IL-10 Neutralization Promotes Parasite Clearance in Splenic Aspirate Cells From Patients With Visceral Leishmaniasis

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The mechanisms underlying the failure to contain the growth of *Leishmania* parasites in human visceral leishmaniasis (VL) are not understood. *L donovani* amastigotes were quantified in cultured splenic aspirate cells to assess the function of IL-10 in lesional tissue ex vivo. In 67 patients with active VL, IL-10 neutralization promoted parasite killing in 73% and complete clearance in 30%, while 18% had more parasites and 9% did not change. The splenic cells secreted increased levels of both tumor necrosis factor α (TNFα) and interferon γ (IFNγ) under IL-10-neutralizing conditions. These findings provide direct support for targeting IL-10 as an approach to therapy in human VL.

Interleukin-10 (IL-10) is an important immunosuppressive cytokine that, in addition to dampening potentially harmful inflammatory responses during chronic infection, can contribute to pathogen persistence [1]. IL-10 suppresses the activation of macrophages and dendritic cells for microbicidal and antigen presentation functions, and can inhibit T-cell activation directly [2]. Infection models in mice have produced unequivocal evidence that IL-10 is responsible, at least in part, for pathogen persistence. Thus, genetic ablation of IL-10 or IL-10 receptor blockade results in more effective clearance of a broad spectrum of microbial pathogens, including *Leishmania* spp., *Plasmodium* spp., *Mycobacterium tuberculosis*, *Chlamydia pneumoniae*, *Candida albicans*, lymphocytic choriomeningitis virus (LCMV), and cytomegalovirus (CMV; reviewed in [1]).

In humans, elevated levels of IL-10 are correlated with important chronic infectious diseases, including malaria [3, 4], tuberculosis [5, 6], and human immunodeficiency virus (HIV) [7]. An association between elevated IL-10 and an advanced stage of disease is especially well documented in patients with visceral leishmaniasis (VL) or kala-azar, who have high concentrations of IL-10 in serum as well as strongly elevated IL-10 mRNA in target organs such as spleen and bone marrow (reviewed in [8]). The evidence that IL-10 contributes to the chronicity of infection in humans is nonetheless indirect and confined to the enhancement of immune correlates of protection when IL-10 function is blocked in vitro [9, 10]. There is so far no direct evidence in any clinical setting that IL-10 inhibition will promote pathogen clearance. In the present studies, we have used the number of viable amastigotes present in splenic aspirate cells from VL patients as a readout to explore the effect of IL-10 neutralization on parasite killing ex vivo. The findings are the first to demonstrate a host protective effect of IL-10 neutralization in lesional tissue, and provide compelling evidence that overproduction of IL-10 directly contributes to the pathogenesis of human VL.

METHODS

Study Subjects

All patients presented with symptoms of VL at the Kala-azar Research Center, Muzafarpur, Bihar, India. In each case, their diagnosis was confirmed by detection of amastigotes in splenic aspirate smears. In total, 67 patients were included in this study. Aggregate clinical data of patients are presented in Table 1. Their median and mean duration of illness were 30 days and 36.6 ± 14.9 days, respectively, with a median/mean splenic infection score of 2/2.07 ± 1.14 and a median/average spleen size of 4/4.59 ± 2.92 centimeters. This work was conducted with ethical approval obtained from institutional review committees in India and the United States, and informed consent was obtained from all patients or their guardians.

Culture of Splenic Aspirate Cells

Splenic needle aspirates (100–200 μL) were collected for diagnostic purposes before treatment. Following preparation of smears, the remaining volume in the syringe was rinsed out with

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Table 1. Aggregate Clinical Data for VL Patients

	n=67
Age (years)	22.6 ± 14.9 (18)[a]
Sex % (M/F)	60/40
Duration of fever (days)	36.6 ± 25.4 (30)
Spleen size (cm)	4.6 ± 2.9 (4)
Splenic score[b]	2.07 ± 1.14 (2)

NOTE. [a] Mean values ± SD of aggregated data are shown; median values given in parentheses.

[b] Scoring of parasite load is on a logarithmic scale from 1 to 6, where 0 is no parasites per 1000 microscopic fields (1000×), 1 is 1–10 parasites per 1000 fields, and 6 is >100 parasites per field.

0.8 mL heparinized Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 20 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). Samples were transported to a central laboratory at 4°C–8°C. A fraction of the cell suspension (150 μL) was inoculated into a 96-well plate containing blood agar for baseline quantification of amastigotes by limiting dilution, as described [11]. The remaining cell suspension was dispensed in duplicate 150-μL volumes into either U-bottom polypropylene culture tubes or into wells of a U-bottom 96-well culture plate. Monoclonal antibodies against human IL-10 or control immunoglobin G (IgG) were added to a final concentration of 20 μg/mL. Two sources of antibodies were used: BD Biosciences, anti-IL-10 clone JES3-19F1, rat IgG2A isotype control clone 54447 and R&D Systems, anti-IL-10 clone 25209, mouse IgG2B isotype control clone 20116. The cells were incubated for 3 days at 37°C under 5% CO_2. Culture supernatant was removed and replaced with promastigote growth medium (cM199) comprising M199 containing 20% heat-inactivated fetal calf serum (FCS), 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 40 mM HEPES, 0.1 mM adenine (in 50 mM HEPES), 5 mg/mL hemin (in 50% triethanolamine), and 1 mg/mL 6-biotin [11]. The splenic cells were transferred directly into a 96-well blood agar plate for titration culture using 2- or 3-fold serial dilutions. The number of viable parasites was determined from the highest dilution at which promastigotes could be grown out after 7–10 days of incubation at 25°C. The levels of IFN-γ and TNF-α in culture supernatants were measured by Aushon Biosystems Searchlight multiplex sample analysis.

Statistical Analyses

Statistical significances were analyzed using PRISM4 (GraphPad Software). Differences among paired treatment groups were compared by the Wilcoxon signed rank test for paired samples.

RESULTS AND DISCUSSION

We have taken advantage of the availability of splenic aspirate cells from VL patients, containing parasitized macrophages as well as IL-10 secreting cells, to directly address the possible function of IL-10 in promoting the survival and growth of *L donovani* in the VL spleen. Direct titration culture of an equivalent fraction of the splenic aspirate cells from the 67 patients, prior to their incubation with anti-IL-10 or control antibodies, revealed a wide range in the number of viable amastigotes present in the tissue aspirate (minimum 9; maximum 4.78×10^6; median 19 683; data not shown). This variability reflects the sampling variance associated with the needle aspiration procedure (ie, the volume and cell concentration of the aspirate), as well as the stage of disease at the time the aspirates were obtained.

Following 3 days of incubation of the splenic cells, the number of viable organisms in the anti-IL-10-treated cultures showed a highly significant reduction compared with the control-treated cultures (control IgG, geometric mean 14 540; anti–IL-10, geometric mean 1182; $P < .0001$; Figure 1*A*). Of the 67 paired cultures analyzed, 49 (73%) had fewer parasites, while 12 (18%) had more and 6 (9%) did not change under IL-10–neutralizing conditions. Furthermore, the IL-10 inhibition resulted in complete clearance of organisms from 20 (30%) of the cultures. In 9 of the 12 paired cultures for which the anti-IL-10 treatment resulted in increased numbers of parasites, the end-point titration differed by only a single well, representing a 2–3-fold increase. The 12 patients from whom these aspirates were obtained did not differ significantly in any clinical parameter from the 49 patients for whom their splenic parasite numbers were reduced by the IL-10 neutralization.

Determination of the concentration of cytokines released by the spleen cells during the 3 days of culture revealed consistently elevated levels of both IFNγ and TNFα in the anti-IL-10-treated cultures (Figure 1*B*). Of the supernatants analyzed from 32 paired cultures, IL-10 neutralization enhanced IFNγ and TNFα secretion in 29 and 31 of the cultures, respectively, compared with the control-treated cells. The wide range of cytokine concentrations again reflects the variability in the number of cells plated in these assays. No association was found between the amount of cytokines released and the amount of killing conferred by the IL-10 neutralization in this sampling of splenic aspirate cultures submitted for analysis.

IL-10 is secreted by multiple cell types, including macrophages, dendritic cells, natural killer (NK) cells, B cells, and various T-cell subsets. We have recently identified $CD4^+CD25^-Foxp3^-$ T cells as the major source of elevated IL-10 mRNA in the VL spleen [12]. Our current findings do not address the source(s) of the IL-10 that promoted the survival of amastigotes present in the splenic aspirate cells, and it is possible that other cell types, such as the parasitized macrophages themselves, are the most potent source of IL-10 in this assay. The cellular target(s) of the suppression is also not addressed, although we favor a direct action of IL-10 on the infected cells to render them refractory to activation signals. Because enhanced secretion of IFNγ and TNFα was also observed, the IL-10 may have also compromised T-cell activation directly, or

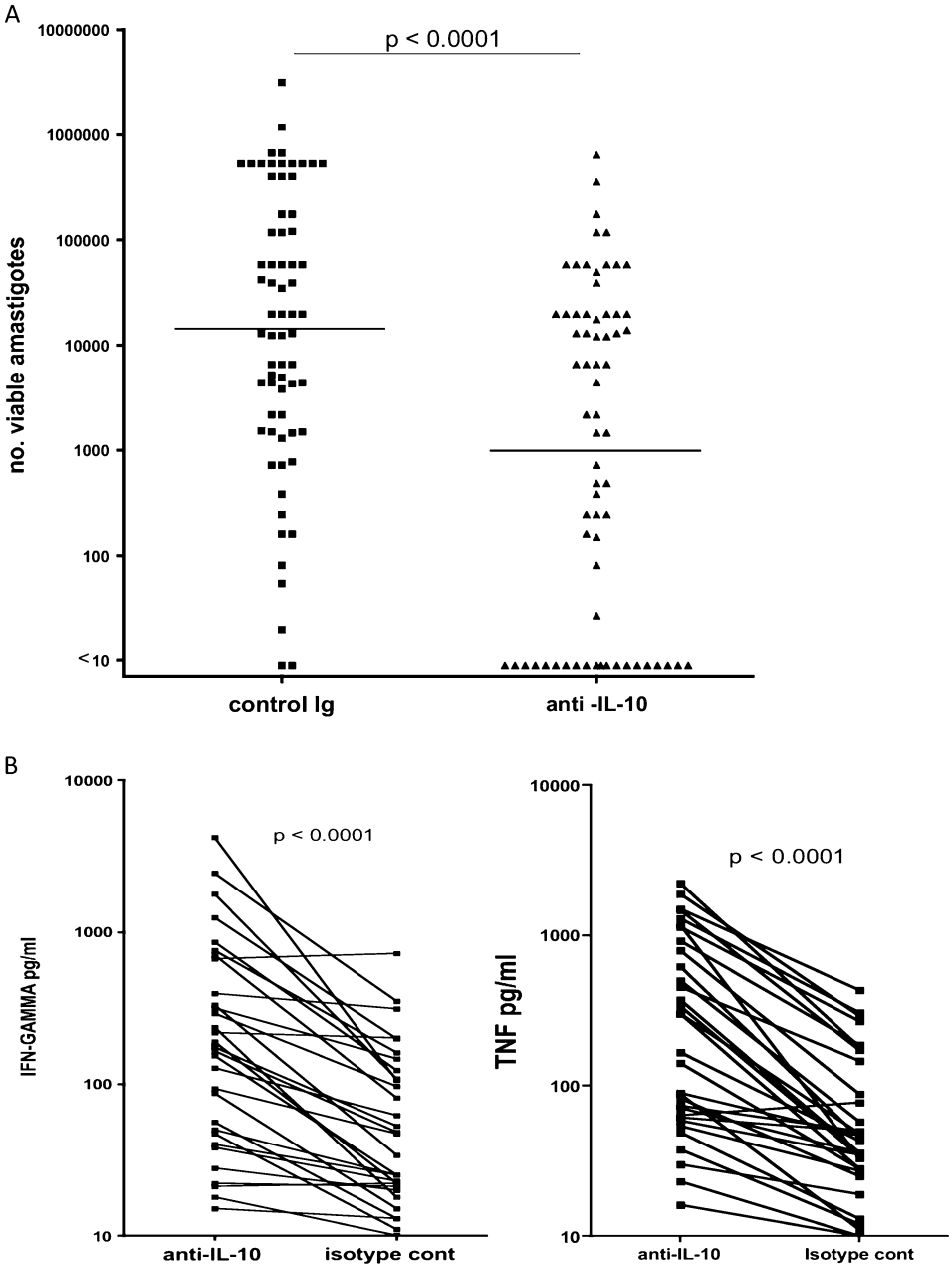

Figure 1. IL-10 neutralization promotes the killing of *L donovani* and enhances IFNγ and TNFα secretion in splenic aspirate cells from patients with active VL. Splenic cells were cultured at 37°C, 5% CO_2 in the presence of anti–IL-10 mAb (20 μg/mL) or control Ig (20 μg/mL). *A,* Values shown are the number of viable amastigotes present after 3 days, as estimated by microtitration culture in blood agar plates. Bars represent the geometric mean number of parasites. Differences between treatment groups were compared by the Wilcoxon signed rank test for paired samples. *B,* Supernatants from splenic aspirate cells cultured for 3 days in the presence of anti–IL-10 mAb or control Ig were analyzed for cytokine concentrations using a multiplex assay. The values of paired samples are shown.

via effects on antigen-presenting cell (APC) function. It seems likely that in the setting of the VL spleen, infected macrophages, APCs, and T cells are all suppressed by IL-10, such that IL-10 inhibition profoundly favors the balance of activating over deactivating cytokines. With regard to the few anti–IL-10–treated cultures that demonstrated increased numbers of parasites, it is possible that they contained extracellular amastigotes, due perhaps to lysis of infected cells during the aspiration procedure. These organisms would be unaffected by IL-10 inhibition and might begin to replicate as extracellular forms.

Finally, these findings provide the most direct support to date for IL-10 as a therapeutic target in human VL. Treatment of kala-azar remains unsatisfactory, and there is an urgent need to develop new treatment strategies to reduce the dose and duration of

chemotherapies, the development of drug resistance, and the persistence of parasites following clinical cure [13]. The dose-sparing and sterilizing effects of IL-10 receptor blockade on treatments involving pentavalent antimony or amphotericin B have been demonstrated in experimental models of VL [14, 15]. Direct manipulation of the immune response via IL-10 inhibition, and in combination with antiparasitic drugs, provides a novel approach to optimizing treatment regimens for human VL.

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