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Reassessment of Immune Correlates in Human Visceral Leishmaniasis as Defined by Cytokine Release in Whole Blood

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Depressed cell-mediated immunity in human visceral leishmaniasis (VL) (also known as kala-azar), revealed as the inability of peripheral blood mononuclear cells (PBMCs) to respond to *Leishmania* antigen, remains a hallmark of and is thought to underlie the progressive nature of this disease. We recently reported the ability of a whole-blood, gamma interferon (IFN-γ) release assay to detect subclinical infections among healthy individuals living in an area where kala-azar is endemic (Bihar, India) and the surprising result that patients with active VL also secreted significant levels of antigen-specific IFN-γ in this assay. We were interested in ascertaining whether these findings would be true for a larger cohort of subjects and in employing the whole-blood assay to detect additional cytokines that might better correlate with the disease status of infected individuals. We evaluated IFN-γ, tumor necrosis factor alpha (TNF-α), and interleukin-10 (IL-10) release in 35 patients with active VL, 54 patients with VL who were cured, 27 patients with other diseases, 52 healthy controls who lived in regions where VL or kala-azar is not endemic (NEHCs [for nonendemic healthy controls]), and 147 healthy controls who lived in regions where kala-azar is endemic (EHCs [for endemic healthy controls]). The cellular responses of the EHCs were correlated with their serological antibody titers against *Leishmania donovani* and *Phlebotomus argentipes* saliva. The whole-blood cells from the majority of both active (80%) and cured (85%) VL patients, as well as 24% of EHCs with presumed subclinical infections, produced significantly elevated levels of IFN-γ. The findings do not support a severe Th1 response defect in kala-azar. Importantly, only the patients with active VL also produced IL-10, which in conjunction with IFN-γ better reflects the immune responses that distinguish individuals with active disease from cured or subclinically infected, immune individuals.

Visceral leishmaniasis (VL), also known as kala-azar, is a potentially fatal disease caused by obligate intracellular parasites of the genus *Leishmania.* The estimated annual global incidence of VL is 500,000 with 90% of these cases occurring in India, Nepal, Bangladesh, Sudan, and Brazil (28). The disease is characterized by persistent low-grade fever, enlarged liver and spleen, weight loss, pancytopenia, and hypergammaglobulinemia. The parasite resides primarily within macrophages of the liver, spleen, and bone marrow, and the progressive nature of the systemic infection implicates an underlying defect in immune control mechanisms. Indeed, a hallmark of human VL is a depressed cell-mediated immune response, characterized by the failure of peripheral blood mononuclear cells (PBMCs) to proliferate or to produce gamma interferon (IFN-γ) in response to *Leishmania* antigens (2, 4, 6, 13, 14, 25, 26). In contrast, PBMCs from the majority of cured patients proliferate and/or produce IFN-γ or tumor necrosis factor alpha (TNF-α) in response to antigen (4, 9, 14, 25, 26), reinforcing the view that these assays provide meaningful immunologic correlates of VL susceptibility or resistance. Elevated expression of the immunosuppressive cytokine interleukin-10 (IL-10), detected as an increased level of circulating protein in plasma or mRNA level in spleen or bone marrow (4, 9, 15, 16, 21, 24), has been another consistent feature of human VL and is thought to underlie many of the immunologic defects in kala-azar (reviewed in reference 22). The ability of IL-10 inhibition to rescue antigen (Ag)-specific responses of PBMCs *in vitro* has not been found consistently (5, 9, 21) however, and secretion of IL-10 by antigen-stimulated PBMCs been detected in only one study (26).

We recently reported preliminary findings that showed how in sharp contrast to assays employing PBMCs, an IFN-γ release assay (IGRA) involving *Leishmania donovani* antigen-stimulated whole-blood cells was able to detect the secretion of IFN-γ by cells from the majority of patients with active VL (12). The IGRA is a modification of the QuantiFERON tube test (Cellestis, Australia), which is a commercial test kit that measures IFN-γ levels released by sensitized T lymphocytes in a venous blood sample stimulated with peptide antigens of *Mycobacterium tuberculosis* (19). Using the same modified assay, we have also recently reported *Leishmania* Ag-specific IL-10 secretion by whole-blood cells from a small number of VL patients (1). These initial findings have challenged the prevailing notion that there is a strong Th1 response defect in patients with active disease, suggesting instead that the profile of multiple cytokine release, and especially IL-10, might better reflect the immune responses associated with disease status in human VL.

In the current studies, we have evaluated the ability of the whole-blood assay to detect antigen-specific release of IFN-γ, TNF-α, and IL-10 by cells in samples of whole blood obtained

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TABLE 1 Demographic characteristics for subjects in the different groups in this study

Group[a]	No. of subjects	Age (yr) (mean ± SD)	No. of males	No. of females
NEHC	52	32.2 ± 11.4	31	21
EHC	147	25.8 ± 11.3	61	86
Active VL	35	21.9 ± 8.2	12	23
Cured VL	54	26.5 ± 13.5	30	24
DD	27	27.5 ± 10.6	19	8

[a] The groups were as follows: subjects who lived in areas where visceral leishmaniasis (VL) is not endemic (NEHC [for nonendemic healthy control] group), subjects who lived in areas where VL is endemic (EHC [for endemic healthy control] group); groups of subjects with active VL or cured VL; and the group of subjects with different diseases (DD).

from a large series of clinically well-characterized subjects, including individuals with active disease and confirmed to have VL, individuals with clinically cured VL, and healthy individuals from areas where VL is endemic and areas where VL is not endemic. The findings strongly support the conclusion that the majority of VL patients produce robust antigen-specific IFN-γ and TNF-α responses and that IL-10 secretion is indeed a signature cytokine response distinguishing active VL from cured or subclinically infected, "immune" individuals.

MATERIALS AND METHODS

Study subjects. The study was approved by the Ethics Committee of Banaras Hindu University, Varanasi, India. Written informed consent was obtained from all of the subjects included in the study, who were divided into 4 clinically well-characterized groups: (i) 35 cases of parasitologically confirmed, active VL; (ii) 54 subjects who were definitively cured of VL and sampled at least 6 months after successful treatment; (iii) 27 subjects suffering from other febrile illnesses like malaria, tuberculosis, typhoid fever, and dengue; (iv) 52 healthy controls living in regions where VL is not endemic (NEHCs [for nonendemic healthy controls]). The groups of cured VL patients and NEHCs were used to estimate sensitivity and specificity, respectively, of the IGRA (see below). Furthermore, we compared the performance of the IGRA with that of serology in a series of 147 healthy controls who lived in areas where VL is endemic (EHCs [for endemic healthy controls]) and who were close contacts of individuals with VL recruited at the community level in Bihar, India, i.e., a group with a high probability of being asymptomatically infected by *L. donovani* (3). We selected 104 subjects from 88 households with at least one confirmed VL case in the past 5 years, as well as 43 neighbors of these individuals. We excluded individuals who had suffered from kala-azar in the past, individuals with a fever within the past month, and children less than 5 years of age. All subjects within the EHC group were monitored 6 and 12 months after enrollment in the study to detect any development of active VL. Aggregate clinical data of the subjects are presented in Table 1.

Whole-blood cytokine release assay (modified QuantiFERON) and serology. The QuantiFERON (Cellestis, Chadstone, Australia) assay was conducted according to the manufacturer's instructions and to modifications described previously (12). Briefly, 1 ml of venous blood was collected in QuantiFERON assay kit tubes, 1 tube with soluble *Leishmania* antigen (SLA) (5 μg/ml), 1 tube with phytohemagglutinin (PHA), and 1 tube with phosphate-buffered saline (PBS) as a negative control, and hand shaken to mix with heparin. The tubes were incubated at 37°C for 16 to 24 h. Incubated samples were centrifuged at 2,000 × *g* for 10 min, and harvested plasma was stored at −20°C until used for cytokine analysis. SLA was prepared from stationary-phase promastigotes of *L. donovani* strain as described previously (23). IFN-γ levels in plasma from stimulated blood samples were measured using the QuantiFERON enzyme-linked immunosorbent assay (ELISA) kit (provided by Cellestis, Australia) per the manufacturer's instructions. IFN-γ levels of each test sample were determined by subtracting background levels measured in the corresponding nonstimulated (PBS) sample. IL-10 and TNF-α levels in plasma from stimulated blood samples were measured using matched antibody pairs (BD Pharmingen) by ELISA. Cytokine concentrations (expressed in international units per milliliter or picograms per milliliter) were calculated using a standard curve generated from recombinant cytokines. A smaller set of plasma samples, randomly selected from the same series used to assay IFN-γ, was used for the analysis of IL-10 and TNF-α, as well as the anti-saliva antibody ELISA described below. The whole-blood cells from an independent series of 10 active VL patients were treated with monoclonal antibodies against human IL-10 (clone 25209; R&D Antibodies) or mouse IgG2B isotype control (clone 25209; R&D Antibodies), each added at the start of the cultures to a final concentration of 20 μg/ml, and in the presence or absence of SLA (5 μg/ml). IFN-γ levels of each test sample were determined after 24 h.

Antibody response against *Phlebotomus argentipes* saliva was measured by ELISA using preadsorption methods as described previously (7). The salivary gland homogenates were provided by Petr Volf, Charles University, Prague, Czech Republic. For measurement of anti-*Leishmania* antibodies, plasma samples were diluted 1:400 in PBS and assayed by ELISA using recombinant antigen rK39, as detailed elsewhere (18) or by direct agglutination test (DAT) as previously described (17).

Statistical analysis. The data were presented as means ± standard deviations (SDs) for continuous variables, and frequencies with their respective percentages were given for categorical variables. The Kolgomorov-Smirnow test was used for testing the normality of the variables. For variables with a non-Gaussian distribution, a comparison of medians was done by the Kruskal-Wallis test or the Mann-Whitney test, depending on the size of the groups. Proportions were compared by chi-square tests. Differences were considered statistically significant if the *P* value was <0.05.

We constructed a receiver operating characteristic (ROC) curve to determine the best cutoff for the assay ("best" being defined as the cutoff maximizing sensitivity and specificity). The sensitivity of the IGRA was estimated in the series of 54 cured VL subjects, as they can be assumed to have a strong cellular immune response, and expressed as a percentage of IGRA-positive individuals, while specificity was given by 100% minus the percentage of IGRA- positive individuals in the 52 NEHCs. We computed 95% exact binomial confidence intervals around the estimates. The calculation and interpretation of ROC results were done using software provided by the manufacturer (Analysis Software v1.51; Cellestis Limited). The agreement between diagnostic tests was assessed by partial agreement indices and adjusted kappa values. The data were analyzed using Graph Pad Prism 5 (San Diego, CA) and SPSS software version 18.0. For the anti-saliva antibody ELISA, the cutoff was defined as 2 standard deviations from the mean of the Indian NEHC group.

RESULTS

Every sample tested in the IGRA produced strongly elevated levels of IFN-γ in response to PHA, confirming the viability of the cells and the suitability of the culture conditions for IFN-γ secretion by suitably activated cells (not shown). Figure 1A compares the mean levels of IFN-γ in the unstimulated cultures and reveals a slight elevation in plasma samples from individuals with active VL, though this difference did not reach significance. Figure 1B shows the mean levels of IFN-γ following stimulation with SLA and subtracting the values in the unstimulated cultures for each sample. The ROC analysis showed that the best cutoff for the IFN-γ concentration was >0.78 IU/ml. Figure 1B compares the results in each study group to this cutoff value. The assay was positive in 46/54 (85.2%) of subjects with cured VL, while none of the NEHCs was positive. Therefore, the IGRA had a sensitivity of 85.2% (95% confidence interval [95% CI], 73.4 to 92.3) in cured

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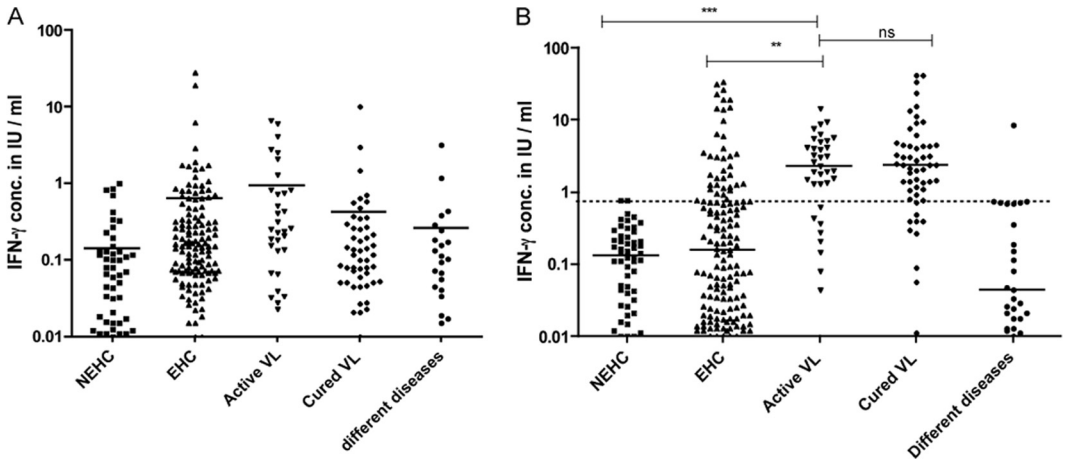

FIG 1 IFN-γ levels in plasma after stimulation of whole-blood cells with SLA. (A) Comparison of unstimulated baseline concentration of IFN-γ (IU/ml) in different groups of subjects. (B) Comparison of SLA-stimulated IFN-γ release (unstimulated value subtracted) for different subject groups. Each symbol represents the value for an individual. The bars represent the median values for the groups. The dotted line represents the cutoff (0.78 IU/ml) for positivity by ROC. Statistically significantly different median values for the groups are indicated by bars and asterisks (**, $P < 0.01$; ***, $P < 0.001$; ns, not significant).

VL patients in detecting the cellular immune response and a specificity of 100% (95% CI, 93.1 to 100.0) in NEHCs at the optimal cutoff. We also found a significant production of IFN-γ in the patients with active VL upon stimulation with SLA (80% [28/35]), with no difference in the mean level of IFN-γ comparing the group with active VL to the group with cured VL. Only 1 of the 27 patients (3.8%) suffering from another febrile illness secreted IFN-γ above the baseline level (Fig. 1B), reinforcing the specificity of the response observed in the active and cured VL cases. Finally, in the EHC group, each of whom were close contacts of a patient with confirmed VL, the IGRA was positive in 35/147 (24%) subjects, suggesting a high rate of asymptomatic infection in these individuals, and confirming the ability of the modified QuantiFERON assay to detect subclinical cases of *L. donovani*-infected individuals.

We also measured the antibody response against salivary gland antigens of the vector *P. argentipes* in IGRA-positive and -negative subjects within the cohort of EHCs, as well as in active VL cases and NEHCs. Figure 2A shows the levels of IFN-γ following stimulation with SLA (background values subtracted) in the selected samples used for the anti-saliva antibody determinations, emphasizing that the respective groups were either wholly negative or positive as defined by the cutoff value calculated for this assay. The anti-saliva antibody ELISAs revealed a significantly higher level of antibodies in the IGRA-positive EHCs than in the EHCs who gave IGRA-negative results, and comparable to the elevated anti-saliva antibody titers observed in the active VL cases (Fig. 2B). Using an optical density (OD) cutoff value defined as 2 standard deviations from the mean of the group of the healthy controls who lived in areas of India where VL is not endemic (broken line), then 38% (8/21) of EHC IGRA-negative cases, 65% (23/35) of EHC IGRA-positive cases, and 79% (19/24) of active VL cases were positive for salivary antibody. To the extent that

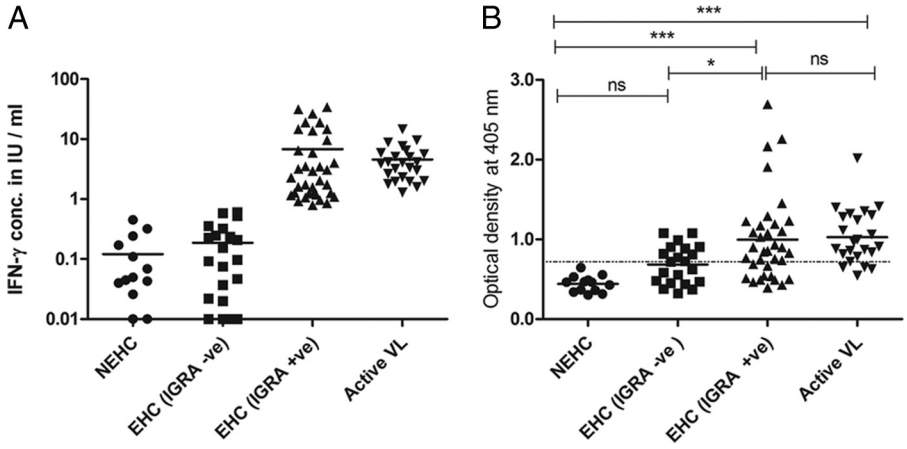

FIG 2 Human IgG response to *Phlebotomus argentipes* saliva. (A) Levels of IFN-γ released by whole-blood cells following stimulation with SLA (background values subtracted) in the samples selected from the groups shown in Fig. 1 and used for the anti-saliva antibody determinations. (B) Anti-saliva antibody levels of NEHC ($n = 13$), EHC IGRA-negative (IGRA -ve) ($n = 21$), EHC IGRA-positive (IGRA +ve) ($n = 35$), and active VL ($n = 24$) groups. The bars represent the median values, and the dotted line indicates the cutoff (0.74 OD) for positivity. Sera were preadsorbed against *Phlebotomus papatasi* saliva and processed for antibodies to *P. argentipes* saliva by a modified ELISA. Statistically significantly different median values for the groups are indicated by bars and asterisks (*, $P < 0.05$; ***, $P < 0.001$; ns, not significant).

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TABLE 2 Positive and negative agreement indices between cellular immunity and serology in 147 healthy controls from areas where VL is endemic

Tests compared	Positive AI[a] (%)	95% confidence interval	Negative AI (%)	95% confidence interval	Adjusted kappa
QuantiFERON vs rK39 ELISA	30.51	0.1521–0.4580	82.55	0.7729–0.8781	0.4422
QuantiFERON vs DAT	40.00	0.2362–0.5638	86.19	0.8153–0.9086	0.5510
DAT vs rK39 ELISA	59.09	0.4185–0.7634	92.80	0.8948–0.9642	0.7551

[a] AI, agreement index.

these elevated titers reflect a greater exposure to sand fly bites and thus a greater risk of exposure to infected flies, then the findings support the conclusion that the IGRA-positive EHCs represent subclinically infected individuals.

Of the 147 subjects in the EHC group, 35 of whom were positive by the IGRA, only 20 (13.6%) and 24 (16.3%) were positive by DAT and rK39 ELISA, respectively, and only 7 (4.8%) were positive by all three tests. Agreement between the IGRA and the serological test was only moderate as shown in Table 2. There was no significant difference between the IGRA positivity in neighbors (20% [9/43]) versus household contacts (25% [26/104]) ($P = 0.584$; χ^2 test). None of the 147 subjects developed clinical VL during the 1-year follow-up.

Since the IGRA failed to distinguish the immune status of individuals with active and cured VL, we assayed the levels of other cytokines released into plasma following stimulation of the whole-blood cells by SLA. Figures 3 and 4 show the concentrations of TNF-α and IL-10, respectively. Every sample tested produced strongly elevated levels of each cytokine in response to PHA compared to unstimulated cells (not shown). The TNF-α response to SLA was significantly elevated in both the individuals with active and cured VL compared to NEHCs, and as with the IFN-γ response, there was no significant difference between the active and cured cases (Fig. 3B). In contrast, the IL-10 levels in response to SLA were highest in the group with active VL and significantly elevated compared to each of the other groups, including the cured VL group and the EHC individuals who had positive results in the IGRA (Fig. 4B). The whole-blood cells from 30 of 33 active VL cases secreted higher levels of IL-10 in response to SLA. Of note, the plasma IL-10 levels from active VL cases were elevated even in the nonstimulated cultures (Fig. 4A), consistent with the elevated circulating levels of IL-10 previously described in patients with active disease (4, 21, 24). Despite the relatively high levels of IFN-γ secretion by SLA-stimulated whole-blood cells from active VL cases, the fact that IL-10 was simultaneously secreted suggests that the IFN-γ response might still have been compromised. Treatment of the whole-blood cell cultures with neutralizing anti-IL-10 antibodies in fact enhanced SLA-stimulated IFN-γ secretion by cells from 9 out of 10 VL patients examined, though in each case the increase was small compared to the values for the control treated cultures (Fig. 5).

DISCUSSION

Our results confirm on a large series of clinically well-defined patient groups and healthy controls that the IGRA detects cell-mediated immunity with high sensitivity and specificity in cured VL patients, as well as in a significant proportion of healthy contacts of individuals with active VL. Moreover, a remarkable finding, confirming a preliminary study on a small number of patients (12), was the production of IFN-γ in the whole-blood samples from patients with active VL upon stimulation with SLA that was comparable to that in individuals with cured VL in both the proportion of individuals responding and the level of IFN-γ produced. This result is in striking contrast to the consistent findings involving PBMCs from VL patients that do not proliferate or release IFN-γ after *Leishmania* antigen stimulation (4, 6, 9, 21, 25, 26). The T cell source of antigen-stimulated IFN-γ produced by the whole-blood cells in these assays has been confirmed (R. Kumar et al., unpublished results). The difference between the T cell responsiveness in whole blood as opposed to PBMC preparations is not yet understood but is under investigation. The antigen-specific IFN-γ response detected in VL patients is consistent with the elevated levels of IFN-γ mRNA expression detected in lesional tissue (9, 15, 16), most of which is associated with $CD4^+$ $CD25^-$ $Foxp3^-$ T cells (21), and the finding that *L. donovani*-infected human myeloid dendritic cells (DCs) instruct Th1 differentiation

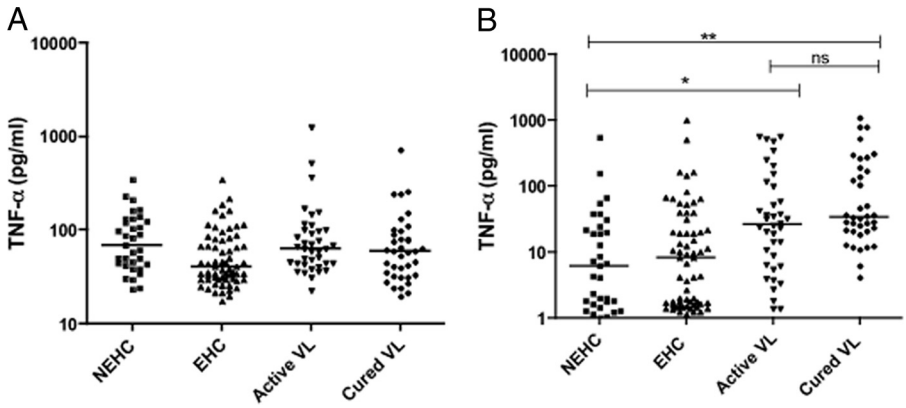

FIG 3 TNF-α levels in plasma after stimulation of whole-blood cells with SLA. (A) Comparison of unstimulated baseline concentration of TNF-α (pg/ml) in different subject groups. (B) Comparison of SLA-stimulated TNF-α release (unstimulated value subtracted) for different subject groups. The bars represent the median values. Statistically significantly different median values for the groups are indicated by bars and asterisks (*, $P < 0.05$; **, $P < 0.01$; ns, not significant).

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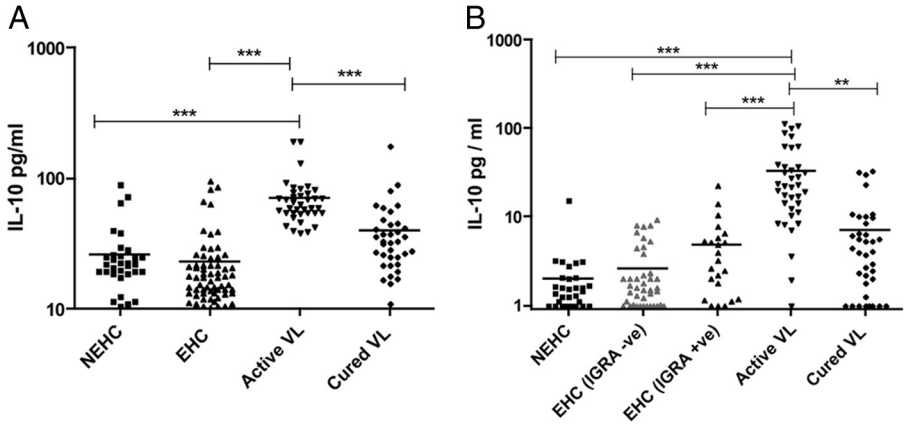

FIG 4 IL-10 levels in plasma after stimulation of whole-blood cells with SLA. (A) Comparison of unstimulated baseline concentration of IL-10 (pg/ml) in different subject groups. (B) Comparison of SLA-stimulated IL-10 release (unstimulated value subtracted) for different subject groups. The bars represent the median values. Statistically significantly different median values for the groups are indicated by bars and asterisks (**, $P < 0.01$; ***, $P < 0.001$).

in $CD4^+$ T cells from kala-azar patients (11). Together, these findings suggest that unfavorable clinical outcomes are not related to a Th1 response defect per se but that other immunosuppressive mechanisms contribute to the pathogenesis of VL.

Our findings lend further support to the view that IL-10 is a key immunosuppressive cytokine in VL patients, since only patients with active disease responded in the whole-blood assay by secreting increased levels of IL-10 upon stimulation with SLA. The association of IL-10 and VL is well established (reviewed in reference 22). Experimentally, IL-10-deficient mice, or mice treated with anti-IL-10 receptor antibody, are highly resistant to *L. donovani* infection. Clinically, patients with active disease have elevated levels of IL-10 in serum as well as enhanced IL-10 mRNA expression in target organs such as the spleen or bone marrow. In the current studies, we were able to demonstrate a small but consistent enhancement of IFN-γ secretion by antigen-stimulated whole-blood cells from VL patients in the presence of neutralizing anti-IL-10 antibodies. Anti-IL-10 antibodies have also been found to promote a small enhancement in the IFN-γ or proliferative responses in two prior studies of PBMCs from VL patients (5, 10). In contrast to the current results involving whole blood in which IFN-γ release was already quite robust, in the prior studies the anti-IL-10 treatment restored IFN-γ responses that were otherwise undetectable in the PBMCs, as was secretion of the IL-10 itself. It should also be noted that while IFN-γ is efficiently released following antigen restimulation of whole-blood cells *in vitro*, this does not necessarily reflect the responsiveness of these cells *in vivo*, or the response of cells in target organs where antigen-presenting cell (APC) function might be more strongly inhibited by localized production of IL-10. Nonetheless, on the basis of the comparable levels of IFN-γ produced by patients with active VL and cured VL that coexpress IL-10 or do not coexpress IL-10, we would argue that the main disease-promoting activity of IL-10 in VL might be to render host macrophages refractory to activation signals in response to IFN-γ, TNF-α, and other immune activating cytokines and chemokines to which they remain exposed during the active stage of disease. Strong evidence that IL-10 prevents the efficient killing of *L. donovani* by infected cells in human VL was recently provided in studies showing that the number of parasites present in splenic aspirate cells was markedly reduced following a short-term culture in the presence of anti-IL-10 monoclonal antibodies (8).

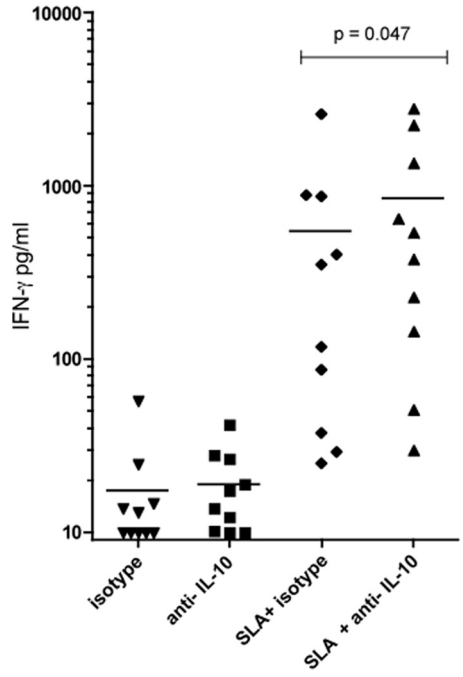

FIG 5 IL-10 neutralization enhances IFN-γ secretion by whole-blood cells from VL patients. Monoclonal antibodies (20 μg/ml) against human IL-10 or control IgG was added to the whole-blood cells, either alone or with SLA (5 μg/ml) at the start of the cultures. IFN-γ concentrations (pg/ml) in plasma after 24 h are shown. The bars represent the mean concentrations for the different groups; differences between treatment groups were compared by the Wilcoxon signed rank test for paired samples.

The ability of the whole-blood assay to detect positive responses in a significant proportion (24%) of healthy contacts of individuals with active cases of VL provides further support for its use to identify asymptomatic cases. As the EHCs were all close contacts of individuals with VL, we would expect a relatively high frequency of *L. donovani* infection (3). We also found that these individuals had significantly elevated serological titers of antibodies against *P. argentipes* saliva compared to the IGRA-negative

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EHCs or the NEHCs and were comparable to the individuals with active VL, presumably reflecting their greater exposure to sand fly bites and their greater risk of having been exposed to an infected sand fly (7). This finding lends some support to the argument that the EHCs who were scored positive in this assay represent a population of subclinically exposed, immune individuals. We acknowledge that this study has some limitations, as the general lack of a gold standard to define *L. donovani* infection in clinically normal individuals seriously hampers the development of and the evaluation of the accuracy of new markers. The specificity of the IGRA was estimated in this study on a valid group of healthy controls recruited in an area where VL and *L. donovani* transmission are not endemic, but it should be further investigated on more-representative control groups recruited from areas where VL is endemic to evaluate the potential use of this marker for epidemiological studies. The group of cured VL used for sensitivity estimation was clinically well characterized and would seem a valid basis for sensitivity estimation, as cure correlates with cellular immunity (20). The IGRA results can be compared only to the imperfect standard of serology. As found in previous studies comparing the Leishmanin sensitization test (LST) with serology (3, 27), we also found a higher positivity rate in the IGRA compared to DAT and rK39 ELISA. The greater sensitivity of IGRA to detect asymptomatic infection might be explained by the fact that antibodies are thought to wane earlier than cellular immunity.

In conclusion, the IGRA, if further validated in larger community-based studies, could become a useful epidemiologic tool to provide a more complete picture of *L. donovani* transmission. The results of our studies make clear, however, that antigen-driven IFN-γ release alone is not an adequate marker for immune status, since all clinically exposed groups produced comparable levels of this cytokine. Thus, kala-azar patients do not appear to have the severe Th1 response deficit that has long been associated with their progressive disease. Instead, coexpression of IL-10, likely produced as a homeostatic mechanism to control strong and persistent infection-induced inflammation, provides a better immune correlate of disease, and its expression might also be of prognostic value in asymptomatic infections.

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