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

Carmel B. Stober, Selma M. B. Jeronimo, Nubia N. Pontes, E. Nancy Miller,[†] and Jenefer M. Blackwell*

Department of Medicine, University of Cambridge School of Clinical Medicine, Cambridge, United Kingdom; Department of Biochemistry, Bioscience Center, Universidade Federal do Rio Grande do Norte, Natal, Rio Grande do Norte, Brazil; Instituto de Ciência e Tecnologia de Doenças Tropicais, Natal, Rio Grande do Norte, Brazil; Cambridge Institute for Medical Research, University of Cambridge School of Clinical Medicine, Addenbrooke's Hospital, Cambridge, United Kingdom; Telethon Institute for Child Health Research, Centre for Child Health Research, University of Western Australia, Subiaco, Western Australia, Australia

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 sub-tropical/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.¹ Traditional use of live parasites (leishmanization) to induce lesions and scars in a preferred body site was continued until relatively recently.¹ 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.^{2–6} 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 (LACK¹⁰) and tryparedoxin peroxidase (TRYP¹¹) 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

*Address correspondence to Jenefer M. Blackwell, Telethon Institute for Child Health Research, University of Western Australia, PO Box 855, Subiaco, Western Australia 6872, Australia. E-mail: jblackwell@ichr.uwa.edu.au

[†]Deceased.

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,⁸ 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 candidature as cross-species vaccine or immunotherapeutic targets.

MATERIALS AND METHODS

Study area and study participants. As reported,¹⁴ 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.¹⁵ 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 Comissão 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.^{14,16} 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 test¹⁵ was assessed by using 25 μ g of *L. chagasi* antigens provided by the Centro de Produção e Pesquisa de Imunobiológicos (Curitiba, Parana, Brazil). Forty-eight to 72 hours after skin test application, the area of induration was measured by using the ball point pen technique.¹⁷ Induration diameter $\geq$ 5 mm is considered a positive response, indicating prior *Leishmania* exposure.¹⁸

Phenotype definition. Study participants were divided into sub-groups as described.^{14,16} 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 $\geq$ 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.^{18,19} 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.²⁰ 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 described¹² 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 μ g/mL of leupeptin, and freezing–thawing three times over liquid nitrogen. Recombinant LACK and TRYP proteins Table 1 were prepared as described,^{11,12} with large-scale preparation, endotoxin removal, and protein estimation outsourced to

TABLE 1
Characteristics of 13 antigens tested for immune responses in *Leishmania chagasi*-exposed persons, Brazil*

Clone	CDS (amino acids)	GeneDB accession no.	Size (kD)	%	% Identity with <i>L. infantum</i>		Putative function
					N/N amino acids	P	
TRYP	199	LmjF15.1040	22.1	91	182/199	2.3×10^{-99}	Tryparedoxin peroxidase
LACK	312	LmjF28.2740, LmjF28.2750	34.4	97	311/319	5.1×10^{-168}	Activated protein kinase C receptor
R71	129	LmjF36.4420, LmjF36.3270	15.0	100	129/129	1.1×10^{-65}	60S ribosomal L22
Q51	165	LmjF30.1750	18.7	100	21/21	2.6×10^{-7}	Hypothetical protein
L37	172	LmjF34.2780	19.4	99	169/170	9.7×10^{-92}	40S ribosomal S19
N52	137	LmjF12.0520	15.5	94	129/137	5.4×10^{-66}	V-ATPase subunit F
L302.06	258	LmjF04.0230	27.7	90	234/258	9.6×10^{-124}	Hypothetical protein
J89	65	LmjF26.1015	7.2	87	57/65	2.1×10^{-25}	Hypothetical protein
M18	199	LmjF08.0770	22.2	78	166/203	4.5×10^{-87}	Amastin-like protein
J41	189	LmjF33.1535	21.0	73	106/144	6.2×10^{-72}	Hypothetical protein
M22	78	LmjF29.1605	8.3	61	32/52	6.8×10^{-11}	Hypothetical protein
M63	47	LmjF33.2725	5.35	54	6/11	0.029	Hypothetical protein
M57	83	LmjF20.1375	9.48	37	18/48	0.27	Hypothetical protein

*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 (GeneDB) genomic sequences for *L. infantum*. M57 and M63 show low amino acid identity (with low confidence) between *L. major* and *L. infantum*.

TABLE 2

IgG responses in recovered VL patients (n = 10), DTH+ asymptomatic contacts (n = 4), or DTH- household contacts (n = 4), Brazil*

Antibody	Recovered VL, mean $\pm$ SD OD	DTH+ contacts, Mean $\pm$ SD OD	DTH- contacts, Mean $\pm$ SD OD	P value, VL vs. DTH+, VL vs. DTH-, DTH+ vs. DTH-
K39 IgG	1.09 $\pm$ 0.793	0.07 $\pm$ 0.045	0.08 $\pm$ 0.024	0.008†, 0.009†, 0.729
CLA IgG	0.17 $\pm$ 0.097	0.04 $\pm$ 0.014	0.05 $\pm$ 0.011	0.034‡, 0.047‡, 0.485

*Serum samples were obtained by venipuncture before intradermal administration of *Leishmania* antigen to determine the delayed-type hypersensitivity (DTH) response. Levels of IgG specific for K39 or crude *Leishmania* antigen (CLA) are given as optical density (OD) readings.

† $P < 0.01$

‡ $P < 0.05$ indicates a significant difference in IgG levels between study groups.

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,⁸ 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 μ g/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 μ g/mL of streptomycin, and 100 IU/mL of penicillin (Gibco BRL, Sao Paulo, Brazil), and 100 μ L was plated into 96-well U-bottomed plates (Nunc, Rochester, NY). Cells were stimulated in quadruplicate with 100 μ L of medium alone (negative control), 10 μ g/mL of recombinant TRYP or LACK antigen, 10 μ g/mL of CLA, 5 μ g/mL of phytohemagglutinin-M (Sigma-Aldrich, St Louis, MO), or pooled peptide representing each antigen at a final concentration of 5 μ g/mL. Cell cultures were incubated at 37°C in an atmosphere of 5% CO_2 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.²¹ 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 data were not normally distributed, statistical differences ($P < 0.05$) between the groups were determined by using nonparametric tests, the Kruskal-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.^{20,22,23} 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.²⁰ 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,^{15,21} 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.^{18,19} 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*

TABLE 3

Correlation of DTH response with IFN- γ , TNF- α , and IL-5, but not with IL-10 release across study participants from the three patient groups (n = 10 recovered from visceral leishmaniasis; n = 4 DTH+; n = 4 DTH-), Brazil*

Source of CLA	Cytokines			
	IFN- γ	TNF- α	IL-5	IL-10
<i>L. chagasi</i>	0.55, $P = 0.021$ †	0.56, $P = 0.020$ †	0.42, $P = 0.092$	0.17, $P = 0.504$
<i>L. major</i>	0.60, $P = 0.011$ †	0.62, $P = 0.008$ ‡	0.50, $P = 0.041$ †	0.13, $P = 0.629$
<i>L. donovani</i>	0.57, $P = 0.017$ †	0.66, $P = 0.004$ ‡	0.41, $P = 0.103$	0.29, $P = 0.252$

*Delayed-type hypersensitivity (DTH) in millimeters was determined 48–72 hours after intra-dermal administration of *Leishmania* antigen. Whole blood cells obtained by venipuncture before administration of skin test antigen were stimulated *ex vivo* with 10 μ g/mL of crude *Leishmania* antigen (CLA) derived from *L. chagasi*, *L. major*, or *L. donovani*. Supernatants were harvested at 72 hours and cytokines measured by enzyme-linked immunosorbent assay. The DTH responses were correlated with cytokine levels across the patient groups, and analyzed by using Pearson's correlation coefficient. IFN- γ = interferon- γ ; TNF- α = tumor necrosis factor- α ; IL-5 = interleukin-5; IL-10 = interleukin-10.

† $P < 0.05$ indicates a significant correlation between DTH response and cytokine of interest.

‡ $P < 0.01$ indicates a significant correlation between DTH response and cytokine of interest.

FIGURE 1. Box and whiskers plots (Tukey) for cytokine responses to crude *Leishmania* antigen (CLA) in recovered visceral leishmaniasis (VL) ($n = 16$) compared with delayed-type hypersensitivity (DTH)+ ($n = 18$) and DTH- ($n = 5$) groups, Brazil. Whole blood cells were stimulated *ex vivo* with 10 $\mu\text{g/mL}$ of CLA derived from *Leishmania chagasi*. Supernatants were harvested at 24 hours for tumor necrosis- α (TNF- α) (A), or at 6 days for interferon- γ (IFN- γ) (B) and interleukin-5 (IL-5) (C), and analyzed by enzyme-linked immunosorbent assay. P values denote between group differences as determined by using the nonparametric Mann-Whitney test. Analysis of variance across all three groups by using the nonparametric Kruskal Wallis test was significant ($P = 0.018$) for IFN- γ .

(Table 3), 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- γ (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 Kruskal-Wallis analysis of variance). A similar trend was observed for TNF- α (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 ($< 50 \text{ pg/mL}$) 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- γ 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- α (Figure 2A)

FIGURE 2. Box and whiskers plots (Tukey) for showing that trypanredoxin peroxidase (TRYP)- and *Leishmania* homolog of the receptor for activated C kinase (LACK)-mediated tumor necrosis- α (TNF- α):interleukin-10 (IL-10) and interferon- γ (IFN- γ):IL-10 is higher in recovered visceral leishmaniasis (VL) patients ($n = 16$) than in delayed-type hypersensitivity (DTH)+ household contacts ($n = 18$), Brazil. Whole blood cells were stimulated *ex vivo* with 10 $\mu\text{g/mL}$ of either recombinant TRYP or LACK. Supernatants were harvested at 24 hours (TNF- α , IL-10) or 6 days (IFN- γ , IL-10) and analyzed by enzyme-linked immunosorbent assay. TNF- α (A) and IL-10 (B) levels measured at 24 hours are presented as ratios in (C). IFN- γ (D) and IL-10 (E) levels measured at 6 days are presented as ratios in (F). For day 6, IFN- γ :IL-10 ratios could not be calculated because of a small number of non-responders, resulting in 13 for VL TRYP, 14 for DTH+ TRYP, 10 for VL LACK, and 11 for DTH+ LACK. P values denote differences between the VL patient and DTH+ groups for each antigen, NS = not significant. Dots represent outliers as determined statistically by using the Tukey method in GraphPad Prism 5 (Graphpad, San Diego, CA). Similar results were obtained for IFN- γ and IL-10 measured at 72 hours.

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 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.²⁵ 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 stand 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 and recombinant vaccine models.^{26,27} Disappointingly, 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

TABLE 4

Interferon- γ response to novel *Leishmania* antigens in persons who recovered from visceral leishmaniasis (V1–V10) and DTH+ (D1–D4) and DTH- (H1–H4) study groups, Brazil*

ID no.	Time†	Mitogen or <i>Leishmania</i> antigen													
		DTH, mm	PHA‡	CLA	R71 100	Q51 100	L37 99	N52 94	L302.06 90	J89 87	M18 78	J41 73	M22 61	M63 54	M57 37
V1	2 mo	ND	++++	–	–	–	++	–	–	+	++	–	–	–	–
V2	3 mo	9	++++	–	–	–	++	–	–	++	+++	–	+	–	–
V3	3 mo	14	++++	++++	++	–	–	+	+	++	+++	++	+++	–	–
V4	3 mo	0	++++	++++	++	+++	+++	–	++	++	+++	++	++	–	–
V5	11 mo	0	++++	+++	–	–	++	–	++	++	++	+	++	–	–
V6	10 y	10	++++	++++	–	–	–	+	–	+++	–	–	–	–	–
V7	10 y	10	++++	+	+++	+	+++	++	+++	+++	+++	++	+++	–	–
V9	11 y	ND	++++	++++	–	–	–	–	–	–	++	+++	+++	–	–
V9	11 y	17	++++	++++	–	–	–	++	–	–	+	–	–	–	–
V10	18 y	15	++++	++++	++	–	+	–	++	+	++	–	+	–	–
D1	5	5	++++	++	–	–	–	++	+	++	–	–	–	–	–
D2	5	5	++++	+++	++	–	+++	+++	++	+++	+++	–	+++	–	–
D3	7	7	++++	+++	–	–	–	–	–	++	–	–	–	–	–
D4	6	6	++++	+++	–	–	–	–	–	++	–	–	–	–	–
H1	0	0	++++	–	–	–	–	+	–	++	–	–	–	–	–
H2	0	0	++++	–	–	–	–	–	–	+	–	–	–	–	–
H3	4	4	++++	–	–	–	+	–	+	++	++	–	+	–	–
H4	1	1	++++	–	–	–	–	–	–	–	–	–	–	–	–
% Responders§			100	86	36	14	50	43	50	86	71	36	57	0	0

* Whole blood cells were stimulated *ex vivo* with phytohemagglutinin (PHA) (5 μ g/mL), crude *Leishmania* antigen (CLA) (10 μ g/mL), or peptide pools (5 μ g/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 pg/mL; + = 20 to < 100 pg/mL; ++ = 100 to < 500 pg/mL; +++ = 500–10,000 pg/mL; ++++ = > 10,000 pg/mL; ND = not determined.

† mo = months; y = years.

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

§ Based on recovered visceral leishmaniasis patients and DTH+ groups.

FIGURE 3. Peptide-specific tumor necrosis- α (TNF- α) (A), interleukin-10 (IL-10) (B), and interferon- γ (IFN- γ) (C) cytokine responses after stimulation of whole blood cells from recovered visceral leishmaniasis (VL) patients ($n = 10$) with 11 novel *Leishmania* antigens, Brazil. Whole blood cells were stimulated *ex vivo* with peptide pools at a final concentration of 5 $\mu\text{g/mL}$. Supernatants were harvested at 72 hours, and analyzed by enzyme-linked immunosorbent assay.

measure of protection observed in persons who convert to skin test positive during the trial (whether or not they received the vaccine).²⁻⁶ We therefore hypothesized that further characterization of immune responses associated with natural conversion to a DTH response to leishmanial antigen in disease-endemic areas, or after drug-induced recovery from clinical disease, could provide important clues to the type of immunity that a successful vaccine will need to elicit. In particular, to determine whether the same correlations of protective immunity that have been observed in successful murine vaccine studies are observed in these groups of naturally exposed humans.

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.^{12,24} 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.,²⁹ in addition to determining that TNF- α concurrent with IFN- γ also provides a good correlation of protection against *L. infantum*.¹³

In the virulent model of visceralizing *L. major* infection used in our studies,^{12,24} 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 TNF- 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 TNF- α contribute to fever and cachexia, and are detrimental.³⁰ Until recently, it was also believed that active VL patients failed to show IFN- γ responses to recall antigen in cells from peripheral blood,³¹ and that this anergy may be the main reason for susceptibility. More recently, whole blood quantiferon assays³² 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.³³ In that study, levels of IFN- γ and TNF- 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. braziliensis* 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 TNA- 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 protective 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,⁸ 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.²⁵ 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,^{37,38} including those reported in our study.

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Authors' addresses: Carmel B. Stober, Department of Medicine, University of Cambridge School of Clinical Medicine, Cambridge, CB2 2QQ, United Kingdom, E-mail: c.stober@doctors.org.uk. Selma M. B. Jeronimo and Nubia N. Pontes, Department of Biochemistry, Bioscience Center, Universidade Federal do Rio Grande do Norte, Natal, Rio Grande do Norte, 59078-970, Brazil; and Instituto de Ciência e Tecnologia de Doenças Tropicais, Natal, Rio Grande do Norte, Brazil, E-mails: smbj@cb.ufrn.br and nnatalir@yahoo.com.br. Jenefer

M. Blackwell, Cambridge Institute for Medical Research, University of Cambridge School of Clinical Medicine, Addenbrooke's Hospital, Cambridge CB2 0XY, United Kingdom; and Telethon Institute for Child Health Research, Centre for Child Health Research, University of Western Australia, Subiaco, Western Australia 6008, Australia, E-mail: jblackwell@icmr.uwa.edu.au.

REFERENCES

- Greenblatt CL, 1988. Cutaneous leishmaniasis: the prospects for a killed vaccine. *Parasitol Today* 4: 53–54.
- Antunes CM, Mayrink W, Magalhaes PA, Costa CA, Melo MN, Dias M, Michalick MS, Williams P, Lima AO, Vieira JB, 1986. Controlled field trials of a vaccine against New World cutaneous leishmaniasis. *Int J Epidemiol* 15: 572–580.
- Khalil EA, El Hassan AM, Zijlstra EE, Mukhtar MM, Ghalib HW, Musa B, Ibrahim ME, Kamil AA, Elsheikh M, Babiker A, Modabber F, 2000. Autoclaved *Leishmania major* vaccine for prevention of visceral leishmaniasis: a randomised, double-blind, BCG-controlled trial in Sudan. *Lancet* 356: 1565–1569.
- Mayrink W, da Costa CA, Magalhaes PA, Melo MN, Dias M, Lima AO, Michalick MS, Williams P, 1979. A field trial of a vaccine against American dermal leishmaniasis. *Trans R Soc Trop Med Hyg* 73: 385–387.
- Momeni AZ, Jalayer T, Emamjomeh M, Khamesipour A, Zicker F, Ghassemi RL, Dowlati Y, Sharifi I, Aminjavaheri M, Shafiei A, Alimohammadian MH, Hashemi-Fesharki R, Nasseri K, Godal T, Smith PG, Modabber F, 1999. A randomised, double-blind, controlled trial of a killed *L. major* vaccine plus BCG against zoonotic cutaneous leishmaniasis in Iran. *Vaccine* 17: 466–472.
- Sharifi I, FeKri AR, Aflatonian MR, Khamesipour A, Nadim A, Mousavi MR, Momeni AZ, Dowlati Y, Godal T, Zicker F, Smith PG, Modabber F, 1998. Randomised vaccine trial of single dose of killed *Leishmania major* plus BCG against anthroponotic cutaneous leishmaniasis in Bam, Iran. *Lancet* 351: 1540–1543.
- Ivens AC, Peacock CS, Worthey EA, Murphy L, Aggarwal G, Berriman M, Sisk E, Hertzfowler C, Quail MA, Harris D, Rajandream M-A, Davies RM, Aert R, Andersson B, Anupama A, Attipoe P, Bason N, Bauser C, Beck A, Beverley SM, Bianchetti G, Borzym K, Bothe G, Bruschi CV, Collins M, Cadag E, Ciarloni L, Clayton C, Coulson RMR, Cronin A, Cruz AC, De Gaudenzi J, Dobson DE, Duesterhoeft A, Fazelina G, Fosker N, Frasch AC, Fraser A, Fuchs M, Gabel C, Goble C, Goffeau A, Hilbert H, Horn D, Huang Y, Klages S, Knights A, Kube M, Larke N, Litvin L, Lord A, Louie T, Marra M, Masuy D, Matthews K, Michaeli S, Mottram JC, Mueller-Auer S, Munden H, Nelson S, Nilsson D, Norbertczak H, Oliver K, O'Neill S, Pentony M, Pohl TM, Price C, Purnelle B, Rabbinoiwitsch E, Reinhardt R, Rieger M, Rinta J, Robben J, Robertson L, Ruiz J, Rutter S, Saunders D, Schaefer M, Schein J, Schwartz DC, Seeger K, Seyler A, Sharp S, Shin H, Sivam D, Squares R, Squares S, Tosato V, Tram A-N, Vogt C, Volckaert G, Wambutt R, Warren T, Wedler H, Woodward J, Zhou S, Zimmermann W, Smith DF, Blackwell JM, Stuart KD, Barrell B, Myler PJ, 2005. The genome of the kinetoplastid parasite, *Leishmania major*. *Science* 309: 436–442.
- Stober C, Lange U, Roberts MT, Gilbmartin B, Francis R, Almeida R, Peacock CS, McCann S, Blackwell JM, 2006. From genome to vaccines for leishmaniasis: screening 100 novel vaccine candidates against murine *Leishmania major* infection. *Vaccine* 24: 2602–2614.
- Campos-Neto A, Porrozzio R, Greeson K, Coler RN, Webb JR, Seiky YA, Reed SG, Grimaldi G Jr, 2001. Protection against cutaneous leishmaniasis induced by recombinant antigens in murine and nonhuman primate models of the human disease. *Infect Immun* 69: 4103–4108.
- Mougeon E, Altare F, Wakil AE, Zheng S, Coppola T, Wang Z-E, Waldmann R, Locksley RM, Glaichenhaus N, 1995. Expression cloning of a protective *Leishmania* antigen. *Science* 268: 563–566.
- Levick MP, Tetaud E, Fairlamb AH, Blackwell JM, 1998. Identification and characterisation of a functional peroxidoxin from *Leishmania major*. *Mol Biochem Parasitol* 96: 125–137.

12. Stober C, Lange U, Roberts MTM, Alcamí A, Blackwell JM, 2005. IL-10 from regulatory T cells determines vaccine efficacy in murine *Leishmania major* infection. *J Immunol* 175: 2517–2514.
13. Dondji B, Perez-Jimenez E, Goldsmith-Pestana K, Esteban M, McMahon-Pratt D, 2005. Prime-boost vaccination using the LACK antigen protects against murine visceral leishmaniasis. *Infect Immun* 73: 5286–5289.
14. Jeronimo SM, Duggal P, Ettinger NA, Nascimento ET, Monteiro GR, Cabral AP, Pontes NN, Lacerda HG, Queiroz PV, Gomes CE, Pearson RD, Blackwell JM, Beaty TH, Wilson ME, 2007. Genetic predisposition to self-curing infection with the protozoan *Leishmania chagasi*: a genomewide scan. *J Infect Dis* 196: 1261–1269.
15. Jeronimo SM, Oliveira RM, Mackay S, Costa RM, Sweet J, Nascimento ET, Luz KG, Fernandes MZ, Jernigan J, Pearson RD, 1994. An urban outbreak of visceral leishmaniasis in Natal, Brazil. *Trans R Soc Trop Med Hyg* 88: 386–388.
16. Jeronimo SM, Holst AK, Jamieson SE, Francis R, Martins DR, Bezerra FL, Ettinger NA, Nascimento ET, Monteiro GR, Lacerda HG, Miller EN, Cordell HJ, Duggal P, Beaty TH, Blackwell JM, Wilson ME, 2007. Genes at human chromosome 5q31.1 regulate delayed-type hypersensitivity responses associated with *Leishmania chagasi* infection. *Genes Immun* 8: 539–551.
17. Howie BN, Donnelly P, Marchini J, 2009. A flexible and accurate genotype imputation method for the next generation of genome-wide association studies. *PLoS Genet* 5: e1000529.
18. Reed SG, Badaro R, Masur H, Carvalho EM, Lorenco R, Lisboa A, Teixeira R, Johnson WD Jr, Jones TC, 1986. Selection of a skin test antigen for American visceral leishmaniasis. *Am J Trop Med Hyg* 35: 79–85.
19. Jeronimo SM, Teixeira MJ, Sousa A, Thielking P, Pearson RD, Evans TG, 2000. Natural history of *Leishmania (Leishmania) chagasi* infection in northeastern Brazil: long-term follow-up. *Clin Infect Dis* 30: 608–609.
20. Braz RF, Nascimento ET, Martins DR, Wilson ME, Pearson RD, Reed SG, Jeronimo SM, 2002. The sensitivity and specificity of *Leishmania chagasi* recombinant K39 antigen in the diagnosis of American visceral leishmaniasis and in differentiating active from subclinical infection. *Am J Trop Med Hyg* 67: 344–348.
21. Jeronimo SM, Duggal P, Braz RF, Cheng C, Monteiro GR, Nascimento ET, Martins DR, Karplus TM, Ximenes MF, Oliveira CC, Pinheiro VG, Pereira W, Peralta JM, Sousa J, Medeiros IM, Pearson RD, Burns TL, Pugh EW, Wilson ME, 2004. An emerging peri-urban pattern of infection with *Leishmania chagasi*, the protozoan causing visceral leishmaniasis in northeast Brazil. *Scand J Infect Dis* 36: 443–449.
22. Badaro R, Benson D, Eulalio MC, Freire M, Cunha S, Netto EM, Pedral-Sampaio D, Madureira C, Burns JM, Houghton RL, David JR, Reed SG, 1996. rK39: a cloned antigen of *Leishmania chagasi* that predicts active visceral leishmaniasis. *J Infect Dis* 173: 758–761.
23. Burns JM Jr, Shreffler WG, Benson DR, Ghalib HW, Badaro R, Reed SG, 1993. Molecular characterization of a kinesin-related antigen of *Leishmania chagasi* that detects specific antibody in African and American visceral leishmaniasis. *Proc Natl Acad Sci USA* 90: 775–779.
24. Stober CB, Lange UG, Roberts MT, Alcamí A, Blackwell JM, 2007. Heterologous priming-boosting with DNA and modified vaccinia virus Ankara expressing trypanothione peroxidase promotes long-term memory against *Leishmania major* in susceptible BALB/c Mice. *Infect Immun* 75: 852–860.
25. Blackwell JM, Jamieson SE, Burgner D, 2009. HLA and infectious diseases. *Clin Microbiol Rev* 22: 370–385.
26. Mauel J, 2002. Vaccination against *Leishmania* infections. *Curr Drug Targets Immune Endocr Metabol Disord* 2: 201–226.
27. Gurunathan S, Prussin C, Sacks DL, Seder RA, 1998. Vaccine requirements for sustained cellular immunity to an intracellular parasitic infection. *Nat Med* 4: 1409–1415.
28. Modabber F, 2010. Leishmaniasis vaccines: past, present and future. *Int J Antimicrob Agents* 36 (Suppl 1): S58–S61.
29. Jayakumar A, Castilho TM, Park E, Goldsmith-Pestana K, Blackwell JM, McMahon-Pratt D, 2011. TLR1/2 activation during heterologous prime-boost vaccination (DNA-MVA) enhances CD8+ T cell responses providing protection against *Leishmania (Viannia)*. *PLoS Negl Trop Dis* 5: e1204.
30. Barral-Netto M, Badaro R, Barral A, Almeida RP, Santos SB, Badaro F, Pedral-Sampaio D, Carvalho EM, Falcoff E, Falcoff R, 1991. Tumor necrosis factor (cachectin) in human visceral leishmaniasis. *J Infect Dis* 163: 853–857.
31. Sacks DL, Lal SL, Shrivastava SN, Blackwell JM, Neva FA, 1987. An analysis of T cell responsiveness in Indian Kala-azar. *J Immunol* 138: 908–913.
32. Gidwani K, Jones S, Kumar R, Boelaert M, Sundar S, 2011. Interferon-gamma release assay (modified QuantiFERON) as a potential marker of infection for *Leishmania donovani*, a proof of concept study. *PLoS Negl Trop Dis* 5: e1042.
33. Follador I, Araujo C, Bacellar O, Araujo CB, Carvalho LP, Almeida RP, Carvalho EM, 2002. Epidemiologic and immunologic findings for the subclinical form of *Leishmania braziliensis* infection. *Clin Infect Dis* 34: E54–E58.
34. Novoa R, Bacellar O, Nascimento M, Cardoso TM, Ramasawmy R, Oliveira WN, Schrieffer A, Carvalho EM, 2011. IL-17 and regulatory cytokines (IL-10 and IL-27) in *L. braziliensis* infection. *Parasite Immunol* 33: 132–136.
35. Marsh SG, Albert ED, Bodmer WF, Bontrop RE, Dupont B, Erlich HA, Fernandez-Vina M, Geraghty DE, Holdsworth R, Hurley CK, Lau M, Lee KW, Mach B, Maiers M, Mayr WR, Muller CR, Parham P, Petersdorf EW, Sasazuki T, Strominger JL, Svejgaard A, Terasaki PI, Tiercy JM, Trowsdale J, 2010. Nomenclature for factors of the HLA system, 2010. *Tissue Antigens* 75: 291–455.
36. Costa CH, Peters NC, Maruyama SR, de Brito EC Jr, Santos IK, 2011. Vaccines for the leishmaniasis: proposals for a research agenda. *PLoS Negl Trop Dis* 5: e943.
37. Goto Y, Bhatia A, Raman VS, Liang H, Mohamath R, Picone AF, Vidal SE, Vedvick TS, Howard RF, Reed SG, 2011. KSAC, the first defined polypeptide vaccine candidate for visceral leishmaniasis. *Clin Vaccine Immunol* 18: 1118–1124.
38. Chakravarty J, Kumar S, Trivedi S, Rai VK, Singh A, Ashman JA, Laughlin EM, Coler RN, Kahn SJ, Beckmann AM, Cowgill KD, Reed SG, Sundar S, Piazza FM, 2011. A clinical trial to evaluate the safety and immunogenicity of the LEISH-F1+MPL-SE vaccine for use in the prevention of visceral leishmaniasis. *Vaccine* 29: 3531–3537.