

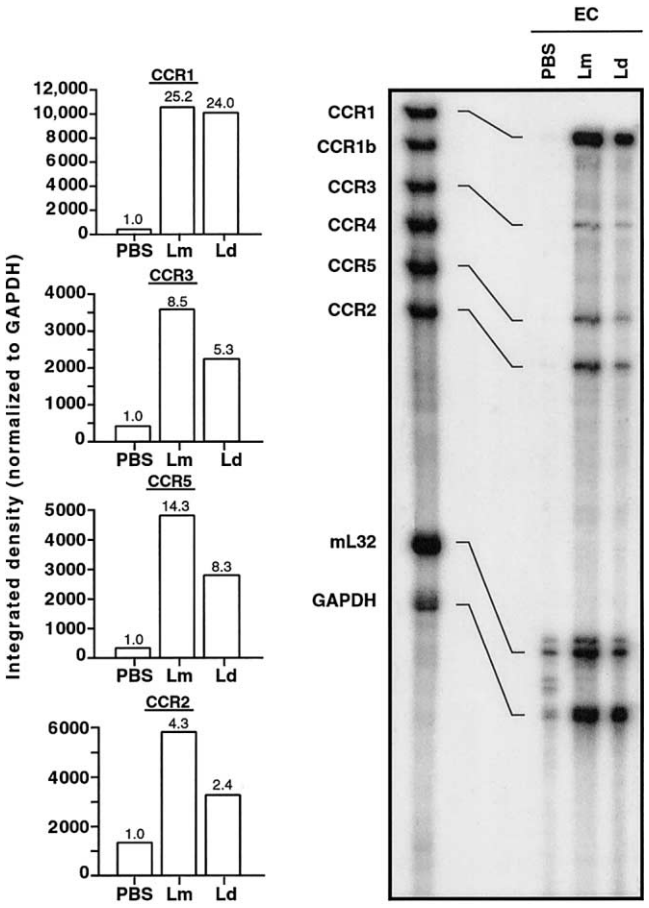



**Figure 6.** Patterns of CC chemokine receptor (CCR) gene expression by leukocyte cells recruited in response to *Leishmania donovani* (Ld) or *Leishmania major* (Lm) infection. Exudate cells (ECs) were collected 6 h after injection. CCR mRNA gene expression was monitored by RNase protection assay. Graphs represent densitometric quantification of bands from CCR mRNA normalized to glutaraldehyde-3-phosphate dehydrogenase (GAPDH) signal. mL32, internal marker.

**Figure 5.** *Leishmania*-induced chemokine gene expression in vitro and in vivo. *A,* In vivo chemokine gene expression in air pouches in response to *Leishmania* inoculation ($10^7$ promastigotes). Exudate cells (ECs) and air pouch lining tissue (AP) were collected 6 h after inoculation. Chemokine mRNA expression was monitored by RNase protection assay. *B,* In vitro chemokine gene expression in B10R murine macrophages in response to *Leishmania* infection. Cells were stimulated with *Leishmania major* (Lm) or *Leishmania donovani* (Ld) (20:1, parasite:cell ratio) for 2, 4, and 8 h. mRNA expression was monitored by RNase protection assay (data not shown). Densitometric quantification of each chemokine mRNA was normalized to glutaraldehyde-3-phosphate dehydrogenase (GAPDH; see histograms). Numbers at top correspond to fold-increase over PBS. IP, interferon-inducible protein; Ltn, lymphotactin; MCP, monocyte chemotactic protein; MIP, monocyte inflammatory protein; RANTES, regulated on activation normal T cell–expressed and –secreted protein; TCA, T cell activation protein. mL32, internal marker.

cated in the containment of the infection at the skin level. In support of this hypothesis, a previous study reported that human macrophages treated with purified recombinant human RANTES and further infected with *Trypanosoma cruzi* showed enhanced uptake and intracellular destruction of trypomastigotes [46]. Although some differences have been reported in vivo, our in vitro study showed that exposure of murine macrophages to both *Leishmania* species led to a similar pattern of chemokine expression (MIP-1$\alpha$, MIP-1$\beta$, MIP-2, and MCP-1). The rapid and transient induction of these chemokines by *Leishmania* is in agreement with the results reported by others [23, 24], who showed the expression of JE/MCP-1, KC/GRO-$\alpha$, and IL-8 in murine macrophages infected with *L. major.* However, the in vitro system did not allow for the discrimination of whether *L. major* and *L. donovani* could differentially modulate these molecules, further reinforcing the use of in vivo system to fully establish the immunologic and inflammatory mechanisms that direct the development of species-specific pathologic disorders.

Finally, our observations led us to determine whether this upregulation marked by *L. major* of RANTES could be correlated to a precise modulation of chemokine receptor expression. Our study showed that *Leishmania* induces the expression of CCR1, CCR3, CCR5, and CCR2, whereas the expression of CCR4 remained undetectable. RANTES and MIP-1$\alpha$ are known ligands for CCR1, whereas CCR3, a specific eosinophil receptor, responds to RANTES and eotaxin. CCR5 is stimulated by RANTES, MIP-1$\alpha$, and MIP-1$\beta$ and, finally, CCR2 binds to MCP-1. It is interesting to note that *L. donovani* is solely able to induce (to a lesser extent) these chemokine receptors, especially the RANTES receptor, which correlates nicely with its reduced capacity to induce leukocyte recruitment. Because lipophosphoglycan of *L. donovani* altered the migration of inflammatory cells by reducing the expression of specific adhesion molecules [44], *L. donovani* must avoid chemokine-attractive actions by altering the expression of their specific receptors or by selectively not leading to RANTES induction. In addition, because *L. major* seems to be a more powerful proinflammatory cytokine inducer than *L. donovani,* it is possible that chemokine receptor expression is modulated by these cytokines, thus allowing the circulating leukocytes to migrate further toward *L. major* inoculation sites.

Collectively, these results demonstrated that *L. major* can act on specific inflammatory mediators, leading to the recruitment of an important number of competent cells that might be responsible for their restriction at inoculation sites and implicated in the development of specific and characteristic cutaneous lesions. On the other hand, *L. donovani* seems to minimally modulate these mediators, resulting in reduced leukocyte accumulation that could favor *L. donovani* progression toward its target organs.

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