Regulation of Impaired Protein Kinase C Signaling by Chemokines in Murine Macrophages during Visceral Leishmaniasis

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The protein kinase C (PKC) family regulates macrophage function involved in host defense against infection. In the case of Leishmania donovani infection, the impairment of PKC-mediated signaling is one of the crucial events for the establishment of parasite into the macrophages. Earlier reports established that C-C chemokines mediated protection against leishmaniasis via the generation of nitric oxide after 48 h. In this study, we investigated the role of MIP-1α and MCP-1 in the regulation of impaired PKC activity in the early hours (6 h) of infection. These chemokines restored Ca2+-dependent PKC activity and inhibited Ca2+-independent atypical PKC activity in L. donovani-infected macrophages under both in vivo and in vitro conditions. Pretreatment of macrophages with chemokines induced superoxide anion generation by activating NADPH oxidase components in infected cells. Chemokine administration in vitro induced the migration of infected macrophages and triggered the production of reactive oxygen species. In vivo treatment with chemokines significantly restricted the parasitic burden in livers as well as in spleens. Collectively, these results indicate a novel regulatory role of C-C chemokines in controlling the intracellular growth and multiplication of L. donovani, thereby demonstrating the antileishmanial properties of C-C chemokines in the disease process.

Leishmania donovani is an obligate intracellular parasite which infects and replicates within mammalian macrophages. The intracellular survival of this protozoan invader within the hostile environment is manifested due to the suppression of the normal microbicidal machineries of the macrophages. During leishmaniasis, the inflammatory reactions attempting to combat the pathogenic entry occur in two stages. First, during the initial uptake and phagocytosis of promastigotes, the macrophage produces toxic free radicals, including superoxide anions (O2−) (13, 44). O2− production is catalyzed by the NADPH oxidase, a heme-containing cytochrome that contains cytosolic and membrane bound components (5, 31). After the assembly of its components, the oxidase transfers electrons from molecular oxygen, producing O2−. Exposure to O2− has been reported to be fatal to the Leishmania promastigotes (13, 42, 44, 59). Second, even after infection is established, the quiescent macrophage can be activated to kill the intracellular amastigote form of leishmania. This second antileishmanial event occurs via nitric oxide (NO) generation after activation of macrophages by gamma interferon, C-C chemokines, or tumor necrosis factor alpha along with lipopolysaccharide (7, 15, 23, 58).

Several signaling molecules have been implicated in the regulation of phagocytosis, including members of the protein kinase C (PKC) superfamily (29, 37, 41, 48). PKC is a calcium- and phospholipid-dependent serine/threonine kinase that exists as a family of different isotypes having closely related structures (18, 29, 37, 41, 48). In the case of leishmaniasis, it was previously reported that infection with L. donovani and the Leishmania-derived glycolipid lipophosphoglycan (LPG) accounted for impaired PKC activity (27, 45). PKC may also participate in the regulation of phagosome maturation, as the isoenzymes PKC-α and -β are associated with phagosomal membrane (1, 2, 11). In this context, the protozoan parasite Leishmania and Leishmania-derived LPG received a great deal of attention because both of them impair PKC-dependent signal transduction in macrophages and thus survive in the macrophage microenvironment (8, 10, 21, 22, 50, 56). Accordingly, BALB/c peritoneal macrophages infected with UR-6, an LPG-deficient attenuated leishmanial parasite (8, 43), enhanced PKC-β activity (8). Moreover, PKC plays a major role in the enhancement of respiratory burst activity and NO induction (9, 16, 19, 36). Recently, it was established that PKC-β is involved in the regulation of macrophage function involved in host defense and is a crucial factor that controls chemotaxis and the respiratory burst mechanism of phagocytes (12, 28, 32). Hence, it has been proposed that activated macrophages can destroy intracellular parasites by generating reactive oxygen species (ROS) and nitric oxide (35, 46).

Chemokines are the key molecules in recruiting immune cells by chemotaxis, which also act in leukocyte activation, inflammatory diseases, and antimicrobial mechanisms (6, 39). Monocyte chemotactic and activating factor have been shown to have a direct antiprotozoal activity for three protozoans: Toxoplasma gondii, L. donovani, and Trypanosoma cruzi (38). Recently, we demonstrated that C-C chemokines, particularly macrophage inflammatory protein 1α (MIP-1α) and macro-
phage chemotaxatractant protein 1 (MCP-1), showed antileishmanial activity via the induction of tumor necrosis factor alpha release and NO generation (7). However, C-C chemokines are also known to attract phagocytic cells both in vivo and in vitro and to induce the respiratory burst mechanism (40, 53, 54), but the precise role of chemokines in the regulation of impaired PKC signaling is yet to be addressed. In the present investigation, we explored for the first time the potential role of the C-C chemokines MIP-1α and MCP-1 in the restoration of the impaired PKC activity during visceral leishmaniasis both in vitro and in vivo, including induction of chemotaxis as well as induction of respiratory burst mechanisms in the early stages of *L. donovani* infection in macrophages.

**MATERIALS AND METHODS**

**Materials.** PKC-α, PKC-β1, PKC-βII, p67phox, and p47phox primary antibodies (rabbit polyclonal, react with mouse origin) were obtained from Santa Cruz Biotechnology. Anti-actin antibody (mouse monoclonal) was obtained from Sigma. Recombinant mouse MIP-1α and MCP-1 were purchased from R&D Systems (DNA sequences encoding the mature mouse MIP-1α or MCP-1 protein sequences were expressed in Escherichia coli; purity of >97% was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE] and visualized by silver staining; endotoxin level was determined by the limulus amebocyte lysate assay, we explored for the first time the potential role of the C-C chemokines MIP-1α and MCP-1 in the restoration of the impaired PKC activity during visceral leishmaniasis both in vitro and in vivo, including induction of chemotaxis as well as induction of respiratory burst mechanisms in the early stages of *L. donovani* infection in macrophages.

ROS detection in cells by fluorescence microscopy. Chemokine-induced ROS generation in macrophages was detected by fluorescence microscopy. Migrated cells were loaded with 6-carboxy-2',7'-dichlorodihydroxy fluorescein diacetate (DCFDA) (10 μM) for 30 min in PBS at 37°C in a 5% CO₂ environment (51). At the end of the incubation, the PBS containing DCFDA was aspirated; cells were washed twice with PBS and examined under a Leica DM LB microscope with blue excitation and emission states set at 490 and 530 nm, respectively. Fluorescence of oxidized DCFDA in cells was captured with a Retiga SR camera.

**Preparation of cell lysate.** The adherent cell population was scraped and centrifuged at 400 × g for 15 min at 4°C. The cells were then resuspended in ice-cold extraction buffer containing 50 mM Tris-HCl (pH 7.5), 50 mM EGTA, antiprotease mixture, and 50 mM β-mercaptoethanol. The contents of antipro- tease mixture are listed above (37). The macrophage-containing suspension was sonicated at 4°C and centrifuged at 4,250 × g for 10 min at 4°C, and then the supernatant was used for experiments.

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ugation on a Histopaque 1077 (Sigma) gradient and splenocytes were collected, washed, and resuspended in RPMI 1640 complete medium supplemented with 10% fetal bovine serum.

**Isolation of RNA and reverse transcriptase PCR (RT-PCR).** RNA was isolated according to the standard protocol (14, 55). Briefly, total RNA was extracted from 4 x 10⁷ splenocytes by using Trizol reagent (Sigma). Isolated total RNA was then reverse transcribed using RevertAid Moloney murine leukemia virus reverse transcriptase (Fermentas). The cDNA (GenBank accession no. X72973) encoding the PKC-ζ gene was amplified using specific primers (forward, 5'-CTCTCCCAAGTAGTGCTGGAAG-3'; reverse, 5'-GAAGGGCATGACAGATTCATCAT-3'; product size, 359 bp) designed by the reported sequences deposited with the GenBank database (8). For PKC-βI PCR amplification, the primers used were β-α, 5'-TTGTAGTGAAGTGGTGAACCGG-3'; and β-αs, 5'-CTC GGCATAAGCCTCTGCAATT-3'; and for amplification of PKC-βII, the primers used were β-α, 5'-TTGTAGTGAAGTGGTGAACCGG-3', and β-αs, 5'-TT CTGGTGTTGAGTGGAGTTGTGAAGCGG-3'. The PCR-amplified product was subsequently size fractioned on a 1% agarose gel, stained with ethidium bromide, and visualized under UV light. In parallel, the expression of the GAPDH gene in the control macrophages and parasites was evaluated using specific primers (5'-AGGTGGAGTTGTAGGAGCGG-3'; and reverse, 5'-CCTCCCAGATGGAGCTGGAAG-3'). The expected size of the PCR product was 242 bp. The PCR-amplified product was subsequently size fractioned on a 1% agarose gel, stained with ethidium bromide, and visualized under UV light. In negative-control experiments with omission of the reverse transcriptase, no PCR product was detected for either set of the PKC and GAPDH primers (data not shown).

**Real-time quantitative RT-PCR.** Real-time RT-PCR was performed on an iCycler (Bio-Rad Laboratories, Hercules, CA) with SYBR green reagent. The PCR mixture (25 μL) contained 10 pmol of each primer (the same combination of forward and reverse primers as for semiquantitative RT-PCR were used), 8 μL of water, 12.5 μL of a commercial SYBR green PCR master mixture (Sigma), and 2.0 μL of cDNA. The samples were placed in 96-well plates (Bio-Rad) that were sealed with optical sealing tape (Bio-Rad). PCR amplifications were performed by using an iCycler IQ multicolor real-time PCR detection system (Bio-Rad). The thermal cycling conditions were as follows: initial activation step (3 min at 95°C) and cycling step (denaturation for 30 s at 94°C, annealing for 30 s at 58°C, and 72°C for 1 min) carried out for 30 cycles. The sizes of amplified products were 788 bp for PKC-βI (β-SI-βαs) and 770 bp for PKC-βII (β-SI-βαs). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the control was PCR amplified using 5'-CAAGGCTGTGGGCAAGGTCA-3' and 3'-AGGTGGAGTTGTAGGAGCGG-5'. The PCR-amplified product was subsequently size fractioned on a 1% agarose gel, stained with ethidium bromide, and visualized under UV light. In negative-control experiments with omission of the reverse transcriptase, no PCR product was detected for either set of the PKC and GAPDH primers (data not shown).

**Densitometric analysis.** Autoradiographs of endogenous-protein phosphorylation and immunoblots were analyzed using a model GS-700 imaging densitometer and molecular analyst (version 1.5; Bio-Rad Laboratories, Hercules, Calif.).

**RESULTS**

Chemokine-mediated changes in PKC activity and expression in infected macrophages. In previous studies, we demonstrated that impairment of PKC isotypes is one of the crucial adaptive strategies which help in the establishment of the parasite within the hostile environment of the macrophages (8). The present study determines whether there was any alteration in the classical PKC-mediated signal transduction within the parasitized macrophages in response to C-C chemokines. Therefore, we measured both Ca²⁺/PS/DG-dependent PKC activity and Ca²⁺/PS-dependent but PS/DG-dependent PKC activity. In control macrophages, a considerable amount of PKC activity was observed in the presence of Ca²⁺/PS/DG (Fig. 1A). In the *L. donovani*-infected macrophages, Ca²⁺/PS/DG-dependent PKC activity was remarkably inhibited whereas Ca²⁺/PS-independent, PS/DG-dependent PKC activity showed enhancement compared to that of control macrophages. Pre-treatment of macrophages with 50 ng of MIP-1α or MCP-1 for 2 h followed by infection led to the induction of the Ca²⁺/PS/DG-dependent PKC activity compared to results for control macrophages (Fig. 1A). We could not detect any significant change of Ca²⁺/PS/DG-dependent PKC activity in the control macrophages treated with either MIP-1α or MCP-1. Furthermore, C-C chemokine treatment abrogated the induction of Ca²⁺/PS-independent, PS/DG-dependent atypical PKC activity in *L. donovani*-infected macrophages.

In continuation with the above observation, it was necessary to investigate whether the PKC activity profile was also reflected at the level of protein expression. In agreement with the PKC activity study, it was observed that attenuated expression levels of PKC-βI and PKC-βII under parasitic stress were markedly recovered when the macrophages were pretreated with 50 ng of MIP-1α or MCP-1 (Fig. 1B).

Our previous studies demonstrated that during visceral leishmaniasis Ca²⁺/PS-independent atypical PKC-ζ is activated (8) and helps parasitic survival within the hostile environment of macrophages. In the present study, we found that when the cells were treated with either MIP-1α or MCP-1 before infection, there was a substantial inhibition of PKC-ζ expression in infected cells (Fig. 1C). This result suggests that C-C chemokines play a significant role in altering the profile of impaired PKC during visceral leishmaniasis.

**Effect of chemokines on endogenous-protein phosphorylation during visceral leishmaniasis.** Chemokine-mediated changes in PKC activity during visceral leishmaniasis prompted us to study the role of chemokines in endogenous-protein phosphorylation in the *L. donovani*-infected macrophages. In the control macrophages, significant phosphorylation of 67-, 54-, 47-, and 36-kDa proteins was observed to occur in a PKC-dependent manner in the presence of activators Ca²⁺/PS/DG (Fig. 2A, lane 3). Infection with *L. donovani* inhibited the Ca²⁺/PS/DG-dependent protein phosphorylation (Fig. 2A, lane 6). It is interesting that when the macrophages were pretreated with MIP-1α or MCP-1, followed by infection, the phosphorylation of 67-, 54-, 47-, and 36-kDa proteins was restored (Fig. 2A, lanes 9 and 12). Densitometric scanning analysis (Fig. 2B) revealed that the percentages of restoration of Ca²⁺/PS/DG-dependent phosphorylation of 67-, 54-, 47-, and 36-kDa proteins were 51.2, 80.4, 40.1, and 75.4%, respectively, for MIP-1α-pretreated infected macrophages and 46.5, 74.6, 46.4, and 78.2%, respectively, for MCP-1-pretreated infected macrophages compared to infected macrophages (100%).

**Effect of C-C chemokines on translocation of p47phox and p67phox proteins in *L. donovani*-infected macrophages.** It is well established that PKC plays a vital role in the assembly of
NADPH oxidase components via phosphorylation (16, 19). Assembly of an active NADPH oxidase and generation of O$_2^{-}$ require translocation of cytosolic factors p47$^{phox}$ and p67$^{phox}$ to the plasma membrane, where they interact with the membrane protein cytochrome $b_{558}$ (5, 20, 31). In our present study, immunoblot analyses of the cytosolic and membrane fractions of $L$. donovani-infected macrophages were performed to detect the expression levels of p47$^{phox}$ and p67$^{phox}$. Our data showed less inhibition of p47$^{phox}$ in the cytosolic fraction of infected cells (Fig. 3A, lanes 1 and 2), since in the membrane fraction, p47$^{phox}$ was not expressed, showing no translocation of the protein (Fig. 3A, lanes 5 and 6). A similar observation was noted in the case of p67$^{phox}$; in spite of the similar expression (Fig. 3B, lanes 1 and 2) of p67$^{phox}$ in the cytosolic fraction of infected cells, there was no translocation to the membrane fraction (Fig. 3B, lane 6). Interestingly, when the macrophages were treated with either MIP-1α or MCP-1, followed by infection, there was significant translocation of both p47$^{phox}$ and p67$^{phox}$ to the membrane (Fig. 3A and B, lanes 7 and 8). Densitometric scanning analysis (Fig. 3C) of p47$^{phox}$ and p67$^{phox}$ in cytosol and membrane fractions clearly showed significant translocations of these proteins to the membrane in response to C-C chemokines in infected cells. These data clearly indicated that C-C chemokines were involved in the...

FIG. 1. Effect of C-C chemokines on PKC activity and expression in $L$. donovani-infected macrophages. Macrophages were pretreated with MIP-1α or MCP-1 for 2 h (at 50 ng/ml) and challenged with $L$. donovani (LD) promastigotes (cell:parasite ratio, 1:10) for 4 h. Noningested promastigotes were removed, and macrophages were cultured for another 2 h. (A) PKC assay. The activity of PKC was assayed with MgCl$_2$, PS, DG, and [$\gamma$-$^{32}$P]ATP, in the presence and absence of CaCl$_2$, by measuring the $^{32}$P incorporation into histone type III-S (in picomoles/min/mg protein). The data represent the means ± SD of data from three independent experiments, which yielded similar results. Asterisks indicate statistically significant inhibition (*, $P < 0.05$) and induction (**, $P < 0.03$) of PKC activity. (B and C) Expression levels of different PKC isotypes in $L$. donovani-infected macrophages in response to C-C chemokines. Cell lysates were prepared and subjected to immunoblotting with PKC-βI, PKC-βII, and PKC-ζ antibodies. The blots were reprobed with anti-mouse-β-actin antibody to confirm equal protein loading. This is the representative of four individual experiments.
translocation of NADPH oxidase components like p47phox and p67phox which might regulate superoxide anion generation in infected cells.

Effect of C-C chemokines on superoxide anion generation during infection. The proteins p47phox and p67phox are known to be the components of NADPH oxidase having a direct effect on superoxide anion generation (8, 36). Hence, we studied the generation of superoxide anions, which are considered to be important oxidative-defense machinery adopted by phagocytes against microbial invasion (4). O$_2^-$ generation in $L. donovani$-infected cells was inhibited compared to that of the control cells (Fig. 4). Pretreatment of cells with 50 ng of MIP-1α or MCP-1 for 2 h followed by infection showed a significant restoration of superoxide anion generation compared to that of $L. donovani$-infected macrophages (Fig. 4).

These results further confirm that C-C chemokines showed antileishmanial properties not only via the NO generation (7) but also by regulating the impairment of PKC activity and O$_2^-$ generation in the early stages of pathogenesis.

Effect of C-C chemokines on the induction of chemotaxis and ROS generation in infected macrophages. During an inflammatory process, microbial products and inflammatory mediators direct the migration of phagocytic cells to the site of microbial invasion. This has been modeled by an in vitro migration assay, where macrophages are allowed to migrate through a porous membrane towards RPMI 1640 medium with MIP-1α as a chemoattractant agent (57). In this assay system, enhanced migration towards MIP-1α was observed for control macrophages. In $L. donovani$-infected macrophages, there was substantial inhibition of chemotactic migration towards MIP-1α. When the macrophages were treated with MIP-1α or MCP-1 prior to infection (see Materials and Methods), there was significant induction of chemotaxis for MIP-1α, while the MCP-1-treated macrophages showed less chemotaxis upon in-
Infection (Fig. 5). Chemokine-induced intracellular generation of ROS of these migratory cells was also studied by DCFDA oxidation. Fluorescence microscopic examination of these cells revealed a level of intracellular fluorescence due to formation of oxidized DCFDA that was enhanced compared to that of the infected macrophages (Fig. 5). These results show that the C-C chemokines induced the migration as well as the ROS generation, which is the most characteristic parameter in phagocytic activation for the clearance of pathogen within *L. donovani*-infected macrophages.

Antileishmanial activity of C-C chemokines in BALB/c mice infected with *L. donovani* in vivo. As both of the C-C chemokines rescued the PKC-mediated signaling impairment in vitro, we administered these C-C chemokines to *Leishmania donovani*-infected BALB/c mice to test its therapeutic potential. Three groups of 8-week-old *L. donovani*-infected BALB/c mice were either left untreated or treated with MIP-1α (5 μg/kg of body weight) or MCP-1 (5 μg/kg of body weight) on the seventh day after infection. It was observed that treatment with MIP-1α or MCP-1 resulted in a condition that was more normalized than with the infected controls, as assessed by liver and spleen parasitic burden. Mice were sacrificed on days 1, 7, 14, 28, and 56 days posttreatment, and levels of parasitic burden were determined (Fig. 6A and B). MIP-1α or MCP-1 treatment could suppress infection by 87% or 66%, respectively, in the liver (*P* < 0.01) or by 69% or 52%, respectively, in the spleen (*P* < 0.01) after 14 days posttreatment. On day 28, parasitic burden diminished about 88% in liver (*P* < 0.01) and 96% in spleen (*P* < 0.05) with MIP-1α treatment and 81% in liver and 94% in spleen (*P* < 0.05) with MCP-1 treatment. The fall in parasitemia induced by C-C chemokine therapy was highly significant compared to the level in infected mice injected with PBS only.

mRNA expression of PKC-β and PKC-ζ in the splenocytes of C-C-chemokine-treated BALB/c mice infected with *L. donovani*. The above in vivo results prompted us to investigate whether the chemokine treatment could regulate the selective impairment of PKC-mediated signal transduction in infected mice. The relative expression levels of PKC mRNA in splenocytes of these infected mice were studied by the semiquantitative RT-PCR method. Like the in vitro results (Fig. 1B), here also we found substantial inhibition of PKC-βII at the level of mRNA expression, which was up-regulated in the case of chemokine-treated infected mice (Fig. 7A). We observed signifi-
cant induction of PKC-ζ mRNA expression in splenocytes of infected mice, but with the C-C chemokine treatment the induction of PKC-ζ expression was substantially inhibited.

We further confirmed the restoration of expression of different PKC isoforms in the splenocytes of chemokine-treated infected mice by using the real-time quantitative RT-PCR method. Quantification was performed by using the comparative cycle threshold method (34), and values were expressed as differences (n-fold) relative to the value for a calibrator cDNA. The results revealed a significant decrease in the expression of PKC-βI and PKC-βII mRNA in infected mice compared to expression in the uninfected mice (Fig. 7B). In infected mice
subjected to MIP-1α treatment, there were 3.8- and 4.7-fold increases in the expression levels of PKC-βI and PKC-βII mRNA, respectively, compared to the expression levels for uninfected mice, while in infected mice under MCP-1 treatment there were 2.9- and 3.6-fold increases in the expression levels of PKC-βI and PKC-βII mRNA, respectively, compared to the expression levels for uninfected mice. We observed sixfold increases in the expression levels of PKC-ζ mRNA in infected mice compared to levels for the uninfected mice. Interestingly, in MIP-1α- or MCP-1-treated mice there was significant reduction of PKC-ζ mRNA expression even under L. donovani infection (Fig. 7B).

**DISCUSSION**

The present study was aimed to evaluate the effect of C-C chemokines on L. donovani-infected macrophages, leading to changes in the signal transduction events. At an early hour of pathogenic invasion, the respiratory burst mechanism is the most crucial event for the host defense. Earlier reports from our laboratory established that C-C chemokines are involved in rendering protection against leishmaniasis via the generation of NO at 48 h (7). It is known that the kinetics of O₂⁻ production is different from that of NO production by macrophages in response to infection (25). It is very interesting to note that the signal transduction events are very useful at the early stages of cellular functioning.

Multiple forms of PKC are well documented in phagocytes like macrophages, which can be differentiated by intracellular distribution, cofactor requirements, and substrate specificity (48). Impairment of classical PKC-β during leishmaniasis, in which normal host cellular functions are affected, has already been established (8, 9). Here for the first time we noted that C-C chemokines MIP-1α and MCP-1 are effective against the impaired PKC activity in leishmaniasis (Fig. 1A). Most strikingly, we observed that the Ca-dependent PKC-βII isotype was impaired under both in vitro and in vivo conditions, whereas Ca-independent isotype PKC-ζ was found to be enhanced during leishmaniasis both in vitro and in vivo. C-C chemokine pretreatment showed withdrawal effects on PKC isotypes during infection (Fig. 1B and C). From this study, it seems that the C-C chemokines MIP-1α and MCP-1 play a crucial role as antileishmanial agents in regulating the disease process.

It has been established that PKC is responsible for the phosphorylation of p47phox and p67phox, which leads to the induction of superoxide anion generation by the NADPH oxidase complex (16). These proteins are reported to be the active NADPH oxidase components, and during the resting condition of the cell, these proteins reside in the cytoplasm. In response to extracellular signals, these components are phosphorylated by PKC and translocated to the membrane (16). Therefore, we attempted to study the PKC-dependent phosphorylation of macrophage-derived proteins during leishmaniasis and the effect of C-C chemokines on this phosphorylation. From the present study, we observed that there was inhibition of 67-, 54-, 47-, and 36-kDa protein phosphorylation in L. donovani-infected macrophages (Fig. 2A, lane 6), but when the macrophages were pretreated with C-C chemokines, followed by infection, phosphorylation of the above proteins was restored. This observation was further confirmed by studying the translocation of p47 (47-kDa) and p67 (67-kDa) proteins to the membrane fraction. We observed less inhibition of p47 protein (Fig. 3A, lanes 1 and 2) and an insignificant change in p67 protein in the cytosolic fraction (Fig. 3B, lanes 1 and 2) of L. donovani-infected cells but no translocation of these proteins to the membrane (Fig. 3A and B, lanes 5 and 6). However, in C-C-chemokine-pretreated cells, followed by infection, there was significant translocation of both p47phox and p67phox to the membrane fraction, indicating the restoration of NADPH oxidase activity.

**FIG. 6.** Effect of in vivo treatment with C-C chemokines on the parasitic load in spleen and in liver. Mice were infected and treated with either a single dose of PBS (controls) or MIP-1α (5 μg/kg of body weight) or MCP-1 (5 μg/kg of body weight) at 7 days postinfection. Mice were sacrificed on days 1, 7, 14, 28, and 56 posttreatment. Levels of parasitic burden in (A) liver and (B) spleen are expressed in Leishman Donovan units (LDU). Results are from three independent experiments and represent the means ± standard errors of the means for four animals per group per time point. *, P of <0.05; **, P of <0.01 (compared to MCP-1-treated infected mice).
membrane (Fig. 3A and B, lanes 7 and 8). These results clearly implicate that C-C chemokines not only withdraw the pathogenic effects of *L. donovani* infection but also are involved in the translocation of NADPH oxidase components, like p47phox and p67phox, from the cytosol to the membrane.

The activation of NADPH oxidase depends on the phosphorylation of p47phox and p67phox by PKC-β1, PKC-βII, and PKC-ζ (19, 36). It was reported earlier that superoxide anion generation is impaired during infection, in contrast with results with UR-6, which did not inhibit the phosphorylation of the abovementioned proteins (rather, this phosphorylation was enhanced compared to levels with control macrophages) (8). This enhanced phosphorylation is in agreement with the enhanced superoxide anion generation by UR-6 treatment (8). In the present study, the impaired superoxide anion generation was restored in the presence of chemokines (Fig. 4). A similar observation was made with the antileishmanial drug sodium stibogluconate (52). Therefore, it clearly indicates that chemokines not only play an antileishmanial role via the NO generation at 48 h (7) but also modulate the early events of signaling via superoxide anion generation.

Additionally, the study investigating the chemotactic index (Fig. 5) of infected macrophages by using MIP-1α gives strong support for the C-C-chemokine-mediated recruitment of phagocytic cells and restoration of respiratory burst of infected macrophages. To our knowledge, this is the first report that infected macrophages under C-C chemokine pretreatment not only enhanced chemotaxis but also induced the generation of total ROS, including superoxide anion, in these infected macrophages in order to clear up the invading pathogens.

Having established the role of C-C chemokines in controlling *L. donovani* infection in vitro, we tested their effect on the regulation of infection in BALB/c mice. We observed that both of the chemokines were capable of suppressing parasitic burden in livers and spleens of BALB/c mice in vivo. On days 14 and 28 posttreatment, MIP-1α was significantly ($P < 0.01$) more effective than MCP-1 in suppressing spleen as well as liver parasite burden (Fig. 6). Since the selective impairment of

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**FIG. 7.** C-C chemokines regulate impaired PKC expression in vivo during infection. Three groups of infected BALB/c mice were treated with chemokines as described in the legend for Fig. 6. All groups of mice were sacrificed on day 28 posttreatment, and the total RNA was isolated. (A) Semiquantitative RT-PCR analyses for PKC-βI, PKC-βII, PKC-ζ, and GAPDH were done with the splenocytes of these mice. Data represented here are from one of three independent experiments, all of which yielded similar results. (B) Effects of C-C chemokine treatment on the expression levels of PKC-βI, PKC-βII, and PKC-ζ mRNA transcripts in splenocytes of *L. donovani* (LD)-infected mice, as measured by quantitative real-time PCR. Data are presented as changes (n-fold) from uninfected control cells. The data represent the means ± SD of data from three independent experiments, which yielded similar results. *, $P$ of <0.01, compared to control mice; **, $P$ of <0.01, compared to infected mice.
PKC isotypes (9, 26) is one of the adaptive strategies of the parasite to evade the host inflammatory responses, we investigated the expression of PKC isotypes by semiquantitative RT-PCR and quantitative real-time RT-PCR (Fig. 7A and B). From the above studies, it is apparent that C-C chemokines play a pivotal role in restricting the survival of L. donovani via the restoration of PKC-dependent signaling mechanisms. Our findings clearly indicate that with C-C chemokines, MIP-1α seems to be more effective than MCP-1 in controlling leishmaniasis in vitro. However, in vivo results showed both C-C chemokines acting similarly to reduce the parasitic burden and splenomegaly in L. donovani-infected BALB/c mice. Thus, our detailed investigations suggest that the application of chemokine therapy might be effective in the treatment of a large number of immunocompromised and/or immunocompetent patients with visceral leishmaniasis.

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