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IL-10- and TGF- β -Mediated Susceptibility in Kala-azar and Post-kala-azar Dermal Leishmaniasis: The Significance of Amphotericin B in the Control of *Leishmania donovani* Infection in India¹

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Visceral leishmaniasis (VL) or kala-azar is known to be associated with a mixed Th1-Th2 response, and effective host defense requires the induction of IFN- γ and IL-12. We address the role of the differential decline of IL-10 and TGF- β in response to sodium antimony gluconate (SAG) and amphotericin B (AmB), the therapeutic success of SAG and AmB in Indian VL, and the significance of IL-10 and TGF- β in the development and progression of post-kala-azar dermal leishmaniasis (PKDL). In the active disease, PBMC from VL patients showed suppressed Ag-specific lymphoproliferation, IFN- γ and IL-12 production, and elevation of IL-10 and TGF- β . Cure corresponded with an elevation in IFN- γ and IL-12 production and down-regulation of IL-10 and TGF- β . Both CD4⁺ and CD8⁺ T cells were involved in IFN- γ and IL-10 production. Interestingly, the retention and maintenance of residual IL-10 and TGF- β in some SAG-treated individuals and the elevation of IL-10 and TGF- β in PKDL, a sequel to kala-azar, probably reflects the role of these cytokines in reactivation of the disease in the form of PKDL. Contrastingly, AmB treatment of VL resulted in negligible TGF- β levels and absolute elimination of IL-10, reflecting the better therapeutic activity of AmB and its probable role in the recent decline in PKDL occurrences in India. Moreover, elucidation of immune responses in Indian PKDL patients revealed a spectral pattern of disease progression where disease severity could be correlated inversely with lymphoproliferation and directly with TGF- β , IL-10, and Ab production. In addition, the enhancement of CD4⁺CD25⁺ T cells in active VL, their decline at cure, and reactivation in PKDL suggest their probable immunosuppressive role in these disease forms. *The Journal of Immunology*, 2007, 179: 5592–5603.

Visceral leishmaniasis (VL),⁴ commonly known as kala-azar, is a disease caused by species of *Leishmania* that are transmitted by the bite of a female sandfly. Most cases occur on the Indian subcontinent and East Africa where *Leishmania donovani* is the causative agent. The parasite selectively infects and multiplies in the macrophages of the liver,

spleen, and bone marrow. Most human infections caused by the visceralizing strains of *Leishmania* run a subclinical and self-healing course (1, 2), but in some a fatal visceral disease evolves for which treatment is required (3). In most parts of the world where VL is endemic, therapy with pentavalent antimony is effective and the acquisition of initial resistance to infection results in the maintenance of immunity and apparent resistance to reinfection (4, 5). Relapse, although not common, does occur in some cases of antimony treatment (6–8). In Bihar, India, a large population of kala-azar patients (34–65%) fail to respond to antimony (6, 8). Since 1990 these patients are being treated with amphotericin B (AmB) (9, 10). The therapeutic success rate is high with no report of either relapse or resistance to AmB in otherwise immunocompetent patients (11). In India, post-kala-azar dermal leishmaniasis (PKDL), a complication of VL, occurs as a sequel to kala-azar in a small percentage of cases (5–10%) after 1–7 years of treatment (12, 13). This chronic skin condition of a wide range of clinical and histopathological manifestations (14) is probably a reservoir of parasites, especially during interepidemic periods of kala-azar (13).

Acquired resistance through antileishmanial chemotherapy with antimony is not direct but depends upon host T cells, cytokines (IFN- γ , IL-2, IL-12), and activated macrophages (4, 15–17). Although AmB has a direct action against *Leishmania* and may not require T cells for its initial activity (4, 18), its immunomodulatory activity is well recognized (19–21) and an intact immunity holds the key to the curative ability also for AmB (22, 23). Moreover, the induction of long-term success even with AmB is dependent upon

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⁴ Abbreviations used in this paper: VL, visceral leishmaniasis; AmB, amphotericin B; CMI, cell-mediated immunity; DTH, delayed-type hypersensitivity; LAg, leishmanial membrane antigen; PKDL, post-kala-azar dermal leishmaniasis; SAG, sodium antimony gluconate; SI, stimulation index; Treg, regulatory T cell; wSLA, whole cell soluble leishmanial Ag.

an effective T cell immunity (24). Despite the acquired resistance, all successfully treated phenotypically normal individuals presumably harbor residual parasites. It is thought that maintenance of this low-level infection provides an ongoing immunization against future infection. However, these persistent parasites often give rise to disease reactivation under conditions of immunosuppression as observed in HIV-associated VL (25, 26) and PKDL after the cure of kala-azar (13, 27) by a mechanism that is still not clear.

Clinical studies of visceral infection have shown that human VL is a complex immunological disease that cannot be interpreted as simple Th1 or Th2-mediated processes, which are characteristics of the murine model of leishmaniasis. Because both IFN- γ and IL-10 are coexpressed, the outcome of infection is likely determined by the balance between the contrasting effects of protective (IL-2, IFN- γ) and nonprotective (IL-4, IL-10) cytokines during the initial phase of infection (15, 28–30). This is reflected in patients unsuccessfully treated with antimony who demonstrate specific elevation in humoral immune responses and enhanced IL-4 over IFN- γ (31, 32). Again, PKDL develops despite cell-mediated immune responses in most patients (33, 34). IL-10 mediated immune suppression probably determines disease reactivation despite IFN- γ production (30, 35). However, the factors that skew the immune response toward Th1 or Th2 dominance are partially understood. It is believed that direct interaction between parasite Ags and cells of the host immunity participate to shape the subsequent pathogenic or protective immune responses. Moreover, depending upon the acquired antileishmanial response through the drug administered, specific requirements for sensitized CD4⁺ and CD8⁺ T cells and the activating cytokines may vary. Further, successful antileishmanial therapies may reflect a distinct host defense mechanism in the recurrence of the disease in the form of PKDL. Prior clinical observations have established that the posttreatment protective host immune responses in VL are primarily T cell mediated. To learn more about these responses, which bear directly on the requirements of therapeutic success especially in antimony resistance and, conversely, on the reactivation and development of varied manifestations of PKDL in treated kala-azar, we investigated the outcome after chemotherapy in *L. donovani*-infected patients, both responsive and unresponsive to antimony. The spectrum of acquired antileishmanial mechanisms to *Leishmania* Ags was extrapolated to the possible mechanisms of disease recurrence in the form of PKDL.

Materials and Methods

Study subjects

Patients with active VL and PKDL, admitted between 2001 and 2007 to the School of Tropical Medicine (Kolkata, India) were mainly from Bihar and West Bengal, the endemic regions of eastern India. VL was diagnosed by the usual clinical presentations such as prolonged fever, hepatosplenomegaly, and pancytopenia and confirmed by a direct agglutination test and the demonstration of *Leishmania* parasites in the splenic or bone marrow aspirate. Two groups of VL patients were distinguished based on their mode of treatment. Group 1 consisted of 10 sodium antimony gluconate (SAG)-treated patients who were treated with SAG by i.v. injections at 20 mg/kg of body weight for 20 consecutive days. These patients were considered SAG responsive on the basis that none of them showed relapse with VL within 6 mo of posttreatment. Some SAG-treated patients did return to the School of Tropical Medicine with a relapse within 6 mo of treatment and were therefore excluded from this study. Group 2 was composed of 10 AmB-treated patients who had either come to the School of Tropical Medicine from an endemic area already known for SAG-resistant infections or who had been taking an insufficient dosage of SAG with an unsuccessful cure. These patients were treated with AmB by i.v. drip in dextrose solution on alternate days. Total dosage of the drug was 20 mg/kg of body weight; treatment started with a low dose (0.1 mg), was gradually increased up to 1 mg/kg/day, and continued until the completion of the full dose. Longitudinal heparinized blood samples from the patients of these two groups

were collected at three time points: before the initiation of treatment with SAG or AmB, at mid-treatment, and immediately after the completion of treatment. Six patients treated with SAG (one at 4 mo, three at 6 mo, one at 4 years, and one at 6 years posttreatment) and four patients treated with AmB (two at 3 mo, one at 4 mo, and one at 4 years) who suffered no relapse or PKDL could be followed up after successful chemotherapy. Longitudinal blood samples from five AmB-treated patients were collected separately for FACS analysis. PKDL patients were diagnosed by the clinical presentation of dermal lesions (macular, papular, nodular, erythematous plaque, or all their possible combinations), their distribution and history of spread (starting from the face and gradually spreading to the trunk and limbs), and the relation to a previous history of VL. All of the PKDL patients except one had a history of VL and were successfully treated with SAG for kala-azar. Blood samples from these patients were collected only before the initiation of treatment for PKDL. Clinical parameters of the patients are summarized in Tables I and III. Blood samples from five healthy donors of the Indian Institute of Chemical Biology (Kolkata, India) were included in this study. All of these healthy individuals were from nonendemic areas of VL and were negative for the recombinant K39 strip test. Moreover, the PBMC of these individuals did not show any significant lymphoproliferation in response to a *L. donovani* promastigote membrane Ag (LAG) (see *Results* and Fig. 1A). The present study was approved by the Ethical Committee on Human Subjects at the Indian Institute of Chemical Biology. Consents were obtained from all patients and donors for blood sampling.

Parasites

L. donovani (MHOM/IN/83/AG83) originally isolated from an Indian kala-azar patient (38) was maintained in BALB/c mice and hamsters. The amastigotes, isolated aseptically from small pieces (~100 mg) of infected animal spleens, were allowed to convert to promastigotes at 22°C in medium 199 (M199) (pH 7.4) supplemented with 20% heat-inactivated FBS, 2 mM glutamine, 25 mM HEPES, 100 U/ml penicillin G-sodium, and 100 μ g/ml streptomycin sulfate (all from Sigma-Aldrich). Parasites were subcultured at an average density of 2×10^6 cells/ml for 72 h in the same medium at 22°C (39).

Preparation of *Leishmania* membrane Ags

LAG were prepared as described (40). In a typical preparation, 2×10^{10} cells (1-g cell pellet) from stationary phase culture were suspended in 5 mM cold Tris-HCl buffer (pH 7.6). The suspension was vortexed six times for 2 min each with a 10-min interval on ice. The parasite suspension was then centrifuged at $2310 \times g$ for 10 min. The crude ghost membrane pellet thus obtained was resuspended in 10 ml of the same buffer and sonicated at 4°C three times for 1 min in an ultrasonicator. The suspension was finally centrifuged at $5190 \times g$ for 30 min. The supernatant containing the membrane proteins was harvested and stored at -70°C . The amount of protein obtained from a 1-g cell pellet, as assayed by the method of Lowry et al. (41), was ~14 mg.

Proliferation assay

Human PBMC were isolated from the heparinized blood samples of VL and PKDL patients as well as healthy controls by density sedimentation (Histopaque-1077; Sigma-Aldrich), washed, resuspended in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μ g/ml). The plasma was separated from the upper layer of the gradient and stored at -20°C until use. Freshly isolated PBMC (1×10^6 /ml) were cultured in triplicate in 96-well flat-bottom tissue culture plates (Nunc) and stimulated with LAG (12.5 μ g/ml), whole cell soluble leishmanial Ag (wcSLA) (12.5 μ g/ml), or cell lysate (12.5 μ g/ml) wherever applicable for 5 days at 37°C in 95% humidified air with 5% CO₂. The cells were pulsed with 1 μ Ci/well [³H]thymidine (sp. act. 5 Ci/mM; Amersham Biosciences) during the last 18–24 h. Radioactive incorporation was measured by a liquid scintillation counter and expressed in cpm (cpm of stimulated cultures – cpm of only medium) for each individual sample. The mean stimulation index (SI) (cpm of stimulated cultures/cpm of unstimulated cultures) of each group is indicated. Cell proliferation was considered significant when cpm and SI were ≥ 1500 and ≥ 2 , respectively.

Cytokine analysis

For cytokine analysis, PBMC of VL patients as well as healthy individuals were cultured in the presence of LAG for 4 days as described above. To investigate the capacity of AmB and SAG to modulate cytokine production, PBMC (1×10^6 /ml) from healthy controls were incubated with various concentrations of AmB (0, 0.031, 0.062, 0.125,

Table I. Clinical characteristics of VL patients

Clinical Characteristics	SAG Treated (n = 10)	AmB Treated (n = 10)
Sex		
Male	7	5
Female	3	5
Age (years), mean \pm SD	21.30 \pm 11.80	17.10 \pm 12.73
Duration of illness (mo), mean \pm SD	5.75 \pm 1.76	5.10 \pm 3.38
Body weight (kg), mean \pm SD		
Pretreatment	39.00 \pm 12.66	33.10 \pm 11.41
Posttreatment	40.50 \pm 12.38	34.20 \pm 10.90
Karnofski score, ^a mean \pm SD		
Pretreatment	73.50 \pm 3.15	71.50 \pm 2.41
Posttreatment	88.50 \pm 5.16	85.00 \pm 4.08
Spleen size (cm), mean \pm SD		
Pretreatment	7.57 \pm 3.74	7.70 \pm 4.82
Posttreatment	1.95 \pm 2.10	3.00 \pm 1.83
Splenic score, ^b mean \pm SD		
Pretreatment	3.60(+) \pm 1.26	4.10(+) \pm 1.49
Posttreatment	Nil	Nil
White blood cell count (cells/ μ l), mean \pm SD		
Pretreatment	2600 \pm 560	2400 \pm 432
Posttreatment	5100 \pm 1221	5200 \pm 1150
Hemoglobin (g/dl), mean \pm SD		
Pretreatment	6.75 \pm 1.43	7.50 \pm 2.29
Posttreatment	7.96 \pm 0.98	9.25 \pm 1.40

^a Ref. 36.^b Ref. 37.

0.25 μ g/ml) or SAG (0, 1, 10, 50, 100 μ g/ml) with or without LPS (1 μ g/ml) or PHA (2.5 μ g/ml) for 48 h at 37°C in 95% humidified air with 5% CO₂. Cell-free supernatants were collected and stored at -70°C until use. IFN- γ (DuoSet ELISA development kit; R&D Systems), IL-12 (p40/p70), and IL-10 (BD OptEIA ELISA kit; BD Biosciences) were measured according to the manufacturers' instructions. For total TGF- β measurement, culture supernatants were acidified to activate the latent TGF- β by adding 1 N HCl for 10 min and neutralized with 1.2 N NaOH. TGF- β was captured with monoclonal anti-TGF- β 1, MAB240, and detected with biotinylated polyclonal anti-TGF- β 1, BAF240 (R&D Systems). The standard curve was prepared using rTGF- β 1 (R&D Systems) suspended in culture medium. The color reaction was performed using avidin-HRP and tetramethylbenzidine and read at OD₄₅₀. IL-4 was measured by DuoSet (R&D Systems). For this, cells were stimulated with LAg for 4 days and then pulsed with 50 ng/ml PMA and 1 μ M ionomycin for 24 h before harvesting the supernatant (42).

LAg-specific IgG and IgA Ab titers

LAg-specific Ab was determined by ELISA based on a previously described assay (31). Briefly, 96-well polystyrene microtiter plates (Tarsos) were coated with LAg at 2 μ g/well/100 μ l of 0.02 M phosphate buffer (pH 7.2) overnight at 4°C. The plates were washed as described previously and blocked with 1% BSA for 3 h at room temperature. After subsequent washes, PKDL sera, serially diluted in PBS containing 1% BSA, were added to the wells and incubated overnight at 4°C. After three washes with PBS supplemented with 0.05% Tween 20, bound Abs were detected with polyclonal goat antihuman IgG or IgA conjugated to HRP (Bangalore Genei, and Sigma-Aldrich, respectively) applied at 1/5000 in PBS. After 3 h of incubation at room temperature, the plates were washed and an *o*-phenylenediamine dihydrochloride (Sigma-Aldrich) substrate was added. The plates were read in a spectrophotometer at OD₄₅₀. Each serum sample was titrated at the dilution required to reach half-maximal absorbance (A₄₅₀ = 1.0).

FACS analysis for surface phenotype and intracellular cytokines

For intracellular detection of IFN- γ and IL-10 in CD4⁺ or CD8⁺ cells, fresh PBMC (10⁶/ml) from active or cured VL patients were stimulated

with LAg (12.5 μ g/ml) in a 24-well flat-bottom plate for 72 h at 37°C. Brefeldin A (10 μ g/ml; ICN Pharmaceuticals) was added to the cultures during the last 3 h to induce the intracellular accumulation of newly synthesized cytokines. After 72 h, the cells were harvested, washed with FACS buffer (0.02 M PBS, 1% FBS, and 0.01% sodium azide), and stained with FITC-conjugated anti-CD4 or anti-CD8 mAb (BD Pharmingen) for 30 min at 4°C. Cells were then washed with FACS buffer, fixed and permeabilized with 1 \times BD Perm2 reagent for 10 min at 4°C, and washed again with 0.1% saponin in FACS buffer. After permeabilization, intracellular staining was performed with PE-conjugated Abs specific for IFN- γ or IL-10 (BD Pharmingen) for 45 min at 4°C. Respective isotype controls were used for all Abs. Cells were then washed and refixed with 1% paraformaldehyde. Analysis was performed using a FACSCalibur cytometer with CellQuest software on at least 10,000 events. Intracellular cytokine expression is represented by the frequencies of positive cells in gated cells. Analysis gates were set for lymphocytes using forward and side scatter properties, and the frequencies of cytokine-producing cells were acquired by phenotype gating using anti-CD4 and anti-CD8. For ex vivo phenotyping of the CD4⁺CD25⁺ T cells, 1 \times 10⁶ PBMC were isolated from a blood sample as described above, washed with FACS buffer, and stained with anti CD4-FITC and anti CD25-PE for 30 min at 4°C. Cells were washed again and fixed with 2% paraformaldehyde and analyzed for the CD4⁺CD25⁺ double-positive cells.

Cocultures and proliferation assays

To verify the regulatory function of CD4⁺CD25⁺ T cells, CD4⁺ T cells were isolated from PBMC by negative selection using magnetic beads (Miltenyi Biotec). CD4⁺CD25⁺ T cells were isolated from the pure, untouched CD4⁺ T cells using CD25 microbeads (Miltenyi Biotec). Purified CD4⁺CD25⁺ T cells from infected VL patients or nonendemic healthy controls were cultured with PBMC (1 \times 10⁵/well) from healthy donors at a ratio of 1:10 in 96-well U-bottom plates in the presence of PHA (1 μ g/ml) at 37°C and 5% CO₂. On day 4, 0.5 μ Ci/well [³H]thymidine was added for the final 16 h of culture performed in triplicate. Incorporation of [³H]thymidine was measured using a liquid scintillation counter. The SI was calculated based on the PHA-induced cell proliferation in the presence

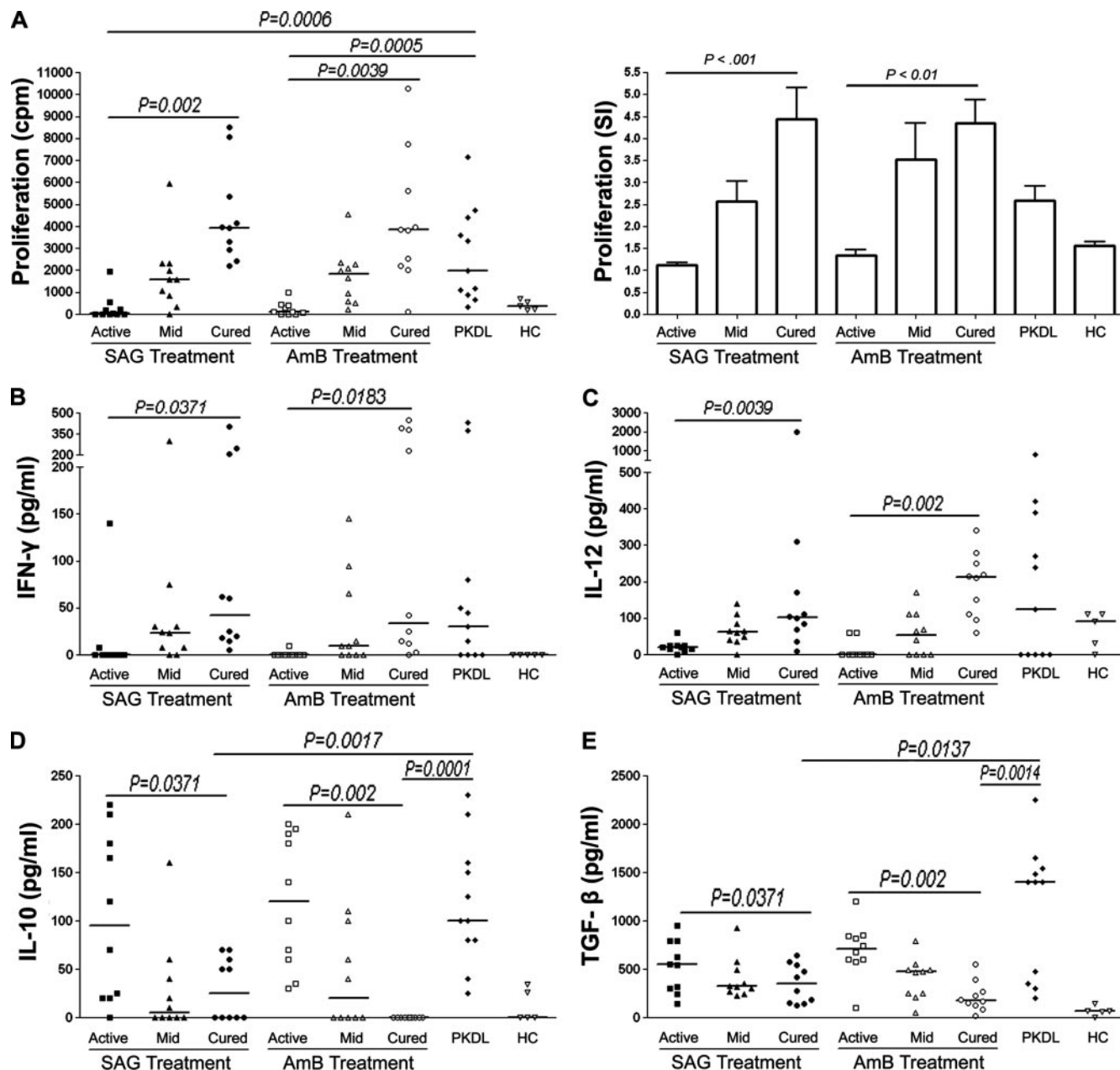


FIGURE 1. *Leishmania*-specific cell-mediated immune response in VL, PKDL, and healthy controls. Longitudinal samples of PBMC were isolated from SAG-treated ($n = 10$) and AmB-treated ($n = 10$) VL patients during the course of chemotherapy. PBMC were also isolated from PKDL patients before the onset of treatment. **A**, Cells were stimulated with 12.5 μ g/ml LAg for 5 days and lymphoproliferation was measured by [3 H]thymidine incorporation for the last 18–24 h of culture. **B–E**, Cytokines were measured by ELISA from the supernatants of similar cultures for 4 days. Data for individual patients are presented in cpm, whereas stimulation indices are presented as mean \pm SE. **B**, IFN- γ . **C**, IL-12. **D**, IL-10. **E**, TGF- β . Each symbol represents the proliferation (in cpm) or cytokine production for one patient. Thick horizontal lines represent the median values of each group. Values for p were calculated using Wilcoxon matched pairs signed rank test for paired samples and the Mann-Whitney U test for unpaired samples; $p < 0.05$ were considered significant.

or absence of CD4 $^+$ CD25 $^+$ T cells divided by the proliferation of unstimulated cells.

Statistical analysis

Statistical analysis was performed using the nonparametric Wilcoxon matched pairs signed rank test for paired samples and the Mann-Whitney U test for unpaired samples. In cases where the data were expressed as mean \pm SE, statistical analysis was performed using ordinary ANOVA followed by Tukey's multiple comparison test (GraphPad Prism). All values of $p < 0.05$ were considered significantly different.

Results

Chemotherapy with both SAG and AmB restores LAg-specific lymphocyte proliferation in VL

Although wcSLA or whole cell lysate/sonicate from the parasites have usually been used by several authors for cell-mediated immunity (CMI) studies in humans, LAg was chosen as the candidate Ag for all of the in vitro experiments in this study. A comparative analysis of LAg-stimulated vs wcSLA-stimulated and total lysate-stimulated (prepared according to published

Table II. *IL-10 and TGF-β profile of VL patients at posttreatment follow-up*

Patient	Treatment Group	Duration of Posttreatment Follow-Up	IL-10 (pg/ml)	TGF-β (pg/ml)
1	SAG treatment	4 mo	32	57.6
2	SAG treatment	6 mo	0	4.5
3	SAG treatment	6 mo	0	3
4	SAG treatment	6 mo	0	70
5	SAG treatment	4 years	65	210
6	SAG treatment	6 years	0	0
7	AmB treatment	3 mo	0	9.8
8	AmB treatment	4 mo	0	45
9	AmB treatment	4 mo	0	19.6
10	AmB treatment	4 years	0	0

methods; Refs. 43 and 44, respectively) in vitro lymphoproliferation showed that, of the three Ags, LAg induced the least nonspecific response in healthy controls and active VL patients and the highest Ag-specific response in cured kala-azar patients (data not shown). Earlier, we reported the better diagnostic ability of LAg (extracted from the promastigote membrane) over whole cell lysate in the diagnosis of VL (45) and described the ability of LAg for differential serodiagnosis of VL and PKDL (46). Moreover, we also investigated the modulation of humoral immune responses in human VL in the course of SAG and AmB treatment (31) using LAg.

Because *L. donovani* infection is known to induce severe cell-mediated immune suppression in VL patients, which is rapidly reversible after successful antimony therapy, we wanted to investigate whether treatment with AmB is effective for a comparable up-regulation of CMI. For this, PBMC of VL patients were collected before, during, and after chemotherapy from both SAG-treated and AmB-treated patients. Severe LAg-specific suppression of CMI at active disease was observed from the lymphoproliferation assays (Fig. 1A; both cpm and SI values are presented). With the onset of chemotherapy, PBMC of both groups of patients showed steady recovery of the immune suppression, and the proliferation was significantly enhanced immediately after the cure. This observation suggests that besides microbicidal activity, both SAG and AmB may have immunomodulatory effect on the T cells. PKDL patients, in contrast, showed variable levels of LAg-specific CMI at active disease (Fig. 1A). The median lymphoproliferation in PKDL patients is significantly higher than that in the active VL of both the SAG-treated and AmB-treated groups

($p = 0.0006$ and 0.0005 , respectively). However, the healthy controls showed negligible lymphoproliferation in response to LAg.

Lag-specific cytokine analyses reveal type 2 dominance at active VL and an absolute type 1 dominance at the cure with AmB treatment

VL is generally known to present a mixed Th1/Th2 response. But critical analysis of the literature suggests that this understanding has been established from studies that did not include *Leishmania* Ag-specific cytokine responses (29, 30, 47–49). Investigations of leishmanial Ag-specific immune modulation in VL showed Th2 bias at active infection and Th1 bias at the cure. We attempted to assess the LAg-specific modulation of the type 1/type 2 response in the course of chemotherapy in both SAG-treated and AmB-treated patients. PBMC collected before, during and after chemotherapy were cultured in vitro in the presence of LAg for 4 days. Levels of type 1 cytokines, IFN-γ and IL-12, and type 2 cytokines, IL-10 and IL-4, secreted in the supernatant were measured by ELISA. We found that in both groups, except for a single SAG-responsive patient, there was almost no IFN-γ production at the active stage of disease (Fig. 1B). With the onset of treatment there was a gradual increase that was significantly elevated at cure. In vitro production of IL-12 was found to have a similar longitudinal pattern (Fig. 1C) with a negligible level at the active stage and a significant elevation at the cure. Conversely, in vitro production of IL-10 was found to be elevated at active disease in most patients (Fig. 1D), and the levels declined significantly after chemotherapy with SAG and AmB ($p = 0.0371$ and 0.002 respectively). IL-4 could not be detected in the supernatant even after 24 h of stimulation with PMA and ionomycin after 4 days of stimulation with LAg (data not shown). No IFN-γ and minimal levels of IL-12 and IL-10 were produced by some healthy controls.

Differential decline of the level of in vitro IL-10 and TGF-β production after chemotherapy with SAG vs AmB

It is known that along with IL-10, TGF-β, an inhibitory cytokine elevated both in experimental VL and cutaneous leishmaniasis, plays an important role in the susceptibility of *Leishmania* parasites by inhibiting IFN-γ production (50–52). To assess its role during disease, we measured the LAg-specific production of total TGF-β in the culture supernatants of PBMC of VL and PKDL patients in comparison to healthy controls, in addition to IL-10. The median levels of IL-10 and TGF-β in antimony-responsive and AmB-treated VL patients at active disease were elevated to comparable levels, respectively, with some variations among the

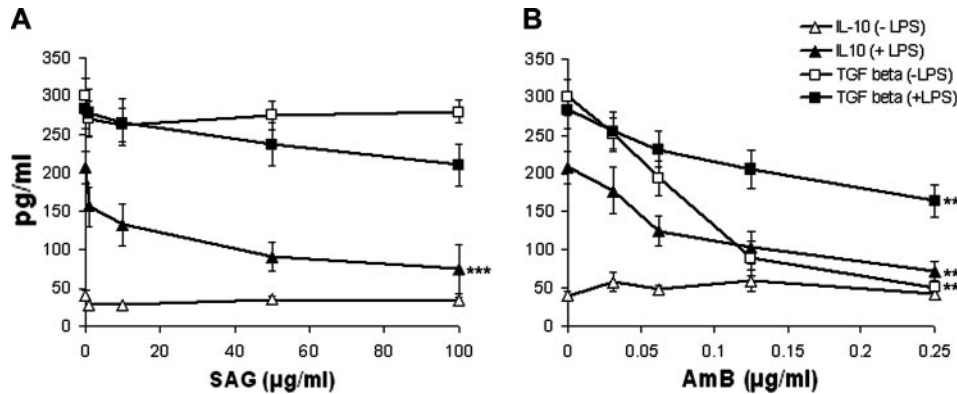


FIGURE 2. Ability of SAG and AmB to modulate TGF-β and IL-10 production of human PBMC. PBMC (1×10^6 /ml) from healthy controls were incubated with various concentrations of SAG (A) or AmB (B) with or without LPS ($1 \mu\text{g/ml}$) for 48 h at 37°C in 95% humidified air with 5% CO_2 . Cytokines were measured by ELISA from supernatants. Each symbol represents mean cytokine level (\pm SEM) at each dose of the drugs ($n = 5$). Values for p were calculated by ordinary ANOVA followed by Tukey's multiple comparison test; $p < 0.05$ were considered significant. **, $p < 0.01$; ***, $p < 0.001$.

patients (Fig. 1, *D* and *E*). However, there was an interesting difference in the longitudinal patterns of IL-10 and TGF- β production in response to SAG and AmB in the two groups. Although total elimination of TGF- β due to either of the therapies was not observed, the decline of TGF- β production in AmB-treated patients was reduced more effectively than in SAG-treated individuals ($p = 0.002$) (Fig. 1*E*). Again, despite significant decline, 50% of the SAG-treated patients retained considerable levels of IL-10 and TGF- β after cure. In contrast, AmB-treated patients demonstrated a steady decline of IL-10 in the course of treatment that led to the absolute elimination of IL-10 upon completion of treatment.

Ability to retain IL-10 and TGF- β by some SAG-treated patients at posttreatment follow-up

A considerable number of the SAG-treated, in contrast to AmB-treated, patients retained IL-10 and TGF- β . It is noteworthy that PKDL patients who were successfully treated with SAG for past VL maintained elevated levels of IL-10 and TGF- β (Fig. 1, *D* and *E*). Therefore, it was important to observe whether the ability of residual IL-10 and TGF- β production by SAG-treated patients is maintained over time after posttreatment. We were able to follow up and collect blood samples from six SAG-treated and four AmB-treated patients at different posttreatment durations (Table II). It was interesting to observe that the ability to produce IL-10 and TGF- β was maintained in two of the SAG-treated patients at different periods of follow-up. Because these patients never faced a relapse of VL within this period, it is tempting to assume that this ability to maintain the production of IL-10 and TGF- β could possibly lead to the reactivation of the disease in the form of PKDL. Contrastingly, at follow-up none of the four AmB-treated patients could produce these cytokines in significantly higher amounts than healthy individuals.

Ability of SAG and AmB to modulate cytokine production of human PBMC

It is known that both SAG and AmB exert direct leishmanicidal activity, and the restoration of IL-12 and IFN- γ response could be a consequence of the reduced parasite burden after treatment. However, differential reduction of IL-10 and TGF- β in patients successfully treated with SAG and AmB suggested an immunoregulatory role of these drugs with differential effects. To investigate the immunomodulatory capacity of SAG and AmB, LPS- and PHA-stimulated cultures of PBMC from healthy individuals were treated with various concentrations of SAG and AmB (Fig. 2). The highest concentrations of drugs used were 90% effective doses for the suppression of parasite infection in macrophages and nontoxic to cells (Ref. 53; D. Banerjee and N. Ali, unpublished observations). It was found that both SAG and AmB were able to down-regulate the enhanced production of IL-10 stimulated by LPS in a dose-dependent manner (Fig. 2). Forty-eight hours of culture with unstimulated PBMC led to the production of some amount of TGF- β that could not be enhanced further in the presence of LPS (1 μ g/ml). Nevertheless, in contrast to the minimal ability of SAG (Fig. 2*A*), AmB was instrumental in down-regulating this inherent production of TGF- β in the unstimulated cells. This ability of AmB, although reduced, was still significant in LPS-stimulated PBMC (Fig. 2*B*). However, neither of these two drugs was effective in significant augmentation of IFN- γ or IL-12 production from the PBMC of healthy individuals stimulated with PHA or LPS, respectively (data not shown).

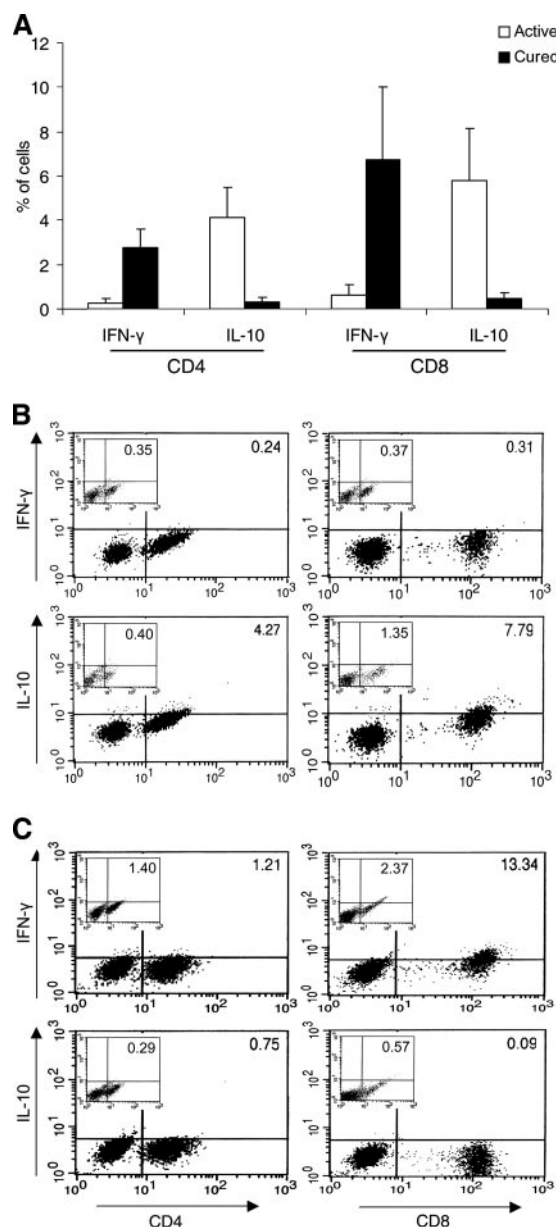


FIGURE 3. Percentage of LAg-stimulated CD4⁺ and CD8⁺ T cells of active and AmB-treated VL patients producing IFN- γ and IL-10. Isolated PBMC were stimulated with LAg (12.5 μ g/ml) for 72 h. Surface phenotyping and intracellular staining were performed as stated in *Materials and Methods* and cells were examined by flow cytometry. T cells were identified by first gating on lymphocytes by light scatter properties and the percentages of double-positive CD4⁺ and CD8⁺ T cells were measured on the gated populations. *A*, Mean percentage of CD4⁺ and CD8⁺ T cells producing IFN- γ and IL-10 (\pm SEM) after culturing with LAg in each group of active ($n = 3$) and cured ($n = 3$) patients. *B*, Data showing one representative of three active VL patients. *C*, Data showing one representative of three cured VL patients. The percentage of IFN- γ and IL-10 producing cells after culturing with LAg is expressed in the upper right portion of each panel. Unstimulated cells are shown as insets.

Cytokine production from the PBMC of VL patients involves both CD4⁺ and CD8⁺ T cells

Although many reports in the literature emphasize the role of Th2 and Th1 cytokines in the susceptibility and cure of human VL respectively, very few have shown the definite source of these cytokines. In a previous report, flow cytometric studies of PBMC from cured VL patients of Kenyan origin showed that CD4⁺ T

Table III. *Clinical and immunological spectrum among PKDL patients*

Patients	Age (years)	Duration (mo)	VL History	Type of Dermal Lesion	Skin LDB Count ^a	IgG Titer	IgA Titer	Proliferation (cpm)	TGF- β (pg/ml)	IL-10 (pg/ml)
1	34	45	Yes (15 years)	Mild macular lesions spread over the body, tiny papules on the chin		2000	35	7153.0	205.0	80
2	14	12	Yes (2 years)	Mild macular lesions		1000	25	4422.6	350.0	100
3	12	7	No	Small macular patches on whole body		1700	24	3590.4	300.0	80
4	40	6.5	Yes (6 years)	Mild papular lesions mainly on face	1+	10000	70	1090.5	480.0	100
5	17	24	Yes (8 years)	Hypopigmented and erythematous maculopapular lesions on the extremities of trunk, and papulonodular lesions on chin, above upper lip, both sides and base of nose, angle of lips	1+ ^b	2600	0	1991.1	1485.0	25
6	33	9	Yes (5 years)	Papulonodular lesions mainly on face	2+	8000	50	4739.7	1400.0	40
7	42	15	Yes (10 years)	Macular patches all over the body, erythematous papules in different parts of the trunk, especially in the axillary regions and neck, papulonodular lesions all over the face, inside the lips	2+	20600	75	3351.0	1400.0	150
8	35	12	Yes (2 years)	Papular and nodular lesions on the nose, lips, chin and ear, mild macular lesion on fore arms	2+	10000	55	1170.6	1400.0	125
9	50	30	Yes (12 years)	Macular, nodular, plaque (almost all types of lesions) on both lower limbs below knee	2+	21000	56	343.3	1650.0	160
10	35	14	Yes (6 years)	Macular, papular, nodular, plaque all over the body, palpable bilateral epitrochlear lymph nodes	3+ ^c	32000	86	903.6	1540.0	230
11	45	15	Yes (5 years)	Severe nodular lesions forming erythematous plaques on face, limbs, and all over the body	3+	17000	60	650.2	2255.0	210

^a Ref. 37; LDB, Leishman-Donovan body.^b Leishman-Donovan bodies found in right epitrochlear lymph node.^c Leishman-Donovan bodies found in both epitrochlear lymph nodes. ("trochlea" is used with respect to elbow joints. The epitrochlear lymph node is positioned at the medial aspect of the anterior surface of the elbow joint just above the head of the ulna. The epitrochlear gland is generally palpable in VL patients).

cells were the main source of these cytokines with the involvement of a very low percentage of CD8⁺ T cells (54). In this study we investigated the percentage of the population of CD4⁺ and CD8⁺ T cells involved in the leishmanial Ag-specific type 1/type 2 cytokine production in both active and cured VL patients treated with AmB. Blood samples from the SAG-treated group could not be collected for FACS analysis because by the time we started these studies, treatment with SAG for VL patients was practically stopped due to increasing occurrences of SAG-resistance. PBMC isolated from these patients were cultured in vitro in the presence of LAg for 72 h, stained with surface markers of anti-CD4 or anti-CD8 and intracellular markers of anti-IFN- γ and anti-IL-10, and then subjected to FACS analysis. Fig. 3A shows the mean percentage of CD4 and CD8 cells from active and cured VL patients producing IFN- γ or IL-10. Fig. 3, B and C are representative of one active and one cured VL patient, respectively. It was observed that both CD4⁺ and CD8⁺ T cells were involved in IL-10 production during the active disease and IFN- γ production at the cure.

Immune response in PKDL patients is definitely correlated with the manifestation of the dermal lesions and reveals a clear spectral pattern among the patients

Disease manifestation in PKDL patients involves different types of dermal lesions indicating different levels of disease aggravation (14, 55, 56). In India, PKDL appears either with hypopigmented macules that may coalesce and spread over the body or in the form of erythematous eruptions that lead to the formation of papules, nodules, and plaques or combinations thereof with progression of the disease (12, 14). A proper understanding of the host immunity elicited in response to chemotherapy in kala-azar will not only help to elucidate the exact mechanisms underlying the development of PKDL, but also identify specific immunological components that might also help to characterize the diverse manifestations and progression of PKDL. Classification based on cellular immune correlates corresponding to the diversity and severity of the disease is well defined in leprosy (57). However, there are few investigations

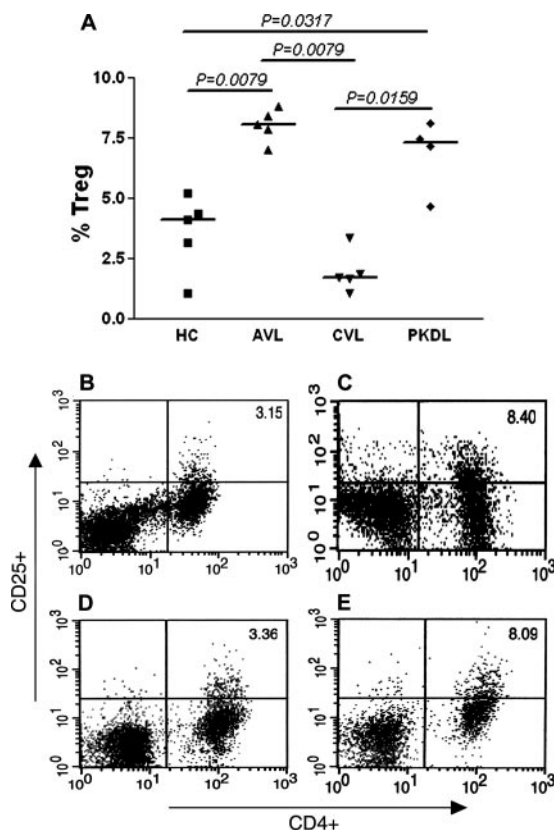


FIGURE 4. Ex vivo percentage of $CD4^+CD25^+$ T cells in PBMC of healthy volunteers (HC), active VL (AVL), cured VL (CVL), and PKDL. PBMC (1×10^6) were freshly isolated from blood samples, stained with anti-CD4-FITC and anti-CD25-PE mAbs, and assessed on the gated lymphocyte fraction by a FACSCalibur flow cytometer. **A**, Percentage of Treg cells in HC ($n = 5$), AVL ($n = 5$), CVL ($n = 5$) and PKDL ($n = 4$). Each symbol represents the percentage of Treg cells for one individual. Thick horizontal lines represent the median values of each group. Values for p were calculated using Mann-Whitney U test; $p < 0.05$ were considered significant. **B–E**, Representative flow cytometry diagrams of $CD4^+CD25^+$ T cell percentages of one individual each from HC (**B**), AVL (**C**), CVL (**D**) and PKDL (**E**).

that correlate the immunological status of the patients and the varied manifestations of the disease, and these demonstrate contrasting observations (33, 58). We collected blood samples from PKDL patients with different degrees of severity at the active stage before the onset of antimony therapy. Table III presents the characteristics of the PKDL patients in the increasing order of disease manifestation. PBMC of these patients were cultured in the presence of LAg as conducted for VL, and cytokine production was measured. Unlike VL, immune suppression in PKDL patients is not a common phenomenon as observed through the proliferation assays by us and others (Fig. 1A and 33). Moreover, median levels of IFN- γ , IL-12, IL-10, and TGF- β demonstrate the elevation of all the four cytokines during disease. The stimulation of both type 1 and type 2 cytokines in PKDL is in contrast to active VL where LAg-specific IL-10 and TGF- β were elevated and levels of IFN- γ and IL-12 were negligible. In contrast, when compared with cured VL, PBMC of PKDL patients produced significantly higher levels of IL-10 ($p = 0.0017$ and 0.0001) and TGF- β ($p = 0.0137$ and 0.0014) than both the SAG-treated and AmB-treated groups, respectively.

Variation of immune responses within the group of PKDL patients led to interesting observations. The CMI response of PKDL patients in terms of LAg-stimulated lymphoproliferation showed

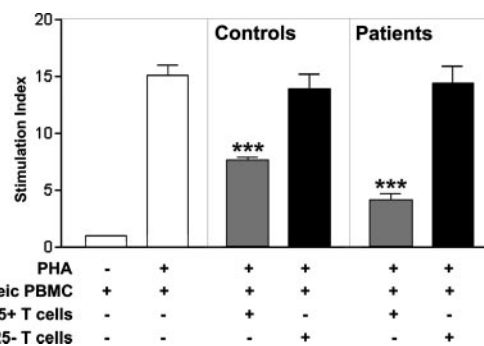


FIGURE 5. Immunosuppressive function of $CD4^+CD25^+$ T cells. Magnetic bead-sorted $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells purified from PBMC from VL patients ($n = 5$) and healthy controls ($n = 5$) were tested for their ability to suppress the proliferation of allogeneic PBMC. Allogeneic PBMC (1×10^5 cells/well) were cultured with medium only, PHA, PHA plus $CD4^+CD25^+$, or $CD4^+CD25^-$ T cells (1×10^4 cells/well) from VL patients and healthy controls. Proliferation was determined after 4 days of culture by [3H] thymidine incorporation for the last 16 h. Results are expressed as the mean \pm SEM of SI; ***, $p < 0.001$, compared with proliferation of allogeneic T cells.

an inverse correlation with the increase in the disease (Table III). Conversely, a comparison of the production of IL-10 and TGF- β and the patients with different degrees of disease manifestation demonstrated a direct correlation with the intensity of the dermal lesions (Table III). Further, TGF- β production increased steadily with the increase in the severity of the disease from mild macular to severe nodular lesions. In addition, LAg-specific serum IgG levels showed direct correlation of the humoral response with the degree of dermal lesions. IL-10 and TGF- β are involved in enhancing IgA production in human cells (59–61). Earlier, we reported an up-regulation of IgA in some of the PKDL patients (46) and postulated that this up-regulation might be correlated with enhanced IL-10 production. In this study we show that PKDL patients with up-regulated serum IgA in fact produce high amounts of IL-10 in vitro under stimulation with LAg (Fig. 1D and Table III).

Increase in percentage of the population of $CD4^+CD25^+$ T cells in VL and PKDL patients and their decline at cure are indicative of their role in disease susceptibility and reactivation

It is recognized that regulatory T cells play a crucial role in the homeostasis of the immune system by suppressing immune responses to self-Ags as well as to bacteria, viruses, parasites, and fungi (62). Besides inducible regulatory T cells such as Tr1 and Th3 cells that develop from conventional $CD4^+$ T cells exposed to specific stimulatory conditions and act through the secretion of IL-10 and TGF- β , respectively, suppression exerted by natural regulatory T (Treg) cells ($CD4^+CD25^+$ T cells) during infection may result in a failure to induce adequate control of infection (63). Evidence from experimental murine models of *Leishmania major* infection suggests that endogenous or natural $CD4^+CD25^+$ Treg cells play an important role in the infectivity of *Leishmania* also (64, 65). In addition to cell-cell contact (62), these cells can execute their immunosuppressive function through the secretion of IL-10 and TGF- β signaling (66, 67). Because we observed high levels of IL-10 and TGF- β in VL as well as in PKDL patients with severe manifestation, we attempted to measure the ex vivo percentage of the population of $CD4^+CD25^+$ T cells in these patients by flow cytometry. For this experiment we collected PBMC from healthy controls, VL patients with active disease and immediately after the cure, and PKDL patients before the onset of SAG therapy.

The cells were stained *ex vivo* with anti-CD4 and anti-CD25 surface markers and subjected to FACS analysis. Gating was set on lymphocytes. Active VL patients showed a significantly higher percentage of the population of CD4⁺CD25⁺ T cells than healthy controls ($p = 0.0079$) (Fig. 4A), and this dropped significantly after the cure ($p = 0.0079$). Interestingly, PKDL patients also showed increase in CD4⁺CD25⁺ T cells during disease and the percent population was significantly higher than both healthy controls and cured VL ($p = 0.0317$ and 0.0159 respectively). Figs. 4, B–E show the *x-y* scattered plot of the flow cytometric analysis of one patient from each of these four groups.

CD4⁺CD25⁺ T cells are able to exert immunosuppressive function

We attempted to confirm whether the purified CD4⁺CD25⁺ T cells had regulatory properties and might therefore be responsible for the immune suppression observed during disease. The CD4⁺CD25⁺ T cells and CD4⁺CD25[−] T cells from active VL patients and healthy controls were immunomagnetically sorted, cultured with allogeneic T cells (ratio of 1:10), and the proliferative response was evaluated. The addition of CD4⁺CD25⁺ T cells significantly inhibited the PHA-induced T cell proliferation. Interestingly, the inhibition induced by CD4⁺CD25⁺ T cells from patients was significantly higher than that observed with CD4⁺CD25⁺ T cells from control subjects (Fig. 5). Conversely, the SI values of the allogeneic T cells were similar in the presence of CD4⁺CD25[−] cells from patients and healthy controls (Fig. 5).

Discussion

Extensive experimental studies have established that an effective immunity against *Leishmania* relies on the differentiation of CD4⁺ T cells toward a Th1 response (68, 69). Conversely, genetic or acquired deficiencies in cytokines like IL-12, IFN- γ and TNF- α , receptors like IL-12R and IFN- γ R, and costimulatory molecules like CD40/CD40L lead to susceptibility (70). The present knowledge emphasizes that immune correlates of human leishmaniasis do not follow the strict dichotomy of Th1 vs Th2 associated with resistance vs susceptibility (29, 44, 47). However, these developments should be viewed and reassessed from the following perspectives. First, the understanding of CMI in kala-azar was chiefly based on the Th1 component, IFN- γ and IL-12, and because the role of IL-4 was less obvious in humans, IL-10 was considered as the Th2 component (15, 16, 30, 33). IL-10, however, rather than being a signature cytokine of the Th2 regime, is a pleiotropic cytokine with diverse effects (71, 72). Moreover, because CD8⁺ T cells can also be differentiated into Tc1 and Tc2 (73, 74), cytokines from CD8⁺ and CD4⁺ T cells would be more rationally assessed as type 1 (Th1/Tc1) and type 2 (Th2/Tc2) responses. Second, as mentioned earlier, the concept of a mixed Th1-Th2 response in kala-azar has been established either from assessments of the total mRNA of cytokines from different tissues or from serum cytokines. This might not focus the disease-specific cytokine scenario, because kala-azar patients often become coinfecting with other pathogens such as mycobacterial (75), malarial, or HIV infections (76). In contrast, studies with leishmanial Ag-stimulated PBMC showed Th2 bias in active VL and Th1 at the cure (15, 16, 28, 77). This strategy is more relevant, because *in vitro* production of cytokines depends on the capability of the *Leishmania*-specific memory cells. Third, IL-10, because of its counter-regulatory effect against IL-12 and IFN- γ (16, 28–30) favors the survival of *Leishmania* by inhibiting NO-mediated killing (78), and TGF- β (also pleiotropic, and not a Th2 cytokine) enhances the susceptibility of *Leishmania* parasites by inhibiting IFN- γ production (50–52). Therefore, effective elimination of IL-10 and TGF- β synchroniz-

ing with the up-regulation of IFN- γ and IL-12 could be key factors for therapeutic success against human VL. Fourth, to date knowledge of the dynamics of the immune mechanisms in kala-azar is based on the therapeutic responses of antimony. Because AmB, in recent years, has become almost a regular first-line drug for kala-azar in India, immune responses due to its effect need special attention to understand the pathogenesis of VL and PKDL in India.

Under the above perspectives the present study characterizes: 1) the pretreatment immune status of active kala-azar patients; 2) immune mechanisms underlying the therapeutic success of SAG and AmB; and 3) the probable relevance of these mechanisms in the development of PKDL. In addition, attempts have been made to identify possible immune correlates in the progression of PKDL. Restoration of LAg-specific lymphoproliferation, elevation of IFN- γ and IL-12, and decline of IL-10 and TGF- β , although variable, occurred in response to successful treatment with both SAG and AmB. Hence, it is evident that, independently of the drugs used, skewing toward a dominant type 1 immune environment is necessary for a successful cure of kala-azar. Our results further showed that both CD4⁺ and CD8⁺ T cells contribute in producing IL-10 during the disease and IFN- γ at a successful cure. IL-10 is produced by many cell types including B cells and macrophages. Subsets of T cells, including CD4⁺ and CD8⁺, have been reported to produce IL-10, and our studies herein confirm these observations in kala-azar (28, 79, 80).

The differential immune regulation observed in kala-azar patients in response to SAG and AmB is noteworthy. Besides direct leishmanicidal activity of AmB through an ergosterol pore mechanism (4, 81), previous workers showed that AmB enhances the production of proinflammatory cytokines like IL-1- β and TNF- α as well as NO by activating macrophages and inhibits production of the anti-inflammatory cytokine IL-1ra in human PBMC (19–21, 82). Our data for the first time showed that AmB and SAG had almost equal ability to decline IL-10, as observed in the LPS-stimulated PBMC of healthy individuals. Interestingly, in contrast to SAG, AmB also had pronounced ability to down-regulate TGF- β . In parallel, successful therapy of kala-azar patients with AmB led to significant down-regulation of TGF- β and absolute elimination of IL-10. In contrast, TGF- β production and persistent IL-10 was observed in 50% of SAG-treated patients. Possibly a more efficient down-regulation of TGF- β by AmB helps in complete elimination of IL-10 in AmB-treated patients. At follow-up, two of the SAG-treated patients maintained elevated IL-10 and TGF- β without any relapse of VL, whereas none of the AmB-treated patients produced significant IL-10 or TGF- β . A recent report of experimental VL emphasizes the requirement of host-protective immune responses for the maintenance of the success of AmB therapy despite its initial direct parasite-killing effect (83). These observations, and the evidence of a blockade in the functions of IL-10 and TGF- β leading to protection (84–86) prompt us to propose that the therapeutic potential against VL is determined by the efficacy of a drug to down-regulate IL-10 and TGF- β . From this perspective, AmB is a more potent antileishmanial drug than SAG. This might clarify the epidemiological fact that despite rapidly emerging antimony unresponsiveness in India (6), treatment with AmB induces a high cure rate of ~100% (11). Unpublished observations of R. Goswami (coauthor of this paper) revealed that to date there has been no case of relapse of VL (except for HIV coinfection) after AmB treatment.

Interestingly, IL-10 has been reported to play an important role in PKDL, a sequel to kala-azar (13, 35). Supportive of this, most of the PKDL patients in this study produced significantly higher levels of IL-10 and TGF- β than cured VL and healthy controls. Therefore, we speculate that the persistence and maintenance of

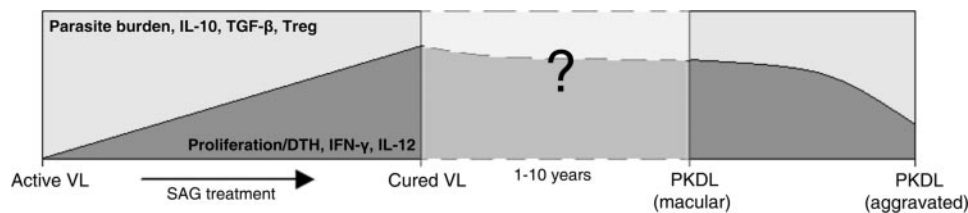


FIGURE 6. A schematic model of dynamic relationship in the clinical and immunological spectrum of Indian VL and PKDL in terms of parasite burden, lymphoproliferation/DTH, and *Leishmania*-specific in vitro cytokine production. *Upper panel* of the figure (shaded light gray) represents responses related to susceptibility in terms of parasite burden, IL-10, and TGF- β production and the percentage of Treg cells, whereas the *lower panel* (shaded dark gray) indicates responses related to protection in terms of lymphoproliferation/DTH, IFN- γ , and IL-12 production. The change in area of the shaded panels represents the extent of modulation of the above responses. The model is indicative of the hypothesis that the residual IL-10 and TGF- β after SAG treatment are somehow responsible for the reactivation of PKDL. The intermediate immunomodulatory sequences during 1–10 years after a successful cure from VL with antimony is yet unclear (hazily shaded, and the probable transition is represented by discontinuous lines). The differences in immune status among PKDL patients with different grades of lesions indicate that the up-regulation of IL-10 and TGF- β production is responsible for the disease aggravation and the resulting clinical and immunological spectrum among PKDL patients.

IL-10 and TGF- β in some patients after SAG therapy and, thereafter, their elevation could lead to disease reactivation in some of these patients in the form of PKDL. To date, to our knowledge all of the reported PKDL cases with a VL history were treated with SAG for past VL. AmB was introduced as a first line drug in Bihar in the early 1990s (9) and, quite interestingly, in the last 4–5 years PKDL cases in Bihar have shown such a dramatic decline that it has caught the independent attention of many physicians (S. Sundar, C. Thakur, and B. Saha, unpublished observation). Because PKDL in India occurs within 1–10 years after successful therapy of VL (87, 88), we further speculate that, due to AmB-treatment, down-regulation of IL-10 and TGF- β were possibly maintained in cured individuals through years, in turn limiting the development of PKDL. In this study it was logistically not practical to follow a cured VL patient till the development of PKDL. However, one limited comparative study in Sudan, where PKDL develops early after VL treatment, showed less PKDL in the AmB (liposomal AmB) group than in the stibogluconate group (89).

CMI responses of different PKDL patients indicate the importance of IL-10 and TGF- β in the progression of PKDL lesions. Most of the PKDL patients, unlike VL patients, show positive delayed-type hypersensitivity (DTH) and T cell proliferation (13). Our results, besides supporting these reports, reveal variations in the extent of lymphoproliferation, IFN- γ , IL-12, IL-10, and TGF- β production among the PKDL patients. Disease manifestation and parasite burden in PKDL showed an inverse correlation with CMI and a direct correlation with TGF- β , IL-10, and humoral response. This reflects a spectral pattern among the PKDL patients in terms of disease manifestation and associated immune responses. A similar spectral pattern is known for leprosy, where a tuberculoid pole (one extreme) leads to a type 1 reaction and a lepromatous pole (another extreme) leads to a type 2 reaction (Ref. 57 and the online Emedicine article “Leprosy” by R. Sridharan, N. Lorenzo, L. N. Ranganathan, and S. Govindarajan at <http://www.emedicine.com/neuro/topic187.htm>). An earlier report from India indicated the association of higher CMI with newly developed PKDL and lower CMI with chronic PKDL (33). The disease manifestation and CMI are probably regulated by TGF- β and IL-10. When the ability to produce these cytokines is low PKDL is less severe, whereas their increased production leads to aggravation of the disease with polymorphic lesion formation.

The association of IL-10 and TGF- β observed in both VL and PKDL prompted us to investigate the role of CD4⁺CD25⁺ T cells in these diseases. Our preliminary observation of a significant elevation of the percentage of the population of CD4⁺CD25⁺ T cells in VL in comparison to healthy controls, its decline at cure,

and further significant elevation in PKDL patients indicate that in vivo expansion and suppressive activity of CD4⁺CD25⁺ T cells is probably influenced by the production of IL-10 and TGF- β . More interestingly, IgA production in the different PKDL patients followed the pattern of enhanced IL-10 and TGF- β production with disease severity and appears to confirm our earlier speculation that the elevation of IgA in some PKDL patients might be induced by IL-10 with help from TGF- β (46).

We therefore propose that the reactivation of PKDL followed by successful antimony therapy and the disease aggravation in PKDL patients are nothing but the sequestration of the same immune orchestra initiated by *L. donovani* parasites in kala-azar patients. *L. donovani* infection in a naive individual leads to a complex host-parasite interaction leading to severe suppression of type 1 immunity and up-regulation of IL-10, TGF- β , and CD4⁺CD25⁺ T cells. Although successful antimony therapy reverses the conditions to a large extent, in some individuals, depending upon various factors including the genetic make up of the individual and environmental conditions, residual IL-10 and TGF- β probably redirect the immunity favorable to the parasite for the reactivation of infection. In continuation, the production of IL-10 and TGF- β and the expansion of CD4⁺CD25⁺ T cells indicate their roles in the aggravation of PKDL. This relationship between VL, PKDL and the resultant immunological consequences are schematically summarized in Fig. 6. However, the reason for the dermatotropic manifestation of *L. donovani* remains unexplored.

In conclusion, the present study is an important attempt to visualize the immunomodulatory sequences in human VL, both in SAG-treated and AmB-treated patients. Further, our data suggest the relevance of SAG treatment in the reactivation of PKDL. In parallel, we focus on the efficacy of AmB as a better leishmanicidal drug than SAG and propose that the resultant effective decline of IL-10 and TGF- β due to AmB treatment might reduce the occurrence of PKDL to a great extent. It is noteworthy that a sufficient duration of AmB treatment for VL is important to optimize the immunomodulation toward a protective one for the maintenance of the curative efficacy of the drug and, thus, the prevention of relapse and reactivation. Because reports from both India and Sudan suggest that short durations or inadequate treatment regimens of VL might be the causative factors for PKDL (12, 90), such treatment regimens for VL with AmB should be avoided.

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Disclosures

The authors have no financial conflict of interest.

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