Experimental validation of multi-epitope peptides including promising MHC class I- and II-restricted epitopes of four known Leishmania infantum proteins

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INTRODUCTION
Leishmaniasis is a significant worldwide health problem for which no vaccine exists. Activation of CD4+ and CD8+ T cells is crucial for the generation of protective immunity against parasite. Recent trend in vaccine design has been shifted to epitope-based vaccines that are more specific, safe, and easy to produce. In the present study, four known antigenic Leishmania infantum proteins, cysteine peptidase A (CPA), histone H1, KMP-11, and Leishmania eukaryotic initiation factor (LeIF) were analyzed for the prediction of binding epitopes to H2Db MHC class I and II molecules, using online available algorithms. Based on in silico analysis, eight peptides including highly scored MHC class I- and II-restricted epitopes were synthesized. Peptide immunogenicity was validated in MHC compatible BALB/c mice immunized with each synthetic peptide emulsified in complete Freund’s adjuvant/incomplete Freund’s adjuvant. CPA_p2, CPA_p3, H1_p1, and LeIF_p6 induced strong spleen cell proliferation upon in vitro peptide re-stimulation. In addition, the majority of the peptides, except of LeIF_p1 and KMP-11_p1, induced IFN-γ secretion, while KMP-11_p1 indicated a suppressive effect on IL-10 production. CPA_p2, CPA_p3, LeIF_p3, and LeIF_p6 induced IFN-γ-producing CD4+ T cells indicating a T11-type response. In addition, CPA_p2, CPA_p3, and H1_p1 induced also the induction of CD8+ T cells. The induction of peptide-specific IgG in immunized mice designated also the existence of B cell epitopes in peptide sequences. Combining immunoinformatic tools and experimental validation, we demonstrated that CPA_p2, CPA_p3, H1_p1, H1_p3, CPA_p2, LeIF_p3, and LeIF_p6 are likely to include potential epitopes for the induction of protective cytotoxic and/or T11-type immune responses supporting the feasibility of peptide-based vaccine development for leishmaniasis.

Keywords: in silico analysis, cysteine peptidase A, histone H1, kinetoplastid membrane protein 11, Leishmania eukaryotic initiation factor, lymphocyte proliferation, CD8+ IFN-γ+ T cells, CD4+ IFN-γ- T cells

1http://www.who.int/en/
testing of overlapping peptides or peptide libraries. T cell epitope prediction by bioinformatic analysis of protein sequences has been proposed as a promising strategy for vaccine development and an increasing number of tools have been developed, based on different algorithms and methods (15, 16). There is great possibility of missing the emergence of the sequence mutants that would potentially escape the vaccine’s protective effect. Moreover, the fact that T cells from genetically distinct populations would recognize and respond to a single peptide epitope, underline the need of identifying one or more epitope(s) that bind to multiple HLA alleles and cover close to 100% of the genetically diverse human population (17). Multi-peptide-based vaccines are designed to generate a diverse immune response to incorporate antigens and to reduce limitations due to MHC restriction into a single entity.

The effectiveness of a vaccine depends on its capacity to ensure long-lasting cell-mediated immunity. In VL, there is evidence that an interplay of T helper cytokines (T(H)1/T(H)2) is observed, whereas resistance or resolution of infection is associated with dominant T(H)1 response and CD8+ T cells (18–20). Furthermore, successful treatment of VL with sodium stibogluconate requires the presence of both CD4+ and CD8+ T cells (21) accompanied with IL-12 and IFN-γ production (22). In contrast, T(H)2 response with IL-4 and IL-10 production results in susceptibility to infection and development of severe disease. Murine models of leishmaniasis have been extensively used to study the pathogenesis of the disease and to test novel therapeutic agents or potent vaccine candidates in pre-clinical studies. One of the most widely studied and commonly used model of VL is the BALB/c strain of mice infected intravenously with *L. infantum*. Although this strain is considered to be susceptible and the infection progresses during the first month, the infection is then controlled by the host immune response. This mouse model is comparable to self-controlled oligosymptomatic cases and therefore is useful for the study of the protective immune response (23).

Several reports demonstrate that different leishmanial antigens elicit desired T(H)1 and CTL responses capable to sustain protection against experimental challenges (24). Among these antigens, cysteine peptidase A (CPA), histone H1, kinetoplastid membrane protein 11 (KMP-11), and *Leishmania* histone H1 (LeIF) are considered important immunogens, as supported by numerous studies. Specifically, CPA induces protection against *L. major* in the experimental model of CL through development of specific T(H)1 immune responses (25–27). Histone H1 and KMP-11, structural highly conserved proteins, are able to trigger specific immune responses (28–33), and immunization with these proteins confer protection against *L. major* or *L. infantum* infections in experimental animal models (34–38). Moreover, LeIF, originally described as a T(H)1-type natural adjuvant, is capable of stimulating IL-12 mediated T(H)1 responses in PBMCs of patients (39). Furthermore, recombinant forms of CPA, histone H1, and KMP-11 act as potent B cell immunogens since they are recognized by sera from either recovered or active cases of CL and VL, as well as by sera from asymptomatic or symptomatic dogs with leishmaniasis (40–45).

In the present study, we applied immunoinformatics using currently available online algorithms in order to identify potentially immunogenic T cell epitopes from the above mentioned *L. infantum* proteins, and design multi-epitope peptides containing both MHC class I and II-restricted epitopes as possible candidate peptide vaccines for VL. Immunogenicity of the synthetic multi-epitope peptides in terms of T cell activation was validated in immunized BALB/c mice by analyzing peptide-specific proliferative responses and cytokine production by CD8α+ and CD4+ T cells.

**MATERIALS AND METHODS**

**PROTEIN SEQUENCE RETRIEVAL AND PREDICTION OF MHC CLASS I AND II BINDING EPITOPES**

Full protein sequences of selected proteins, CPA, Histone H1, KMP-11, and LeIF, were retrieved from GenBank database on JPCM5 strain (MCAN/ES/98/LLm-887) and analyzed by SignalP3 for the prediction of signal peptides and transmembrane domains (Table 1). Potential MHC class I and II binding epitopes derived from the four *L. infantum* proteins, were predicted by *in silico* analysis, using three online available, binding algorithms named SYFPEITHI4, BIMAS5, and NetMHCII6. The cut-off score was adjusted to ≥18 for SYFPEITHI, ≥100 for BIMAS, and a default prediction threshold (binding affinity <500 nM) depicting accuracy >85% was used for NetMHCII (Tables 2 and 3).

**SYNTHETIC MULTI-EPI-TOPE PEPTIDES**

Based on the prediction results of the algorithms used, 9-mer epitopes MHC class I-restricted and 15-mer epitopes MHC class II-restricted giving high score against H2d alleles were extracted and combined in order to generate multi-epitope peptides for each *L. infantum* protein. Thus, 8 peptides, 20–30 amino acid (aa) length, were designed in a way that each peptide included at least one MHC class I-restricted epitope scored very high, as well as adjacent or overlapping MHC class II-restricted epitopes scored also high. Sequence homology between each multi-epitope peptide and mouse proteome were analyzed on BLAST database2 and peptides with 100% identity were excluded or re-designed to

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2http://www.ncbi.nlm.nih.gov/genbank
3http://www.cbs.dtu.dk/services/NetMHCII
4http://www.cbs.dtu.dk/services/NetMHCII
5http://syfpeithi.de
6http://www.bimas.cit.nih.gov/molbio/hla_bind
7http://www.ncbi.nlm.nih.gov/BLAST
cp

| Table 1 | *L. infantum* proteins selected as candidate antigens for epitope mapping |
| **Protein abbreviation** | **Protein name** | **Protein length (aa)/mass (kDa)** | **GenBank accession number** |
| CPA | Cysteine peptidase A | 354/39.0 | CAM67356 |
| Histone H1 | Histone H1 | 111/11.1 | ABN54817 |
| KMP-11 | Kinetoplastid membrane protein 11 | 92/11.2 | CAA64883 |
| LeIF | Leishmania eukaryotic initiation factor | 403/45.3 | CAM65231 |
Table 2 | *In silico* predicted MHC class I-restricted 9-mer epitopes of *L. infantum* proteins.

| Protein     | No. of epitope | Epitope sequence     | SYFPEITHI | BIMAS |
|-------------|---------------|----------------------|-----------|-------|
|             |               |                      | H2-K<sup>d</sup> | H2-L<sup>d</sup> | H2-K<sup>d</sup> | H2-L<sup>d</sup> | H2-D<sup>d</sup> |
| CPA         | 1             | 8-FFAIVVTIL-18       | 23        | –     | 1152   | –     | –     |
|             | 2             | 84-HYDVGKVPA-92      | 23        | –     | –      | –     | –     |
|             | 3             | 273-LYFGGVTIL-281    | 23        | –     | 2400   | –     | –     |
|             | 4             | 254-AYGKNGPV-262     | 22        | –     | 1200   | –     | –     |
|             | 5             | 23-SALIALQTPI-31     | 20        | –     | –      | –     | –     |
|             | 6             | 102-LYLNPYYA-110     | 20        | –     | 120    | –     | –     |
|             | 7             | 334-NYVTATID-343     | 20        | –     | –      | –     | –     |
|             | 8             | 7-FFAIVVVTI-15       | –         | –     | 1152   | –     | –     |
|             | 9             | 165-QWALKNHSI-173    | –         | –     | 240    | –     | –     |
|             | 10            | 62-RFNAFKONM-70      | –         | –     | 144    | –     | –     |
|             | 11            | 319-GYIRLAMGS-327    | –         | –     | 120    | –     | –     |
|             | 12            | 179-QVLYSCDN1-187    | –         | –     | 115.20 | –     | –     |
|             | 13            | 68-QNMQTAYFL-76      | –         | –     | 115.20 | –     | –     |
|             | 14            | 160-GNIEGQWAL-168    | –         | –     | 115.20 | –     | –     |
| Histone H1  | 15            | 46-KKAGAKKAV-54      | 18        | –     | –      | –     | –     |
|             | 16            | 2-SSDOSAVAAL-10      | 17        | 19    | –      | –     | –     |
| KMP-11      | 17            | 4-TYEEFSAKL-12       | 20        | –     | 2400   | –     | –     |
|             | 18            | 47-HYFEKFERMI-55     | 19        | –     | 2000   | –     | –     |
|             | 19            | 72-HFKGFAE1-79       | 18        | –     | 960    | –     | –     |
|             | 20            | 69-HSEHKFKF-77       | –         | 18    | –      | –     | –     |
|             | 21            | 7-EFSALQDR-15        | –         | –     | 960    | –     | –     |
| LeIF        | 22            | 385-HYHTQIDEL-393    | 24        | –     | 2000   | –     | –     |
|             | 23            | 360-RYGRKGVAIL-368   | 23        | –     | 2000   | –     | –     |
|             | 24            | 8-APQQDQSFL-16       | –         | 22    | –      | 225   | –     |
|             | 25            | 25-IPSFDQML-33       | –         | 22    | –      | 150   | –     |
|             | 26            | 199-LPKDIQAL-207     | –         | 22    | –      | –     | –     |
|             | 27            | 344-LPTKNENLY-352    | –         | 22    | –      | 150   | –     |
|             | 28            | 393-LPVDFAAYL-401    | –         | 22    | –      | 150   | –     |
|             | 29            | 14-SFLDDQPOV-22      | 21        | –     | 576    | –     | –     |
|             | 30            | 197-RFLPKDQV-205     | 21        | –     | 480    | –     | –     |
|             | 31            | 100-LSPTRLAL-108     | –         | 21    | –      | –     | –     |
|             | 32            | 187-GDQYIE1-195      | 20        | –     | 960    | –     | –     |
|             | 33            | 318-SRVLVTVDL-326    | 20        | –     | –      | –     | –     |
|             | 34            | 23-RPQPSFDM-31       | –         | 20    | –      | 150   | –     |
|             | 35            | 123-NSSFCEFET-131    | –         | 20    | –      | –     | –     |
|             | 36            | 265-VSIAOSVIF-273    | –         | 20    | –      | –     | –     |
|             | 37            | 222-KFMRDPVRI-230    | –         | –     | 2304   | –     | –     |
|             | 38            | 195-IFRFLPKDI-203    | –         | –     | 960    | –     | –     |
|             | 39            | 272-FANTRIKV-280     | –         | –     | 288    | –     | –     |
|             | 40            | 312-TFRSGSSRV-320    | –         | –     | 288    | –     | –     |
|             | 41            | 164-RGALSEESL-172    | –         | –     | –      | –     | 120   |

*The cut-off score was adjusted to $\geq 18$ for SYFPEITHI, $\geq 100$ for BIMAS, and $<500$ for NetMHCII.*

To avoid potential autoimmunity (Table 4), two multi-epitope peptides of CPA (160–189 and 273–302 aa) and Histone H1 (1–20 and 43–61 aa), one peptide of KMP-11 (4–23 aa), and three peptides of LeIF (6–35, 181–210, and 371–400 aa) were synthesized by GeneCust (Labbx, Dudelange, Luxembourg) with purity $\geq 95\%$.

Synthetic peptides were dissolved in DMSO, acetic acid (10% in $dH_2O$), or $dH_2O$ according to their hydrophobicity, by vigorous pipetting and stored in aliquots, in $-80^\circ C$ until use. Peptide solutions were found endotoxin free, since LPS concentration was $<5$ EU/mg as determined by LAL Test Cartridges Portable Test.
Table 3 | *In silico* predicted MHC class II-restricted 15-mer epitopes of *L. infantum* proteins.

| Protein | No. of epitope | Epitope sequence | Score
|---------|---------------|------------------|-------|
| **CPA** | 1 | 149-MCGSCWFATTGNGIE-163 | 28 | – | – |
| | 2 | 246-PHDEEIAAYVGKNG-260 | 27 | – | – |
| | 3 | 114-KDYKEHVHVDDSVRS-128 | 26 | – | – |
| | 4 | 257-GKNGPVAVAVDATTW-271 | 26 | – | – |
| | 5 | 32-GVDDFIASAHYGRFK-46 | 25 | – | – |
| | 6 | 312-GSSWGKEKGYIRLAMG-326 | – | 24 | – |
| | 7 | 4-RNPFFAIVVTILFV-18 | 23 | – | – |
| | 8 | 5-NPFFAIIVVTILFV-19 | 23 | – | – |
| | 9 | 13-VTLFVCYGSALIA-27 | 22 | – | – |
| | 10 | 172-SLVLSEQOLVSCDN-186 | 22 | – | – |
| | 11 | 17A-VSLEQDVCLSDNID-188 | 22 | – | – |
| | 12 | 12-VTLFVCYGSALIA-26 | 21 | – | – |
| | 13 | 260-GPVAHADTWWLQ-274 | 21 | – | – |
| | 14 | 279-VTLCFLSSLNHSVVL-293 | 21 | – | – |
| | 15 | 67-KOMQFAFLNHAP-81 | 20 | – | – |
| | 16 | 273-LYFGGVTLICFGLSL-287 | 20 | – | – |
| | 17 | 301-KPPYWIVKNSWGSS-315 | 20 | – | – |
| | 18 | 328-NOCLLKNYVVTATID-342 | 20 | – | – |
| | 19 | 216-SYPYTSAGGTRPPCH-230 | – | 20 | – |
| | 20 | 308-KNSWGSSWGKEKGYIR-322 | – | 20 | – |
| **Histone H1** | 21 | 1-MSSDSAVALSAAMT-15 | 31 | – | 88.3 |
| | 22 | 27-KTAAKAAKAAKAAK-41 | 29 | – | 182.6 |
| | 23 | 32-KAAKAAKAAKAKAG-46 | 29 | – | 239.9 |
| | 24 | 37-KAAKAAKAKAKAG-51 | 29 | – | – |
| | 25 | 42-KAGAKAAKAKAKVRK-56 | 29 | – | – |
| | 26 | 2-SSDSSAIALSAAMTS-16 | 28 | – | 123.7 |
| | 27 | 56-KVATPKPKAAKAKK-70 | 24 | – | – |
| | 28 | 36-KKAAKAKAGAKKAGA-50 | – | 24 | – |
| | 29 | 41-KKAGAKAGAKKAVR-55 | – | 24 | – |
| | 30 | 71-AAKPAAKKAKKPAK-85 | – | 24 | – |
| | 31 | 16-SPOKSPRSPPKKTA-30 | – | 22 | – |
| | 32 | 21-PRSSPKTKAAKAAA-35 | – | 22 | – |
| | 33 | 26-KTAAKAAKAKAAA-40 | – | 22 | 163.5 |
| | 34 | 31-KKAAKAAKAAKAKA-45 | – | 22 | 190.3 |
| | 35 | 45-AKKAGAKKAVRKKAT-59 | – | 22 | – |
| | 36 | 51-KAVRKVATPKPK-65 | – | 22 | – |
| | 37 | 55-RKVATPKPKAAKAK-69 | – | 22 | – |
| | 38 | 59-TPKPAKKAUAAKKA-73 | – | 22 | – |
| | 39 | 63-PKKAAKAAKAAKPAK-77 | – | 22 | – |
| | 40 | 67-AAAKAAKPKKPKKAV-81 | – | 22 | – |
| | 41 | 75-PKKVAKKAAKPAK-89 | – | 22 | – |
| | 42 | 79-VAKKAAKAAKPAK-93 | – | 22 | – |
| | 43 | 83-PKKAAKAAKPKPK-97 | – | 22 | – |
| | 44 | 87-AKKAAKAAKAAK-101 | – | 22 | – |
| | 45 | 91-PKKAAKAAKAAK-105 | – | 22 | – |

(Continued)
| Protein | No. of epitope | Epitope sequence | SYFPEITHI | NetMHCII |
|---------|---------------|------------------|------------|----------|
|         |               |                  | H2-IA<sup>d</sup> | H2-IE<sup>d</sup> | H2-IA<sup>d</sup> |
| KMP-11  | 62            | 4-TYEEFSAKLRLDEE-18 | 23          | –         | –         |
|         | 63            | 75-OXFAELLEQQKAQN-89 | –          | 19        | –         |
|         | 64            | 45-KEHYEKFERMIKHE-59 | –          | 18        | –         |
|         | 65            | 74-KOFAELLEQQKAA-88 | –          | 18        | –         |
| LeIF    | 66            | 100-LSPTRERALQTAEEVI-114 | 28         | –         | 264.2     |
|         | 67            | 320-VLVTDLVARGLCVH-334 | 28         | –         | –         |
|         | 68            | 199-LPKDIOVALFSATMP-213 | 27         | –         | –         |
|         | 69            | 387-HTQIDEELPVDFAYL-401 | 27         | –         | –         |
|         | 70            | 138-ODDLRLQAGVIVAV-152 | 26         | –         | 205.3     |
|         | 71            | 166-ALRTESLRLVLEDEA-180 | 26         | –         | –         |
|         | 72            | 169-TESLRLVLEADEAM-183 | 26         | –         | –         |
|         | 73            | 62-RGDIAQAOSQSTGK-76 | 25         | –         | –         |
|         | 74            | 140-DLRKQAGVIVAVG-154 | 24         | –         | 256.8     |
|         | 75            | 142-RKLQAGVIVAVGTP-156 | 24         | –         | –         |
|         | 76            | 259-MDLYETVSIAQPSF-273 | 24         | –         | –         |
|         | 77            | 223-FMRDPVRILVKSRESL-237 | 24         | –         | –         |
|         | 78            | 325-DLVARQIDVHVNV-339 | 23         | –         | –         |
|         | 79            | 168-RTESLRLVLEADEAM-182 | 22         | –         | –         |
|         | 80            | 263-ETVSIAQSVIFANTR-277 | 22         | –         | –         |
|         | 81            | 293-TVSSMHAEMPKSDRE-307 | 22         | –         | –         |
|         | 82            | 314-RSGSRLVLVTLVA-328 | 22         | –         | –         |
|         | 83            | 268-AQSVIFANTRRKVDW-282 | –          | 22        | –         |
|         | 84            | 16-LDDQPGVRIPSPFDD-30 | 20         | –         | –         |
|         | 85            | 71-QSGTKTGAFAISGL-85 | 20         | –         | –         |
|         | 86            | 107-ALQATAVIRSEGFL-121 | 20         | –         | –         |
|         | 87            | 174-VLVLEADEAMLSQGF-188 | 20         | –         | –         |
|         | 88            | 255-LDTLMQVLYETVSIAC-269 | 20         | –         | –         |
|         | 89            | 376-VELLHEIEAHYHTIQ-390 | 20         | –         | –         |
|         | 90            | 77-TGAFSIQLLORLDR-91 | –          | 20        | –         |

(Continued)
Table 3 | Continued

| Protein | No. of epitope | Epitope sequence | SYFPEITHI | NetMHCIIC |
|---------|----------------|------------------|-----------|-----------|
|         |                |                  | H2-IA<sup>d</sup> | H2-IA<sup>d</sup> | H2-IE<sup>d</sup> |
| 91      | 81-SIGLQLRLDFRHNL-95 | –             | 20        | –         |
| 92      | 158-VSDV/KRGALRTESL-172 | –             | 20        | –         |
| 93      | 102-PRELALQAEISR-116 | –             | –         | 95.3      |
| 94      | 103-PRELALQAEISRI-117 | –             | –         | 95.4      |
| 95      | 101-PRELALQAEVIS-115 | –             | –         | 163.8     |
| 96      | 139-DLRLKAOQAVAVG-153 | –             | –         | 249.0     |
| 97      | 99-YLSPTRELALQAEV-113 | –             | –         | 263.1     |
| 98      | 98-LVLSPTRELALTAE-112 | –             | –         | 2876.6    |
| 99      | 141-LKAOQAVAVG-153 | –             | –         | 296.7     |
| 100     | 49-PSSIQRAAPIFTRG-63 | –             | –         | 320.9     |
| 101     | 48-KPSIQRAAPIFTR-62 | –             | –         | 378.0     |
| 102     | 50-PSSIQRAAPIFTRGG-64 | –             | –         | 434.6     |
| 103     | 97-GLVLSPTRELALTA-111 | –             | –         | 452.0     |
| 104     | 290-SNHTVSSMHAIEMPSK-304 | –             | –         | 489.1     |

<sup>a</sup>The cut-off score was adjusted to ≥ 18 for SYFPEITHI, ≥ 100 for BIMAS, and < 500 for NetMHCIIC.

**System** (Endosafe, Charles River Laboratories, USA). Synthetic multi-epitope peptides were also checked for the presence of 9-mer or/and 15-mer epitopes able to bind to HLA alleles (A2, A3, A24, B7, B15, DP, DQ, DR supertypes) using the above mentioned algorithms (Table 4). In addition, data on the crystal structure of HLA-A2 and HLA-DRB1 molecules were obtained from Protein Data Bank (PDB, codes 1HHG and 2SEB, respectively) and multi-epitope peptides of length 30 aa were transformed into PDB files using SWISS-MODEL, and each of them was docked with the HLA-A2 or HLA-DRB1 molecule using the ClusPro program<sup>8</sup> for structure-based analysis (46–49).

**IMMUNIZATION OF BALB/C MICE**

Eight groups of female BALB/c mice (n = 8/group), 6–8 weeks old, were immunized subcutaneously at upper and lower dorsal region, with 100 µl emulsion consisting of 50 µg of each synthetic multi-epitope peptide in complete Freund’s adjuvant (CFA). Mice were also received a second immunization with 100 µg of the same peptide in incomplete Freund’s adjuvant (IFA), and a third immunization with 50 µg of the same peptide in PBS at 2 weeks intervals. Two sex and age matched groups of mice (n = 8/group) immunized similarly either with the adjuvant or with PBS alone, were served as control groups.

Animals were obtained from the breeding unit of the Hellenic Pasteur Institute (Athens, Greece) and reared in institutional facilities under specific pathogen-free conditions, receiving a diet of commercial food pellets and water <i>ad libitum</i>. All experimental procedures had been approved by the institutional Animal Bioethics Committee regulating according to the EU Directive 2010/63 and the National Law 2013/56.

**CULTURE OF LYMPHOCYTES AND PROLIFERATION ASSAYS**

Fifteen days post the third immunization, spleens from immunized and control mice (n = 3/group) were collected in aseptic conditions and used for the preparation of single cell suspensions in RPMI-1640 medium (Biochrom AG, Berlin, Germany) supplemented with 2 mM L-glutamine, 10 mM HEPES, 24 mM NaHCO<sub>3</sub>, 0.05 mM β-mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% (v/v) heat inactivated fetal bovine serum (FBS; Gibco, Paisley, UK) at a density of 1 × 10<sup>6</sup> cells/ml. Cell viability was >95% as determined by trypan blue exclusion. A volume of 200 µl/well were placed in triplicate into 96-well U-bottomed plates in the presence of various concentrations of each synthetic multi-epitope peptide ranging from 5 to 40 µg/ml and incubated for 96 h in 5% CO<sub>2</sub> at 37°C in a humidified atmosphere. The optimal concentration for each peptide was determined at 10 µg/ml and used thereafter for recall stimulation. Cells cultured in medium alone or in the presence of Concanavalin A (6 µg/ml) were served as negative or positive control, respectively. Cells were pulsed with 1 µCi/ml of <sup>3</sup>H-TdR (GE Healthcare, Buckinghamshire, UK) for the final 18 h of the culture period. Cells were harvested and <sup>3</sup>H-TdR incorporation was determined on a liquid scintillation counter (Wallac, Turku, Finland). The results were expressed as ∆cpm (cpm of cells from immunized mice stimulated with peptide – cpm of immunized mice cultured in medium alone). Proliferative response against each synthetic peptide giving ∆cpm > 2000 was considered as positive.

**CYTOKINE DETECTION AND FLOW CYTOMETRY**

Spleen cells from immunized and control mice (n = 5/group) were also used for cytokine detection and flow cytometry. Briefly, 1 ml/well of lymphocytes in complete RPMI-1640 medium at a density of 2 × 10<sup>6</sup> cells/ml were placed in triplicate into 24-well
Table 4 | Synthetic multi-epitope peptides including MHC class I and II-restricted epitopes.

| Peptide name | Synthetic multi-epitope peptide sequence | Included epitopes | HLA supertype |
|--------------|------------------------------------------|------------------|---------------|
| CPA_p2       | 160-GNIEGWALKNHSLVSVSEQLVSLCDNID-189     | 165-QWALKNHSL-173, 179-GVLSVCDNI-187, 160-GNIEGQWAL-168, 172-SVLSVSEQLVSLCDNID-189 | HLA-A2 (A*0201), HLA-A3 (A*0301), HLA-DRB1, HLA-DPA1, HLA-DQA1 |
| CPA_p3       | 273-LYFGGVTLCFGLSLNHGVLVGFNMQAPK-302    | 273-LYFGGVTL-281, 279-VTLCFGLSLNHGVLV-293, 273-LYFGGVTLCFGLSL-287 | HLA-A2 (A*0201), HLA-A3 (A*0301), HLA-DPA1, HLA-DQA1 |
| H1_p1        | 1-MSSDSAVAALSAAMTSPOK-20                | 2-SSDSAVAAL-10, 1-MSSDSAVAALSAAMTS-15, 5-SSDSAVAALSAAMTSPOK-19 | HLA-A2 (A*0201), HLA-A3 (A*0301), HLA-DRB1, HLA-DQA1 |
| H1_p3        | 43-AGAKKAGAKKAVRKVATPKK-61             | 46-KKAGAKKAV-54, 42-KAGAKKAGAKKAVRK-56, 45-KKAGAKKAGAKKAVRKAT-59 | HLA-A2 (A*0201), HLA-A3 (A*0301), HLA-DRB1 |
| KMP-11_p1    | 4-TYEEFSAKLDRLDEEFNRKMK-23             | 4-TYEEFSAKL-12, 7-FFSACLDR-15, 4-TYEEFSAKLDRLDEE-18 | HLA-A3 (A*0301), HLA-DPA1, HLA-DQA1 |
| LeIF_p1      | 6-KIAPQDQDSFLDDQGVRPIPSFDDMLHQ-35      | 8-APQDQDSFL-16, 25-IPSFDDML-33, 14-SFLDDQGVRPIPSFDD-31, 16-LDDQGVRPIPSFDD-30 | HLA-B7 (B*5101, B*5102), HLA-B15 (B62), HLA-DRB1, HLA-DPA1, HLA-DQA1 |
| LeIF_p3      | 181-DEMLOQDQFADQIYEIFRFLPKQVALFS-210   | 199-LPKQVAL-207, 197-RFLPKQVAL-205, 187-GFQDQIYEI-196, 195-IRFLPKQDI-203, 199-LPKQVALFQ-213 | HLA-B7 (B*3501, B*5101), HLA-DPA1, HLA-DQA1 |
| LeIF_p6      | 371-VTEKDVELLHEIEAHYHTQIDELPVDNAAY-400 | 385-HYHTQIDEL-393, 393-LPVDNAAYL-401, 320-VLTTDLVARGICVH-334, 387-HTQIDELPVDNAAYL-401, 376-VELLHEIEAHYHTQIDELPVDNA-390 | HLA-A3 (A*0301), HLA-DPA1, HLA-DQA1 |

plates and stimulated with 10 µg/ml of each synthetic multi-epitope peptide. Cells were cultured for 72 h in 5% CO₂ at 37°C in a humidified atmosphere. At the end of the incubation period, culture supernatants were collected and stored at −80°C until analyzed for their cytokine content. The concentrations of IFN-γ and IL-10 in the supernatants were determined by sandwich ELISA kits (900-K98, 900-K53; PeproTech, Rocky Hill, NJ, USA) according to the manufacturer’s instructions. The cytokine concentrations were calculated by reference to standard curves; detection threshold for IFN-γ and IL-10 was 23 and 47 pg/ml, respectively.

In parallel, at 48 h of culture period, similarly cultured cells were exposed for 4 h to 2.5 µg/ml brefeldin A (Fluka, Buchs, Germany), washed in FACS buffer (PBS-2% FBS) and stained with anti-CD4 and anti-CD8 monoclonal antibodies (mAbs) conjugated either with FITC (anti-CD4-FITC, clone RM4-5) or PE (anti-CD8-PE, clone H129.19; anti-CD8-PE, clone 53-6-7) for 30 min. For the identification of intracellular cytokine production, cells were permeabilized using FACS buffer supplemented with 0.1% (v/v) saponin (Sigma) and stained for 30 min on ice with anti-IFN-γ conjugated with FITC (clone XMG1.2) or anti-IL-4 conjugated with PE (clone BVD4-1D11) mAbs. In all cases, control cells were processed similarly using matched isotype control. All mAbs used in the study, were purchased from BD Biosciences (Erembodegem, Belgium). For each sample, 20,000 cells were analyzed on a FACSCalibur (Becton-Dickinson, San Jose, CA, USA) and the data were processed with Cell Quest Software (Becton-Dickinson). The percentage of specific cytokine-producing CD4+ or CD8+ T cells relative to total numbers of CD4+ or CD8+ T cells was determined by analysis of FACS data using the FlowJO software package (Tree Star, Inc., Ashland, OR, USA). The percentage...
of peptide-specific cytokine-producing cells was normalized to their respective proportion in unstimulated cells from mice immunized with CFA/IFA alone, in order to allow for comparison among all the synthetic multi-epitope peptides.

**ENZYME LINKED IMMUNOSORBENT ASSAYS**

Blood collected from each group of mice (n = 8/group) at fifteenth day post the third immunization, were centrifuged at 4000 x g for 5 min and separated sera were aliquoted for the detection of specific antibodies against each synthetic multi-epitope peptide by specific ELISAs as previously described (50). In brief, 96-well microtiter plates were coated with 5 µg/ml of each individual peptide in carbonate buffer (15 mM Na₂CO₃, 35 mM NaHCO₃), pH 9.6 and left overnight at 4°C. For the detection of total IgG antibodies, 10-fold dilutions of each serum sample in 1% BSA in PBS-T were added and incubated with HRP-labeled goat anti-mouse IgG (1/10000 dilution; GE Healthcare, Buckinghamshire, UK). For the detection of IgG1 and IgG2a isotypes, serum samples were added and plates were similarly incubated either with biotin-labeled rat anti-mouse IgG1 (500 ng/ml; AbD Serotec, Oxford, UK) or IgG2a (250 ng/ml; AbD Serotec) followed by the addition of streptavidin–HRP (1/5000 dilution; AbD Serotec) and incubation for 1 h at 37°C. The cut-off value was determined as the mean OD value of normal mouse serum in a 1/100 dilution + 2SD.

**STATISTICAL ANALYSIS**

Data were expressed as the mean value with the standard deviation (SD) indicated. Statistical significant differences of the mean values between groups of mice immunized with synthetic multi-epitope peptide emulsified in CFA/IFA and mice immunized with CFA/IFA alone were assessed by unpaired Student’s t-test. The probability (p) of <0.05 was considered to indicate statistical significance.

**RESULTS**

**IN SILICO PREDICTION OF PROMISING EPITOPES OF *L. INFANTUM***

**PROTEINS BIND TO MHC CLASS I AND II MOLECULES**

CPA, Histone H1, KMP-11, and LeIF have already been defined as candidate antigens. CPA, a protein significantly up-regulated in mature amastigotes (40), is predicted as a secretory protein by SignalP (cleavage site between position 24 and 25 residue), while Histone H1, an also highly expressed protein in mature amastigotes (51), KMP-11, a cytoskeleton-associated protein, and LeIF constitutively expressed in both promastigotes and amastigotes (45, 52), are predicted as non-secretory. In silico analysis of proteins for the prediction of binding epitopes to H₂-K and H₂-IE alleles, respectively, were predicted by BIMAS and SYFPEITHI for CPA. These peptides were spanning throughout the protein amino-acid sequence and they covered the 69.2% of its length. In addition, 2 and 41 binding peptides to H₂-K/L and H₂-IE alleles, respectively, were predicted by SYFPEITHI and NetMHCII for Histone H1. The peptides were also spanning throughout Histone H1 amino-acid sequence, covering the entire length of the protein. Five and four binding peptides to H₂-K/L and H₂-IE alleles, respectively, were predicted by BIMAS and SYFPEITHI for KMP-11. Peptides were gathered in the middle, as well as in the amino- and carboxy-terminal region of KMP-11 sequence, and covered the 55.4% of its length. In regards to LeIF, 20 and 39 binding peptides to H₂-K/L and H₂-IE alleles, respectively, were predicted by BIMAS, SYFPEITHI, and NetMHCII. Peptides were spanning throughout LeIF amino-acid sequence and covered the 79.4% of the entire protein.

**SYNTHETIC MULTI-EPITOPE PEPTIDES CONTAINING BOTH MHC CLASS I- AND II-RESTRICTED EPITOPES**

Based on the above data, eight peptides, 20–30 amino-acid length, were designed and synthesized. At least, one MHC class I-restricted epitope scored very high, as well as adjacent or overlapping MHC class II epitope scored also high were nested in each synthetic peptide (Table 4). These multi-epitope peptides included CPA_p2 (160-GNIEGQWALKNHSLVLSERYLVDSCNIDD-189) and CPA_p3 (273-LYFGGYVTLCGFSLNIIHGLVLVGVNQRKAP-302) from CPA, H1_p1 (1-MISSDSAVALSAMTSPKS-20) and H1_p3 (43-AGAKKAGAKKAVRKVATPCKK-61) from Histone H1, KMP-11_p1 (4-TYEEFSLKLDDLDEEFNKKM-23) from KMP-11, LeIF_p1 (6-KIAQPDQDSFLDDPQGVPSPFDMPHQ-35), LeIF_p3 (181-DEMLSQGFAQDQIEFIRLPKDKQVALFSA-210), and LeIF_p6 (371-VTEKDVELLHEAEHYHTIQTIDELVPDAAY-400) from LeIF. Synthetic peptide sequences showed to be retrieved from highly conserved regions of *L. infantum* proteins, since protein BLAST analysis revealed up to 95% residue identity to homologous sequences of corresponding proteins of strains belonging to *L. major* and *L. donovani* complexes. In addition, promiscuous 9-mer and 15-mer epitopes bound to HLA alleles (A2, A3, A24, B7, B15, DP, DQ, DR supertypes) were also nested in synthetic multi-epitope peptides as predicted by *in silico* analysis using the above mentioned algorithms.

**VALIDATION OF SYNTHETIC MULTI-EPITOPE PEPTIDES IMMUNOGENICITY IN MICE**

Immunogenicity of the eight synthetic multi-epitope peptides was validated in BALB/c mice (H₂-K haplotype) immunized with each synthetic peptide in combination with CFA/IFA, 15 days post third immunization. Specific proliferative T cell responses induced by synthetic multi-epitope peptides were firstly assessed. As shown in Figure 1A, CPA_p2, CPA_p3, H1_p1, and LeIF_p6 induced strong proliferation of spleen cells upon *in vitro* re-stimulation (Acpm > 1128 ± 165) at the optimal dose of 10 µg/ml. Of these, CPA_p2 induced the strongest proliferation, followed by LeIF_p6, CPA_p3, and H1_p1. The results indicated that four of the eight candidate peptides could effectively induce spleen cell proliferation.

To validate the profile of cytokines secreted in response to the eight synthetic multi-epitope peptides, spleen cell culture supernatants from immunized mice were analyzed for their content in IFN-γ and IL-10 at 72 h post respective peptide *in vitro* re-stimulation. Quantitation by ELISA revealed that all peptides, except from LeIF_p1 and KMP-11_p1, induced the secretion of
immunized with PBS alone, stimulated in vitro. Section “Materials and Methods.” Spleen cells derived from mice immunized with CFA/IFA alone are indicated by * (with each synthetic peptide emulsified in CFA/IFA and the group of mice ± as pg/ml secretion. Cytokines were detected in culture supernatants of spleen cells from immunized BALB/c mice (Figure 1B). CPA_p3, H1_p1, CPA_p2, and H1_p3 were able to induce the highest secretion of IFN-γ, followed by LeIF_p3 and LeIF_p6. In contrast, unstimulated spleen cells from immunized mice produced low levels of IFN-γ spontaneously, similar to those measured in the culture supernatants of spleen cells from mice immunized with CFA/IFA alone. The results suggest that the majority of the candidate peptides could induce IFN-γ secretion.

In addition, low levels of IL-10 were detected in the supernatants of spleen cells stimulated in vitro with each synthetic multi-epitope peptide (Figure 1C). These levels were comparable to those detected in the supernatants of unstimulated spleen cells, as well as in the supernatants of spleen cells from mice immunized with CFA/IFA alone. In particular, KMP-11_p1 indicated a rather suppressive effect on IL-10 production.

To further confirm the pattern of cytokines induced by each synthetic multi-epitope peptide, intracellular cytokine production was determined in spleen cells from immunized mice at 48 h post peptide in vitro re-stimulation using flow cytometry. As shown in Figure 2A, none of the peptides tested were able to stimulate important peptide-specific IL-4 production by CD4+ T cells, although a certain predisposition in BALB/c mice has been documented by previous studies (53). In contrast, CPA_p2, CPA_p3, H1_p3, LeIF_p3, and LeIF_p6 were able to stimulate important peptide-specific IFN-γ production by CD4+ T cells, indicating a TTH cell driven toward the TTH type.

Regarding the ability of the synthetic multi-epitope peptides to induce the production of IFN-γ by CD8+ T cells, flow cytometry revealed that one of them, H1_p1 strongly induced the production of IFN-γ by splenic CD8+ T cells of immunized mice. H1_p3, CPA_p2, and CPA_p3 were also able to stimulate peptide-specific IFN-γ production by CD8+ T cells in a lower level than that detected in H1_p1 (Figure 2C). Flow cytometry overall results indicated that most of the peptides tested induced IFN-γ production from CD4+ and/or CD8+ T cells confirming the results obtained with in silico analysis (Figures 2B,D).

Furthermore, specific antibodies of IgG class, as well as of IgG1 and IgG2a isotypes, were detected in the serum of mice immunized with each synthetic peptide emulsified in CFA/IFA, 15 days post third immunization, in order to evaluate peptide effect on humoral response. According to the results, all the synthetic multi-epitope peptides were able to induce the secretion of specific IgG antibodies (Figures 3A,B). Of these, CPA_p2, CPA_p3, LeIF_p3, and LeIF_p6 induced the highest secretion, followed by KMP-11_p1, LeIF_p1, H1_p1, and H1_p3. Analysis of isotype pattern showed that CPA_p2 strongly induced the production of both IgG2a and IgG1 isotypes, followed by CPA_p3, while LeIF_p3 induced the production of IgG2a > IgG1 (Figure 3C). In contrast, LeIF_p6 strongly induced the production of IgG1 isotype and weakly the production of IgG2a isotype. The other four peptides, KMP-11_p1, LeIF_p1, H1_p1, and H1_p3, had insignificant effect on the production of these two IgG isotypes.

Next, we employed a structure-based method for further analysis of the tertiary structure of the most promising synthetic peptides (CPA_p2, CPA_p3, H1_p3, LeIF_p3) that bound to HLA-A2 or HLA-DRB1 molecule, since HLA-restricted epitopes were also nested in peptide sequences according to algorithms prediction.
FIGURE 2 | Multi-epitope peptide-specific cytokine production by CD4\(^+\) and CD8\(^+\) T cells. Spleen cells from BALB/c mice (n = 5/group) immunized either with individual peptide emulsified in CFA/IFA or PBS alone, were re-stimulated in vitro with the respective peptide (10\(\mu\)g/ml) for 48h, and analyzed for CD4\(^+\) and CD8\(^+\) T cells producing IL-4 and IFN-\(\gamma\). (A-C) Representative FACS plots of intracellular staining used to define IL-4- and IFN-\(\gamma\)-expressing CD4\(^+\) and CD8\(^+\) T cells in spleens derived from immunized mice. Values represent the percentages of (A) IL-4\(^+\) cells among CD4\(^+\) T cells and (B,C) IFN-\(\gamma\)^+ cells among CD4\(^+\) and CD8\(^+\) T cell populations. (D) Quantification of IL-4 and IFN-\(\gamma\) producing T cells in spleens derived from immunized mice. All data were normalized to CFA/IFA control group. Each bar represents the mean proportion of IL-4\(^+\) and IFN-\(\gamma\)^+ T cells induced by the respective peptide.

Selection of HLA-A2 and HLA-DRB1 molecules was based on published data demonstrating high frequency of these supertypes in human population (54, 55). The ClusPro program was run to predict docked conformations presenting good surface complementarity with the two MHC molecules mentioned above. The most probable 3D models according to algorithm analysis indicating peptides located onto the peptide-binding cleft of the MHC molecules with good surface complementarity are presented in Figure 4.

DISCUSSION

In the perspective of second generation vaccines, a variety of different parasite molecules, such as secretory or transmembrane proteins, including enzymes and receptors, has been tested to date as candidate antigens for anti-Leishmania vaccine development (56). Among them, CPA, Histone H1, KMP-11, and LeIF were found to be highly immunogenic as described in murine experimental models, cured VL patients and L. infantum infected dogs and have been considered as potential vaccine candidates (39–41, 44, 45, 57). The induction of an effective T cell response against vaccine antigens requires antigen processing and peptide presentation by antigen-presenting cells (APCs), and it is well-established that T cells recognize the peptide sequence in association to appropriate MHC molecules. The discovery of MHC-binding motifs in proteins has led to the development of several algorithms predicting MHC class I- and II-restricted epitopes for presentation to CD8\(^+\) or CD4\(^+\) T cells, respectively, accelerating research related to peptide-based vaccine approach (15).

In the present study, we investigated the use of three algorithms, SYFPEITHI, BIMAS, and NetMHCII to predict sequences in CPA, Histone H1, KMP-11, and LeIF able to bind to MHC class I and II molecules of the H2\(^d\) haplotype. Furthermore, combining this approach with experimental validation in MHC compatible BALB/c mice, we determined epitopes in each protein and designed multi-epitope peptides capable to induce peptide-specific T cell proliferation and cytokine production by CD4\(^+\) and/or CD8\(^+\) T cells.

The analysis of protein sequences yielded a significant number of possible epitopes from all four proteins, but only few of them were predicted by all algorithms used with binding efficiency to more than one supertypes or alleles. Interestingly, comparison of predicted peptides for each MHC class I and II alleles showed a low overlapping level between the results obtained from different algorithms used in the study, indicating the significant differences existing in the database source of building matrix motifs and different forms of scoring function of each algorithm. Also, it was observed an antigenic region clustering. Based on these findings and to the fact that prediction of MHC class I-restricted epitopes is considered more reliable (>85%) than that of MHC class II-restricted epitopes, we designed eight multi-epitope peptides for all proteins, based predominantly on highly scored MHC class I-restricted epitopes. Adjacent or overlapping MHC class II-restricted epitopes scored high were also nested in each synthetic peptide. These multi-epitope peptides contained epitopes recognized also by HLA class I and II molecules as defined by in silico analysis. Until now, very few vaccine antigens against different pathogens such as viruses, bacteria, and parasites, contain promiscuous T cell epitopes that have the ability to induce T cell-mediated protective immune responses both in mice and human by binding
FIGURE 3 | Multi-epitope peptide-specific antibody production. BALB/c mice (n = 9/group) immunized either with individual peptide emulsified in CFA/IFA or PBS alone, were bled 15 days post third immunization and sera were separated. (A,B) total IgG Abs, and (C) IgG1 and IgG2a Abs against each peptide were assessed by ELISA. The results are expressed as OD_{450} ± SD. Significant differences between groups of mice immunized with each synthetic peptide emulsified in CFA/IFA and the group of mice immunized with CFA/IFA alone are indicated by * (P < 0.05).

to several alleles of a supertype or between different supertypes (58, 59). Thus, these promiscuous epitope-driven vaccines could have the capacity of increasing the frequency of responders in genetically variable species, such as human populations (60).

The success of many vaccines is dependent on IFNγ-secreting CD4+ T cells recruitment for long term protection. This accounts for better immunologic memory leading to sustained immunity after healing of live infections (61, 62). CD4+ T cells are activated in terms of recognition of peptides-MHC class II complexes in the surface of APCs after protein processing in cells’ endocytic compartment. Activation of IFN-γ-producing CD4+ T cells plays a pivotal role in protective immune responses against leishmaniasis. Specifically, IFN-γ mediates macrophage activation against both the promastigote and amastigote forms in H2O2-dependent manner (63, 64) and nitric oxide production for parasite killing (65). According to our results, CPA_p2, CPA_p3, LeIF_p3, and LeIF_p6 induced peptide-specific IFN-γ production from CD4+ T cells in immunized mice indicating the processing and recognition of MHC class II-specific epitopes by CD4+ T cells. CPA and LeIF are considered significant candidate proteins for vaccine design against leishmaniasis. In the case of CPA, it has been shown that administration of plasmid encoding CPA induced specific T11H immune responses resulting to partial protection against L. major in the experimental model of CL. However, protection was significantly enhanced when co-administered with CPB or as CPA/B hybrid protein (25–27), indicating the need of CPA co-administration with another protein or adjuvant. On the other hand, LeIF was originally described as a T11H-type natural adjuvant and as an antigen inducing an IL-12 mediated T11H response in the PBMCs of leishmaniasis patients (39). LeIF is also capable of inducing the secretion of cytokines IL-12, IL-10, and TNF-α by APCs from healthy individuals (52, 66, 67). Furthermore, recombinant trifusion vaccines (leish111; leish110f) were developed by incorporating the amino-terminal region of LeIF antigen. These vaccines were shown to be efficient in experimental or clinical trials for vaccination or immunotherapy (68).

Existing data suggest that secretory and surface exposed proteins strongly induce specific CD8+ T cell responses (69, 70). A previous study applying in silico analysis revealed that a high number of peptides derived from L. major secretome could bind to H2 BALB/c molecules (71). Several studies have shown the great role played by CD8+ T cells in protective immune responses against parasite in the susceptible BALB/c strain (72–74). Specifically, CD8+ T cells either contributed in the destruction of Leishmania-infected cells by activating macrophages to oxidative burst via cytokines produced upon antigen stimulation (75, 76), or regulating CD4+ T cell-mediated immune responses (77, 78). In our study, both synthetic multi-epitope peptides of CPA, CPA_p2, and CPA_p3, except from CD4+ IFN-γ+ T cells activation, induced significant IFN-γ production by CD8+ T cells. CPA_p2 and CPA_p3 belong to the secreted region of CPA as SignalP analysis showed (30, 31). Interestingly, another study applying in silico analysis in CPA sequence with MULTIPRED algorithm indicated the existence of four highly immunogenic regions recognized by the
agallou et al. validation of multi-epitope l. infantum peptides

FIGURE 4 | Synthetic multi-epitope peptides docking. Ribbon diagram of 3D structural analysis of interactions between (A) HLA-A2 molecule (PDB-code: 1HHG) and the synthetic peptides CPA_p2, CPA_p3, H1_p3, and LeF_p3, and (B) HLA-DRB1 molecule (PDB-code: 2SEB) and the synthetic peptides CPA_p2 and LeF_p3. Candidate peptides were predicted to locate onto the peptide-binding cleft of the HLA molecules by using ClusPro program. The side and top view are shown, the α strands were shown in green, the β strands in blue, and the multi-epitope peptides in magenta.

HLA-A2 supertype (79), which harbored parts from our CPA multi-epitope peptides.

As for KMP-11, KMP-11_p1 belonged to the amino-terminal region of the protein and in contrast to previous observations this synthetic peptide was proved to be poorly immunogenic, indicated by the absence of peptide-specific proliferative response and cytokine secretion in immunized mice. Previous studies concerning the identification of T cell epitopes using infected macrophages or DCs as APCs, revealed the existence of potential HLA class I- and II-restricted T cell epitopes in the amino-terminal region, characterizing a dominant cluster between position 1 and 33 of KMP-11 sequence that could trigger specific cellular immune responses in L. donovani- or L. panamensis-infected volunteers (80, 81). Furthermore, hybrid-cell, DNA-based or heterologous KMP-11-DNA/rVV based vaccination exhibited immunoprotective capacity in susceptible VL murine models. Protection was accompanied with generation of antigen specific CD4+ and CD8+ T cells that produced effector cytokines such as IFN-γ, IL-2, and TNF-α (36–38, 82, 83). Also in a previous work, we demonstrated that vaccination with ex vivo pulsed bone marrow-derived dendritic cells with the Leishmania histone H1 elicited significant protection in the experimental model of VL, with a pronounced enhancement of parasite-specific IFNγ-producing CD8+ T cells (88). The protective effect of Leishmania histone H1 against L. major or L. infantum infections was also shown in different experimental animal models (34, 35) suggesting that it is also a promising vaccine candidate against leishmaniasis. In contrast, none of the LeF peptides tested could evoke specific CD8+ T cell responses. This finding was in agreement with the study of Rafati et al. showing that PBMCs from patients recovered from L. major failed to elicit HLA-A2-restricted CD8+ T cell responses against three synthetic nonamer peptides.
of LeIF, suggesting that these peptides are not able to induce a CD8+ T cell-induced protective immunity (86).

The relative low concentrations of IL-10 detected in the supernatants of immune lymphocytes compared to IFN-γ after peptide re-stimulation were consistent with the suggestion of a dynamic reciprocal relationship between these two cytokines. IL-10 primarily down-modulates innate as well as acquired immunity leading to parasite establishment or disease progression. In experimental model of VL, IL-10 prevents DCs migration in spleen to activate T cells (89, 90) and suppresses both Th1 and Th12 cells (91). Also, the CD4+IL-4+ T lymphocytes detected in the presence of all peptides may be attributed different to BALB/c intrinsic feature to induce the production of type-2 cytokines, such as IL-4 (53), since there was not any significant difference of IL-4 levels between peptide-immunized mice and control mice receiving the adjuvant alone. Furthermore, IFA adjuvant has a propensity to induce preferentially Th12 cytokines (92, 93). Similar study for the evaluation of immunoreactivity of in silico predicted Th1 epitopes of Schistosoma japonicum showed that high levels of IL-4 were attributed to Freund’s adjuvant and BALB/c strain used (94). Therefore, in terms of proportion of intracellular cytokine production of recall CD8+ and CD4+ T cells, it is concluded that CPA_p3, H1_p1, H1_p3, CPA_p2, LeIF_p3, and LeIF_p6 synthetic multi-epitope peptides are likely to include potential epitopes for the induction of protective cytotoxic (CTL) and Th1-type immune responses. Taken into account that the sequences of these synthetic peptides are highly conserved and bind in a promiscuous manner to murine or human MHC molecules according to in silico analysis and structure-based techniques, make them candidate vaccines against leishmaniasis. Based on these results, it would be worthwhile conducting future investigations for the verification of peptides’ possible ability to induce protection in common or humanized mouse models of leishmaniasis. The incorporation of alternative and/or additional epitopes, the use of modern adjuvants and new antigen delivery systems should be combined. Conclusively, these findings give complementary data on epitope mapping for Leishmania proteins and demonstrate that combination of immunoinformatic approaches with experimental validation enables peptide identification with greater accuracy contributing to rational epitope-based vaccine development.

AUTHOR CONTRIBUTIONS

Evita Athanasiou and Olga Koutsoni contributed equally to this work. Conceptualized and designed the experiments: Evdokia Karagouni. Performed computational analysis: Maria Agallou, Evita Athanasiou, Olga Koutsoni, Evdokia Karagouni. Performed the experiments: Maria Agallou, Olga Koutsoni, Evita Athanasiou. Analyzed the data: Maria Agallou, Evita Athanasiou, Olga Koutsoni, Evdokia Karagouni, Eleni Dotsika. Wrote the paper: Evdokia Karagouni, Maria Agallou.

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