Evaluation of serological markers to monitor the disease status of Indian post kala-azar dermal leishmaniasis

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ABSTRACT

Post kala-azar dermal leishmaniasis (PKDL), a dermal sequel of visceral leishmaniasis presents with macular or polymorphic lesions. As immunological variations between these two forms have not been delineated, we evaluated levels of antileishmanial total Ig, IgG and its subclasses, IgA, IgE, IgG avidity, cytokines IL-10, IL-4, IL-13 and expression of CD19.

The levels of Ig and IgG in polymorphic PKDL were higher than macular PKDL, while significant curtailment in levels of Ig, IgM and IgG following treatment was evident only in polymorphic PKDL. With regard to IgG subclasses, IgG1 and IgG3 were significantly raised in polymorphic PKDL whereas in macular PKDL only IgG1 was elevated; treatment decreased levels of IgG1, IgG2 and IgG3 only in polymorphic PKDL; IgE levels were raised in both groups but no marked alterations occurred following treatment. The avidity of IgG was higher in polymorphic PKDL and correlated with duration of disease. IL-10 was higher in polymorphic PKDL and decreased significantly after treatment, whereas in macular PKDL, IL-4 predominated. Taken together, in PKDL, the humoral immune response was greater in the polymorphic variant than the macular form suggesting that serological markers may have a role in monitoring polymorphic PKDL.

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1. Introduction

Leishmaniasis is a complex vector borne disease having diverse clinical manifestations ranging from a self healing cutaneous to a fatal visceral form. The disease globally affects about 12 million people with an increasing incidence of 1.5-2 million new cases diagnosed every year and 350 million people at risk.1 Post kala-azar dermal leishmaniasis (PKDL), a dermal sequel of visceral leishmaniasis (VL) or kala-azar is confined mainly to the Indian subcontinent and Sudan along with its adjoining areas; importantly, owing to the absence of any zoonotic reservoirs, patients with PKDL are considered as reservoirs for kala-azar in India,2 thereby assuming greater clinical relevance from an Indian perspective, where eradication of VL is presently a national priority.3

Based on their clinical features, patients with PKDL in the Indian subcontinent can be categorized into two broad subgroups: macular PKDL, where the patients primarily present with hypopigmented macules and patches, and polymorphic PKDL where in addition to hypopigmented macules, the patients also have papules/plaques and/or nodules.5 The polymorphic group is predominant, ranging from 45-85%5 of all PKDL cases. Although considerable
work has been done regarding the histopathological variations of macular and polymorphic PKDL,\textsuperscript{2} humoral responses of PKDL have always been studied as a single entity and studies regarding their immunological differences, if any, are notably absent.\textsuperscript{6,7} All studies regarding humoral responses in PKDL have shown elevated levels of either antileishmanial Ig and/or IgG and its subclasses, but differences between the macular and polymorphic variants have not been explored.\textsuperscript{6,7} It has been documented that patients with macular PKDL respond more slowly to antileishmanial drugs than the polymorphic variant.\textsuperscript{8} Another confounding variable is the inability to measure clinical cure in patients with macular PKDL as regeneration of melanocytes usually takes 3–6 months.\textsuperscript{8}

With a view towards identifying a potential serological marker for monitoring the disease status of polymorphic and macular PKDL, the aim of this study was to dissect the humoral response in macular and polymorphic PKDL in terms of the antileishmanial Ig, IgG and their subclasses, and to establish whether a correlation existed between cytokines responsible for immunoglobulin class switching and associated IgG subclasses.

2. Materials and Methods

2.1. Reagents

All immunological reagents (anti human CD3, anti human CD19 and ELISA kits for IL-4, IL-10, IL-13) were from Immunotools, (Friesoythe, Germany) and others were from Sigma-Aldrich (St. Louis, MO, USA), except protease inhibitors and 2,2’-azino-bis-[3-ethylthiazoline-6-sulfonic acid] (ABTS, Roche Applied Science, Penzberg, Germany), rK39 immunochromatographic test strips (In Bios International, Seattle, WA, USA), para nitro phenyl phosphate (PNPP) and urea (Sisco Research Laboratories, Mumbai, India), tetra methyl benzidine (TMB) from Himedia (Mumbai, India). Polystyrene coated maxisorp strips were obtained from Nunc Immunomodules (Roskilde, Denmark).

2.2. Study population

From 2008–2011, 57 patients clinically diagnosed with PKDL were recruited from the Dermatology Outpatient Department, School of Tropical Medicine, Kolkata, India.

The diagnosis of PKDL was primarily clinical and corroborated with history of prior VL or residence in an endemic zone for VL and confirmed using LDA as the coating antigen in the polystyrene wells. From 2008-2011, 57 patients clinically diagnosed with PKDL were recruited from the Dermatology Outpatient Department, School of Tropical Medicine, Kolkata, India. Additionally, in cases where the interval between onset of VL and appearance of PKDL was short, we confirmed our diagnosis by doing an IFA based PCR from dermal biopsies. In cases where parasite isolates were obtained, they were typed as \textit{L. donovani}.\textsuperscript{6} None of the patients suffered from any infections or had any pre-existing disease. As controls, 15 age and sex-matched healthy volunteers were recruited from non-endemic areas and were seronegative for anti-leishmanial antibodies. Patients were randomly allocated to receive either sodium antimony gluconate (SAG; 20 mg/kg body weight/day intramuscularly for 4 months) or miltefosine (100 mg/day per oral for 4 months). Among them, 28 were followed up successfully and received SAG (n=14) or miltefosine (14) and were either polymorphic (22) or macular (6). Samples were collected at disease presentation and on completion of treatment.

2.3. Ethics statement

All patients provided informed written consent (in case of a minor, the parent/guardian provided the same) and the study received approval from the Institutional Ethical Committee of School of Tropical Medicine, Kolkata, India and Institute of Post Graduate Medical Education & Research, Kolkata, India.

2.4. Preparation of crude Leishmania lysate

Crude \textit{L. donovani} antigen (LDA) lysate was prepared from a \textit{L. donovani} strain MIHOM/RN/00/GE1/FBRK using log phase promastigotes harvested in ice-cold phosphate buffered saline (0.02 M, pH 7.2, PBS); the resultant pellet was resuspended in lysis buffer (20 mM Tris-HCl, 40 mM NaCl, pH (7.4), 5 mM EDTA and protease inhibitor cocktail). After several freeze-thaw cycles and centrifugation (1000 rpm x 5 min), the supernatant was collected and protein concentration determined.\textsuperscript{11} lysates were stored at -20°C until use. The LDA was diluted in phosphate buffer (0.02 M, pH 7.2) and served as the coating antigen (1 µg/well/100 µl).

2.5. ELISA for total antileishmanial immunoglobulin

LDA was added to polystyrene coated wells, incubated overnight at 4°C and followed by three washings with PBS supplemented with 0.05% Tween-20 (PBS-T); the nonspecific binding sites were blocked by PBS supplemented with 2% FBS (PBS-FBS) for 2 h (200 µl/well); sera (diluted 1:500 in PBS-FBS; 100 µl/well) was added and incubated overnight at 4°C. The wells were finally incubated with horse radish peroxidase (HRP) conjugated protein-A (diluted 1:5000 in PBS-FBS; 100 µl/well) at 37°C for 30 min and after five washes with PBS-T, binding was detected using ABTS (100 µl/well), optical density (OD 405 nm) being measured on an ELISA reader (Model 680, Bio-Rad, California, USA).

2.6. Determination of antileishmanial IgG, IgM and IgE

ELISA for antileishmanial IgG, IgM and IgE was measured using LDA as the coating antigen in the polystyrene coated wells. After an overnight incubation at 4°C followed by three washings with PBS-T, the remaining reactive sites were blocked with PBS-FBS (200 µl/well) for 2 h; sera (diluted 1:500 for IgG/IgM and 1:3 for IgE in PBS-FBS; 100 µl/well) were added and incubated overnight at 4°C. After three washes with PBS-T, the wells were incubated with anti human HRP-IgG or IgM (diluted 1:5000 and 1:25000 respectively in PBS-FBS, 100 µl/well) or with alkaline phosphatase conjugated anti human IgE (diluted 1:25000 respectively in PBS-FBS, 100 µl/well).
1:5000 in PBS-FBS, 100 μl/well) at room temperature (20–25°C) for 30 min. Following extensive washing with PBS-FBS, binding was detected using anti-mouse IgG (1:10000 in PBS-FBS) followed by addition of streptavidin-HRP (diluted 1:5000, 100 μl) and binding detected using HRP conjugated anti mouse IgG (diluted 1:5000 in PBS-FBS) with ABTS (100 μl) as the substrate at 450 nm. The IgG avidity of each sample was calculated as the ratio between its OD450 in Row B/OD450 in Row A.

2.11. Statistical analysis

The avidity of antileishmanial IgG was measured by indirect ELISA using polystyrene coated wells. Briefly, LDA coated polystyrene wells after being blocked with PBS-FBS (200 μl) were incubated overnight with human sera (diluted 1:50 in PBS-FBS) followed by incubation with mouse anti human IgG (diluted 1:5000) and biotinylated anti human IgG2, IgG3 and IgG4 (diluted 1:30,000, 1:4000 and 1:15,000 respectively, in PBS-FBS). Binding was detected using HRP conjugated anti mouse IgG (diluted 1:10,000 in PBS-FBS) for IgG1 and streptavidin-HRP (1:5000 in PBS-FBS) for IgG2, IgG3 and IgG4 using anti-h Human IgG2, IgG3 and IgG4 (diluted 1:4000 and 1:15,000 respectively, in PBS-FBS). Binding was detected using HRP conjugated anti mouse IgG (diluted 1:10,000 in PBS-FBS) for IgG1 and streptavidin-HRP (1:5000 in PBS-FBS) for IgG2, IgG3 and IgG4 using anti-h Human IgG2, IgG3 and IgG4 (diluted 1:4000 and 1:15,000 respectively, in PBS-FBS). 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Table 1
Clinical features of study population.

<table>
<thead>
<tr>
<th></th>
<th>Polymorphic PKDL Mean±SD (range)</th>
<th>Macular PKDL Mean±SD (range)</th>
<th>Controls Mean±SD (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years (range)</td>
<td>31.93±14.03 (12-65)</td>
<td>21.87±13.29 (8-55)</td>
<td>27.87±3.33 (23-34)</td>
</tr>
<tr>
<td>Sex ratio (M:F)</td>
<td>5:1</td>
<td>3:2</td>
<td>3:2</td>
</tr>
<tr>
<td>Number</td>
<td>41</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>Disease duration, years</td>
<td>5.84±7.37 (0-45)</td>
<td>2.78±2.68 (0-12)</td>
<td>NA</td>
</tr>
<tr>
<td>Interval between cure of VL and onset, years (range)</td>
<td>9.68±12.39 (0-65)</td>
<td>2.99±3.59 (0-12)</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA: Not applicable; PKDL: post kala-azar dermal leishmaniasis.

*p<0.01, **p<0.05: significantly differs from polymorphic PKDL. For statistical analysis, Mann Whitney test was applied as the data was non parametric.

in this study, the mean age was 29 years; however, the mean age of patients with macular PKDL was significantly lower than in those with polymorphic PKDL (Table 1).

3.2. Elevated levels of antileishmanial immunoglobulin in patients with PKDL

Measurement of antileishmanial antibody levels has been used for diagnosis and levels shown to correlate with disease severity accordingly, we studied whether any differences existed between polymorphic and macular PKDL. Higher levels of antileishmanial immunoglobulin (Ig) were detected in both polymorphic and macular PKDL patients vs non endemic healthy controls, with the polymorphic group being 8.4 fold and the macular group 4.3 fold higher than healthy controls (Figure 1A). Furthermore, the Ig levels in the polymorphic group was significantly higher than in the macular group, the median (IQ range) of OD450 being 0.60 (0.36-0.93) vs 0.31 (0.20-0.45) respectively (p<0.05; Figure 1A). This indicated that in polymorphic PKDL, the humoral response was stronger than in the macular variant, possibly attributable to the
relatively longer duration of disease or higher antigen load. Upon treatment, polymorphic patients showed a dramatic regression in levels of Ig (p<0.001), but a similar response was notably absent in the macular group (Figure 1B).

3.3. IgG was the major contributor towards the elevated Ig response in patients with PKDL

The levels of IgM, IgG, and IgE as determined by ELISA indicated that IgG was not significantly elevated in patients with PKDL, as compared to healthy controls, irrespective of the clinical variant (Figure 1C). A similar finding was reported in Sudan25 and is not surprising, considering the chronicity of the disease (Table 1). However, Haldar et al.18 reported increased levels of IgM in Indian patients with PKDL. Treatment caused a significant decrease in levels of IgM in patients with polymorphic lesions (p<0.05), but not in patients with macular lesions (Figure 1D). The role of IgG in patients with leishmaniasis is debatable, as it may either have no role in parasite elimination as parasites reside within macrophages. Alternatively, IgG may enhance lesion development as passive administration of antileishmanial IgG to BALB/c mice resulted in appearance of larger lesions along with increased amounts of IL-10.17 This was attributed to the binding of IgG to Fc- receptors on macrophages and it was proposed that IgG-opsonised parasites survived better in the host.18 It is also possible that IgG is lethal for Leishmania promastigotes following activation of the complement pathway.19 In human leishmaniasis, increased levels of IgG have been consistently reported20 and similarly in PKDL, we observed that levels of IgG were significantly elevated in both polymorphic (0.62 [0.27–0.92]; p<0.001) and macular PKDL (0.36 [0.17–0.56]; p<0.05) as compared to healthy controls (0.03 [0.00–0.02]; Figure 2).

3.4. Loss of CD19 expression supports the activation of antibody secreting plasma cells in PKDL

As proliferation and differentiation of B lymphocytes is regulated by T cells and soluble cytokines, measurement of individual IgG subclasses may indirectly reflect polarization of immune responses and thereby serve as surrogate markers of T cell responsiveness. Although this relationship has been reported in several pathological conditions including leishmaniasis, information regarding polymorphic and macular PKDL is limited and even contradictory.6,22 Treatment of IgG1 and IgG3 in patients with PKDL

CD19 is expressed on B cells from early B cell lineage to formation of blasts but is lost upon maturation to antibody secreting plasma cells. As levels of antileishmanial antibodies in patients with PKDL were higher, we aimed to establish whether this rise was attributable to pre-existing stable antibodies which can be present for years27 or appeared following formation of antibody secreting plasma cells which would translate into a decreased expression of CD19. The significant decrease in CD19 expression in patients with polymorphic PKDL as compared to healthy controls (10.76±6.07 vs 17.95±5.20, p<0.05; Figure 2) indicated that in PKDL, B cells were being transformed into antibody secreting plasma cells. This data also corroborated with higher antileishmanial antibody levels in patients with polymorphic PKDL (Figure 1). On the other hand CD3 expression, a pan T lymphocyte marker, was not altered in either forms of PKDL when compared with healthy controls (Figure 2).

3.5. Raised IgG1 and IgG3 in patients with PKDL

As proliferation and differentiation of B lymphocytes is regulated by T cells and soluble cytokines, measurement of individual IgG subclasses may indirectly reflect polarization of immune responses and thereby serve as surrogate markers of T cell responsiveness. Although this relationship has been reported in several pathological conditions including leishmaniasis, information regarding polymorphic and macular PKDL is limited and even contradictory.6,22 This could be due to ethnic variation, variable parasite genotype, antigen used for detection of antibodies22 or simply because of the smaller number of patients in these studies which ranged from 16–27. However, as our study population was larger, we felt it relevant to re-evaluate the status of antileishmanial IgG subclasses in PKDL. Among the subclasses, IgG1 and IgG3 levels in polymorphic PKDL was 14.5 and 4.5 fold higher than controls (0.41 [0.26–0.87] vs 0.03 [0.01–0.08]; p<0.001 and 0.55 [0.20–1.61] vs 0.16 [0.14–0.19], p<0.01 respectively; Figure 3A and 3E). With treatment, both IgG1 and IgG3 levels in polymorphic patients decreased significantly (p<0.01 and p<0.001; Figure 3B and 3F respectively). However, in the macular variant, only IgG1 was 7-fold higher than in healthy controls (0.33 [0.12–0.41] vs 0.03 [0.01–0.08]; p<0.05; Figure 3A) but not IgG3 (Figure 3E); with treatment, neither IgG1 nor IgG3 levels decreased in these patients (Figure 3B and 3F respectively). With regard to IgG2 and IgG4, minimal changes were found in both the polymorphic (0.08 [0.00–0.16] and 0.13 [0.02–0.26] respectively) and macular (0.11 [0.04–0.22] and 0.13 [0.02–0.26] respectively).
Healthy Control

6.98

Macular

13.98

Figure 2. Expression of CD19 on B cells in patients with post kala-azar dermal leishmaniasis (PKDL).

A. Comparison of CD19 expression in patients with polymorphic PKDL, macular PKDL and healthy controls.

B. Representative profile of CD19 expression on healthy controls, patient with polymorphic PKDL and macular PKDL. Whole blood was stained with fluorochrome labelled anti CD19 and CD3 with their respective isotypes after which lymphocytes were initially gated and fluorescence quantified as described in Materials and methods.

Importantly, IgG1 and IgG3 subclasses possess high levels of complement fixing and opsonising activities that are potentially useful for persistence of infection, and may well account for the disease chronicity. However, IgG2 has poor complement fixing and opsonising properties and is possibly less likely to contribute to the disease pathology.

3.6. Serum cytokines in patients with PKDL

It is a well accepted axiom in immunology that T cells exert a differential control over the immunoglobulin isotype switching and one of the molecular switch factors behind immunoglobulin class switching is cytokines. IL-10 caused an increase in IgG1 and IgG3, whereas IL-4 and IL-13 from activated T cells caused switching to IgG4 and IgG3. IFN-γ in conjunction with IL-6 caused increased production of IgG2. Previous reports with other forms of leishmaniasis suggested mixed immune responses are evident in Indian VL wherein the increased IFN-γ was proposed to account for the observed high IgG2 while raised IL-4 accounted for the increased IgG4, along with increased IL-10 accounting for augmented levels of IgG1 and IgG3.

As levels of IgG1 and IgG3 were increased in patients with PKDL (Figure 3A and E), we checked whether circulating levels of IL-10 are raised, accounting for the elevated IgG1 and IgG3. In polymorphic PKDL, levels of IL-10 were raised, accounting for the elevated IgG1 and IgG3.
IgG subclass levels were measured by ELISA as described in Materials and methods.

$^\text{•}p<0.05$, $^\text{•}p<0.01$, $^\text{•}p<0.001$.

For Figures 3 A, C, E and G where comparison was done between three groups, Kruskal-Wallis test followed by Dunn’s multiple comparison test was performed while for Figure 3 B, C, F and H which are paired representation of macular and polymorphic PKDL, Wilcoxon signed rank paired test was performed as the variables were normally distributed.

A significant 2.6-fold higher than healthy controls ($p<0.05$) which was curtailed significantly after treatment ($p<0.01$; Table 2). Furthermore, as IgG was significantly increased in this group, we measured IL-4 and IL-13 but their levels were not significantly higher than healthy controls (2.1 and 3.1 fold respectively). Interestingly, treatment caused levels of IL-4 to decrease significantly in patients with polymorphic PKDL ($p<0.05$), but IL-13 remained unaltered (Table 2). Patients with macular PKDL had a 3.1 fold higher level of IL-4 than healthy controls ($p<0.05$), which changed minimally after treatment (Table 2); levels of IL-10 and IL-13 were not significantly different from healthy controls (2.1 and 2.5 fold raised respectively), and treatment caused no changes (Table 2).

3.7. Correlation between cytokines and their surrogate immunoglobulin markers

A relationship between Th1/Th2 cytokines and immunoglobulin isotype levels has been shown in experimental studies but their correlation, if any, in vivo has not been established and the same holds.

Table 2

<table>
<thead>
<tr>
<th>Cytokine*</th>
<th>Healthy Controls</th>
<th>Polymorphic PKDL (before treatment)</th>
<th>Polymorphic PKDL (after treatment)</th>
<th>Macular PKDL (before treatment)</th>
<th>Macular PKDL (after treatment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10</td>
<td>11.69 (7.14-18.22)</td>
<td>22.34 (13.95-37.07) p&lt;0.05</td>
<td>22.69 (14.57-44.74)</td>
<td>36.81 (11.45-56.39)</td>
<td></td>
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<tr>
<td>IL-4</td>
<td>62.19 (43.7-74.77)</td>
<td>126.50 (58.59-182.00)</td>
<td>88.50 (48.94-145.37) p&lt;0.05</td>
<td>108.00 (77.70-189.00) p&lt;0.05</td>
<td>147.26 (105.30-189.20)</td>
</tr>
<tr>
<td>IL-13</td>
<td>33.40 (18.78-52.79)</td>
<td>24.96 (9.88-66.60)</td>
<td>24.10 (6.51-96.64)</td>
<td>79.88 (21.94-156.40)</td>
<td>22.52 (5.31-33)</td>
</tr>
</tbody>
</table>

*Serum cytokines, median (IQR) in pg/ml were measured in healthy controls and patients with PKDL (polymorphic and macular) at presentation and after treatment as described in Materials and methods.

$^\text{p}<0.05$: significantly different than healthy controls.

$^\text{p}<0.01$: significantly different than at presentation.

$^\text{p}<0.001$: significantly different than at presentation.
true for leishmaniasis where only an up regulation of cytokines and Leishmania specific immunoglobulins has been reported.28-34 We obtained a relatively weak negative correlation between IgG3 and IL-10 and none between IgG4 vs. IL-4 or IL-13 (Table 3). However, with regard to IgG, it showed a significant correlation with IL-13 (r=0.45, p<0.05; Table 3), not with IL-4. Taken together, our study has established that the rise in Leishmania specific IgG (Figure 1E) and its subclasses (IgG1, IgG3; Figure 3A and 3E) are not demonstrably driven by IL-10, necessitating analysis of other putative immunoregulatory cytokines such as TGF-β.

3.8. Raised levels of IgG avidity in polymorphic PKDL

IgG produced at the early stage of infection are known to have low avidity which increases with duration of disease and this phenomenon has been utilized to pinpoint the time of infection.35 As determination of IgG avidity can be used to indicate the time course and duration of disease, we studied this in patients with PKDL, wherein patients with polymorphic lesions had significantly higher IgG avidity than those with macular lesions (69.00 [57.30-81.41] vs 54.27 [50.50-64.75], p<0.05; Figure 4A). Furthermore, IgG avidity correlated with duration of disease of polymorphic PKDL (r=0.42, p<0.05; Figure 4B), not with macular PKDL (r=0.19; Figure 4C). There is a single report on IgG avidity regarding leishmaniasis which dealt mainly with acute and chronic form of VL, not with different forms of PKDL, thereby emphasizing the need to measure IgG avidity in different variants of PKDL.

4. Conclusion

Monitoring of the disease status and their response towards antileishmanial chemotherapy is essential for patients with PKDL because these patients are potential reservoirs for VL and elimination of leishmaniasis has been targeted at 2015.3 Although several markers have been used for diagnosis of PKDL, none have proved effective and one is still dependent on clinical evaluation. Furthermore, as SAG, which is notorious for its high propensity towards unresponsiveness, is still used for treatment of PKDL, it emphasises the need for developing prognostic markers. In cases of Indian kala-azar, Chattjee et al. showed that in antimony unresponsive cases, levels of IgG subclasses were unaltered or increased slightly compared to levels at presentation wherein in SAG responsive cases, curtallment of IgG subclasses were observed.10

In our efforts to identify a potential serological prognostic marker for PKDL, we propose that patients with polymorphic PKDL can be effectively monitored by measurement of antileishmanial Ig, IgG, IgG1 and IgG3 as these parameters significantly increased at disease presentation as also decreased substantially after treatment (Figure 1A,B,E,F and Figure 3A,B,E,F). However, for patients with macular PKDL, monitoring of antileishmanial antibody levels is not a plausible option.

Table 3

<table>
<thead>
<tr>
<th>Cytokine*</th>
<th>IgG1</th>
<th>IgG3</th>
<th>IgG4</th>
<th>IgE</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10</td>
<td>0.001</td>
<td>-0.29</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>IL-4</td>
<td>NA</td>
<td>NA</td>
<td>0.04</td>
<td>-0.21</td>
</tr>
<tr>
<td>IL-13</td>
<td>NA</td>
<td>NA</td>
<td>-0.22</td>
<td>0.40</td>
</tr>
</tbody>
</table>

NA: not applicable.
* Serum cytokines and IgG subclasses and IgG levels were measured in healthy controls and patients with post kala-azar dermal leishmaniasis at presentation as described in Materials and methods.

Authors' contributions: Conception and design of the study: DM, NKD, MC. Analysis and interpretation of data: DM, SDS, AM, AH, MC. Drafting the article: DM, DNG, JNB, AKM, MC. All authors read and approved the final version of the manuscript. MC is guarantor of the paper.

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Competing interests: None declared.

Ethical approval: All patients provided informed written consent (in case of a minor, the parent/guardian provided the same) and the study received approval from the Institutional Ethical Committee of School of Tropical Medicine, Kolkata, India and Institute of Post-Graduate Medical Education & Research, Kolkata, India.

References

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Targets for immunochemotherapy in leishmaniasis


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"...our quest for expanding the limited therapeutic armamentarium against Leishmania should focus on identifying compounds having direct parasiticidal and/or indirect immunomodulatory activity."

A pivotal pathogenic event in leishmaniasis is the harboring of the causative Leishmania parasite within phagolysosomes of macrophages for which the parasite deviously initiates mechanisms to modulate the macrophage microbial machinery. As macrophages are also critical sentinels of the immune system, establishment of infection critically hinges on the parasite's ability to modulate the host's signaling systems, the end point being immunosuppression. Therefore, our quest for expanding the limited therapeutic armamentarium against Leishmania should focus on identifying compounds having direct parasiticidal and/or indirect immunomodulatory activity. Current challenges in anti-leishmanial chemotherapy include the increasing resistance of parasites to the front-line drug, sodium antimony gluconate (SAG), high costs and drug toxicity, and also a limited repertoire of new drugs. Combinational therapy with immunomodulators is being strongly advocated to enhance the host immune response, the challenge being to identify key targets that the parasite can modulate.

Global burden of leishmaniasis

Leishmaniasis, a vector-borne disease, is endemic in large areas of the tropics, subtropics and the Mediterranean basin, and is one of the important neglected tropical diseases. It has a diverse range of clinical manifestations from a self-healing localized form (cutaneous leishmaniasis (CL)), to the life-threatening systemic visceral leishmaniasis (VL) [1]. More importantly, all forms of leishmaniasis are consistently characterized with modulation of host immune responses, necessary for parasite persistence and, therefore, the role of host immune responses on chemotherapeutic effectiveness cannot be ignored. There are at least 20 Leishmania species, they affect 1.5-2 million people per year and presents as four main clinical syndromes namely CL, mucocutaneous leishmaniasis, VL along with its dermal sequel, post kala-azar dermal leishmaniasis [3,4]. In the Indian subcontinent, the vector is anthropoctic in transmission and VL predominates, the estimated annual incidence being 500,000 with 90% of these cases being confined to five countries – namely India (especially the state of Bihar and its adjoining states), Bangladesh, Nepal (Terai region), Sudan and north eastern Brazil [5].

Immunopathogenesis of leishmaniasis

Conventionally, macrophages, upon encountering a pathogen, respond by phagocytosis, phagosome–lysosome fusion and, eventually, elimination of the engulfed pathogen by generating toxic reactive oxygen and nitrogen intermediates [6]. By contrast, the engulfed Leishmania parasite cleverly prevents phagolysosome formation, suppresses MHC-II-mediated antigen presentation and thereby prevents activation of IFN-γ-producing CD4+ Th1

KEYWORDS: immunomodulation • leishmaniasis • MAPK • NF-kB • PKC • PI3K • Toll-like receptor

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cells, thus guaranteeing its survival and replication. To achieve this, a series of complex interactions occur between the Leishmania parasite and host macrophages wherein the parasite drives the immune system towards a Th2 profile by interfering with several macrophage signaling pathways. This is mediated by Leishmania surface molecules, primarily lipophosphoglycan, gp63 and sphingomyelinase. Initially lipophosphoglycan prevents complement-mediated lysis and subsequently triggers and enhances complement receptor-mediated opsonization. This translates into the abrogation of the oxidative burst and inhibition of an IL-12 mediated Th1 response [7]. Furthermore, lipophosphoglycan inhibits activation of classical PKC-α, β, while Leishmania surface sphingomyelinase activates the atypical PKC-ε. This results in disruption of the lipid rafts necessary for cytokine receptor assembly (especially IFN-γ), inhibition of antigen presentation and ceramide generation [7]. This activation of PKC-ε and induction of ceramide in turn suppresses p58 MAPK-dependent NF-κB mediated Th1 responses, culminating in the attenuated generation of free radicals and skewing of the cytokine balance towards a Th2 response [8]. Additionally, gp63 enhances protein tyrosine phosphatase-1B and activates SHP-1 which, in turn, inhibits Th1 induced production of IL-12 and TNF; furthermore, SHP-1 by interacting strongly with JAK2, additionally impairs interferon signaling pathways. Taken together, the cumulative effort of these signaling molecules translates into a negative regulation of host protective Th1 response (IFN-γ and IL-2), concomitant with an upregulation of parasite protective Th2 responses (IL-4, -10 and -13). Therefore, it is logical to conclude that the activation of macrophages could be a de novo chemotherapeutic strategy to improve therapeutic regimens.

Targeting phagocytic cells, safe havens for the parasite Following uptake of the parasites into neutrophils, infected neutrophils are then silently phagocytosed by macrophages and subsequently generate an anti-inflammatory milieu, thereby promoting phagocytosis [9]. Therefore, one is tempted to extrapolate that compounds capable of increasing the phagocytic activity and generating an oxidative burst within Leishmania-infected neutrophils should be considered. Indeed, meglumine antimoniate is a Th1-based immunomodulator that increases the phagocytic capacity of monocytes and neutrophils along with enhanced generation of superoxide and production of TNF and nitric oxide (NO) [10]. As Leishmania evades the immune response by selectively attenuating proinflammatory signaling pathways, the immunomodulatory potential of an anti-leishmanial compound is documented by its influence on enhancing macrophage derived proinflammatory cytokines (IFN-γ, IL-12 and TNF-α) or decreasing levels of IL-10. Indeed, this is corroborated by the majority of the conventional anti-leishmanial drugs and compounds tested in experimental models of leishmaniasis [10,11]. However, this modus operandi of available anti-leishmanial drugs has only been established in hindsight, but has fuelled a growing interest in developing combined immunotherapy. Sadly, stand-alone immunomodulators are yet to find clinical application, a sole example being imiquimod (imidazoquinoline) and its analogue S-28463, which stimulates production of NO [12] and is effective in human CL [13].

Combinatorial treatment with immunomodulators is synergistic In view of the emerging resistance of parasites to SAG, combination therapy with immunomodulators is being tested as an alternative therapeutic option against VL. In suboptimal doses, SAG along with diperoxovanadate reduced the parasitic load in BALB/c mice, achieved by expanding the anti-leishmanial Th-cell repertoire, along with increased generation of superoxide and NO and an altered ratio of IFN-γ:IL-10 [14]. Several attempts have been made to combine pentavalent antimony with immunomodulators, such as IFN-γ or IL-2, and although it proved beneficial in T cell-deficient hosts, it only showed moderate improvement in clinical studies [15]. Generally, approaches applied have aimed at overcoming the overwhelming immunosuppressive environment but an underlying fear exists of generating an overdrive proinflammatory scenario. In experimental VL, another approach attempted was the removal of the endogenous immunosuppression by the use of anti-IL-10 combined with SAG/amphotericin B, and the results were promising [15,16]. Dendritic cell-based immunotherapy combined with antimony was effective against murine VL [17], as was the addition of GM-CSF with antimeta­nials in American CL and mucocutaneous leishmaniasis [18]. Imiquimod combined with SAG or paromomycin also showed marked improvement in patients with CL [19].

"The scientific advances in our knowledge of the immune response in leishmaniasis have allowed for immunomodulatory options to be effectively validated in animal models."

Th1-based immunomodulation enhanced the efficacy of paromomycin-associated stearylamine-bearing liposomes in experimental VL [20], as did picroliv combined with paromomycin or miltefosine, both being via increased oxidative stress [21]. A similar scenario was reported when miltefosine was combined with azoles (fluconazole or ketoconazole) [22,23] or with liposomal Cpg ODN, the end point being a strong enhancement of Th1 cytokines, along with raised levels of NO, reactive oxygen species and H2O2 [24]. These results indicate that immunomodulatory drugs show promise as an adjunct to conventional chemotherapy warranting pharmacological consideration.

Targeting the signaling pathways of Leishmania-infected macrophages As T-cell mediated regulation of immune responses is intimately associated with costimulatory molecules present on APCs, modulating the interaction of CD28 with CD80/CD86 influences parasite survival. Miltefosine in circulating monocytes of patients with post kala-azar dermal Leishmaniasis caused an upregulation of CD16 and CD86 along with reduced expression of CD14 [25]. Another important costimulatory molecule is CD40, whose weaker interaction with CD40L increased expression of

---

IL-10, concomitant with decreased expression of IL-12 [7]. This occurred subsequent to enhanced phosphorylation of ERK1/2, which increased IL-10 and inhibited IL-12. The production of NO hinges on inducible NO synthase expression which too is linked to the MAPKs signaling pathway. Taken together, as ERK and p38 MAPKs differentially regulate induction of macrophage effector molecules, which dictate the course of infection, one is tempted to propose that these kinases should be considered as potential targets to combat leishmaniasis, as demonstrated by miltefosine [8] and antimonials [27].

To potentiate activation of the MAP kinase pathways, Toll-like receptors following MyD88 activation recognize a pathogen-associated molecular pattern, which is then recruited to the TIR domain of IRAK-1 and IRAK-2 to trigger IRAK-mediated signaling. IRAK activation then leads to upregulation of Toll-like receptor signaling and a pro-inflammatory cytokine. As Leishmania infection inhibits this IRAK-mediated signaling, upregulation of Toll-like receptor signals is an attractive therapeutic option as endorsed by imiquimod, monophosphoryl lipid A and CpG [28].

Cytokines determine the host immune response in Leishmania infection by triggering a cascade of intracytoplasmic proteins known as JAK and STATs, which in turn influence the biological effects of IFN-γ. As Leishmania infection inhibits the JAK2/STAT1 signaling cascade, activation of this pathway would boost parasite elimination, as evidenced with miltefosine [28]. Another attractive option is targeting the NF-κB pathway, which upon activation by the MAPKs, causes increased expression of inducible NO synthase and TNFα cytokines [29]. Therefore, preventing degradation of IκB and its downstream events is a strategy employed by Leishmania and its abrogation by anti-leishmanial compounds such as 18β-glycyrrhetinic acid corroborates its potential as a putative target [30].

Another key player in several signal transduction cascades is PKC wherein Leishmania donovani infection selectively inhibits the Ca²⁺-dependent classical PKC activity (PKC-α and -β) via IL-10, while enhancing the Ca²⁺-independent atypical PKC (PKC-ζ) activity; reversal of this phenomenon is achieved by amphotericin B [31]. PKCζ signaling is activated during Leishmania infection leading to downregulation of IL-12 production enabling disease progression [10]. Therefore, its selective blockade by AS-605240 has endorsed the fact that development of PI3K inhibitors could facilitate parasite elimination [32].

Conclusion

The scientific advances in our knowledge of the immune response in leishmaniasis have allowed for immunotherapeutic options to be effectively validated in animal models. However, as resources in drug development are diverted towards diseases directly affecting the developed world, clinical trials to validate the potential of immunotherapy are few and far between. Alternative strategies may be used as exogenous information from other pathogens (e.g., antifungals or antitrypanosomal drugs) to obtain lead molecules by studying target-based libraries, which can then be validated by high throughput screening approaches. An underlying fear of unleashing an overwhelming pro-inflammatory response is also a deterrent, but it has consistently been observed that anti-leishmanial drugs do not induce a pronounced proinflammatory response and instead simply revert the altered milieu to baseline levels, thus meritizing pharmacological consideration [34].

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References

12. Buelles S, Marleshewski G. Treatment of experimental leishmaniasis with the...
immunomodulators imiquimod and S-28463: efficacy and mode of action.


Murray HW, Lu CM, Mauze S.


Sane SA, Shukla, Gupta S.


Ukul A, Burew A, Das T, Dar PK.


Review

Immunomodulation by chemotherapeutic agents against Leishmaniasis

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Abstract

Leishmaniasis is caused by protozoan parasites of the genus Leishmania and causes a wide spectrum of clinical manifestations ranging from self-healing cutaneous lesions to the fatal visceral form. The use of pentavalent antimony, the mainstay of therapy of Leishmaniasis is now limited by its toxicity and alarming increase in unresponsiveness, especially in the Indian subcontinent. Furthermore, other anti-leishmanial drugs are unsatisfactory in many affected countries and as vaccination based approaches have not yet proved to be effective, chemotherapy remains the only alternative, emphasizing the need for identifying novel drug targets. In this review, we have described the different host immune signaling pathways that could be considered as potential drug targets for Leishmania chemotherapy.

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often associated with rigor and chills, hepatosplenomegaly, lymphadenopathy, progressive anemia, weight loss and hyperammonaemia (mainly lactic acidosis from polyclonal B cell activation) and concomitant hyperalaninaemia [2]. Even after recovery, African and Indian VL patients may present with a secondary form called Post Kala-azar Dermal Leishmaniasis [3].

2. Disease Immunopathogenesis

Survival of the parasite within macrophages is crucial, which it ensures by deviously manipulating the macrophage related immune functions [4 and references therein]. The engulfed pathogens prevent formation of phagolysosomes, and thereby suppresses MHC-II mediated presentation of the parasite antigen to CD4⁺ T cells, eventually preventing macrophages from eliminating the phagocytosed pathogen [4]. Furthermore, the parasite prevents initiation of a respiratory burst, ensuring that the macrophage is safe haven for the parasite. Therefore, it is only logical to conclude that activation of macrophages would be a de novo chemotherapeutic strategy for Leishmaniasis.

The outcome of leishmanial infections is determined by two functionally distinct T helper (Th) cell populations, Th1 (IFN-γ, IL-2) and Th2 (IL-4, IL-10 and IL-13). Generally, uncontrolled, non healing disease presentation showed increased levels of both IL-10 and IFN-γ type, as measurement of splenic and bone marrow cytokine mRNA at disease resolution was associated with a simultaneous decrease in both IL-10 and IFN-γ, indicating that both Th1 and Th2 are components of the immune system during active disease and both regress with effective treatment [8].

Patients with VL usually demonstrate a negative skin test to Leishmania antigens and peripheral blood mononuclear cells fail to proliferate and produce IFN-γ when exposed to specific antigen in vitro, indicative of anergy; addition of anti-IL-10R antibody to T cells harvested from these patients, restored cytokine responses, corroborating a role for IL-10 in suppressing T-cell responses [9]; furthermore, cure was associated with a fall in mRNA levels of IL-10 [8,9]. Effective parasite elimination also requires restoration of macrophage function for production of toxic nitrogen and oxygen metabolites, necessary to kill resident amastigotes [11 and references therein].

3. Chemotherapy of Leishmaniasis

Currently, drugs used to treat Leishmaniasis are handicapped by emergence of strains resistant to conventional antimonials, associated toxicities and high cost especially regarding liposomal formulations of amphotericin B [12], Table 1. A matter of concern is that Miltefosine, the only orally effective drug can potentially become ineffective, as studies with resistant amastigotes have shown the presence of mutant drug transporters [13]. Taken together, the current armamentarium of Leishmaniasis is limited and alternative therapies are strongly warranted. An area of growing interest is the potential of natural plant-derived products of diverse structural classes having anti-leishmanial properties [14-16] and includes naphthylisoquinoline alkaloids and synthetic analogs, Luteolin, Quassia, Curcumin, Artocarpus and several others [14,17-23].

4. Targeting of host immunity by anti-leishmanial drugs

Within the mammalian host, Leishmania reside as amastigotes in phagocytic cells that include neutrophils, macrophages and dendritic cells (DCs); therefore, an immunomodulatory compound could be potentially leishmanicidal by virtue of its potential to activate phagocytic cells.

4.1. Role of neutrophils

The best characterized function of polymorphonuclear neutrophils (PMNs) is their preeminent role in phagocytosis and killing of invading microorganisms via generation of reactive oxygen species (ROS) and release of lytic enzymes. Following entry of Leishmania into the mammalian host, PMNs are recruited immediately to the site of infection within 24 h, implying that they possibly serve as host cells for Leishmania in the very early phase of infection [21-24]. Neutrophils being inherently short-lived undergo apoptosis [23], while Leishmania parasites are known to delay their apoptosis, possibly by interfering with production of ROS, which importantly facilitates their survival [25,26]. To trigger apoptosis, neutrophils utilize a mitogen activated protein kinase (MAPK) signaling pathway, p38 MAPK being a key player [27]. Recent data suggests a critical role for neutrophils in the early protective response against L. donovani, both as effector cells involved in parasite killing and for influencing development of a protective Th1 response [28].

Importantly, Leishmania parasites that enter macrophages via the uptake of infected, apoptotic PMNs then survive and multiply effectively [23]. The amount of TGF-β secreted by macrophages following uptake of infected PMNs was higher than after direct uptake of L. major promastigotes [23] indicating that uptake of infected, apoptotic PMNs creates a more effective anti-inflammatory milieu, beneficial for Leishmania survival. Therefore, as neutrophils harbor and transport Leishmania, targeting pathogens residing in neutrophils should be taken into consideration when designing novel anti-leishmanial compounds.

Therefore, it is tempting to extrapolate that a compound capable of increasing phagocytic activity and generating an oxidative burst within Leishmania infected, neutrophils would effectively eliminate parasites. Indeed, antimonials increase the phagocytic capacity of neutrophils along with increased production of superoxide [29]. Berberine chloride (Table 2) also promoted parasite elimination via enhancement of apoptosis in L. donovani infected neutrophils, subsequent to modulation of the MAP kinase pathways [30].

4.2. Monocytes and macrophages

To sustain infection, it is necessary that Leishmania parasites establish themselves in macrophages, but considering the potent antinfectious functions of macrophages, the subject of how Leishmania survive is a subject of intense research.

(i) Phagocytosis: C3b is a complement protein that following binding to Leishmania surface glycoprotein gp63 increases parasite uptake into macrophages as gp63 cleavages C3b into iC3b, which then favors phagocytosis, yet prevents lytic clearance [31]. Antimoniais [29], Pounoura guineensis [pleuromutiline acid, 32 and Upphyllil isolated from Haplophyllum bucharicum Liv [33] influence the phagocytic activity of macrophages as do Cpg oligodeoxynucleotides (Cpg GDN) and miltefosine [34].

(ii) Acidification: Generally, following fusion of the phagosome with the endosomal compartment, a significant drop in pH ensues. However, Leishmania produce a surface acid phosphatase that inhibits the oxidative burst within macrophages, and additionally is an active proton pump keeps the intracellular pH close to
Table 1
Currently available anti-leishmanial drugs: their mechanism of action on parasites, dosage, advantages and limitations.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mode(s) of action</th>
<th>Dosage (for VL)</th>
<th>Advantages</th>
<th>Limitations</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentamidine isostate</td>
<td>Activated within the amastigote/macrophage after conversion to the infective form</td>
<td>20 mg/kg b.w., i.m., daily (40 mg total) for 30 days in India</td>
<td>Easly availability and low cost</td>
<td>May cause tissue necrosis and toxicity</td>
<td></td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>Complexes with 24-substituted sterol, such as ergosterol in the cell membrane, causing pores which allow ion balance, increase membrane permeability resulting in cell death; also acts as an inhibitor of ergastoplasm</td>
<td>0.25-1.0 mg/kg for 15-20 infections either daily or alternate days in India</td>
<td>Primary resistance unknown</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid formulation of amphotericin B</td>
<td>Anti-T cell/anti-B/Amphotericin</td>
<td>Ambisone: 2.0 mg/kg x 5 days, i.v. in India 16 mg/kg x 21 days, i.m.; 20 mg/kg x 17 days, i.m.</td>
<td>Highly effective, low toxicity</td>
<td>Need for prolonged hospitalisation High cost, high fever with rigor, chills, hypokalaemia, renal dysfunction High cost</td>
<td></td>
</tr>
<tr>
<td>Paromomycin (aminoglycoside antibiotic)</td>
<td>Also known as antimonials or monomycin</td>
<td>Effective, well tolerated and relatively cheap, acts synergistically with antimabials</td>
<td>Lack of efficacy in East Africa</td>
<td>Lack of efficacy in East Africa</td>
<td></td>
</tr>
<tr>
<td>Mifepristone (progesterone/cholesterol)</td>
<td>Unknown, possibly affects mitochondrial electron transport chain</td>
<td>100-150 mg for four weeks, p.o. in India</td>
<td>Effective and safe</td>
<td>Vomiting and diarrhoea, nephrotoxic, teratogenic</td>
<td></td>
</tr>
<tr>
<td>Sulfone (diaminobenzene, originally WR8028)</td>
<td>Unknown, possibly affects mitochondrial electron transport chain</td>
<td>1.75-2 mg/kg/day for 28 days in India</td>
<td>Little is known about its efficacy and toxicity</td>
<td></td>
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4.3. Role of reactive oxygen species (ROS) and reactive nitrogen species (RNS)

As nitric oxide (NO) is an effector molecule critical for elimination of intracellular Leishmania parasites, disease progression is ensured via enhancement of Th2 responses that causes deactivation of pathogen’s macromolecules such as DNA leading to strand breaks. Following parasite engulfment by macrophages, NAD(P)H oxidases are activated, which transform the reducing equivalents from NAD (P)H to molecular oxygen leading to formation of extremely reactive superoxide [11]. These then react with parasite membrane phospholipids leading to increased permeabilization as also react with the pathogen’s macromolecules such as DNA leading to strand breaks. However, when the infection is sustained, macrophages are deactivated causing a decreased production of superoxide which is now beneficial for parasite survival [1]. Therefore, it is anticipated that if a similar pro-oxidant scenario is recreated by anti-leishmanial drugs, they can effectively eliminate the parasite [21,39].

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4.4. Role of DCs

The interaction of Leishmania parasites with DCs is complex, as depending upon the species of Leishmania, the DC subset and other exogenous stimuli involved, there can either be control of infection or disease progression [71 and references therein]. The first study with murine skin DC implicated epidermal Langerhans cells as important cells for detection, uptake and transport of Leishmania to lymph nodes [72]. Dermal DCs efficiently incorporate parasites into vacuoles and

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<td>Ambisone: 2.0 mg/kg x 5 days, i.v. in India 16 mg/kg x 21 days, i.m.; 20 mg/kg x 17 days, i.m.</td>
<td>Highly effective, low toxicity</td>
<td>Need for prolonged hospitalisation High cost, high fever with rigor, chills, hypokalaemia, renal dysfunction High cost</td>
<td></td>
</tr>
<tr>
<td>Paromomycin (aminoglycoside antibiotic)</td>
<td>Also known as antimonials or monomycin</td>
<td>Effective, well tolerated and relatively cheap, acts synergistically with antimabials</td>
<td>Lack of efficacy in East Africa</td>
<td>Lack of efficacy in East Africa</td>
<td></td>
</tr>
<tr>
<td>Mifepristone (progesterone/cholesterol)</td>
<td>Unknown, possibly affects mitochondrial electron transport chain</td>
<td>100-150 mg for four weeks, p.o. in India</td>
<td>Effective and safe</td>
<td>Vomiting and diarrhoea, nephrotoxic, teratogenic</td>
<td></td>
</tr>
<tr>
<td>Sulfone (diaminobenzene, originally WR8028)</td>
<td>Unknown, possibly affects mitochondrial electron transport chain</td>
<td>1.75-2 mg/kg/day for 28 days in India</td>
<td>Little is known about its efficacy and toxicity</td>
<td></td>
<td></td>
</tr>
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</table>
Table 2
Antileishmania compounds having Immunomodulatory activity.

<table>
<thead>
<tr>
<th>Compound</th>
<th>dose in mice, b.w./day</th>
<th>% reduction</th>
<th>Mode(s) of action</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B</td>
<td>0.097x10⁸ (spleen)</td>
<td>53.7±11.9</td>
<td>Increased TNF-a and expression of IL-12 and IFN-γ.</td>
<td>[39,66,141-143]</td>
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<tr>
<td>Artemether</td>
<td>86x10⁸ (spleen)</td>
<td>78.3±8.3</td>
<td>Increased production of NO and expression of IL-12 and IFN-γ.</td>
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<td>Artemether</td>
<td>0.033x10⁶ (spleen)</td>
<td>54.5±10.1</td>
<td>Reduced parasite no. from 1.67x10⁸ to 8.48x10⁷.</td>
<td>[43,136-139]</td>
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<tr>
<td>Artemether</td>
<td>22 µM</td>
<td>81.7%</td>
<td>Phosphorylated p38 MAPK and abrogated the atypical PKC and restored impaired classical PKC.</td>
<td>[39,144,145]</td>
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<td>Artemether</td>
<td>1 nM</td>
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<td>Artemether</td>
<td>416 mg/kg</td>
<td>35.4±10.1</td>
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</table>
Table 2 (continued)

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<tr>
<th>Compound</th>
<th>Leptimania spp.</th>
<th>L amazonensis</th>
<th>L donovani</th>
<th>L tropica</th>
<th>Patients with CL</th>
<th>L max</th>
<th>L major</th>
</tr>
</thead>
<tbody>
<tr>
<td>Momordica charantia L</td>
<td>10 mg/kg b.w, p.o. x30 days</td>
<td>Reduced parasite burden</td>
<td>51%</td>
<td>51%</td>
<td>Increased production of NO</td>
<td>153</td>
<td>153</td>
</tr>
<tr>
<td>Clycyrrhizza glabra</td>
<td>300 mg/kg b.w, p.o. x5</td>
<td>Suppressed antibody production</td>
<td>58%</td>
<td>58%</td>
<td>Reduced histiocytic cellular aggregation</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Compounds</td>
<td>L amazonensis</td>
<td>L donovani</td>
<td>L major</td>
<td>L max</td>
<td>L max</td>
<td>L max</td>
<td>L max</td>
</tr>
<tr>
<td>PMSO</td>
<td>ADP</td>
<td>ATP</td>
<td>ATP</td>
<td>ATP</td>
<td>ATP</td>
<td>ATP</td>
<td>ATP</td>
</tr>
<tr>
<td>OIPeroxovanadate</td>
<td>ADP</td>
<td>ATP</td>
<td>ATP</td>
<td>ATP</td>
<td>ATP</td>
<td>ATP</td>
<td>ATP</td>
</tr>
<tr>
<td>Glycolipids and other constituents</td>
<td>ADP</td>
<td>ATP</td>
<td>ATP</td>
<td>ATP</td>
<td>ATP</td>
<td>ATP</td>
<td>ATP</td>
</tr>
<tr>
<td>(ii) Sb-resistant (SbR-LD)</td>
<td>(ii) 63.48%</td>
<td>(ii) 49.9 ± 224%</td>
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</tr>
<tr>
<td>(i) Sb-sensitive (SbS-LD)</td>
<td>(i) 72%</td>
<td>(i) 72.7</td>
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</tr>
<tr>
<td>Desmodium gangeticum L</td>
<td>EtOH extract</td>
<td>Reduced parasite burden</td>
<td>87%</td>
<td>87%</td>
<td>Increased secretion of pro-inflammatory cytokines</td>
<td>153</td>
<td>153</td>
</tr>
<tr>
<td>O. gratissimum</td>
<td>Hexane extract</td>
<td>Reduced parasite burden</td>
<td>98%</td>
<td>98%</td>
<td>Reduced histiocytic cellular aggregation</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Briophyllum pinnatum</td>
<td>Aqueous extract</td>
<td>Reduced parasite burden</td>
<td>82%</td>
<td>82%</td>
<td>Reduced histiocytic cellular aggregation</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>(i) Leafy aqueous extract</td>
<td>(i) 320 mg/kg b.w, p.o. x30 days</td>
<td>Suppressed antibody production</td>
<td>58%</td>
<td>58%</td>
<td>Reduced histiocytic cellular aggregation</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>(ii) Quercetin 3-O-α-L-rhamnopyranoside</td>
<td>(ii) 300 mg/kg b.w, p.o. x5</td>
<td>Suppressed antibody production</td>
<td>58%</td>
<td>58%</td>
<td>Reduced histiocytic cellular aggregation</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>(iii) Free quercetin</td>
<td>(iii) 300 mg/kg b.w, p.o. x5</td>
<td>Suppressed antibody production</td>
<td>58%</td>
<td>58%</td>
<td>Reduced histiocytic cellular aggregation</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>(i) EtOH extract</td>
<td>(i) 300 mg/kg b.w, p.o. x5</td>
<td>Suppressed antibody production</td>
<td>58%</td>
<td>58%</td>
<td>Reduced histiocytic cellular aggregation</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>(ii) Hexane extract</td>
<td>(ii) 300 mg/kg b.w, p.o. x5</td>
<td>Suppressed antibody production</td>
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<td>100</td>
<td>100</td>
</tr>
<tr>
<td>(iii) Aqueous extract</td>
<td>(iii) 300 mg/kg b.w, p.o. x5</td>
<td>Suppressed antibody production</td>
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<td>58%</td>
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<td>100</td>
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</tr>
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</table>
### Table 2 (continued)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Leishmania spp.</th>
<th>In vivo</th>
<th>Model(s) of action</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrazinamide</td>
<td>L. major 10.2 μg/ml</td>
<td>–</td>
<td>Increased IL-12, TNF-α, and production of NO.</td>
<td>[61]</td>
</tr>
<tr>
<td>Quinolone</td>
<td>L. donovani 25 μg/ml</td>
<td>–</td>
<td>Enhanced NO generation and iNOS expression both at a protein and mRNA level and up-regulated pro-inflammatory cytokines such as TNF-α and IL-12.</td>
<td>[63]</td>
</tr>
<tr>
<td>Quinolone acid glycocones and cadamamine isolated from Neodesma deidem.</td>
<td>L. infantum, 1.0 μM</td>
<td>–</td>
<td>Induced NO production.</td>
<td>[63]</td>
</tr>
<tr>
<td>Terpenoids, sesquiterpene, and pentaterepene</td>
<td>L. enriettii 100 μg/ml, caused B85 inhibition</td>
<td>–</td>
<td>Inhibited production of NO and TGF-β.</td>
<td>[70]</td>
</tr>
<tr>
<td>Tannins</td>
<td>L. amebicida 11.1 μg/ml</td>
<td>(i) Proanthocyanidins (i) L. donovani 0.2 μM; (ii) &lt;0.4-125 μg/ml</td>
<td>Induced rapid elimination of parasitophorous vacuoles.</td>
<td>[56,156,157]</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>(i) Butanol fraction (i) L. chagasi 2.4 ± 0.3 μM</td>
<td>L. chagasi, hamsters, 20 mg/kg b.w., ip, × 15 days decreased parasite load by 90-95%.</td>
<td>Activated macrophages and up-regulated NO, TNF-α, INF-γ, IL-10, IL-12 and IL-18 expression.</td>
<td>[47,80,81,158,159]</td>
</tr>
<tr>
<td>Tannin</td>
<td>Quassin</td>
<td>L. amazonensis</td>
<td>Increased production of NO</td>
<td>[55]</td>
</tr>
<tr>
<td>Tannin</td>
<td>Tabernaemontana catharinensis L</td>
<td>L. amazonensis</td>
<td>Increased production of NO</td>
<td>[55]</td>
</tr>
<tr>
<td>Tannin</td>
<td>Tinospora sinensis</td>
<td>L. amazonensis</td>
<td>Increased production of NO</td>
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are proposed to act as principal APCs in Leishmaniasis, while others suggest that lymph node resident DCs are initiators of the immune response [71].

Leishmania have cleverly devised several strategies to avoid DCs, as in humans, L donovani blocks maturation of DC [72] and production of IL-12, essential for initiation of a protective immune response. Accordingly, Miltefosine in turn can activate DCs [74] as also does Pyrazinamide via increased secretion of proinflammatory molecules and enhanced co-stimulatory molecules (61, Table 2).

### 4.5. Lymphocytes

T lymphocytes are generally responsible for intracellular pathogen elimination whereas B lymphocytes eliminate extracellular bacteria. In order to eliminate Leishmania, the macrophage needs to be activated by antigen specific T lymphocytes who by secreting IFN-γ, upregulate production of NO from macrophages. Both CD4 and CD8 cells are required for resolving the infection, along with a balance between Th1 and Th2, preferably a Th1 skewed response [5]. Therefore, essential prerequisites of an effective immunomodulatory, anti-leishmanial drug
Fig. 1. Schematic representation of Leishmaniasis induced signaling events within macrophages: The different targets (1-8) within the macrophages modulated by Leishmania are potential sites that can be targeted by the anti-leishmanial compounds. (1) Increased interaction of CD4/CD45R; (2) Activation of MAPKs; (3) Upregulation of TLRs; (4) Activation of NF-kB; (5) Enhanced nuclear translocation of NF-κB; (6) Down regulation of PTPs; (7) Upregulation of PKC ε, δ and enhanced maturation of phagolysosomes leading to increased assembly of NADPH oxidase complex and (8) Downregulation of PTPs which in turn would upregulate expression of pro-inflammatory mediators. IFN-γ R: IFN-γ receptor; iNOS: Inducible nitric oxide synthase; IL-10: IL-10 associated kinase 1; AKAP: A kinase anchoring protein; MAPK: Mitogen Activated Protein Kinase; PV: Parasitophorous vacuole; STAT: Signal Transducer and Activator of Transcription; TLR: Toll-like receptor 1 receptor (T1RAP) domain containing adapter protein; TRAF: TNF receptor associated factor 6.

should be its potential to tilt the Th1-Th2 imbalance in favor of Th1. Furthermore in VL, T cell proliferation is impaired possibly due to loss of co-stimulatory molecule(s) [5], and so this too can be an additional target. Restoration of the lymphoproliferative capacity is achieved by Miltefosine [74], AmB in association with a suboptimum dose of streptomycin-bearing cationic liposomes [75], a monoclonal dipheroxovanadate compound K [NO (O2)2(H2O)] [73] along with SAG [44] and a human placental extract [52], Table 2).

Although Leishmaniasis is characterized by the appearance of anti-leishmanial antibodies, B cells and antibodies are unimportant, as parasites tend to hide within the macrophage parasitophorous vacuole [5]. In experimental models, it has been observed that anti-leishmanial antibodies play a contributory role as IgG coated parasites upon ligation with Fc receptors on macrophages [1] or dendritic cells [76] induce increased production of IL-10, a key cytokine for disease persistence. Furthermore, this was corroborated by studies with B cell depleted animals that were found to be highly resistant to Leishmaniasis [77]. Liposomal Z-100 and Kokenshoe pinnata decreased IgG and its subclasses [56,78], Table 2).

4.6. Macrophage derived cytokines as a measure of immunomodulatory activity

The immunomodulatory potential of anti-leishmanial drugs has been established by measuring its influence on macrophage derived cytokines, mainly IFN-γ, IL-12, TNF-α and IL-10. Miltefosine [41,42,74], glucamine [79], and Amphotericin B [43] along with experimentally effective compounds such as 18 Beta-glycyrhetinic acid [46], tannins [47,80,81], artemisinin [22], quassin [19], aconse extract of human placenta [52], garcic [82], pyrazinamide [61], CPG containing oligodeoxynucleotide [64], Berberine chloride [60] and 2,3,7,8-Tetrachlorodibenzo-P-dioxin [65], collectively support the notion that upregulation of the Th1 response is an effective strategy for parasite elimination. IL-6 and IL-1β are potent pro-inflammatory cytokines involved in the generation of NO and macrophage activation which are increased by antimlaus [83], tannins and related compounds [47,80,81] as also sage phenolics [84].

Chemokines, a superfamily of low MW cytokines recruit distinct subsets of leukocytes and by activating them play an important role in Leishmaniasis. TNF-α and IL-1β together with NF-κB (Macrophage inflammatory protein 1α) regulates transport of Leishmania from infected sites to lymph nodes [85 and references therein]. During Leishmaniasis, IFN-γ together with macrophage chemotactic protein 1 (MCP-1) eliminates L. major while conversely, IL-4 antagonizes production of MCP-1 [86]. Essential oil and extracts from Xylopia discoidea induces differential production of MCP-1 in leishmaniasis [87]. IL-8 is another chemokine that controls the early infection of Leishmania via recruitment of neutrophils [88] and release of NO along with pro-inflammatory cytokines (from macrophages [89]): SAG in fact induces IL-8 synthesis in patients with CL [83]. It has been shown that co-incubation of Leishmania parasites with PMNs inhibits the CXC-chemokine and interferon-γ inducible protein-10 (IP-10), accounting for its Th1 inhibiting activity [88], Furthermore, as IP-10 and OXCL-10 induce NK cells [85] it suggests that induction of chemokines within Leishmania infected cells could also be an effective strategy.

4.7. Effect on co-stimulatory molecules

T cell mediated regulation of immune responses is intimately associated with co-stimulatory molecules present on APCs, as they can modulate the TCR-MHC Interaction. Among them CD86, plays a pivotal role as their enhanced or diminished expression causes immune activation or anergy respectively [90] owing to their interaction with B7.1 (CD80) or B7.2 (CD86) present on monocyte/macrophages and/or B cells. In PKDL, increased levels of circulating CD86*28 lymphocytes confers immune anergy, evidenced by their non proliferating nature which gets reversed following treatment [91]. The impaired expression of CD86 on monocytes as evidenced in
PKDL was markedly increased following treatment with Miltefosine and SAG, the effect of Miltefosine being greater [52]. Pyrazinamide and SAC, expression of iNOS and CD68 in Leishmania infected BALB/C mice [46] as did an aqueous extract of human placenta, evidenced by an increased expression of MHC molecules on APCs [52, Table 2].

5. Modulation of signaling events in Leishmania infection; role of chemotherapy

5.1. Effect on expression of CD40 and MAPK signaling pathways

An important co-stimulatory molecule that determines the outcome of macrophage-Leishmania interactions is CD40 as the CD40-CD40L interaction helps increase the Th1 immune response [93]. With regard to Leishmania infection, CD40 mediated MAPKs have been reported to promote parasite survival by modulating the expression of IL-10 and IL-12 in macrophages [94]. MAPKs, a group of serine/threonine kinases are responsible for phosphorylation of cellular proteins which in turn triggers signals necessary for cell proliferation, differentiation and survival [95]. The CD40 of macrophages interacts with CD40L of T cells and passes the signal onwards to produce IL-12 via p38MAPK and NF-κB (nuclear factor κB, Fig. 1). The released IL-12 then binds to IL-12 receptors present on macrophages, increases their production of IFN-γ, which then acts on infected macrophages to induce parasite killing. However, this CD40-CD40L interaction has been proposed to exert a dual effect, as when CD40 signaling is associated with depletion of cholesterol and TRAF-6, it causes activation of ERK1/2, higher levels of IL-10 follow along with decreased levels of IL-12p40. Conversely, if the CD40 signalosome is associated with normal levels of cholesterol and TRAF-6, it causes p38 MAPK activation which is accompanied with increased leishmanicidal IL-12-p40 and accompanying pro-inflammatory responses [93,96]. It has been proposed that Leishmania lipophosphoglycan stimulate the ERK pathway, which in turn inhibits macrophage production of IL-12 [94 and references therein]. It has also been demonstrated that parasites can modulate the TLR2-stimulated MAPK pathway by suppressing phosphorylation of p38 MAPK along with enhanced phosphorylation of ERK1/2 [97]. Nitric oxide, a crucial mediator for leishmanicidal activity, also undergoes autophosphorylation; subsequently, dissociation of IRAK-1 from MyD88 follows so that it can now interact with TRAF6, which finally activates various cascades, leading to activation of MAP kinase pathways, transcription of NF-κB to nucleus and secretion of proinflammatory cytokines [102]. As Leishmania infection is associated with inhibition of IRAK mediated signaling (Fig. 1), the control of Leishmania parasites in vivo requires the adapter protein MyD88 [103] as a genetically resistant C57BL/6 mice became susceptible to Leishmania in the absence of MyD88 [104, Fig. 1]. Furthermore, silencing of TLR4, TLR3, IRAK-1 and MyD88 expression by RNA interference also led to decrease production of NO and TNF-α by macrophages in response to L. donovani promastigotes [105]. Studies showed that TLR4 signaling can enhance the microbicidal activity of macrophages harboring parasites [106] and Bhattacharya et al., 2010 [106] have demonstrated that the leishmanicidal potential of Arabonitrilelated Lipoproteinmannan was mediated through upregulation of TLR2 signals corroborating its importance as an important chemotherapeutic strategy.

5.2. Toll like receptors and their responsiveness in Leishmania infection

Toll like receptors (TLRs) have been identified as ancient receptors that play a critical role in determining the nature of the host immune response in Leishmania infection as they trigger a signaling pathway through a cascade of intracytoplasmic proteins known as Janus Kinase and signal transducer and activator of transcription (STATs) [107]. The biological effects of IFN-γ are dependent upon activation of STAT1 transcription factors as ligation of IFN-γ with IFN-γ receptor (IFN-γR) activates JAK1/JAK2 kinase which then phosphorylates STAT1-3; the STAT1 then translocates to the nucleus and further enhances transcription of IFN-γ-induced genes (Fig. 1, 108). The induction of IFN-γ is up-regulated by LPS and IFN-γ is primarily controlled by two regulatory regions present in the IFN-γ promoter that contains binding sequences for two transcription factors, NF-κB and IFN regulatory factor 1 (IRF-1) [109]. Both L. donovani and L. mexicana showed defective phosphorylation of JAK1, JAK2, and STAT1 [110]. Both L. major and L. mexicana suppressed expression of IFN-γRα; reduced levels of total JAK1 and JAK2, and downregulated IRF-1 induced JAK1, JAK2, and STAT1 activation, the effects being more profound with L. mexicana than L. major (Fig. 2, 112).

Wadhonne et al., 2009 [42] demonstrated that miltefosine effectively modulates host cell-dependent signaling pathways, restores responsiveness to IFN-γ via enhancement of IFN-γ receptors, IFN-γ induced STAT-1 phosphorylation and reduced activation of SHP-1 (the phosphatase implicated in down-regulation of STAT-1 phosphorylation). Another immunomodulatory anti-leishmanial cytokin, along with IFN-γ, modulated production of NO in macrophages that was partly dependent on activation of the JAK/STAT pathway [51].
5.4. Modulation of NF-κB signaling pathways by Leishmania

The NF-κB family includes five members of which p50, p65 (RelA) and c-Rel, have been demonstrated in macrophages, p50-p65 being the commonest [113]. In resting cells, NF-κB is retained in the cytoplasm complexed with its inhibitory subunit IkBα; following agonist stimulation, an enhanced serine phosphorylation of IkBα triggers its proteasomal degradation, resulting in subsequent activation of NF-κB; the MAPK signaling pathways have been identified as the upstream kinases that induce NF-κB activation via phosphorylation of its inhibitor, IkBα [114]. These signals induce IkBα kinase (IKK) and after activation of the IKK complex, specific IkBα phosphorylation/degradation causes subsequent release of NF-κB and its translocation to the nucleus activates transcription of multiple pro-inflammatory genes, including iNOS and TNF receptor 1 [115]. Therefore, preventing the degradation of IkBα and its downstream events is a strategy used by L. donovani promastigotes to effectively shut down the NF-κB-dependent expression of proinflammatory cytokines, ultimately translating into lowered promastigotes to effectively shut down the NF-κB-dependent expression of proinflammatory cytokines, ultimately translating into lowered pro-inflammatory cytokine levels and activation of NF-κB [51].

5.5. Alterations of host cell kinases and phosphatases by Leishmania

Protein kinase C (PKC) is a family of 10 isoenzymes involved in controlling the function of other proteins through phosphorylation of hydroxyl groups of their serine and threonine residues. PKCs play an important role in several signal transduction cascades and are activated by increased concentration of diacylglycerol (DAG) or Ca2+ [117]. During leishmaniasis infection, activation of PKC is inhibited and subsequent intracellular signaling PIP₃ being a key determinant [118] as also other glycosylphospholipids [119,120]. Bhattacharyya et al. [2001] have reported that L. donovani infection selectively inhibited Ca2+-dependent PKC activity (PKCζ) via β 10 while Ca2+ independent PKC (PKCα, γ) activity was enhanced [Fig. 1, [121]]. L. major is known to inhibit PKC-dependent c-fos and TNFα gene expression [122]. Infection of macrophages with L. donovani enhanced levels of intracellular caspase which in turn downregulated classical PKC activity, upregulated Ca2+-independent atypical PKCɛ expression [123]. Furthermore, PKC β is known to activate the assembly of NADPH oxidase subunit complex whereas Leishmania infection by causing downregulation of PKC β expression, inhibited the assembly of NADPH oxidase subunit complex and subsequently attenuated generation of ROS [Fig. 1, 93]. It is also known that PKC inform α is responsible for F actin mediated phagolysosomal maturation; once again, Leishmania by downregulating the expression of PKC α inhibited phagosomal maturation [93] and ref therein). Furthermore, as Leishmania induced PKC ε expression, which is known to inhibit the MAPKs, caused decreased production of proinflammatory mediators [Fig. 1, 124 and ref therein].

It has been seen that C2 chemokines, macrophage inflammatory protein (MIP-1 alpha) and macrophage chemotactic protein (MCP-1) can restrict the parasitic burden via restoration of impaired PKC signaling and induction of free-radical generation in macrophage Leishmaniasis [125]. The key signaling event, Ca2+-dependent PKC activity and inhibited Ca2+-independent atypical PKC activity both in vivo and in vitro in L. donovani-infected macrophages [125]. Subsequently, these events have been demonstrated that amphotericin B can restore the impaired classical PKC and abrogate the atypical PKC pathways [43].

During Leishmania infection, activation of (phospho)protein kinase (PKK), PKR, signaling caused a down-regulation of β-12 [123]. SHP-1/FTP is a protein Tyrosine Phosphatase (PTP) is an important negative regulatory molecule of signaling pathways, related to the actions of interferons [127].

Macrophages infected with Leishmania in vitro have elevated SHP-1/PTP activity induced by gp 63, which led to colocalization of SHP-1 and p72, and thereby prevented IFN-γ-induced tyrosine phosphorylation of JAK2 (Fig. 1, [128]). Leishmania induced PTPs are known to inhibit MAPKs leading to inhibition of nuclear translocation of NF-κB [Fig. 1, [93,124]]. It has been shown that SAC induces ERK1/2 phosphorylation through activation of P38, protein kinase C, and while p38 MAPK phosphorylation occurs through activation of PI3K and Akt [46].

6. Conclusions

The key pathogenic event in leishmaniasis is harboring of the causative Leishmania parasite within phagolysosomes of macrophages. Therefore, to establish infection, Leishmania invariably develop mechanisms to neutralize the microbial machinery of macrophages. Hence, establishment of infection critically hinges on whether the balance tilts towards the host’s ability to activate its antiparasitic ammunitions or the parasite’s ability to escape or evade this host immune response. Macrophages are host cells for the parasite, but also importantly, sentinels of the immune system. The parasite interferes with the signaling system of the host, such that effector functions triggered by various cell surface receptors are either actively suppressed or are altered so as to result in immune suppression that will promote parasite survival. Therefore, our quest for anti-leishmanial drugs should focus on their direct parasiticidal and/or indirect immunomodulatory activity, achieved via restoration of impaired host signaling pathways.

In this review, we have highlighted the participation of various immune cells, microbial molecules and altered signaling mechanisms in Leishmaniasis, together with the influence of anti-leishmanial drugs upon various immune cells like neutrophils, macrophages, DCs and lymphocytes. The different immune mechanisms impacted upon include increased generation of ROS and RNS, activation of co-stimulatory molecules and signaling pathways e.g. TRL, MAPK, JAK-STAT, PKC, and translocation of NF-κB. Taken together, screening for compounds having the propensity to modulate the host defense signaling pathways alone or in combination with existing anti leishmanial drugs [129] may well prove to be an effective immunomodulator strategy in Leishmaniasis worthy of pharmacological consideration.

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References


Barroso PA, Marco JD, Calvopina M, Kato H, Korenaga M, Hashiguchi Y. A trial of
fructose 1-2-fructose 1-phosphate against Leishmaniasis, Int Immunopharmacol
2011;11:10.1016/j.intimp.2011.05.002

chondrocyte derived anilic acid for protection during chemotherapy. J Diabetes
2011:10.1016/j.jdiab.2011.05.004

Martin-Quitin Z, del Rosario Garcia-Mile M, Most-Martin M, Mateo-Moo A, Torres-Tapia DA, Peraza-Sanchez SR. The leishmaniaidal effect of [35S]-16,17-

Baneijee D, Griewank K, Gazeau C, Eichhorn A, von Stebut E. Miltefosine efficiently
blocks schistosome eggs by chemotherapeuticagents against Leishmaniasis, Int Immunopharmacol
2011).10:1016/j.intimp.2011.05.002

Smelt SC, Cotterell SE, Engwerda CR, Kaye PM, B cell-deficient mice are highly
susceptible to Leishmania major infection associated with a polarized Th2 response. J Infect

Bhattacharya P, Bhattacharjee S, Gupta G, Majumder S, Adhikari A, Mukherjee A


Mathur RK, Awasthi A, Wadhone P, Ramanamurthy B, Saha B. Reciprocal CD40

Yamauchi S, Ito H, Miyajima A. IkappaBeta, a nuclear IkappaB protein, positively


Kropf P, Freundenberg MA, Misdul F, Price HP, Herrmann C, Antonioli S, et al. Toll-

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Linsley PS, Ledbetter JA. The role of the CD28 receptor during T cell responses to


Yamauchi S, Ito H, Miyajima A. IkappaBeta, a nuclear IkappaB protein, positively


Kropf P, Freundenberg MA, Misdul F, Price HP, Herrmann C, Antonioli S, et al. Toll-

Linsley PS, Ledbetter JA. The role of the CD28 receptor during T cell responses to


Rahland A, Kima PL. Activation of PI3K/Akt signaling has a dominant negative effect on IL-12p35 production by macrophages infected with Leishmania donovani. J Biol Chem 1995;270:18179-82.


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 8, and interleukin 10) 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.
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-1β 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-naphthal)hydroxylamine dihydrochloride (Loba Chemie, Mumbai, India), trichloro acetic acid (TCA), sulphanilamide, 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 µg/mL), and 10% heat-inactivated fetal bovine serum. Viability was confirmed using trypan blue (>99%).

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 µg/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-10, IL-13, TGF-β, and IL-1β 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.
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% isomitrosopropiophenone (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 to 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, 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 curtailing 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 1; Table 2). CD28 was restored following chemotherapy [13]. Miltefosine upregulated Monocyte-Associated Pro-Inflammatory Cytokines

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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SAG</th>
<th>Miltefosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>30.69 (4.11)</td>
<td>30.31 (4.35)</td>
</tr>
<tr>
<td>Sex ratio, male:female</td>
<td>11:1</td>
<td>11:1</td>
</tr>
<tr>
<td>Lesional type, no. of patients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macular</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Polymorphic</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Disease duration, years</td>
<td>8.16 (1.41)</td>
<td>4.71 (1.08)</td>
</tr>
<tr>
<td>Interval between cure of VL and onset, years</td>
<td>1.78 (0.39)</td>
<td>5.08 (3.28)</td>
</tr>
<tr>
<td>Lesional type, no. of patients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macular</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Polymorphic</td>
<td>9</td>
<td>9</td>
</tr>
</tbody>
</table>

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.
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-1β, 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

<table>
<thead>
<tr>
<th>Monocyte surface marker</th>
<th>Healthy control individuals</th>
<th>Patients with PKDL at presentation</th>
<th>Patients with PKDL after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14*</td>
<td>56.70 (4.39)</td>
<td>58.60 (2.68)</td>
<td>56.16 (0.97)</td>
</tr>
<tr>
<td>CD16*</td>
<td>25.81 (3.39)</td>
<td>24.35 (2.60)</td>
<td>34.84 (5.47)</td>
</tr>
<tr>
<td>CD14<em>CD80</em></td>
<td>1.83 (0.91)</td>
<td>1.87 (0.47)</td>
<td>6.18 (2.59)</td>
</tr>
<tr>
<td>CD14<em>CD86</em></td>
<td>52.11 (6.58)</td>
<td>26.42 (2.59)*</td>
<td>48.88 (7.40)</td>
</tr>
</tbody>
</table>

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. Abbreviations: SAG, sodium antimony gluconate.

* P < .01 compared with healthy control individuals.

* P < .05 compared with presentation.

* 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-CD86, and anti-GD38 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).

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Table 3. Serum Levels of Cytokines in Study Population

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Patients with PKDL after treatment, pg/mL</th>
<th>Patients with PKDL at presentation, pg/mL</th>
<th>SAG</th>
<th>Miltefosine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healthy control individuals, pg/mL</td>
<td>Patients with PKDL at presentation, pg/mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean (SEM)</td>
<td>Median (range)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>6.70 (2.44)</td>
<td>81.43 (13.14)</td>
<td>289.40 (132.20)</td>
<td>381.20 (143.10)</td>
</tr>
<tr>
<td></td>
<td>Median (range)</td>
<td>Mean (SEM)</td>
<td>125.9 (72.7-204.9)</td>
<td>120.8 (49.3-270.3)</td>
</tr>
<tr>
<td>IL-6</td>
<td>53.94 (9.26)</td>
<td>62.10 (10.21)</td>
<td>238.40 (78.94)</td>
<td>296.80 (92.86)</td>
</tr>
<tr>
<td></td>
<td>Median (range)</td>
<td>Mean (SEM)</td>
<td>159.7 (12.4-359.2)</td>
<td>217.9 (45.1-570.3)</td>
</tr>
<tr>
<td>IL-10</td>
<td>57.4 (10.5-81.8)</td>
<td>55.5 (10.6-50.7)</td>
<td>328.90 (132.20)</td>
<td>381.20 (143.10)</td>
</tr>
<tr>
<td></td>
<td>Median (range)</td>
<td>Mean (SEM)</td>
<td>125.9 (72.7-204.9)</td>
<td>120.8 (49.3-270.3)</td>
</tr>
<tr>
<td>IL-13</td>
<td>7.20 (2.87)</td>
<td>7.44 (1.96)</td>
<td>103.20 (48.96)</td>
<td>96.35 (30.37)</td>
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<td>Median (range)</td>
<td>Mean (SEM)</td>
<td>5.7 (4.4-19.3)</td>
<td>31.0 (9.3-222.4)</td>
</tr>
<tr>
<td>IL-6</td>
<td>12.68 (4.46)</td>
<td>34.74 (1.46)</td>
<td>22.18 (5.32)</td>
<td>17.10 (4.96)</td>
</tr>
<tr>
<td></td>
<td>Median (range)</td>
<td>Mean (SEM)</td>
<td>16.8 (6.9-38.9)</td>
<td>12.1 (4.9-20.9)</td>
</tr>
<tr>
<td>IL-10</td>
<td>11.7 (11.2-18.5)</td>
<td>26.1 (13.9-50.4)</td>
<td>86.6 (56.4-142.6)</td>
<td>55.9 (20.5-116.4)</td>
</tr>
<tr>
<td>IL-13</td>
<td>32.9 (11.5-143.8)</td>
<td>336.2 (16.3-1424.2)</td>
<td>1668.0 (1440.2-4160.0)</td>
<td>1398.0 (787.8-4786.0)</td>
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<tr>
<td></td>
<td>Median (range)</td>
<td>Mean (SEM)</td>
<td>56.2 (45.8-76.4)</td>
<td>35.1 (18.9-222.4)</td>
</tr>
<tr>
<td>IL-10</td>
<td>6989.00 (10.12)</td>
<td>12239.00 (489.20)</td>
<td>8938.00 (142.00)</td>
<td>365.80 (28.07)</td>
</tr>
<tr>
<td></td>
<td>Median (range)</td>
<td>Mean (SEM)</td>
<td>12239.00 (489.20)</td>
<td>365.80 (28.07)</td>
</tr>
<tr>
<td>IL-13</td>
<td>4540.0 (2369.0-10.561)</td>
<td>12465.0 (895.7-30.000)</td>
<td>11779.0 (6703.0-16.0)</td>
<td>365.80 (28.07)</td>
</tr>
<tr>
<td></td>
<td>Median (range)</td>
<td>Mean (SEM)</td>
<td>12239.00 (489.20)</td>
<td>365.80 (28.07)</td>
</tr>
<tr>
<td>IL-10</td>
<td>61.36 (8.50)</td>
<td>133.30 (12.48)</td>
<td>56.6 (18.20)</td>
<td>109.90 (15.87)</td>
</tr>
<tr>
<td></td>
<td>Median (range)</td>
<td>Mean (SEM)</td>
<td>28.2 (13.9-50.4)</td>
<td>15.8 (6.9-38.9)</td>
</tr>
<tr>
<td>IL-13</td>
<td>62.2 (8.7-76.8)</td>
<td>127.4 (85.5-215.7)</td>
<td>71.8 (67.5-163.0)</td>
<td>122.5 (45.1-370.3)</td>
</tr>
<tr>
<td></td>
<td>Median (range)</td>
<td>Mean (SEM)</td>
<td>71.8 (67.5-163.0)</td>
<td>122.5 (45.1-370.3)</td>
</tr>
<tr>
<td>IL-10</td>
<td>36.13 (16.60)</td>
<td>108.40 (67.74)</td>
<td>103.5 (57.24)</td>
<td>36.49 (16.31)</td>
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<td></td>
<td>Median (range)</td>
<td>Mean (SEM)</td>
<td>73.2 (21.0-362.5)</td>
<td>17.6 (7.8-38.0)</td>
</tr>
<tr>
<td>IL-13</td>
<td>39.4 (18.9-52.6)</td>
<td>73.2 (21.0-362.5)</td>
<td>42.3 (6.0-279.8)</td>
<td>17.6 (7.8-38.0)</td>
</tr>
</tbody>
</table>

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-10, interleukin 10; IL-13, interleukin 13; TGF-β, transforming growth factor β; THF-α, tumor necrosis factor α.

* P < .01 compared with healthy control individuals.

* P < .001 compared with healthy control individuals.

* P < .05 compared with presentation.

** 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 leishmanial activity, we evaluated serum nitrite levels, which were significantly higher in patients with PKDL than in healthy controls (mean [± SEM], 9.46 ± 0.88 vs 6.36 ± 0.19 μmol/L; P < .05). SAG caused no change (mean [± SEM], 8.86 ± 1.22 μmol/L), whereas miltefosine caused a marginal increase (mean [± SEM], 10.32 ± 1.06 μmol/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 [± SEM], 559.60 ± 56.45 vs 365.80 ± 28.07 U/L; P < .05), which was minimally decreased with miltefosine (mean [± SEM], 461.40 ± 76.26 U/L) and SAG (mean [± 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 1β [IL-1β], 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.

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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) or miltefosine.

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

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Antigen specific</th>
<th>Antigen nonspecific</th>
<th>Antigen specific</th>
<th>Antigen nonspecific</th>
<th>Antigen specific</th>
<th>Antigen nonspecific</th>
<th>Antigen specific</th>
<th>Antigen nonspecific</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>428.0 (127.6)</td>
<td>365.9 (82.7)</td>
<td>595.4 (144.0)</td>
<td>2399.7 (644.1)</td>
<td>68.920 (24345.21)</td>
<td>867.739 (72599.6)</td>
<td>68.920 (24345.21)</td>
<td>867.739 (72599.6)</td>
</tr>
<tr>
<td>IL-6</td>
<td>38.0 (5.8)</td>
<td>35.5 (7.7)</td>
<td>75.1 (6.8)</td>
<td>77.1 (1.8)</td>
<td>5.1 (12.9)</td>
<td>47.3 (1.7)</td>
<td>5.1 (12.9)</td>
<td>47.3 (1.7)</td>
</tr>
<tr>
<td>IL-10</td>
<td>10.4 (11.7)</td>
<td>11.9 (1.3)</td>
<td>10.1 (1.7)</td>
<td>11.6 (2.1)</td>
<td>29.6 (2.3)</td>
<td>27.4 (5.2)</td>
<td>29.6 (2.3)</td>
<td>27.4 (5.2)</td>
</tr>
<tr>
<td>IL-13</td>
<td>4.9 (4.9)</td>
<td>7.8 (4.0)</td>
<td>3.6 (4.9)</td>
<td>5.5 (1.5)</td>
<td>5.6 (3.3)</td>
<td>6.0 (2.3)</td>
<td>5.6 (3.3)</td>
<td>6.0 (2.3)</td>
</tr>
</tbody>
</table>

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.

* P < .01 compared with healthy control individuals.
* P < .05 compared with presentation.

Figure 4: Serum levels of nitrite and arginase activity in patients with PKDL at presentation and after treatment with SAG or miltefosine.

In Figure 4, nitrite and arginase activity were measured in serum samples of patients with PKDL at presentation and after treatment with SAG or miltefosine. Nitrite levels were significantly lower in patients treated with miltefosine compared to SAG.

Table 4: Cytokine Levels in Culture Supernatants of Patients With PKDL

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Antigen specific</th>
<th>Antigen nonspecific</th>
<th>Antigen specific</th>
<th>Antigen nonspecific</th>
<th>Antigen specific</th>
<th>Antigen nonspecific</th>
<th>Antigen specific</th>
<th>Antigen nonspecific</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
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<td>365.9 (82.7)</td>
<td>595.4 (144.0)</td>
<td>2399.7 (644.1)</td>
<td>68.920 (24345.21)</td>
<td>867.739 (72599.6)</td>
<td>68.920 (24345.21)</td>
<td>867.739 (72599.6)</td>
</tr>
<tr>
<td>IL-6</td>
<td>38.0 (5.8)</td>
<td>35.5 (7.7)</td>
<td>75.1 (6.8)</td>
<td>77.1 (1.8)</td>
<td>5.1 (12.9)</td>
<td>47.3 (1.7)</td>
<td>5.1 (12.9)</td>
<td>47.3 (1.7)</td>
</tr>
<tr>
<td>IL-10</td>
<td>10.4 (11.7)</td>
<td>11.9 (1.3)</td>
<td>10.1 (1.7)</td>
<td>11.6 (2.1)</td>
<td>29.6 (2.3)</td>
<td>27.4 (5.2)</td>
<td>29.6 (2.3)</td>
<td>27.4 (5.2)</td>
</tr>
<tr>
<td>IL-13</td>
<td>4.9 (4.9)</td>
<td>7.8 (4.0)</td>
<td>3.6 (4.9)</td>
<td>5.5 (1.5)</td>
<td>5.6 (3.3)</td>
<td>6.0 (2.3)</td>
<td>5.6 (3.3)</td>
<td>6.0 (2.3)</td>
</tr>
</tbody>
</table>
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-1β [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 [35], and after chemotherapy, a 4.7-fold increase was demonstrated (Table 3). The scenario with IL-1β 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-1β, 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 a 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 [28]. 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-1β, 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 3D).

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 [32], 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 [13]. 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.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

Leishmaniasis in India resulting from infection of the hemoflagellate protozoan parasite *Leishmania donovani* manifests in two different forms, visceral leishmaniasis (VL; also known as kala-azar) and its dermatologic sequel post-kala-azar dermal leishmaniasis (PKDL; discussed in ref. 1 and references therein). PKDL was first described by Brahmsachari in 1922 in cured VL patients with eruptions and plaques in the skin, it was confirmed by demonstration of Leishman–Donovan bodies (LD) in slit skin smears, and it was termed dermal leishmanoid (discussed in ref. 1 and references therein). Later, the disease was renamed PKDL because eruptions follow the visceral form, commonly called kala-azar. PKDL manifests in a variety of clinical forms ranging from hypopigmented macules to infiltrated plaques and nodules. Diagnosing PKDL has always been a challenge, because its geographical pocket is always been a challenge, because its geographical pocket is

because of its endemicity and its diagnostic approaches have been developed ranging from serological tests and immunohistochemistry to polymerase chain reaction (PCR). Among the serological tests, the immunochromatographic strip test using recombinant kinesin 39 (rK39) is considered as a rapid, convenient, and useful test for diagnosis of Indian leishmaniasis. The K39 epitope is highly conserved in the visceralizing species of *Leishmania* and was used to develop a diagnostic test for VL and PKDL. In cases of VL, the rK39 nitrocellulose-based dipstick test showed high sensitivity and specificity and therefore, has gained substantial popularity for its ease of use, especially in the field setting. For detection of polymorphic PKDL, comprising both macular and papulo-nodular skin lesions, the sensitivity of the rK39 strip test is 95.6%, and for macular PKDL, the sensitivity is 80.3%. This report deals with three cases where relying on the rK39 strip test led to misdiagnosis and subsequent therapeutic delay. The study was approved by the Institutional Ethical Committee, and peripheral blood or skin biopsy was collected after obtaining informed consent from the parent/guardian of the minors.

**CASE 1**

A 9-year-old boy from the Godda district of Jharkhand, India, presented with multiple hypopigmented macules occupying the face and upper torso as well as the upper and lower limbs (Figure 1). Five years ago, he had suffered from VL, for which he was treated with sodium stibogluconate (SSO). He complained of photosensitivity but had no sensory abnormality and no thickened or tender peripheral nerves. Cervical and axillary lymphadenopathy was present; however, no mucosal or genital lesions were present, and also, no systemic abnormality was found. Because the rK39 strip test was negative, he was not considered as a case of PKDL. Silt skin smear using modified Ziehl–Neelsen (ZN) stain and Giemsa was also negative for Acid Fast Bacilli (AFB) and LD body, respectively. However, on the grounds of a strong clinical suspicion of PKDL, indirect enzyme-linked immunoelectrophoretic assay (ELISA) was performed using crude *Leishmania* antigen as the coating antigen. The presence of antileishmanial antibody in serum was detected using horseradish peroxidase-conjugated Protein-A (Sigma–Aldrich Chemicals, St. Louis, MO), which reacted with substrate 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS; Roche Diagnostics, Mannheim, Germany), and optical density was measured at 405 nm (OD405) by a microplate reader (Model 680; Bio-Rad, Hercules, CA). The titer was considered as positive when the OD405 of the suspected case was at least twofold higher than the composite mean of 15 non-endemic controls (mean ± standard deviation [SD] = 0.08 ± 0.06). Because the OD405 was 0.26 and 3.3-fold higher, it was considered positive. We further confirmed the result by PCR (i.e., tested for parasite DNA in the skin biopsy sample [3-mm punch biopsy] using *Leishmania*-specific primers LITSR [5'-CTGGATCATrTTCCGATG-3'] and L5.8S [5'-TGATA CCACTATTGCACACT-3'] using Platinum Taq polymerase (Invitrogen, Carlsbad, CA), PCR buffer, and deoxyribonucleotide triphosphates (dNTPs; Fermentas, Glen Burnie, MD) in a Master cycler (Eppendorf, Hamburg, Germany). The PCR products were visualized by agarose gel electrophoresis (1.3%) and analyzed in G-BOX gel doc (Syngene, Cambridge, UK) using Gene Tools (version 4.01.04) software. DNA was isolated from both peripheral blood and skin biopsy by the QiAamp DNA mini kit (Qiongen, Hilden, Germany) and eluted in 200 μL elution buffer. For the PCR assay, a positive control
Figure 1. A 9-year-old boy who presented with multiple macular lesions.

(DNA isolated from *L. donovani* promastigotes obtained from a patient with PKDL [SS10]) and a negative control (water) were used. The PCR product length of the skin biopsy sample was 306 bp, which was comparable with the PCR product of the PKDL isolate (product length = 312 bp) (Figure 2). PCR was also performed in a peripheral blood sample obtained from a non-endemic human control, and it gave no band. The patient received SSG (20 mg/kg body weight per day intramuscularly), because the patient was not from an area associated with resistance.5 Outcome of the treatment was assessed after 4 months and showed considerable improvement.

Figure 2. Polymerase chain reaction (PCR) assay with clinical samples of post-kala-azar dermal leishmaniasis (PKDL) and visceral leishmaniasis (VL). Lane M = 100-bp ladder. Lane 1 = PCR control (water). Lane 2 = VL isolate (DD8). Lane 3 = VL isolate (NS2). Lane 4 = VL isolate (YR03). Lane 5 = PKDL isolate (SS10). Lane 6 = non-endemic control (peripheral blood). Lane 7 = PKDL skin biopsy (case 1). Lane 8 = PKDL skin biopsy (case 2). Lane 9 = VL peripheral blood (case 3).

CASE 2

A 15-year-old girl from Murshidabad district of West Bengal, India, presented with multiple hypopigmented patches that were restricted to the face (Figure 3). The lesions were asymptomatic, and there was no sensory abnormality; importantly, they appeared 1 month after cure from VL. She had previously suffered from VL 1.5 years ago and had been treated with SSG. She gave no history of atopy, and peripheral nerves were not thickened. The slit skin smear did not reveal any AFB or LD body, and because rK39 was negative, the results posed a diagnostic dilemma; diagnosing PKDL solely on the basis of a positive history of VL was unjustified, especially because her lesions were restricted to the face. Indeterminate leprosy presenting with multiple lesions was also unlikely, and accordingly, the patient was offered ketoconazole shampoo and topical emollient, because seborrheic dermatitis was considered the most plausible cause. She was asked to return after 1 month but instead, returned after 2 months when the lesions had increased in size and number; again, there was no sensory abnormality or peripheral nerve thickening noted. Histopathology was performed, and it showed non-specific perivascular mononuclear infiltrate with no evidence of perineural/perappendageal localization that would be suggestive of leprosy.

With a strong clinical suspicion of PKDL, we performed indirect ELISA; the antileishmanial antibody titer was negative, OD405 was 0.05, and the composite mean OD405 of the control group was 0.08. PCR was done as previously described from a skin biopsy (3-mm punch biopsy) and was positive (having two bands of length of 318 and 415 bp) (Figure 2). Understandably, this was a case of false-negative rK39 strip.

Figure 3. A 15-year-old girl with few hypopigmented patches present primarily on the face.
test and ELISA, which delayed the diagnosis and led to unneccessary treatment for seborrheic dermatitis.

CASE 3

A 7-year-old boy from Canning, South 24 Parganas district of West Bengal, India, presented with fever without chill and rigor that was not associated with cough. He had previously suffered from VL and had received SSQ, but for economic reasons, he was unable to complete the treatment. He remained afebrile for 4 months, and when he presented at the outpatient unit of the School of Tropical Medicine, he had been suffering for 7 months from a low-grade fever along with generalized weakness. There was no lymphadenopathy or hepatomegaly; spleen was enlarged 6 cm below the costal margin. The malaria parasite was not found, a chest radiograph showed no abnormalities, tests for human immunodeficiency virus (HIV) were negative, and there was no lymphadenopathy or hepatomegaly. Laboratory examination showed hemoglobin of 7.5 g/dL, hematocrit of 28.3%, mean corpuscular volume (MCV) of 74.9 fL, mean corpuscular hemoglobin (MCH) of 19.8 pg, and mean corpuscular hemoglobin concentration (MCHC) of 26.5 g/dL. The white blood cell count was 2,300 cells/mm³ (30% neutrophils, 62% lymphocytes, and 0.5% monocytes), platelet count was 210,000 cells/mm³, erythrocyte sedimentation rate (ESR) was 62 mm in the first hour, and prothrombin time was 11.0 seconds. Kidney function tests (area = 15 mg/dL and creatinine = 0.7 mg/dL) and liver function tests (aspartate aminotransferase = 44 IU/L, alanine aminotransferase = 12 IU/L, alkaline phosphatase = 158 IU/L, total bilirubin = 1.0 mg/dL, and conjugated versus unconjugated = 0.4/0.6) were within normal limits. Total protein was 9.3 g/dL, and the albumin/globulin ratio was 2.7/6.6, indicating hypoalbuminemia and hypergammaglobulinemia. Blood sodium and potassium levels were normal as was blood sugar. Serological tests using dipstick rK39 showed no reactivity; however, the clinic and laboratory profile and the fact that the patient resided in an area where occurrence of VL has been reported prompted us to do ELISA, bone marrow aspiration for transformation of parasite, and PCR. The indirect ELISA OD at 550 nm was 0.77, which was 9.6-fold higher than the composite mean of non- endemic healthy controls (0.08). Subsequently, bone marrow aspiration was done using a Sahli’s needle, whereas the posterior superior iliac spine was chosen as the site for aspiration. The aspirated material was collected, diluted 1:1 with Schneider’s insect medium supplemented with 20% heat-inactivated fetal calf serum, penicillin G (50 IU/mL), and streptomycin (50 µg/ml), and kept in a tissue-culture flask (25 cm²) for transformation of parasite. Parasite transformation was evident 6 days later and was typed as L. donovani using a species-specific monoclonal antibody. Giemsa stain of bone marrow aspirate revealed 1-4 LD bodies per 10 oil immersion fields. Further confirmation was achieved by performing PCR using peripheral blood of this patient along with positive controls, which comprised of DNA isolated from promastigotes of three VL isolates (DD8, NS2, and YR08) confirmed to be L. donovani by ELISA using a species-specific monoclonal antibody along with negative control (DNA isolated from a peripheral blood sample of a healthy control from a non-endemic area of VL). The PCR product length of this patient was 306 bp, whereas the lengths of the L. donovani VL isolates DD8, NS2, and YR08 were 324, 422, and 419 bp, respectively (Figure 2).

The observed variation can, therefore, be attributed to a sequence variation known to occur between strains of L. donovani. The patient received a daily injection of amphotericin B (0.75 mg/kg body weight) for 4 weeks.

In case 1, it was very important to clinically differentiate it from lepromatous leprosy, because infiltration of peripheral nerves is symmetrical and asymptomatic, making the diagnosis difficult. Other tell-tale signs like photosensitivity and lymphadenopathy were important parameters to clinically differentiate PKDL from leprosy. In case 2, because both rK39 and indirect ELISA were negative, it suggests that the patient may have been immunocompromised (however, the patient tested negative for HIV) and therefore, only an antigen- or DNA-based test would be effective. In case 3, the clinical presentation prompted us to additionally evaluate by ELISA, PCR, and for the most definitive evidence, parasite transformation. These three cases emphasize the need for relying on a high index of suspicion and clinical acumen in dealing with patients of VL and PKDL. The laboratory tests are always a supplement to clinical methods but relying too much on tests can be counterproductive. Silt skin smear, for detection of both AFB and LD bodies, lacks sensitivity and here, the predictive value of a negative test is low. The rK39 is undoubtedly a useful test, especially in a field setting; however, if the rK39 is negative but clinical suspicion is high, one may consider performing a DNA-based test. In the Indian perspective, eradication of VL is presently a national priority. Because it is widely accepted that PKDL serves as the disease reservoir, it would, therefore, be pertinent to consider development of a referral system, where cases of PKDL can receive appropriate attention, to achieve the goal of eliminating VL.

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REFERENCES


Enhanced Lesional Foxp3 Expression and Peripheral Anergic Lymphocytes Indicate a Role for Regulatory T Cells in Indian Post-Kala-Azar Dermal Leishmaniasis

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Indian post-kala-azar dermal leishmaniasis (PKDL) is a low-frequency (5–10%) dermal sequela of visceral leishmaniasis (VL) caused by Leishmania donovani; Importantly, affected individuals are speculated to be parasite reservoirs. Insight into its immunopathogenesis could translate into rational immunomodulatory therapeutic approaches against leishmaniasis. In patients with PKDL (n = 21), peripheral lymphocytes were analyzed for surface markers, intracellular cytokines, and lymphoproliferative responses using flow cytometry. In lesional tissue biopsies (n = 12), expression of counter-regulatory cytokines (IFN-γ and IL-10) and the T-regulatory transcription factor forkhead box protein 3 (Foxp3) was analyzed using reverse transcriptase-PCR, which would sustain disease pathology. Treatment. CD8+ lymphocytes showed impaired proliferative responses to antigen; mRNA, messenger RNA; PBMC, peripheral blood mononuclear cell; PerCP, peridinin chlorophyll protein; PKDL, post-kala-azar dermal leishmaniasis; VL, visceral leishmaniasis.

INTRODUCTION

Post-kala-azar dermal leishmaniasis (PKDL), first described by Brahmanachri (1922), presents as a dermal rash after cure of visceral leishmaniasis (VL) and is caused by the protozoan parasite Leishmania donovani (Zijlstra et al., 2000; Ramesh et al., 2007). PKDL is primarily reported from two VL-endemic regions, the Indian subcontinent and Sudan and its adjoining areas. In the Indian variant, only 5–10% of patients with VL acquire PKDL, as opposed to 50–60% in Sudan (Zijlstra et al., 2003; Ramesh et al., 2007); Importantly, with zoonotic hosts yet to be defined, patients with PKDL are the proposed parasite reservoirs (Zijlstra et al., 2003). In the Indian subcontinent, immunological factors predisposing patients with VL to PKDL remain poorly characterized, with the disease pathology often being attributed to parasite-specific cell-mediated immune (CMI) responses (Ramesh et al., 2007). Sudanese patients with PKDL respond to Leishmania antigen (Ismail et al., 1999), whereas studies on immune responses in Indian PKDL are not so clearly defined and even contradictory (Haldar et al., 1983; Neogy et al., 1988). Our previous study on intracellular cytokine expression within circulating lymphocytes of patients with PKDL indicated that generalized CMI was restored but antigen-specific CMI was restricted to CD3+ CD8+ lymphocytes producing IL-10 (Ganguly et al., 2008). In this study, we describe downstream CMI responses in patients with PKDL.
vis-à-vis cured VL patients and healthy controls by measuring mitogen-induced and antigen-specific proliferation in circulating CD4+ and CD8+ lymphocytes.

Lesions in PKDL typically present as hyperpigmented macules, nodules, and papules. In Sudanese PKDL, the incidence of papular or nodular rash is maximal followed by maculopapular, micropapular, and macular lesions (Zijlstra et al., 2000), whereas Indian patients report erythema and induration on the face or numerous, hypopigmented macules (Ramesh and Mukherjee, 1995; Zijlstra et al., 2003). Regardless of the differences, the nodular, macular, and maculopapular lesions predominate and are construed as hallmarks of PKDL (Zijlstra et al., 2003).

The sustained lesional expression of counter-regulatory cytokines in patients with PKDL (Ansari et al., 2006) suggests a role for regulatory T cells (TRegs) who by maintaining immune homeostasis can regulate immunity to infection (Sakaguchi, 2004). TRegs, comprise 5–10% of the circulating CD4+ T-cell population and uniquely express the transcription factor, Forkhead Box P3 (Foxp3), which is indispensable for their development and function (Shevach et al., 2006). In acute infections, TRegs benefit the host by thwarting immune-mediated pathology after pathogen eradication, whereas in chronic infections their activity is detrimental to the host, as they promote parasite survival amidst an active immune response (Belkaid, 2007). Therefore, in PKDL, the chronic nature of the disease warrants an analysis into the possible role of TRegs.

RESULTS

Study population

Patients with PKDL (n = 21) were classified as polymorphic, macular, or papulonodular. All patients tested positive with the rK39 test and ELISA for anti-leishmanial antibodies; only two patients (one macular and one papulonodular) tested negative for L. donovani bodies in skin smears. The majority presented with polymorphic (n = 18, 85%) followed by papulonodular (n = 2, 10%) and macular lesions (n = 1, 5%) (Table 1); five patients (24%) gave no previous history of VL attributable to poor documentation, inability to recall past incidence of VL, or the subclinical nature of VL. However, they were included as they had demonstrable L. donovani bodies and tested positive by rK39 and ELISA. In the remaining 16 patients, the time interval between cure of VL and onset of PKDL ranged from 1 to 49 years (Table 1). The time interval often extends to decades (Ramesh and Mukherjee, 1995); only one patient reported an interval of 49 years, four had an interval within 17–19 years, whereas the majority (n = 11) reported an interval from 1 to 3 years. However, no correlation was evident between time interval and lesional profiles.

Circulating CD8*CD28− lymphocytes elevated in patients with PKDL

In patients with PKDL, proportions of circulating CD8*CD28− lymphocytes, representing natural T regulatory cells, were comparable with healthy controls (Table 2). However, CD8*CD28− regulatory lymphocytes were significantly raised in patients with PKDL when compared with healthy controls; treatment effected a significant decrease, values being comparable with healthy controls (Table 2).

With regard to the costimulatory molecule CD28, both CD4* and CD8* lymphocytes were significantly raised in patients with PKDL when compared with healthy controls; treatment effected a significant decrease, values being comparable with healthy controls (Table 2).

Table 1. Clinical features of study population

<table>
<thead>
<tr>
<th>PKDL (n=21)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>30.0±14.28</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>90 (12-65)</td>
<td></td>
</tr>
<tr>
<td>Sex ratio</td>
<td>1:3</td>
<td></td>
</tr>
<tr>
<td>Lesional type</td>
<td>Polymorphic:18</td>
<td>Macular:1</td>
</tr>
<tr>
<td>History of VL</td>
<td>16% (16/21)</td>
<td></td>
</tr>
<tr>
<td>Interval between cure of VL and onset (year)</td>
<td>7.57±13.21 (2:149)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: T, temperature; PKDL, post-kala-azar dermal leishmaniasis; VL, visceral leishmaniasis.

Values are mean ± standard deviation. Median and range values are in parentheses.

Table 2. Surface marker and intracellular cytokine profiles of patients with PKDL

<table>
<thead>
<tr>
<th>Surface marker/ intracellular cytokine</th>
<th>PKDL</th>
<th>Pretreatment</th>
<th>Post treatment</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+CD25+</td>
<td>3.72±2.38</td>
<td>13.1±1.74</td>
<td>2.72±1.10</td>
<td></td>
</tr>
<tr>
<td>CD4+CD8+CD28−</td>
<td>52.18±1.86</td>
<td>32.03±1.18</td>
<td>30.3±6.9</td>
<td></td>
</tr>
<tr>
<td>CD8+CD28−</td>
<td>31.63±5.64</td>
<td>32.62±1.70</td>
<td>27.88±3.55</td>
<td></td>
</tr>
<tr>
<td>CD8+CD28+</td>
<td>15.64±4.64</td>
<td>17.95±14.9</td>
<td>13.04±4.93</td>
<td></td>
</tr>
<tr>
<td>CD3+IL-10* (LAC stimulated)</td>
<td>13.86±2.58</td>
<td>13.17±5.26</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>CD3+TFN- (LAC stimulated)</td>
<td>0.45±0.21</td>
<td>0.14±0.04</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>CD3+IL-10* (LDA stimulated)</td>
<td>15.36±2.51</td>
<td>11.65±3.77</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>CD3+TFN- (LDA stimulated)</td>
<td>17.55±2.34</td>
<td>16.9±4.19</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: LAC, leukocyte activation cocktail; LDA, Leishmania donovani antigen; ND, not determined; PBMC, peripheral blood mononuclear cell; PKDL, post-kala-azar dermal leishmaniasis.

Values are mean ± standard deviation. Abbreviations: LAC, leukocyte activation cocktail; LDA, Leishmania donovani antigen; ND, not determined; PBMC, peripheral blood mononuclear cell; PKDL, post-kala-azar dermal leishmaniasis.

*Peripheral blood from patients with PKDL (n=21) and healthy controls (n=10) was stained with surface marker antibodies.

**PBMCs were fixed, permeabilized, and stained with cytokine antibodies.

Cells were acquired using flow cytometry as described in Materials and Methods, with lymphocytes gated by forward and side scatter.

Values are mean ± standard deviation. Values of cells were determined as follows: **P<0.05, ***P<0.01, and **P<0.001 when compared with controls; **P<0.01 and ***P<0.001 when compared with pretreatment.
compared with healthy controls (Table 2). After treatment, restoration of CD28 expression was restricted to the CD4+ subset (Table 3).

Peripheral lymphocytes from patients with PKDL were unresponsive to *L. donovani* antigen (LDA). Specific and generalized CMI were analyzed in terms of lymphoproliferative responses to LDA and phytohemagglutinin (PHA), respectively. Proliferating cells showed an expected dilution in carboxyfluorescein diacetate succinimidyl ester fluorescence, whereas unstimulated cells retained maximum fluorescence, indicating absence of proliferation.

With regard to LDA-induced proliferation, patients with PKDL showed a poor proliferation index (PI) in both CD4+ (1.30 ± 0.15) and CD8+ lymphocytes (1.18 ± 0.03). After treatment, CD4+ lymphocytes showed significantly greater proliferation than healthy controls (1.95 ± 0.43 vs 1.02 ± 0.01, P<0.05), comparable with cured VL patients (2.13 ± 0.64). In the CD8+ subset, the post-treatment PI were more pronounced relative to healthy controls (3.27 ± 1.06 vs 1.01 ± 0.01, P<0.01) and cured VL patients (1.47 ± 0.41, Figure 1a and c).

In response to PHA, CD4+ lymphocytes from healthy individuals proliferated strongly, with the PI being 6.70 ± 0.23 (Figure 1b and d). Similarly, patients with PKDL showed comparable PI before (3.60 ± 0.32) and after treatment (4.26 ± 0.94), whereas patients with VL who had been cured showed restoration of their characteristically impaired PI (2.85 ± 0.52). In patients with PKDL, the CD8+ subset showed impaired proliferation when compared with controls (4.35 ± 0.65 vs 9.66 ± 0.63, P<0.05), which increased to 7.99 ± 3.17 with treatment, whereas in cured VL patients the PI remained lower than controls (4.48 ± 1.57, P=0.09; Figure 1b and d).

**Table 3. Immunohistochemistry scores and lesional profiles of patients with PKDL before and after treatment**

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Clinical features at presentation</th>
<th>Foxp3 score</th>
<th>CD3 score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>1</td>
<td>Papulonodular lesions emerged chronologically on the face, trunk, and lower extremities and hypopigmented macules on the trunk and extremities over a period of 7 years.</td>
<td>++ (1.72 ± 0.62)</td>
<td>+++ (5.28 ± 1.05)</td>
</tr>
<tr>
<td>2</td>
<td>Erythematous papules and nodules on the face for 1 year; papules and macules on extremities for 2-3 months.</td>
<td>++ (1.05 ± 1.10)</td>
<td>+++ (5.18 ± 1.0)</td>
</tr>
<tr>
<td>3</td>
<td>Papulonodules appeared chronologically on the face, and upper arms and hypopigmented macules on the trunk and back over a 7-year period.</td>
<td>++ (1.62 ± 1.0)</td>
<td>+++ (6.17 ± 1.0)</td>
</tr>
<tr>
<td>4</td>
<td>Nodular lesions with papules and few disseminated macules over face, extremities, and upper back for the past 10 years.</td>
<td>++ (1.72 ± 0.62)</td>
<td>+++ (5.28 ± 1.05)</td>
</tr>
<tr>
<td>5</td>
<td>Erythematous plaques and nodules on face, trunk, extremities, and genitalia for the past 2 and a half years; hypopigmented macules on trunk and back without sensory abnormality for the past 2 years.</td>
<td>++ (1.05 ± 1.10)</td>
<td>+++ (5.18 ± 1.0)</td>
</tr>
<tr>
<td>6</td>
<td>Hypopigmented macules dispersed all over the body, predominantly on the face, trunk, and extremities for the past year, microabscesses and a few nodules on the facial region.</td>
<td>++ (1.72 ± 0.62)</td>
<td>+++ (5.28 ± 1.05)</td>
</tr>
<tr>
<td>7</td>
<td>Nodules and papules emerged on the nasal chin, and pinnae 3 years ago.</td>
<td>++ (1.05 ± 1.10)</td>
<td>+++ (5.18 ± 1.0)</td>
</tr>
<tr>
<td>8</td>
<td>Erythematous plaques and nodules emerged on the nasal chin, and back 3 years ago.</td>
<td>++ (1.05 ± 1.10)</td>
<td>+++ (5.18 ± 1.0)</td>
</tr>
</tbody>
</table>

Abbreviations: Foxp3, forkhead box protein 3; PKDL, post-kala-azar dermal leishmaniasis.
Figure 1. Specific and generalized proliferative responses of lymphocytes. Lymphocytes from patients with post-kala-azar dermal leishmaniasis (PKDL) before (■, n=21) and after (□, n=12) treatment, from patients with visceral leishmaniasis (VL) after cure (□□, n=5), and from healthy controls (□□□, n=6) were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE), incubated with/without Leishmania donovani antigen (LDA; 5 µg/ml, a) or phytohemagglutinin (PHA; 10 µg/ml, b), and CFSE fluorescence of gated CD3+CD4+ and CD3+CD8+ lymphoblasts was determined using flow cytometry. Columns represent mean proliferation indices of at least five individuals per group, with error bars denoting SEM. *P<0.05, **P<0.01, significantly different from controls; &P<0.05, significantly different from VL after treatment (c) Representative profile of proliferative responses of CD3+CD4+ and CD3+CD8+ lymphocytes to LDA in patients with PKDL before and after treatment, cured VL patients, and healthy individuals. (d) Representative profile of proliferative responses of CD3+CD4+ and CD3+CD8+ lymphocytes to PHA in patients with PKDL before and after treatment, cured VL patients, and healthy individuals.
IL-10-producing CD3+ cells were elevated, which were significantly reduced after treatment (Table 2).

Upregulated lesional expression of IFN-γ and IL-10 decreased with treatment

To elucidate counter-regulatory cytokine profiles in lesional tissue from patients with PKDL, messenger (mRNA) expression of IFN-γ and IL-10 was quantitated in paired samples. At presentation (n=12), a significant upregulation in IFN-γ expression was observed when compared with healthy controls (1.21±0.08 vs 0.21±0.02, P<0.001); similarly, IL-10 transcripts were significantly increased (0.44±0.07 vs 0.005±0.002, P<0.001; Figure 2a and c). After treatment, a significant reduction in both IFN-γ (0.50±0.11, P<0.001) and IL-10 (0.04±0.01, P<0.001) was evident (Figure 2a and b).

Elevated lesional Foxp3 mRNA expression in patients with PKDL

To determine whether increased expression of IL-10 in lesional tissue was associated with an enhanced presence of TReg cells, lesional mRNA expression of the TReg transcription factor, Foxp3, was quantitated. Patients with PKDL, when compared with healthy controls, had significantly upregulated Foxp3 transcripts (0.19±0.05 vs 0.02±0.01, P<0.01; Figure 3a and c); treatment effected a modest reduction in Foxp3 mRNA levels (0.14±0.05) (Figure 3a-c). On an individual basis, decreased expression was evident in 9 out of 12 patients, the percentage reduction ranging from 6.13 to 86.49%; one patient retained the same level of expression, whereas two showed higher mRNA levels (Figure 3b).

Increased lesional accumulation of T regulatory cells in patients with PKDL regressed with treatment

To confirm whether upregulated expression of Foxp3 translated into increased protein, immunohistochemistry was performed on lesional tissue from patients with PKDL (n=8), healthy controls (n=3), and one patient with erythema nodosum leprosum; in addition, sections were stained for CD3. In PKDL lesions, microscopic analysis showed an accumulation of Foxp3+ cells within a dense and predominantly CD3+ lymphocytic infiltrate (Figure 4a and b), whereas healthy skin showed virtually no Foxp3+...
Figure 3. Lesional forkhead box protein 3 (Foxp3) expression in patients with post-kaiza-azar dermal leishmaniasis (PKDL) at presentation and after treatment. 
(a) A representative profile of Foxp3 mRNA expression in lesional tissue from patients with PKDL (n=5) before and after treatment and normal skin tissue from three healthy Individuals. Expression was quantified using reverse transcriptase-PCR (RT-PCR) and subsequent densitometric analysis, as described in Materials and Methods. (b) Before-after plot of Foxp3 expression in patients with PKDL (n=12) before (▲) and after completion of treatment (A). (c) Scatter plot showing Foxp3 expression values in patients with PKDL (n=12) before (▲) and after treatment (A) and in healthy controls (♦, n=3). Horizontal lines indicate mean values. *P<0.01, significantly different from controls.

Discussion

In PKDL, enhanced secretion of IL-10 and transforming growth factor-β by antigen-stimulated peripheral blood mononuclear cells (PBMCs) has been associated with disease severity (Saha et al., 2007). Subsequently, we proposed IL-10-producing antigen-specific circulating CD8+ lymphocytes together with increased serum IL-10, IgG1, and IgG3 as markers of Indian PKDL (Ganguly et al., 2008). This study was undertaken to show the presence of both circulatory and lesional T<sub>r</sub>regs in a substantial number of patients with PKDL (Table 1).

In Sudanese PKDL, antigen-specific CMI responses have been documented (Ismail et al., 1999; Gasim et al., 2000), whereas in India, reports are varied. Haldar et al. (1983) reported specific CMI in five out of six patients with newly acquired PKDL, vis-à-vis three out of six patients with chronic PKDL, whereas Neogy et al. (1988) recorded an absence of Leishmania-specific CMI. In this study, patients with PKDL showed no antigen-induced proliferation of CD4+ lymphocytes that was marginally restored after treatment (Figure 1a and c). However, in cured VL patients, a higher degree of proliferation was observed, attributable to greater proportions of antigen-specific memory and residual effector lymphocytes (Figure 1a and c). With respect to antigen-primed CD8+ lymphocytes, patients with PKDL again showed an impairment that was also restored after treatment, attributable to the elevated proportion of CD8+CD28<sup>+</sup> regulatory lymphocytes (Table 2).

However, no impairment of generalized CMI has been reported in Indian PKDL (Haldar et al., 1983; Neogy et al., 1988), which is corroborated by robust intracellular IFN-γ and IL-2 production (Ganguly et al., 2008). In previous studies, proliferative responses were examined in bulk lymphocyte populations (Haldar et al., 1983; Neogy et al., 1988); thereby overlooking differences in effector lymphocyte subsets. Indeed, whereas Neogy et al. (1988) showed an overall PHA-induced proliferation in Indian patients with PKDL, this study, using carboxyfluorescein diacetate

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In comparison with their CD4+CD25+ major histocompatibility complex class II-restricted counterparts, the class I-restricted CD8+ Tregs remain poorly characterized (Siegmund et al., 2009). Induced or naturally occurring CD8+ Tregs include the CD8+CD28+ population that suppress T-cell activation, not by cell-cell contact, but by interfering with antigen-presenting cell functions (Liu et al., 1998). Interestingly, the Treg marker, Foxp3, although not detectable in peripheral CD8+ cells, can be induced by in vitro stimulation with anti-CD3 and anti-CD28 or transforming growth factor-β (Siegmund et al., 2009). In mice infected with L. major, studies on DNA immunization have proposed an immunoregulatory role for CD8+ lymphocytes (Gurunathan et al., 2000); however, information on clinical leishmaniasis remains limited.

As knowledge of the interplay of cytokines within the lesional site is critical to understanding the immunopathology of PKDL, gene expression studies were undertaken on paired lesional biopsies from patients with PKDL. Our study corroborated reports of high lesional expression of both IFN-γ and IL-10 in Indian PKDL (Ansari et al., 2006); on an individual basis, all 12 patients showed a significant reduction in mRNA levels of both cytokines after treatment (Figure 2), pointing to their involvement in disease pathogenesis, with IFN-γ possibly being directed against skin-resident parasites, whereas IL-10 curtailed excessive IFN-γ-driven immune responses. Gene expression studies cannot establish the cellular source(s) of altered cytokines, more so for a pleiotropic cytokine such as IL-10, which is secreted by a plethora of immune cells (O'Garra and Vieira, 2007). The magnitude of the problem increases manifold if one considers the cellular profile of the lesional site in PKDL in which its often-dense inflammatory cell infiltrate can theoretically host several immune cells. The question of which cellular subset accounts for the increased lesional IL-10 expression in PKDL has remained unanswered, thus stoking our interest in inducible TRegs.

In chronic infections, TRegs are primary sources of IL-10 (Belkaid, 2007). As no information exists on TReg involvement in PKDL, we examined the role (if any) of TRegs in the localized immune milieu of PKDL. Expression of the TReg-exclusive transcription factor, Foxp3, studied both at the mRNA (Figure 3) and protein levels (Figure 4), yielded a finding that TRegs feature prominently in the inflammatory infiltrate (Figures 3 and 4a and b and Table 3); to our knowledge, this finding has not been reported in PKDL.
This accumulation of Foxp3+ Treg was not simply an accompaniment of infiltrating CD3+ lymphocytes, as lesions of a patient with erythema nodosum leprosum, a granulomatous reversal reaction of leprosy, showed barely detectable Foxp3+ cells in a CD3+ lymphocyte-rich lesional milieu (Supplementary Figure S1 online). Treatment curtailed accumulation of Treg as evident by the reduced expression of Foxp3 (Figure 4a and Table 3) affirming their contribution to lesional pathology, which however was not evident in the marginal decrease in mean lesional Foxp3 mRNA levels, suggesting a deficiency in translational mechanisms. Although natural Treg have a negligible role in VL with IL-10 expression limited to splenic T cells other than CD4+CD25+Foxp3+ Treg (Nylen et al., 2007), lesional Treg downregulate effector T-cell responses in cutaneous leishmaniasis caused by Leishmania braziliensis, (Campanelli et al., 2006). In addition, in localized cutaneous leishmaniasis caused by L. guanensis, treatment refractoriness was associated with high lesional Foxp3 expression (Bourreau et al., 2009a) and lesional Treg were shown to suppress IFN-γ production by antigen-stimulated peripheral CD4+CD25+ T lymphocytes (Bourreau et al., 2009b), suggesting that accumulating Treg by dictating impairment of local immunity contribute to disease persistence in leishmaniasis.

Collectively, we propose that in patients with PKDL, the circulating antigen-specific IL-10-producing CD4+ lymphocytes (Ganguly et al., 2008) are anergic and endowed with regulatory ability. These cells upon infiltration into the lesional site potentially sustain disease progression by producing IL-10 to counter IFN-γ-mediated responses against parasites. However, specialized immunohistology is necessary to pinpoint whether Treg are the cellular sources of IL-10 in PKDL, considering that Foxp3 is nuclear in location whereas IL-10 is cytoplasmic. Future investigations dealing with their functional profiles, specifically their interaction with their functional profiles, specifically their interaction with target immune cells within the localized PKDL milieu, are necessary for the development of immunomodulatory therapies against PKDL and by extension, the leishmaniasis.

MATERIALS AND METHODS

Study subjects

Patients diagnosed with PKDL (n = 21) or treated for VL (n = 5) were recruited from the School of Tropical Medicine, Kolkata and Medical College, Kolkata from 2006 to 2009. Diagnosis was based on clinical features, a past history of VL, rK39 positivity (Sundar et al., 2006), ELISA using crude LDA (Chatterjee et al., 1998), and presence of L. donovani bodies in skin smears. Patients with PKDL received either sodium stibogluconate (20 mg/kg/day intramuscular for 3 months) or miltefosine (100 mg/day orally for 2 months), whereas patients with VL had received amphotericin B (1 mg/kg/day intramuscular for 1 month). Remission of clinical features was a primary criterion of cure; samples were collected at presentation and 1 month after completion of treatment after obtaining informed consent from the patient or parent/guardian in case of minors. Non-steroidal anti-inflammatory drugs were included. The study received previous approval from the institutional human ethical committee and was conducted according to the Declaration of Helsinki principles.

Collection of samples

Heparinized blood (12 ml) was collected and skin biopsies (4 mm) were taken from lesional sites for (a) immunohistochemistry, (b) isolation of RNA stored in RNA Later (Ambion, Austin, TX) at −20°C, and (c) parasite transformation (placed in Schneider’s insect medium (Sigma-Aldrich, St Louis, MO) supplemented with 20% heat-inactivated fetal calf serum). 

Surface marker analysis

Whole blood (100 µl) was surface stained with fluorochrome-conjugated antibodies (CD4 and CD25, CD8 and CD28); lymphocytes were gated on a linear forward versus side scatter dot plot and fluorescence was measured (Ganguly et al., 2008) using CellQUEST Pro software (BD Biosciences, San Jose, CA).

Isolation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells were isolated as previously described (Ganguly et al., 2008) and resuspended in RPMI-1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), and 10% heat-inactivated fetal calf serum. Cell viability (>95%) was confirmed using Trypan blue exclusion.

Preparation of L. donovani antigen (LDA)

Antigen was prepared as previously described (Ganguly et al., 2008) and stored at −20°C until use.

Lymphoproliferation assay

Peripheral blood mononuclear cells were incubated with carboxyfluorescein diacetate succinimidyl ester (10 µM in serum-free medium) at 37°C in 5% carbon dioxide for 10 minutes; the reaction was terminated on ice for 5 minutes, and the cells were washed, resuspended in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum (1 × 10^6/ml well−1), and incubated with PHA (10 µg/ml) or LDA (5 µg/ml) for 6 days at 37°C in 5% carbon dioxide. They were then harvested, stained with fluorochrome-conjugated antibodies to CD3 and CD4 for 15 minutes, and acquired on a BD FACSCalibur flow cytometer using BD CellQuest Pro software (BD Biosciences). Lymphoblasts were specifically gated on the blast-characteristic population on a forward versus side scatter dot plot and further gated on an FL3 (CD3 fluorescence) versus FL2 (CD4 fluorescence) dot plot and a minimum of 10,000 CD3+ blasts were acquired. Proliferation indices were determined by curve-fitting analysis on a proliferation model of ModFit LT software (Verity Software House, Topsham, ME) in which the marker was set on the parent population in unstimulated cells.

Intracellular cytokine staining

Peripheral blood mononuclear cells, which were proliferating in response to PHA (10 µg/ml) or LDA (5 µg/ml), were either restimulated for the last 4 hours of treatment with leukocyte activation cocktail containing phorbol myristate acetate (10 ng/ml), ionomycin (1 µM), brefeldin A (10 µg/ml), or exposed to brefeldin A alone. After harvesting, PBMCs were surface-stained with anti-human CD3-PerCP, fixed and permeabilized, stained with fluorochrome-conjugated antibodies against IFN-γ and IL-10, acquired on a flow cytometer, and analyzed as previously described (Ganguly et al., 2008).
Reverse transcriptase-PCR analysis
Total RNA was isolated from skin lesions according to the manufacturer's instructions (RNAsqueez-PCR Kit, Ambion). Reverse transcriptase-PCR was performed on isolated RNA (100 ng) with the one-step reverse transcriptase-PCR kit (Qiagen, Hilden, Germany) using gene-specific primers for β-actin, IFN-γ, IL-10, and Foxp3 (Supplementary Table S1 online). For reverse transcription, samples were subjected to an initial incubation at 50°C for 30 minutes followed by an initial PCR activation (95°C for 15 minutes). The amplification cycle comprised 35 cycles of denaturing (94°C for 30 seconds), annealing for 30 seconds (varying temperatures for each primer set; Supplementary Table S1 online), and extension (72°C for 60 seconds), and a final extension at 72°C (10 minutes). Products were resolved on agarose gels (2% containing ethidium bromide (0.5μgml⁻¹) and quantified using Total lab Nonlinear Dynamic Image analysis software (Newcastle, UK), with the values being normalized to β-actin.

Immunohistochemistry
Immunohistochemical staining was performed as previously described (Little et al., 2001). In brief, slide-mounted sections were boiled in citrate buffer (pH 6.0; for CD4 staining) or Tris-EDTA buffer (pH 9.0; for Foxp3 staining) for antigen retrieval. Sections were incubated overnight at 4°C with a 1:75 dilution of anti-human Foxp3 (eBioscience, San Diego, CA) or for 1 hour at room temperature with a 1:100 dilution of anti-human CD3 (DAko, Glostrup, Denmark) and binding was detected by Super Sensitive Polymer-horseradish peroxidase Detection system (BioGenex, San Ramon, CA), following the manufacturer's instructions. After addition of the substrate 3-diaminobenzidine tetrahydrochloride and counterstaining with hematoxylin, the cells that were positive for DAB or hematoxylin were counted, with three representative fields per section. A positivity score for Foxp3/CD3 was allocated on the basis of a scoring index as follows: 0, negative; 1, <1% positive cells; 2, 1-5% positive cells; 3, 5-10% positive cells; 4, 10-20% positive cells; 5, 20-40% positive cells; and 6, >40% positive cells. Sections were evaluated independently by two observers and all samples were coded to minimize bias.

Statistical analysis
For parametric data (determined with the Shapiro-Wilk normality test), one-way analysis of variance was performed and the differences between groups were analyzed using Bonferroni's multiple comparison test. For non-parametric data, Kruskal-Wallis test was used to compare data for individual groups. All analyses were described (Little et al., 2001). Immunohistochemical staining of skin lesions with cutaneous leishmaniasis exhibit phenotypic and functional characteristics of natural regulatory T cells. J Infect Dis 193:1593-9


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**REFERENCES**


Boureau E, Ronet C, Danisacar E et al. (2009a) In Leishmania gypenesis infection, distal intronatal interleukin-10 and Foxp3 mRNA expression are associated with unresponsiveness to treatment. J Infect Dis 199:576-9


Ganguly S, Das NK, Panja M et al. (2008) Increased levels of interleukin-10 and IgG3 are hallmarks of Indian post-kala-azar dermal leishmaniasis. J Infect Dis 197:1762-71


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Vieira PL, Christensen JR, Minaee S et al. (2004) IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4+CD25+ regulatory T cells. J Immunol 172:5986-93