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Miltefosine Promotes IFN-γ-Dominated Anti-Leishmanial Immune Response[1]

Pallavi Wadhone, Moitrayee Maiti, Reena Agarwal, Vanita Kamat, Sunil Martin, and Bhaskar Saha[2]

***Leishmania donovani*, a protozoan parasite, resides and replicates as amastigotes within macrophages. The parasite inflicts the disease visceral leishmaniasis by suppressing host cell function. Neither a therapeutic vaccine nor an effective anti-leishmanial drug to reverse the immunosuppression is available. Although miltefosine (hexadecylphosphocholine or HPC) is a promising orally bioavailable anti-leishmanial drug, its efficacy is seriously compromised by contra-indications in pregnant women. Further rational redesigning of the drug requires studies on its mechanism of action, which is unknown at present. Because miltefosine is proposed to have immunomodulatory functions, we examined whether miltefosine exerts its anti-leishmanial functions by activating macrophages. We observed that miltefosine's anti-leishmanial function was significantly compromised in IFN-γ-deficient macrophages suggesting the importance of endogenous IFN-γ in miltefosine-induced anti-leishmanial functions of macrophages. Miltefosine induced IFN-γ, neutralization of which reduced the anti-leishmanial functions of macrophages. IFN-γ responsiveness is reduced in *L. donovani*-infected macrophages but is significantly restored by miltefosine, as it enhances IFN-γ receptors and IFN-γ induced STAT-1 phosphorylation but reduced activation of SHP-1, the phosphatase implicated in the down-regulation of STAT-1 phosphorylation. Miltefosine induced protein kinase C-dependent and PI3K-dependent p38MAP kinase phosphorylation and anti-leishmanial function. Miltefosine promotes p38MAP kinase-dependent anti-leishmanial functions and IL-12-dependent Th1 response. *Leishmania donovani*-infected macrophages induced Th2 response but miltefosine treatment reversed the response to Th1-type. Thus, our data define for the first time the mechanistic basis of host cell-dependent anti-leishmanial function of miltefosine. *The Journal of Immunology,* 2009, 182: 7146–7154.**

Visceral leishmaniasis is caused by *Leishmania donovani*, *L. infantum* and *L. chagasi*. The disease is of increasing concern due to worldwide occurrence of 0.5 million new cases/per annum, increase in resistance to standard antimony-based drugs, and HIV-*Leishmania* coinfection (1, 2). Treatment of HIV- *Leishmania* coinfections is almost impossible, as the conventional antimonial treatment is less effective in immunocompromised patients (3). High-dose antimonial treatment with a combination of liposomal amphotericin B has limited efficacy due to high recurrence and requirement for sustained therapy. Therefore, an immunomodulatory drug with anti-leishmanial functions such that it not only kills the parasite but also promotes the host-protective immune response is necessary for the treatment of the disease.

Recently, miltefosine (hexadecylphosphocholine or HPC),[3] an alkyl-phosphocholine originally developed as an anticancer drug (4) has proved to be an effective treatment for *L. donovani* infection in macrophages in vitro (5), in experimental animals (6) and in patients (7). The molecular mechanism of action of HPC against cancer cells has been linked to apoptosis as well as lipid-dependent cell signaling pathways (8). Similarly, rapid accumulation of HPC by *Leishmania* and subsequent apoptosis of the parasite suggest a direct action of the drug on the parasite, perhaps by impairing the membrane synthesis (9). The finding that HPC retains its anti-leishmanial effect in *Leishmania*-infected immunodeficient mice also supports a direct parasite killing by HPC (10). However, these findings do not exclude the possibility that HPC may have macrophage-activating functions (11) that aid parasite killing. Indeed, HPC has been shown to have significant immunomodulatory properties in other models (12–14). Among the immunomodulatory activities important for eliminating *Leishmania* are IL-12-dependent production of IFN-γ (15), which activates macrophages for inducible nitric oxide synthetase 2 (iNOS2)-dependent elimination of *Leishmania* (16). In contrast, highly infected macrophages do not respond to IFN-γ, suggesting that IFN-γ responsiveness is lowered in *L. donovani* infection (17). Therefore, we have tested whether HPC can restore IFN-γ responsiveness and synergize with IFN-γ eliminating the parasite more efficiently. The mechanism of the immunomodulatory functions of HPC is also examined. Our experimental data suggest that HPC induces IFN-γ, TNF-α, and IL-12 production from macrophages. Although IFN-γ and TNF-α induced *Leishmania* killing in macrophages, IL-12 induced Th1 response. *Leishmania*-infected macrophages induced Th2 cytokines whereas the same macrophages treated with HPC induced Th1 cytokines. Such Th1-inducing property was neutralized significantly by IL-12 neutralization. The immunomodulatory functions of HPC may at least partially be dependent upon its ability to activate several cell signaling intermediates. The observations suggest HPC can function as an important immunomodulator even in an immunocompromised state.

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[3] Abbreviations used in this paper: HPC, hexadecylphosphocholine; PKC, protein kinase C; CSA, crude soluble leishmanial Ag; DC, dendritic cell; DTH, delayed-type hypersensitivity; iNOS2, inducible nitric oxide synthetase 2.

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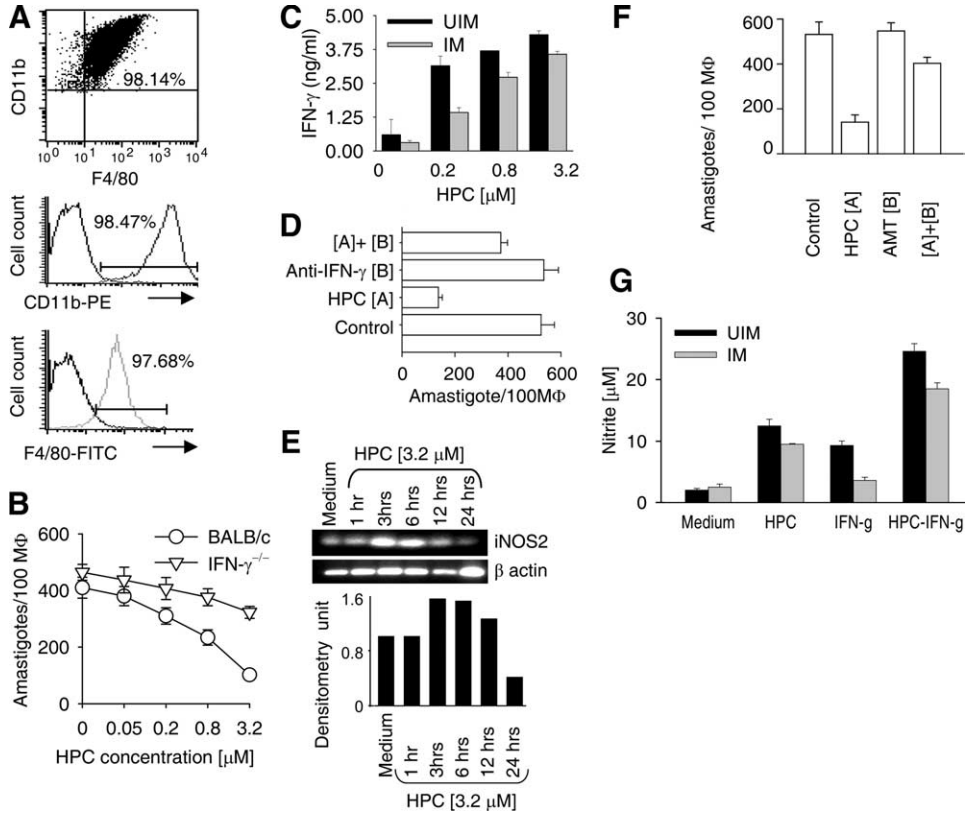

FIGURE 1. Miltefosine requires endogenous IFN-γ for its anti-leishmanial action. *A*, Purity of peritoneal macrophages. Thioglycolate-elicited peritoneal macrophages were washed and adhered for 3 h. The adherent cells were stained with F4/80-FITC and CD11b-PE and analyzed on a flow cytometer. *B*, Dose-dependent reduction in amastigote number by miltefosine. BALB/c (circle) and IFN-$\gamma^{-/-}$ (inverted triangle) mouse-derived peritoneal macrophages were infected with *L. donovani* promastigotes at a macrophage: parasite ratio of 1:10. The macrophages were infected for 48 h, followed by miltefosine treatment with the indicated doses for 24 h. The cells were fixed in methanol, stained with Giemsa stain and counted under a light microscope. *C*, Miltefosine induces IFN-γ production from macrophages. Thioglycolate-elicited peritoneal macrophages from BALB/c mice were infected with *L. donovani* promastigotes for 48 h as described above. The macrophages were treated with the indicated doses of miltefosine (HPC) for the last 24 h. The cytokine in the supernatant was assessed by ELISA using paired Abs from BD Pharmingen ($p < 0.001$ for both uninfected macrophages (UIM) and infected macrophages (IM), as compared between 0 and 3.2 mM HPC). *D*, Endogenous IFN-γ is crucial for miltefosine to exert its anti-leishmanial effects. BALB/c-derived peritoneal macrophages were infected with *L. donovani* promastigotes for forty-eight hours as described above, followed by 24 h treatment with HPC (3.2 μM), as described above, in presence or absence of anti-IFN-γ Ab (clone R46A2; 10 μg/ml). At the end of 72 h, the cultures were fixed, stained with Giemsa stain and the number of amastigotes per 100 macrophages was counted under a microscope. HPC treatment reduced the parasite load ($p < 0.001$) whereas IFN-γ neutralization reduced the leishmanicidal efficacy ($p < 0.001$). *E*, Miltefosine induces iNOS2 expression. BALB/c-derived peritoneal macrophages were treated with miltefosine (3.2 μM) for the indicated time period. The RNA was extracted, followed by the reverse transcription of the RNA and PCR using iNOS2-specific primers, as described earlier (Ref. 19). *F*, iNOS2 inhibition resulted in less parasite killing by HPC. The *L. donovani*-infected macrophages were treated with HPC (3.2 mM) with or without AMT, an inhibitor of iNOS2 (Ref. 19). The macrophages were cultured for 72 h and the amastigotes numbers per 100 macrophages were counted as described above. *G*, AMT showed a significant decrease in anti-leishmanial function of miltefosine, suggesting a role for NO induced by the drug. The cultures were set in triplicates and the experiments were done a minimum of three times. The data shown are from one experiment. The error bars represent mean ± SD.

Materials and Methods

Animals, parasite infection, and reagents

BALB/c and IFN-γ-deficient mice on BALB/c background were originally obtained from Jackson ImmunoResearch Laboratories and were subsequently bred in the Institute's experimental animal facility in Thoren Caging systems. *Leishmania donovani* (strain AG83) was maintained in vitro in RPMI 1640 medium, supplemented with 10% FCS (Life Technologies), and the virulence was maintained by passage through BALB/c mice as described earlier (18). Promastigotes in the stationary phase were used for i.v. infection of mice (2×10^7 per mouse). Parasite burden in mice was assessed by stamp-smear method and was expressed as Leishman-Donovan Unit as described earlier (18). Miltefosine was procured from Sigma-Aldrich and AG Scientific. Radicicol, SB203580, Ly294002, and protein kinase C (PKC) inhibitor peptide were procured from Calbiochem.

Macrophage culture and L. donovani *infection in vitro*

BALB/c and IFN-$\gamma^{-/-}$ mice were injected with 2 ml of 3% thioglycolate i.p. The peritoneal cells were harvested 5 days after the thioglycolate injection and were rested for 24 h before any treatments on these cells. In the meanwhile, the nonadherent cells were washed out to generate >98% pure macrophages. The thioglycolate-elicited macrophages (98% pure as shown in Fig. 1*A*) thus isolated were cultured in 16-well tissue culture slides and were infected with promastigotes at a macrophage: promastigote ratio of 1:10 for 12 h. The cultures were incubated for another 60 hours without treatment. In some cases, 48-h infected cultures were treated with the indicated doses of miltefosine for 24 h. The cultures were terminated after 72 h, fixed with methanol, stained with Giemsa stain and counted under a light microscope (E600, Nikon). The parasite load was expressed as the number of amastigotes per hundred macrophages as described earlier (19).

Isolation of T cells and coculture with macrophages

BALB/c-derived peritoneal macrophages were infected with *L. donovani* promastigotes for 48 h as described above. The uninfected and infected macrophages were treated with HPC for 24 h. The macrophages were washed and pulsed with CSA (10 μg/ml). In the presence or absence of anti-IL-10 Ab, these macrophages were cocultured for another 24 h with the $CD4^+$T cells derived from the BALB/c mice infected with *L. donovani*

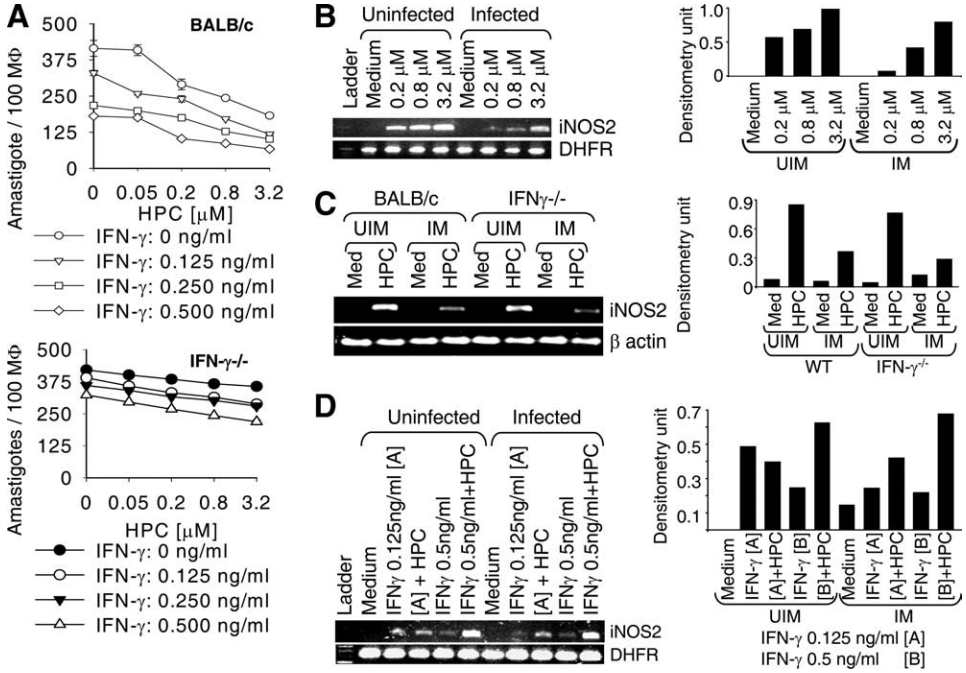

FIGURE 2. Miltefosine enhances the IFN-γ-induced parasite killing in macrophages. *A*, BALB/c and IFN-$\gamma^{-/-}$ mice-derived peritoneal macrophages were infected with *L. donovani* promastigotes as described above. The macrophages were treated with the indicated doses of HPC and IFN-γ for 24 h. The parasites were counted and expressed as amastigotes per 100 hundred macrophages. *B–D*, Miltefosine augments IFN-γ-induced iNOS2 and increases IFN-γ responsiveness in uninfected (UIM) and *L. donovani*-infected (IM) macrophages. The macrophages were infected with *L. donovani* promastigotes for 72 h, as described earlier, followed by treatment with the indicated doses of IFN-γ or HPC for 6 h. RNA was extracted and the iNOS2 PCR was done with the reverse transcribed cDNA. On the *right panels* (*B–D*), respective densitometry data are shown. The data shown are for one of three experiments.

for 10 days (D10T). The D10T cells were used as these cells were earlier found to be primed with leishmanial Ags and were able to respond to challenge with leishmanial Ags in vitro (20, 21). The cell culture supernatants were assessed for IL-4 and IFN-γ productions by ELISA.

Delayed type hypersensitivity assay

Crude soluble leishmanial Ag (CSA) was prepared as described earlier (20). In brief, following six cycles of freezing and thawing, the promastigotes were sonicated for ten cycles, each cycle of 30 s. The extract was clarified by centrifugation (5471R Eppendorf, Germany) at 10,000 rpm for 30 min at 4°C. The supernatant was collected and the protein was estimated by a standard protein assay kit (Pierce). CSA was injected s.c. into the left hind footpad of different groups of mouse, as indicated. Using a digital micrometer (Mitituyo), footpad swelling was assessed 24 h after CSA injection.

Splenocyte culture and cytokine ELISA

Mice were sacrificed and the spleens were collected and weighed. A part of spleen was used for stamp-smear and the rest was used for proliferation assays. RBC-depleted splenocytes were cultured in 96-well plates at a density of 3×10^5 cells per well in presence or absence of CSA (10 μg/ml). Sixty hours later, supernatants were harvested and [^{3}H]thymidine (1.0 μCi/well, Bhaba Atomic Research Centre) was added to each well. The cultures were pulsed for 12 h, harvested, and counted in a beta-counter by liquid scintillation. IFN-γ in the CSA-stimulated splenocyte cultures were measured by ELISA as described elsewhere (22).

RT-PCR for iNOS2 and IFN-γ receptors

Thioglycolate-elicited peritoneal macrophages from BALB/c, uninfected, or *L. donovani*-infected, were treated with the anti-CD40 Ab or IFN-γ (0.5 ng/ml) in presence or absence of miltefosine (3.2 μM) or with miltefosine (3.2 μM) alone for 6 hours. Total RNA was extracted using TRIzol (Life Technologies). For cDNA synthesis, 1 μg of total RNA from each sample was incubated with random primer, 0.1 M DTT, 500 μM dNTPs, 40 U RNase inhibitor, and 1 μl (200U) of Moloney murine leukemia virus-reverse transcriptase (Life Technologies). Samples were incubated at 37°C for 1 h, followed by 5 min incubation at 95°C. cDNA from each sample was amplified with *Taq*DNA Polymerase (Life Technologies-BRL) in 50 μl under following conditions: 95°C for 2 min, 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min for a total of 35 cycles. Specific primers (GenoMechanix) were designed to amplify the mouse iNOS2 (sense: 5′-AGC TCC TCC CAG GAC CAC AC-3′; antisense:5′-ACG CTG AGT ACC TCA TTG GC-3′). Each sample was amplified for mouse DHFR (sense: 5′-CTC AGG GCT GCG ATT TCG CGC CAA ACT-3′; antisense 5′-CTG GTA AAC AGA ACT GCC TCC GAC TAT C-3′) to ensure equal cDNA input. Similarly, thioglycolate elicited peritoneal macrophages from BALB/c, uninfected, or *L. donovani*-infected, were treated with the anti-CD40 Ab or IFN-γ in presence or absence of miltefosine (3.2 μM) or with miltefosine (3.2 μM) alone for 10 h. Total RNA was extracted and RT-PCR was performed as described earlier. Specific primers (GenoMechanix) were designed to amplify the mouse IFN-γ Rα coding regions (sense: 5′-TAC ACT TCT CCC CTC CCT TTG-3′; antisense 5′-ACA TCA TCT CGC TCC TTT TCT-3′). β-actin, GAPDH, or DHFR amplification was also done as described earlier to ensure equal cDNA input.

Western blot analyses

Western blots were performed as described earlier (23). Peritoneal macrophages from BALB/c or IFN-γ-deficient mice were stimulated with the indicated amount of HPC in presence or absence of different inhibitors (Calbiochem) as indicated. The Abs to the signaling intermediates- recognizing the total and the phosphorylated forms and the secondary Abs, viz., anti-mouse IgM HRP, anti-goat IgG HRP, anti-mouse IgG HRP, and anti-rat IgG HRP were purchased from Santa Cruz Biotechnology.

Miltefosine-induced nitrite production by uninfected and Leishmania*-infected macrophages*

The induction of nitrite production by miltefosine was tested following our earlier protocol (19). In brief, BALB/c-derived peritoneal macrophages (5×10^5 cells/ml), uninfected, or *Leishmania*-infected were primed with HPC (3.2 μM) for 24 h. The macrophages were then treated with IFN-γ (20 ng/ml) for another 24 h before the culture supernatants were collected. The nitrite concentration was measured by Griess reagent (Sigma-Aldrich), as described earlier (19).

FACS analyses for IFN-γ receptor expression

Peritoneal macrophages from BALB/c mice were infected with *L. donovani* promastigotes at a macrophage: parasite ratio of 1:10 for 72 h, as described above. The macrophages were washed and stained with Abs to IFN-γRα and IFN-γRβ (BD Pharmingen), as shown in the figure. The cells were analyzed using FACSVantage flow cytometer (BD Biosciences) (24).

Dendritic cell (DC) generation in vitro and treatment with miltefosine

DC were generated in vitro following established protocol (25, 26). In brief, BALB/c-derived femoral cells were fractionated on Ficoll-Hypaque. The interface cells were depleted of B and T cells and were plated in a 24-well plate (5×10^5 cells/well) with GM-CSF (20 ng/ml) plus IL-4 (15 ng/ml) for 7 days, replenishing medium on third day. The DC were infected with *L. donovani* promastigotes for 72 h and stimulated with HPC, as indicated. At the end of culture, the cells were washed, fixed, Giemsa-stained, and analyzed for parasite load under an oil immersion (×100) in a light microscope (E-600, Nikon).

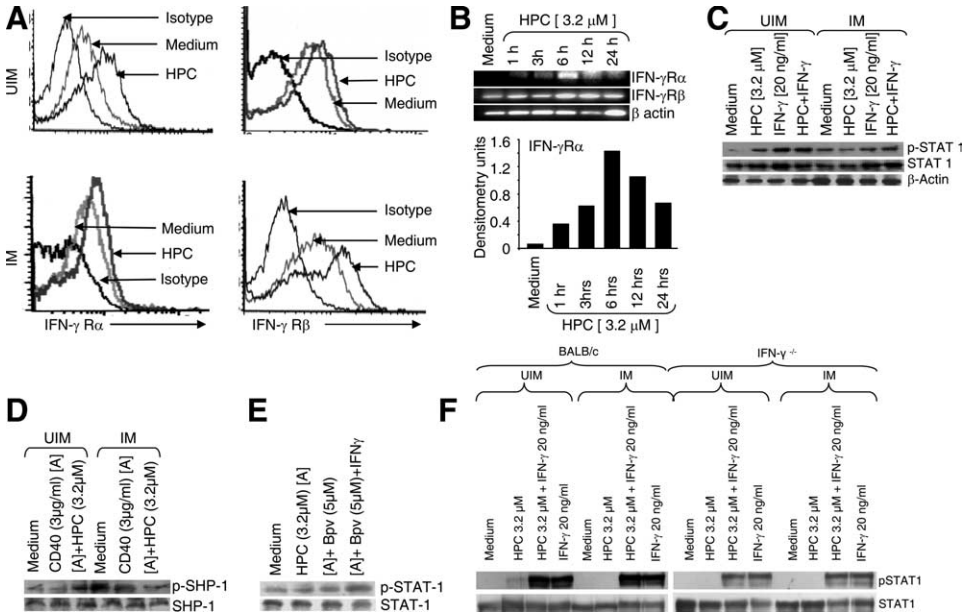

FIGURE 3. Miltefosine enhances IFN-γ responsiveness in macrophages. *A* and *B*, Miltefosine augments the expression of both IFN-γRα and IFN-γRβ. BALB/c-derived macrophages were infected with *L. donovani,* as descried above for 48 h, followed by miltefosine (HPC) treatment for 12 h. The time-point was fixed by the kinetics shown in *B*. The macrophages were stained with the Abs to IFN-γRα and IFN-γRβ, followed by FACS analysis on a FACSVantage flow cytometer. Densitometry data for IFN-γRα from the bottom panel in *B* is shown. *C*, In *L. donovani*-infected macrophages, IFN-γ-induced STAT-1 activation is impaired but is augmented by miltefosine (HPC). The BALB/c-derived macrophages were infected with *L. donovani* for 48 h, followed by 24-h miltefosine (3.2 μM) treatment, as described above. The uninfected (UIM) and the infected (IM) macrophages were treated with IFN-γ for 45 min, followed by cell lysate preparation and Western blot for STAT-1 and phospho-STAT-1, as indicated. *D*, Miltefosine down-regulates SHP-1 activity in infected macrophages (IM). BALB/c-derived peritoneal macrophages, uninfected (UIM) or *Leishmania*-infected (IM) and HPC treated or untreated, as described above, were stimulated with anti-CD40 Ab for 15 min, followed by cell lysate preparation and Western blot for SHP-1 activation. *E*, HPC-induced STAT-1 phosphorylation was augmented by Bpv, an inhibitor of SHP-1, a phosphotyrosine phosphatase inhibitor. *F*, HPC-induced STAT-1 phosphorylation was completely inhibited in IFN-$\gamma^{-/-}$ macrophages. Each of these experiments was performed for at least three times. Results from one experiment are shown here.

Statistical analysis

Each of the experiments was performed at least three times. The data from one representative experiment are shown. The in vitro cultures were set in triplicates and the in vivo experiments were performed with five mice per group. Student's *t* test was performed to ascertain the significance of the differences between the means of the control and the experimental groups.

Results

The anti-leishmanial function of miltefosine requires endogenous IFN-γ

To test whether miltefosine required host cell activation for its full anti-leishmanial function, the thioglycolate-elicited peritoneal macrophages were isolated from IFN-γ-deficient and wild-type BALB/c mice. In general, the purity of macrophages was 98% (Fig. 1*A*). These macrophages were infected with *L donovani* promastigotes and were treated with different doses of miltefosine. It was observed that miltefosine restricted the growth of *L. donovani* in BALB/c peritoneal macrophages in a dose-dependent manner (Fig. 1*B*). Interestingly, the number of amastigotes per hundred macrophages was significantly higher in IFN-γ-deficient macrophages than that observed in BALB/c macrophages despite miltefosine treatment (Fig. 1*B*; $p < 0.001$) suggesting that endogenous IFN-γ is required for optimal anti-leishmanial function of miltefosine. Consistent with this finding, it was observed that miltefosine induced IFN-γ in a dose-dependent manner (Fig. 1*C*). At lower doses of HPC, the IFN-γ induction was significantly less in *Leishmania*-infected macrophages (Fig. 1C; $p < 0.001$). Addition of the anti-IFN-γ Ab reduced the efficacy of the anti-leishmanial function of HPC significantly (Fig. 1*D*; $p < 0.001$ (HPC vs HPC plus αIFN-γ)). Because killing of *Leishmania* amastigotes requires iNOS2, the enzyme that catalyzes the generation of NO, the free radical responsible for killing of *Leishmania* (19), we tested whether HPC induced iNOS2 in macrophages. It was observed that HPC induced iNOS2 expression that peaked around 3–6 h after HPC treatment (Fig. 1*E*). Corroborating to the HPC-induced iNOS2 expression, inhibition of iNOS2 by an inhibitor resulted in reduction of HPC-induced leishmanial killing (Fig. 1*F*), perhaps executed by nitrite production (Fig. 1*G*). Therefore, these observations suggest that miltefosine exerts the anti-leishmanial effects via endogenous IFN-γ and implemented by iNOS2.

Miltefosine enhances the IFN-γ-induced parasite killing in macrophages

We demonstrated earlier that successful chemotherapy restores the host's cell-mediated immunity especially Th1 response (24). Because BALB/c mice reduced the intracellular parasite load more efficiently than the IFN-γ-deficient macrophages, miltefosine may exert its anti-leishmanial effects more effectively in synergism with IFN-γ. Therefore, we tested possible synergy between miltefosine and IFN-γ. It was observed that the anti-leishmanial function of suboptimal dose of miltefosine was enhanced by IFN-γ (0.5 ng/ml) (Fig. 2*A*; $p < 0.001$). However, the synergy between miltefosine and exogenous IFN-γ exerted significantly less anti-leishmanial effect on IFN-γ-deficient macrophages than on BALB/c macrophages (Fig. 2*A*; $p < 0.001$) indicating an essential role of endogenous IFN-γ in restricting the parasite load in macrophages.

Because amastigote elimination requires iNOS2 induction (16, 19), we tested whether HPC and IFN-γ synergized to induce iNOS2 and thereby cooperated in amastigote elimination. It was

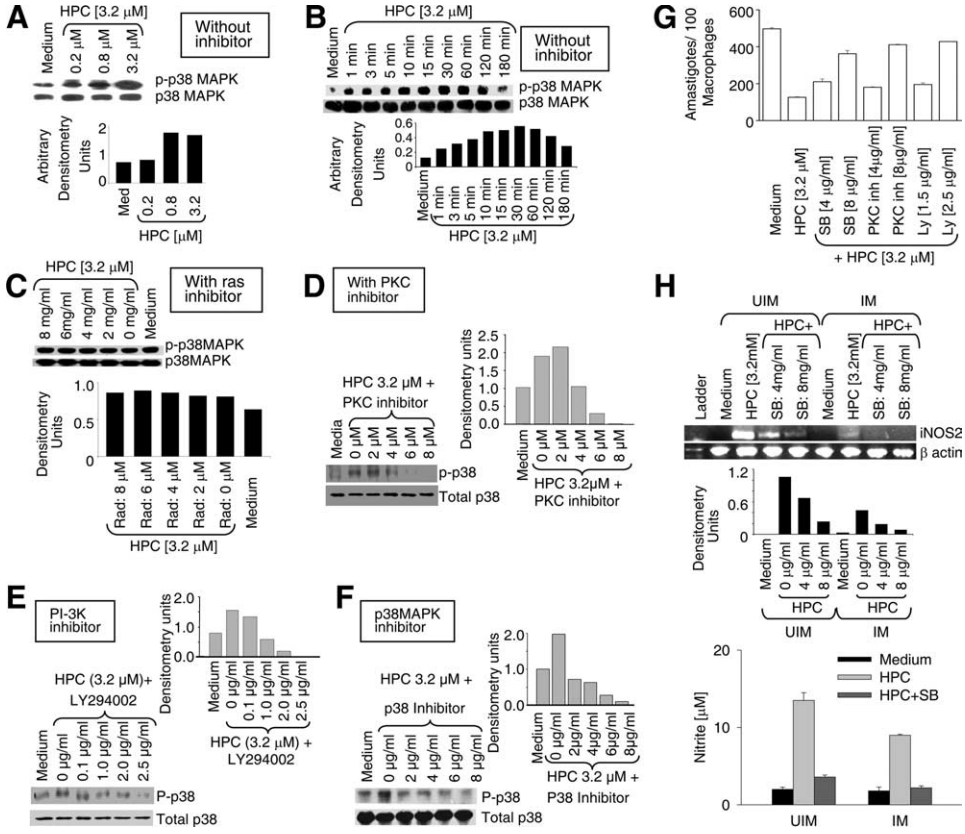

FIGURE 4. Miltefosine induces p38MAP kinase phosphorylation, p38MAP kinase-dependent iNOS2 expression and leishmanicidal activity in BALB/c-derived peritoneal macrophages. *A*, The macrophages were stimulated with the indicated doses of miltefosine (HPC) for fifteen minutes, followed by Western blot for phospho- and total p38MAP kinase, as described earlier (Ref.19). *B*, The macrophages were stimulated with the indicated dose of miltefosine for the indicated time-period, following which the cell extracts were blotted for the phospho- and total p38MAP kinase. *C–F*, Miltefosine-induced p38MAP kinase phosphorylation in presence of different inhibitors as indicated. The cells were treated with miltefosine (HPC) for 15 min. The inhibitors- radicicol (*C*, ras-raf), a PKCβ peptide inhibitor (*D,* PKC-β), Ly294002 (*E*, PI3K), and SB203580 (*F*, p38MAP kinase inhibitor) were used at the indicated concentration. *G*, PKC inhibitor and PI3K inhibitor prevent miltefosine-induced anti-leishmanial function. Anti-leishmanial functions were assessed by the parasite load assay as described above. *H*, p38MAP kinase is required for miltefosine-induced iNOS2 expression (*upper panel*) and NO production (*bottom panel*) in BALB/c macrophages in vitro. The experiments were performed three times and one set of representative data are shown.

observed that miltefosine induced iNOS2 in a dose-dependent manner in both uninfected and *Leishmania*-infected macrophages (Fig. 2*B*); the iNOS2-inducing ability of HPC was compromised in IFN-$\gamma^{-/-}$ macrophages (Fig. 2*C*). Although IFN-γ alone could not induce a strong iNOS2 expression, HPC increased the level of IFN-γ-induced iNOS2 expression (Fig. 2*D*).

Miltefosine enhances IFN-γ responsiveness of macrophages

The observed enhancement of IFN-γ-induced parasite killing and iNOS2 induction by HPC suggested that the drug possibly primed the macrophages to respond to IFN-γ. Because macrophages would respond to IFN-γ through its receptor and signaling, we first tested whether HPC treatment enhanced IFN-γ receptor expression. It was observed that IFN-$\gamma R\alpha$ expression was reduced on *Leishmania*-infected macrophages (Fig. 3*A*) and that miltefosine enhanced the expression of both IFN-$\gamma R\alpha$ and IFN-$\gamma R\beta$ in macrophages (Fig. 3, *A* and *B*), most probably by transcriptional activation. Thus, these results indicate that besides the reported direct effect on the amastigotes, miltefosine does have an immune interface whereby it enhances IFN-γ responsiveness and facilitates IFN-γ-mediated amastigote killing.

Because IFN-γ responsiveness depends not only on IFN-γ receptor expression but also on IFN-γ signaling through STAT-1, we tested IFN-γ-induced STAT-1 expression in miltefosine-treated and untreated macrophages. We observed that *L donovani* infection suppressed the IFN-γ-induced STAT-1 activation, which was partially restored by miltefosine (Fig. 3*C*). Because the IFN-γ-induced STAT-1 was less in infected macrophages (Fig. 3*C*) it could be due to its increased dephosphorylation of its phosphorylated tyrosine residues by SHP-1, a tyrosine phosphatase. Indeed, in *L. major* infection, IFN-γ receptor signaling through STAT-1 decreases due to hyperactivation of protein tyrosine phosphatases (27). Therefore, we tested whether in *L. donovani* infection, the SHP-1 activation was higher and whether miltefosine reduced the SHP-1 activation. We observed that in *L. donovani*-infected macrophages, both the basal and the CD40-induced SHP-1 phosphorylation increased; miltefosine reduced such activation (Fig. 3*D*). In fact, inhibition of SHP-1 by bpv, an inhibitor of SHP-1, augmented the miltefosine-induced STAT-1 activation in macrophages (Fig. 3*E*) suggesting that miltefosine regulates the phosphotyrosine phosphatase activity also to enhance the STAT-1 phosphorylation. In addition, the HPC-induced STAT-1 phosphorylation was completely inhibited in IFN-$\gamma^{-/-}$ macrophages suggesting that the endogenous IFN-γ is required for the HPC-induced STAT phosphorylation (Fig. 3*F*). This result suggests that HPC induces STAT-1 phosphorylation through IFN-γ induction. HPC itself may not be able to induce STAT-1 directly.

Miltefosine induces PKC- and PI3K-dependent p38MAP kinase phosphorylation

Because we have shown previously that p38MAP kinase is required for the induction of iNOS2 (23), we tested whether miltefosine induced p38MAP kinase phosphorylation in macrophages. We observed that at the 3.2 μM concentration, miltefosine induced the peak p38MAP kinase phosphorylation (Fig. 4*A*) and that with that concentration of miltefosine, the peak p38MAP kinase phosphorylation was observed 15 min after stimulation (Fig. 4*B*). Because ras-raf system is implicated in MAP kinase activation, we tested the effect of radicicol, an inhibitor of raf-1 kinase (28), on miltefosine-induced p38MAP kinase phosphorylation. We observed that radicicol had no effect on miltefosine-induced p38MAP kinase phosphorylation (Fig. 4*C*). In contrast, inhibitor peptides of PKC-βII (29) and LY 294002, an inhibitor PI3K (30) prevented the miltefosine-induced p38MAP kinase phosphorylation, respectively (Fig. 4, *D* and *E*). In addition, SB203580, an inhibitor of p38MAP kinase (19), also inhibited the miltefosine-induced p38MAP kinase phosphorylation (Fig. 4*F*). As the inhibitors of PI3K, PKC-βII and P38MAP kinase reduced miltefosine's antileishmanial function significantly (Fig. 4*G*; $p < 0.001$) and as the inhibition of p38MAP kinase also reduced the iNOS2 induction and nitrite production (Fig. 4*H*), these data indicate that miltefosine triggers signaling through PKC-βII and PI3K-dependent p38MAP kinase, resulting in iNOS2 induction and amastigote elimination. Although the increase in PI3K phosphorylation with HPC is modest (Fig. 4*E*), the functional data in Fig. 4*G* shows that the PKC and PI3K inhibitors significantly reduce the antileishmanial function of HPC. In addition, our dose-response and kinetics data show a clear up-regulation of p38MAPK activation by HPC; we also show that HPC induces p38MAP kinase and that its inhibition reduces HPC-induced iNOS2 expression. Thus, miltefosine's one mechanism of action can be linked to its ability to trigger the activation of multiple signaling intermediates in macrophages that eventuates in the expression of host-protective functions.

Miltefosine enhances CD40-induced IL-12 production

In both *L. major* and *L. donovani* infections, IL-12 expression was reported to be another host-protective function of the APCs such as macrophages or DCs (31–33). We showed previously that CD40-induced IL-12 production by macrophages was down-regulated due to impaired p38MAP kinase activation (19, 23). Because miltefosine induced p38MAP kinase activation, we tested whether miltefosine would induce IL-12 in a p38MAP kinase-dependent manner. We observed that miltefosine induced IL-12 production in a dose-dependent manner and that the inhibition of p38MAP kinase by SB203580 reduced HPC-triggered IL-12 production (Fig. 5*A*). Importantly, miltefosine partially restored the IL-12 production by infected macrophages. We had shown previously that CD40 stimulation also induced IL-12 expression in macrophages and that CD40-induced IL-12 production was less in *L. major*-infected macrophages. Because HPC was observed to induce IL-12 production from macrophages, we tested whether miltefosine would synergize with CD40 to enhance IL-12 production from *L. donovani*-infected macrophages. We observed that miltefosine enhanced CD40-induced IL-12 production (Fig. 5*B*), which could be associated with enhanced CD40 expression or CD40-induced p38MAPK phosphorylation. Altogether, activation of these signaling intermediates and their involvement in the leishmanicidal functions of miltefosine suggest a novel mechanism of miltefosine's host cell-dependent anti-leishmanial functions.

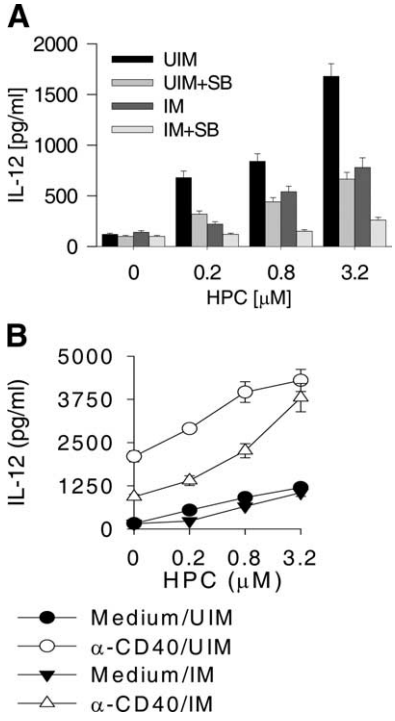

FIGURE 5. Miltefosine enhances CD40-induced IL-12 production. *A*, Miltefosine induces p38MAP kinase-dependent IL-12 production. BALB/c-derived peritoneal macrophages were infected with *L. donovani* promastigotes, as described above and some sets were pretreated with the p38MAP kinase inhibitor, SB203580, as described earlier (Ref. 19). The cells were treated with the indicated doses of HPC for 24 h, followed by IL-12 ELISA in the supernatant. *B*, Miltefosine enhances CD40-induced IL-12 production. The macrophages, uninfected or 48-h infected, were cotreated with anti-CD40 (3 μg/ml) and the indicated doses of HPC for 24 h, followed by IL-12 ELISA in the supernatant. The cultures were set in triplicates and the data shown here are representatives from one of three individual experiments. Error bars represent mean $\pm$ SD.

Miltefosine enhances Th1 response

It was reported that *Leishmania major*-infected macrophages induce Th2 response (34). The uninfected macrophages cultured with Ag-specific T cells induced predominantly IFN-γ production whereas the same macrophages when infected with *L. major* induced primarily IL-4 (31). Because miltefosine induced IL-12 production even from *L. donovani*-infected macrophages and IL-12 is known to induce Th1 response, we tested whether the infected but miltefosine-treated macrophages could induce a Th1 response. It was observed that the *L. donovani*-infected macrophages induced primarily IL-4 whereas the infected but miltefosine-treated macrophages induced primarily IFN-γ (Fig. 6). In addition, inclusion of anti-IL-10 Ab in the culture reduced IL-4 but increased IFN-γ production (Fig. 6). Thus, these results indicate that miltefosine can induce Th1 response.

Miltefosine induces Th1 response in susceptible BALB/c mice

IFN-γ-deficient and BALB/c mice were infected with *L. donovani* promastigotes. Two weeks after infection, mice were treated orally with miltefosine (20 mg/kg body weight) for 5 days. The dose of miltefosine was fixed based on the study reported by Escobar et al. (10). Mice were assayed for delayed-type hypersensitivity (DTH) reactions toward leishmanial Ags and were sacrificed 3 days after the completion of drug treatment. The splenic parasite load, T cell proliferation and cytokine production were assessed. It was observed that miltefosine had significant anti-leishmanial effect in

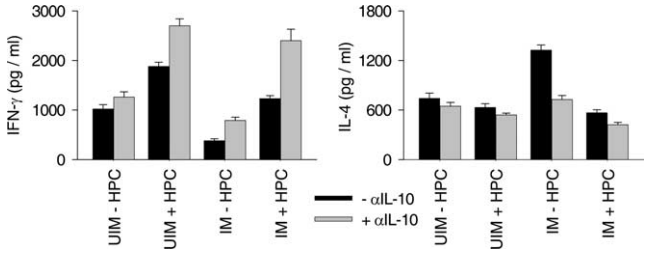

FIGURE 6. Miltefosine enhances Th1 response. BALB/c-derived peritoneal macrophages were infected with *L. donovani* promastigotes for 48 h as described above. The uninfected and infected macrophages were treated with HPC for 24 h. The macrophages were washed, pulsed with CSA (10 μg/ml). In presence or absence of anti-IL-10 Ab, these macrophages were cocultured for another 24 h with the $CD4^+$T cells derived from the BALB/c mice infected with *L. donovani* for 10 days. The cell culture supernatants were assessed for IL-4 and IFN-γ productions by ELISA.

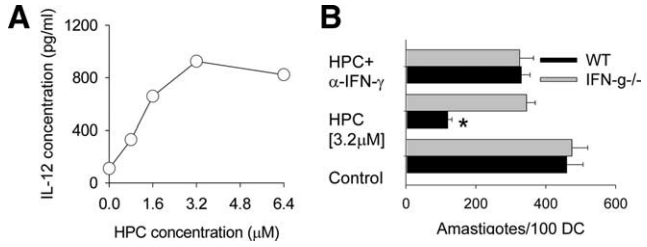

FIGURE 8. Miltefosine (HPC) induces anti-leishmanial functions in DCs. *A*, Miltefosine induces IL-12 from the bone marrow-derived DCs in a dose-dependent manner. The generation of the DCs is described in *Materials and Methods*. *B*, Miltefosine exerts less anti-leishmanial activity in IFN-$\gamma^{-/-}$ DCs, similar to that described for macrophages. The experiments are performed as described in the *Materials and Methods* section. Data from one of the three experiments are presented here. Error bars represent mean ± SD.

BALB/c mice (Fig. 7*A*; $p < 0.005$) but not in IFN-γ-deficient mice (Fig. 7*A*; $p \geq 0.1632$). DTH reactions were also found to be significantly different between the drug-treated and untreated groups (Fig. 7*B*; $p < 0.005$) and as compared with the DTH in BALB/c mice, the IFN-γ-deficient mice mounted significantly less DTH response toward the leishmanial Ags (Fig. 7*B*; $p < 0.001$). The T cell proliferation assay showed no significant difference between the treated and untreated uninfected BALB/c mice but the miltefosine-treated IFN-γ-deficient mice showed significantly higher proliferation as compared with the untreated IFN-γ-deficient mice (Fig. 7*C*; $p < 0.001$). Similarly, although IFN-γ secretion by the splenocytes were comparable between the miltefosine-treated and untreated uninfected BALB/c mice, the IFN-γ secretion was much higher in the miltefosine-treated *L. donovani*-infected BALB/c mice (Fig. 7*D*; $p < 0.001$). In striking contrast, the splenocytes from both the miltefosine-treated BALB/c and IFN-γ-deficient mice secreted significantly more IL-12 than the untreated controls (Fig. 7*E*; $p < 0.001$). Thus, the present study describes for the first time the importance of endogenous IFN-γ, particularly the miltefosine-induced IFN-γ from macrophages, in establishing a host-protective Th1 response.

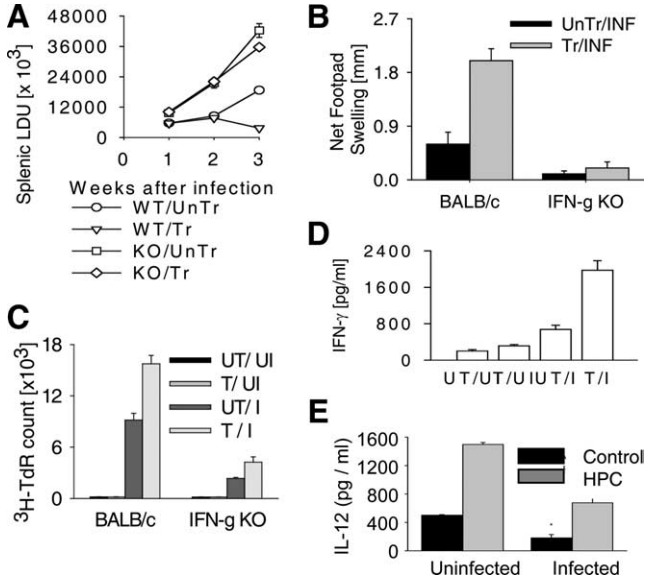

FIGURE 7. Miltefosine and IFN-γ synergize in vivo. *A*, Splenic Leishman-Donovan Unit Assay. IFN-γ-deficient mice (KO) and BALB/c mice (WT) were infected with *Leishmania*. Following 2 wk of infection, some mice were treated with miltefosine (Tr) at 20 mg/kg body weight for 5 days and some mice were left untreated (UnTr). On the third day after the miltefosine treatment, the mice were sacrificed and splenic parasite load was determined. *B*, Delayed-type hypersensitivity assay. The infected BALB/c and IFN-γ-deficient mice (INF), miltefosine treated (Tr) or untreated (UnTr), were injected s.c. in one of the hind footpads with leishmanial Ag (CSA; 40 μg/mouse). Twenty-four hours later, footpad swelling was measured. *C*, T cell proliferation assay. Splenocytes from uninfected (UI) and *L. donovani*-infected (I) BALB/c and IFN-γ-deficient mice, miltefosine treated (T) or untreated (UT) were stimulated with CSA (10 μg/ml) for 72 h. During the last 12 h, the cultures were pulsed with [^{3}H]thymidine. The counts in the cultures with medium alone were very low. *D*, The supernatants from cultures parallel to the above cultures were harvested and IFN-γ contents were assessed by ELISA. *E*, Splenocytes from the above mice, as described in *A* were stimulated with anti-CD40 Ab for 72 h in vitro. The culture supernatants were harvested and assayed for IL-12 production by ELISA. Data from one of the three experiments are presented here. Error bars represent mean ± SD.

Miltefosine also activates DCs

Because DCs also possess the ability to produce IL-12-dependent IFN-γ and can be infected by *Leishmania* parasites, we tested miltefosine's ability to induce these anti-leishmanial functions in DCs. We observed that miltefosine induced IL-12 in a dose-dependent manner (Fig. 8*A*) and endogenous IFN-γ-dependent parasite killing (Fig. 8*B*; *, $p < 0.0001$), as happened in macrophages. Thus, in addition to its direct effects on the parasite (6), miltefosine exerts anti-leishmanial functions also by modulating the functions of various immunocompetent cells such as macrophages and DCs.

Discussion

Visceral leishmaniasis is fatal, if untreated. The first line of treatment is with antimony-based drugs that suffer from toxic side effects in host and resistance of the parasite (35). In addition, antimony-based drugs require assistance from the host immune system to be effective (36). Although T cells are suppressed in *L. donovani* infection, they are required for responsiveness to conventional anti-leishmanial chemotherapy with pentavalent antimony (Sb^V), as it is entirely inactive in T cell-deficient athymic (nude) mice (36). The other drugs available are Amphotericin B (37), particularly in lipid formulation, and paromomycin (38) but none of them is orally bioavailable; as a consequence, the treatment becomes difficult, increasing patient noncompliance. The situation is further aggravated by the coinfection with HIV that cripples the immune system (39–41). Therefore, availability of an orally administrable drug such as miltefosine has been a pressing need for the treatment of visceral leishmaniasis patients.

Miltefosine was originally described as an anti-cancer drug (4). Although looking for its mode of action, it was proposed that the drug may interfere with the lipid synthesis and thereby alters the cellular survival (9). Because the drug has a long fatty acid chain, it was also proposed that the drug might directly go into the lipid layers of the membrane and interfere with cellular signaling (8). When the drug was first observed to be active against *Leishmania* in vitro, it was proposed that the drug might act directly on the parasite (5) causing parasite apoptosis (42). Indeed, studies on cellular uptake of the drug suggested that it went to the parasitophorous vesicle where the amastigotes reside. Although all these observations supported the direct action of the drug on the parasite, there were other observations that indicated to an indirect action of the drug. By the latter mode, miltefosine stimulates the immune system (12–14) that may eliminate *Leishmania* from macrophages. However, neither the macrophage activation resulting in *Leishmania* elimination nor a detailed mechanism of the anti-leishmanial function of the drug in vivo was demonstrated to support the notion.

A report by Murray and Delph-Etienne (6) previously suggested that miltefosine did not require any immune contribution to exert its anti-leishmanial functions in mice. Previous reports on the role of miltefosine as an immunomodulator and our current observations appear to add a new dimension to the conclusion drawn upon the mode of action of the drug (6). Although Murray and Delph-Etiene were right about the direct action of the drug on the parasite, the same did not rule out that the drug could not have any immunomodulatory component that may promote anti-leishmanial functions of macrophages. Thus, ours and previous reports on the immunomodulatory role of miltefosine are not necessarily contradictory to the report by Murray and Delph-Etiene (6).

In this report, we describe the host cell-dependent mechanism of anti-leishmanial action of miltefosine. We have demonstrated that miltefosine activates the macrophages to induce iNOS2 that catalyzes the generation of NO, the reactive radical that kills the parasite (16). One basic immune mechanism that eventuates in leishmanial killing is IFN-γ production from the Th1-type $CD4^+$T cells (43, 44). However, it was reported that IFN-γ failed to work on infected macrophages due to reduced IFN-γ responsiveness (17, 45), which might be due to enhanced STAT-1 inactivation by phosphatases (27) but not necessarily due to less IFN-γ receptor expression (45). In fact, a previous study showed that, in contrast with the wild-type or heterozygous knockout mice, IFN-γ-deficient animals were unable to restrict the growth of the parasite and suffered severe infection over 6–8 wk (46). Because we observed that miltefosine enhanced the IFN-γ-induced anti-leishmanial function of macrophages, we tested whether miltefosine could restore the IFN-γ responsiveness of the infected macrophages. Our observations indicate that miltefosine enhances the IFN-γ receptor expression and IFN-γ-induced STAT-1 activation. Indeed, miltefosine treatment of macrophages decreased SHP-1 phosphorylation and inhibition of SHP-1 by bpV increased the IFN-γ-induced STAT-1 phosphorylation in miltefosine-treated *Leishmania*-infected macrophages. Although the inhibition of SHP-1 by HPC may be direct, the induction of STAT-1 phosphorylation appears to be indirect through the induction of host cell IFN-γ, explaining the absence of HPC-induced STAT-1 phosphorylation in IFN-γ-deficient macrophages (Fig. 3*F*). These observations indicate that miltefosine exerts a direct immune activation such that the miltefosine-treated macrophages are able to respond to IFN-γ and eliminate the parasite.

Next, we examined the mechanism of the modulation of such effector functions in the macrophages. Our data demonstrate a novel p38MAP kinase-dependent host cell-targeted mechanism of miltefosine's anti-leishmanial functions. How miltefosine activates p38MAP kinase in macrophages is unknown but multiple possibilities exist. Firstly, it is possible that miltefosine goes into the cell and interacts with the cell signaling intermediates such PI3K. Secondly, miltefosine incorporates itself into the macrophage membrane and trigger cell signaling. Thirdly, miltefosine works through platelet activating factor receptor, as it shares the structural features with platelet activation factor (PAF). Or, miltefosine induces PAF secretion and PAF executes a part of the anti-leishmanial functions. Indeed, treatment of macrophages with PAF results in better anti-leishmanial control (47) and PAF receptor-deficient mice are susceptible to *L. amazonensis* infection (48). However, none of these reports suggested the mechanism of anti-leishmanial action of PAF, PAF receptor, or miltefosine.

Like miltefosine and PAF, there are other alkyl lysophospholipids who share similar chemical structures and are reported to have anti-leishmanial functions (49–53). These are edelfosine, ilmofosine, and perifosine. The studies on the structure-activity relationship for the anti-leishmanial functions of these compounds are very much limited (54), but all of these are shown to possess anti-leishmanial activity in vitro and in vivo (49–53). However, the sensitivity of *Leishmania* to these drugs may vary with the species (54). As all these drugs are actively taken up by the macrophages, perhaps with a "translocase" on the cell membrane (55), these drugs can have intracellular host cell modifying functions as shown in this study. However, detailed studies with functional group modifications in each of these alkyl lysophospholipids are required to compare their efficacy in modulating host cell functions and anti-leishmanial activity.

Our observations show not only the mechanism of the immunomodulatory action of the drug for the first time but also indicate both the superiority of the drug to the antimony-based drugs and its limitations mechanistically. The apparent superiority of miltefosine to antimony-based drugs lies in the former's ability to induce IFN-γ from macrophages and to enhance IFN-γ responsiveness by inducing IFN-γ receptor in macrophages. STAT-1 phosphorylation in IFN-γ-deficient macrophages was less than that observed in BALB/c macrophages. The reduced STAT-1 phosphorylation in *Leishmania*-infected macrophages was consistent with the reduced IFN-γRα expression on infected macrophages. The fact that miltefosine-induced STAT-1 phosphorylation in IFN-γ-deficient macrophages was not affected by *Leishmania* infection suggests a key role for endogenous IFN-γ in miltefosine-induced STAT-1 phosphorylation. Altogether, these data suggest that miltefosine has at least two basic mechanisms of increasing STAT-1 phosphorylation; one of them is direct and independent of IFN-γ and the other is indirect through endogenous IFN-γ. The indirect component is impaired in IFN-γ-deficient macrophages. Thus, miltefosine activates p38MAP kinase in a PKC-βII- and PI3K-dependent manner. This leads to enhanced IFN-γ responsiveness and expressions of iNOS2 and IL-12. Although enhanced IFN-γ responsiveness promotes the iNOS2-dependent anti-leishmanial functions of IFN-γ on macrophages, enhanced IL-12 production skews the T cell response to Th1-type. The IFN-γ-dominated response further helps clear the parasite in a susceptible host. Thus, using miltefosine as a model anti-leishmanial drug, we define for the first time the host cell-modifying functions of an anti-leishmanial drug.

Disclosures

The authors have no financial conflict of interest.

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