Cytokine Responses to Novel Antigens in an Indian Population Living in an Area Endemic for Visceral Leishmaniasis

Om Prakash Singh1, Carmel B. Stober2, Abhishek Kr. Singh1, Jenefer M. Blackwell3,4*, Shyam Sundar1+*

1 Infectious Disease Research Laboratory, Department of Medicine, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India, 2 Department of Medicine, University of Cambridge School of Clinical Medicine, Cambridge, United Kingdom, 3 Cambridge Institute for Medical Research, University of Cambridge School of Clinical Medicine, Addenbrooke’s Hospital, Cambridge, United Kingdom, 4 Telethon Institute for Child Health Research, Centre for Child Health Research, The University of Western Australia, Subiaco, Western Australia, Australia

Abstract

Background: There are no effective vaccines for visceral leishmaniasis (VL), a neglected parasitic disease second only to malaria in global mortality. We previously identified 14 protective candidates in a screen of 100 Leishmania antigens as DNA vaccines in mice. Here we employ whole blood assays to evaluate human cytokine responses to 11 of these antigens, in comparison to known defined and crude antigen preparations.

Methods: Whole blood assays were employed to measure IFN-γ, TNF-α and IL-10 responses to peptide pools of the novel antigens R71, Q51, L37, N52, L302.06, J89, M18, J41, M22, M63, M57, as well as to recombinant proteins of tryparedoxin peroxidase (TRYP), Leishmania homolog of the receptor for activated C kinase (LACK) and to crude soluble Leishmania antigen (SLA), in Indian patients with active (n = 8) or cured (n = 16) VL, and in modified Quantiferon positive (EHC+ve, n = 20) or modified Quantiferon negative (EHC−ve, n = 9) endemic healthy controls (EHC).

Results: Active VL, cured VL and EHC+ve groups showed elevated SLA-specific IFN-γ responses, but only active VL patients produced IL-10 and EHC+ve did not make TNF-α. IFN-γ to IL-10 and TNF-α to IL-10 ratios in response to TRYP and LACK antigens were higher in cured VL and EHC+ve exposed individuals compared to active VL. Five of the eleven novel candidates (R71, L37, N52, J41, and M22) elicited IFN-γ and TNF-α, but not IL-10, responses in cured VL (55–87.5% responders) and EHC+ve (40–65% responders) subjects.

Conclusions: Our results are consistent with an important balance between pro-inflammatory IFN-γ and TNF-α cytokine responses and anti-inflammatory IL-10 in determining outcome of VL in India, as highlighted by response to both crude and defined protein antigens. Importantly, cured VL patients and endemic Quantiferon positive individuals recognise 5 novel vaccine candidate antigens, confirming our recent data for L. chagasi in Brazil, and their potential as cross-species vaccine candidates.

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* E-mail: jblackwell@ichr.uwa.edu.au (JMB); drshyamsundar@hotmail.com (SS)
+ These authors contributed equally to this work.

Introduction

Visceral leishmaniasis (VL), also known as kala-azar, is a potentially fatal disease caused by obligate intracellular parasites of the Leishmania donovani species complex. VL is a serious public health problem in indigenous and rural populations in India, accounting for enormous morbidity and mortality, as well as major costs to both local and national health budgets. The estimated annual global incidence of VL is 200,000 to 400,000, and >90% of these cases occur in India, Bangladesh, Sudan, South Sudan, Ethiopia and Brazil [1]. Interestingly, 80 to 90% of human infections are subclinical or asymptomatic, and this asymptomatic infection is associated with strong cell-mediated immunity [2,3,4,5,6]. Only a small percentage of infected individuals develop severe disease [7,8], and patients who recover from VL display resistance to reinfection [5]. This suggests the development of protective immunity and provides a rational basis for the development of vaccines that impart potent cell-mediated immune responses. Furthermore, the factors that skew the immune response toward T helper 1 (Th1) or Th2/T regulatory (Treg) cell dominance are partially understood, and it is believed that direct interaction between parasite antigens and host immune cells participate to shape the subsequent pathogenic or protective immune responses [9,10].
A key mechanism by which T cells mediate their effector functions is through the production of cytokines. However, heterogeneity of CD4+ T-cell cytokine responses has made it difficult to define immune correlates of protection after vaccination in leishmaniasis. In murine cutaneous leishmaniasis, the degree of protection in vaccinated mice was predicted by the frequency of CD4+ T cells simultaneously producing interferon-γ (IFN-γ), interleukin (IL)-2 and tumour necrosis factor (TNF, formerly TNF-α) [11]. These multi-functional effector CD4+ T cells elicited by all vaccines tested were unique in producing high amounts of IFN-γ [11]. In our own studies comparing vaccines with different efficacies in mice, we found that the balance between antigen-specific CD4 T cell-derived pro-inflammatory IFN-γ and regulatory IL-10 (and to a lesser extent IL-4 and IL-5), rather than magnitude of IFN-γ per se, provided the best correlate of a protective immune response [12]. A strong tumour necrosis factor-α (TNF-α) response concurrent with IFN-γ has also been shown to be important in models of VL [13]. A crucial step in vaccine development against human disease requires improved understanding of the functional heterogeneity of T-cell cytokine responses generated by candidate vaccine antigens. For example, one study in malaria reported that peptide-specific IFN-γ to a conserved epitope of the circumsporozoite surface protein was strongly associated with protection of immune individuals who make strong cytokine responses in people with active disease, in those drug-cured from the disease, and in the naturally resistant individuals. We show that immune individuals make strong cytokine responses to five of eleven novel vaccine candidates that were tested, making them ideal candidates to take forward in the development of a defined vaccine against leishmaniasis.

**Materials and Methods**

**Study subjects**

The study was approved by the Ethics Committee of the Banaras Hindu University, Varanasi, India. Written informed consent was obtained from all adult subjects included in the study, or from the parents or guardians of individuals less than 18 years of age. Subjects belonged to 4 clinically well characterized groups: (i) active VL: cases of parasitologically confirmed, active VL (n = 8); (ii) cured VL: subjects who were definitively cured of VL and shown to have no parasites in splenic aspirates at least 6 months after treatment (n = 16); (iii) EHC with a positive antigen-specific IFN-γ response measured by modified Quantiferon (Cellestis, Chadstone, Australia) assay (cf. below) (EHCIII+, n = 20); and (iv) EHC testing negative by modified Quantiferon assay (EHCIII−, n = 9). Subjects having fever within the past month, and children less than five years of age, were excluded. Follow up visits were made to the homes of the EHCIII− and cured subjects 6 and 12 months after enrolment to monitor for the development of active VL. Demographic and clinical characteristics of participants enrolled in the vaccine study are summarized in Table 1. None of the cured VL or EHC subjects developed clinical VL during the 1 year follow-up.

**Preparation of soluble leishmania antigen (SLA)**

SLA from an Ethiopian strain of *L. donovani* (LV9) or *L. major* (LV39) were prepared at the Cambridge Institute for Medical Research, University of Cambridge School of Clinical Medicine, UK as described previously [12]. SLA from an Indian strain of *L. donovani* was prepared at the Infectious Disease Research Laboratory, Banaras Hindu University, according to the published protocol of Scott and co-workers [26]. The protein concentration was estimated using the BCA method [27]. SLA was stored at −80°C until use.

**Vaccine antigens**

Recombinant LACK and TRYP proteins were prepared as described [11,12], with large-scale preparation, endotoxin removal
Blood (5 mL) was collected into heparinised tubes, and samples were harvested, pooled and stored at −80°C until analysed by ELISA. The limit of detection for these ELISAs was 31 pg/mL. Background levels in non-stimulated control wells were deducted from antigen-stimulated values to determine antigen specific cytokine responses (with negative values recorded as zero). To control for inter-plate and intra-plate variation, a positive-control supernatant (1:4 and 1:8 dilution of PHA stimulated Non Endemic Healthy Controls (NEHC) whole blood pooled supernatant) was used in duplicate on each ELISA plate. The mean variability of these duplicate measurements was 2.53% (intra-plate variation).

Statistical analysis

Because data were generally not normally distributed (as determined using the Kolgomorov-Smirnov test), data are plotted using box and whiskers (Tukey) plots, and statistical differences (P<0.05) between pairs of groups were determined using nonparametric 2-tailed Mann-Whitney tests. Nominal P-values are presented throughout (i.e. without correction for multiple testing). Plots were generated using GraphPad Prism 5 (San Diago, USA), and statistical analyses were performed using GraphPad Prism 5 or SPSS software v18.0.

Results

SLA cytokine responses correlate with disease status

To investigate antigen specific production of IFN-γ, TNF-α and IL-10 cytokines, diluted whole blood from different patient groups was initially stimulated with SLA from an Indian L. donovani strain. Comparison of responses over time post stimulation in active VL

| Table 1. Demographic and clinical characteristics of participants. |
|---------------------------------------------------------------|
| **Post treatment (Cured VL)** | **Active VL** | **ENDEMIC HEALTHY CONTROLS** |
| N | 16 | 08 | 20 | 09 |
| Age (year) | 23.81±10.57 | 21.13±7.93 | 26.96±11.74 | 25.35±11.73 |
| Sex % (M/F) | 45.55 | 25.75 | 37.63 | 34.56 |
| Weight (Kg) | 41.94±12.77 | 43.00±10.73 | 40.21±8.135 | 44.67±11.42 |
| RBC* (×10^12/mm^3) | 4.29±0.537 | 3.06±0.339 | 4.22±0.581 | 4.03±0.79 |
| Platelets (×10^9/mm^3) | 2.50±0.85 | 1.18±0.77 | 2.3±1.17 | 2.6±1.07 |
| WBC* (×10^3/mm^3) | 10.33±2.68 | 3.8±1.84 | 9.97±2.56 | 11.56±4.37 |
| Haemoglobin (g/dl) | 11.69±1.25 | 7.87±1.752 | 11.79±1.229 | 11.53±1.19 |
| Lymphocyte (×10^3/mm^3) | 2.69±0.72 | 1.70±0.63 | 2.86±0.91 | 2.76±0.51 |
| Granulocyte (×10^3/mm^3) | 6.95±2.76 | 1.76±1.33 | 6.32±2.23 | 7.8±3.8 |
| SGOT* (IU/ml) | ND | 53.0±43.79 | ND | ND |
| SGPT* (IU/ml) | ND | 48.13±44.12 | ND | ND |
| Creatinine (mg/dl) | ND | 0.85±0.23 | ND | ND |
| Splenic Score* | NA | 2.75±1.28 | NA | NA |

*RBC = Red Blood Cells; WBC = White Blood Cells; SGOT = Serum glutamic oxaloacetic transaminase; SGPT = Serum glutamic pyruvate transaminase.
*Spilic score was graded on a conventional logarithmic scale of 0 (indicating no parasites per 1000 oil-immersion fields) to 6 (indicating ≥100 amastigotes per 1000 fields) at ×1000 magnification.

Note: Mean value ± SD of aggregated data are shown throughout, N/D = not done, N/A = not applicable.

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cases showed that all 3 cytokines were highest, and less variable, at the 24 hour time point (Fig. 1A–C). Active cases made variable responses to PPD reflecting prior exposure to Mycobacterium and indicating that ability to make a response to mycobacterial antigens is not compromised in active VL patients. Cured cases made similarly variable responses to PPD (Fig. S1). Between group comparisons at 24 hours post stimulation showed that active VL, cured VL, and EHC$$^{+ve}$$ study groups all made higher IFN-$$\gamma$$ responses relative to EHC$$^{-ve}$$ subjects (Fig. 1D). The observation that active VL cases make a significant amount of IFN-$$\gamma$$ is in line with our recent observations for whole blood assays using undiluted blood in a modified Quantiferon assay [6,23]. Of interest, while cured VL and active VL groups generated TNF-$$\alpha$$ concomitant with IFN-$$\gamma$$, the EHC$$^{+ve}$$ group did not (Fig. 1E), suggesting that production of this cytokine might relate to the pathogenic role of TNF-$$\alpha$$ in VL disease [29]. Note, however, that this was not true for responses to putative vaccine candidates outlined below, which all elicited TNF-$$\alpha$$ concomitant with IFN-$$\gamma$$ in the EHC$$^{+ve}$$ group. Importantly, only the active VL group made IL-10 in response to Indian L. donovani SLA (Fig. 1F), supporting previous data indicating that IL-10 is a key regulatory cytokine in VL patients [10,30].

Cytokine responses to 3 different Leishmania strains, an Indian L. donovani strain (designated SLA), an Ethiopian L. donovani strain (LV9) and L. major strain (LV39) were compared (Fig. 2). For IFN-$$\gamma$$ responses the two L. donovani preparations stimulated equivalent responses (Fig. 2A). Interestingly, SLA prepared from the local Indian L. donovani strain elicited significantly stronger 24 h TNF-$$\alpha$$ (Fig. 2B) and IL-10 responses (Fig. 2C) compared to Ethiopian L. donovani ($$p = 0.0003;$$ $$p = 0.004$$) or the L. major strain ($$p = 0.028;$$ $$p = 0.007$$). The L. major antigen was more variable in eliciting responses across all cytokines and time points.

The ability of diluted whole blood assay samples to respond to mitogenic stimulation with PHA (Tables S1 and S2) confirmed the viability of the cells from all donors.

Cytokine responses to TRYP and LACK recombinant proteins

We previously demonstrated that high CD4-derived IFN-$$\gamma$$ to low IL-10 ratios predicted vaccine success in mice when comparing TRYP and LACK as DNA with/without Modified Vaccinia Ankara vaccines [12,31]. Here, we examined immune responses to these potential vaccine antigens in clinically well characterized groups of human subjects. The full set of results for IFN-$$\gamma$$, TNF-$$\alpha$$ and IL-10 responses to TRYP (Fig. S2) and LACK (Fig. S3) at 24 hours, 72 hours and 6 days post stimulation in active VL, cured VL, EHC$$^{+ve}$$ and EHC$$^{-ve}$$ study groups is provided in the figures S2 and S3. Of note, although the EHC$$^{-ve}$$ group comprised negative responders to Indian SLA by the modified Quantiferon assay, their cytokine responses to TRYP and LACK was rarely significantly different as a group from cured VL and EHC$$^{+ve}$$ groups. Hence, we conclude that there are

**Figure 1. SLA-specific IFN-$$\gamma$$, TNF-$$\alpha$$ and IL-10 responses.** Box plots (Tukey) for cytokine production by peripheral whole blood cells in response to SLA (an Indian L. donovani strain, 10$$\mu$$g/mL) or PPD (5$$\mu$$g/mL) examined in active VL (n = 8), cured VL (n = 16), EHC$$^{+ve}$$ (n = 20) and EHC$$^{-ve}$$ (n = 9) groups by ELISA. (A–C) provide comparisons of cytokine levels over 24 hours, 72 hours and 6 days post stimulation with SLA or PPD in the active VL group (parallel data for IFN-$$\gamma$$ responses in the cured VL group are provided in Fig. S1). (D–F) compare cytokine responses at 24 hours post stimulation across the 4 study groups. Antigen-stimulated cytokine responses are provided after subtraction of the non-stimulated control wells. Statistical differences between groups determined using the non-parametric Man-Whitney test are indicated by bars above columns, * indicates $$p < 0.05$$, ** $$p < 0.01$$, and *** $$p < 0.001$$. doi:10.1371/journal.pntd.0001874.g001
exposed individuals amongst this EHC group, although we did not test non-endemic healthy control responses to these antigens. As this exposure status is variable and equivocal, we exclude them from further analysis of between group responses.

Results for active VL, cured VL and EHC\textsuperscript{ve} groups are summarised in figures 3 (TRYP) and 4 (LACK). For both antigens, the pattern of IFN-\(\gamma\) and TNF-\(\alpha\) responses across the 3 groups is generally established at 24 hours, and clear cut by 72 hours and 6 days, post stimulation. For these two cytokines, responses were significantly lower in active VL compared to cured VL and EHC\textsuperscript{ve} groups at 72 hours and 6 days post stimulation. The pattern of responses for IL-10 was similar (i.e. higher in cured VL and EHC\textsuperscript{ve} compared to active VL), but more clearly apparent at 24 hours post-stimulation. This led to interesting between group differences in the ratios of IFN-\(\gamma\) to IL-10 and TNF-\(\alpha\) to IL-10 at 24 hours, when ratios were significantly higher in the active VL group compared to cured VL and EHC\textsuperscript{ve} groups (particularly for TRYP), compared to 6 days of stimulation where the reverse was true for both antigens. For both antigens, the ratios of IFN-\(\gamma\) to IL-10 and TNF-\(\alpha\) to IL-10 were highest in the EHC\textsuperscript{ve} group, suggesting that a potent pro-inflammatory response relative to modest levels of IL-10 may correlate with protection from disease in this confirmed QuantiFeron positive exposed EHC group.

Antigen specific cytokine release in response to novel vaccine antigens

We previously identified 14 protective Leishmania antigens in a screen of 100 candidates delivered as DNA vaccines to susceptible BALB/c mice [25]. We measured IFN-\(\gamma\) and TNF-\(\alpha\) as effector pro-inflammatory cytokine responses to peptide pools for each of 11 of these antigens in diluted whole blood assays, and IL-10 as a measure of their ability to elicit a regulatory cytokine response. A full summary of responder status on a categorical scale (\(\approx\) 20 ng/mL above background) with time post stimulation, we observed maximal IFN-\(\gamma\) responders at 24 hours and 72 hours post stimulation (Fig. 5A), with 55–87.5% responders to 5 of the novel antigens (R71, L37, N52, J41 and M22; of these L37 exceptional in eliciting the highest sustained IFN-\(\gamma\) responses at day 6 post stimulation, see also Table S2). Comparing across groups for the 72 hour time point (Fig. 5B), we observed 40–65% responders to these 5 novel antigens in the EHC\textsuperscript{ve} group, with \(\pm\)25% of active VL cases also making IFN-\(\gamma\) responses to these antigens. Looking across cytokine responses for these 5 antigens (Fig. 6), we observe a similar profile of TNF-\(\alpha\) responses in cured VL and EHC\textsuperscript{ve} groups as we observed for IFN-\(\gamma\), but no IL-10. Even amongst active VL cases, N52 was the only antigen to elicit IL-10 responses (Fig. 6L). As for TRYP and LACK, a small number (22–33%) of responders was observed in the EHC\textsuperscript{ve} group, consistent with evidence of exposure in these individuals despite their negative response in the modified QuantiFeron assay. Alternatively, these might represent non-specific responses to these antigens as we did not include non-endemic controls in our study. In summary, we have identified five Leishmania antigens from 11 putative vaccine candidates tested that stimulate potent pro-inflammatory recall responses in exposed but protected individuals (cured VL patients and EHC\textsuperscript{ve}) in the absence of regulatory IL-10, providing potential immunotherapeutic or vaccine targets for future investigation.

Discussion

A variety of defined antigens have been investigated as vaccine antigen candidates for VL in animal models [32,33,34], but few have advanced to human clinical trials [35,36]. One limitation in the search for an effective vaccine for leishmaniasis is the lack of information on immunological correlates of natural and vaccine-mediated protection in humans. In recent studies we have highlighted the use of a modified QuantiFeron assay to screen for naturally exposed resistant individuals in the Indian study area [6]. That assay relies on 3 mL of undiluted whole blood. Here we show that individuals positive by the modified QuantiFeron assay are also positive in our 96-well plate assays using diluted whole blood, providing the means to more efficient screening in large-scale epidemiological studies as has been used previously in studies of mycobacterial diseases [37,38,39]. Importantly too, our 96-well plate assay also showed that active VL patients were positive for IFN-\(\gamma\) in these diluted whole blood 96-well plate assays. Our initial
demonstration [23] that active VL patients are positive for IFN-γ in the modified Quantiferon assay was remarkable given the numerous previous studies that had failed to observe cellular proliferation or IFN-γ release after stimulation of peripheral blood mononuclear cells from active VL patients with crude Leishmania antigen [10,40,41,42]. Ability to measure this IFN-γ response in the diluted whole blood assay described here will also facilitate more efficient screening of active VL cases using smaller blood volumes in a 96-well plate format.

In human and murine cells infected in vitro, and in mice in vivo, clearance of Leishmania parasites requires IFN-γ. However, IFN-γ alone does not predict vaccine-mediated protection in mice [12,31,43,44]. Rather, the simultaneous production of IFN-γ, IL-2 and TNF-α by a particular subset of CD4 T cells [11], and/or the balance between pro-inflammatory IFN-γ/TNF-α and regulatory IL-10 [12,31,44,45], have been variously shown to be predictive of vaccine outcome. Epidemiological studies indicate that patients drug-cured from L. donovani infection are protected against subsequent clinical disease [46], and it is thought that exposed individuals who test as positive to crude leishmanial antigens in the modified Quantiferon assay employed in our study area in India are infected asymptomatic individuals who are resistant to developing active VL disease [6]. Therefore, in the analysis of human immune responses to known and novel antigens presented here, we hypothesized that ability to stimulate IFN-γ, TNF-α and IL-10 in cured VL and EHC+ve individuals, compared to active VL cases, would provide some insight into their potential as vaccine candidates.

Our investigations focused initially on the known vaccine candidates TRYP and LACK. Although others have found LACK protective in murine models of cutaneous leishmaniasis [47], in the virulent model of visceralising L. major LV39 infection in mice we found that TRYP was protective but LACK was not [12]. Although the vaccine-induced IFN-γ responses were similar between the two antigens in mice, lower IL-10 was elicited by TRYP than LACK, resulting in higher IFN-γ to IL-10 ratios as correlates of protective immunity. In the human studies described here, we found that TRYP and LACK were equivalent to each other in the magnitudes of IFN-γ, TNF-α and IL-10 responses elicited, and in generating higher IFN-γ to IL-10 and TNF-α to IL-10 ratios in putatively protected cured VL and EHC+ve individuals than in active VL cases. It was of interest that in India, the asymptomatic EHC+ve group had equivalent responses to the cured VL group, whereas in our recent study [28] of the same antigens (and antigen preparations) in Brazil, we found that asymptomatic DTH+ve individuals had lower ratios of IFN-γ to IL-10 and TNF-α to IL-10 compared to cured VL patients. This was due to higher IL-10 responses in the DTH+ve group compared to the cured VL group, leading us to suggest that a measure of modulation of the pro-inflammatory response by IL-10 in the figure.
DTH\(^{\text{ve}}\) might contribute to the protective response. In active VL disease, high levels of TNF-\(\alpha\) contribute to fever and cachexia, and are detrimental [29], and it is not yet known what role in pathogenesis is played by the strong 24 hour IFN-\(\gamma\) responses observed in whole blood assays in active VL [6,23].

In our analysis of novel vaccine candidates, we found that 5 antigens (R71, L37, N52, J41 and M22) elicited IFN-\(\gamma\) and TNF-\(\alpha\) responses in a high percentage of cured VL (55–87.5%) and EHC\(^{\text{ve}}\) (40–65%) subjects. This represents remarkable replication of recent findings from an area endemic for \(L.\) infantum chagasi in

Cytokine Responses to Novel Leishmanial Antigens

Figure 4. LACK-specific cytokine release in whole blood assays. Box plots (Tukey) for LACK recombinant protein (10 \(\mu\)g/mL) stimulated cytokine release at (A–E) 24 hours, (F–G) 72 hours, and (K–O) 6 days post stimulation in active VL (n = 8), cured VL (n = 20) and EHC\(^{\text{ve}}\) (n = 20) study groups (full data for all study groups are provided in Fig. S3). Data are presented for each cytokine response at the different time points (A,F,K IFN-\(\gamma\); B,G,L TNF-\(\alpha\); C,H,M IL-10) as well as for the ratios of IFN-\(\gamma\) to IL-10 (D,I,N) and TNF-\(\alpha\) to IL-10 (E,J,O). Antigen-stimulated cytokine responses are provided after subtraction of the non-stimulated control wells. Statistical differences between groups determined using the non-parametric Mann-Whitney test are indicated by bars above columns, * indicates \(p<0.05\), ** \(p<0.01\), and *** \(p<0.001\).

Figure 5. IFN-\(\gamma\) responses to peptide pools for 11 novel defined leishmania antigens. Bar graphs indicating the percentage of responders to peptide pools (5 \(\mu\)g/mL) for each of 11 novel antigens (A) at 24 hours, 72 hours and 6 days post stimulation for the cured VL group, and (B) across active VL (n = 8), cured VL (n = 16), EHC\(^{\text{ve}}\) (n = 20) and EHC\(^{\text{ve}}\) (n = 9) groups at the 72 hour time point. Numbers immediately under the bars indicate the percent identity between the originally sequenced \(L.\) major proteins and their \(L.\) infantum orthologues. See also tables S1 and S2.

Figure 5. IFN-\(\gamma\) responses to peptide pools for 11 novel defined leishmania antigens. Bar graphs indicating the percentage of responders to peptide pools (5 \(\mu\)g/mL) for each of 11 novel antigens (A) at 24 hours, 72 hours and 6 days post stimulation for the cured VL group, and (B) across active VL (n = 8), cured VL (n = 16), EHC\(^{\text{ve}}\) (n = 20) and EHC\(^{\text{ve}}\) (n = 9) groups at the 72 hour time point. Numbers immediately under the bars indicate the percent identity between the originally sequenced \(L.\) major proteins and their \(L.\) infantum orthologues. See also tables S1 and S2.
northern Brazil, where 4 of these antigens (R71, L37, N52 and M22; same preparations of peptide pools) also elicited strong IFN-γ and TNF-α responses in both cured VL and exposed asymptomatic DTH+ individuals [28]. In Brazil, responses to J41 were only observed in the cured VL group, but the sample size for DTH+ individuals was small (n = 4). Strong responses were also observed in Brazil to two additional antigens, L302.06 and M18, for which a lower percentage (<30%) of responders were observed in India. This may reflect small samples sizes, differences in amino acid sequences of the parasites, and/or differences in HLA alleles between the two populations. On balance, all of these antigens remain strong candidates in the context of a multivalent cross-species vaccine against leishmaniasis. R71 and L37 are ribosomal proteins with high (100 and 99%, respectively) percentage identity at the amino acid level between L. major and L. infantum [28]. N52 is a V-ATPase subunit F which also has high (94%) identity across the two species. J41 and M22 are hypothetical proteins of unknown function which, despite lower percent identities (73% and 61%, respectively) between L. major and L. infantum, appear to provide cross-reactive epitopes that are recognised in both Brazil [28] and India. An important contrast between the two endemic regions was the almost complete lack of IL-10 responses to these novel antigens (same preparations of peptide pools) in the Indian study in both cured VL and EHC-ve groups, whereas in the Brazilian study cured VL subjects who were positive for IFN-γ and TNF-α responses also produced IL-10. N52 was also unique in being the only antigen to stimulate IL-10 responses in active VL patients, suggesting that responses to this antigen might provide an important early diagnostic biomarker for disease-associated IL-10 in VL. Further studies are needed to evaluate more carefully the differences in cytokine responses to individual antigens in active compared to cured VL groups, as well as between cured VL and exposed asymptomatic DTH+ or modified Quantiferon positive groups. Unlike Brazil [4,5], DTH responses have not provided a sensitive means of evaluating cell mediated immune response in cured VL or exposed individuals in India [48], pointing to potential differences in cell-mediated responses between DTH+ compared to Quantiferon positive exposed asymptomatic individuals that might hold the key to uncovering the true correlates of vaccine-induced immunity in leishmaniasis.

Results of our study demonstrate that only a percentage of individuals respond to vaccine antigens that have individually been shown to be protective in mice. This suggests that defined vaccine for use in humans will need to be complex multi-epitope/antigens...
vaccines. To date, only one multicomponent vaccine, Leish-111f, has been assessed in a large clinical trial [49]. Our recent small-scale clinical trial in a L. donovani endemic area showed Leish-F1-MPL-SE was safe and well tolerated in people with and without prior VL exposure and induced strong antigen-specific T cell responses [36]. The data presented here, and in our earlier study from Brazil [28], provide evidence to support a number of novel candidates that could be taken forward as vaccines against human leishmaniasis.

Supporting Information

Figure S1 Box plots (Tukey) for IFN-γ production by peripheral whole blood cells in response to SLA (an Indian L. donovani strain, 10 μg/mL), or PPD (5 μg/mL) as measured by ELISA. The IFNγ responses in (A) the active VL group (n = 8) are compared with (B) the cured VL group (n = 16), over 24 hours, 72 hours and 6 days post stimulation with SLA or PPD. The data presented in main figure 1 are a subset of the data presented here. Statistical differences between groups determined using the non-parametric Man-Whitney test are indicated by bars above columns, * indicates p<0.05, ** p<0.01, and *** p<0.001.

Figure S2 Box plots (Tukey) for TRYP recombinant protein (10 μg/mL) stimulated cytokine release at (A–E) 24 hours, (F–G) 72 hours, and (K–O) 6 days post stimulation in active VL (n = 8), cured VL (n = 20), EHC+ve (n = 20) and EHC-ve (n = 9) study groups. Data are presented for each cytokine response at the different time points (A,F,K IFN-γ; B,G,L TNF-α; C,H,M IL-10) as well as for the ratios of IFN-γ to IL-10 (D,L,N) and TNF-α to IL-10 (E,J,O). Statistical differences between groups determined using the non-parametric Man-Whitney test are indicated by bars above columns, * indicates p<0.05, ** p<0.01, and *** p<0.001.

Figure S3 Box plots (Tukey) for LACK recombinant protein (10 μg/mL) stimulated cytokine release at (A–E) 24 hours, (F–G) 72 hours, and (K–O) 6 days post stimulation in active VL (n = 8), cured VL (n = 20), EHC+ve (n = 20) and EHC-ve (n = 9) study groups. Data are presented for each cytokine response at the different time points (A,F,K IFN-γ; B,G,L TNF-α; C,H,M IL-10) as well as for the ratios of IFN-γ to IL-10 (D,L,N) and TNF-α to IL-10 (E,J,O). Statistical differences between groups determined using the non-parametric Man-Whitney test are indicated by bars above columns, * indicates p<0.05, ** p<0.01, and *** p<0.001.

Figure S4 Dot plots showing individual cytokine responses in subjects from active VL (n = 8), cured VL (n = 16), EHC+ve (n = 20) and EHC-ve (n = 9) groups 72 hours post stimulation of whole blood assays with peptide pools (5 μg/mL) for the 5 antigens R71, L37, N52, J41, and M22. Data are presented for each cytokine response (A–E IFN-γ; F–J TNF-α; K–O IL-10). Bars indicate the mean group response. Statistical differences between groups determined using the non-parametric Man-Whitney test are indicated by bars above columns, * indicates p<0.05, ** p<0.01, and *** p<0.001.

Table S1 IFN-γ responses in individuals cured from VL at 24 hours, 72 hours, and 6 days after stimulation of whole blood ex vivo with candidate vaccine antigens (peptide pools; 5 μg/mL), SLA (10 μg/mL), PPD (5 μg/mL), or mitogen PHA (5 μg/mL). IFN-γ levels were measured by ELISA. Percent identity with L. infantum sequence at the amino acid level is indicated below candidate vaccine antigens (R71, Q51, etc.), which were originally derived from L. major.

Table S2 IFN-γ responses in EHC +ve (EC-01 to EC-20), active VL (VI-1 to VL-8) and EHC-ve (EC-42 to EC-50) individuals at 72 hours after stimulation of whole blood ex vivo with candidate vaccine antigens (peptide pools; 5 μg/mL), SLA (10 μg/mL), PPD (5 μg/mL), or mitogen PHA (5 μg/mL). IFN-γ levels were measured by ELISA.

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Author Contributions

Conceived and designed the experiments: CBS JMB. Performed the experiments: OPS CBS JMB. Analyzed the data: OPS JMB. Contributed reagents/materials/analysis tools: CBS JMB. Wrote the paper: OPS CBS JMB. Supervised the research: SS.

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