

Immunogenicity and immune modulatory effects of in silico predicted *L. donovani* candidate peptide vaccines

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Visceral leishmaniasis (VL) is a serious parasitic disease for which control measures are limited and drug resistance is increasing. First and second generation vaccine candidates have not been successful. The goal of the present study was to select possibly immunogenic *L. donovani donovani* GP63 peptides using immunoinformatics tools and to test their immunogenicity in vitro. The amino acid sequence of *L. donovani donovani* GP63 [GenBank accession: ACT31401] was screened using the EpiMatrix algorithm for putative T cell epitopes that would bind to the most common HLA class II alleles (DRB1*1101 and DRB1*0804) among at-risk populations. Four T cell epitopes were selected from nine potential candidates. Stimulation of whole blood from healthy volunteers using the peptides separately produced mean IFN- γ and IL-4 levels that were not significantly different from negative controls, while the pooled peptides produced a moderate IFN- γ increase in some volunteers. However, mean IL-10 levels were significantly reduced for all individuals compared with controls. The immunogenicity of these epitopes may be harnessed most effectively in a vaccine delivered in combination with immune-modulating adjuvants.

Introduction

The leishmaniasis are an increasingly recognized major public health problem in 88 countries in four continents, with an incidence of 1.5–2 million cases per year.¹ Visceral leishmaniasis (VL; kala-azar) is the most fatal disease phenotype, especially in African children.^{2,3} Case detection and treatment with antimonial drugs is the only available form of VL control. Anti-leishmanial drugs are expensive and are associated with major toxicities and increased drug unresponsiveness.^{2,4} Recently, regimens that combine Sodium stibogluconate with Paromomycin have been developed so as to reduce treatment duration and toxicities.⁵⁻⁷

VL protective immune responses depend entirely on the production of IFN- γ by Th1 cells. The activation of CD4⁺ helper T cells is essential for the development of adaptive immunity against pathogens; a critical step in this activation is the recognition of epitopes presented by HLA class II molecules.⁸⁻¹² Patients with active VL usually exhibit transitory anergy to *Leishmania* antigens with production of large amounts of IL-10 and reciprocal inhibition of IFN- γ secretion. Addition of anti-IL-10R

antibody to T cells harvested from VL patients restores cytokine responses, indicating a role for IL-10 in suppressing T cell responses in active disease.¹³⁻¹⁸ On the other hand, elevated IL-4 levels in VL patients have been associated with treatment failure or disease relapse.^{19,20}

The development of life-long immunity following convalescence or subclinical infection provides a rational basis for vaccine development. First generation vaccines for VL have produced a wealth of data but were limited in efficacy. Alternative vaccines might include T cell epitopes derived from *L. donovani* proteins with adjuvants that will trigger more effective immune responses.²¹⁻²⁷

T cell epitopes are linear peptides recognized by effector T cell receptors (TCR) when the epitopes are presented in the binding groove of HLA molecules on the surface of antigen presenting cells (APC). T cell epitopes which bind to class I HLA molecules have an optimal size of 9 amino acids with a range of 8–11, while those binding to class II HLA have an optimal size range of 12–25 amino acids. The set of HLA molecules which are expressed by an individual determines which peptide fragments will be presented as epitopes.^{28,29}

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The prediction and analysis of HLA-binding sequences within protein antigens is central to modern vaccine development. The goal of T cell epitope prediction is to accurately identify peptide sequences within any protein that will elicit a protective T cell response when presented to the immune system in the context of an HLA molecule on the cell surface.³⁰

It is well documented that small fragments of an antigen can effectively lead to induction of immune responses against the whole antigen. Traditionally, the whole antigen sequence is screened by synthesizing overlapping peptide fragments and assaying their immunogenic potentials. The development of a number of in silico epitope-mapping tools has made it easier to locate T cell epitopes that are central to T cell activation and regulation, but HLA allelic polymorphism and promiscuity still pose problems to the development of the most effective set of peptides for vaccine design.³¹ Thus a suite of tools has been developed by collaborators of De Groot, termed the iVAX system, which is now available for use with Neglected Tropical Diseases. This system is comprised of several tools that can be used to compare sequences (Conservatrix), map epitopes (EpiMatrix), and identify epitope clusters that are recognized in the context of more than one HLA (ClustiMer). Associated tools such as EpiAssembler and VaxCAD can also be used to design vaccines.³¹⁻³³

Peptides derived from genomic sequences can be rapidly synthesized and tested for immunogenicity in vitro using peripheral mononuclear cells or whole blood culture systems. These peptides can then be further developed as 'string-of-beads' constructs in any number of vaccine delivery platforms (DNA, protein, live vaccine vectors) for further study in pre-clinical models (in animal vaccination studies and human artificial immune models).^{32,33}

Synthetic peptide vaccines have a number of advantages that include: absence of infectious materials, economical production on a large scale, dose-standardization and the ability to manufacture vaccines for pathogens difficult or impossible to culture in the laboratory and reduced chance of stimulating autoimmunity. Under some circumstances, immune responses to subsets of antigens and epitopes derived from infectious agents may be sufficient for competent protection.³³ On the other hand; some peptide vaccines can be poorly immunogenic, perhaps due to ineffective delivery systems or lack of potent adjuvants.³⁴

Two *Leishmania* surface molecules have been characterized and studied extensively: the glycoprotein-63 (GP63) and lipophosphoglycan (LPG), both of which are involved in the attachment of the parasite to the macrophage.^{35,36} GP63 protein has been shown to induce protective immune responses against experimental leishmaniasis in murine models and can be a good target for T cell epitopes prediction for human VL vaccines.^{37,38}

This study aimed to identify potentially immunogenic *Leishmania donovani donovani* (GP63) T cell epitopes as possible candidate peptide vaccines for visceral leishmaniasis, using the EpiMatrix epitope mapping tool, and to test their immunogenicity in an in vitro whole blood system.

Results

Nine clusters of GP63 protein were identified as potential T cell epitopes. Four clusters were selected for synthesis based on their

EpiMatrix score, EpiBar content, hydrophobicity and degree of homology to human proteins. The selected clusters were synthesized and used for in vitro immunogenicity testing. The four predicted and synthesized peptides were assigned the following numbered letters: P1, P2, P3 and P4 for the sake of simplicity.³⁹

- Cluster 7 [STHRHRSVAARLVRLAAAGAAVIA] **P1**
- Cluster 151 [DILVKHLIPQALQLH] **P2**
- Cluster 205 [TDFVMYVASVPSEGDVL] **P3**
- Cluster 496 [SHGIIKSYAGLCANVRCDT] **P4**

NB. The actual peptide sequence is shown in **bold letters**. The flanking residues are helpful to stabilize the peptide in class II HLA molecules, and ease synthesis. The predicted peptides bear little or no homology to human antigens.

Heparinized whole venous blood was collected from consenting healthy volunteers with a mean age of 30 ± 11.7 y (median 25.5) and a male: female ratio of 1. Twelve volunteers were leishmanin skin test (LST) non-reactive (induration = 0 mm), while the rest ($n = 10$) were LST-reactive with a mean LST induration of 12.6 ± 6 mm.

IFN- γ production ($n = 22$). The mean IFN- γ production induced in total (LST-reactive and non-reactive volunteers) by soluble *Leishmania* antigen (sLAg), P1, P2, P3 and P4 peptides, or the peptide pool was not significantly different from that of negative control samples (PBS, no peptide) ($p = 0.3$ to 0.95 , **Tables 1 and 2**). The peptides combined as a pool produced moderate increase that is not statistically significant in the group of LST non-reactive volunteers (402 ± 340 pcm/ml compared with 307 ± 259 pcm/ml, **Table 3**).

IL-4 production ($n = 22$). IL-4 production levels were not elevated and were similar across all groups of volunteers in response to stimulation by the sLAg, peptides and pool as compared with the negative control (PBS) samples ($p = 0.4$ to 0.8 , **Tables 1-3**).

IL-10 production. P1 peptide produced significantly higher IL-10 levels in the group of LST-reactive volunteers compared with the sLAg/PBS control samples ($p = 0.0097$, **Table 2**), while P2 and P3 peptides did not induce statistically significant levels of IL-10 ($p = 0.7$ to 0.9). On the other hand, P4 peptide reduced IL-10 production in 90% (9/10; $p = 0.016$) of the volunteers in the LST-reactive group (**Table 2**). Similarly, the peptide pool significantly reduced IL-10 production in the group of LST-reactive volunteers as well as the total group of volunteers ($p = 0.004$ and $p = 0.0001$, respectively). IL-10 production was not statistically significant, when compared with sLAg and PBS control samples, in the group of LST non-reactive volunteers following stimulation with P1, P2, P3, P4 or the peptide pool ($p = 0.07$, **Table 3**).

In summary. • **IFN- γ** was not significantly increased in any of the volunteer groups. The peptides combined as a pool produced moderate increase that is not statistically significant in the group of LST non-reactive volunteers.

• **IL-4** was not significantly increased for any of the volunteer groups (LST-positive and LST-negative).

• **IL-10** was significantly increased following stimulation with the P1 peptide in the LST-reactive volunteer group and the total group of 22 volunteers. A similar increase was not observed in the LST non-reactive group. Significant IL-10 production was observed following stimulation with the P3 peptide in the LST

Table 1. Means of IFN- γ , IL-4 and IL-10 levels for all study volunteers (n = 22) from whole blood stimulation

Antigenic stimulant	IFN- γ pcm/ml	IL-4 pcm/ml	IL-10 pcm/ml
Control (No antigens/No peptides)	154 \pm 222 (65)	16.5 \pm 26.0 (4)	825 \pm 580 (705)
Soluble <i>Leishmania</i> antigen	177 \pm 306 (68)	13.5 \pm 30.0 (8)	827 \pm 448 (832)
P1 peptide	126 \pm 209 (50)	17.7 \pm 24.5 (8.5)	1223 \pm 678 (1287)*
P2 peptide	120 \pm 175 (53)	19.7 \pm 25.9 (13)	940 \pm 538 (763)
P3 peptide	133 \pm 213 (51)	19.4 \pm 24.0 (9.5)	895 \pm 508 (821)
P4 peptide	165 \pm 274 (71)	17.2 \pm 22.4 (12)	544 \pm 652 (217)

P1, P2, P3 and P4 the predicted and synthesized peptides. Continuous variables are expressed as means \pm SD (median). pcm/ml = picogram/ml. *p = 0.03.

Table 2. Leishmanin skin test (LST) reactivity, mean IFN- γ , IL-4 and IL-10 levels in the study volunteers

Leishmanin skin test induration/mm	IFN- γ /pcm/ml	IL-4 pcm/ml	IL10
LST non-reactive (00 mm) (n = 12):			
Control (No antigens/peptides)	239 \pm 251 (133)	26 \pm 32 (16)	993 \pm 693 (681)
Soluble <i>Leishmania</i> antigen	183 \pm 126 (222)	18 \pm 22 (14)	947 \pm 445 (1004)
P1 peptide	199 \pm 259 (139)	25 \pm 30 (17)	1085 \pm 651 (1210)
P2 peptide	187 \pm 222 (128)	25 \pm 31 (18)	1095 \pm 590 (1271)
P3 peptide	214 \pm 261 (132)	26 \pm 28 (16)	1958 \pm 489 (940)*
P4 peptide	253 \pm 345 (180)	23 \pm 28 (17)	824 \pm 716 (510)
LST-reactive (\geq 8 mm) (n = 10):			
Control (No antigens/peptides)	52 \pm 76 (14)	05 \pm 10 (1)	624 \pm 337 (740)
Soluble <i>Leishmania</i> antigen	169 \pm 397(31)	08 \pm 10 (6)	683 \pm 429 (573)
P1 peptide	40 \pm 67 (12)	09 \pm 12 (5)	1339 \pm 706 (1430)*
P2 peptide	40 \pm 60 (15)	13 \pm 18 (3)	754 \pm 422 (727)
P3 peptide	37 \pm 54 (13)	12 \pm 16 (4)	698 \pm 481 (701)
P4 peptide	58 \pm 82 (24)	10 \pm 12 (7)	208 \pm 369 (102)**

Continuous variables are expressed as mean \pm SD (median). pcm/ml = picogram/ml. P1, P2, P3 and P4 the predicted and synthesized peptides.

*p = 0.0097 **p = 0.016.

non-reactive volunteer group. IL-10 was significantly decreased following stimulation with the P4 peptide in the LST-reactive volunteer group. The peptide pool significantly decreased IL-10 production in the entire group as well as the LST-reactive volunteer group specifically.

Discussion

Whole *Leishmania* parasite vaccines showed some promise as being safe and immunogenic but are plagued by the issue of dose standardization. Implementation of the Immunome-derived vaccines approach, coupled with in vitro immunogenicity testing of predicted peptides using exposed human blood samples may accelerate the development of candidate vaccines for leishmaniasis.^{40,41} The *Leishmania* parasite surface proteins are good candidates for vaccine development, since they undergo minimal genetic change over time.

This study was designed to identify potentially immunogenic T cell epitopes of *L.donovani donovani* GP63 surface protein using the EpiMatrix tool kit. The predicted peptides were commercially synthesized and tested for immunogenicity. This study

was performed under field conditions, thus neither ELISpot nor flow-cytometry were available for more precise definition of T cell response and phenotype.^{42,43} In addition, the use of whole blood, the relatively low concentration of the peptide (100 μ g/ml) and the short duration of incubation may have contributed to the type of response that was measured here. Furthermore, cytokine ELISA on supernatant collected from whole blood may markedly underestimate immune responses at the T cell level. Finally, HLA typing was not performed for the tested volunteers, thus while the peptides were predicted to be promiscuous, the HLA-restriction of the immune response to some of the peptides may have diminished or altered the measured immune responses. Despite these caveats, there are some interesting immune responses that were observed to these peptides that deserve further consideration.

The in vitro immuno-modulatory effects of the peptides, studied as individual peptides and as pool were very different. Whereas P1 peptide induced significantly heightened IL-10 production in all volunteers and especially in LST-reactive volunteers, P3 produced a similar effect in LST non-reactive volunteers. The P4 peptide significantly reduced IL-10 production in the majority of the LST-reactive and a good percentage of LST

Table 3. Means of IFN- γ , IL-4 and IL-10 levels following stimulation with P1, P2, P3 and P4 peptide mixture (polytope)

Leishmanin skin test induration/mm	IFN- γ pcm/ml	IL-4 pcm/ml	IL-10 pcm/ml
All volunteers (n = 10)			
Control (No antigens/peptides)	242 \pm 266 (181)	24 \pm 36 (6)	624 \pm 496 (472)
Soluble <i>Leishmania</i> antigen	194 \pm 242 (131)	18 \pm 24 (14)	915 \pm 508 (934)
P1, P2, P3, P4 peptides mixture	249 \pm 321 (177)	28 \pm 34 (17)	285 \pm 315 (196)*
LST-reactive (\geq 5 mm) (n = 4)			
Control (No antigens/peptides)	47 \pm 94 (0.5)	0.5 \pm 0.6 (0.5)	506 \pm 445 (433)
Soluble <i>Leishmania</i> antigen	25 \pm 28 (11.5)	3.8 \pm 3.8 (3.5)	906 \pm 588 (875)
P1, P2, P3, P4 peptides mixture	21 \pm 22 (16.5)	9 \pm 16 (1.5)	79 \pm 36 (68)**
LST non-reactive (= 0 mm) (n = 6)			
Control (No antigens/peptides)	372 \pm 267 (304)	40 \pm 40 (25)	702 \pm 554 (472)
Soluble <i>Leishmania</i> antigen	307 \pm 259 (245)	27 \pm 27.3 (21)	921 \pm 507 (940)
P1, P2, P3, P4 peptides mixture	402 \pm 340 (213)	40 \pm 38 (28)	423 \pm 347 (315)

Continuous variables are expressed as mean \pm SD (median). pcm/ml = picogram/ml. P1, P2, P3 and P4 = the predicted and synthesized peptides.

* $p = 0.004$ ** $p = 0.00$.

non-reactive volunteers. A similar IL-10 reduction was seen when the peptides were pooled, whereas IL-10 production was reduced significantly in the total subject population and especially in the LST-reactive volunteer group.

The increased IL-10 production seen with P1 and P3 peptides stimulation could be perceived as a mechanism by which parasite peptides might reduce immune responses to the whole parasite in VL-susceptible individuals. If this hypothesis is accurate, T cells and monocytoic cells may work together to produce IL-10 to suppress antigen presentation and IFN- γ secretion, facilitating parasite propagation. This hypothesis is supported by studies that have shown IL-10 as a major cytokine in progression of *Leishmania* infection to visceral disease in susceptible individuals.⁴⁴⁻⁵²

Leishmania surface proteins have been shown to modulate host immune responses allowing establishment or propagation of themselves or other organisms. This could be achieved through inhibition of cell signaling or increased IL-10 production.⁵³⁻⁵⁵ Musa and colleagues have shown that alum-precipitated autoclaved *L. major* (alum/ALM) vaccine and sodium stibogluconate (SSG) can cure patients with chronic persistent post kala-azar dermal leishmaniasis by suppressing IL-10 expression and upregulating IFN- γ expression.⁴⁴

The immune-modulatory effects of P4 and the peptides pool can be exploited as an immune therapeutic tool to inhibit IL-10 production⁵⁶ in diseases with IL-10 over production. Visceral leishmaniasis and persistent post-kala-azar dermal leishmaniasis (PKDL) are good starting points.

The lack of clear immunogenicity of the peptides is not surprising since peptide epitopes are not expected to be particularly immunogenic, but the lack of significant differences in cytokine production in some of the subjects may be correlated with their stage of the immune response, especially the LST-reactive volunteers as previously reported.^{40,44}

In conclusion, although our synthetic peptides produced no clear Th1-response individually, in a pool they marginally increased IFN- γ secretion in whole blood assays, suggesting that they could be the basis of a sub-unit vaccine in combination

with a potent adjuvant. There was no significant IL-4 production upon peptide stimulation alone or pooled. Some of the peptides (P1 and P3) have a strong IL-10 upregulatory effect, making them good targets for future anti-pathogenesis tools. P4 and the peptides pool have marked IL-10 reduction effects, making them probable immune therapies for *Leishmania*-associated diseases with increased IL-10 levels. Macrophages may present different peptides preferentially in different individuals resulting in IL-10 production or inhibition. Differences in the immune responses to the peptides, as reported here, both between peptides and between disease types, deserve further investigation in future studies. In this study we used immunoinformatics tools and described some epitopes that appear to suppress immune responses.

Methods

T cell epitope prediction and peptide synthesis. The *L. donovani donovani* GP63 amino acid sequence (GenBank accession: ACT31401) was analyzed using the EpiMatrix algorithm for T cell epitope identification (www.immunome.org/iVAX/, Institute for Immunology and Informatics, University of Rhode Island). The protein was parsed into overlapping 9-mer frames, and each frame was predicted for its binding affinity for DRB1*1101 and DRB1*0804, the most commonly prevalent alleles in VL-at risk population in Eastern Sudan. In parallel, the 9-mer frames were predicted against a panel of 8 HLA-DR "supertype" alleles whose coverage spans > 90% of human populations worldwide. A 9-mer frame predicted to bind at least 4 such HLA alleles is considered an EpiBar. EpiBars may be the signature feature of highly immunogenic, promiscuous class II epitopes. Epitopes containing EpiBars tend to be more immunogenic than epitopes that do not. ClustiMer was employed to identify regions of high epitope density called clusters, occurring across frames and alleles. Hydrophobicity analysis was performed to identify hydrophobic epitopes that have low water solubility and are technically difficult to synthesize. Basic Local Alignment Search Tool (BLAST)

analysis was executed to confirm that none of the predicted clusters had any significant homology to the human genome. The choice of clusters selected for synthesis was based on the EpiMatrix cluster score, number of EpiMatrix hits in classical DR allotypes, hydrophobicity and number of EpiBars. The selected peptides were synthesized by the 21st Century Biochemicals.³⁶

Whole blood stimulation and cytokine measurement. Following informed consent, a group of 10 healthy volunteers ≥ 15 y of age from VL endemic areas in Eastern Sudan, with or without a history of VL, and leishmanin skin induration of ≥ 8 mm, were enrolled. Another group of 12 healthy volunteers aged ≥ 15 y from VL non-endemic areas was selected, without a clinical history of VL and absence of LST reactivity (induration = 0 mm). Eight ml of heparinized venous blood was collected from each volunteer. The lyophilized peptides were dissolved in de-ionized double distilled water. One ml aliquots derived from each of the samples were stimulated with either 100 μ l Phytohaemagglutinin (PHA), 100 μ l PBS, 100 μ g soluble *L. donovani* antigen (sLAg), 100 μ g from each of the 4 predicted peptides or 100 μ g of a pool composed of equal proportions of each peptide. The tubes were incubated at 37°C in a Cellestis incubator (Cellestis International) for 16–24 h. Following centrifugation at 4,000 rpm, the supernatants were stored in cryovials at -20°C for later analysis. Cytokine ELISA was used to measure IFN- γ , IL-4 and IL-10 as per manufacturer's instruction (Komabiotech Inc.). Statistical analyses were performed using Epidemiological Information (EpiInfo v3.5.3) software. The mean levels of cytokines were compared using Student's t-test and chi-square (GraphPad Software; www.graphpad.com). *P* values of < 0.05 were considered significant.

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