Miltefosine Efficiently Eliminates *Leishmania major* Amastigotes from Infected Murine Dendritic Cells without Altering Their Immune Functions[▽]

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Received 18 July 2009/Returned for modification 18 August 2009/Accepted 20 November 2009

As a treatment for leishmaniasis, miltefosine exerts direct toxic effects on the parasites. Miltefosine also modulates immune cells such as macrophages, leading to parasite elimination via oxidative radicals. Dendritic cells (DC) are critical for initiation of protective immunity against *Leishmania* through induction of Th1 immunity via interleukin 12 (IL-12). Here, we investigated the effects of miltefosine on DC in *Leishmania major* infections. When cocultured with miltefosine for 4 days, the majority of *in vitro*-infected DC were free of parasites. Miltefosine treatment did not influence DC maturation (upregulation of major histocompatibility complex II [MHC II] or costimulatory molecules, e.g., CD40, CD54, and CD86) or significantly alter cytokine release (IL-12, tumor necrosis factor alpha [TNF- α], or IL-10). Further, miltefosine DC treatment did not alter antigen presentation, since unrestricted antigen-specific proliferation of CD4⁺ and CD8⁺ T cells was observed upon stimulation with miltefosine-treated, infected DC. In addition, miltefosine application *in vivo* did not lead to maturation/emigration of skin DC. DC NO⁺ production, a mechanism used by phagocytes to rid themselves of intracellular parasites, was also unaltered upon miltefosine treatment. Our data confirm prior studies indicating that in contrast to, e.g., pentavalent antimonials, miltefosine functions independently of the immune system, mostly through direct toxicity against the *Leishmania* parasite.

Infections with *Leishmania* spp. represent a serious health problem in large parts of the world. *Leishmania* are obligate intracellular parasites, and after being passed on from an infected sand fly, they primarily locate to the phagolysosomes of skin macrophages (M Φ). According to the latest World Health Organization report (2000), 12 million people are currently suffering from leishmaniasis, and an estimated 1 to 1.5 million new cases every year and a death toll of around 70,000 annually are observed (47). Due to these numbers, the development of new drugs or vaccines against leishmaniasis has recently received more attention.

Leishmaniasis patients are mainly treated with pentavalent antimonials. These well-known drugs have considerable disadvantages (30). They need to be given intravenously (i.v.), require 3 to 4 weeks of hospitalized treatment, and have serious side effects. Most importantly, treatment efficacy in certain regions has declined due to an increasing number of resistant strains. Amphotericin B is an effective alternative, but it also requires i.v. administration, is costly, and has severe side effects. The most promising drug that was developed in recent years is miltefosine, an alkylphospholipid (hexadecylphosphocholine). Miltefosine was originally developed as an anticancer drug (38); however, its efficacy in the treatment of murine visceral leishmaniasis was reported soon thereafter (5, 19). Clinical trials led to very promising therapeutic results, resulting in the registration of miltefosine for the treatment of vis-

ceral and cutaneous leishmaniasis (18). Approval for the treatment of cutaneous leishmaniasis is under way.

A direct toxic effect of miltefosine on promastigote and amastigote forms of *Leishmania* in peritoneal macrophages has been well documented (5, 6, 16). The sensitivity to miltefosine appears to be strain dependent. Two *in vitro* studies documented sensitivities with the following decreasing orders of sensitivity: (i) *L. donovani*, *L. aethiopica*, *L. tropica*, *L. mexicana*, *L. panamensis*, and *L. major* (9) and (ii) *L. donovani*, *L. braziliensis*, *L. guyanensis*, and *L. mexicana* (48). A range of clinical studies has shown miltefosine to be particularly effective against *L. major* (24, 28) and *L. panamensis* (32, 33) in studies using human or murine cells. The results were mixed and less impressive in patients infected with *L. braziliensis*/*L. mexicana* (34, 35).

A number of reports have also shown altered cytokine secretion from different types of cells, including human mononuclear cells and murine peritoneal M Φ upon *in vitro* culture with miltefosine (2, 12, 15, 50). In physiological *in vivo* infections, the *Leishmania* parasite interacts with a number of immune cells. M Φ are the predominant host cells taking up *Leishmania* promastigotes shortly after inoculation. The promastigotes bind complement components such as C3b, leading to CR3-mediated uptake. M Φ produce a number of proinflammatory cytokines and, if an effective immune response is generated, ultimately eliminate the parasite through NO. M Φ , however, do not induce protective T-cell responses. Neutrophils are another early player in *L. major* infection. Large numbers migrate to the site of infection and take up parasites (20, 27). Apoptotic infected neutrophils are taken up by M Φ , thus transferring infection (27). However, the key mediators for induction of protective T-cell-mediated immunity against *Leishmania* are dendritic cells (DC) (39, 45). *L. major*-infected, activated DC produce large amounts of interleukin 12 (IL-12)

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[▽] Published ahead of print on 7 December 2009.

and thus initiate a Th1-type response (29, 31, 43). The ensuing gamma interferon (IFN- γ) production from CD4 and CD8 T cells then leads to activation of M Φ , enabling them to produce NO and eliminate the intracellular parasites (21). Recently, the main inducible nitric oxide synthase (iNOS)-producing cells in *L. major* lesions were shown to be inflammatory CD11b⁺ CD11c⁺ major histocompatibility complex class II-positive (MHC II⁺) DC (8).

Even though the effects of miltefosine on M Φ have been studied, no information on whether miltefosine modulates DC function in the context of *Leishmania* infections is available. Since DC are crucial for conferring immunity, we analyzed the influence of miltefosine on both immature and *L. major*-infected, activated, bone marrow-derived DC (BMDC). We found that miltefosine efficiently kills intracellular amastigotes of *L. major* independently of NO⁻ and that miltefosine treatment does not otherwise impede the ability of *L. major*-infected DC to be activated (either *in vitro* or *in vivo*), to release proinflammatory cytokines such as IL-12, and to strongly stimulate antigen-specific CD4 and CD8 T cells.

MATERIALS AND METHODS

Animals and cells. C57BL/6 mice (8 to 12 weeks old) were bred in the Animal Care Facility in Mainz, Germany. Animal experimentation was conducted in accordance with current state and federal guidelines. BMDC were generated in granulocyte-macrophage colony-stimulating factor (GM-CSF)- and IL-4-containing media as described previously (17). BMDC were stimulated with miltefosine (5 to 500 μ M; kind gift from Zentaris AG, Frankfurt, Germany), lipopolysaccharide (LPS)-IFN- γ (100 ng/1,000 U/ml; Sigma/Peprotec) or infected with *L. major* amastigotes.

Parasites and DC infections. For *in vitro* infections of DC, amastigotes of *L. major* clone VI (MHOM/IL/80/Friedlin) were prepared as described previously (5). Isolated parasites were opsonized with 5% normal BALB/c mouse serum and washed before use. DC (2×10^5 cells/ml) were infected for 18 h with amastigotes at a parasite/cell ratio of 5:1, as described previously (46). Cells were then washed extensively to remove adherent, extracellular parasites. Infection rates of DC and the number of intracellular parasites were determined on samples subjected to centrifugation in a Cytospin and stained with DiffQuick (Dade Behring) using light microscopy (magnification, $\times 100$).

Cell survival and apoptosis. To determine the percentage of surviving cells, BMDC incubated with miltefosine were harvested at various time points and incubated with 10 μ M propidium iodide (PI) and annexin V (0.5 mg/ml) for several minutes before the number of viable, PI-excluding/annexin-negative cells was determined using flow cytometry.

Assessment of DC maturation. DC (infected or uninfected) were incubated at 1×10^5 cells/100 μ l and treated with miltefosine (50 μ M) or LPS (100 ng/ml). After 18 h, cells were harvested and washed, and cell surface expression of MHC class II and the costimulatory molecules CD40, CD54, and CD86 was determined as described previously using flow cytometry (41, 43). Cytokine release into 18-h supernatants was determined using an enzyme-linked immunosorbent assay (ELISA) specific for IL-10, IL-12p40, IL-12p70, and tumor necrosis factor alpha (TNF- α). Release of nitrite was determined after 72 h using the Griess reagent (42).

To assess DC activation *in vivo*, 10 μ l of either LPS (100 and 1,000 ng), miltefosine (20 μ g and 200 μ g), or phosphate-buffered saline (PBS) was injected intradermally into both ears. After 48 h, ears were harvested, washed, split, and incubated with 2 mg/ml liberase as described previously (46). After 2 h, cells were dissociated mechanically and counted, and the frequency of CD11c⁺ (clone HL3; Pharmingen)/MHC class II⁺ (clone 2G9; Pharmingen) DC was determined by flow cytometry by gating on monocytes.

Antigen-specific T-cell proliferation. To obtain *Leishmania*-specific T cells, groups of five mice were infected with 2×10^5 infectious-stage metacyclic promastigotes in the skin. T cells were enriched from spleens of >6-week-infected C57BL/6 mice using microbeads against CD4 or CD8. Cells were added to 96-well plates at 10^5 lymph node cells/200 μ l in RPMI-5% fetal calf serum. Irradiated (infected) DC were added at various concentrations. After 48 h, cells were pulsed with 1 μ Ci [³H]thymidine for the final 18 h of culture. Thymidine incorporation was determined using a liquid scintillation counter.

Statistics. Statistical analysis was evaluated using the unpaired Student *t* test.

RESULTS

Viability of DC after treatment with miltefosine. Pharmacokinetic studies in cutaneous leishmaniasis patients with miltefosine treatment revealed a plasma level of 30,800 ng/ml during the last week of a 4-week treatment course. This translates to approximately 75 μ M in culture medium (9); however, in skin, less miltefosine will be present. Thus, we first tested different concentrations of miltefosine in DC cultures to determine at which dosage miltefosine affects DC survival. To do this, we generated immature bone marrow-derived DC from C57BL/6 mice as described previously (16). Cells were then incubated with different doses of miltefosine. After 48 h, the effect of miltefosine on cell survival became statistically significant starting at 25 μ M, with 25% fewer cells surviving than in the untreated control (Fig. 1A). Treatment with 50 μ M led to a 50% decrease, and concentrations of 100 μ M or more led to almost complete cell death. In addition to necrosis, miltefosine-treated DC may also undergo early apoptosis. We thus determined the number of annexin V⁺ DC after 18 and 72 h of culture (Fig. 1B). We did not detect annexin⁺ PI⁻ DC in response to miltefosine treatment above the baseline, indicating that most DC die of necrosis upon coculture with miltefosine. We also assessed the effects of 5 μ M miltefosine over time (Fig. 1C). We detected an increase in cell death at 72 to 96 h. These data are consistent with those of other *in vitro* studies demonstrating similar effects in infected M Φ at comparable doses (1).

Miltefosine does not affect DC maturation. We next analyzed the effect of miltefosine on DC maturation. Maturing DC upregulate a number of activation markers, such as MHC class II, as well as the costimulatory molecules CD40, CD54, and CD86. The expression patterns in *Leishmania*-infected and uninfected DC treated for 18 h with either miltefosine (5, 25, and 50 μ M), LPS (100 ng/ml), or nothing (controls) was examined (Fig. 2). We observed that treatment with miltefosine did not lead to increased DC maturation, as the activation markers showed even lower expression in miltefosine-cocultured DC than both the control (untreated) and LPS-activated DC groups (Fig. 2A and B). In contrast, dose-dependent inhibition of spontaneous DC maturation was observed. Similarly, unaltered DC activation markers were found on *L. major*-infected and miltefosine-treated DC (Fig. 2C).

Next, we studied the effect of miltefosine treatment of primary DC *in vivo* (Fig. 3). Control PBS, LPS (100 ng or 1,000 ng), and miltefosine (20 and 200 μ g) were injected intradermally into ear skin. Forty-eight hours later, we assessed the frequency of CD11c⁺ MHC class II⁺ DC in the skin by flow cytometry. As expected, we detected a considerable decrease in the local CD11c⁺ MHC class II⁺ DC population in LPS (100- and 1,000-ng)-treated groups, while miltefosine (20 and 200 μ g) and sham-treated groups showed no decrease in the skin DC subtypes *in vivo* (Fig. 3).

Cytokine secretion by DC is essential for DC-induced immune responses. To investigate if miltefosine treatment alters mediator production by DC, we determined the cytokine production of DC cocultured either with miltefosine, LPS, or nothing (control). TNF- α , IL-12p40, IL-12p70, and IL-10 levels were determined after 18 h by ELISA (Fig. 4A). As expected, untreated DC produced very little cytokine, whereas

FIG. 1. Survival of DC after treatment with miltefosine. DC were generated as immature cells from bone marrow of C57BL/6 mice and incubated with various doses of miltefosine (0 to 500 μ M) at 1×10^5 cells/100 μ l. (A) After 48 h, the percentage of surviving DC was determined using propidium iodide (PI) exclusion via flow cytometry (20 to 100 ng/ml). Data are means plus standard errors of the means (SEM) ($n \geq 3$; * and ***, $P < 0.05$ and $P < 0.002$ compared to untreated cells after 48 h). (B) Early apoptosis was determined by staining of miltefosine-treated DC with PI and annexin V after 18 and 72 h using flow cytometry. (C) DC were incubated with 5 μ M miltefosine, and cell survival was monitored over the following 4 days. Data are percent PI⁻ cells relative to untreated controls at each time point (mean $\pm$ SEM; $n \geq 2$; ***, $P < 0.002$).

LPS-stimulated cells released considerable amounts of all cytokines studied. Miltefosine-treated DC produced cytokine levels comparable to those produced by the untreated control group, arguing against miltefosine treatment's having a modulating effect on DC cytokine production. In the context of

treatment purposes, it is more interesting to know whether miltefosine has an effect on the cytokine release from *L. major*-infected DC. Thus, we infected DC with *L. major* amastigotes 24 h prior to treatment with either miltefosine, LPS, or nothing (Fig. 4B). As expected, *Leishmania*-infected DC produced higher levels of cytokines than uninfected, untreated DC. LPS addition further increased the cytokine secretion of infected DC; however, infected DC incubated with miltefosine showed no alterations in their cytokine production compared with untreated, infected DC. We therefore conclude that miltefosine does not alter the cytokine production of uninfected or *Leishmania*-infected bone marrow-derived DC.

Miltefosine efficiently eliminates *L. major* amastigotes from DC. *Leishmania*-infected DC were prepared as described above and separated into a control group and a miltefosine-treated group (50 μ M). After harvesting the DC at different time points, we prepared DiffQuick-stained samples after centrifugation in a Cytospin and examined the DC by light microscopy to determine the number of infected cells (Fig. 5A). The number of cells not containing visible intracellular parasites increased with time and rose considerably in the miltefosine-treated group, leading to the majority of DC being free of the parasite after 96 h. In addition, the number of detectable amastigotes per infected DC decreased with time (Fig. 5B). One of the mechanisms allowing phagocytes (e.g., infected M Φ and DC) to rid themselves of intracellular *Leishmania* parasites is the production of NO (2). We next tested whether miltefosine allowed DC to eliminate intracellular amastigotes by leading to increased NO production. Even though LPS- and IFN- γ -treated DC produced considerable amounts of NO as expected (36), miltefosine-treated DC released very small amounts of nitrite comparable to the untreated DC control group (Fig. 5C).

Antigen-specific restimulation of T cells by infected DC is unaltered by miltefosine. One of the main contributions of DC to inducing protective immunity against *Leishmania* is priming and restimulation of antigen-specific T cells and thus initiation of adaptive immunity (4, 40). Thus, we assessed if miltefosine alters DC-mediated expansion of both antigen-specific CD4⁺ and CD8⁺ T cells. After purification of T cells from infected mice, they were cocultured with *Leishmania*-infected DC (T-cell/DC ratio, 10:1) which had been treated with or without miltefosine (50 μ M) (Fig. 6). As expected, ³H incorporation in antigen-specific T cells incubated with *L. major*-infected DC was dramatically increased compared to that in T cells cocultured with uninfected DC. No significant differences in the expansion of *Leishmania*-specific CD4 or CD8 cells were observed when miltefosine-treated versus untreated *L. major*-infected DC were used. These data speak against miltefosine having an influence on DC-mediated T-cell proliferation.

DISCUSSION

DC are crucial for the induction of protective immunity against leishmaniasis. To study whether therapeutic effects of miltefosine rely in part on an alteration of DC function, we analyzed various functional parameters of (*L. major*-infected) DC in the presence of miltefosine. We show that miltefosine does not influence DC function *in vitro* or *in vivo*, and thus the DC are still able to promote protective immunity.

FIG. 2. Miltefosine treatment abrogates *L. major*-induced upregulation of activation markers. (A and B) Bone marrow DC were incubated with 5, 25, or 50 μ M miltefosine or 100 ng/ml LPS for 18 h (1×10^5 cells/100 μ l). Cell activation by miltefosine or LPS was assessed by determination of surface expression levels of MHC class II and the costimulatory molecules CD40, CD54, and CD86 (solid line, isotype staining; filled curve, specific-MAb staining). (C) Prior to miltefosine or LPS treatment, DC were infected overnight with amastigotes of *L. major* (5 parasites/cell; mean infection rate, $23.6\% \pm 1.4\%$) and washed. Subsequently, DC were stimulated with miltefosine or LPS as described above. Data are percent change in surface expression relative to untreated controls (mean $\pm$ SEM; $n \geq 3$; *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.002$).

The direct toxic effect of miltefosine on *Leishmania* promastigotes is well described. However, *in vivo*, *Leishmania* exists primarily in the obligate intracellular amastigote form. The effect of miltefosine on the amastigote life stage has been repeatedly tested in the well-established mouse peritoneal MΦ model. The effects seen were impressive, proving miltefosine more effective than conventional pentavalent antimonials (5, 6, 12). This indicates that miltefosine has a direct toxic effect on the amastigote form; however, the model cannot exclude the possibility that miltefosine has additional beneficial effects on the function of parasite-harboring MΦ, contributing to parasite elimination.

In contrast to $M\Phi$, DC are essential players in initiating and

maintaining a successful adaptive immune response. DC present antigens on both MHC class I and II molecules, leading to priming and activation of CD4 and CD8 T cells. At the same time, their secretion of cytokines, such as IL-12 (3, 4, 37, 41, 45), other IL-12 family members (IL-23 and IL-27) (16), and proinflammatory cytokines (e.g., IL-1 α / β) (13, 44), is crucial for initiating and maintaining a protective anti-*Leishmania* Th1 response. Recently, De Trez et al. also demonstrated that the recruitment and activation of iNOS-producing inflammatory DC in *L. major* infections are associated with resistance against progressive disease (8). In addition, we and others have previously shown differences between the responses of infected DC from *Leishmania major*-resistant and -susceptible mouse

FIG. 3. *In vivo* application of miltefosine does not lead to maturation of resident DC. (A) LPS, miltefosine, or PBS was injected intradermally as indicated. After 48 h, MHC II⁺ CD11c⁺ DC were enumerated by fluorescence-activated cell sorting (FACS) analysis. Representative dot blots from one of two experiments are shown. (B) Pooled data. The percentages of cells in the monocyte gate were calculated (mean $\pm$ SEM; $n = 2$).

strains. Infected DC from resistant C57BL/6 mice produced more IL-1 α and less of the blocking IL-12 homodimer IL-12p80 than DC from susceptible BALB/c mice (26). Therapeutically altering the cytokines produced by DC would certainly be helpful in shifting from a susceptible Th2 toward a protective Th1 response.

In the present study, we observed a significant anti-*Leishmania* effect of miltefosine on amastigotes within infected DC, with a considerable number of cells being completely free of the parasite after two days, as well as fewer parasites in those still infected. This confirms the strong amastigote-killing effect of miltefosine against *L. major*, which has been reported to be one of the species that are least susceptible to miltefosine (32). Our findings fit well with former studies, which showed 50% effective miltefosine doses of 5 to 13 μ M for *L. major* promastigotes and 32 to 37 μ M for amastigotes (10). The doses we used (50 μ M or higher) were well above both of these concentrations.

FIG. 4. Incubation with miltefosine does not significantly alter DC cytokine production. Untreated (A) or *L. major*-infected (B) DC were incubated with miltefosine or LPS at 1×10^5 cells/100 μ l. Cytokine levels were determined in supernatants harvested after 18 h using ELISA specific for TNF- α , IL-12p40, IL-12p70, and IL-10. Data are means plus SEM ($n \geq 3$; *, $P < 0.05$).

Even though miltefosine treatment led to lower parasite numbers or complete eradication of amastigotes from infected DC, it was not by causing an increase in the production of nitric oxide radicals. In light of the recent discovery that the majority of iNOS-producing cells in leishmaniasis lesions are of inflammatory DC origin (8), this might be especially important. Thus, it appears that the primary therapeutic mechanism of miltefosine is direct toxicity to the parasite, which also affected intracellular amastigotes in phagolysosomes. Although effects of miltefosine on phosphatidylinositol metabolism (22) and the alkyl-acyl-coenzyme A acyltransferase (23) in *Leishmania* have been reported, the exact mechanism of miltefosine-induced parasite toxicity is still unknown.

Previously, various groups have reported increased production of factors such as IFN- γ , GM-CSF, TNF- α , and NO when treating various cell types with miltefosine, including human mononuclear cells and murine peritoneal M Φ (2, 11, 15, 50). Miltefosine applied in micellar, liposomal, or multilamellar vesicle form led to TNF- α and NO secretion from rat liver and mouse peritoneal M Φ (49, 50) as well as the human histiocytic cell line U937 (11). By using Northern blotting, Beckers et al. showed transcription of receptors for IL-3, GM-CSF, and FcR1 in KG1 cell lines treated with miltefosine (2). Hochhuth et al. reported considerable increases in IFN- γ and GM-CSF

FIG. 5. *L. major* amastigotes are eliminated from infected DC by miltefosine treatment despite absent NO production. C57BL/6 DC were infected overnight with amastigotes at a cell/parasite ratio of 1:5 (A and B) or 1:10 (C). Some cells were left untreated. (A and B) Infected cells were washed thoroughly to remove extracellular parasites and plated at 1×10^5 cells/100 μ l. Half of the wells were additionally incubated with 50 μ M miltefosine. Every 24 h, cells were harvested and subjected to centrifugation in a Cytospin, and the percentage of infected cells (A) as well as the number of intracellular parasites (B) was determined by light microscopy. Data are means plus SEM ($n \geq 3$; ***, $P < 0.002$; *, $P < 0.05$). (C) After washing, cells were plated at 2×10^6 cells/ml and incubated with 50 μ M miltefosine or LPS (100 ng/ml) and IFN- γ (1,000 U/ml). Nitrite production was assessed after 72 h using the Griess reagent (means plus SEM; $n = 7$; LPS/IFN- γ , $n = 4$; *, $P < 0.05$).

gene expression as well as IFN- γ secretion in IL-2-stimulated human mononuclear cell cultures when miltefosine was added (15). These reports support the idea that miltefosine may achieve its therapeutic success by positively modulating the M Φ response to *Leishmania*. In contrast, according to our data, miltefosine treatment does not significantly modulate DC

FIG. 6. Unaltered antigen presentation by miltefosine-treated, *L. major*-infected DC. CD4⁺ (A) and CD8⁺ (B) T cells were enriched from C57BL/6 mice that had been infected with *L. major* at least 6 weeks earlier. DC were incubated with *L. major* overnight or left untreated, washed, and incubated for 72 h in the presence or absence of miltefosine (50 μ M). T cells (1×10^5) were incubated with various concentrations of irradiated DC (starting with a T-cell/DC ratio of 10:1). After 48 h, cells were pulsed with 1 μ Ci [³H]thymidine for the last 18 h of incubation. Thymidine incorporation was determined using a liquid scintillation counter. Data are means plus SEM from ≥ 3 independent experiments.

function in immune responses against LPS or *L. major* infection. Miltefosine did not lead to DC maturation in terms of altered expression of costimulatory markers and MHC class II molecules or cause an increase or alteration in cytokine production in *L. major*-infected or uninfected DC. Miltefosine appears to slightly downmodulate DC activation and cytokine release *in vitro*, but only at higher doses. In addition, *in vivo* application of miltefosine did not lead to maturation and emigration of dermal DC. Miltefosine also showed no effect on the DC-induced proliferation of antigen-specific T cells.

Two groups have reported that miltefosine was effective in mice independent of the presence of T cells (11, 25). In addition, various immune cell types, including natural killer cells and cytotoxic spleen cells, as well as M Φ activity and antibody production were unaffected by miltefosine treatment (14). Our data confirm these findings. A number of reports have observed that M Φ produce more radical nitrogen intermediates (RNI) and radical oxygen intermediates (ROI) upon miltefosine treatment (2, 11, 49, 50). In our case, we did not see an augmentation of RNI production in DC in the presence of miltefosine. Our findings do, however, fit very well with the fact

that miltefosine treatment was also effective in mice unable to produce RNI, ROI, or both (25). Thus, in conjunction with other reports, particularly the *in vivo* experiments showing that miltefosine treatment is effective in various knockout mice lacking either T cells, IFN- γ , ROI, or NOI, our data support the notion that miltefosine primarily exerts an immune system-independent effect. Although we have tested the effect of miltefosine on DC only in *L. major* infections, it is likely that the situation is similar in the context of infections with other *Leishmania* strains.

In conclusion, our findings have implications for the clinical setting. The standard treatment for leishmaniasis is pentavalent antimonials. Escobar et al. showed that successful treatment of *L. donovani*-infected mice with pentavalent antimonials was dependent on T cells (11), implying that immunocompromised patients will not respond as well to treatment. This was also noted clinically, with high relapse rates of *Leishmania* infections in HIV-coinfected individuals treated with antimonials (7). In contrast, the results of the present study would argue for miltefosine's being a good treatment option even for immunocompromised patients, since it appears to function independently of the immune system.

ACKNOWLEDGMENTS

This work was funded by SFB490 and STE833/6-1 of the Deutsche Forschungsgemeinschaft (DFG) to E.V.S.

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