Cytokine Responses to Novel Antigens in a Peri-Urban Population in Brazil Exposed to *Leishmania infantum chagasi*

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Abstract. Visceral leishmaniasis (VL) is fatal if untreated, and there are no vaccines for this disease. High levels of CD4-derived interferon-γ (IFN-γ) in the presence of low levels of interleukin-10 (IL-10) predicts vaccine success. Tumor necrosis factor-α (TNF-α) is also important in this process. We characterized human immune responses in three groups exposed to *Leishmania infantum chagasi* in Brazil: 1) drug-cured VL patients (recovered VL); 2) asymptomatic persons with positive *Leishmania*-specific delayed-type hypersensitivity skin reactions (DTH+); and 3) DTH-negative household contacts. Magnitude of DTH correlated with crude *Leishmania* antigen–driven IFN-γ, TNF-α, and IL-5, but not IL-10. DTH+ persons showed equivalent levels of IFN-γ, but higher levels of IL-10, to tryparedoxin peroxidase and *Leishmania* homolog of receptor for activated C kinase compared with recovered VL patients. The IFN-γ:IL-10 and TNF-α:IL-10 ratios were higher in recovered VL patients than in DTH+ persons. Seven of 11 novel candidates (R71, L37, N52, L302.06, M18, J41, and M22) elicited cytokine responses (36–71% of responders) in recovered VL patients and DTH+ persons. This result confirmed their putative status as cross-species vaccine/immunotherapeutic candidates.

INTRODUCTION

*Leishmania* are protozoan parasites that cause severe and debilitating cutaneous, and fatal visceral disease in subtropical/tropical regions of Old and New Worlds. Leishmaniasis affects 12 million persons, and there are an estimated 1.5 million new cases annually. Overall disease burden is calculated by World Health Organization as 1.98 million disability-adjusted life years. There are no vaccines available. Chemotherapy is toxic and expensive, and a limited number of drugs are available to which drug resistance is documented. Spontaneous or drug-induced recovery from leishmaniasis is usually accompanied by immunity against re-infection, which provides a rational basis for developing vaccines against human disease.† Traditional use of live parasites (leishmanization) to induce lesions and scars in a preferred body site was continued until relatively recently. However, adverse reactions in susceptible persons and the increase in human immunodeficiency virus–associated immunocompromised patients necessitated caution against its continued use as a vaccine regimen. Similarly, the use of any live attenuated strain of *Leishmania* as a vaccine could be problematic, given an increase in incidence of human immunodeficiency virus infection in disease-endemic countries.

Alternative strategies using killed organisms administered with/without Bacille Calmette-Guérin with or without alum have been used with limited success. The only common thread to all of these killed parasite vaccine trials has been a measure of protection observed in persons who show conversion of skin test results during the trial (whether or not they received the vaccine), but no overall statistically significant projection has been associated with any trial.‡–⁶ Therefore, further characterization of immune responses associated with natural conversion to a delayed-type hypersensitivity (DTH) response to leishmanial antigen in disease-endemic areas, or with drug-induced recovery from clinical disease, could provide important clues to the type of immunity that a successful vaccine will need to elicit.

The approximately 33.6-Mb genome (approximately 8,300 protein-coding genes) of *Leishmania major* has been sequenced⁷ and provides a rich source of potential vaccine candidates. We recently described the identification of novel *Leishmania* antigens delivered as DNA vaccines to susceptible BALB/c mice, having undertaken a screen of 100 amastigote-expressed genes.⁸ Antigens selected using this high-dose footpad challenge model of infection have been shown to confer protection against *L. major* in non-human primates.⁹ We have also explored immunologic correlations of protection elicited by vaccination with the *Leishmania* homolog of the receptor for activated C kinase (LACK)¹⁰ and tryparedoxin peroxidase (TRYP)¹¹ in the BALB/c mouse model. We showed that high pre-challenge with interleukin-10 (IL-10) in the presence of high interferon-γ (IFN-γ) levels predicted vaccine failure after vaccination with LACK.¹² In contrast, low levels of antigen-specific IL-10 and equivalent IFN-γ responses correlated with protection from disease. These differences were further magnified after challenge infection, with TRYP-vaccinated mice showing high IFN-γ to low IL-10 levels, and LACK-vaccinated mice showed low IFN-γ to high IL-10. A strong TNF-α response, concurrent with IFN-γ, has also been shown to be an important correlate of protective immunity after prime-boost vaccination with the LACK antigen in a low-dose intradermal model of *L. infantum* infection in mice.¹³

Previous human studies have characterized persons living in disease-endemic communities according to clinical visceral leishmaniasis (VL), and those who have been exposed but are resistant to disease on the basis of a positive *Leishmania*-specific Montenegro DTH skin test response.¹⁴ A third group of persons includes those who are DTH negative in response to *Leishmania* skin test antigen, i.e., show no evidence of exposure despite having resided for an extended period (e.g., >3 years) in households with VL cases or DTH-positive persons.¹⁴
In this study, immune responses to a crude preparation of crude *Leishmania* antigen (CLA), recombinant LACK and TRYp proteins, and pools of overlapping peptides from each of 11 novel *Leishmania* antigens are examined in these three groups. We show that magnitude of DTH responses across all groups correlated with CLA-driven IFN-γ and TNF-α, and to a lesser extent, IL-5 responses, but not with IL-10. Recovered VL and DTH+ groups had equivalent CLA-driven TNF-α, IFN-γ, and IL-5 responses. The DTH+ group had equivalent IFN-γ responses to TRYp and LACK, but had higher IL-10 responses compared with recovered VL patients. Thus, IFN-γ: IL-10 and TNF-α:IL-10 ratios are higher in recovered VL patients than in DTH+ persons. Finally, we show that 7 of 11 novel candidates (R71, L37, N52, L302.06, M18, J41, and M22) elicited cytokine responses (36–71% responders) in recovered VL and DTH+ groups, confirming their putative candidacy as cross-species vaccine or immunotherapeutic targets.

**MATERIALS AND METHODS**

**Study area and study participants.** As reported, the study site was the peri-metropolitan area surrounding Natal, Brazil, a city with 700,000 persons within the state of Rio Grande do Norte, which has been a disease-endemic focus for VL since the mid-1980s. Patients with VL and neighborhoods with ongoing transmission were identified by reviewing hospital admission notes of patients admitted to the public hospitals, or medical records maintained by the National Brazilian Foundation for Health.

**Ethical approval.** All patients and contacts participating in the study provided written consent if adults, or this was supplied by a parent or guardian if <18 years of age. Research protocols were approved by the Institutional Review Boards of the Universidade Federal do Rio Grande do Norte, the Comission Nacional de Ética em Pesquisa, the University of Iowa, Johns Hopkins University, the University of Virginia and the National Human Genome Research Institute, National Institutes of Health. The Brazilian Institutional Review Board approved by the National Institutes of Health. Persons with medical conditions detected during the study were treated by the study team or referred or taken to the appropriate medical facility in Natal.

**Delayed-type hypersensitivity or Montenegro skin test.** The DTH response or Montenegro skin test was assessed by using 25 µg of *L. chagasi* antigens provided by the Centro de Produção e Pesquisa de Immunobiológicos (Curitiba, Paraná, Brazil). Forty-eight to 72 hours after skin test application, the area of induration was measured by using the ball point pen technique. Induration diameter ≥5 mm is considered a positive response, indicating prior *Leishmania* exposure.

**Phenotype definition.** Study participants were divided into sub-groups as described. The first group included persons who had recovered from VL (recovered VL, n = 16), which was verified by clinical history, laboratory validation such as positive bone marrow smear for parasites, positive anti-*Leishmania* serologic results, and response to treatment. Only treated patients were included in the study, and these patients had median of 7.5 years (interquartile range = 0.8–11.0 years) between disease treatment and study participation. The second group included DTH positive (DTH+, n = 18) persons who had a negative history of VL and a positive Montenegro skin test result, defined as ≥5 mm induration 48–72 hours after administration of *Leishmania* antigen. Previous studies show that a positive test result correlates with disease resolution and protection against subsequent re-infection. This DTH+ group represented exposed but asymptomatic household contacts. The third group were persons who had negative *Leishmania* serologic results and a negative DTH response (DTH−, n = 5) living in a household with a VL case or DTH+ persons. Not all study participants in these groups were included for all investigations, as detailed in the figures. A fourth group of persons who were DTH− but had positive serologic results has been described, possibly reflecting acute infection. However, this group was not evaluated in this study.

**Preparation of crude, recombinant, and peptide antigens.** Crude freeze-thawed *Leishmania* parasite antigen was prepared as described from stationary phase promastigotes of *L. chagasi* strain MHOM/BR/00/1669, *L. major* strain MRHO/SU/59/P (LV39), or *L. donovani* strain MHOM/ET/ 67/HU3 (LV9) by resuspension in 10 mM Tris-HCl, pH 8.5, 0.51 M NaCl, 1 mM phenylmethylsulfonyl fluoride, and 50 µg/mL of leupeptin, and freezing-thawing three times over liquid nitrogen. Recombinant LACK and TRYp proteins Table 1 were prepared as described with large-scale preparation, endotoxin removal, and protein estimation outsourced to

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*CDs = crude *Leishmania* antigen. Sizes of the full-length CDs are as predicted for the *L. major* Friedlin genomic sequence and amino acid sequences for which are compared with public domain (GenoDB) genomic sequences for *L. infantum*. M57 and M63 show low amino acid identity (with low confidence) between *L. major* and *L. infantum*. 

**664 STOBER AND OTHERS**
Novexin Ltd. (Cambridge, United Kingdom). Overlapping 13–20-mer peptides (minimal overlap of 12 amino acids, 7–31 peptides/antigen depending on amino acid length of the protein) representing 11 (Table 1: R71, Q51, L37, N52, L302.06, J89, M18, J41, M22, M63, M57) of 14 novel antigens identified, were synthesized commercially (Peptide2.0, Chantilly, VA), initially solubilized in dimethylsulfoxide, and pooled (for peptides within each antigen) in endotoxin-free phosphate-buffered saline at a final concentration of 50 μg/mL per individual peptide. Peptides pools were stored at −80°C.

Whole blood assay. Whole blood was collected into heparinized tubes and samples were diluted 1 in 5 in serum-free complete medium comprising RPMI 1640 medium supplemented with 2 mM l-glutamine, 100 μg/mL of streptomycin, and 100 IU/mL of penicillin (Gibco BRL, Sao Paulo, Brazil), and 100 μL was plated into 96-well U-bottomed plates (Nunc, Rochester, NY). Cells were stimulated in quadruplicate with 100 μL of medium of low (negative control), 10 μg/mL of recombinant TRYP or LACK antigen, 10 μg/mL of CLA, 5 μg/mL of phytohemagglutinin-M (Sigma-Aldrich, St Louis, MO), or pooled peptide representing each antigen at a final concentration of 5 μg/mL. Cell cultures were incubated at 37°C in an atmosphere of 5% CO2 for 24 hours, 72 hours, or 6 days. Supernatants were harvested and stored at −80°C until analysis by enzyme-linked immunosorbent assay (ELISA).

IgG and cytokine ELISA. Antibodies (IgG) to L. chagasi were detected by using an ELISA with Brazilian L. chagasi strain MHOM/BR/00/1669 or recombinant rK39 (kindly provided by Steven Reed; Infectious Disease Research Institute, Seattle, WA) as the source of antigen as described. Results are expressed as optical densities. Control known positive (active patient) and negative (non- endemic donor) serum samples were run on each plate, and the cut-off for a positive response was determined to be 3 SD above the control negative response. Levels of IFN-γ, TNF-α, IL-10, or IL-5 were measured by using matched antibody pairs (BD Pharmingen, Franklin Lakes, NJ) and an ELISA.

Statistical analysis. Because date were not normally distributed, statistical differences (P < 0.05) between the groups were determined by using nonparametric tests, the Kruskall-Wallis test for analysis of variance across multiple groups, and a two-tailed Mann-Whitney test for comparisons between pairs of groups. Correlation was estimated by using Pearson’s correlation coefficient.

RESULTS

Correlation of K39-specific IgG levels with clinical disease in symptomatic VL patients. Clinical disease is associated with hypergammaglobulinemia, which has been used to develop diagnostic assays. Emergence of antibodies to the Leishmania antigen K39 is highly sensitive and predictive of the onset of acute disease, and antibody levels decrease after successful treatment, only being detected in approximately 3% of DTH-asymptomatic persons. In concordance with this finding, K39- and CLA-specific antibodies were detected only in the recovered VL patient group Table 2. Across the entire recovered VL group, time since active disease showed a negative correlation with antibody levels to K39 (R = 0.62, P = 0.099) and CLA (R = 0.69, P = 0.058) antigens. Thus, as in previous studies, antigen-specific IgG responses correlated with past clinical disease in exposed persons, and the magnitude of the IgG response decreased with time since active disease.

Correlation of CLA-mediated cytokine release with DTH. Studies have shown that a positive DTH response is associated with protection from leishmaniasis. We determined whether DTH responses to intra-dermal Leishmania antigen (measured in millimeters) correlated with cytokine release (measured in pg/mL) from whole blood cells stimulated ex vivo with CLA. The correlation of DTH responses with IFN-γ, TNF-α, IL-5, or IL-10 release across all study participants is shown in Table 3. IFN-γ (R = 0.55, P = 0.021), TNF-α (R = 0.56, P = 0.020), and IL-5 (R = 0.42, P = 0.092) release from cells stimulated with L. chagasi CLA showed a positive correlation with DTH, but IL-10 (R = 0.17, P = 0.504) did not show a positive correlation. The magnitude and pattern of cytokine release was comparable independently of whether CLA was derived from L. chagasi, L. major, or L. donovani.
suggesting sharing of common dominant antigens across the Old and New World *Leishmania* species.

Within the three study groups, there was significant release of the pro-inflammatory cytokine IFN-$\gamma$ (Figure 1B) by cells derived from recovered VL patients ($P = 0.039$) and asymptomatic DTH+ contacts ($P = 0.005$) in pairwise comparisons relative to the DTH- household contact group ($P = 0.018$ across the three groups, by nonparametric Kruskall-Wallis analysis of variance). A similar trend was observed for TNF-$\alpha$ (Figure 1A; $P = 0.09$ for VL patients and asymptomatic DTH+ contacts pairwise comparisons relative to DTH–persons). In contrast, IL-5 responses were not significantly different among study groups (Figure 1C), and IL-10 levels were equivalently low (< 50 pg/mL) in all groups in response to stimulation with CLA.

**Cytokine responses to TRYP and LACK in recovered VL and asymptomatic DTH+ study groups.** We have demonstrated that high CD4-derived IFN-$\gamma$ to low IL-10 predicted vaccine success in mice when comparing TRYP and LACK as vaccine candidates.12,24 We examined immune responses to these well-characterized antigens in recovered VL patients and DTH+ contacts. The magnitude of TNF-$\alpha$ (Figure 2A)
and IFN-γ (Figure 2D) was equivalent for VL patients relative to DTH+ contacts after stimulation with TRYP or LACK. In contrast, IL-10 responses (Figure 2B and E) were higher for DTH+ contacts than for treated VL patients, resulting in higher ratios of TNF-α:IL-10 (Figure 2C) and IFN-γ:IL-10 (Figure 2F) for the recovered VL group than for DTH+ contacts. The magnitude of cytokine responses to LACK and TRYP did not differ significantly from each other, resulting in similar ratios of TNF-α:IL-10 (Figure 2C) and IFN-γ:IL-10 (Figure 2F).

Antigen-specific cytokine release in response to novel vaccine antigens. We have identified 14 protective Leishmania antigens during a screening of 100 candidates delivered as DNA vaccines in mice. 8 We examined cytokine responses to 11 of the 14 antigens delivered as peptide pools in whole blood assays for the recovered VL, DTH+, and DTH− groups Table 4. Nine of the 11 antigens elicit variable levels of release of all three cytokines in recovered VL patients (Figure 3). In general, the same 11 antigens elicited responses for all three cytokines. Failure to observe cytokine responses (Figure 3 and Table 4) to M63 and M57 is consistent with low amino acid sequence identity (Table 1) for these proteins when the L. infantum sequence is compared with the L. major sequence on which design of the peptide pools was based. In this respect, they act as good negative controls for peptide preparations. However, ability to elicit a strong cytokine response was not directly related to identity at the amino acid level. For example, two of the most potent antigens (M18 and M22) showed only 78% and 61% identity, respectively, although with high confidence (P value) compared with M63 and M57 (Table 1). This finding indicates that there are strong cross-reactive epitopes across Leishmania species, which is of importance in relation to the potential for development of a cross-species vaccine.

There was clear individual variability in response to peptide pools from the novel vaccine candidates (Figure 3) that relates in part to history of exposure (Table 4), but this finding could also reflect important genetic differences in ability of HLA class II molecules to bind epitopes from these antigens for presentation to T cells. 20 Certain persons showed strong responses across the full range of antigens (e.g., persons V4, V7, and D2) (Table 4). These persons were not necessarily those with the strongest DTH responses, and included one person with a recent history of disease (<12 months) but DTH negative, one person with a long-term previous case (10 years) with a strong DTH+ response, and one (D2; Table 4) of the DTH+ asymptomatic persons. Across all recovered VL and DTH+ study groups, antigens R71, L37, N52, L302.06, M18, J41, and M22 stood out as potential candidate antigens because they elicited IFN-γ (Figure 3C and 36–71% responders; Table 4), TNF-α (Figure 3A) and IL-10 (Figure 3B) responses. Antigen J89 was of interest in eliciting responses across all study groups, suggesting that it might be more mitogenic than antigenic in eliciting non-specific responses. Of interest, however, one donor (H3, Table 4, DTH = 4 mm) classified as DTH− by World Health Organization criteria (i.e., DTH < 5 mm) showed responses to several peptide pools, including L302.06, J89, M18, and M22. An alternative explanation is that J89 might be more sensitive for early detection of exposure to infection, which could only be resolved by studying a larger sample of exposed DTH− persons.

DISCUSSION

Vaccination against leishmaniasis has met with great success in mice by using killed whole parasites and crude recombinant vaccine models. 26,27 Disappointedly, some of the same vaccine protocols have met with limited success in humans. 28 However, the potential for vaccination of humans has always been considered high, given the observation that spontaneous or drug-induced recovery from leishmaniasis is usually accompanied by immunity against re-infection. 1 A common thread in killed whole parasite vaccine trials in humans has been a

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*Whole blood cells were stimulated ex vivo with phytohemagglutinin (PHA) (1 μg/mL), crude Leishmania antigen (CLA) (10 μg/mL), or peptide pools (5 μg/mL). Supernatants were harvested at 72 hours, and IFN-γ levels were determined by enzyme-linked immunosorbent assay. Antigen names are indicated with % amino acid identity (see Table 1) between L. infantum and L. major indicated beneath them. Time corresponds to time from clinical diagnosis/treatment commencement; mm = millimeters of induration for the DTH response; ++ = 20–100 pg/mL; ++ = 100–500 pg/mL; +++ = 500–1,000 pg/mL; +++ = > 1,000 pg/mL; ND = not determined.

†No significant difference between PHA responses when donor groups were compared.

§Based on recovered visceral leishmaniasis patients and DTH+ groups.
Few doubt the need for a pro-inflammatory cytokine response, specifically IFN-γ and TNF-α, to cure persons with leishmaniasis. Accordingly, we observed strong responses for these two cytokines to crude and recombinant antigen preparations in persons exposed to L. chagasi infection in Brazil, and the response to crude antigen showed a direct correlation with the magnitude of the DTH response. However, although these cytokines are necessary for a protective immune response, they alone are not good predictors of vaccine success or failure in murine models. Instead, the degree of modulation of their action by the regulatory cytokine IL-10 appears to be crucial. Thus, in a model in which we protected highly susceptible BALB/c mice against virulent visceralizing high-dose L. major infection with TRYP antigen delivered as a DNA or DNA/modified vaccine Ankara prime-boost vaccine, magnitude of IFN-γ alone was not the important predictor of vaccine success.

Instead, the ratio of IFN-γ to IL-10 (and to a lesser extent, IL-4 or IL-5) provided the best correlate of protective immunity. Others investigators have also observed this finding in murine models of vaccination against Leishmania spp., in addition to determining that TNF-α concurrent with IFN-γ also provides a good correlation of protection against L. infantum.

In the virulent model of visceralizing L. major infection used in our studies, we found that TRYP was protective but LACK was not protective. Although the magnitude of pre-challenge IFN-γ was equivalent after vaccination with these two antigens, levels of IL-10 were higher in LACK-vaccinated mice. Thus, the best correlate of protection was the higher ratio of IFN-γ/IL-10 with TRYP vaccination. In the work presented here, magnitudes of IFN-γ and IL-10 cytokine responses to TRYP and LACK were equivalent to each other within recovered VL and asymptomatic DTH+ groups studied, indicating that both groups might provide equivalent potential as vaccine candidates for human disease.

Of perhaps greater interest was the observation of higher ratios of IFN-γ to IL-10 and TNA-α to IL-10 for LACK and TRYP recombinant antigens in recovered VL patients than in DTH+ asymptomatic persons. This finding suggests a measure of modulation of the immune response to the parasite in the DTH+ group, who did not succumb to clinical disease. In human active VL, high levels of TNA-α contribute to fever and cachexia, and are detrimental. Until recently, it was also believed that active VL patients failed to show IFN-γ responses to recall antigen in cells from peripheral blood, and that this anergy may be the main reason for susceptibility. More recently, whole blood quantiferon assays have demonstrated high levels of antigen-driven IFN-γ in active VL patients.

Although it is clear that high levels of IL-10 may mediate susceptibility by interfering with IFN-γ/TNF-α-mediated parasite killing by macrophages, a balanced response that includes IL-10 may also modulate pathologic changes associated with a too potent pro-inflammatory immune response. Asymptomatic DTH+ persons might have the correct magnitude of response and cytokine balance. A similar conclusion was drawn in comparing immune responses in 104 persons with asymptomatic infection after L. braziliensis infection compared with 29 patients with untreated cutaneous leishmaniasis. In that study, levels of IFN-γ and TNA-α in crude antigen-driven lymphocyte responses were lower in DTH+ persons with asymptomatic infections than in cutaneous
leishmaniasis patients, but IL-5 responses were higher. At the other extreme of *L. bразiliensis* infection, an excess pro-inflammatory response in the absence of sufficient IL-10 was associated with mucosal disease.34

In developing novel vaccines for leishmaniasis, one of the major issues is that of eliciting long-term memory responses that provide the correct balance of pro-inflammatory to regulatory cytokine responses.27 In the continuing search for potential vaccine candidates, we examined cytokine responses to a series of novel vaccine candidates in persons with a positive DTH response by testing persons with asymptomatic infections or recovered VL patients. We found that 7 of 11 novel candidates (R71, L37, N52, L302.06, M18, J41, and M22) elicited cytokine responses (36–71% responders). In all instances, positive IFN-γ and TNF-α responses were accompanied by IL-10 responses, indicating the potential for vaccine antigens to elicit a balance of pro-inflammatory and regulatory cytokine responses. Other researchers have also demonstrated type 1 pro-inflammatory IFN-γ responses to novel vaccine candidates in recovered VL patients, and antigens (cysteine proteinase B and sterol 24-c-methyltransferase) were the most protected in mice and showed the strongest signals in recovered VL patients.35

In our study M18, a high copy number tandemly arrayed member of the amastin gene family,6 was the most potent antigen in terms of percent responders and magnitude of immune response. However, a number of DTH+ persons and recovered VL patients also showed strong responses to almost the full array of our novel vaccine candidates, which include ribosomal proteins and hypothetical proteins of unknown function. Individual variability in response to different antigens is also an important observation, especially in relation to potential differences in the ability of HLA class II molecules to bind epitopes from different antigens, and the type of immune responses that might be driven by different class II molecules.25

Studies are in progress to examine immune responses to these candidate vaccine antigens in persons typed for HLA DRB1 and DQB1, which have been shown to influence susceptibility to primary infection.27 More research is also needed to understand how to deliver defined antigen vaccine candidates to humans to elicit protection associated with a balanced cytokine response.27,36 Nevertheless, it is clear that there is no shortage of potential vaccine candidates that have been identified through studies in mice and can now be used in human trials,7,28 including those reported in our study.

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