Splenic accumulation of IL-10 mRNA in T cells distinct from CD4+CD25+ (Foxp3) regulatory T cells in human visceral leishmaniasis

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Visceral leishmaniasis (VL) is a life-threatening disease characterized by uncontrolled parasitization of the spleen, liver, and bone marrow. Interleukin (IL)-10 has been implicated in the suppression of host immunity in human VL based on the elevated levels of IL-10 observed in plasma and lesional tissue, and its role in preventing clearance of *Leishmania donovani* in murine models of VL. The aim of this study was to identify the cellular source of IL-10 in human VL and determine if $CD4^+CD25^+$ ($Foxp3^{high}$) regulatory T (T reg) cells are associated with active disease. We analyzed surface marker and gene expression in peripheral blood mononuclear cells and splenic aspirates from Indian VL patients before and 3–4 wk after treatment with Amphotericin B. The results did not point to an important role for natural $CD4^+CD25^+$ ($Foxp3^{high}$) T reg cells in human VL. They did not accumulate in and were not a major source of IL-10 in the spleen, and their removal did not rescue antigen-specific interferon γ responses. In contrast, splenic T cells depleted of $CD25^+$ cells expressed the highest levels of IL-10 mRNA and were the predominant lymphocyte population in the VL spleen. The elevated levels of IL-10 in VL plasma significantly enhanced the growth of *L. donovani* amastigotes in human macrophages. The data implicate IL-10–producing $CD25^-Foxp3^-$ T cells in the pathogenesis of human VL.

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Abbreviations used: EC, endemic control; HOD, healthy organ donor; SEB, staphylococcal enterotoxin B; SLA, soluble *Leishmania donovani* antigen; T reg, regulatory T; VL, visceral leishmaniasis.

Visceral leishmaniasis (VL), or kala-azar, the most severe form of leishmanial disease, is a chronic infectious disease characterized by fever, enlargement of the spleen and liver, weight loss, anemia, and leucopenia. If left untreated, VL is generally fatal. Most cases of kala-azar occur in India, Sudan, Nepal, and Bangladesh, where it is caused by the transmission of *Leishmania donovani*. Every year, >100,000 cases of VL occur in India alone, with the state of Bihar accounting for the majority of these cases, followed by West Bengal and Eastern Uttar Pradesh. Pentavalent antimonial compounds (Sb^v) have remained the first-line treatment for VL in most endemic regions. In Bihar, however, there has been a growing incidence of refractoriness to Sb^v, such that infusion with amphotericin B remains the only option available to most patients (1). Overall, treatment of VL remains unsatisfactory, and there is an urgent need to develop new therapies to reduce drug toxicity and long-term hospitalization, and to prevent drug resistance. As successful treatment of kala-azar is thought to depend, at least in part, on alterations in the host immune response to the parasite, direct manipulation of the immune response, either alone or in combination with drugs, may be a useful strategy for improving treatment regimens for VL.

A key immunologic feature of VL is the inability of PBMCs to mount curative, antigen-specific immune responses (2, 3), as reflected by their failure to proliferate or to produce IFN-γ in response to leishmanial antigens. There appears to be no inherent defect in antigen-induced Th1 responsiveness, however, because cured individuals are resistant to reinfection,

become leishmanin skin test positive, and mount antigen-specific IFN-γ responses in vitro (4, 5). Furthermore, even during the acute phase of disease, elevated levels of IFN-γ mRNA have been found in lesional tissue, such as the spleen and bone marrow (6–8).

The regulatory cytokine, IL-10, has repeatedly been implicated as an immunosuppressive factor in both human and experimental leishmaniasis. Patients with an advanced stage of disease have elevated levels of IL-10 in serum (9, 10) as well as enhanced IL-10 gene expression in lesional tissue (6–8). In mice, IL-10–deficient BALB/c and C57BL6 mice are highly resistant to *L. donovani* infection, and treatment of wild-type mice with anti–IL-10 receptor antibody promotes rapid control of *L. donovani* infection and dramatically enhances the leishmanicidal activity of Sb^v (11–13).

IL-10 has pleiotropic, primarily deactivating effects on target cells, including antagonizing dendritic cell functions and rendering macrophages unresponsive to activation signals (14). Importantly, the source(s) of IL-10 in human VL has not been defined. IL-10 can be produced by many cell types, including B cells, macrophages, and $CD4^+$ T cells (14). Several IL-10–producing $CD4^+$ T cell subpopulations have been described that have in common their ability to inhibit the response of other T cells (15, 16). The best characterized regulatory $CD4^+$ T cell subset is defined by its constitutive expression of IL-2R-α chain (CD25) and by expression of the transcriptional regulator Foxp3. Naturally occurring $CD4^+CD25^+Foxp3^+$ regulatory T (T reg) cells constitute 5–10% of peripheral $CD4^+$ T cells in naive mice and humans, and suppress several potentially tissue-damaging responses in vivo, most notably T cell responses directed against self-antigens. T reg cells may also suppress potentially beneficial immune responses, such as those directed against tumors and microbial pathogens. Naturally occurring T reg cells have been shown to produce high amounts of IL-10 and/or TGF-β, which in some experimental systems is responsible for their suppressive activity in vivo. Experimental models of cutaneous leishmaniasis have shown that IL-10 produced by naturally occurring T reg cells is crucial for persistent *Leishmania major* infection (17). In humans, dysregulation in the equilibrium between natural T reg cells and effector T cells has been associated with impaired immune responses to chronic infections, such as *Helicobacter pylori*, hepatitis C virus, and HIV (18–20), and depletion of $CD4^+CD25^+$ cells from PBMCs has been shown to increase antigen-specific proliferative and IFN-γ responses against several microbial antigens (21–23). Finally, T reg cells from hepatitis C virus patients have been demonstrated to be a source of antigen-specific IL-10 (22). The objective of the present study was to reveal the cellular source of the elevated IL-10 in VL, to determine whether accumulation or activation of T reg cells is associated with VL pathology, and to demonstrate an immunosuppressive role for IL-10.

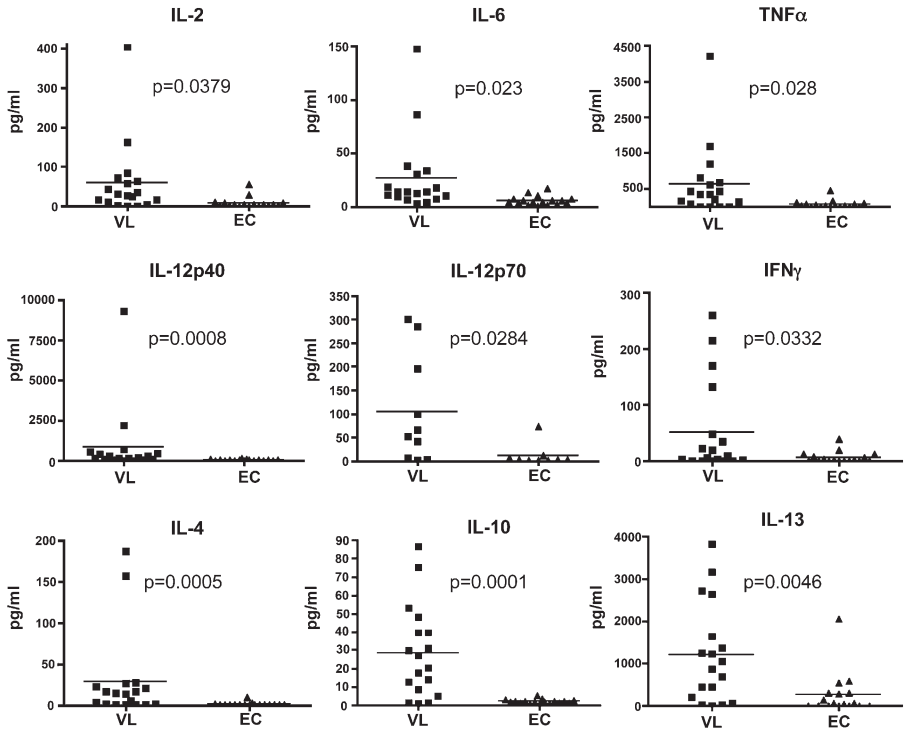

Figure 1. Cytokines in plasma from VL patients. Multiplex analysis of cytokines in plasma of VL patients before treatment ($n = 18$) and ECs ($n = 17$). 10 samples from each group were used for IL-12p70 analysis. Significant differences are indicated with p-values using Student's *t* test or Mann-Whitney U-test, when a normality test failed (IL-4 and IL-12p40).

RESULTS

Active VL is associated with elevated plasma levels of multiple cytokines

Screening of plasma using a multiplex assay showed that VL patients (see Table I) had elevated levels of circulating IL-2, IL-4, IL-6, IL-10, IL-12p40, IL-12p70, IL-13, IFN-γ, and TNF-α (Fig. 1). There was no difference in total TGF-β between the groups (not depicted). Thus, it would appear that Th1- and Th2-inducing as well as suppressive signals are induced in VL. The difference between VL patients and endemic controls (ECs) was especially striking in the levels of plasma IL-10, which was elevated in 15 of the 18 VL patients studied, but barely detectable in all 17 of the ECs.

Plasma IL-10 from VL patients promotes intracellular growth of *L. donovani* in human macrophages

The comparison of *L. donovani* infection levels in macrophages from a single normal human donor after a 3-d incubation in 20% plasma from VL patients or ECs, and in the presence of anti–human IL-10 or isotype control antibodies, is shown in Fig. 2. Neutralization of IL-10 resulted in reduced numbers of intracellular amastigotes in all cultures containing the plasma from the 13 different VL patients tested, and the mean infection levels in the anti–IL-10–treated VL group was significantly reduced compared with the control-treated group ($P < 0.001$). In contrast, no significant difference in infection levels was observed after neutralization of IL-10 in cultures containing plasma from any of the six ECs tested, and the mean number of organisms was comparable to that observed after culture in the VL plasma treated with anti–IL-10. The ability of recombinant human IL-10 to promote *L. donovani* infection in these macrophages is also shown, and the ability of the anti–IL-10 to neutralize this effect is confirmed. Thus, the elevated levels of circulating IL-10 in VL patients detected by the multiplex assay can be shown to have a direct effect on enhancing infection. It is important to note that because the VL plasma was added after the uptake of amastigotes and removal of any remaining extracellular parasites, it is unlikely that the source of IL-10 in these cultures was from macrophages after activation by immune complexes, as has been described (24).

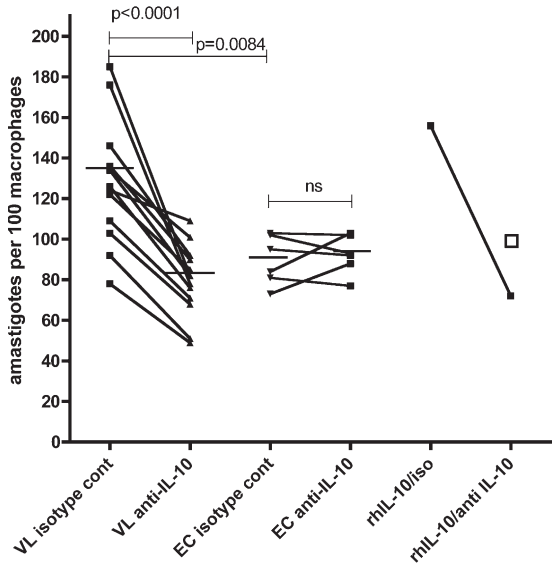

Figure 2. IL-10 in plasma from VL patients enhances *L. donovani* growth in human macrophages. Monocyte-derived macrophages from a normal human donor were infected with *L. donovani* amastigotes for 6 h, and after the removal of extracellular parasites, cultures were incubated for an additional 72 h in the presence of 20% plasma from VL patients or ECs, and treated with either anti–IL-10 or isotype control antibodies. Infected macrophages cultured in 20% normal human AB serum and treated with recombinant human IL-10 plus anti–IL-10 or control antibodies are also shown, as is the control of infected macrophages cultured in AB serum alone (□). Symbols shown represent the mean of duplicate assays, and bars designate the means for each treatment group.

PBMC profile in VL patients and ECs

Assessment of surface marker expression on gated PBMC lymphocytes (see Table II) showed no differences between VL patients and ECs in the percentage of cells expressing CD3, CD4, CD8, or CD19, nor any changes in B cell/T cell or CD4/CD8 T cell ratios. In line with previous reports (25, 26), alterations in the CD4 lymphocytes CD45RA/RO ratio were evident, which suggested a reduction in the percentage of memory (CD45RO) CD4 cells in VL patients. Expression of NK cell–associated markers NKp46 and CD16 was slightly lower in VL patients compared with ECs, but the frequency of $CD3^-CD56^+$ did not differ.

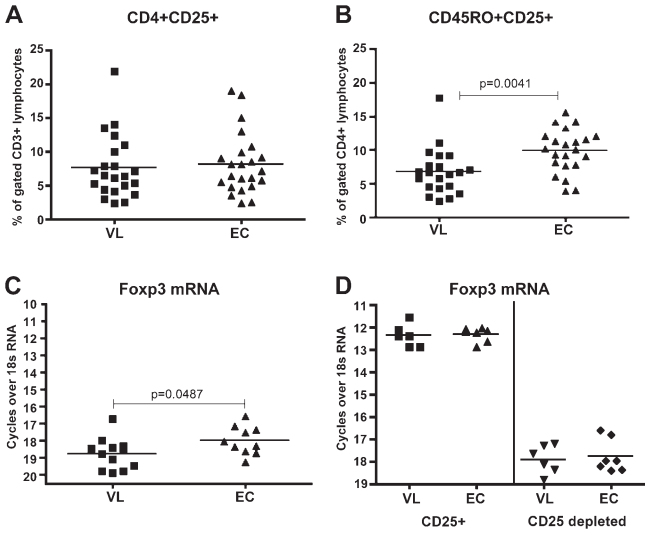

Figure 3. Natural T reg cells in PBMCs from VL patients. Analysis of T reg cells in PBMCs from VL patients (pretreatment) and ECs. (A) Percentage $CD4^+CD25^+$ of gated T ($CD3^+$) lymphocytes ($n = 22/23$). (B) Percentage $CD25^+CD45RO^+$ cells of gated $CD4^+$ lymphocytes ($n = 22/23$). Relative Foxp3 mRNA expression in (C) whole PBMCs ($n = 12/10$) and (D) positively selected $CD25^+$ cells ($n = 6$) and CD25-depleted PBMC populations ($n = 6$). Donors analyzed were the same in A and B, but not in C and D. Significant differences are indicated with p-values using Student's *t* test.

Regulatory CD4$^+$CD25$^+$ (Foxp3) T cells in PBMCs of VL patients and ECs

Analysis of the percentage of T cells expressing surface markers CD4$^+$CD25$^+$ in PBMCs revealed no difference between VL patients and ECs (Fig. 3 A). Because in the mouse natural CD4$^+$CD25$^+$ T reg cells are also CD45RB^{low}, a comparable definition of human T reg cells as CD4$^+$CD25$^+$CD45RO$^+$RA$^-$ indicated a frequency slightly lower in pretreatment VL PBMCs compared with ECs ($P = 0.013$; Fig. 3 B). Analysis of Foxp3 mRNA supported this finding, again showing a slightly lower expression in whole PBMCs from VL patients compared with ECs ($P = 0.0487$; Fig. 3 C). Analysis of Foxp3 expression in positively selected CD25$^+$ cells showed that, although highly enriched in this population, there was no difference between the level of Foxp3 mRNA in VL patients and ECs when normalized for comparable numbers of cells, nor was there a difference in Foxp3 expression in the CD25-depleted PBMCs (Fig. 3 D).

Depletion of CD25$^+$ cells does not rescue *Leishmania*–specific PBMC responses

To test if T reg cells mediated the antigen-specific unresponsiveness observed in PBMCs from VL patients, we evaluated the effect of CD25 depletion on IFN-γ and IL-10 secretion by PBMCs in 3-d culture supernatants after stimulation with staphylococcal enterotoxin B (SEB) or soluble *L. donovani* antigen (SLA; Fig. 4). Consistent with prior observations, PBMCs from VL patients did not respond to stimulation with leishmanial antigen, whereas ∼30% of the ECs produced IFN-γ in response to SLA, again consistent with prior studies involving family members of active cases in Bihar (3). The response of VL PBMCs to the superantigen SEB remained intact. With the exception of one patient, SLA-specific IFN-γ responses in VL PBMCs were not revealed by removal of CD25$^+$ cells. The IFN-γ responses to SEB were significantly enhanced in CD25-depleted EC-PBMCs (9 of 12 donors), which validates the depleted CD25$^+$ cells as functional suppressors of an effector response. Interestingly, removal of CD25$^+$ cells from VL PBMCs enhanced SEB-induced IFN-γ secretion in only two patients. Detectable IL-10 secretion was only induced by SEB stimulation, and removal of CD25$^+$ cells strongly reduced IL-10 production by the EC-PBMCs (Fig. 4, C and D), validating these cells as a potential source of IL-10. Moreover, activation of CD25-depleted T cells for proliferation or IFN-γ production could not be induced by co-culture with CD14-derived dendritic cells stimulated with SLA or live stationary phase promastigotes (not depicted). Based on these results, the inability of PBMCs to respond to leishmanial antigen is unlikely to be mediated by T reg cells. If anything, these cells appear to be somewhat reduced in both frequency and suppressive activity in PBMCs from VL patients.

We also made repeated attempts to recover *Leishmania*-specific proliferative and IFN-γ responses by blocking IL- 10. However, in line with the finding that no IL-10 could be measured over background in antigen-stimulated cultures, no effect of the blocking antibodies was observed. IL-10 blockade was only effective in the SEB-stimulated cultures in which IL-10 induction was also observed (not depicted).

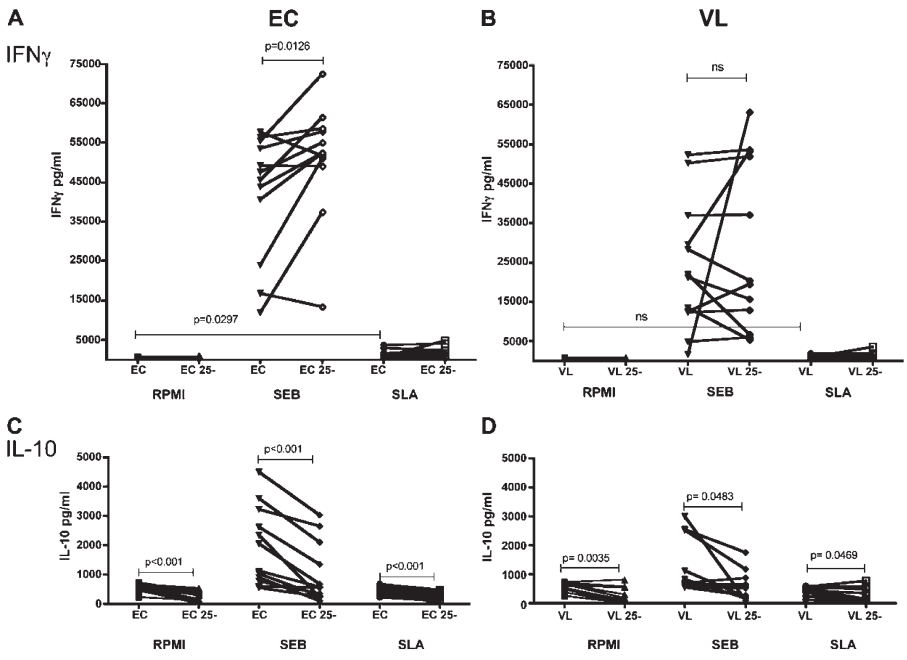

Figure 4. Cytokine production by whole or CD25-depleted PBMCs in response to SEB or SLA. IFN-γ (A and B) and IL-10 (C and D) in three-dimensional culture supernatant from whole or CD25-depleted PBMCs isolated from EC ($n = 12$; A and C) or VL patients before treatment ($n = 11$; B and D).

Lymphocyte profile in human spleen samples

To address the possibility that T reg cells are recruited to and accumulate in target organs, we undertook a comprehensive analysis of the frequency and cytokine profiles of lymphocyte subsets in VL spleens before and after curative treatment with amphotericin B and in comparison with splenic cells from healthy organ donors (HODs). To our knowledge, this is the first flow cytometry–based analysis of lymphocyte populations in VL spleen before and after treatment, and the first to conduct cytokine mRNA expression analysis on defined subsets of splenic cells. As only 50–200 μl of aspirated tissue, containing ~0.5–3 × 10^6 (median 1.7 × 10^6) white cells, was available for analysis, the spleen cells were processed either for FACS analysis or real-time PCR, but not both. Differential counting of stained splenic smears indicated that the majority (70%) of the nucleated cells were lymphocytes, 4% were plasma cells, 7% were monocytes/macrophages, and 10% were neutrophils. Blast morphology was frequently observed; thus, a broad gate was used for FACS analysis of lymphocyte subsets (Fig. 5 A). Based on profiles of PBMCs or whole blood, and on back-gating of analyzed samples and subsequent annexin V–7ADD (apoptotic: dead) staining of splenic aspirates (not depicted), the cells gated on forward-side scatter were mainly viable lymphocytes (small and blast), but also included most of the $CD14^+$ monocytes present in the aspirate. Cells/events scattered to the left of the gate are RBCs, debris, and a few dead cells. Granulocytes were scattered above the gate.

Commonly used lymphocyte surface markers were assessed in the aspirates from patients with active VL and compared with posttreatment samples and healthy spleen cells obtained from HODs (Fig. 5, B–G). The most striking change in the VL spleen was the B cell (CD19)/T cell (CD 3) ratio, which implicated splenic T cell infiltration/proliferation and/or selective loss or differentiation of B cells during active VL. 3 wk after initiation of treatment, an increase in the B/T cell ratio could already be observed, concomitant with a decrease in

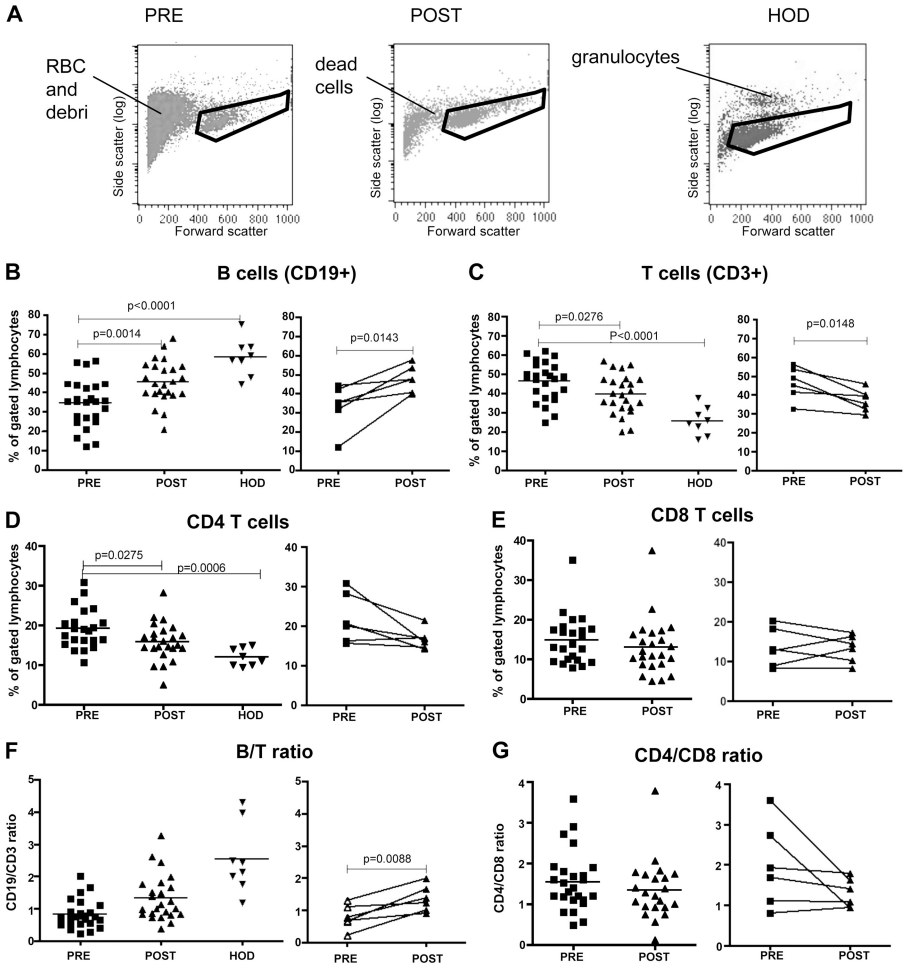

Figure 5. Flow cytometry analysis of lymphocyte subsets in VL. (A) Forward (linear) side (log) scatter profiles of splenic aspirates from the same VL patient before and after treatment, and a single healthy spleen sample from an organ donor (HOD). Cells in the gates demarcated in A were analyzed for cell surface marker expression in before (n = 25) and after treatment (n = 19–24), and in HOD (n = 8) samples. The paired samples (n = 6) shown separately were also included in the group analyses. Percentages (B–E) and ratios (F and G) between analyzed subsets are shown. Significant differences are indicated with p-values in graphs.

Table I. Aggregate clinical data for VL patients and ECs

		VL (pretreatment)[a]	VL (posttreatment)	EC	Control spleen
n		106	33	33	8
Age		22.3 ± 13 (19)[b]	23.4 ± 13.8 (19.5)	40.7 ± 13.5 (40)	ND
Sex % (M/F)		70:30	80:20	59:41	ND
Duration of illness (mo)		2.1 ± 2.5 (1)	2.3 ± 2.7 (1.5)	N/A	N/A
Infection score[c]		2 ± 1.2 (2)	1.8 ± 1.1 (1)	N/A	N/A
Spleen size (cm)	Day 0	5.8 ± 4.3 (5)	5.7 ± 3.1 (6)	N/A	N/A
	Day 15	2.9 ± 3.1 (2)	3.4 ± 2.8 (2)	N/A	N/A
WBC ($\times 10^3$/mm^3)	Day 0	3.1 ± 1.6 (2.7)	3 ± 1.4 (2.6)	ND	N/A
	Day 15	4.9 ± 2.3 (4.7)	4.2 ± 1.8 (3.7)	ND	N/A

N/D, not done; N/A, not applicable.
[a]Includes 30 donors from whom posttreatment samples were also available (column three).
[b]Mean values ± SD of aggregated data are shown, and median values are given within parenthesis.
[c]Scoring of parasite load is on a logarithmic scale from 1 to 6, were 0 is no parasites per 1,000 microscopic fields (1,000×), 1 is 1–10 parasites per 1,000 fields, and 6 is >100 parasites per field.

spleen size (Table I). Although no significant change in the CD4/CD8 T cell ratio pre- compared with posttreatment was observed, a posttreatment decrease in the percentage of $CD3^+CD4^+$ lymphocytes was apparent, whereas the effect on $CD3^+CD8^+$ cells was less so (Fig. 5, E and F).

CD45RA/RO expression on CD4 cells did not change between pre- and posttreatment (not depicted). Less than 3% of the splenic lymphocytes analyzed were NK cells ($NKp46^+CD3^-$), with no difference between pre- and posttreatment samples (not depicted). Plasma cells ($CD138^+$) were only detectable at low levels in aspirates from 2 of 16 patient samples analyzed. Expression of CD14, a monocyte-associated cell surface marker, was evaluated in 14 patients before treatment, 11 patients after treatment, and 8 HOD samples and found to be low on the gated cells (before: 2.7 ± 2.6%; after: 3.8 ± 4.4%; HOD: 2.5 ± 0.9%), with no significant differences between the groups.

No accumulation of T reg cells in splenic aspirates from VL patients before treatment

In experimental and human hosts, tissue-specific accumulation or proliferation of T reg cells at sites of chronic infection or tumor metastasis has been observed (27–29). To test if active VL is associated with accumulation of T reg cells in lesional tissue, splenic aspirates were analyzed before and after treatment for $CD4^+CD25^+$ cells and expression of Foxp3 mRNA (Fig. 6). On average, only 2.3% of the gated splenic lymphocytes, or 10% of the $CD4^+$ cells, from VL patients with active VL were $CD4^+CD25^+$, with no difference between pre- and posttreatment samples. Although CD25 expression correlates well to cells with regulatory activity in the blood, its usefulness as a marker of T reg cells at other sites has been questioned (29, 30). Nonetheless, splenic Foxp3 mRNA expression levels reflected the CD25 data, showing no significant difference in VL patients before and after treatment (Fig. 6 B). Foxp3 mRNA was also not elevated in comparison with HODs, suggesting that the pretreatment samples did not reflect sustained elevated levels that had not yet returned to the expression levels typical of a healthy spleen. Hence, there would not appear to be a selective recruitment or expansion of T reg cells in the primary target organ of VL patients during active disease.

Cytokine mRNA expression in VL spleen

Real-time PCR analysis clearly indicated that IL-10 mRNA levels in splenic aspirates were, as anticipated, significantly higher in pre- compared with posttreatment and HOD spleen samples (Fig. 7). This difference was reflected in 8 of 10 paired samples. As shown previously (7, 8), pretreatment samples also expressed

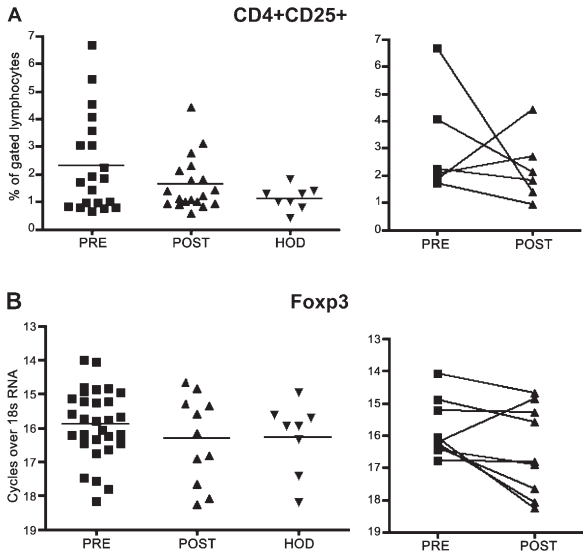

Figure 6. Evaluation of natural T reg cells in VL spleen. (A) Frequency of $CD4^+CD25^+$ cells as a percentage of gated splenic lymphocytes from VL patients before ($n = 20$) and after treatment ($n = 19$), and in HOD spleen samples ($n = 8$). (B) Relative expression of Foxp3 mRNA in splenic aspirates from VL patients before ($n = 31$) and after treatment ($n = 12$), and in HOD spleen samples ($n = 8$). Paired samples are included in groups but are also shown separately. Surface marker and Foxp3 expression analyses were not performed on the same VL patients.

Table II. Surface marker expression on PBMC lymphocytes

CD marker	VL pretreatment ($n = 22$)	EC ($n = 23$)
% of gated lymphocytes[a]		
$CD3^+$	56.7 ± 25.6	63.3 ± 14.1
$CD19^+$	5.1 ± 3	6.1 ± 3.1
Ratio CD3/CD19	13.7 ± 7.8	14.8 ± 11
$CD16^+$	6.5 ± 4.6[b]	10.1 ± 5.9
$NKp46^+$ ($n = 16/17$)	3.5 ± 3[b]	6.1 ± 3.8
$CD56^+CD3^-$ ($n = 10$)	3.25 ± 2.5	4.5 ± 1.7
% of gated $CD3^+$ lymphocytes		
$CD4^+$	50.9 ± 9.9	46.6 ± 12
$CD8^+$	37.5 ± 9	41.8 ± 10.1
Ratio CD4/CD8	1.5 ± 0.7	1.2 ± 0.5
% of gated $CD4^+$ lymphocytes		
CD45RA	45 ± 17.1[c]	29.6 ± 9.6
CD45RO	47.6 ± 15.8[d]	71.2 ± 10.7
Ratio RA/RO	1 ± 1	0.4 ± 0.2

[a]Lymphocytes were gated by forward side scatter, including blast/large cells.
[b]$P < 0.05$.
[c]$P < 0.01$.
[d]$P < 0.001$.

significantly more IFN-γ mRNA than posttreatment samples. IL-4 mRNA was expressed only at very low levels in each of the groups, and along with TNF-α mRNA, it did not change with treatment. A slight elevation in TGF-β mRNA was observed in the pretreatment samples. As this cytokine is constitutively expressed by many cell types, and bioactive TGF-β requires posttranscriptional processing, the significance of this finding is difficult to assess. Finally, the expression levels of CD3ε were similar in all groups, and normalization with CD3ε did not change the results (not depicted).

Cellular source of IL-10 in the spleen

Because elevated pretreatment levels of IL-10 may be a key factor in the pathogenesis of VL, we were interested in which cells were responsible for IL-10 production. Analysis of positively selected splenic cell subsets obtained after sequential enrichment using CD19, CD25, and CD3 MACS beads showed no correlation between Foxp3 mRNA and IL-10 mRNA expression. Although Foxp3 mRNA was, as expected, highly enriched in the positively selected $CD25^+$ cells, IL-10 mRNA was expressed at the highest levels in the CD25-depleted, $CD3^+$-enriched cells (Fig. 8, A–D). Moreover, FACS analysis together with morphological evaluation of splenic smears implicated $CD3^+$ ($CD25^-$) lymphocytes as a major cell subset in the aspirates (Fig. 4) and thus likely to be the main source of IL-10 mRNA in the VL spleen. Importantly,

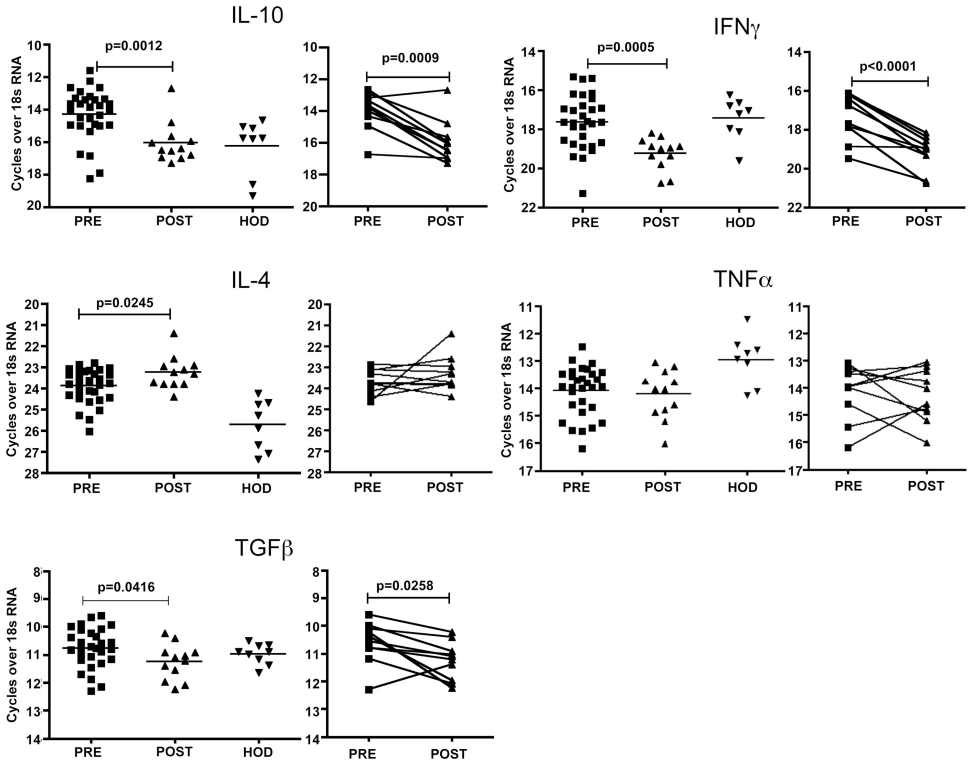

Figure 7. Cytokine mRNA expression in VL spleen. Ex vivo analysis of relative cytokine mRNA levels in splenic aspirates from VL patients before ($n = 31$) or 21 d after treatment ($n = 12$), and in HOD spleen samples ($n = 8$). Paired samples ($n = 10$) are included in groups but are also shown separately. Significant differences are indicated with p-values.

a significant decrease in IL-10 mRNA levels within the $CD3^+$ ($CD25^-$) subset was observed after treatment (Fig. 8 D), reflecting the decrease in paired samples when whole aspirates were compared (Fig. 7).

The same subset was also the main source of IFN-γ mRNA expression, and a similar decrease was seen in IFN-γ mRNA levels with treatment (Fig. 8, E and F). Real-time PCR of CD3ε mRNA was included to monitor the enrichment of T cells in the samples because there was not sufficient numbers of selected cells to analyze by FACS. CD3ε mRNA expression was highest in the $CD3^+$ selected cells. However, CD3ε expression was significantly lower in the $CD25^+$ cells than in the $CD3^+$ cells, which could reflect down-regulation of CD3ε expression, but it could also indicate that the selected CD25 cells are not a pure T cell population. Using a similar positive selection protocol on PBMCs, we found that 85–90% of the selected $CD25^+$ cells were T cells ($CD3^+$). In any event, as the $CD25^+$ population was clearly enriched for cells expressing Foxp3, then if natural T reg cells were a major source of IL-10, they should also be enriched for IL-10 mRNA, and this was not the case. And although the $CD3^+$ CD25-depleted population appeared to contain a significant number of $Foxp3^+$ cells relative to the $CD19^+$ and "depleted" fractions, these cells are unlikely to contribute substantially to the high levels of IL-10 mRNA in this population because this would again be reflected by a strong association of IL-10 within the Foxp3-enriched $CD25^+$ cells.

The depleted, negatively selected population remaining after the removal of $CD19^+$, $CD25^+$, and $CD3^+$ cells expressed significantly less IL-10 mRNA compared with the positively selected $CD3^+$ population ($P = 0.0273$). Cell surface phenotyping of five depleted samples that contained sufficient numbers of cells for FACS analysis revealed a heterogenous population of cells and essentially confirmed the Foxp3 and

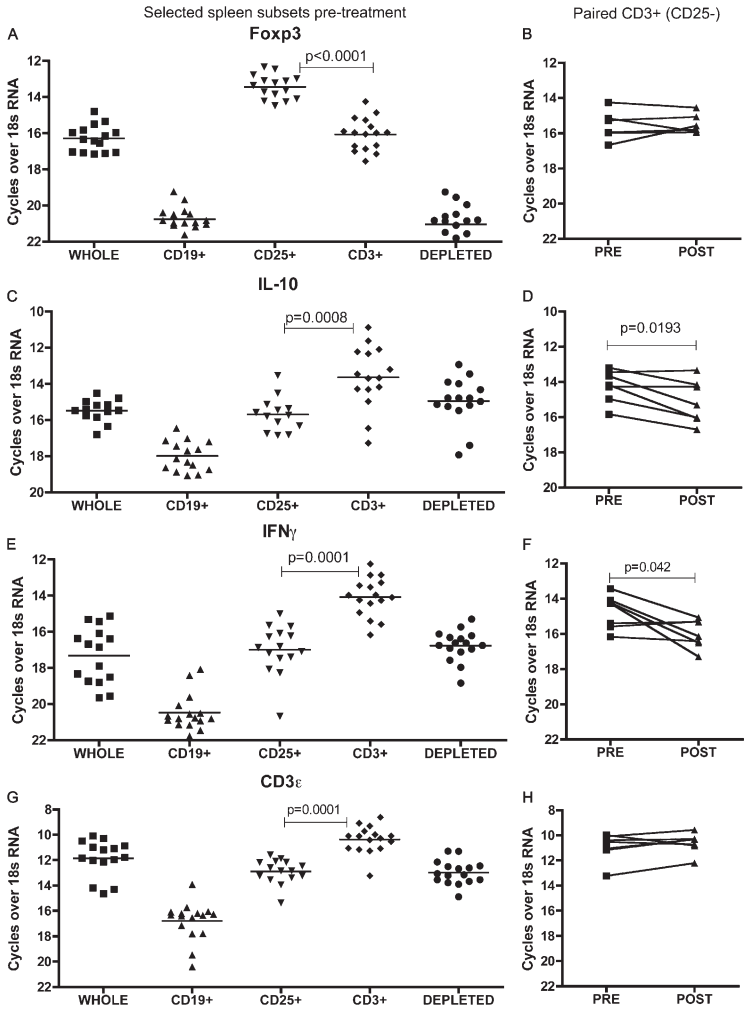

Figure 8. Ex vivo analysis of mRNA expression in splenic lymphocyte subsets. Relative expression of Foxp3 (A and B), IL-10 (C and D), IFN-γ (E and F), and CD3ε (G and H) mRNA in sequential, positively selected $CD19^+$, $CD25^+$, and $CD3^+$ cells, and in the remaining depleted spleen cell populations before treatment ($n = 16$; A, C, E, and G). mRNA expression in pre- and posttreatment paired samples ($n = 7$) enriched for $CD3^+$ (CD19, CD25 depleted) spleen cells (B, D, F, and H). Significant differences are indicated with p-values.

CD3ε mRNA expression analysis, indicating that while the $CD25^+$ cells had been efficiently removed, $CD3^+$ cells were still present (22 ± 7% of gated lymphocytes), although much reduced compared with the whole splenic aspirates.

DISCUSSION

Elevated production of IL-10 has also been a consistent finding in human VL (6–8, 26, 31, 32) and is reinforced in the present studies with respect to the levels detected in plasma and spleen. IL-10 is presumed to play a determinant role in the pathogenesis of VL based on experimental models in which IL-10–deficient or anti–IL-10 receptor–treated mice display enhanced resistance or clinical cure (11, 13) and reports that anti– IL-10 treatment may revert the antigen-specific unresponsiveness of PBMCs from VL patients in vitro (33, 34). Importantly, the source of IL-10 in human VL is not known. As IL-10 produced by naturally occurring T reg cells has been shown to promote chronic infection in mouse models of cutaneous leishmaniasis (17, 27), and as accumulating findings pertaining to chronic infections in humans have implicated natural T reg cells in the down-modulation of protective immunity (18, 22, 23, 35), the primary objective of the present studies was to evaluate the contribution of natural T reg cells to IL-10 production in human VL and their association with the pathogenesis of this disease. The extensive data generated, however, does not support a major role for natural T reg cells in human VL. Active disease was not associated with a higher frequency of $CD4^+CD25^+$ T cells or higher expression of Foxp3 in the blood or a target organ, the spleen. Moreover, depleting PBMCs of $CD25^+$ cells did not reconstitute antigen-specific IFN-γ responses. Importantly, natural T reg cells did not appear to contribute to the elevated levels of IL-10 present in the VL spleen. Instead, the main source of IL-10 was identified as a T cell population depleted of $CD25^+Foxp3^{high}$ cells, a finding that implicates adaptive T reg cells in the immunosuppression of potentially curative antileishmanial responses in kala-azar.

Prior studies characterizing T cell responses in kala-azar have been confined to peripheral blood, from which an adequate number of lymphocytes can be routinely obtained for T cell subset purification, depletion, and functional analyses. The present studies are the first to address the potential contribution of natural T reg cells to the antigen-specific unresponsiveness observed in PBMCs from VL patients. If anything, the results point to a loss of T reg cells and T reg function in the blood. A lower frequency of $CD4^+CD25^+CD45RO^+$ cells was observed, and expression of Foxp3 was slightly lower in whole PBMCs from VL patients compared with ECs. Furthermore, CD25 depletion, although clearly unable to reconstitute the antigen-specific response, also did not significantly enhance the SEB-induced IFN-γ response in VL patients as it did in the PBMCs from ECs. However, removal of $CD25^+$ cells from PBMCs did reduce the SEB-induced IL-10 production in PBMCs from both EC and VL patients, confirming that T reg cells may be an important physiologic source of IL-10, particularly if there is selective recruitment, activation, or expansion of T reg cells in target organs such as the spleen.

Despite the limiting cell numbers in residual splenic aspirates available for study, the submission of the cells to phenotypic characterization by FACS, sequential subset purification, and cytokine mRNA expression profiling by real-time PCR yielded remarkably consistent findings regarding the association of specific cells and cytokines to VL pathology. Morphologic, together with flow cytometric, analysis of splenic aspirates indicated that T cells were the main leukocyte subset in VL patients before treatment, consistent with prior observations (25). This is in contrast to healthy donors in whom B cells appear to dominate the spleen (Fig. 5 and reference 36). Thus, similar to what has been observed in experimental VL (37), T cells, including $CD4^+$ T cells with specificity for heterologous antigens (38), may accumulate in the VL spleen. There did not appear to be selective recruitment or expansion of T reg cells, as the frequency of $CD4^+CD25^+$ T cells, or the expression of Foxp3 mRNA, was not significantly increased in the VL spleen. Much of the splenic response appears to be proinflammatory, as indicated by the elevated splenic transcript levels of IFN-γ and the elevated plasma protein levels of IFN-γ, IL-12, IL-6, and TNF-α. These clinical findings indicate that unfavorable infection outcomes are not related to Th2 dominance or a Th1 response defect per se, but to concomitant production of counterregulatory cytokines, in particular IL-10, that may be triggered as a homeostatic response to limit immune-mediated pathology.

The results clearly showed that on a population basis comparing different subsets of positively selected splenic cells, and normalized for levels of 18S mRNA, $CD25^+$ cells expressed significantly less IL-10 mRNA than the $CD3^+$ CD25-depleted cells, and Foxp3 expression did not correlate with IL-10 expression. Gene expression of Foxp3 and CD3ε was used as a control for enrichment because there was insufficient material to perform FACS analysis on the positively selected cells. The cells that appeared to be the most important source of IL-10, both on a population basis comparing roughly equivalent numbers of positively selected cells and taking into account the relative frequencies of cells in the whole aspirate, were the $CD3^+$ CD25-depleted cells, which by far out number the $CD25^+$ cells in the VL spleen.

Previous reports, based primarily on findings in *L. major*–infected BALB/c mice (24), have suggested monocytes/macrophages as an important source of IL-10 in VL, and examination of lymphoid organs of VL patients has revealed an increase in the number of these cells (39–41). Our analysis cannot exclude that VL may produce changes in the frequencies of splenic macrophages or that these cells secrete high levels of IL-10. Macrophages, however, represented only a low percentage of the cells in the aspirates we scored. Moreover, IL-10 mRNA expression in the negatively selected splenic population depleted of $CD19^+$, $CD25^+$, and $CD3^+$ cells was significantly lower compared with the positively selected $CD3^+$ cells. Although this population still contained a substantial number of lymphocytes, it was presumably enriched

for monocytes/macrophages. Collectively, these results do not implicate monocytes/macrophages as the main source of splenic IL-10. It should be noted that the patients included in this study mostly had a low parasite burden and had only experienced symptoms of VL for 1–2 mo when splenic aspirates were obtained for diagnosis. Thus, in comparison with other studies, our data may reflect an earlier phase of the disease. The finding that splenic accumulation of $CD25^-Foxp3^-$ T cells expressing elevated levels of IL-10 mRNA occurs at a relatively early stage of disease argues that it is not simply a secondary outcome of chronic exposure to high parasite burdens in the spleen, but it may be causally related to the evolution of the noncure response.

We have been unable to demonstrate that IL-10 is directly responsible for *Leishmania*-specific unresponsiveness of PBMCs in vitro, as we were unable to rescue antigen-induced proliferation or IFN-γ production in the presence of anti–IL-10 antibodies, even when cultures were optimized for T cell activation using antigen-pulsed or infected autologous dendritic cells. Although anti–IL-10 antibodies have been found to promote these responses in two prior studies of VL, the number of patients studied were small, the effects were modest, and/or control antibodies were not compared (6, 33). It is possible that the peripheral blood of Indian VL patients during their early stage of disease is relatively depleted of antigen-specific cells, which are recruited to and accumulate in lesional tissue. Our studies confirm that the splenic $Foxp3^-$ T cells are enriched for and express elevated levels of IFN-γ mRNA. It has so far not been feasible to examine the influence of IL-10 on antigen-specific responses in the few splenic cells available for analysis. Our studies strongly suggest, however, that apart from suppressing the generation or maintenance of a Th1 response, the main disease-promoting activity of IL-10 might be to condition host macrophages for enhanced survival and growth of the parasite. A fivefold dilution of VL plasma from each of 13 patients tested showed an IL-10–dependent enhancement of *L. donovani* intracellular growth in human macrophages. The consequences of prolonged exposure to elevated, circulating levels of IL-10 on promoting intracellular parasitism, and impairing the ability of macrophages to kill in response to activation signals, is apt to be substantial.

Based on the results presented here, we propose that T cells, variably referred to as adaptive T reg or Tr1 cells (42), that arise from $CD25^-Foxp3^-$ T cells in the periphery, are antigen driven, and can produce large amounts of IL-10, are more important than naturally occurring T reg cells in the suppression of anti-leishmanial immunity in human VL. As the $CD3^+$ CD25-depleted fraction was also enriched for the cells producing IFN-γ in the spleen, it is possible that at least some of the cells produce both cytokines. Simultaneous production of IFN-γ and IL-10 by human T cell clones can be induced by IL-12 (43), and T cell subsets secreting both cytokines have been described in mice infected with *L. major* (44, 45) or *Toxoplasma gondii* (46), and in humans infected with *Borrelia burgdorferi* (47) or *Mycobacteria tuberculosis* (48).

Antigen-specific, IL-10–producing T reg cells arising from $CD25^-Foxp3^-$ cells have been described in several experimental systems involving, for example, immunosuppressive drugs or repeated administration of an antigenic peptide (41, 49). They are critical in regulating colitis induced by *Helicobacter hepaticus* infections in mice (50) and are thought to regulate immune responses in tuberculosis patients (51). Most critically, IL-10 production by splenic $CD4^+CD25^-Foxp3^-$ cells has recently been associated with disease progression in *L. donovani*–infected mice (52), and $CD25^-Foxp3^-$ CD4 T cells have been shown to be the critical source of IL-10–mediated suppression in chronic cutaneous murine leishmaniasis (44). As recently proposed (53), IL-10–producing adaptive T reg cells may be preferentially induced and required to control tissue damage in sites of strong inflammation associated with antimicrobial responses. Therapies that specifically target IL-10–producing T reg cells, or directly inhibit IL-10 function, might shift the balance of effector and regulatory cytokines to favor immune clearance of the parasite and enhance the efficacy of current drugs against VL.

MATERIALS AND METHODS

Study subjects. Aggregated clinical data for the patients and ECs enlisted in this study are provided in Table I. All patients presented with symptoms at the Kala-azar Research Center, Muzaffarpur, Bihar, India, and were confirmed to be VL^+ by detection of amastigotes in splenic aspirates and/or by detection of antibodies against the recombinant antigen, K39 (54). Patients positive for HIV were excluded from the study. In total, 106 patients were included in the study. Their median and average duration of illness were 1 and 2.1 mo, respectively, with a median/average splenic infection score of 2.

Splenic needle aspirates were collected for diagnostic purposes before treatment and 3–4 wk after initiation of Amphotericin B treatment to evaluate parasitologic cure. Venous blood (heparinized) was obtained from adult VL patients (16 yr and older) before treatment and from EC volunteers ($n = 33$), who were in each case healthy adult household family members of an active case. Control healthy spleen cells ($n = 8$), obtained from necro organ transplantation donors (HODs) at Karolinska University Hospital, Huddinge, Stockholm, Sweden, were isolated on a Ficoll gradient, frozen in 10% DMSO, 90% FCS, and stored at −150°C until used. The study of human volunteers followed recommendations outlined in the Helsinki Declaration. Ethical approval for the study was obtained from appropriate institutional review committees in India, Sweden, and the United States.

Detection of cytokines in plasma. Plasma levels of IL-2, IL-4, IL-6, IL-10, IL-12p40, IL-12p70, IL-13, IFN-γ, TNF-α, and TGF-β were measured by Pierce Biotechnology/Perbio Science multiplex sample analysis service.

Biological activity of plasma IL-10. The effect of human plasma from VL patients and ECs on intracellular infection was assayed using human macrophages generated from $CD14^+$ peripheral blood–derived monocytes obtained from a normal donor, as described previously (55). Macrophages were plated in eight-chamber Lab-Tek Permanox tissue culture slides (Nalge Nunc) at 10^5 cells/well in a volume of 0.4 ml RPMI 1640 complete. Hamster-derived tissue-purified amastigotes of *L. donovani* strain 9515 (MHOM/IN/95/9515), isolated from a patient with VL in Bihar, India, were added to macrophages at a 2:1 ratio and incubated for up to 6 h at 35°C. Remaining extracellular parasites were removed by extensive washing, and the infected cells were incubated for an additional 72 h in complete RPMI containing 20% human plasma and either 20 μg/ml anti–IL-10 (MAB2171) or IgG2b isotype control (MAB004) antibodies. Recombinant human IL-10 was used at 10 ng/ml (all from R&D Systems). Slides were stained with Diff-Quik solutions, and the number of intracellular amastigotes per 100 macrophages was

calculated based on the percent of macrophages infected and the number of amastigotes per infected cell. All conditions were performed in duplicate, and at least 300 cells were counted for each culture.

Real-time PCR. Whole splenic needle aspirates (~100–150 μl) were directly placed in 1 ml RNAlater (QIAGEN) and stored at −70°C until used. Total RNA was isolated using the RNeasy minikit and Qiashredder homogenizers (QIAGEN) according to the manufacturer's protocol. Quality of RNA was assessed by denaturing agarose gel electrophoresis and ethidium bromide staining. Reverse transcription was performed in 40-μl reactions on 0.1–0.4 μg RNA (samples compared were of the same concentrations) using Superscript III First-Strand synthesis system for RT-PCR (Invitrogen) according to the manufacturer's instructions.

To isolate RNA from selected lymphocyte subsets, the viability of cells in the splenic aspirates were maintained during the 18–24-h transportation period in chilled heparinized tubes containing 1 ml RPMI 1640 supplemented with 10% normal human AB^+ serum and 5 U/ml IL-2 (Sigma-Aldrich). Thereafter, erythrocytes were lysed by a 5-min incubation in cold ACK-lysis buffer, washed with PBS, and diluted in PBS containing 0.5% BSA and 2 mM EDTA. Cell subsets were enriched by sequential positive selection of $CD19^+$ cells, followed by $CD25^+$ cell selection from the CD19-depleted fraction and $CD3^+$ cell selection from the CD19- and CD25-depleted cells using MACS beads and MS columns (Miltenyi Biotec) according to the manufacturer's instructions, but using twice the volume of CD19 beads. Cells remaining after the removal of $CD19^+$, $CD25^+$, and $CD3^+$ cells are referred to as depleted. The positively selected and the depleted cells were resuspended in 25–50 μl PBS, followed by mixing with 200–300 μl RNAlater. They were stored at −70°C until used. RNA was isolated as described above, except that the isolated cell subsets were processed as liquid samples. Real-time PCR was performed on an ABI Prism 7900HT sequence detection system (Applied Biosystems) using cDNA-specific FAM-MGB–labeled primer/probe sets for Foxp3, IFN-γ, IL-10, IL-4, TGF-β, and TNF-α with VIC-MGB–labeled 18S mRNA (primer limited) as control for the relative amount of mRNA in each sample. CD3ε expression was measured using the same method and used as an estimate of the varying percentages of T cells in pre- and posttreatment samples. All reagents were purchased from Applied Biosystems. The relative quantification of products was determined by the number of cycles over 18S mRNA endogenous control required to detect the gene expression of interest.

Analysis of cell surface marker expression. PBMCs were purified by Ficoll-Hypaque (Amersham) gradient centrifugation. Splenic aspirates were collected in 1 ml RPMI 1640 supplemented with 10% normal human AB^+ serum and 5 U/ml IL-2 (Sigma-Aldrich). The red blood cell content was reduced by a 5-min incubation in cold ACK lysis buffer. PBMCs and spleen cells from EC and/or VL patients were stained with combinations of the following fluorochrome-labeled antibodies: CD3-FITC or PECy5, CD4-PECy5, CD8-PECy5 or allophycocyanin (APC), CD14-PE, CD16-FITC, CD19-APC, CD45RA-FITC, CD45RO-APC, CD56–Alexa 488, NKp46-PE, CD138-FITC (BD Biosciences), and CD25-PE (Miltenyi Biotec). HOD spleen cells were stained with CD3-APC, CD4-FITC, CD14-FITC, CD19-PE (BD Biosciences), and CD25-PE (Miltenyi Biotec). Fluorescence was measured on lymphocytes and blast cells gated by forward (linear scale) and side (log scale) light scatter using FACSCalibur and CELLQuest software (BD Biosciences).

Cell culture. Whole PBMCs or PBMCs depleted of CD25 cells (MACS) were cultured for 3 d with 25 μg/ml SLA (prepared as described previously [56]), 10 μg/ml SEB, or in the absence of antigen. Dendritic cells were generated from $CD14^+$ peripheral blood–derived monocytes as described previously (55). Anti–IL-10 and isotype control antibodies were used at 10 μg/ml and recombinant human IL-10 was used at 10 ng/ml. Production of IL-10 and IFN-γ was measured in culture supernatants by ELISA (MabTech) according to the manufacturer's instructions. The detection limits for IFN-γ ranged from 15 to 30 pg/ml and for IL-10 from 30 to 60 pg/ml.

Statistical analysis. Statistical significances were analyzed using PRISM4 (GraphPad Software). Differences between groups or paired pre- and post-treatments were compared by two-tailed *t* test for unpaired and paired samples, respectively. Mann-Whitney U-test was applied if samples did not pass a normality test.

We would like to thank the hospital staff at the Kala-azar Medical Research Center; Muzaffarpur for their assistance in the collection of patient samples; and Dr Amar Singh Chandel, Brijesh Kumar, Anshuman Mishra, Karnlesh Gidwani, Subodh Gupta, and Pankaj Srivastava for their kind help in the processing of samples.

This work was supported by the Intramural Research Program of the NIH, NIAID. L. Eidsmo was funded by Svenska Sällskapet för Medicinsk Forskning and Svenska Läkare Sällskapet. The care of the patients was supported by the Sitaram Memorial Trust, Muzaffarpur, India.

The authors have no conflicting financial interests.

Submitted: 30 May 2006
Accepted: 28 February 2007

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