Evaluation of *Ex Vivo* Human Immune Response against Candidate Antigens for a Visceral Leishmaniasis Vaccine

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Abstract. People cured from visceral leishmaniasis (VL) develop protection mediated by Th1-type cellular responses against new infections. We evaluated cytokine responses against 6 defined candidate vaccine antigens in 15 cured VL subjects and 5 healthy endemic controls with no evidence of previous exposure to *Leishmania* parasites. Of the 6 cytokines examined, only interferon-gamma (IFN- γ) differentiated cured VL patients from non-exposed individuals, with cured patients mounting a significantly higher IFN- γ response to a crude parasite antigen preparation. Among candidate vaccine antigens tested, the largest number of cured subjects recognized cysteine proteinase B, leading to heightened IFN- γ responses, followed by sterol 24-c-methyltransferase. These two antigens were the most immunogenic and protective antigens in a murine VL model, indicating a relationship between T cell recall responses of humans cured from VL and protective efficacy in an experimental model. Further studies may help prioritize antigens for clinical development of a subunit vaccine against VL.

INTRODUCTION

Leishmaniasis causes the second highest number of deaths because of parasitic infection globally, and is overwhelmingly associated with poverty.¹ Because of the lack of effective, affordable, and minimally toxic treatments; an effective vaccine to combat this disease is needed.

Visceral leishmaniasis (VL) is the most severe form of leishmaniasis, characterized by fever, anemia, and hepatosplenomegaly. Although this disease is fatal if left untreated, patients successfully cured by chemotherapy are immune to new infections. Such protective immunity is associated with antigen-specific cell-mediated responses represented by lymphoproliferation and delayed type hypersensitivity² and production of Th1 cytokines like interferon-gamma (IFN- γ) and interleukin (IL)-2 on antigen recall.³ In contrast, IL-10, which is associated with T cell hyporesponsiveness, is the predominant cytokine during active VL.^{4–6}

Although significant progress has been made recently to understand mechanisms of VL immunity in humans, there is no effective vaccine available for humans against any form of leishmaniasis. Nonetheless, there have been efforts to identify antigens that show protective efficacy against *Leishmania donovani* or *Leishmania infantum* infection in experimental VL models. Such antigens include kinetoplastid membrane protein-11 (KMP11),⁷ sterol 24-c-methyltransferase (SMT),⁸ A2,⁹ cysteine proteinase B (CPB),¹⁰ K26/HASPB,¹¹ and nucleoside hydrolase (NH).¹² Here, we evaluate the ability of these 6 preclinical vaccine candidates to stimulate peripheral blood T cells of cured VL patients by measuring *ex vivo* the release of Th1 and Th2 cytokines.

MATERIALS AND METHODS

Study site. Enrollment of subjects and whole blood assay (WBA) was performed at the Kala-azar Medical Research Center (KAMRC) in Muzaffarpur, and cytometric bead assay (CBA) was performed at Banaras Hindu University (BHU),

Varanasi, India. This study was approved by the ethical committee of KAMRC, and written informed consent was obtained from the study subjects.

Study population. Fifteen individuals cured from VL (3–6 months post-treatment, numbered 101–115) and 5 healthy individuals from the endemic region (no. 201–205) who were free of previous *Leishmania* exposure were enrolled. For the cured group, individuals who had presented at the local hospital with clinical symptoms consistent with VL, had been parasitologically confirmed with the presence of amastigotes in the splenic smears, and had been treated by chemotherapy were included in the study. Twelve of the 15 patients had been treated with amphotericin B, while 3 (nos. 107, 112, and 113) had been treated with paromomycin. The rK39 rapid test (Kalazar Detect, InBios International, Inc., Seattle, WA) and direct agglutination test (DAT) were used for screening the non-exposed group. Healthy individuals who had no history of VL were considered to be free of previous exposure to *Leishmania* parasites and eligible for inclusion in the study if their rK39 test was negative and DAT titer was $\leq 1:400$. Among cured subjects, 9 were male and the median age was 30.5 (minimum 18, maximum 50) years. Of the healthy subjects, 3 were male and the median age was 25.2 (min 18, max 38) years.

Antigens. Six previously characterized antigens were used for WBA: KMP11 (GenBank: XP_001469032.1), SMT (GenBank: XP_001469832.1), A2 (GenBank: S69693.1), CPB (GenBank: CAD12393.1), K26 (GenBank: AAD55244.1), and NH (GenBank: XP_001464969.1). A gene coding NH was cloned from the *L. infantum* genome by polymerase chain reaction (PCR) using a set of primers, 5' CAA TTA CAT ATG CAT CAC CAT CAC CAT CAC ATG CCG CGC AAG ATT CTC, and 3' AA TTA AAG CTT TCA TTG AGG ATC GCC GAT GCG. The amplified PCR product was inserted in-frame into the *NdeI/HindIII* site of the vector pET-17 (EMD Biosciences, San Diego, CA) and the recombinant protein expressed and purified using Ni-NTA agarose (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. To remove endotoxin, the Ni-NTA agarose bound with the protein was washed with 0.5% sodium deoxycholate followed by 60% isopropanol before elution with imidazole. Recombinant proteins of the other genes were produced in previous studies.^{8,13,14} Soluble *Leishmania* antigen (SLA) was

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prepared from *L. donovani* promastigotes by sonication, as previously described.¹³ Endotoxin content of all the proteins used in WBA was less than 100 EU/mg. Phytohaemagglutinin (PHA) and phosphate-buffered saline (PBS) were used as positive and negative controls, respectively.

Collection of blood and WBA. Using a QuantiFERON tube (Cellestis Inc., Valencia, CA), 1 mL of venous blood was collected for each antigen tested and for positive and negative controls. All the antigens except SLA were used at a concentration of 10 µg/mL, whereas SLA and PHA were used at a concentration of 7.5 µg/mL. After adding antigens, tubes were incubated at 37°C for 24 hr and centrifuged at 2000 × g for 10 min. Supernatants were collected and transported to Banaras Hindu University (BHU) for cytokine analysis.

Cytometric bead array. Levels of IL-2, IL-4, IL-5, IL-10, tumor necrosis factor α (TNF-α), and IFN-γ in supernatant from each WBA tube were measured using a BD CBA Human Th1/Th2 Cytokine Kit (cat no.550749, Becton Dickinson, Franklin Lakes, NJ) according to the manufacturer's instructions. Beads incubated with WBA supernatant (50 µL) were run on a BD FACSCalibur, and cytokine levels were determined using BD CBA software. The lower and upper detection limits of a cytokine were 20 and 5,000 pg/mL.

Mouse experiments for immunogenicity and protection. Immunogenicity and protective efficacy of the 6 defined antigens were evaluated with a C57BL/6—*L. infantum* model as previously described.⁸ Briefly, mice were immunized with 10 µg of antigen plus 20 µg of monophosphoryl lipid A in stable emulsion (GlaxoSmithKline Biologicals, Rixensart, Belgium) for IFN-γ assay using spleen cells and for challenge. A control group received saline. Female C57BL/6 mice, 5–7 wks old, were purchased from Charles River (Wilmington, MA). All mice were maintained in the Infectious Disease Research Institute (IDRI) animal care facility under specific

pathogen-free conditions and were treated in accordance with the regulations and guidelines of the IDRI Animal Care and Use Committee.

Statistical analysis. The Mann-Whitney test was used for statistical analyses between two groups in the WBA assay. These statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA). Statistical difference was considered significant if the *P* value was less than 0.05.

RESULTS

Cytokine responses of peripheral blood to SLA. In the absence of stimulation with exogenous leishmanial antigens, one of the post-treatment individuals showed IFN-γ production of 34 pg/mL in WBA; all the other study participants had IFN-γ below the detection limit (20 pg/mL). Antigen-specific cytokine levels produced in response to *Leishmania* antigen stimulation were determined by subtracting background levels measured in the non-stimulated (PBS) samples. The PHA, an antigen-non-specific activator of T cells, induced all 6 cytokines (IFN-γ, TNF-α, IL-2, IL-4, IL-5, and IL-10) with comparable levels between cured and non-exposed groups (Figure 1).

Twelve of the 15 post-treatment individuals with a history of kala-azar showed SLA-specific stimulation of IFN-γ production (> 20 pg/mL). This compared with only 1 of 5 healthy individuals responding to SLA (Figure 2). Significantly higher IFN-γ production with SLA stimulation was found in the cured group than in the non-exposed group (*P* = 0.03, Figure 2). In contrast, none of the other 5 Th1/Th2 cytokines examined differentiated cured and healthy individuals to the same degree as IFN-γ (Figure 2). Although both mean and median values of TNF-α were higher in the cured group, the difference between the groups was not statistically significant (*P* = 0.96). The IL-10 was detected in 3 of the cured individuals and 1 of the

FIGURE 1. Phytohaemagglutinin (PHA)-induced production of Th1/Th2 cytokines in cured and non-exposed (NE) subjects. Cytokine production by peripheral blood in response to either phosphate-buffered saline (PBS) or PHA was examined in cured and NE subjects by whole blood assay (WBA)/cytometric bead assay (CBA). Cytokine concentration values of PHA-stimulated sample (minus the individual's non-stimulated value) are shown. Bars represent median values. Dotted lines represent the assay detection limit (20 pg/mL).

FIGURE 2. Soluble *Leishmania* antigen (SLA)-specific Th1/Th2 cytokine production in cured and non-exposed (NE) subjects. Cytokine production by peripheral blood in response to either phosphate-buffered saline (PBS) or SLA was examined in cured and NE subjects by whole blood assay (WBA)/cytometric bead assay (CBA). Cytokine concentration values of SLA-stimulated sample (minus the individual's non-stimulated value) are shown. Bars represent median values. Dotted lines represent the assay detection limit (20 pg/mL).

non-exposed. Only 2 study participants showed IL-2 levels above the detection limit; none of the IL-4 and IL-5 levels were above the detection limit. The ability of all blood samples to respond to stimuli was demonstrated using PHA, which induced detectable levels for the 6 cytokines in all 20 individuals with the exception of IL-5 response in one cured subject (Figure 1).

When patterns of multiple cytokine production were analyzed in individuals in the cured group, the top three IFN- γ producers (nos. 105, 103, and 101) were also positive for TNF- α and IL-10 (Table 1). The next 3 were IFN- γ /TNF- α double-positive and IL-10-negative. The remaining six responders were IFN- γ single-positive. Levels of IFN- γ and TNF- α induced by stimulation with SLA showed a positive correlation ($r = 0.69$, $P < 0.01$ by the Spearman correlation test).

TABLE 1

Cytokine production patterns of cured subjects in response to SLA*

Study ID	Cytokines		
	IFN- γ	TNF- α	IL-10
105	+++	++	++
103	+++	+	+
101	++	+	+
111	++	+	-
112	++	+	-
115	++	++	-
107	++	-	-
110	++	-	-
114	+	-	-
108	+	-	-
102	+	-	-
109	+	-	-
113	-	-	-
106	-	-	-
104	-	-	-

* (-) 0–< 20 pg/mL; (+) 20–< 100 pg/mL; (++) 100–< 500 pg/mL; (+++) 500 pg/mL $\leq$.

IFN- γ production in response to defined antigens. IFN- γ production by cured and non-exposed individuals in response to six defined antigens was analyzed. One of the non-exposed individuals (no. 204) showed IFN- γ response (≥ 20 pg/mL) against SLA. However, neither this person nor any other non-exposed individuals responded to any of the recombinant antigens tested (Table 2). Cured subjects who did not respond to SLA were uniformly non-responsive to other antigens

TABLE 2

IFN- γ response to defined *Leishmania* antigens in individuals cured from VL*

Group	Study ID	Antigens						
		SLA	KMP11	SMT	A2	CPB	K26	NH
Cured	105	+++	-	++	++	++	-	+++
	103	+++	-	++	+	++	-	+
	101	++	-	-	-	-	-	-
	111	++	-	-	-	+	-	-
	112	++	-	-	-	+	-	-
	115	++	-	+	-	-	-	-
	107	++	-	-	-	+	-	-
	110	++	+	+	-	++	-	-
	114	+	-	-	-	+	-	-
	108	+	-	-	-	-	-	-
	102	+	-	-	-	-	-	-
	109	+	-	-	-	+	-	-
	113	-	-	-	-	-	-	-
	106	-	-	-	-	-	-	-
	104	-	-	-	-	-	-	-
Non-exposed	201	-	-	-	-	-	-	-
	202	-	-	-	-	-	-	-
	203	-	-	-	-	-	-	-
	204	++	-	-	-	-	-	-
	205	-	-	-	-	-	-	-
	206	-	-	-	-	-	-	-

* (-) 0–< 20 pg/mL; (+) 20–< 100 pg/mL; (++) 100–< 500 pg/mL; (+++) 500 pg/mL $\leq$. SLA = soluble *Leishmania* antigen; KMP11 = kinetoplast membrane protein-11; SMT = sterol 24-c-methyltransferase; CPB = cysteine proteinase B; NH = nucleoside hydrolase.

FIGURE 3. Immunogenicity and protective efficacy of vaccine candidate antigens in mice. (A) Spleen cells from mice inoculated with an antigen plus an adjuvant were stimulated *in vitro* with medium alone, con A, or the antigen. Culture supernatants were collected after 72 hrs stimulation and the levels of IFN- γ in the supernatants were measured by sandwich enzyme-linked immunosorbent assay (ELISA). Mean and SEM of three mice in each group are shown. (B) Mice, either immunized or unimmunized, were challenged with 5×10^6 promastigotes of *Leishmania infantum* and the numbers of parasites in the liver were measured by limiting dilutions 4 weeks after the infection. Reductions in parasite numbers compared with the unimmunized control are shown here with mean values and SEM of at least three independent experiments for individual antigens.

tested. The CPB was the antigen most recognized by the cured individuals, with 8 of the 12 SLA responders showing IFN- γ production. Mean values of IFN- γ production to CPB in cured and non-exposed groups were 63 and 4 pg/mL, respectively, and median values were 20 and 2 pg/mL, respectively. Four of the cured subjects responded to SMT, and 2—those who responded most strongly to SLA—responded to A2 and NH. There was no cytokine response in any post-treatment samples to the K26 antigen and only one of the cured subjects responded to KMP-11 with an IFN- γ level above the limit of detection.

Immunogenicity/protective efficacy in a mouse VL model.

Spleen cells from C57BL/6 mice immunized with one of the 6 antigens were stimulated *ex vivo* with the corresponding antigen to see the induction of antigen-specific T cell response. With the exception of KMP11, all the antigens induced antigen-specific cellular responses, showing IFN- γ production on antigen recall (Figure 3A). Although antigen-specific response was detectable in K26-immunized mice, its magnitude was weaker than that seen in mice immunized with the other four antigens (Figure 3A).

To evaluate the protective efficacy of the 6 leishmanial antigens against VL, immunized mice were challenged by intravenous injection of 5×10^6 *L. infantum* promastigotes. Significant reduction of parasites in the liver was seen in mice immunized with SMT, CPB, and NH, and, to a lesser extent, K26 (Figure 3B). In contrast, KMP11 and A2 provided only limited levels of protection in this experimental model (Figure 3B).

DISCUSSION

Animal models of VL have been used to test protective efficacy of vaccine candidates. The purpose of this study was to assess six of those VL vaccine candidates for their abilities to elicit an *ex vivo* cellular immune response in whole blood taken from human subjects with and without evidence of

prior infection with *Leishmania*. Use of a CBA assay allowed screening for multiple cytokines in a small amount of blood without the need for preparing peripheral blood mononuclear cells. The rationale is that antigens that elicit cellular immune responses correlating with protection or cure (e.g., IFN- γ production in previously exposed and cured individuals) may be good candidates for prophylactic and therapeutic vaccines. In our study, CPB and SMT induced IFN- γ production in more cured individuals (8 and 4 out of 12 SLA responders, respectively) than did other defined antigens. Although all six proteins have been previously characterized in animal models as protective against VL, in our mouse model SMT and CPB were also the two most protective antigens, followed by NH, suggesting linkages between protective efficacy in mice and antigenicity in humans.

Twelve of the 15 cured individuals responded to SLA with IFN- γ production, indicating responses to SLA proteins of still-circulating memory T cells that were first stimulated during the original *L. donovani* infections. One of the healthy individuals also responded to SLA. This could be caused by cross-reactivity of memory T cells, or it could be the result of prior or current sub-clinical infection and cure of the healthy individual in the absence of producing DAT-reactive antibodies. The other cytokines examined after SLA stimulation—IL-2, IL-4, IL-5, and TNF- α —did not clearly differentiate between the cured and non-exposed groups, with levels ranging from low to undetectable. In contrast, all the cytokines were produced with PHA stimulation, indicating that the study participants did not have genetic defects in producing these cytokines.

Six of 12 SLA responders produced both IFN- γ and TNF- α , and those double-positive responders produced higher levels of IFN- γ than did IFN- γ single-positive subjects. This pattern resembles that seen in multifunctional Th1 cells, which produce a higher level of IFN- γ than do IFN- γ single-positive cells.¹⁵ These two cytokines synergize in inducing NADPH oxidase,¹⁶ the product of which, superoxide, is involved in killing *Leishmania* parasites in human macrophages, as is nitric oxide in mice.¹⁷ The capability of the cured subjects to simultaneously express these two cytokines may be beneficial for protection against new infections. Intriguingly, the three strongest IFN- γ producers also secreted IL-10 and TNF- α in response to SLA. IL-10 is generally associated with immune suppression during active VL, negatively regulating Th1 responses.⁶ In contrast, Kemp and others¹⁸ have demonstrated that people cured of VL possess T cells simultaneously expressing IFN- γ and IL-10 on antigen stimulation. Recent studies showed the majority of IL-10 produced during human visceral leishmaniasis or murine cutaneous leishmaniasis is not from T-regulatory, but rather from IFN- γ -producing, Th1 cells,^{19,20} possibly serving as a mechanism of feedback control. Although further single-cell analyses are needed to address individual T cell cytokine expression patterns, IL-10 production seen in people with strong IFN- γ responses may indicate homeostatic processes during cure.

In this study, KMP-11 and K26/HASPB did not show Th1-related IFN- γ production in cured VL subjects. These were also the two least immunogenic antigens in the mouse model used here. However, previous studies with experimental animal models have shown a protective role for KMP-11 and K26/HASPB.^{7,11} The protective efficacy of these two antigens largely relies on CD8⁺ T cells, not CD4⁺ cells.^{11,21} This may

explain the discrepancy in mouse protection and human IFN- γ production for these two antigens, as the majority of antigen-specific IFN- γ -producing T cells in people cured of VL are CD4⁺ cells.¹⁸ In general, soluble antigens are not efficient for *ex vivo* recall of CD8⁺ T cells unless they are presented at high concentrations or with an efficient antigen delivery system.²² Hence, the dose of recombinant antigens (10 μ g/mL) used for WBA may not be sufficient, and the assay may be biased against detecting responses to CD8⁺ cytotoxic T cell (CTL) antigens. Alternatively, the lack of response to KMP11 in mice may be the result of different processing and presentation by antigen presenting cells of antigen delivered as a recombinant protein as in this study and the DNA vaccine in a previous study.⁷ The lack of T cell response could also be caused by the lack of natural adjuvants, as association with lipophosphoglycan (LPG) may enhance the immunogenicity of KMP-11 for human lymphocytes.²³ Further studies including antigen dosing are definitely required to improve this method to apply to selection of vaccine candidates.

In summary, this study indicates a relationship between T cell recall responses of humans cured from VL and protective efficacy in an experimental model. It is not anticipated that a single antigen will be recognized by T cells from every human donor with an immunologically and genetically divergent background after disease cure, or was that found to be the case. This study is very preliminary, as the number of study participants was limited and only a single dose of antigen was evaluated; further studies with improvements on those counts are needed to propose our approach—looking at the across-population ability of antigens to elicit an IFN- γ response and the strength of the response elicited—as one means of translating the efficacy of vaccine antigens from homogeneous experimental models to divergent human populations.

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