Prediction of T Cell Epitopes from *Leishmania major* Potentially Excreted/Secreted Proteins Inducing Granzyme B Production

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Abstract

*Leishmania*-specific cytotoxic T cell response is part of the acquired immune response developed against the parasite and contributes to resistance to reinfection. Herein, we have used an immune-informatic approach for the identification, among *Leishmania major* potentially excreted/secreted proteins previously described, those generating peptides that could be targeted by the cytotoxic immune response. Seventy-eight nonameric peptides that are predicted to be loaded by HLA-A*0201 molecule were generated and their binding capacity to HLA-A2 was evaluated. These peptides were grouped into 20 pools and their immunogenicity was evaluated by *in vitro* stimulation of peripheral blood mononuclear cells from HLA-A2*-immune individuals with a history of zoonotic cutaneous leishmaniasis. Six peptides were identified according to their ability to elicit production of granzyme B. Furthermore, among these peptides 3 showed highest affinity to HLA-A*0201, one derived from an elongation factor 1-alpha and two from an unknown protein. These proteins could constitute potential vaccine candidates against leishmaniasis.

Introduction

Leishmaniasis represents a heterogeneous group of diseases with an estimated incidence of 2 million cases annually worldwide [1]. They are caused by protozoan parasites of the genus *Leishmania* and are transmitted by the bite of infected sand flies. The disease is characterized by a spectrum of clinical manifestations determined by the species of *Leishmania* and the immune response of the host to the parasite [2]. It ranges from asymptomatic infections to cutaneous or fatal visceral forms. Most individuals who developed leishmaniasis or symptomless infection are resistant to subsequent infections, which makes vaccine development rational [3]. Studies of anti-*Leishmania* vaccine candidates have advanced in recent years due to the
understanding of the cell-mediated immunological mechanisms for controlling infection. However, no efficient vaccine is available for human use as of today and *Leishmania* vaccine development has proven to be a difficult and challenging task.

In common with other intracellular pathogens, cellular immune responses are critical for protection against leishmaniasis [4]. Considerable evidence suggests that *Leishmania major* infection induces the development of a Th1 response that not only controls the primary infection but also results in a lifelong immunity to reinfection. Protection against *Leishmania* infection has been shown to involve CD4+ and CD8+ T cells [5–9]. Indeed, peripheral blood mononuclear cells (PBMCs) obtained from individuals with active or healed localized cutaneous leishmaniasis proliferate and produce Th1 type cytokines, when stimulated *in vitro* with *Leishmania* antigens [10–12]. However, previous reports indicate the implication of CD8+ T cells in immunoprotective mechanisms in CL as well as the establishment of a Th1 response, mainly through the production of IFN-γ [12]. Although cytokine production is thoroughly analyzed, the involvement of cytotoxic activity in protection remains undefined.

Previously, we have shown that cytotoxic activity specific of *Leishmania major* (*L. major*) is developed by individuals living in areas of *L. major* transmission [13] and seems to play a crucial role in resistance to re-infection (Louzir H, 2005, unpublished data). Similar data suggest that CD8+ T cells may have a protective role in subclinical infection [14]. Contrastingly, evidence has been accumulated regarding the role of CD8+ T cells in the pathophysiology of CL. Indeed, these cells have been involved in the chronicity of *Leishmania* infection by exacerbating the tissue lesions, as described in mucocutaneous leishmaniasis caused by *L. braziliensis* [14–16]. Such controversy regarding the role of cytotoxicity in the pathogenesis of human leishmaniasis indicates that the functions of CD8+ T cells remain to be established. Furthermore, conflicting data about the route of activation of CD8+ T cells in leishmaniasis exist, since *Leishmania* resides within the parasitophorous vacuole of the macrophage and it is not clear how these cells present *Leishmania* antigens to CD8+ T cells through class I MHC [17–19]. Such controversy indicates that CD8+ T cells may have a protective role in subclinical infection [14]. Contrastingly, evidence has been accumulated regarding the role of CD8+ T cells in the pathophysiology of CL. Indeed, these cells have been involved in the chronicity of *Leishmania* infection by exacerbating the tissue lesions, as described in mucocutaneous leishmaniasis caused by *L. braziliensis* [14–16]. Such controversy regarding the role of cytotoxicity in the pathogenesis of human leishmaniasis indicates that the functions of CD8+ T cells remain to be established. Furthermore, conflicting data about the route of activation of CD8+ T cells in leishmaniasis exist, since *Leishmania* resides within the parasitophorous vacuole of the macrophage and it is not clear how these cells present *Leishmania* antigens to CD8+ T cells through class I MHC [17–19]. Several data suggest that external or secreted *Leishmania* antigens are able to reach macrophage cytosol to be presented by class I HLA molecules, which is a prerequisite for CD8+ T cell activation [17–19].

Previously, we also have characterized a set of 33 *Leishmania* proteins that are potentially secreted by the parasite in the phagolysosomal vacuole [20].

Herein, we have first used immuno-informatic tools to select nonameric peptides derived from the 33 *Leishmania major* excreted/secreted (*LmES*) proteins previously described based on the binding motifs of the class I MHC: HLA-A*0201*, which is the most frequent HLA allele in the Tunisian population (32.5%) [21]. Potentially ES proteins have been reported to contain antigens highly immunogenic and protective in vaccine models [17, 22–25]. Evidence has been shown regarding the immunogenicity of *Leishmania* ES proteins recovered from human cutaneous leishmaniasis [26]. *In silico* peptide prediction was followed by experimental validation of the capacity of these peptides to bind to HLA-A2 and the analysis of their immunogenicity in naturally-exposed individuals.

**Materials and Methods**

**Selection of study subjects**

Peripheral blood was obtained from 6 HLA-A*0201* positive and 6 HLA-A*0201* negative donors recovered from zoonotic cutaneous leishmaniasis (ZCL) living in an area of high transmission of *L. major* parasite (Central Tunisia). These individuals were selected based on (i) clinical criteria showing the presence of ZCL scars, (ii) positivity of the leishmanin skin test (LST) reactivity, and/or (iii) positive lymphoproliferative response to soluble *Leishmania* antigens (SLA) [immune individuals]. Screening of HLA-A*0201* positive individuals was done
using a lymphocytotoxicity test. HLA subtype A*0201 was confirmed by PCR using HLA SSP ABC Typing Kit (One Lambda Inc., Canoga Park, CA). HLA-A*0201 positive healthy individuals living outside endemic areas without any lymphoproliferative response to SLA were included as control groups. The main clinical and laboratory features of the selected individuals are described in Table 1. This study has obtained the Ethical Committee approval of the Pasteur Institute of Tunis (protocol number 07–0018). Individuals were included in the study after providing informed written consent.

### Epitope prediction and peptide synthesis

A set of 33 *L. major* genes encoding proteins that are potentially ES proteins by the parasite have previously been described in our laboratory [20]. All protein sequences were submitted to analysis by computerized HLA-binding prediction based on the freely accessible online databases: "Syfpeithi": [http://www.syfpeithi.de/bin/MHCServer.dll/EpitopePrediction.htm](http://www.syfpeithi.de/bin/MHCServer.dll/EpitopePrediction.htm), HLA-peptide binding prediction site supplied by: "BIMAS": [http://www-bimas.cit.nih.gov/molbio/hla_bind](http://www-bimas.cit.nih.gov/molbio/hla_bind), "RANKPEP": [http://www.http://bio.dfci.harvard.edu/MIF/RANKPEP](http://www.http://bio.dfci.harvard.edu/MIF/RANKPEP), and "NetMHC": [http://www.cbs.dtu.dk/services/NetMHC](http://www.cbs.dtu.dk/services/NetMHC). "Syfpeithi", "BIMAS", and "NetMHC" programs provide peptide sequences that are likely to be presented by the HLA-A*0201 molecules.

The probability for the peptides to be cleaved in the proteasome was predicted by "RANKPEP" along with a ranking or score. All peptides predicted with at least 3 softwares were selected and purchased from Intavis Bioanalytical Instruments (Cologne, Germany). Stock solutions of single peptides (20mg/mL) were produced by dissolving freeze-dried peptides in DMSO (Sigma-Aldrich, St. Louis, MO) and kept at -80°C until use.

### Parasites

*L. major* (Zymodeme MON25; MHOM/TN/94/CLC94) isolated from skin lesions of patients with CL was used in the present study. Parasites were cultivated on NNN medium at 26°C and

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**Table 1. Clinical and laboratory main features of the study subjects.**

| Sex | Age (years) | LCZ scars (Y/N) | LST (mm) | Proliferation (SLA) SI | HLA-A Typing |
|-----|-------------|-----------------|----------|-----------------------|-------------|
| ZCL04 | F | 58 | Y | 8.5 | 41.87 | A2/01 A24 |
| ZCL05 | F | 50 | Y | 7 | 63.22 | A2/01 A24 |
| ZCL23 | F | 40 | Y | ND | 20.42 | A2/01 A1 |
| ZCL25 | M | 41 | N | 7 | 44.62 | A2/01 A26 |
| ZCL29 | M | 32 | N | 10 | 45.4 | A2/01 A30 |
| ZCL34 | M | 46 | N | ND | 35.68 | A2/01 A30 |
| ZCL01 | M | 39 | Y | 12 | 109 | A1 A28 |
| ZCL03 | F | 42 | Y | 7.5 | 79 | A11 A32 |
| ZCL07 | F | 41 | Y | 8.5 | 14 | A26 A30 |
| ZCL14 | F | 24 | Y | 14 | 19 | A1 A23 |
| ZCL22 | F | 20 | N | ND | 12.5 | A24 A11 |
| ZCL24 | F | 24 | Y | ND | 6.8 | A3 A26 |
| T1 | F | 50 | N | ND | 1.6 | A2/01 A25/01 |
| T2 | F | 42 | N | ND | 2.02 | A2/01 A30/01 |

ND: Not determined; SI: Stimulation Index; SLA: Soluble *Leishmania* Antigens

LST: Leishmanin Skin Test; F: Female; M: Male; Y/N: Yes/No.

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then were progressively adapted to RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) containing 2mM L-Glutamine (Sigma-Aldrich, St. Louis, MO), 100U/mL Penicillin (Sigma-Aldrich, St. Louis, MO), 100mg/mL Streptomycin (Sigma-Aldrich, St. Louis, MO), and 10% heat-inactivated fetal calf serum (Sigma-Aldrich, St. Louis, MO). Stationary-phase metacyclic promastigotes were used to infect macrophages.

**Cell line**

The T2 cell line is a human tumor cell line that expresses HLA-A*0201 and lacks TAP1 and TAP2 transporters [T2 (174 x CEM.T2), (ATCC® CRL-1992™)]. It was kindly provided to us by Dr. Salem Chouaib (Gustave Roussy Institute, France).

**Detection of peptides binding to HLA-A*0201 molecules on T2 cells**

The affinity of peptides for HLA-A*0201 molecules was evaluated by using the stabilization assay as previously described [27]. Briefly, T2 cells were incubated with human β2-microglobulin at a final concentration of 10μg/mL in the presence or not of peptides at 10μg/mL for 16h at 37°C in 5% CO2. Cells were then incubated with 5μg/mL Brefeldin A (Sigma-Aldrich, St. Louis, MO) for 2h at 37°C. Expression of HLA-A*0201 on T2 cells was then determined by staining with fluorescein isothiocyanate-labelled anti-HLA-A2 antibody (BD Biosciences, San Jose, CA) and analyzed by flow cytometry using FACScan (BD Biosciences, San Jose, CA). Results were expressed in relative fluorescence intensity (RFI) calculated as the percentage increase of the mean fluorescence above that of the negative controls [28].

**In vitro stimulation of PBMCs with peptides**

To assess whether the selected peptides could stimulate or not CD8+ T cells, we have analysed the induction of GrB and IFN-γ by stimulated PBMCs from healed ZCL individuals. PBMCs separated from heparinized blood samples using Ficoll-Hypaque (Sigma-Aldrich, St. Louis, MO) density gradient centrifugation were resuspended in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 2mM L-Glutamine (Sigma-Aldrich, St. Louis, MO), 1mM sodium pyruvate (Gibco, Invitrogen, Grand Island, NY), 100U/mL Penicillin (Sigma-Aldrich, St. Louis, MO), 1μg/mL Streptomycin (Sigma-Aldrich, St. Louis, MO), 10μg/mL HEPES (Gibco, Invitrogen, Grand Island, NY), 20μg/mL Gentamicin (Gibco, Invitrogen, Grand Island, NY), 1X non-essential amino acids (Gibco, Invitrogen, Grand Island, NY), 2-mercaptoethanol (Gibco, Invitrogen, Grand Island, NY), and 10% (v/v) heat-inactivated human AB serum (Sigma-Aldrich, St. Louis, MO), [complete medium] at a concentration of 1.0x10⁶ cells/mL. Peptide pools were prepared instantly by dilution with phosphate buffered saline and then added to the cell culture at a final concentration of 1μg/mL. In some experiments, peptides were added separately to the culture at a concentration of 20μg/mL. As positive control, PBMCs were stimulated with 10ng/mL of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO) and 50ng/mL Ionomycin (Sigma-Aldrich, St. Louis, MO). All cultures were incubated at 37°C in 5% CO2 for 5 days. Culture supernatants were then harvested and frozen at -80°C until use.

**Granzyme B, IFN-γ, and IL-10 ELISA assays**

Granzyme B (GrB), IFN-γ, and IL-10 levels in culture supernatants were quantified with an enzyme-linked immunosorbent (ELISA) assay (MABTECH AB, Nacka Strand, Sweden) for the first one and OptEIA set ELISA assay kit (BD Biosciences, San Jose, CA) for the others. The results were expressed as pg/mL based on the standards provided by the kits. Quantification thresholds were fixed to 100pg/mL for GrB, 45pg/mL for IFN-γ, and 20pg/mL for IL-10.
Statistical analyses

Statistical analyses were carried out by using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). Mann-Whitney test was used to compare the induction of GrB and IFN-γ between the different study groups. Correlation between GrB and IFN-γ levels induced by peptide pools or individual peptides were estimated by use of Spearman’s rank order correlation coefficient. A classification of the peptide pools according to their induction of GrB was achieved using Matlab 7.0 (Mathworks, Inc., Natick, MA). A Kruskal-Wallis test was performed to compare the rank of peptide pools.

Results

Selection of potential HLA-A*0201-binding peptides within LmES proteins

The sequence of 33 different clones of potentially ES proteins has been used. Based on computer software predictions, putative class I HLA-restricted T cell epitopes were identified. Twenty proteins were able to generate a total of 78 nonameric peptides that could be loaded by HLA-A’0201 molecule (Table 2). Subsequently, we have evaluated the binding affinity of these peptides to HLA-A’0201 molecules by class I HLA stabilization assay. This assay measures the increase of HLA-A’0201 molecules induced on T2 cells following exposure to exogenous HLA-A’0201 binding peptides, with high affinity peptides inducing HLA-A’0201 up-regulation more strongly than low-affinity peptides. Individual results are shown on Fig 1A. The 78 tested peptides were classified into 3 groups regarding the percentage of RFI. Six peptides namely, D6, E1, F6, G1, G2, and G3 showed the highest percentage of RFI increase (RFI > 200%), 50 peptides showed intermediate affinity (RFI ranges from 100 to 200%), and 22 peptides had a weak affinity (RFI < 100%) (Fig 1B).

Stimulation with peptide pools induces production of GrB

Given their large number and to test their immunogenicity in vitro, the predicted peptides were compiled into 20 pools as shown in Table 3. Each pool contains peptides belonging to the same protein. Pools were tested for their ability to induce GrB secretion by PBMCs obtained from 5 HLA-A’0201”-immune donors and 2 HLA-A’0201” healthy donors. Surprisingly, low IFN-γ levels, not exceeding 45pg/mL (quantification threshold), were detected in culture supernatants of PBMCs obtained from immune individuals, stimulated with the different peptide pools. Similar results were obtained for IL-10, which was detected at low levels (ranging from 20 to 120pg/mL) in only one immune individual. Stimulation of PBMCs from these individuals with SLA or PMA/Ionomycin showed high levels of IFN-γ (data not shown).

As shown in Fig 2A, peptide pools induce variable levels of GrB in culture supernatants of PBMCs obtained from immune individuals. In contrast, for healthy donors no GrB production could be induced. Considering the variability of detected GrB levels, we have resorted to a ranking method (Fig 2C). Classification of the 20 peptide pools corresponding to the 20 different LmES proteins was done according to their capacity to induce GrB production. The concept consists of computing the rank of the different pools for each individual and then calculating the mean rank of each pool for the 5 individuals tested. Interestingly, the Kruskal-Wallis test has revealed that the highest GrB levels were induced by the peptide pools P19, P20, P13, P18, P12, and P17 corresponding respectively to the proteins Pr78, Pr90, Pr38, Pr77, Pr37, and Pr74. GrB levels measured in culture supernatants of PBMCs stimulated with these peptide pools were significantly higher compared to those induced by the other ones (p = 0.0002). Taken together, these results allow us to rank 6 proteins among the potentially ES proteins as best generators of peptides that are recognized by PBMCs of HLA-A’0201”-immune individuals.
Table 2. Characteristics of *in silico* predicted HLA-A*0201*-restricted peptides for *LmES* proteins.

| Protein          | peptide    | Start Position | SEQUENCE       | RANKPEP<sup>a</sup> | SYFPEITY<sup>b</sup> | BIMAS<sup>a</sup> | NET MHC MATRIX<sup>x</sup> | NET MHC ANN<sup>b</sup> |
|------------------|------------|----------------|----------------|----------------------|----------------------|----------------|---------------------------|------------------------|
| **Pr 9.1 (LmjF.14.0820)** | A1         | 85             | ALQEETHVL     | 82                   | 75%                  | 35.9102        | 25.522                    | 101                    |
|                  | A2         | 427            | YMAQKAEVE     | 74.05                | 69.44%               | 113.2229       | 20.067                    | 48                     |
|                  | A3         | 259            | KLTVSAAA      | 93                   | -                    | -              | 23.091                    | 1580                   |
|                  | A4         | 92             | VLGSHVQTL     | 86                   | 75%                  | 83.5270        | 25.372                    | -                      |
|                  | A5         | 182            | LLRQETARL     | 82                   | 72.22%               | -              | 23.016                    | 1468                   |
|                  | A6         | 362            | HLMGQNLK      | 79                   | 83.33%               | -              | 25.566                    | 274                    |
| **Pr 9.2 (Ribosomalprotein S18)** | A7         | 107            | RLDDLERL      | 70                   | 69.44%               | 7.5019         | 20.177                    | 1437                   |
|                  | A8         | 45             | YLLDVSTLL     | 94                   | 6.44%                | 1490.7110      | 26.521                    | 100                    |
|                  | A9         | 198            | NLIDFNFKL     | 93                   | -                    | 23.016         | 1580                      | 20.772                 | 152                    |
| **Pr 12 (Ubiquitin protein ligase: LmjF.07.0280)** | A10        | 185            | LLKDSFAFL     | 85                   | 66.66%               | -              | 22.603                    | 653                    |
|                  | A11        | 230            | CLLDSFKEL     | 75                   | 66.66%               | 615.7285       | 22.474                    | -                      |
|                  | A12        | 167            | VLLEENRTTL    | 73                   | 66.66%               | -              | 20.196                    | -                      |
| **B1**           | B1         | 9              | VLCALLFCV     | 68                   | 72.22%               | 1577.3003      | 25.092                    | 562                    |
| **B2**           | B2         | 387            | KLHPYDVKV     | 66                   | 69.44%               | 178.9225       | 24.676                    | 318                    |
| **Pr 13 (LmPDI)** | B3         | 259            | ALKGSDEV      | 91                   | 63.88%               | -              | 22.517                    | 344                    |
|                  | B5         | 157            | EMASMITKV     | 88                   | 63.88%               | -              | -                         | 1171                   |
|                  | B6         | 63             | DMLAGIALT     | 69                   | 80.55%               | -              | -                         | 716                    |
|                  | B7         | 321            | LLSAQIARL     | 93                   | 77.77%               | 83.5270        | 23.94                     | 771                    |
|                  | B8         | 256            | ALKGSLVAV     | 84                   | 80.55%               | 1267.1043      | 24.159                    | 174                    |
| **Pr 15 (LmjF.15.0410)** | B9         | 737            | RLMQCVQVL     | 81                   | 69.44%               | 181.7940       | 23.118                    | 1445                   |
|                  | B10        | 33             | SLVVSASL      | 88                   | 72.22%               | -              | 23.241                    | 5167                   |
|                  | B11        | 29             | SLCRSLVVV     | 86                   | 77.77%               | -              | 23.281                    | 5111                   |
|                  | B12        | 395            | ALNDALWAV     | 96                   | 80.55%               | 4919.0652      | 27.307                    | 45                     |
| **C1**           | C1         | 77             | RLLVQDLAQ    | 83                    | 77.08%               | -              | 23.214                    | 5167                   |
| **Pr 20.1 (Chaperonin subunit alpha: LmjF.32.3270)** | C2         | 175            | IVVDAIMS      | 101                  | -                    | 366.6129       | 21.354                    | -                      |
|                  | C4         | 367            | VIAGTSNAV     | 77                   | 69.44%               | -              | -                         | 835                    |
|                  | C5         | 128            | AMREALRYL    | 76                   | 72.22%               | -              | -                         | 349                    |
|                  | C6         | 213            | GVFAAISI      | 82                   | 72.22%               | -              | 13.8482                   | -                      |
| **Pr 20.2 (LmjF.36.2650)** | C7         | 69             | QVGFLEG       | 50                   | 55.55%               | 8.0051         | -                         | 600                    |
|                  | C8         | 145            | GLDYESEEL     | 46                   | 55.55%               | 4.1870         | -                         | -                      |
| **Pr 22 (LmjF.05.0710)** | C9         | 111            | RVAASVAA      | 95                   | 63.88%               | 13.9973        | -                         | 10621                  |
|                  | C10        | 221            | GTDDTVAA      | 64                   | 63.88%               | 3.6438         | -                         | 8253                   |
|                  | C11        | 141            | TIPSFIVR      | 88                   | 63.88%               | 83.5841        | -                         | 2164                   |
|                  | C12        | 68             | RLLEGSAI      | 79                   | 61.11%               | 30.8995        | -                         | 582                    |
| **Pr 22.1 (Ribosomal protein S9: LmjF.36.1250)** | D2         | 127            | LIQQRHIAV     | 66.05                | 58.33%               | 16.2578        | -                         | 1987                   |
|                  | D3         | 134            | AVAKIVTI      | 95                   | 66.66%               | -              | -                         | 8403                   |
| **Pr 27 (similar to LAEL147_000045800)** | D4         | 24             | NMMAVQGLL     | 81                   | 63.88%               | 17.0684        | -                         | 6564                   |
|                  | D5         | 836            | KLEDDEVV      | 83                   | 72.22%               | 261.7205       | 23.158                    | 170                    |
|                  | D6         | 892            | ELLGNLEE      | 79                   | 75%                  | 21.7519        | 24.38                     | 535                    |
| **Pr 31 (LmjF.34.0680)** | D7         | 690            | RMADEVQRL     | 77                   | 69.44%               | 145.4898       | 20.464                    | 520                    |
|                  | D8         | 135            | RAVSLHEL      | 81                   | 80.55%               | 49.1335        | 22.162                    | -                      |
|                  | D9         | 648            | LLPGAYQSI     | 78                   | 69.44%               | 26.6036        | -                         | 729                    |
|                  | D10        | 781            | VIAEPLYV      | 77                   | -                    | 366.6129       | -                         | 1130                   |

(Continued)
Evaluation of GrB and IFN-γ production by PBMCs stimulated with individual peptides

All peptides belonging to the selected proteins were tested separately for their capacity to induce GrB and IFN-γ. PBMCs obtained from 3 HLA-A*0201+ and 3 HLA-A*0201--immune donors were stimulated with the different individual peptides, then GrB and IFN-γ levels were measured in culture supernatants. We have used PBMCs obtained from 2 HLA-A*0201- healthy individuals as negative controls. It should first be mentioned that levels of IFN-γ

Table 2. (Continued)

| Protein                        | peptide | Start Position | SEQUENCE | RANKPEP | SYFPEITY | BIMAS | NET MHC MATRIX | NET MHC ANN
|-------------------------------|---------|----------------|----------|---------|----------|-------|----------------|---------|
| D11                           | 40      | PLASAVISPV     | 81       | -       | -        | 25.673| 641            |         |
| D12                           | 320     | LLPAPLVSV      | 90       | 86.11%  | 271.9483 | 26.986| 504            |         |
| Pr 37 (LmjF.36.3860)          | E1      | 575            | MLLWTAHAV | 82     | 69.44%   | 437.482| 25.381         | 162     |
| Pr 38 (similar to LTRL590_180019400) | E5      | 69             | VVAGMLRWV | 65     | 63.88%   | 26.1750| -              | 1295    |
| Pr 57 (Ribosomal protein S16: LmjF.26.0880/ LmjF.26.0890) | E9      | 115            | FLAYDKFL | 115    | 61.11%   | 569.9488| -              | 343     |
| Pr 66 (LmjF.26.0880/ LmjF.26.0890) | E10     | 461            | HIFDRVAGV | 78     | 75%      | -      | -              | 361     |
| Pr 68 (Ribosomal protein L7/L12-like protein: LmjF.07.0500) | F1      | 136            | GLQEVTAR | 83     | 66.66%   | 8.5549 | -              | 317     |
| Pr 74 (elongation factor proteasome 1-alpha: LmjF.17.0082) | F3      | 211            | TLKLNIR | 87     | 72.22%   | -      | -              | 735     |
| Pr 77 (Probable regulatory ATPase (L. major): LmjF.13.1090) | F9      | 404            | ALRERRMK | 80     | 72.22%   | 21.6724| 20.701         | 677     |
| Pr 78                          | F3      | 187            | GLEOQIQEI | 81     | 66.66%   | -      | -              | 623     |
| Pr 90 (Ribosomal protein L3: LmjF.32.3130) | G3      | 83             | MLVQSCTSI | 90     | 55.55%   | -      | -              | 1781    |
| G4                            | 110     | VVSVLTHS | 69     | 58.33%   | 21.087 | 2809 |
| G5                            | 106     | WIPPVVS | 56     | 66.66%   | -      | -    | 5645          |
| G7                            | 282     | QLNNKIKYQI | 88     | 69.44%   | 23.9954| -    |

a: Results expressed as score.  
b: Results expressed as percentage calculated according to the highest score (= 36).

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detected in culture supernatants were very weak (<45 pg/mL) in the individuals tested with most of the peptides. Regarding GrB, no production could be detected in supernatants of PBMCs obtained from negative controls in response to stimulation with any of these peptides (data not shown). Interestingly, variable levels of GrB were detected in HLA-A*0201+ and HLA-A*0201--immune donors (Fig 3A). However, there was no statistically significant difference between HLA-A*0201+ and HLA-A*0201--immune individuals (p > 0.05 for all tested peptides). Taken together, 6 peptides (E2, E6, F6, G2, G3, and G4) among the 24 tested have been shown to induce the highest levels of GrB (Fig 3B). Furthermore, 5 peptides out of the 6 selected ones stabilized HLA-A2 molecule on T2 cells with high (F6, G2, and G3) or intermediate (E2 and G4) affinity. Only E6 showed no affinity for HLA-A2 molecule.

Discussion

For a long time, it has been a consensus that a Th1 dominant response promotes IFN-γ production, induces lesion healing, and controls parasite burden [7]. Based on this, different vaccine candidates have been selected. CD8+ T cells play a major role in controlling leishmaniasis, since growing evidence did prove their participation in the immune response against different Leishmania species studied in experimental models and humans [29, 30]. Few studies have focused on the identification of Leishmania epitopes that can be presented by class I MHC molecules to CD8+ T cells [31, 32]. Currently, there are no well-defined Leishmania CD8+ T cell epitopes, which has made it difficult to investigate how CD8+ T cell activation occurs in leishmaniasis. Antigen-presenting cells, such as macrophages and dendritic cells have been shown to be able to capture, process, and present in a class I MHC-restricted manner various exogenous antigens including those derived from intracellular pathogens like Leishmania parasites [17, 33].

Previously, we have characterized 33 Leishmania genes coding for proteins that are probably released by the parasite in the phagolysosomal vacuole [20].

Herein, we have analyzed these potentially LmES proteins in an attempt to identify HLA-A*0201-binding peptides able to activate CD8+ T cells. We have identified 6 epitopes: E2,

Table 3. Setup of peptide pools.

| Protein | 9.1 | 9.2 | 12 | 13 | 15 | 20.1 | 20.2 | 22 | 22.1 | 27 | 31 | 37 | 38 | 57 | 66 | 68 | 74 | 77 | 78 | 90 |
|---------|-----|-----|----|----|----|------|------|----|------|----|----|----|----|----|----|----|----|----|----|----|----|
| Pool*   | 1   | 2   | 3  | 4  | 5  | 6    | 7    | 8  | 9    | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
| A1      | A7  | A8  | B1 | B7 | C1 | C6   | C9   | C11| D4   | D5 | D12| E3  | E8  | E10 | F4  | F7  | F12 | G6  |
| A2      | A9  | A2  | B3 | B8 | C2 | C7   | C10  | C12| D6   | E1 | E4  | E9  | E12 | F5  | F8  | G1  | G7  |
| A3      | A10 | B4  | B9 | C3 | C8 | D1   | D7   | E2 | E5   | F1 | F6  | F9  | G2  |     |     |     |     |
| A4      | A11 | B5  | B10| C4 | D2 | D8   | E6   | E5 | F1   | F6 | F9  | G2  |     |     |     |     |     |
| A5      | A12 | B6  | B11| C5 | D3 | D9   | E7   | F3 | F1   | F3 | G4  |     |     |     |     |     |     |
| A6      | B12 | D10 | D11|     |     |      |      |    |      |    |     | G5  |     |     |     |     |     |
| n       | 6   | 1   | 5  | 6  | 5  | 3    | 2    | 5  | 1    | 3  | 5   | 2   | 1   | 5   | 3   | 5   | 6   | 2   |

* Numbers 1 to 20 refer to peptide pools.

n: Number of peptides made up for each protein.
E6, F6, G2, G3, and G4 that are able to induce GrB production by PBMCs obtained from immune individuals. These peptides derived from the sequence of the Pr37, Pr38, Pr78, and Pr74 proteins. Our study is not exhaustive since the choice of the 33 potentially ES protein sequences was made out of more than 8,000 parasite protein-coding genes. In fact, there are probably additional *Leishmania* ES proteins that have not been described as of yet. Moreover, it is quite possible that non-excreted parasitic antigens able to generate CD8+ T cell epitopes do also exist.

Fig 2. Peptides grouped in pools induced GrB production. (A) PBMCs from 5 HLA-A*0201+-donors with a history of ZCL in response to stimulation with peptide pools at a final concentration of 1μg/mL per peptide or (B) SLA (10μg/mL) and PMA/ionomycin (10ng/mL and 50ng/mL, respectively) as positive controls. GrB production was assessed in culture supernatants using ELISA. (C) Rank of the peptide pools. +: mean pool rank, -: median.

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Our hypothesis is clear and our approach is simple. We have assumed that *LmES* proteins may generate peptides that could be presented to CD8\(^+\) T cells. This approach oriented us towards 4 proteins of interest. Pr74 corresponding to elongation factor-1 alpha (EF-1\(\alpha\)), which is a multifunctional protein essentially involved in protein biosynthesis and parasite survival in infected macrophages \[34–39\]. Indeed, the presence of *Leishmania* EF-1\(\alpha\) in the cytosol of infected macrophages has also been demonstrated \[37, 38\]. Interestingly, this protein was one of the leishmanial antigens that was used for construction of the vaccine LeishDNAvax composed of MIDGE-TH1 vectors encoding 5 conserved leishmanial antigens: KMP11, TSA, CPA, CPB, and Pr74 \[40\]. The 3 remaining proteins were described as potentially secreted by the parasite but do not correspond to any proteins described in sequence libraries. Among them, 2 proteins Pr37 and Pr78 contain signal peptide sequences and one, Pr38 was predicted to be secreted via non-classical pathways \[20\].

In this study, the selection of peptides was performed using the computer-based prediction method, which constitutes a useful tool for peptide identification. However, this method is unable to predict all peptide sequences. Thus, some interesting and immunogenic peptides could be missed out and prevented from being tested in the immune response, only because *in silico* methods could not predict them. So the best way is to use overlapping peptides to scan all
protein sequences as performed by Basu and collaborators regarding peptide identification belonging to the protein Kmp11 [31]. Nonetheless, this method could not be applied in our study because it would have given us thousands of peptides to test, which was currently not feasible for all proteins tested here. Moreover, Pelte and collaborators have previously identified one single stimulating peptide, which did not stabilize HLA-A’0201 expression on T2 cells and could therefore not be presented by HLA-A’0201 [41]. The paradox of T cell recognition of a peptide that fails to bind to HLA-A2 could be explained by the fact that peptides could be recognized after binding to other class I HLA molecules carried out by the patients, which could subsequently present the epitope to specific T cells.

The next step was to analyse the immunogenicity of the antigenic peptides in naturally-infected individuals. In addition to their capacity to bind to class I MHC molecules, we have assumed that these peptides exist in large quantities in the intracellular phagolysosomal vesicle. Consequently, in natural infection some peptides predicted to have high affinity in theoretical and functional tests could fail to induce significant immune response since they are not secreted or because they are in different cellular structures. By contrast, some low-affinity peptides can still be presented by class I MHC molecules because of their abundance in the intracellular phagolysosomal vesicle. For these reasons, all predicted peptides were compiled in pools and their immunogenicity tested in HLA-A’0201+-ZCL recovered individuals. Pooling peptides has been used in many previous studies [28, 32] and does not seem to be a limiting factor [42, 43] considering that all peptides are predicted with almost equal affinity for HLA binding and with same stimulatory concentrations in cultures. Unexpectedly, weak levels of IFN-γ were detected in culture supernatants of PBMCs stimulated with the different peptide pools. This cannot be attributed to the inhibition of IFN-γ production by IL-10, which was not detected in these culture supernatants. This could rather be explained by the possibility of low frequency of memory CD8+ T cells due to stimulation conditions. In fact, in the present study PBMCs were stimulated with peptide pools without adding IL-2 or anti-CD48 as done by Seyed and collaborators [32]. Our results are similar to those described in other studies using different read out systems for the IFN-γ detection in T cells, such as flow cytometry [41] or ELISPOT [28]. Results of these two studies showed a weak production of IFN-γ induced by only few peptides among those selected by using bioinformatics.

Further, we have shown here that variable levels of GrB were induced by the different peptide pools, which led us to rely on the ranking method. Thus, we have selected 6 proteins as the best generators of peptides recognized by PBMCs obtained from HLA-A’0201-immune individuals. Consequently, we have analyzed separately the immunogenicity of all peptides belonging to these proteins. The highest GrB levels were detected in supernatants of PBMCs stimulated with the peptides E2, E6, F6, G2, G3, and G4. Unexpectedly, these peptides have also induced GrB production in HLA-A’0201-negative immune individuals. Similar results have been reported by Seyed and collaborators [32]. As discussed by the authors, this could be explained by specificity overlap between supertypes of HLA molecules and would need to be further confirmed in a larger population of individuals bearing other HLA-A alleles [32, 44]. To achieve that, we will be extending our study to map potential CD8+ T cell epitopes restricted to other common class I HLA alleles.

To better trigger the specific response to our peptides, several experiments are planned, i.e., establishing “short-term” cell lines specific of the selected peptides and analyzing their ability to induce the production of GrB, IFN-γ, IL-2, and IL-10 when co-cultured in the presence of the T2 cell line pulsed with each of the peptides, and used as antigen-presenting cells.

In conclusion, we have identified novel HLA-A’0201-restricted immunogenic CD8+ T cell epitopes derived from potentially LmES proteins using in silico prediction and functional
studies on PBMCs obtained from immune individuals. Proteins we have identified here could constitute potential candidate vaccine antigens.

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Author Contributions

Conceived and designed the experiments: IN TB HL. Performed the experiments: IN TB MC RB YG. Analyzed the data: IN TB HL NBJ. Contributed reagents/materials/analysis tools: ABS NBH. Wrote the paper: IN TB. Critical review of the manuscript: SG MBA.

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