

Miltefosine Effectively Modulates the Cytokine Milieu in Indian Post Kala-Azar Dermal Leishmaniasis

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Background. The increasing incidence of unresponsiveness to antimonials in leishmaniasis prompted the use of newer drugs such as miltefosine. Miltefosine influences macrophage effector functions, but its effect on patients with post kala-azar dermal leishmaniasis (PKDL) has not been evaluated.

Methodology. The immunomodulatory activity of miltefosine was evaluated in patients with PKDL by studying the expression of activation markers (CD14 and CD16) and costimulatory molecules (CD80 and CD86) on circulating monocytes, levels of pro-inflammatory cytokines (tumor necrosis factor α , interleukin 6, interleukin 1 β , and interleukin 8) and anti-inflammatory cytokines (interleukin 10, transforming growth factor β , interleukin 4, and interleukin 13) in serum and peripheral blood mononuclear cell culture supernatants, and serum nitrite and arginase activity.

Results. Miltefosine on circulating monocytes upregulated expression of CD16 and CD86 and reduced that of CD14. Miltefosine also induced a significant increase in circulating levels of pro-inflammatory cytokines with a concomitant decrease in anti-inflammatory cytokines. Its macrophage activating potential was evidenced by its ability to decrease serum arginase activity and increase serum nitrite.

Conclusions. Miltefosine increased the proportion of monocytes that have a pro-inflammatory phenotype, which was accompanied by an enhanced secretion of pro-inflammatory cytokines and increased levels of serum nitrite. The decrease in anti-inflammatory cytokine levels and serum arginase activity collectively indicated that miltefosine triggered a robust T-helper 1 response that facilitated parasite elimination.

Post kala-azar dermal leishmaniasis (PKDL) is caused by persistence of the protozoan parasite *Leishmania donovani* in the skin following successful treatment of visceral leishmaniasis (VL) or kala-azar. Because transmission of leishmaniasis in India is anthroponotic, patients with PKDL are potential reservoirs of the parasite, especially during interepidemic periods of VL [1] (and references therein). The disease occurs mainly in

Sudan and its adjoining areas as well as in the Indian subcontinent, but the disease pathology varies: in Sudanese PKDL, the immune mechanisms have been established [2]; whereas in Indian PKDL, underlying immunopathological mechanisms have only partially been established and remain restricted to production of immunoglobulins and their subclasses [1, 3], as well as the cell-mediated immune response mediated by T lymphocytes [1, 3]. Notably, information related to the status of circulating pro-inflammatory monocytes and associated cytokines remains limited [1] (and references therein).

The outcome of leishmanial infections is determined by 2 functionally distinct T-helper (Th) cell populations, namely, a pro-inflammatory Th1 population (eg, interleukin 2 [IL-2], interferon γ [IFN- γ], interleukin 6 [IL-6], and tumor necrosis factor α [TNF- α]) and an anti-inflammatory Th2 population (eg, interleukin 4 [IL-4], interleukin 10 [IL-10], interleukin 13 [IL-13],

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and transforming growth factor β [TGF- β]). The cutaneous form of the disease, cutaneous leishmaniasis (CL), is considered to be the best documented example of the differential activation of Th1 and Th2 subsets [4], wherein disease susceptibility is associated with Th2 proliferation whereas expansion of IFN- γ -producing CD4⁺ Th1 cells occurs in healing responses. However in VL, the disease is associated with marked impairment of macrophage functions [5] and a mixed Th1/Th2 immune profile [6].

In chronic parasitic diseases, one of the major effector mechanisms necessary for parasite clearance is activation of pro-inflammatory monocytes and production of pro-inflammatory cytokines [7]. In CL, monocytes are activated even during the disease process, which possibly accounts for their self-healing nature [8], and chemotherapy is accompanied by higher circulating levels of pro-inflammatory cytokines [7]. However, information regarding the scenario in Indian VL or PKDL remains limited; this study aimed to delineate whether in PKDL, chemotherapy caused expansion of circulating pro-inflammatory monocytes and pro-inflammatory cytokines, along with reduction in anti-inflammatory cytokines, leading to resolution of disease.

MATERIALS AND METHODS

Reagents

All immunological reagents were from BD Biosciences (San Jose, CA) except anti-human CD16 phycoerythrin (PE) and CD14 fluorescein isothiocyanate (FITC), enzyme-linked immunosorbent assay (ELISA) kits for IL-4, IL-6, IL-8, IL-10, IL-13, and TNF- α (Immunotools, Friesoythe, Germany), and Quantikine immunoassay kits for IL-1 β and TGF- β (R&D systems, Minneapolis, MN). All other reagents were from Sigma-Aldrich (St. Louis, MO) except protease inhibitors (Roche, Penzberg, Germany); rK39 strips (InBios International, Seattle, WA); *N*-(1-naphthyl)ethylenediamine dihydrochloride (Loba Chemie, Mumbai, India), trichloro acetic acid (TCA), sulphanimide, L-arginine, manganese chloride, and urea (Sisco Research Laboratories, Mumbai, India); and HiSep 1073 (Himedia, Mumbai, India).

Study Population

From 2008 through 2010, patients who received clinical diagnoses of PKDL ($n = 35$) were recruited from the Dermatology Outpatient Department, School of Tropical Medicine, Kolkata, India. Clinical diagnosis was based on clinical features and a prior history of VL or if they hailed from an area endemic for VL. PKDL was confirmed by rK39 strip test, Giemsa staining, and ELISA for antileishmanial antibodies. Patients ($n = 32$) were randomly allocated to receive either sodium antimony gluconate (SAG; 20 mg/kg body weight/day intramuscular for 4 months) or miltefosine (100 mg/day per oral for 4 months),

whereas 3 patients aged <10 years were specifically treated with SAG, to avoid potential adverse effects of miltefosine; 24 patients completed treatment with SAG ($n = 12$) or miltefosine ($n = 12$). Age- and sex-matched healthy volunteers ($n = 10$) were recruited from nonendemic areas and were seronegative for antileishmanial antibodies. Samples were collected at disease presentation and on completion of treatment. The study received approval from the institutional ethical committee of the School of Tropical Medicine, Kolkata, and Institute of Post Graduate Medical Education and Research, Kolkata. All individuals gave written informed consent, and it was provided by the parent or guardian for a minor.

Preparation of Crude *Leishmania* Lysate

Crude *Leishmania* antigen lysate was prepared from *L. donovani* strain MHOM/IN/90/GE1F8R [3].

Isolation of Monocytes

Monocytes were isolated from peripheral blood samples with use of monocyte isolation medium (HiSep LSM 1073); cells were finally resuspended in Roswell Park Memorial Institute 1640 medium supplemented with penicillin (100 U/mL), streptomycin (100 μ g/mL), and 10% heat-inactivated fetal bovine serum. Viability was confirmed using trypan blue (>95%).

Monocyte Activation Markers in Patients With PKDL

Isolated monocytes were stained with fluorochrome-conjugated antibodies against cell surface CD antigens CD16-PE, CD14-FITC/PerCP, CD80-PE, and CD86-FITC with appropriate isotype controls [3]. Monocytes were gated on their forward versus side scatter characteristics followed by fluorescence; 5000 monocytes were acquired and analyzed using CellQuest Pro software (BD Biosciences, San Jose, CA).

Production of Cytokines From Peripheral Blood Mononuclear Cells

Isolated peripheral blood mononuclear cells (PBMCs) [3] were incubated with or without *Leishmania* antigen (20 μ g/mL) at 37°C in 5% carbon dioxide for 6 days to determine levels of pro- and anti-inflammatory cytokines; after centrifugation (4000 rpm for 5 minutes), supernatants were collected and stored at -20°C. Circulating and culture supernatant levels of TNF- α , IL-6, IL-8, IL-4, IL-10, IL-13, TGF- β , and IL-1 β were quantified by ELISA.

Determination of Serum Nitrite Levels

The serum nitrite levels, a stable representative of nitric oxide (NO), was determined by a modified Griess assay [9]: serum was mixed with 5% TCA (1:9 dilution) and centrifuged (8000 rpm for 5 minutes), supernatants were mixed with an equal volume of Griess reagent and incubated for 30 minutes at 37°C, and absorbances were measured at an optical density of 546 nm

(OD₅₄₆). The specific OD₅₄₆ value was calculated by subtracting the OD₅₄₆ of TCA from that of serum; the nitrite concentration was determined using a standard curve of sodium nitrite (0–100 µmol/L).

Measurement of Serum Arginase Activity

Arginase activity was measured [10] wherein the reaction mixture containing L-arginine (0.5 mol/L), manganese chloride (1.0 mmol/L), and serum (100 µL diluted 1:1 with phosphate-buffered saline) was incubated at 37°C for 20 minutes; the reaction was stopped by an acidic solution (sulfuric acid, phosphoric acid, and water diluted 1:3:7 vol/vol; 800 µL), followed by 100 µL of 3% isonitrosopropiophenone (in absolute ethanol). The samples were heated (95°C) for 40 minutes and absorbance values were measured at 540 nm. The calibration curve was prepared using urea (1–200 µg), and arginase activity expressed in units per liter, where 1 unit (U) is defined as the amount of enzyme that catalyzes the formation of 1.0 µmol of urea in 1 minute.

Statistical Analysis

Data were analyzed between groups with a Kruskal-Wallis test followed by Dunn multiple comparison tests for nonparametric data and a Wilcoxon signed rank test for paired data, using Graph Pad Prism software (version 5.0). Results for which $P < .05$ were considered to be significant.

RESULTS

Study Population

The majority of patients with PKDL ($n = 35$; age, 8–65 years) were male, with the ratio of male and female being 4:1. This is possibly attributable to the underlying social bias of men being accorded greater access to medical care, rather than the disease process. The disease was considered to be macular (only hypopigmented lesions) or polymorphic (hypopigmented lesions along with nodules and/or papules) on the basis of clinical features. The polymorphic group predominated ($n = 22$ [62.86%]) over the macular group ($n = 13$ [37.14%]). The interval between VL and PKDL was 0.4–41 years ($n = 30$ [85.71%]), whereas 5 patients (14.29%) gave no prior history of VL; all patients tested positive for rK39 and ELISA. Both treatment arms were evenly distributed for age, disease duration, disease interval, and lesional status, except for the male-to-female ratio (Table 1). Assessment of cure was based on clinical features, wherein the clinical improvement was more evident in patients with polymorphic PKDL.

Expression of Activation Markers of Monocytes in Patients With PKDL

Because monocytes are pivotal for the pathogenesis of leishmaniasis, it is implied that parasite removal should entail its activation. To measure the degree of activation of monocytes,

Table 1. Clinical Features of Study Population

Parameter	SAG	Miltefosine
Age, years	30.69 (4.11)	30.31 (4.35)
Range	8–60	10–60
Sex ratio, male:female	5:1	11:1
Lesional type, no. of patients		
Polymorphic	9	9
Macular	3	3
Disease duration, years	5.16 (1.41)	4.71 (1.08)
Interval between cure of VL and onset, years	1.78 (0.39)	5.08 (3.26)
Range	0–4.5	0–33

Data are mean (SEM) values, unless otherwise indicated. Patients with post kala-azar dermal leishmaniasis were treated with either sodium antimony gluconate (SAG) or miltefosine as described in the Materials and Methods. Abbreviation: VL, visceral leishmaniasis.

the expression levels of CD14 and CD16 were determined [11]. At disease presentation, CD14 positivity was comparable to that in healthy control individuals. Significant differences appeared after treatment, and as with miltefosine, the proportion of CD14⁺-monocytes significantly decreased compared with the proportion at presentation (Figure 1; Table 2). However, SAG had minimal influence on curtailment of CD14 expression (Figure 1; Table 2). CD16 levels at presentation were comparable with those in controls; these levels increased significantly with miltefosine, but not with SAG (Figure 1; Table 2). The influence of miltefosine on monocytes through an increase in CD16 positivity concomitant with a reduction in CD14 positivity collectively indicated the potential of miltefosine to enhance the monocyte population having a pro-inflammatory phenotype.

Expression of Costimulatory Molecules on Monocytes

The role played by monocytes as major antigen-presenting cells skews the immune system toward Th1 or Th2 responses and also strengthens the interaction between T-cell receptor (TCR) and major histocompatibility complex (MHC) via the costimulatory molecules B7.1 (CD80) and B7.2 (CD86) [12]. At presentation, patients with PKDL show a downregulation of CD28 [13], a costimulatory molecule present on CD8⁺ T cells, which interacts with CD80 and CD86. Importantly, CD28 is restored following chemotherapy [13]. Immunophenotypic analysis of these costimulatory molecules revealed that expression of CD86 on CD14⁺ monocytes was significantly decreased at presentation as compared with controls, and reverted following miltefosine treatment (Figure 2; Table 2). With regard to CD80, no changes were evident (Figure 2; Table 2).

Miltefosine Upregulated Monocyte-Associated Pro-Inflammatory Cytokines

Serum levels of pro-inflammatory cytokines TNF- α , IL-6, IL-1 β , and IL-8, the primary source of which are monocytes and

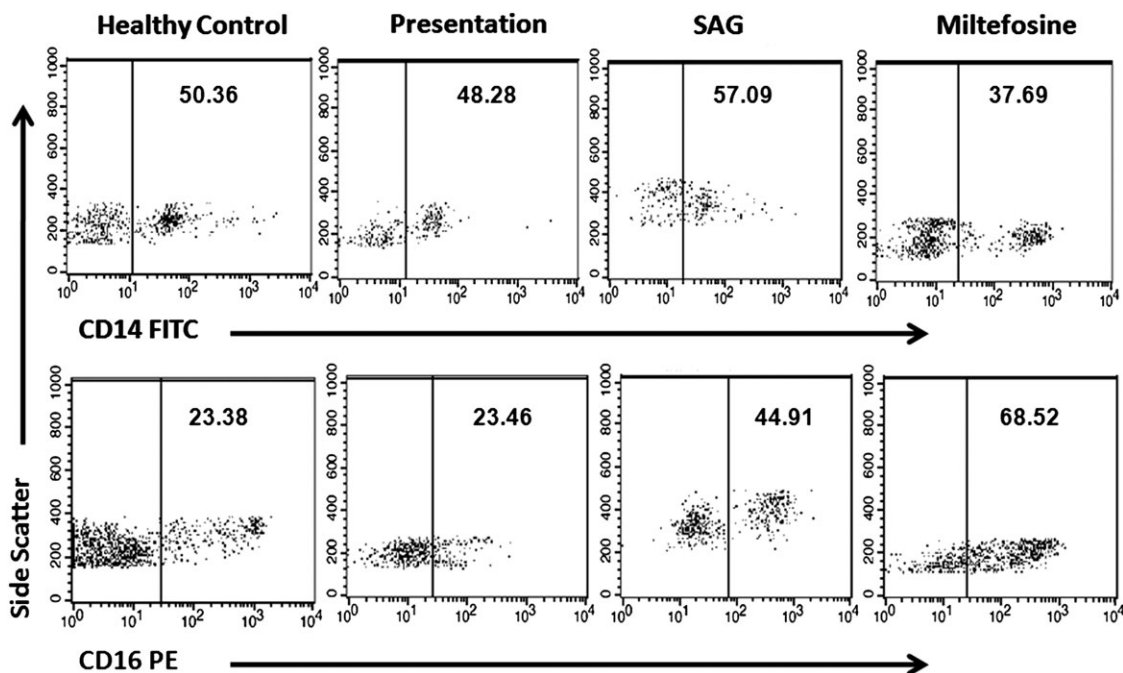


Figure 1. Flow cytometric representation of CD14⁺ and CD16⁺ monocytes in a representative sample of healthy control individuals, patients with post kala-azar dermal leishmaniasis at presentation, and those patients after treatment with sodium antimony gluconate (SAG) or miltefosine. Monocytes were stained with fluorochrome-labeled anti-CD14 and anti-CD16, monocytes were gated, and fluorescence was measured as described in the Materials and Methods. Abbreviations: FITC, fluorescein isothiocyanate; PE, phycoerythrin.

macrophages, were quantified. At presentation, levels of TNF- α were significantly elevated compared with those in controls (Table 3). In patients treated with SAG, a 3.6-fold increase was evident in the level of TNF- α , whereas miltefosine caused a 4.7-fold increase (Table 3). On an individual basis, SAG induced a greater increase (Figure 3A).

With regard to IL-6, levels at presentation were comparable to those in controls; following treatment, a 3.8-fold increase occurred in patients receiving SAG, whereas miltefosine caused a significant 4.7-fold increase (Table 3). On an individual basis, miltefosine alone significantly increased levels of IL-6 (Figure 3A).

Similarly with IL-1 β , levels at presentation were comparable to those in controls; following treatment, a 13.9-fold and 13.1-fold increase was observed with SAG and miltefosine, respectively (Table 3). These data were corroborated when paired samples were analyzed (Figure 3A). Another cytokine, IL-8, showed substantially higher levels (11.3-fold increase compared with controls) in patients with PKDL at presentation (Table 3). Treatment with SAG or miltefosine caused a significant 2.1-fold and 2.6-fold increase, respectively (Table 3); analysis on an individual basis indicated that miltefosine significantly increased IL-8 levels (Figure 3A). Miltefosine consistently enhanced levels

Table 2. Activation Markers of Monocytes in Patients With Post Kala-Azar Dermal Leishmaniasis (PKDL) at Presentation and After Treatment

Monocyte surface marker	Healthy control individuals	Patients with PKDL at presentation	Patients with PKDL after treatment	
			SAG	Miltefosine
CD14 ⁺	64.70 (4.39)	58.60 (2.65)	56.16 (0.97)	40.19 (2.51) ^{a,b}
CD16 ⁺	25.81 (3.39)	24.35 (2.60)	34.84 (5.47)	42.01 (5.19) ^b
CD14 ⁺ CD80 ⁺	1.83 (0.91)	1.47 (0.47)	6.18 (2.30)	3.83 (2.29)
CD14 ⁺ CD86 ⁺	52.11 (6.58)	25.42 (2.59) ^c	48.88 (7.40)	52.78 (7.17) ^b

Data are mean (SEM) no. of monocytes stained with surface marker antibodies and acquired on a flow cytometer as described in the Materials and Methods. Abbreviation: SAG, sodium antimony gluconate.

^a $P < .01$ compared with healthy control individuals.

^b $P < .05$ compared with presentation.

^c $P < .05$ compared with healthy controls.

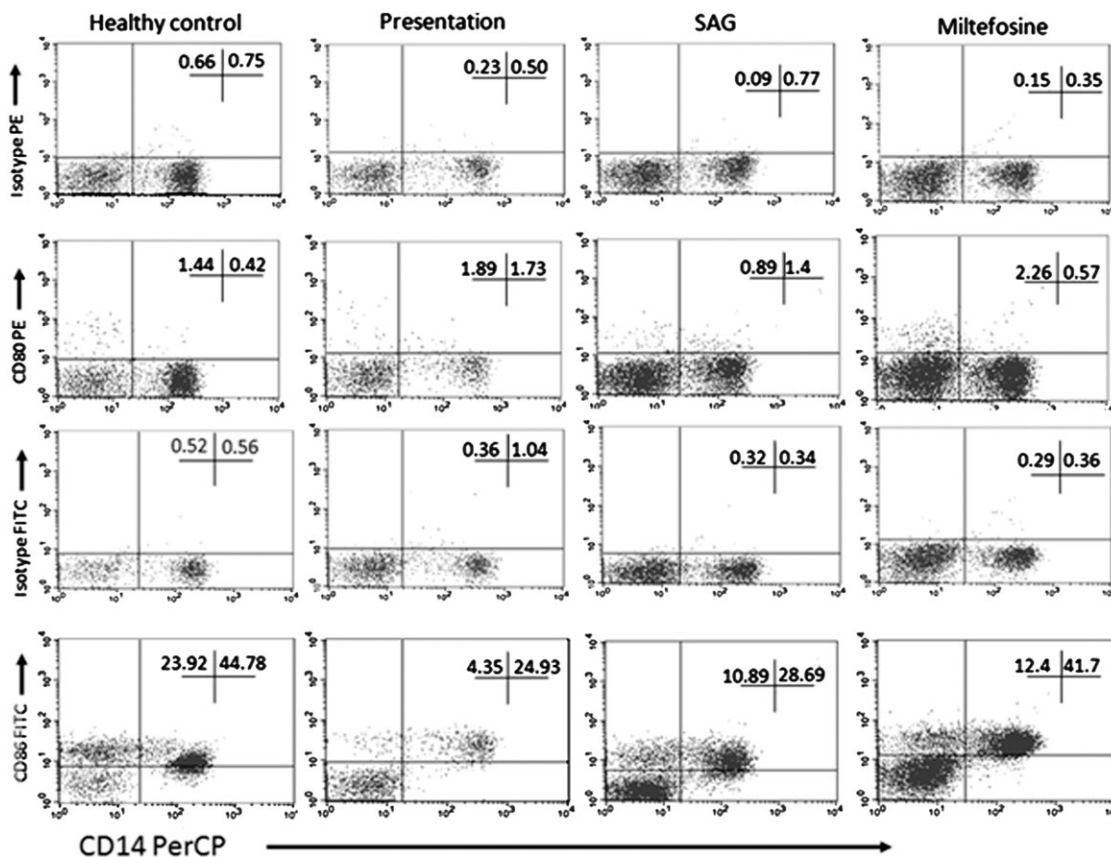


Figure 2. Expression of costimulatory molecules on monocytes in a representative profile of healthy controls individual, patients with post kala-azar dermal leishmaniasis at presentation, and those patients after treatment with sodium antimony gluconate (SAG) or miltefosine. Monocytes were stained with fluorochrome-labeled anti-CD14, anti-CD80, and anti-CD86 with their respective isotypes. Monocytes were initially gated and fluorescence was quantified as described in the Materials and Methods. Abbreviations: FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin chlorophyll protein complex.

of pro-inflammatory cytokines, indicating that its immunomodulatory effect was more pronounced than that of SAG.

Miltefosine Decreased Levels of Anti-Inflammatory Cytokines

Circulating levels of anti-inflammatory cytokines (IL-10, TGF- β , IL-4, and IL-13) were enumerated by sandwich ELISA. IL-10 levels were significantly higher in patients with PKDL at presentation than those in healthy controls and regressed significantly with miltefosine, whereas SAG was less effective (Table 3; Figure 3B).

TGF- β , an established anti-inflammatory and regulatory cytokine, plays an important role in disease progression in leishmaniasis [14], but its status in PKDL remains unknown. TGF- β levels were significantly increased in patients with PKDL at presentation compared with those in healthy controls (Table 3). Treatment caused minimal changes of a 1.49-fold and 1.95-fold decrease with SAG and miltefosine, respectively (Table 3; Figure 3B).

Levels of IL-4, another important anti-inflammatory cytokine, increased 2.2-fold more in patients with PKDL than those in healthy controls, and treatment caused a marginal decrease (Table 3). Upon individual analysis, it was found that miltefosine significantly decreased serum IL-4 levels (Figure 3B).

Similarly, with IL-13, a 5.7-fold increase at presentation was evident as compared with healthy controls (Table 3). Treatment with SAG or miltefosine translated into a 1.5-fold and 5.4-fold decrease, respectively (Table 3), but upon individual analysis, neither treatment was effective (Figure 3B).

Production of Cytokines by Cultured PBMCs

Evaluation of the production of cytokines following stimulation with *Leishmania* antigen in PBMCs showed that only the pro-inflammatory cytokine TNF- α was increased by miltefosine via an antigen-specific upregulation. Levels of IL-6 were significantly increased at presentation in both the stimulated and unstimulated populations, and treatment caused minimal changes, with levels remaining significantly higher than in healthy controls (Table 4). SAG induced IL-8 secretion only in the antigen-specific population, whereas Miltefosine induced it in the antigen-specific and nonspecific population (Table 4).

In the anti-inflammatory cytokine milieu, the increased levels of IL-10 at presentation were effectively decreased by miltefosine irrespective of antigenic stimulation, whereas SAG-mediated curtailment was only evident in the unstimulated population. Changes in IL-4 and IL-13 levels were minimal (Table 4).

Table 3. Serum Levels of Cytokines in Study Population

Cytokine	Healthy control individuals, pg/mL	Patients with PKDL at presentation, pg/mL	Patients with PKDL after treatment, pg/mL	
			SAG	Miltefosine
TNF-α				
Mean (SEM)	6.70 (2.44)	81.43 (13.14) ^a	289.40 (132.20) ^b	381.20 (143.10) ^b
Median (range)	4.1 (0.0–13.3)	53.0 (20.5–116.4)	125.9 (27.2–204.9)	120.8 (49.3–750.3)
IL-6				
Mean (SEM)	53.94 (9.26)	63.10 (10.21)	238.40 (78.94)	296.60 (92.56) ^c
Median (range)	57.4 (30.5–81.6)	55.3 (10.6–90.7)	159.7 (12.4–369.2)	217.9 (45.1–370.3)
IL-1β				
Mean (SEM)	7.20 (2.87)	7.44 (1.99)	103.20 (48.98)	96.95 (30.37) ^c
Median (range)	2.7 (0.0–16.0)	2.4 (0.1–9.8)	5.7 (4.4–193.5)	31.0 (9.3–222.4)
IL-8				
Mean (SEM)	82.98 (35.34)	938.00 (227.00) ^d	1929.00 (559.80) ^b	2421.00 (628.00) ^b
Median (range)	32.8 (15.8–143.8)	336.2 (62.3–1542.0)	1568.0 (400.2–4160.0)	1396.0 (767.8–4276.0)
IL-10				
Mean (SEM)	12.68 (2.05)	34.74 (4.86) ^d	22.18 (5.32)	17.10 (4.95) ^c
Median (range)	11.7 (7.1–18.2)	26.1 (13.8–50.4)	15.8 (6.9–38.9)	12.1 (4.9–20.9)
TGF-β				
Mean (SEM)	5980.00 (1436.00)	19 229.00 (3993.00) ^d	12 924.00 (2664.00)	9858.00 (1427.00)
Median (range)	4540.0 (2369.0–10 561.0)	12 480.0 (855.7–30 300.0)	11 779.0 (5703.0–16.0)	9631.0 (7767.0–12 000.0)
IL-4				
Mean (SEM)	61.35 (6.36)	133.30 (12.46) ^d	96.61 (18.20)	109.90 (15.87)
Median (range)	62.2 (43.7–74.8)	127.4 (65.2–185.7)	71.6 (52.5–163.0)	122.5 (69.0–150.4)
IL-13				
Mean (SEM)	35.13 (6.86)	198.40 (47.74)	135.10 (57.24)	36.49 (16.31)
Median (range)	33.4 (18.9–52.8)	73.2 (21.0–352.5)	42.3 (5.0–279.9)	17.6 (7.8–48.0)

Serum cytokine levels were measured in healthy control individuals and patients with post kala-azar dermal leishmaniasis (PKDL) at presentation and after treatment with sodium antimony gluconate (SAG) or miltefosine as described in the Materials and Methods.

Abbreviations: IL-1 β , interleukin 1 β ; IL-4, interleukin 4; IL-6, interleukin 6; IL-8, interleukin 8; IL-10, interleukin 10; IL-13, interleukin 13; TGF- β , transforming growth factor β ; TNF- α , tumor necrosis factor α .

^a $P < .01$ compared with healthy controls individuals.

^b $P < .001$ compared with healthy control individuals.

^c $P < .05$ compared with presentation.

^d $P < .05$ compared with healthy control individuals.

Miltefosine Increased Serum Nitrite Levels and Decreased Arginase Activity

Because of the importance of reactive nitrogen intermediates in macrophage-mediated leishmanicidal activity, we evaluated serum nitrite levels, which were significantly higher in patients with PKDL than in healthy controls (mean [\pm SEM], 9.46 ± 0.88 vs 6.36 ± 0.19 $\mu\text{mol/L}$; $P < .05$). SAG caused no change (mean [\pm SEM], 8.86 ± 1.22 $\mu\text{mol/L}$), whereas miltefosine caused a marginal increase (mean [\pm SEM], 10.32 ± 1.06 $\mu\text{mol/L}$) although this increase was better evident upon paired analysis (Figure 4A).

Arginase is an important immunomodulatory enzyme that negatively influences macrophage function [15]; therefore, measurement of arginase levels in leishmaniasis is pertinent. At presentation, serum arginase activity was significantly higher in patients with PKDL than in healthy controls (mean [\pm SEM], 559.60 ± 56.45 vs 365.80 ± 28.07 U/L; $P < .05$),

which was minimally decreased with miltefosine (mean [\pm SEM], 461.40 ± 76.26 U/L) and SAG (mean [\pm SEM], 481.20 ± 99.47 U/L). However, on a paired basis, miltefosine significantly decreased arginase activity (Figure 4B).

DISCUSSION

Irrespective of the clinical variant of leishmaniasis, establishment of a successful host-parasite relationship is mandatory for disease pathogenesis. The *Leishmania* parasite ensures its survival within macrophages by deviously inhibiting generation of oxidative burst [16]. Therefore, for parasite elimination, activation of macrophages via preferential production of pro-inflammatory cytokines and production of NO would be beneficial, and indeed in CL, antimonials induced a pro-inflammatory response [7].

Miltefosine has been proposed to exert its effect through a direct antiparasitic and indirect immunomodulatory activity

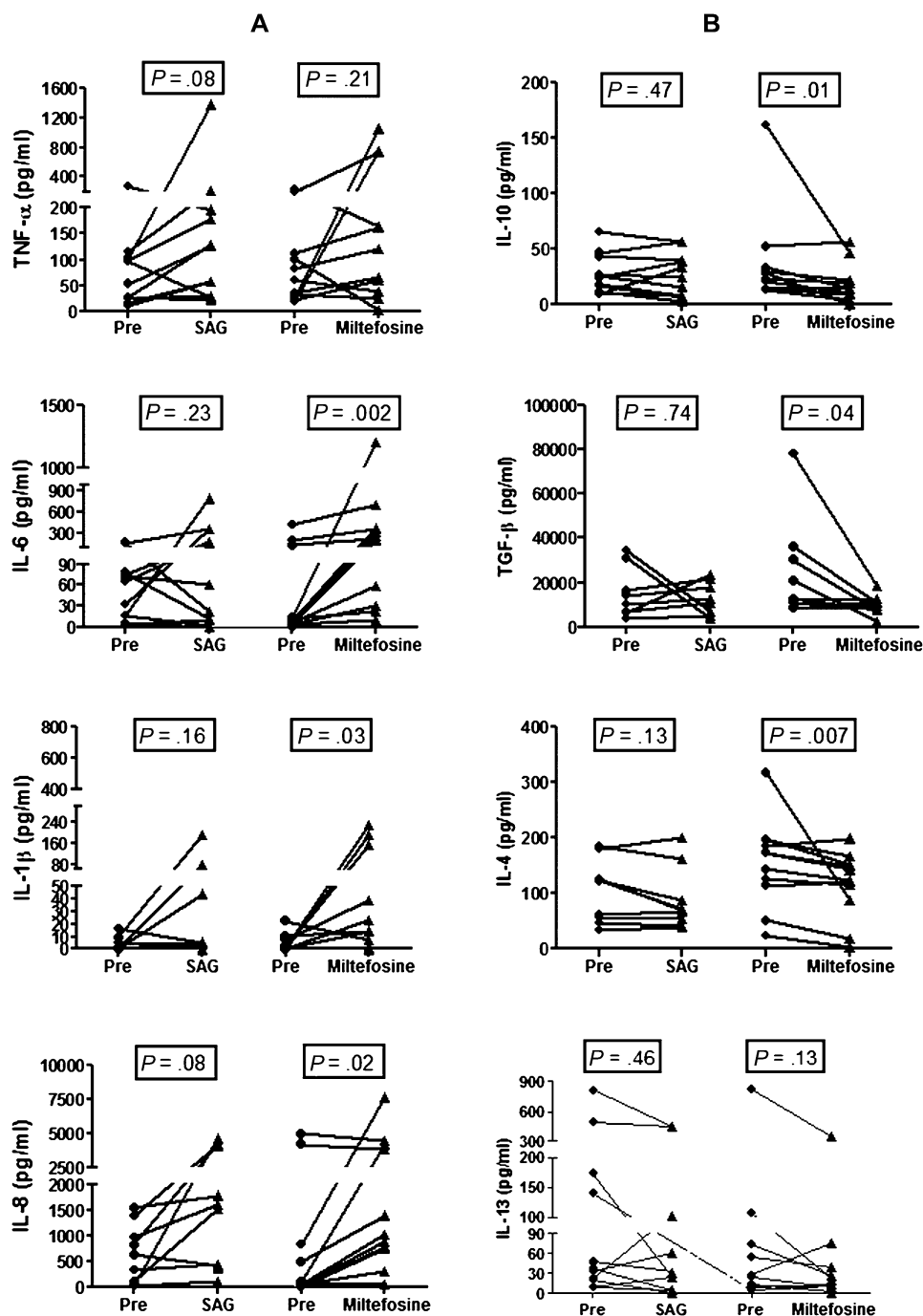


Figure 3. Effect of sodium antimony gluconate (SAG) and miltefosine on serum levels of cytokines in patients with post kala-azar dermal leishmaniasis (PKDL) A, Before and after plots of serum levels of pro-inflammatory cytokines (tumor necrosis factor α [TNF- α], interleukin 6 [IL-6], interleukin 1 β [IL-1 β], and interleukin 8 [IL-8]) in patients with PKDL at presentation (Pre; $n = 12$) (circles) and after completion of treatment (triangles). Serum levels were measured by enzyme-linked immunosorbent assay as described in the Materials and Methods. B, Same as panel A, but for serum levels of anti-inflammatory cytokines (interleukin 10 [IL-10], transforming growth factor β [TGF- β], interleukin 4 [IL-4], and interleukin 13 [IL-13]).

[17–20]. With the use of SCID mice, the leishmanicidal effect of miltefosine was shown to be immune-independent whereas SAG was shown to be immune-dependent [20]. Conversely, endogenous IFN- γ has been shown to be essential for the antileishmanial activity of miltefosine [17]. However, this

immunomodulatory status has only been defined in animal models and normal human PBMCs [21], and observations do not necessarily extrapolate to PKDL. In this study involving patients with PKDL, we propose that the host immune system is critical for the leishmanicidal action of miltefosine.

Table 4. Cytokine Levels in Culture Supernatants of Patients With Post Kala-Azar Dermal Leishmaniasis (PKDL) at Presentation and After Treatment

Cytokine	Healthy control individuals, pg/mL			Patients with PKDL at presentation, pg/mL			Patients with PKDL after treatment, pg/mL		
				SAG			Miltefosine		
	Antigen specific	Antigen nonspecific		Antigen specific	Antigen nonspecific		Antigen specific	Antigen nonspecific	
TNF- α	337.0 (121.06)	783.0 (201.4)	597.6 (224.5)	1003.1 (269.6)	580.9 (202.7)	259.9 (54.92)	2040.9 (715.7) ^{a,b}	1127.2 (355.0)	
IL-6	428.0 (127.6)	165.3 (82.5)	1054.4 (159.7) ^c	950.4 (144.0) ^a	895.0 (372.7)	1024.5 (350.3) ^c	859.3 (161.3)	806.3 (165.6) ^c	
IL-8	4019.7 (1063.8)	4659.4 (2006.2)	36669.1 (755.2)	23997.7 (6044.8)	96920.9 (24348.2) ^c	88737.8 (27585.5)	104599.5 (23168.6) ^{a,d}	97460.3 (21467.8) ^{c,d}	
IL-10	38.0 (5.6)	35.5 (6.7)	75.1 (6.6) ^c	77.1 (7.6) ^c	57.4 (12.3)	47.3 (7.7) ^d	36.2 (11.1) ^d	22.6 (9.0) ^b	
IL-4	10.8 (1.7)	11.9 (1.7)	10.1 (3.7)	16.5 (3.2)	29.6 (6.4)	27.4 (5.2)	11.7 (4.0)	11.6 (2.4)	
IL-13	4.9 (4.9)	7.8 (4.0)	3.6 (1.9)	5.6 (1.5)	5.6 (3.3)	5.7 (2.0)	6.1 (3.2)	6.5 (1.8)	

Data are mean (SEM) values. Peripheral blood mononuclear cells from healthy control individuals and from patients with PKDL at presentation and after treatment with sodium antimony gluconate (SAG) or miltefosine were cultured for 6 days, after which culture supernatants were collected and secreted cytokines were measured by sandwich enzyme-linked immunosorbent assay as described in the Materials and Methods.

- ^a $P < .01$ compared with healthy control individuals.
- ^b $P < .01$ compared with presentation.
- ^c $P < .05$ compared with healthy control individuals.
- ^d $P < .05$ compared with presentation.

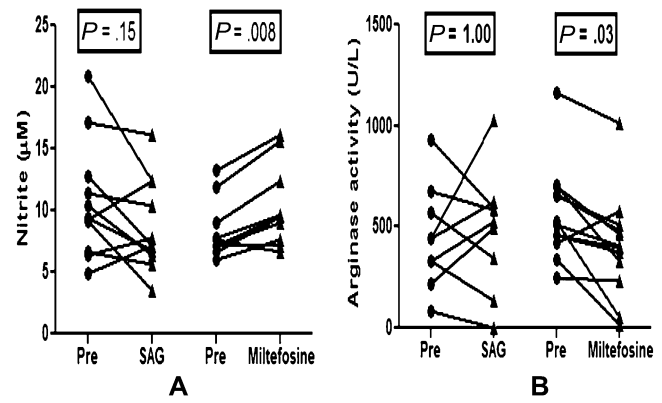


Figure 4. Serum levels of nitrite and arginase activity in patients with post kala-azar dermal leishmaniasis (PKDL) at presentation and after treatment with sodium antimony gluconate (SAG) and miltefosine. *A*, Before and after plots of serum levels of nitrite in patients with PKDL at presentation (Pre; $n = 12$) (circles) and after completion of treatment (triangles). *B*, Same as panel *A*, but for serum levels of arginase activity. μM , $\mu\text{mol/L}$.

In monocytes, increased expression of CD16 with a concomitant reduction of CD14, along with increased production of pro-inflammatory cytokines, are surrogate markers of the pro-inflammatory response [11] and are generally accompanied by increased production of reactive oxygen species and nitrogen intermediates. This phenotype has been reported in several diseases including cancers, asthma, human immunodeficiency virus (HIV) infection, rheumatoid arthritis, and diabetes mellitus [22]. In leishmaniasis, information regarding the status of the CD16⁺CD14^{low} phenotype appears contradictory, as patients with CL showed increased expression of CD16 monocytes at presentation [8], possibly reflective of the self-limiting nature of CL. In the nonhealing diffuse CL (DCL) variant, CD16⁺ monocyte levels were low at presentation [23]; whereas in PKDL, levels of CD16⁺ monocytes at presentation were comparable with those in healthy controls (Figure 1; Table 2). The healing lesions of DCL showed an 8-fold increase in circulating CD16⁺ and CD14⁺ monocytes, and our results are in agreement because effective chemotherapy with miltefosine caused a 1.7-fold expansion of the CD16⁺ population (Figure 1; Table 2).

Impairment of costimulatory molecules on T cells and macrophages has been reported in leishmaniasis [1], and in patients with PKDL, the impaired peripheral antigen-specific CD8⁺ T-cell proliferation was attributed to their loss of CD28, a costimulatory molecule, which importantly was restored following chemotherapy [13]. Apart from a MHC-TCR interaction, the interaction of the CD80 and CD86 costimulatory molecules with CD28 is necessary for T-cell activation [12]. The impaired CD86 expression on CD14⁺ monocytes in PKDL (Figure 2; Table 2) may well account for the observed immunosuppression [13], which interestingly was markedly increased by miltefosine whereas SAG was less effective (Figure 2; Table 2). The expression of CD80 in monocytes is inherently lower [24];

therefore, a reduction, if any, of CD80 would not be measurable (Figure 2; Table 2).

In patients with CL, VL, and mucocutaneous leishmaniasis (MCL) at presentation, levels of circulating pro-inflammatory cytokines are higher [6, 7]. In PKDL, the significant increase of TNF- α level at presentation (Table 3), akin to that seen in previous studies [25], suggests the host's attempt to eliminate the parasite. IL-6 is another pro-inflammatory marker primarily secreted by monocytes [7], the synthesis of which can be induced by IL-1 β [7]. In human VL and CL, higher levels of IL-6 have been reported [7, 25, 26]; whereas in PKDL, levels were comparable to those in healthy controls [25], and after chemotherapy, a 4.7-fold increase was demonstrated (Table 3). The scenario with IL-1 β was similar (Table 3). IL-8, a potent chemotactic cytokine in polymorphonuclear neutrophils, stimulates chemotaxis and generation of reactive oxygen metabolites, and is additionally synthesized by monocytes, macrophages, chondrocytes, and fibroblasts [7]. TNF- α stimulates release of IL-8, which in turn enhances release of pro-inflammatory cytokines IL-1 β , IL-6, and TNF- α , thereby reinforcing the inflammatory cascade [27]. The increase in pro-inflammatory cytokines in leishmaniasis suggests that infection per se does promote an inflammatory response, but not enough to eliminate the parasites. It may be envisaged that chemotherapy is effective by exacerbating this inflammatory response, which has been already triggered by infection. Indeed, miltefosine provided the necessary boost to the inflammatory response (Table 3). In leishmaniasis, there appears to be controversy over the status of IL-8, as it was initially proposed that IL-8 is beneficial for the host [28] but subsequently demonstrated that *Leishmania*-infected human polymorphonuclear neutrophils or PMNs secrete more IL-8 which, by increasing recruitment of PMNs, eventually aids disease progression [29]. The status of IL-8 in PKDL was unknown, and this study demonstrated a marked increase in IL-8 levels (Table 3), thus providing corroborative evidence that a host-driven pro-inflammatory burst helps in parasite elimination. Studies have indicated that chemotherapy increases pro-inflammatory cytokine levels [7, 30], and our study indicated the same, as levels of all pro-inflammatory cytokines studied (TNF- α , IL-6, IL-1 β , and IL-8) increased following treatment (Table 3). On an individual basis, miltefosine exerted a more potent immunostimulatory effect (Figure 3A).

A consistent feature of leishmaniasis is an increase in anti-inflammatory cytokines, which was also evident in PKDL [31] along with increased levels of IL-13 and TGF- β [31, 32], but the status of IL-4 in VL appears to be contradictory [9, 33]. In patients with PKDL, levels of serum IL-10, TGF- β , and IL-4 were significantly increased at presentation (Table 3), suggesting a predominant Th2 immune response. Although antileishmanial compounds have been consistently reported to curtail anti-inflammatory cytokines, our study showed that SAG was

ineffective in decreasing the levels of anti-inflammatory cytokines, whereas miltefosine effectively decreased levels of IL-10, TGF- β , and IL-4, once again reinforcing the immunomodulatory superiority of miltefosine (Table 3; Figure 3B).

Our understanding of alterations in the immune system during leishmaniasis is better explained by the cytokine production of in vitro cultured PBMCs. Among the pro-inflammatory cytokines secreted primarily by monocytes, independent of antigen stimulation, IL-8 and TNF- α were significantly increased by miltefosine (Table 4); IL-6 too was significantly increased at presentation, and increased further with treatment (Table 4), confirming activation of the host immune response. Among the anti-inflammatory cytokines, the effect on IL-10 (the major source of which is monocytes) was most prominent, evident in its significant decrease by SAG and miltefosine (Table 4).

Nitric oxide (NO) is an effector molecule necessary for elimination of intracellular *Leishmania* parasites; understandably, *Leishmania* infection causes deactivation of macrophages resulting in decreased production of NO [34, 35]. Conversely, cure necessitates increased production of NO [35], generally mediated by upregulation of inducible NO synthase (iNOS) [36]. Earlier studies have demonstrated the ability of miltefosine to increase intracellular NO levels [35], and because miltefosine induced secretion of all pro-inflammatory cytokines, the status of serum nitrite was examined. Serum nitrite levels were increased at presentation and increased further with miltefosine treatment (Figure 4). Serum nitrite has multiple sources such as monocytes, macrophages, endothelial cells, and erythrocytes and possibly is reflective of the host's attempt at parasite elimination.

Arginase contributes toward parasite persistence by reducing NO levels and helping in polyamine synthesis, which is necessary for parasite growth [15]. Furthermore, arginase is induced by anti-inflammatory cytokines IL-4, IL-13, IL-10, and TGF- β [37], and increased expression of arginase has been reported in experimental VL [38]. The 1.5-fold higher serum arginase activity in PKDL at presentation was decreased by miltefosine (Figure 4), which is corroborative of the immunomodulatory superiority of miltefosine over SAG. A major source of IL-8 and arginase is neutrophils, but one cannot exclude another important source, namely, the monocyte-macrophage. Because the levels of IL-8 and arginase were quantified in serum, we cannot pinpoint their cellular source, but it is expected that because macrophage functions are primarily altered in leishmaniasis, the observed changes are reflective of macrophage function, in which, importantly, a differential secretion of NO and arginase is expected [37]. In the future, measurement of intracellular levels of NO, arginase, and associated cytokines would provide a better insight; such studies are underway.

Studies evaluating the effect of drug treatment on PKDL are limited to 3 studies wherein emphasis was placed on the lesional pathology and the status of pro- and anti-inflammatory cytokines was measured [13, 39, 40]. These studies consistently indicated

an increase in pro-inflammatory cytokines at presentation that decreased after treatment [13, 39, 40]. However, the present study has focused on the systemic effects of SAG and miltefosine on Th1 and Th2 modulation and has established the immunological superiority of miltefosine over SAG. Future investigations should be directed toward studying the impact of antileishmanial drugs on signaling mechanisms involving host-pathogen interactions; such studies are underway.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

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