

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-1 α 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 ex-

ists 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 macrophageal 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 1 α (MIP-1 α) and macro-

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phage chemoattractant 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-1 α 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 mechanism in the early stages of *L. donovani* infection in macrophages.

MATERIALS AND METHODS

Materials. PKC- ζ , PKC- β I, PKC- β II, p47^{phox}, and p67^{phox} primary antibodies (rabbit polyclonal, reacts with mouse origin) were obtained from Santa Cruz Biotechnology. Anti- β -actin antibody (mouse monoclonal) was obtained from Sigma. Recombinant mouse MIP-1 α and MCP-1 were purchased from R&D Systems (DNA sequences encoding the mature mouse MIP-1 α 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 <1.0 endotoxin unit per microgram of the cytokine; specific activity was measured by ability to chemoattract cultured human monocytes and mouse BaF3 cells transfected with human CCR5 and was expressed as a 50% effective dose [the 50% effective doses for these chemokines are 5 to 25 ng/ml and 0.4 to 2.0 ng/ml, respectively]). Oligonucleotides for semiquantitative PCR and real-time PCR were purchased from Bangalore Genei and [γ -³²P]ATP from JONAKI, DAE, India. SYBR green jumpstart *Taq* ready mix for quantitative PCR was from Sigma and was a gift from Susanta Roy Chowdhury (Indian Institute of Chemical Biology, India). All other chemicals were purchased from either Sigma or Merck.

Animals and parasites. BALB/c mice were purchased from the National Center for Laboratory Animal Sciences, India. For each experiment, 8 to 10 mice (4 to 6 weeks old) were used, regardless of sex. *L. donovani* strain AG-83 (MHOM/IN/1983/AG-83; gift of Ananta Ghosh, National Institute of Environmental Health Sciences, Research Triangle Park, N.C.) was maintained in vitro in medium 199 (Sigma) containing 10% fetal calf serum. Amastigotes were prepared as described elsewhere (30). Promastigotes were obtained by suitable transformation. Experiments were done with promastigotes of stationary phase.

Peritoneal macrophage preparation. Mouse macrophages were isolated by peritoneal lavage with ice-cold phosphate-buffered saline (PBS) 48 h after intraperitoneal injection of 1.0 ml of sterile 4% thioglycolate broth (Difco). Cells were cultured as described by Fahey et al. (24). The adherent cell population was cultured for 48 h prior to any treatment in order to achieve the resting state.

PKC assay. PKC activity was assayed in a phosphatidyl serine/diacylglycerol (PS-DG)- and a Ca²⁺/PS-DG-dependent manner by measuring the incorporation of γ -³²P (BARC, India) into histone type III-S (Sigma), as described by Majumdar et al. (37). Aliquots equivalent to 5 \times 10⁴ cells were incubated in a 50- μ l reaction mixture consisting of 35 mM Tris-HCl (pH 7.5), 0.01% Triton X-100, 10 mM β -mercaptoethanol, antiprotease mixture (0.33 mM leupeptin, 0.2 mM phenylmethylsulfonyl fluoride, 0.35 mM antipain, 0.24 mg/ml of chymostatin, 0.35 mM pepstatin, and 4.8 trypsin inhibitor units of aprotinin/ml), 0.4 mM EGTA, 10 mM MgCl₂, 20 μ g/ml PS, 2 μ g/ml DG, 160 μ g/ml histone type III-S, and 50 μ M (1 μ Ci) [γ -³²P]ATP in the presence or absence of 0.6 mM CaCl₂. Incubation was done at 30°C for 20 min, and the reaction was stopped by adding 5 μ l of 75 mM ice-cold H₃PO₄. The reaction mixture was spotted on Whatman no. P-81 phosphocellulose paper (Thomas Scientific, Philadelphia, Pa., and a generous gift from D. K. Chatterjee, BRL). The Whatman papers (P-81) were washed with 75 mM H₃PO₄ three times to remove the unbound counts, followed by absolute ethanol, and ³²P incorporation was determined with a liquid scintillation counter (Wallac 1409 DSA; Finland). The activity of PKC was expressed as picomoles of ³²P incorporated/min/mg protein. The non-phospholipid-dependent phosphorylation was subtracted from the total amount of ³²P incorporated in order to determine total PS/DG- as well as Ca²⁺/PS/DG-dependent PKC activity.

Endogenous-protein phosphorylation. Macrophages were sonicated in EGTA-containing buffer and centrifuged at 6,000 rpm for 10 min at 4°C. The supernatants were used as the source of both endogenous substrates and enzymes. The

reaction mixture contained 20 mM Tris-HCl (pH 7.5), 6 mM MgCl₂, 0.25 mM EGTA, 1 mM NaF, 0.1 mM sodium vanadate, 20 μ g/ml PS, 2 μ g/ml DG, 50 μ M (γ -³²P)ATP, and 100 μ g of endogenous protein in the presence and absence of 0.6 mM CaCl₂, in a total volume of 50 μ l. Incubation was carried out for 30 min at 30°C (9, 37). The reaction was stopped by adding Laemmli buffer; the mixture was then boiled for 5 min, separated by SDS-PAGE (5 to 20% gradient) gel electrophoresis, and subjected to autoradiography.

Superoxide anion generation. Superoxide anion (O₂⁻) generation was monitored using the superoxide dismutase inhibitable cytochrome *c* reduction method (3) Briefly, an aliquot of 2 \times 10⁶ cells was immediately resuspended in 10 mM HEPES buffer and O₂⁻ generation was measured spectrophotometrically in the presence of 10⁻⁷ (M) *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) at 550 nm.

Chemotaxis assay. Chemotaxis assays were performed with normal, infected, and C-C-chemokine-pretreated infected macrophages (57). After 6 h of incubation, macrophages were washed three times with RPMI 1640 to wash out any residual chemokine. Macrophages (4 \times 10⁶/ml) were suspended in RPMI 1640 medium and added to the upper chamber in a volume of 200 μ l. RPMI 1640 medium (1 ml) containing 50 ng/ml of MIP-1 α was placed in the lower chamber of a 24-well Transwell plate (Costar, Bodenheim, Germany). A polycarbonate filter with a pore size of 5 μ m separated the two chambers. The chambers were incubated for 90 min at 37°C in a humidified air atmosphere containing 5% CO₂. At the end of the incubation, the filter facing the lower compartment was scraped with a cell scraper. Migration of cells was quantified by microscopically counting the cells under high-power field magnification. The assay was performed in triplicate. The chemotactic index was calculated by dividing the number of cells that migrated towards a stimulus by the number of cells that migrated in medium alone.

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 μ M) 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 the excitation and emission states set at 490 and 530 nm, respectively. Fluorescence of oxidized DCFDA in cells was captured with a Ricoh SLR camera.

Preparation of cell lysate. The adherent cell population was scraped and centrifuged at 400 \times 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 antiprotease mixture are listed above (37). The macrophage-containing suspension was sonicated at 4°C and centrifuged at 4,250 \times g for 10 min at 4°C, and then the supernatant was used for experiments.

Subcellular fractionation. After the required time of incubation, cells were suspended in ice-cold extraction buffer containing 50 mM Tris-HCl (pH 7.5), 50 mM EGTA, antiprotease mixture (contents listed above), and 50 mM β -mercaptoethanol. The suspension was then sonicated (three times for 10 s each). Nuclei, granules, and unbroken cells were removed by centrifugation at 1,000 \times g for 10 min at 4°C. Supernatants were ultracentrifuged at 100,000 \times g for 30 min at 4°C to separate cytosol and membrane fractions. Supernatants (cytosol) were collected, and pellets (membrane) were resuspended in the extraction buffer containing 0.1% NP-40.

Electrophoresis and immunoblotting. Proteins were quantified with a Bio-Rad protein assay reagent, using bovine serum albumin as a standard. Equal amounts of protein in each lane were subjected to SDS-10% PAGE and transferred to a nitrocellulose membrane (17). The membrane was blocked overnight with 3% bovine serum albumin in Tris-saline buffer (pH 7.5), and immunoblotting was done as described by Ghosh and coworkers (26).

Determination of antileishmanial activity of C-C chemokines in vivo. BALB/c mice (8 weeks old) were injected in vivo with 1 \times 10⁷ *L. donovani* amastigotes obtained from infected hamster spleen homogenates (47). These groups of infected mice were treated with C-C chemokines (MIP-1 α and MCP-1) on the seventh day after infection. Groups of *L. donovani*-infected mice ($n = 4$) were treated intravenously, via the tail vein, either with a single dose of phosphate-buffered saline (controls) or with MIP-1 α (5 μ g/kg of body weight) or MCP-1 (5 μ g/kg of body weight). Mice were sacrificed at 1, 7, 14, 28, and 56 days post-treatment. The course of visceral infection was determined microscopically using stained liver and spleen tissue imprints after Giemsa staining. Results, expressed as Leishman Donovan units, were calculated as follows: number of amastigotes/1,000 cell nuclei \times organ weight (g) (47). After 28 days of chemokine treatment, spleens from infected BALB/c mice were removed aseptically, and a single-cell suspension was prepared. Briefly, spleen homogenate was subjected to centrif-

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 4×10^6 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'-GT CCTCCAGATGGAGCTGGAAG-3'; reverse, 5'-GAAGGCATGACAGAAT CCAT-3'; product size, 359 bp) designed from the reported sequences deposited with the GenBank database (8). For PKC- β I PCR amplification, the primers used were β -SI, 5'-TTGTGATGGAGTTGTGAACGGG-3', and β I-as, 5'-CTC GGCAATGGACTGTCATT-3'; and for amplification of PKC- β II, the primers used were β -SI, 5'-TTGTGATGGAGTTGTGAACGGG-3', and β II-as, 5'-TT AGCTCTGAATTCAGGTTTTAAAAATTC-3' (33). For PKC- ζ , PCR amplification was conducted in a reaction volume of 50 μ l by use of a Perkin Elmer Gen Amp PCR 2400 system and 0.5 U of *Taq* polymerase set for 35 cycles (denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s). For PKC- β I and PKC- β II, PCRs at 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min were carried out for 30 cycles. The sizes of amplified products were 788 bp for PKC- β I (β -SI/ β I-as) and 770 bp for PKC- β II (β -SI/ β II-as). Glyceroldehyde-3-phosphate dehydrogenase (GAPDH) as the control was PCR amplified using 5'-CAAGGCTGTGGCAAGGTCA-3' and 5'-AGGTGGAA GAGTGGGAGTTGCTG-3' oligonucleotides located in different exons as sense and antisense primers, respectively. The expected size of the PCR product was 242 bp. The PCR-amplified product was subsequently size fractionated 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 μ l) contained 10 pmol of each primer (the same combination of forward and reverse primers as for semiquantitative RT-PCR were used), 8 μ l of water, 12.5 μ l of a commercial SYBR green PCR master mixture (Sigma), and 2.0 μ l 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 (5 min at 95°C) and cycling step (denaturation for 30 s at 94°C, annealing for 30 s at 58°C, and then extension for 1 min at 72°C; 40 cycles) followed by melt curve analysis. An internal control, GAPDH, was amplified in separate tubes. We used the comparative cycle threshold method ($\Delta\Delta C_T$ method) for relative quantitation of gene expression (34). Briefly, the cycle threshold (C_T) for the target amplicon and the C_T for the internal control (GAPDH) were determined for each sample. The ΔC_T for each experimental sample was subtracted from the calibrator. This difference was called the $\Delta\Delta C_T$ value. Finally, the arithmetic calibrator ($2^{-\Delta\Delta C_T}$) was used to calculate the amount of target normalized to the amount of an internal control and relative to the amount of the calibrator. Thus, all values for experimental samples were expressed as differences (n -fold) between the sample mRNA and the calibrator mRNA. The data were represented as the means \pm standard deviations (SD) of data from three independent experiments, which yielded similar results.

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.).

Statistical analysis. The in vitro cultures were in triplicate, and a minimum of four mice was used per group for in vivo experiments. The data, represented as means \pm SD, are from one experiment, which was performed at least three times. Student's *t* test was employed to assess the significance of the differences between the mean values of control and experimental groups. A *P* value of less than 0.05 was considered significant, and a *P* value of less than 0.01 was considered highly significant.

RESULTS

Chemokine-mediated changes in PKC activity and expression in infected macrophages. In previous studies, we demonstrated that impairment of PKC isoforms 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 Ca^{+2} /PS/DG-dependent PKC activity and Ca^{+2} -independent but PS/DG-dependent PKC activity. In control macrophages, a considerable amount of PKC activity was observed in the presence of Ca^{+2} /PS/DG (Fig. 1A). In the *L. donovani*-infected macrophages, Ca^{+2} /PS/DG-dependent PKC activity was remarkably inhibited whereas Ca^{+2} -independent, PS/DG-dependent PKC activity showed enhancement compared to that of control macrophages. Pretreatment of macrophages with 50 ng of MIP-1 α or MCP-1 for 2 h followed by infection led to the induction of the Ca^{+2} /PS/DG-dependent PKC activity compared to results for control macrophages (Fig. 1A). We could not detect any significant change of Ca^{+2} /PS/DG-dependent PKC activity in the control macrophages treated with either MIP-1 α or MCP-1. Furthermore, C-C chemokine treatment abrogated the induction of Ca^{+2} -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- β I and PKC- β II under parasitic stress were markedly recovered when the macrophages were pretreated with 50 ng of MIP-1 α or MCP-1 (Fig. 1B).

Our previous studies demonstrated that during visceral leishmaniasis Ca^{+2} -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-1 α 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 Ca^{+2} /PS/DG (Fig. 2A, lane 3). Infection with *L. donovani* inhibited the Ca^{+2} /PS/DG-dependent protein phosphorylation (Fig. 2A, lane 6). It is interesting that when the macrophages were pretreated with MIP-1 α 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 Ca^{+2} /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-1 α -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 p47^{phox} and p67^{phox} proteins in *L. donovani*-infected macrophages. It is well established that PKC plays a vital role in the assembly of

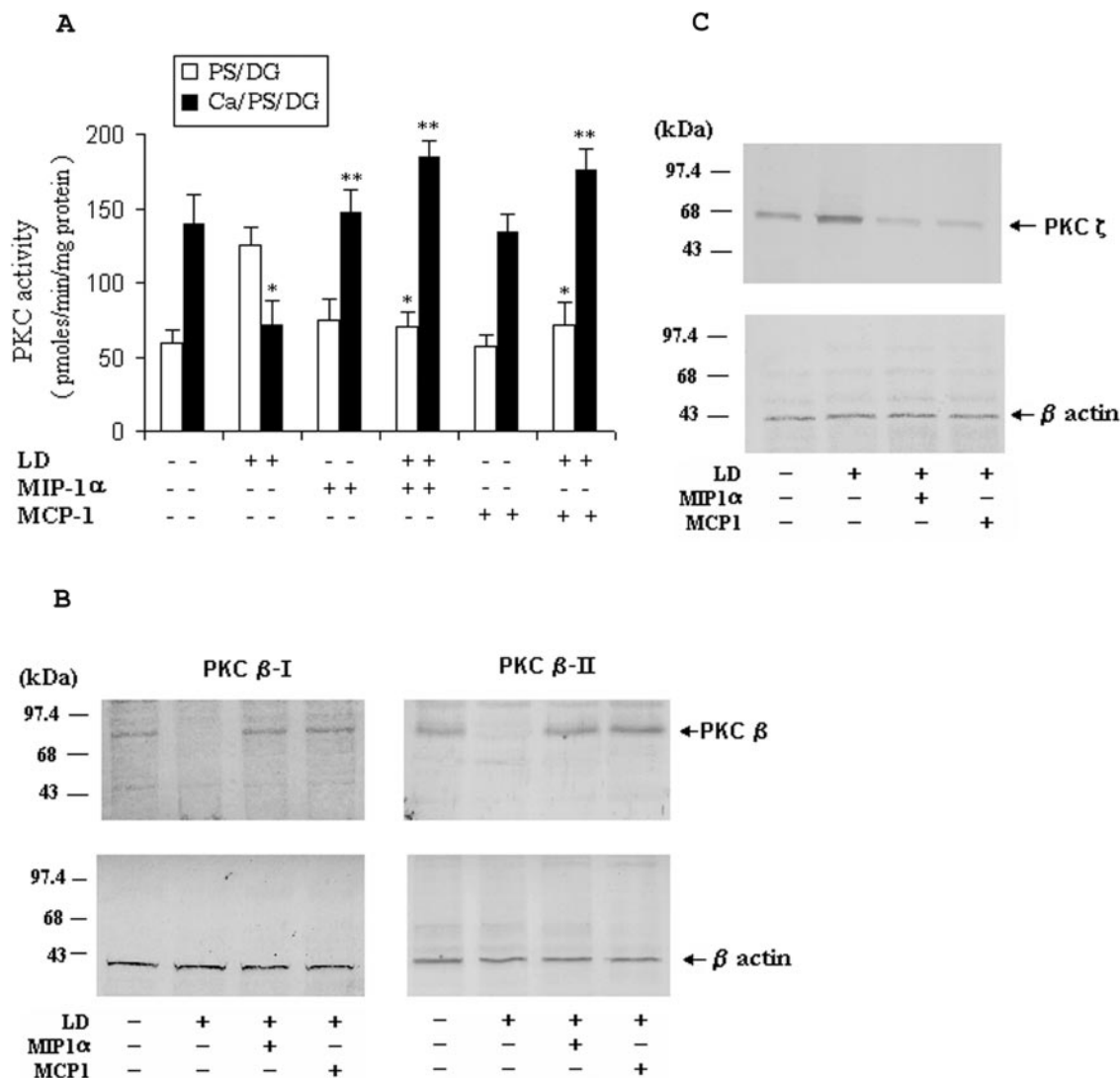


FIG. 1. Effect of C-C chemokines on PKC activity and expression in *L. donovani*-infected macrophages. Macrophages were pretreated with MIP-1α or MCP-1 for 2 h (at 50 ng/ml) and challenged with *L. donovani* (LD) promastigotes (cell:parasite ratio, 1:10) for 4 h. Noningested promastigotes were removed, and macrophages were cultured for another 2 h. (A) PKC assay. The activity of PKC was assayed with MgCl₂, PS, DG, and [³²P]ATP, in the presence and absence of CaCl₂, by measuring the ³²P 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 reprobbed with anti-mouse-β-actin antibody to confirm equal protein loading. This is the representative of four individual experiments.

NADPH oxidase components via phosphorylation (16, 19). Assembly of an active NADPH oxidase and generation of O₂⁻ require translocation of cytosolic factors p47^{phox} and p67^{phox} to the plasma membrane, where they interact with the membrane protein cytochrome b₅₅₈ (5, 20, 31). In our present study, immunoblot analyses of the cytosolic and the 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

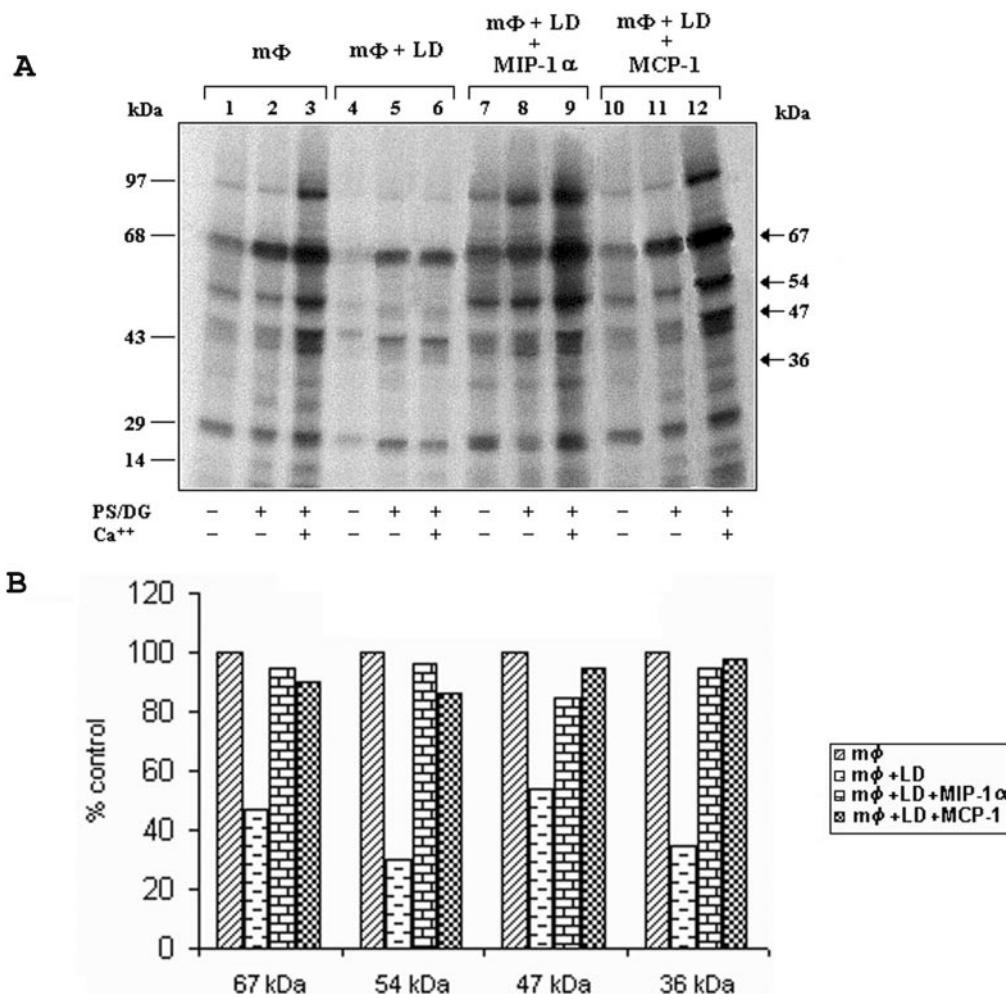


FIG. 2. (A) Endogenous-protein phosphorylation of BALB/c peritoneal macrophages. Macrophages (M Φ) were cultured and treated with chemokines and *L. donovani* (LD) promastigotes as described in the legend for Fig. 1. After 6 h of culture, cell sonicate was used as the source of both endogenous substrates and kinases. The phosphorylation reaction was carried out with MgCl₂, PS, DG, and [γ -P³²]ATP in the presence (+) or absence (-) of CaCl₂. Proteins were solubilized in Laemmli buffer and subjected to 5 to 20% gradient SDS-PAGE followed by autoradiography. (B) Densitometric scanning analysis for the relative phosphorylation levels of 67-, 54-, 47-, and 36-kDa proteins, considering 100% phosphorylation of each individual protein in control macrophages. The autoradiogram is from one of three independent experiments; all of them yielded similar results.

translocation of NADPH oxidase components like p47^{phox} and p67^{phox} which might regulate superoxide anion generation in infected cells.

Effect of C-C chemokines on superoxide anion generation during infection. The proteins p47^{phox} and p67^{phox} 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). O₂⁻ 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-1 α 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 O₂⁻ 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-1 α as a chemoattractant agent (57). In this assay system, enhanced migration towards MIP-1 α was observed for control macrophages. In *L. donovani*-infected macrophages, there was substantial inhibition of chemotactic migration towards MIP-1 α . When the macrophages were treated with MIP-1 α or MCP-1 prior to infection (see Materials and Methods), there was significant induction of chemotaxis for MIP-1 α , while the MCP-1-treated macrophages showed less chemotaxis upon in-

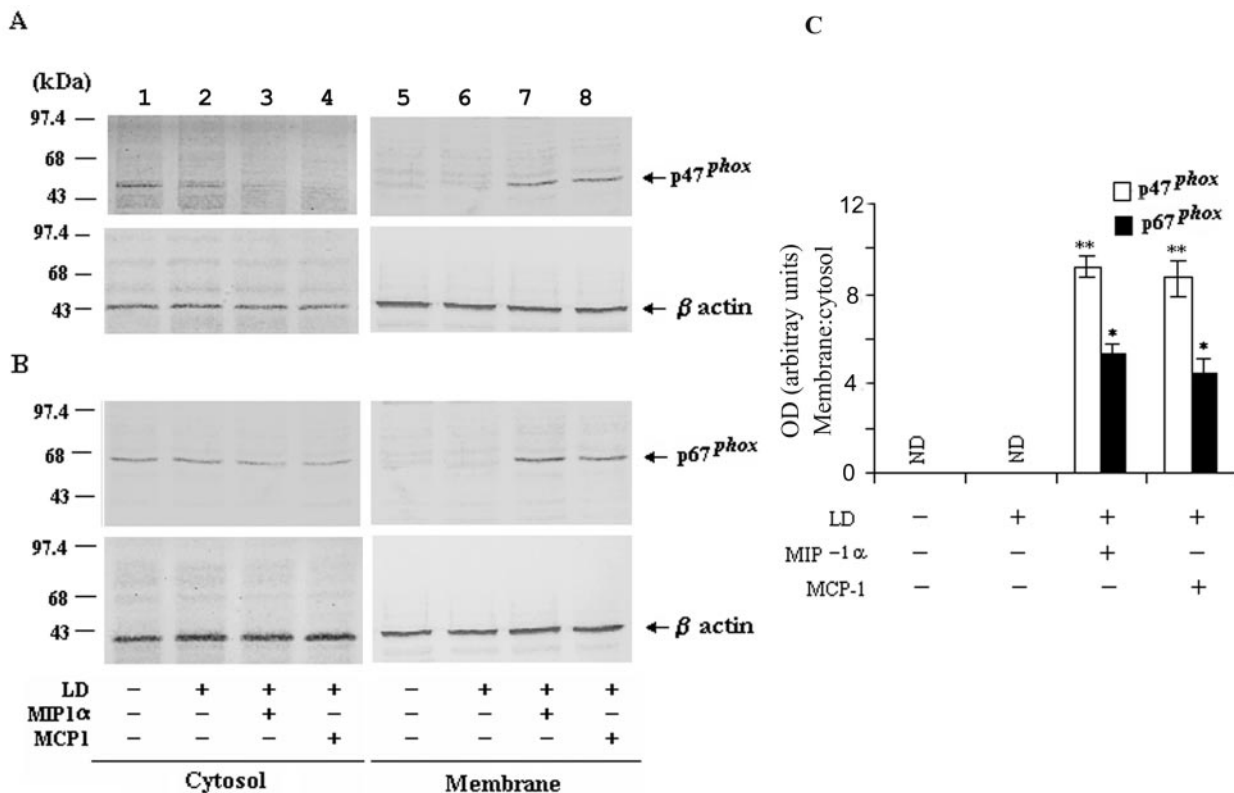


FIG. 3. Effect of C-C chemokines on the activation of NADPH oxidase components in *L. donovani*-infected BALB/c peritoneal macrophages. Macrophages were pretreated with MIP-1α or MCP-1 (50 ng/ml) for 2 h and then challenged with *L. donovani* (LD) promastigotes (cell:parasite ratio, 1:10) for 4 h. Noningested promastigotes were removed, and macrophages were cultured for another 2 h. (A) Effect of C-C chemokines on translocation of p67^{phox} and p47^{phox} in *L. donovani*-infected macrophages. After incubation, cells were processed for membrane and cytosol preparation and Western blot analysis as described in Materials and Methods. The blots were reprobbed with anti-mouse-β-actin antibody to confirm equal protein loading. Data presented are from one of four independent experiments, which yielded similar results. (B) Densitometric scanning analysis of p47^{phox} and p67^{phox} in the membrane fraction and cytosolic fraction were performed and are represented as ratios of relative intensities of respective proteins between the membrane and cytosol. The data represent the means ± SD from four independent experiments, which yielded similar results. OD, optical density; ND, not detected. *, *P* of <0.05; **, *P* of <0.01; significant differences from unstimulated control cells.

fection (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-

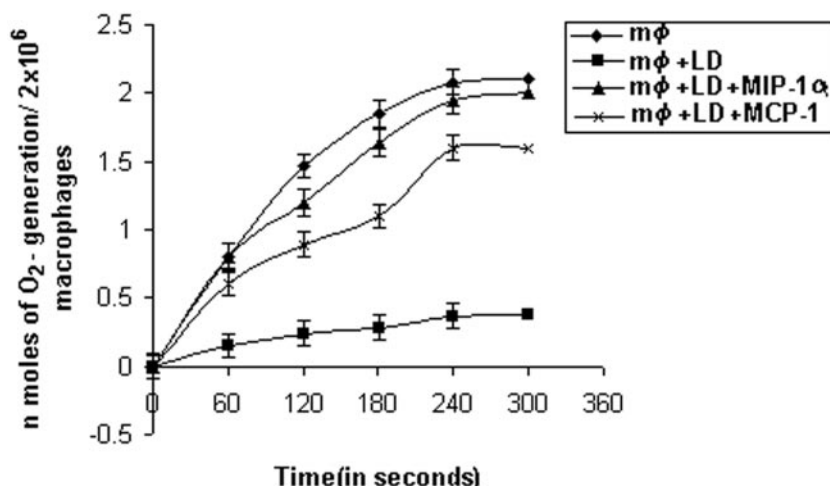


FIG. 4. C-C chemokines induce superoxide anion generation in *L. donovani* (LD)-infected macrophages (Mφ). Generation of O₂⁻ anions was measured as the superoxide dismutase inhibitable cytochrome *c* reduction at 550 nm, and change in optical density was monitored every 30 s up to 5 min. Results are expressed as the means ± SD from four independent experiments, all of which showed similar results, and are expressed as nanomoles of O₂⁻/2 × 10⁶ cells.

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

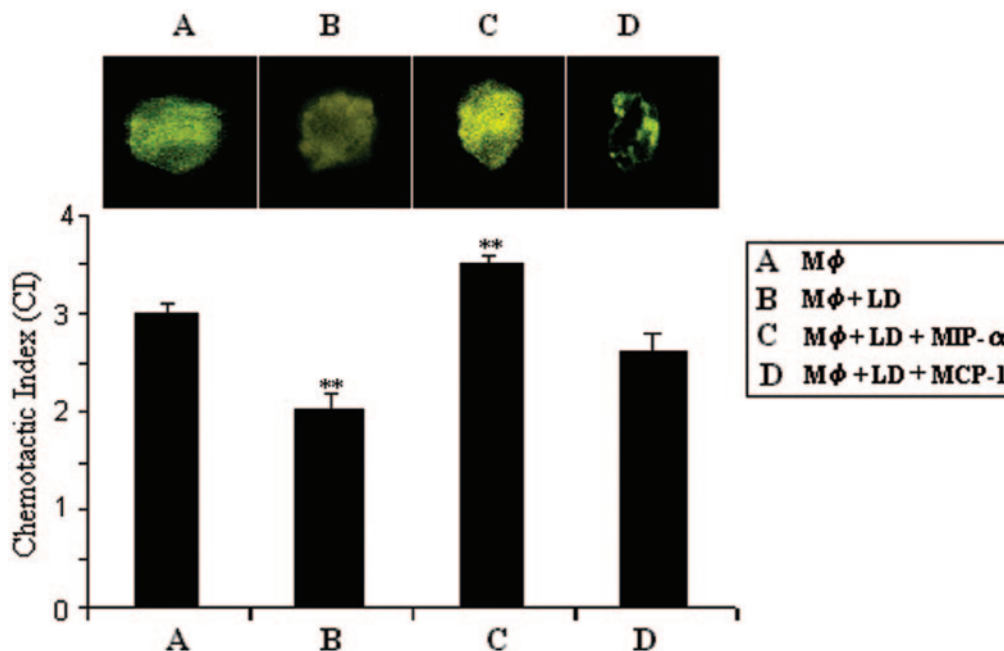


FIG. 5. C-C chemokines induce chemotaxis and ROS generation in *L. donovani*-infected macrophages. (A) Macrophages (Mφ) were cultured and treated with chemokines and *L. donovani* (LD) promastigotes as described in the legend for Fig. 1. After the unbound parasites were washed away, infected cells were cultured for another 2 h. Cells were washed three times with RPMI 1640 to wash out any residual chemokine, which may disrupt the chemokine gradient. Chemotaxis indexes were assessed as described in Materials and Methods. Chemokine-induced ROS formation in cells was detected by fluorescence microscopy (top). Migrated cells were loaded with DCFDA (10 μM) for 30 min in PBS at 37°C in a 5% CO₂ environment. At the end of the incubation, the PBS containing DCFDA was aspirated; cells were washed twice with PBS and examined under a microscope as described in Materials and Methods. The data represent the means ± SD from three independent experiments, which yielded similar results. **, *P* of <0.01; significant differences from control cells.

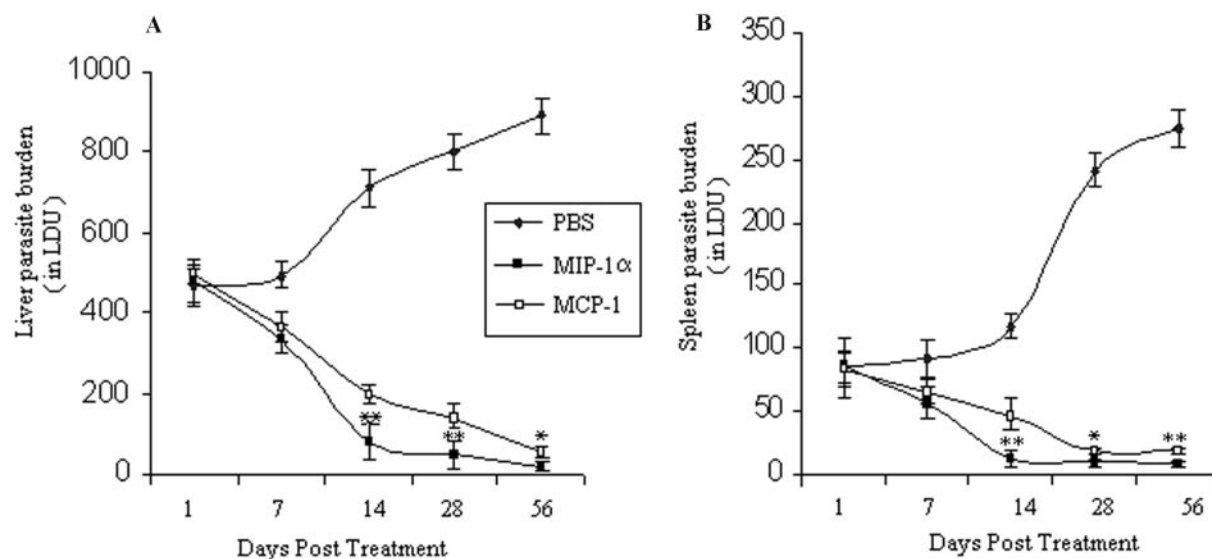


FIG. 6. Effect of in vivo treatment with C-C chemokines on the parasitic load in spleen and in liver. Mice were infected and treated with either a single dose of PBS (controls) or MIP-1 α (5 μ g/kg of body weight) or MCP-1 (5 μ g/kg of body weight) at 7 days postinfection. Mice were sacrificed on days 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 \pm 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).

subjected to MIP-1 α treatment, there were 3.8- and 4.7-fold increases in the expression levels of PKC- β I and PKC- β II 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- β I and PKC- β II 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-1 α - 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-1 α and MCP-1 are effective against the

impaired PKC activity in leishmaniasis (Fig. 1A). Most strikingly, we observed that the Ca-dependent PKC- β II 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-1 α 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 p47^{phox} and p67^{phox}, 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 in the cytosolic fraction (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 p47^{phox} and p67^{phox} to the

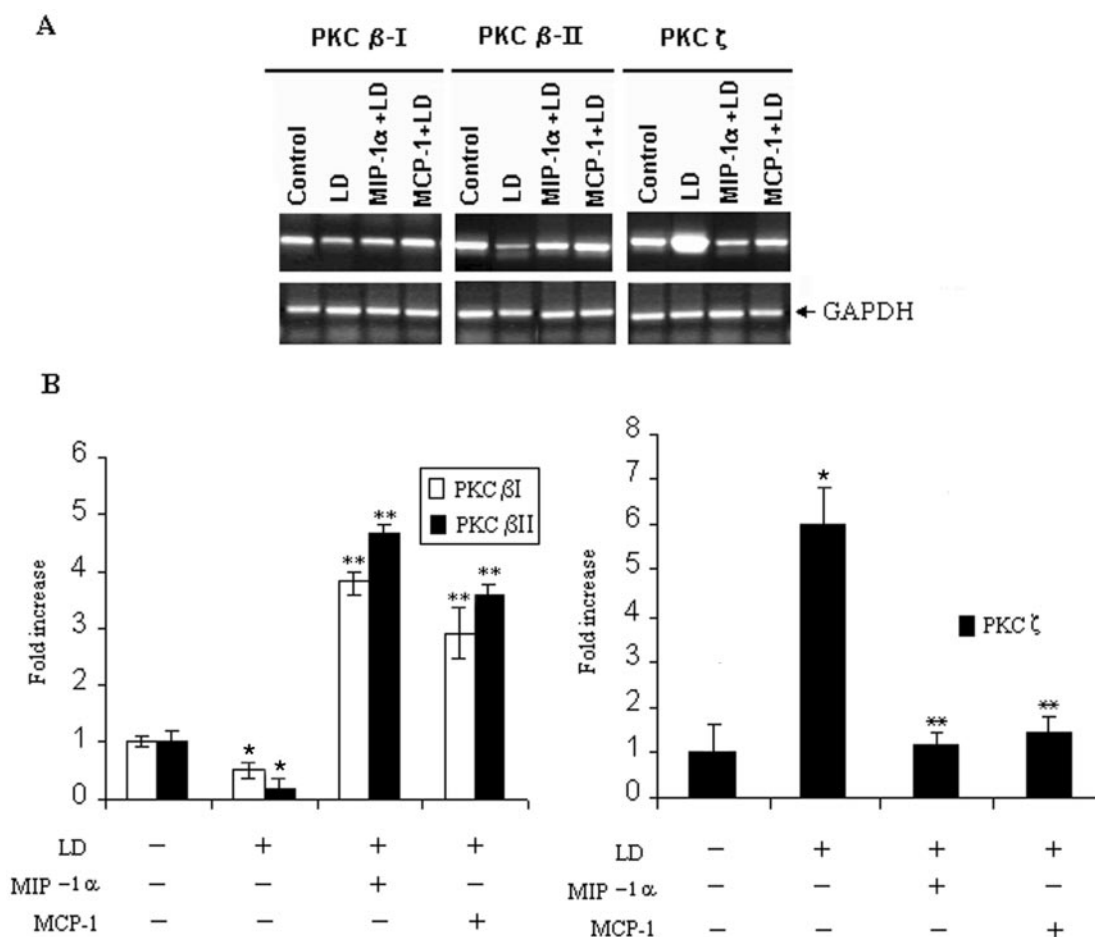


FIG. 7. C-C chemokines regulate impaired PKC expression in vivo during infection. Three groups of infected BALB/c mice were treated with chemokines as described in the legend for Fig. 6. All groups of mice were sacrificed on day 28 posttreatment, and the total RNA was isolated. (A) Semiquantitative RT-PCR analyses for PKC- β I, PKC- β II, 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- β I, PKC- β II, 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 \pm 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.

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 p47^{phox} and p67^{phox}, from the cytosol to the membrane.

The activation of NADPH oxidase depends on the phosphorylation of p47^{phox} and p67^{phox} proteins by PKC- β (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 gener-

ation 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-1 α 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-1 α 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

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-1 α 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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REFERENCES

- Allen, L.-A. H., and A. Aderem. 1996. Molecular definition of distinct cytoskeletal structures involved in complement- and Fc receptor-mediated phagocytosis in macrophages. *J. Exp. Med.* **184**:627–637.
- Allen, L.-A. H., and A. Aderem. 1995. A role for MARCKS, the α isozyme of protein kinase C and myosin I in zymosan phagocytosis by macrophages. *J. Exp. Med.* **182**:829–840.
- Babior, B. M. 1978. Oxygen-dependent microbial killing by phagocytes (first of two parts). *N. Engl. J. Med.* **298**:659–668.
- Babior, B. M. 1999. NADPH oxidase: an update. *Blood* **93**:1464–1476.
- Babior, B. M., R. S. Kipnes, and J. T. Curnutte. 1973. Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. *J. Clin. Invest.* **52**:741–744.
- Baggiolini, M., B. Dewald, and B. Moser. 1997. Human chemokines: an update. *Annu. Rev. Immunol.* **15**:675–705.
- Bhattacharyya, S., S. Ghosh, B. Dasgupta, D. Mazumder, S. Roy, and S. Majumdar. 2001. Chemokine-induced leishmanicidal activity in murine macrophages via the generation of nitric oxide. *J. Infect. Dis.* **185**:1704–1708.
- Bhattacharyya, S., S. Ghosh, P. L. Jhonson, S. K. Bhattacharya, and S. Majumdar. 2001. Immunomodulatory role of interleukin-10 in visceral leishmaniasis: defective activation of protein kinase C-mediated signal transduction events. *Infect. Immun.* **69**:1499–1507.
- Bhattacharyya, S., S. Ghosh, P. Sen, S. Roy, and S. Majumdar. 2001. Selective impairment of protein kinase C isotypes in murine macrophage by *Leishmania donovani*. *Mol. Cell. Biochem.* **216**:47–57.
- Buchmüller-Rouiller, Y., and J. Mauel. 1987. Impairment of the oxidative metabolism of mouse peritoneal macrophages by intracellular *Leishmania* spp. *Infect. Immun.* **55**:587–593.
- Buchwalow, I. B., M. Brich, and S. H. Kaufmann. 1997. Signal transduction and phagosome biogenesis in human macrophages during phagocytosis of *Mycobacterium bovis* BCG. *Acta Histochem.* **99**:63–70.
- Carnevale, K. A., and M. K. Cathcart. 2003. Protein kinase C β is required for human monocyte chemotaxis to MCP-1. *J. Biol. Chem.* **278**:25317–25322.
- Channon, Y. J., M. B. Roberts, and J. M. Blackwell. 1984. A study of the differential respiratory burst activity elicited by promastigotes and amastigotes of *Leishmania donovani* in murine resident peritoneal macrophages. *Immunology* **53**:345–355.
- Chomezynski, P., and N. Sacchi. 1987. A single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156–159.
- Cunha, F. Q., J. Assereuy, D. Xu, I. Charles, S. Moncada, and F. Y. Liew. 1993. Phagocytosis and induction of nitric oxide in murine macrophages. *Immunology* **79**:408–411.
- Dang, P. M.-C., A. Fontayne, J. Hakim, J. El Benna, and A. Périanin. 2001. Protein kinase C ζ phosphorylates a subset of selective sites of the NADPH oxidase component of p47^{phox} and participates in formyl peptide-mediated neutrophil respiratory burst. *J. Immunol.* **166**:1206–1213.
- Das, S., S. Bhattacharyya, S. Ghosh, and S. Majumdar. 2000. Signal transduction mechanism in human neutrophil: comparative study between the ζ and β protein kinase isotypes. *Mol. Cell. Biochem.* **203**:143–151.
- Dekker, L. V., and J. P. Parker. 1994. Protein kinase C is a question of specificity. *Trends Biochem. Sci.* **19**:73–77.
- Dekker, L. V., M. Leitges, G. Altschuler, N. Mistry, A. McDermott, J. Roes, and A. W. Segal. 2000. Protein kinase C- β contributes to NADPH oxidase activation in neutrophils. *Biochem. J.* **347**:285–289.
- DeLeo, F. R., and M. T. Quinn. 1996. Assembly of the phagocyte NADPH oxidase: molecular interaction of oxidase proteins. *J. Leukoc. Biol.* **60**:677–691.
- Descoteaux, A., and S. J. Turco. 1993. The lipophosphoglycan of *Leishmania* and macrophage protein kinase C. *Parasitol. Today* **9**:468–471.
- Descoteaux, A., G. Matashewski, and S. J. Turco. 1992. Inhibition of macrophage protein kinase C mediated protein phosphorylation by *Leishmania donovani* lipophosphoglycan. *J. Immunol.* **149**:3008–3015.
- Diefenbach, A., H. Schindler, N. Donhouser, E. Lorenz, T. Laskay, J. MacMicking, M. Rollinghoff, I. Gresser, and C. Bogdan. 1998. Type 1 interferon (IFN α/β) and type 2 nitric oxide synthase regulated the innate immune response to a protozoan parasite. *Immunity* **8**:77–87.
- Fahey, J. T., K. J. Tracey, P. T. Olson, L. S. Cousins, W. G. Jones, G. T. Shires, and B. Sherry. 1992. Macrophage inflammatory protein 1 modulates macrophage function. *J. Immunol.* **148**:2764–2769.
- Gantt, R. K., T. L. Goldman, M. L. McCormick, M. A. Miller, S. M. B. Jeronimo, E. T. Nascimento, B. E. Britigan, and M. E. Wilson. 2001. Oxidative response of human and murine macrophages during phagocytosis of *Leishmania chagasi*. *J. Immunol.* **167**:893–901.
- Ghosh, S., S. Bhattacharyya, M. Sirkar, G. S. Sa, T. Das, D. Majumdar, S. Roy, and S. Majumdar. 2002. *Leishmania donovani* suppresses activated protein 1 and NF- κ B in host macrophages via ceramide generation: involvement of extracellular signal-regulated kinase. *Infect. Immun.* **70**:6828–6838.
- Giorgione, J. R., J. S. Turco, and M. R. Epand. 1996. Transbilayer inhibition of protein kinase C by the lipophosphoglycan from *Leishmania donovani*. *Proc. Natl. Acad. Sci. USA* **93**:11634–11639.
- Graca-Souza, V. A., M. A. B. Arruda, M. S. Freitas, C. B. Fidalgo, and P. L. Oliveira. 2002. Neutrophil activation by heme: implications for inflammatory process. *Blood* **99**:4160–4165.
- Greenberg, S. 1995. Signal transduction of phagocytosis. *Trends Cell Biol.* **5**:93–99.
- Hart, T. D., K. Vickerman, G. H. Coombs, and A. Quick. 1981. Simple method for purifying *L. mexicana* amastigotes in large numbers. *Parasitology* **82**:345–355.
- Huang, J., and M. E. Kleinberg. 1999. Activation of the phagocyte NADPH oxidase protein p47^{phox}. Phosphorylation controls SH3 domain-dependent binding to p22^{phox}. *J. Biol. Chem.* **274**:19731–19737.
- Korchak, H. M., M. W. Rossi, and L. E. Kilpatrick. 1998. Selective role for protein kinase C signaling for O₂⁻ generation but not degranulation or adherence in differentiated HL60 cells. *J. Biol. Chem.* **273**:27292–27299.
- Lee, S. W., H. B. Kwak, W. J. Chung, H. Cheong, H. H. Kim, and Z. H. Lee. 2003. Participation of protein kinase C- β in osteoclast differentiation and function. *Bone* **32**:217–227.
- Leite, F., S. O'Brien, M. J. Sylte, T. Page, D. Atapattu, and C. J. Czuprynski. 2002. Inflammatory cytokines enhance the interaction of *Mannheimia haemolytica* leukotoxin with bovine peripheral blood neutrophils in vitro. *Infect. Immun.* **70**:4336–4343.
- Liew, F. Y., S. Millot, C. Parkinson, R. M. Palmer, and S. Moncada. 1990. Macrophage killing of *Leishmania* parasite *in vivo* is mediated by nitric oxide from L-arginine. *J. Immunol.* **144**:4794–4797.
- Majumdar, S., L. H. Kane, W. M. Rossi, B. D. Volpp, M. W. Nauseef, and H. M. Korchak. 1993. Protein kinase C isotypes and signal-transduction in human neutrophils: selective substrate specificity of calcium-dependent β -PKC and novel calcium-independent nPKC. *Biochim. Biophys. Acta* **1176**:276–286.
- Majumdar, S., M. W. Rossi, T. Fujiki, W. A. Phillips, S. Disn, C. F. Queen, R. B. Jhonston, O. M. Rosen, B. E. Corkey, and H. M. Korchak. 1991. Protein kinase C isotypes and signaling in neutrophils. *J. Biol. Chem.* **266**:9285–9294.
- Mannheimer, S. B., J. Hariprasad, Y. M. Stoeckle, and H. W. Murray. 1996. Induction of macrophage antiprotozoal activity by monocyte chemoattractant and activating factor. *FEMS Immunol. Med. Microbiol.* **14**:59–61.
- Matsukawa, A., C. M. Hogaboam, N. W. Lukacs, P. M. Lincoln, H. L. Evanoff, and S. L. Kunkel. 2000. Pivotal role of the CC chemokine, macrophage-derived chemokine, in the innate immune response. *J. Immunol.* **164**:5362–5368.
- Matsushima, K., C. G. Larsen, G. C. DuBois, and J. J. Oppenheim. 1989. Purification and characterization of a novel monocyte chemoattractant and activating factor produced by a human myelomonocytic cell line. *J. Exp. Med.* **169**:1485–1490.
- Mellor, H., and P. J. Parker. 1998. The extended protein kinase C superfamily. *Biochem. J.* **332**:281–292.
- Miller, M., S. E. McGown, K. R. Gantt, M. Champion, S. Novick, K. A. Andersen, C. J. Bacchi, N. Yarlett, B. E. Britigan, and M. E. Wilson. 2000.

- Inducible resistance to oxidant stress in the protozoan *Leishmania chagasi*. *J. Biol. Chem.* **275**:33883–33889.
43. **Mukhopadhyay, S., P. Sen, S. Bhattacharyya, S. Majumdar, and S. Roy.** 1999. Immunoprophylaxis and immunotherapy against experimental visceral leishmaniasis. *Vaccine* **17**:291–300.
 44. **Murray, H. W.** 1982. Cell-mediated immune response in experimental visceral leishmaniasis. II. Oxygen-dependent killing of intracellular *Leishmania donovani* amastigotes. *J. Immunol.* **129**:351–357.
 45. **Murray, H. W.** 1982. Pretreatment with phorbol myristate acetate inhibits macrophage activity against intracellular protozoa. *J. Reticuloendothel. Soc.* **31**:479–487.
 46. **Murray, H. W., and C. F. Nathan.** 1999. Macrophage microbicidal mechanism in vivo: reactive nitrogen versus oxygen intermediates in the killing of intracellular visceral *Leishmania donovani*. *J. Exp. Med.* **189**:741–746.
 47. **Murray, W. H., G. D. Miralles, M. Y. Stoeckle, and D. F. McDermott.** 1993. Role and effect of IL-12 in experimental visceral leishmaniasis. *J. Immunol.* **151**:929–938.
 48. **Nishizuka, Y.** 1988. The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature* **334**:662–665.
 49. **Nishizuka, Y.** 1995. Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J.* **9**:484–496.
 50. **Olivier, M., R. W. Brownsey, and N. E. Reiner.** 1992. Defective stimulus-response coupling in human monocytes infected with *Leishmania donovani* is associated with altered activation and translocation of protein kinase C. *Proc. Natl. Acad. Sci. USA* **89**:7481–7485.
 51. **Parinandi, N. L., M. A. Kleinberg, V. P. Usatyuk, J. R. Cummings, A. Pennathur, J. A. Cardounel, L. J. Zweier, G. J. Garcia, and V. Natarajan.** 2003. Hyperoxia-induced NADPH oxidase activation and regulation by MAP-kinases in human lung endothelial cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **284**:L26–L38.
 52. **Rais, S., A. Perianin, M. Lenoir, A. Sadak, D. Rivollet, M. Paul, and M. Deniau.** 2000. Sodium stibogluconate (Pentostam) potentiates oxidant production in murine visceral leishmaniasis and in human blood. *Antimicrob. Agents Chemother.* **44**:2406–2410.
 53. **Ritter, U., and H. Moll.** 2000. Monocyte chemotactic protein-1 stimulates the killing of *Leishmania major* by human monocytes, acts synergistically with IFN- γ and is antagonized by IL-4. *Eur. J. Immunol.* **30**:3111–3120.
 54. **Rollins, B., A. Walz, and M. Baggiolini.** 1991. Recombinant human MCP-1/JE induces chemotaxis, calcium flux, and the respiratory burst in human monocytes. *Blood* **78**:1112–1116.
 55. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. *Molecular cloning: a laboratory manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 56. **Turco, S. J.** 1999. Adversarial relationship between the leishmania lipophoglycan and protein kinase C. *Parasite Immunol.* **21**:597–600.
 57. **van Zandbergen, G., N. Hermann, H. Laufs, W. Solbach, and T. Laskay.** 2002. *Leishmania* promastigotes release a granulocyte chemotactic factor and induce interleukin-8 release but inhibit gamma interferon-inducible protein 10 production by neutrophil granulocytes. *Infect. Immun.* **70**:4177–4184.
 58. **Woods, M. S., II, J. Mayer, J. T. Evans, and J. B. Hibbs, Jr.** 1994. Antiparasitic effects of nitric oxide in an in vitro murine model of *Chlamydia trachomatis* infection and an in vivo murine model of *Leishmania major* infection. *Immunol. Ser.* **60**:179–195.
 59. **Zarley, J. H., B. E. Britigan, and M. E. Wilson.** 1991. Hydrogen peroxide-mediated toxicity for *Leishmania donovani chagasi* promastigotes: role of hydroxyl radical and protection by heat shock. *J. Clin. Investig.* **88**:1511–1521.

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