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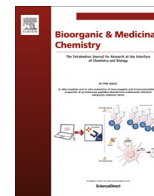


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In silico analysis and *in vitro* evaluation of immunogenic and immunomodulatory properties of promiscuous peptides derived from *Leishmania infantum* eukaryotic initiation factor



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ABSTRACT

It is generally considered as imperative the ability to control leishmaniasis through the development of a protective vaccine capable of inducing long-lasting and protective cell-mediated immune responses. In this current study, we demonstrated potential epitopes that bind to H2 MHC class I and II molecules by conducting the *in silico* analysis of *Leishmania infantum* eukaryotic Initiation Factor (Lief) protein, using online available algorithms. Moreover, we synthesized five peptides (16–18 amino acids long) which are part of the N-terminal portion of Lief and contain promising MHC class I and II-restricted epitopes and afterwards, their predicted immunogenicity was evaluated *in vitro* by monitoring peptide-specific T-cell responses. Additionally, the immunomodulatory properties of these peptides were investigated *in vitro* by exploring their potential of inducing phenotypic maturation and functional differentiation of murine Bone-Marrow derived Dendritic Cells (BM-DCs). It was revealed by our data that all the synthetic peptides predicted for H2 alleles; present the property of immunogenicity. Among the synthetic peptides which contained T-cell epitopes, the peptide 52–68 aa (Lief₂) exhibited immunomodulatory properties with the larger potential. Lief₂-pulsed BM-DCs up-regulated the expression of the co-stimulatory surface molecules CD80 and CD86, as well as the production of the proinflammatory cytokine TNF- α and of the Th1-polarizing cytokines IL-12 and IFN- γ . The aforementioned data suggest that selected parts of Lief could be used to develop innovative subunit protective vaccines able to induce effective immunity mediated by MHC class I-restricted as well as class II-restricted T-cell responses.

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1. Introduction

Leishmaniasis encompasses a group of diseases which are caused by the protozoan parasites of the genus *Leishmania*.¹ It is placed second in mortality and fourth in morbidity among the tropical infections and its burden is expressed as 2,357,000 disability-adjusted life years (DALY's) lost.^{2–4} A total of 98 countries are

Abbreviations: Lief, *Leishmania infantum* eukaryotic initiation factor; aa, amino acids; BM-DCs, bone-marrow derived dendritic cells; DALY's, disability-adjusted life years; CL, cutaneous leishmaniasis; VL, visceral leishmaniasis; MHC, major histocompatibility complex; APCs, antigen presenting cells; IL-12, interleukin-12; IL-10, interleukin-10; TNF, tumor necrosis factor; SLA, soluble *Leishmania* antigen; cpm, radioactivity counts per minute; TCR, T-cell receptor; PDCD4, programmed cell death protein 4; FACS, fluorescence-activated cell sorting.

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reported as endemic and the geographical spread of the disease is detected in all continents, except Oceania⁵. Its main clinical manifestations are cutaneous (CL) and visceral (VL) leishmaniasis and the official annual cases count more than 220,000 and 58,000, respectively.⁶

Although leishmaniasis is a major public health problem, there is no efficient vaccine against *Leishmania* parasites, delivering the necessary level of protection.⁷ The identification of the appropriate antigen is a major drawback for the successful vaccine formulation.⁸ Other significant difficulties for the production of vaccine assays based on the use of native antigens are the great variation between *Leishmania* species and the elevated requirements in time and financial resources.⁹ Nevertheless, the application of genome analysis to vaccine development, emerged a concept termed as *Reverse Vaccinology*, which reduces the time and cost spent on the search of new vaccine targets.^{10,11}

Peptide-based subunit vaccines contain a specific and well-defined fragment of a pathogen and are able to induce very specific cell-mediated and humoral immunity and to diminish the stimulation of an autoimmune response.¹² The development of a successful subunit vaccine requires the identification of antigenic and/or immunogenic regions in protein antigens. Consequently, the first step in designing synthetic peptides for vaccine application is the immunoinformatics analysis of the candidate protein.⁹ Up to this direction, *in silico* tools and databases have been developed and are used in order to identify and characterize promising epitopes, the minimal essential protein units that are recognized by T- and B-lymphocytes and consequently stimulate cellular and humoral immune responses.¹³ The output of numerous T-cell epitope predictive tools consists of a list of peptides and their predicted scores, indicating their possibility of binding or being epitopes.¹⁴ Presentation of exogenous antigens, like *Leishmania* spp., is associated with MHC class II molecules. Nevertheless, *Leishmania* antigens can be also processed for cross-presentation via MHC class I molecules.¹⁵ Therefore, the scientific interest is oriented to the mapping of MHC class I and II-restricted epitopes.

Moreover, a protective vaccine should generate the required effective long-term immunity against antigens and this is associated not only with the activation of T-cells, as mentioned above, but also with the maturation of the main antigen presenting cells (APCs), the dendritic cells (DCs).¹⁶ DCs are identified as key cellular mediators between innate and adaptive immunity¹⁷ and sense pathogens via several kinds of receptors.¹⁸ DCs migrate to T-cell areas after receiving microbial antigens and differentiate into mature DCs.¹⁹ This maturation process is associated with several events such as up-regulation of co-stimulatory molecules (CD86, CD80 and CD40) and change in morphology.²⁰ Mature DCs become capable of eliciting the adaptive immune responses by presenting processed antigenic peptides via MHC molecules to the TCR of naïve T-cells, providing the first signal (Signal 1) for T-cell activation.¹⁶ However, antigenic stimulation *per se* cannot activate T-cells effectively unless a second signal (Signal 2) is received. Ligands on DCs called co-stimulatory molecules interact with specific receptors on T-cells, providing the second signal which afterwards leads to the effective activation of T-cells.^{21,22} The activation of DCs results also in the production of various cytokines, such as IL-1, IL-6 or IL-12, which signal via cytokine receptors on T-cells (Signal 3) in order to polarize them toward an effector phenotype.^{19–23}

The *Leishmania* eukaryotic initiation factor (LeIF) is among the proteins of *Leishmania* spp. parasites that have been characterized as immunogenic and potential protective against leishmaniasis.^{24–26} It was originally described as a natural adjuvant and antigen which induces the protective Th1 immune response against leishmaniasis.²⁷ LeIF induces the production of the Th1-type cytokines (IL-12 and IFN- γ) by human peripheral blood monocytes (PBMC) both from patients suffering from leishmaniasis and from healthy volunteers.²⁷ Moreover, LeIF regulates the production of IL-12p70, TNF- α and IL-10 from macrophages derived from monocytes of healthy volunteers.^{28,29} Furthermore, the N-terminal portion of the protein (1–226 aa) is used as part of the Leish-111f polyprotein vaccine, proven as protective in experimental murine and hamster models.^{30,31} Additionally, *Leishmania infantum* eukaryotic initiation factor (Lief), has been identified as a novel anti-infective therapeutic molecule in an *in vitro* macrophage model.³²

In the present study, in order to screen Lief antigen for *Leishmania*-specific epitopes that could be presented via H-2 class I and II alleles, immunoinformatics tools were used with the selection of public available algorithms. Moreover, we proceeded to the synthesis of selected peptides derived from *L. infantum* eIF protein that could be potentially used as parts of subunit vaccines. We

then evaluated their immunogenicity in an experimental model of cutaneous leishmaniasis and their immunomodulatory properties in an *ex vivo* system of bone marrow-derived DCs (BM-DCs).

2. Materials and methods

2.1. Experimental animals and parasites

Female BALB/c and C57BL/6 mice, 6–8 weeks of age, were obtained from the breeding unit of the Hellenic Pasteur Institute (HPI, Athens, Greece). Mice were reared in institutional facilities under specific pathogen-free conditions, receiving a diet of commercial food pellets and water *ad libitum*. Experimental protocols were approved by the Animal Bioethics Committee of the HPI, following the regulations of the EC Directive 2010/63 and the National Law 1992/2015.

The *Leishmania major* parasite (strain MRHO/SU/59/P, zymodeme LV39) was used in soluble *Leishmania* antigen (SLA) preparation and in infection challenge. Parasites were maintained virulent by monthly passage in BALB/c mice. Promastigotes were grown in complete RPMI-1640 medium (Biochrom AG, Berlin, Germany) supplemented with 2 mM L-glutamine, 10 mM Hepes, 24 mM NaHCO₃, 0.05 mM of 2-mercaptoethanol, 100 U/mL penicillin, 100 μ g/mL streptomycin and 10% v/v heat-inactivated fetal bovine serum (FBS; Gibco, Paisley, UK), as previously described.³²

2.2. Preparation of soluble *Leishmania* antigen

The Soluble *Leishmania* Antigen (SLA) was prepared from *L. major* stationary phase promastigotes, following a published protocol with some modifications.³³ Briefly, 10⁹ promastigotes/mL were washed three times in 5 mL of cold and sterile phosphate-buffered saline (PBS). The parasites were lysed using repeated freeze-thaw cycles. More specifically, after five cycles of freezing (liquid N₂) and thawing (37 °C), the lysate was exposed to probe sonication in an ice bath. The crude lysate was centrifuged at 8000 \times g for 60 min at 4 °C and the supernatant containing SLA, was collected and stored at –80 °C. The protein concentration of the SLA was determined using the BCA protein assay kit (Thermo Scientific, USA).

2.3. Protein selection

The antigens selected for this study included LeIF proteins of the strains *L. infantum*, *L. major* and *L. braziliensis*. The full amino acid sequences of the proteins were extracted from Uniprot/Swiss-Prot protein database (<http://www.ebi.ac.uk/uniprot>). The accession numbers for each protein sequence are presented in Table 1.

The SignalP (<http://www.cbs.dtu.dk/services/SignalP>) analysis confirmed that LeIF proteins are not secretory and their full amino acid sequences are presented in Fig. 1.

Table 1

Leishmania eukaryotic initiation factor 4A. The amino acid sequences of *Leishmania* eukaryotic initiation factor 4A of three *Leishmania* strains. Extracted from Uniprot/Swiss-Prot protein database with the following Swiss-Prot Accession numbers: IF4A_LEIIN, IF4A_LEIMA and IF4A_LEIBR.

Protein name	Abbreviation	Accession number
<i>Leishmania infantum</i> eukaryotic initiation factor 4A	Lief	IF4A_LEIIN (A4HRK0)
<i>Leishmania major</i> eukaryotic initiation factor 4A	Lmef	IF4A_LEIMA (O62591)
<i>Leishmania braziliensis</i> eukaryotic initiation factor 4A	Lbief	IF4A_LEIBR (Q25225)

id=396 nSeq=3

```

M.Q.D+#APQDQDSFLDDQPGVRPIPSFDDMPLHQNLLRGIYSYGFEKPSISIQ
IF4A_LEIIN 1 MAQNDKIAFPDQDSEFLDDQPGVREIPEFDDMPLHQNLLRGIYSYGFEKPSISIQ
IF4A_LEIMA 1 MAQNDKIAFPDQDSEFLDDQPGVREIPEFDDMPLHQNLLRGIYSYGFEKPSISIQ
IF4A_LEIBR 1 MSQQDRVAFDQDSEFLDDQPGVREIPEFDDMPLHQNLLRGIYSYGFEKPSISIQ

QRAIAPFTRGGDIIAQASGTGKTGAFSIGLLQRLDFRHNLIQGLVLSPTREL
IF4A_LEIIN 54 QRAIAPFTRGGDIIAQASGTGKTGAFSIGLLQRLDFRHNLIQGLVLSPTREL
IF4A_LEIMA 54 QRAIAPFTRGGDIIAQASGTGKTGAFSIGLLQRLDFRHNLIQGLVLSPTREL
IF4A_LEIBR 54 QRAIAPFTRGGDIIAQASGTGKTGAFSIGLLQRLDFRHNLIQGLVLSPTREL

ALQTAEVISRIGEFLSNS.KFCETFVGGTRVQDDLRKLQAGV#VAVGTPGRVS
IF4A_LEIIN 107 ALQTAEVISRIGEFLSNSKFCETFVGGTRVQDDLRKLQAGVIVAVGTPGRVS
IF4A_LEIMA 107 ALQTAEVISRIGEFLSNSKFCETFVGGTRVQDDLRKLQAGVIVAVGTPGRVS
IF4A_LEIBR 107 ALQTAEVISRIGEFLSNSAKFCETFVGGTRVQDDLRKLQAGVVAVGTPGRVS

DVIKRGALRTESLRVVLVLEADEMELSQGFADQIYEIFRFLPKDIQVALFSATM
IF4A_LEIIN 160 DVIKRGALRTESLRVVLVLEADEMELSQGFADQIYEIFRFLPKDIQVALFSATM
IF4A_LEIMA 160 DVIKRGALRTESLRVVLVLEADEMELSQGFADQIYEIFRFLPKDIQVALFSATM
IF4A_LEIBR 160 DVIKRGALRTESLRVVLVLEADEMELSQGFADQIYEIFRFLPKDIQVALESATM

PEEVLELTKKFMRDVPRILVKRESLTLEGIKQFFIAVEEEHKLDTLMDLYETV
IF4A_LEIIN 213 PEEVLELTKKFMRDVPRILVKRESLTLEGIKQFFIAVEEEHKLDTLMDLYETV
IF4A_LEIMA 213 PEEVLELTKKFMRDVPRILVKRESLTLEGIKQFFIAVEEEHKLDTLMDLYETV
IF4A_LEIBR 213 PEEVLELTKKFMRDVPRILVKRESLTLEGIKQFFIAVEEEHKLDTLMDLYETV

SIAQSVIFANTRRKVDWIAEKLNSNHTVSSMHAEMPKSDRERVMNTRFRSGSS
IF4A_LEIIN 266 SIAQSVIFANTRRKVDWIAEKLNSNHTVSSMHAEMPKSDRERVMNTRFRSGSS
IF4A_LEIMA 266 SIAQSVIFANTRRKVDWIAEKLNSNHTVSSMHAEMPKSDRERVMNTRFRSGSS
IF4A_LEIBR 266 SIAQSVIFANTRRKVDWIAEKLNSNHTVSSMHAEMPKSDRERVMNTRFRSGSS

RVLVTTDLVARGIDVHHVNIVINFDLPTNKENYLHRIGRGGRYGRKGVAINFV
IF4A_LEIIN 319 RVLVTTDLVARGIDVHHVNIVINFDLPTNKENYLHRIGRGGRYGRKGVAINFV
IF4A_LEIMA 319 RVLVTTDLVARGIDVHHVNIVINFDLPTNKENYLHRIGRGGRYGRKGVAINFV
IF4A_LEIBR 319 RVLVTTDLVARGIDVHHVNIVINFDLPTNKENYLHRIGRGGRYGRKGVAINFV

TEKDVELLHEIEI.EAHYHTQIDELPVDFAAYLGE
IF4A_LEIIN 372 TEKDVELLHEIEI.EAHYHTQIDELPVDFAAYLGE
IF4A_LEIMA 372 TEKDVELLHEIEI.EAHYHTQIDELPVDFAAYLGE
IF4A_LEIBR 372 TEKDVELLHEIEI.EGHYHTQIDELPVDFAAYLGE

```

Fig. 1. Alignment of three Lief proteins. Comparison of amino acid sequences of three Lief proteins derived from *L. infantum*, *L. major* and *L. braziliensis*. The full amino acid sequences of the proteins were extracted from Uniprot/Swiss-Prot protein database (<http://www.ebi.ac.uk/uniprot>), with the following accession numbers: IF4A_LEIIN (A4HRK0), IF4A_LEIMA (O62591), IF4A_LEIBR (Q25225), respectively. The positions of amino acid substitutions are marked with ., + and ≠.

Table 2

MHC alleles that are expressed in mice breeds. https://www.proimmune.com/ecommerce/page.php?page=MHC_alleles.

Strain	Appearance	Haplotype	H2 CLASS I			H2 CLASS II	
			K	D	L	IA	IE
BALB/c	albino	d	K ^d	D ^d	L ^d	IA ^d	IE ^d
C57BL/6	black	b	K ^b	D ^b	-	IA ^b	-
CBA	agouti	k	K ^k	D ^k	-	IA ^k	IE ^k

2.4. Peptide prediction and synthesis

In order to map the promising epitopes of Lief, three different public available algorithms were used: SYFPEITHI (<http://www.syfpeithi.de>), NetMHCII.2 (<http://www.cbs.dtu.dk/services/NetMHCII>) and NetMHC3.0 (<http://www.cbs.dtu.dk/services/NetMHC-3.0/>). We focused on peptides presenting high affinity to mouse MHC class I and class II alleles (Table 2) of three strains: BALB/c which are genetically susceptible against leishmaniasis, as well as C57BL/6 and CBA which are genetically resistant against leishmaniasis. The complementarity of 15-mer and 9-mer long epitopes was evaluated against four MHC class II alleles (H2-IA^d, H2-IA^b, H2-A^k, H2-E^k) and six MHC class I alleles (H2-K^d, H2-K^b, H2-L^d, H2-D^b, H2-D^d, H2-K^k), respectively.

For SYFPEITHI the cut-off score was adjusted above 20 and for NetMHC the default prediction threshold was <500 nM of binding affinity for weak binders and <50 nM of binding affinity for strong

binders. Peptides with 100% similarity to mice proteins were excluded from the selected list based on BLASTp analysis (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

The selected peptides (16–18 amino acids long), Lief₁ 48-KPSSIQRAIAPFTRGG-64, Lief₂ 52-IQRAIAPFTRGGDII-68, Lief₃ 103-TRELALQTAEVISRIGE-119, Lief₄ 15-FLDDQPGVRPIPSFDDM-31 and Lief₅ 200-PKDIQVALFSATMPEEVL-216, were synthesized using automated Fmoc (N-(9 fluorenyl) methoxycarbonyl) solid-phase synthesis (Bio-Synthesis, Lewisville, TX). The peptides were purified by high-pressure liquid chromatography (HPLC) and their identity and purity (>75%) were confirmed by mass spectroscopy (MS). More specifically, the defined purity levels of the synthetic peptides were: >95% for Lief₁ and Lief₅, 95% for Lief₂ and 91% for Lief₃ and Lief₄. The charge of the peptides was calculated using the program <http://www.designerbio-science.com/Calculator.asp>. Synthetic peptides were dissolved in sterile dH₂O, or acetic acid (7% in dH₂O), or DMSO according to their hydrophobicity.

2.5. Protein structure and localization of epitopes

The model of Lief crystal structure was based on template structure of human eukaryotic initiation factor 4A-I (EIF4A-I, PDB: 2zu6) that shares 60.65% homology with Lief. The model was built using Promod-II version 3.70. The localizations of peptides are depicted using surface-electrostatic potential meshes. The blast analysis of human PDCD4 protein with *Leishmania* proteins was performed using the following online available tools: www.genedb.org/ and <http://tritypdb.org/tritypdb/>.

2.6. T-cell proliferation assay

Female BALB/c mice were subcutaneously infected with 2×10^6 *L. major* stationary phase promastigotes. One month post infection, splenocytes were isolated and cultured, as has been previously reported³⁴. Briefly, cells (2×10^6 cells/mL) were plated in triplicate; in round bottom 96-well plates (Corning® Costar®, Sigma-Aldrich, Germany), in a volume of 200 μ L/well of complete RPMI-1640 medium and incubated in 5% CO₂, at 37 °C for 96 h. Cells were incubated in the presence of each one of the synthetic peptides (1, 5 and 10 μ g/mL) or in the presence of SLA (10 μ g/mL). Cells of the positive control group were cultured with 3 μ g/mL of Concanavalin A (Sigma-Aldrich, Germany) and cells of the negative control group were cultured only with complete RPMI-1640 medium (spontaneous proliferation). Cells were then pulsed with 2 μ Ci/mL of ³H-thymidine (Amersham Radiochemicals, UK) for the last 18 h of culture. ³H-thymidine incorporation was measured by liquid scintillation counting (Microbeta Trilux, Wallac, Finland). Results are expressed as $\Delta\text{cpm} = \text{peptide-stimulated mean}_{\text{cpm}}$ in test wells – mean_{cpm} in wells with media only (spontaneous proliferation). Proliferative responses against to each synthetic peptide with $\Delta\text{cpm} > 2000$ and stimulation index > 2 (SI = peptide-stimulated mean_{cpm} /unstimulated mean_{cpm}) were considered as positive, as previously reported.^{34–37}

2.7. Generation of bone-marrow derived DCs

Bone marrow-derived DCs (BM-DCs) were generated from pluripotent bone marrow stem cells, as previously described.³⁸ Briefly, bone marrow cells were isolated from the femur and tibia bones of naïve BALB/c mice and placed in 100 mm petri dish (3×10^6 cells per plate) in 10 mL of complete RPMI-1640 medium supplemented with 20 ng/mL of recombinant GM-CSF (Sigma-Aldrich, Germany), for an overall period of 9 days. On days 3 and 6, non-adherent cells were harvested and resuspended in fresh complete RPMI-1640 medium also supplemented with 20 ng/mL of recombinant GM-CSF. On day 9, non-adherent cells were collected by gentle pipetting and characterized; cell viability was $> 95\%$ as determined by trypan blue exclusion dye and the percentage of CD11c⁺CD8a⁻ cells was $> 75\%$ as assayed by R-phycoerythrin-conjugated (R-PE) hamster anti-mouse CD11c (clone HL3; BD Biosciences, Belgium) or FITC-conjugated rat anti-mouse CD8a (clone Lyt2; BD Biosciences) mAbs and flow cytometry (FACS) analysis. Samples were analyzed on a FACS Calibur (Becton–Dickinson, San Jose, CA, USA) and the acquired data were analyzed with FlowJo V.10.0.8 (Tree Star Inc, Ashland, OR, USA) software.

2.8. Stimulation of BM-DCs and determination of their maturation and cytokine production

Non-adherent T-cells were harvested on day 9, as mentioned in section 2.7, and co-cultured *in vitro* with each one of the synthetic peptides (10 μ g/mL). Cells of the positive control group were stimulated with LPS (1 μ g/mL) derived from *Escherichia coli* (Sigma-

Aldrich, Germany) whereas the cells of the negative control group (ctrl) were cultured with complete RPMI-1640 medium.

After 24 h, the phenotype of the CD11c⁺ CD8a⁻ cells was evaluated by staining with fluorochrome-labeled antibodies for cell surface markers (CD80, CD86, CD40) and for I-A/I-E molecules (MHC class II) (BD Pharmingen) and intracellular staining for the cytokines IL-12p40/p70, IFN- γ , IL-10 and IL-4 (BD Pharmingen). 10,000 events were acquired for each sample in a FACS Calibur cytometer and the data were analyzed with FlowJo V.10.0.8 software.

2.9. Detection of TNF- α expression by semi-quantitative PCR

The Lief_2 peptide was further evaluated *in vitro* for its ability to cause the production of the proinflammatory cytokine tumor necrosis factor alpha (TNF- α) by BM-DCs. More specifically, adherent BM-DCs (5×10^6 BM-DCs per group) were harvested on day 9 of culture, as described above, and co-cultured *in vitro* with the Lief_2 peptide (10 μ g/mL) for 2, 6 and 12 h. Cells of the positive control group were stimulated with LPS (1 μ g/mL) whereas cells of the negative control group were cultured with complete RPMI-1640 medium.

At the end of the designed timepoints, the total RNA of BM-DCs was extracted using an RNeasy Mini Kit (Qiagen, Germany) following the manufacturer's instructions.³⁹ Then, a 1 μ g aliquot of total RNA was reverse-transcribed using M-MLV Reverse Transcriptase and the oligo(dT) 15 primer, according to the manufacturer's instructions (Promega, USA). The reverse transcription step was performed at 40 °C for 60 min and 90 °C for 5 min. Semi-quantitative PCR was performed with Taq polymerase (Invitrogen, Life Technologies, USA) and the specific primer pairs for TNF- α and β -actin were derived from a published work.⁴⁰ Analysis of PCR products obtained from three independent experiments was carried out by electrophoresis in a 2% agarose gel stained with ethidium bromide and visualized by UV-illumination. The levels of PCR products were determined by scanning densitometry using the Image Quant IQ 5.2 software and data are presented upon normalization to the products of β -actin.

2.10. Statistical analysis

The *in vitro* experimental procedures were repeated at least three times, and each sample was placed in triplicate in any experimental procedure. Results are expressed as mean \pm SD (standard deviation). In the *in vivo* procedures, we used three animals per group and the experiments were repeated three times. Results are expressed as mean \pm SD (standard deviation). Statistical significance of differences between means was evaluated by the test Mann-Whitney (*U* test) and the probability $p < 0.05$, was taken as indicating significance.

3. Results

3.1. In silico analysis of Lief protein for prediction of potential peptides binding to MHC class I and II molecules

We orientated our research interest to the mapping of Lief in order to determine immunogenic epitopes that act as possible TCR ligands for CD4⁺ and CD8⁺ T-cell subpopulations; capable to regulate *L. major* induced infection.

At first, the full sequences of the eukaryotic initiation factors 4A of three different *Leishmania* strains; *L. infantum* (Lief), *L. major* (Lmief) and *L. braziliensis* (Lbief) were downloaded (Table 1). The Lief protein exhibits 100% and 98% identity with Lmief and Lbief proteins, respectively. Lief presents amino acid substitutions at the positions 2, 4, 6, 7, 125, 149 and 384, compared to Lbief

Table 3
In silico predicted MHC class II-restricted 15-mer epitopes of *L. infantum* eIF protein.

15-mer	protein	peptide position	SYFPEITHI			NetMHCII	
			H2-IA ^d	H2-IA ^k	H2-IE ^k	H2-IA ^d	H2-IA ^b
			Score ^a				
1	LiIF	178		32			
2	LiIF	100	28			264,2 (WB)	
3	LiIF	320	28				
4	LiIF	199	27	24			
5	LiIF	387	27				
6	LiIF	138	26		20	205,3 (WB)	
7	LiIF	166	26				
8	LiIF	169	26				
9	LiIF	62	25				
10	LiIF	140	24			256,8 (WB)	
11	LiIF	142	24			296,7 (WB)	
12	LiIF	259	24				
13	LiIF	27		24			
14	LiIF	146			24		
15	LiIF	243			24		
16	LiIF	349			24		
17	LiIF	325	23				
18	LiIF	168	22				
19	LiIF	263	22				
20	LiIF	293	22				
21	LiIF	314	22				
22	LiIF	61		22			
23	LiIF	285		22			
24	LiIF	111		22	20		
25	LiIF	255	20	22	20		
26	LiIF	373		22	20		
27	LiIF	16	20				
28	LiIF	71	20				
29	LiIF	107	20				
30	LiIF	174	20				
31	LiIF	171			20		
32	LiIF	190			20		
33	LiIF	202			20		240,4 (WB)
34	LiIF	236			20		
35	LiIF	277			20		
36	LiIF	333			20		
37	LiIF	365			20		
38	LiIF	376	20		20		
39	LiIF	288		20			
40	LiIF	37					417,9 (WB)
41	LiIF	38					209,8 (WB)
42	LiIF	39					170,5 (WB)
43	LiIF	40					149,2 (WB)
44	LiIF	41					139,3 (WB)
45	LiIF	42					208,8 (WB)
46	LiIF	203					176,9 (WB)
47	LiIF	204					158,6 (WB)
48	LiIF	205					152,8 (WB)
49	LiIF	206					265,2 (WB)
50	LiIF	48				378,0 (WB)	
51	LiIF	49				320,9 (WB)	
52	LiIF	50				434,6 (WB)	
53	LiIF	97				452,0 (WB)	
54	LiIF	98				287,6	

Table 3 (continued)

15-mer	SYFPEITHI			NetMHCII			
	protein	peptide position	H2-IA ^d	H2-IA ^k	H2-IE ^k	H2-IA ^d	H2-IA ^b
			Score [*]				
55	LieIF	99				(WB) 263,1	
56	LieIF	101				(WB) 163,8	
57	LieIF	102				(WB) 95,3	
58	LieIF	103				(WB) 95,4	
59	LieIF	139				(WB) 249,0	
60	LieIF	141				(WB) 256,8	
61	LieIF	290				(WB) 489,1	

* The cut-off score was adjusted to ≥ 20 for SYFPEITHI and < 500 nM for NetMHCII

(Fig. 1). Thereafter, the sequence was analyzed with the Signal IP program, in order to investigate the subcellular positioning of LieIF and was found that LieIF has no leader peptide and therefore it should not be qualified as a secreted protein, as it had been previously reported.³⁴

Subsequently, LieIF was mapped with antigenicity prediction algorithms. Immunoinformatics analysis revealed sixty-one (61) 15-mer peptides as MHC class II binders with absolute scores above the cut-off value of each algorithm used. The 44.26% (27 peptides) was suggested by NetMHCII algorithm and the 63.93% (39 peptides) by the SYFPEITHI algorithm (Table 3). Accordingly, forty-one (41) 9-mer peptides were predicted as MHC class I binders with absolute scores above the cut-off value of each algorithm used. The 58.54% (24 peptides) was suggested by NetMHC algorithm and the 41.46% (17 peptides) by SYFPEITHI (Table 4).

The 15-mer peptides exhibited connectivity to the H2-IA^d, H2-IA^k, H2-IE^k and H2-IA^b molecules while the 9-mer peptides exhibited connectivity to the H2-L^d, H2-K^d, H2-K^k, H2-D^b and H2-K^b molecules. A significant fact is that only a small number of the epitopes predicted by all the used algorithms, with binding activity to multiple alleles.

Among the total number of 15-mer and 9-mer peptides of LieIF nominated by the algorithms, we selected five peptides (16–18 amino acids long), which met the following criteria: (i) were situated to the N-terminal region of the protein (1–226 aa) which according to bibliographical references is the region presenting the ability to induce the production of IL-12 and IFN- γ ,⁴¹ (ii) showed high absolute scores and (iii) contained 9-mer and 15-mer epitopes. Finally, the selected peptides were blasted with human and mice proteome and no one of these peptides showed 100% similarity. Table 5 summarizes the candidate epitope characteristics.

3.2. LieIF structure and localization of synthetic peptides

The model of LieIF structure was based on template structure of human eukaryotic initiation factor 4A-I (EIF4A-I, PDB: 2zu6). The localizations of the LieIF_1, LieIF_2, LieIF_3, LieIF_4 and LieIF_5 peptides are represented as surface meshes with different colors (LieIF_1 in cyan, LieIF_2 in yellow, LieIF_3 in purple, LieIF_4 in orange and LieIF_5 in blue) (Fig. 2). The LieIF_1 and LieIF_2 peptides are overlapped and their common region is represented in light green. The LieIF_2 peptide is located away from EIF4A-I's interaction sites with either Eukaryotic Initiation Factor 4G (EIF4G)

or Programmed Cell Death Protein 4 (PDCD4). On the contrary, the LieIF_5 peptide is found to be localized in the interaction site with the Programmed Cell Death Protein 4 (PDCD4). Blast analysis of the sequence of human PDCD4 revealed 50%, 43%, 42% and 40% similarity with hypothetical proteins of *L. mexicana*, *L. major*, *L. infantum* and *L. donovani*, respectively.

3.3. In vitro validation of immunogenicity prediction of synthetic peptides

The predicted immunogenicity of synthetic peptides was validated in *L. major*-infected BALB/c (H2^d haplotype) mice, 30 days post-infection. We monitored *in vitro* the peptide-specific T-cell responses by measuring their proliferation by radiolabeled thymidine incorporation. As shown in Fig. 3, all the synthetic peptides, tested at various concentrations, induced proliferative response of splenocytes upon *in vitro* re-stimulation (Δ cpm > 2000).

3.4. Evaluation of immunomodulatory properties of LieIF synthetic peptides

The ability of the 16–18 amino acid long synthetic peptides, to induce phenotypic maturation and functional differentiation of monocytic cells was assessed *in vitro* using BM-DCs as the most “professional” APCs that critically determine the adaptively induced T-cell responses. To this end, we tested the expression of co-stimulatory (CD80, CD86, CD40) and MHC class II molecules, as well as the production of cytokines capable of inducing the polarization of the protective Th1 immune response, by FACS.

The five selected peptides, were incubated with BM-DCs in a concentration of 10 μ g/mL. As shown in Fig. 4, most of the synthetic peptides induced a modest increase in the expression of co-stimulatory molecules, while the LieIF_2 peptide showed a notable exception by exhibiting a significant increase in the percentage of BM-DCs that express the surface CD80 and CD86 molecules (43% and 77% vs 26% and 58%, respectively, $p < 0.05$), compared to the negative control group of unstimulated cells (Fig. 4). The LieIF-2 peptide also caused up-regulation of the expression of co-stimulatory molecules in BM-DCs derived from resistant C57BL/6 mice (data not shown).

Consequently, the effect of the synthetic peptides over the production of cytokines which orchestrate the protective Th1-type (IL-12, IFN- γ) or the Th2-type (IL-4, IL-10) immune response was

Table 4
In silico predicted MHC class I-restricted 9-mer epitopes of *L. infantum* eIF protein.

9-mer	protein	peptide/position	SYFPEITHI				NetMHC					
			H2-L ^d	H2-K _d	H2-D ^b	H2-K ^k	H2-K _d	H2-D ^d	H2-L ^d	H2-K ^b	H2-D ^b	H2-K ^k
1	LieIF	43		26								
2	LieIF	385		24								
3	LieIF	360		23								
4	LieIF	17				23						
5	LieIF	239				22						
6	LieIF	8	22									
7	LieIF	25	22									
8	LieIF	199	22									
9	LieIF	344	22									
10	LieIF	393	22									
11	LieIF	100	21									
12	LieIF	14		21								
13	LieIF	197		21								
14	LieIF	234				21						
15	LieIF	382				21						
16	LieIF	23	20									
17	LieIF	123	20									
18	LieIF	265	20									
19	LieIF	187		20								
20	LieIF	318		20								
21	LieIF	259				20						
22	LieIF	80			20							
23	LieIF	119			20							
24	LieIF	211			20							
25	LieIF	384					132,0 (WB)					
26	LieIF	7						125,0 (WB)				
27	LieIF	22						51,0 (WB)				
28	LieIF	57						164,0 (WB)				
29	LieIF	343						160,0 (WB)				
30	LieIF	392						227,0 (WB)				
31	LieIF	163							241,0 (WB)			
32	LieIF	29							318,0 (WB)			
33	LieIF	99							423,0 (WB)			
34	LieIF	381									6,0 (SB)	
35	LieIF	238									11,0 (SB)	
36	LieIF	233									21,0 (SB)	
37	LieIF	373									88,0 (WB)	
38	LieIF	250									104,0 (WB)	
39	LieIF	168									189,0 (WB)	
40	LieIF	249									300,0 (WB)	
41	LieIF	180									333,0 (WB)	

^aThe cut-off score was adjusted to ≥ 20 for SYFPEITHI and < 500 nM for NetMHC.

determined. It was observed that the majority of the synthetic peptides (LieIF_1, LieIF_3, LieIF_4 and LieIF_5), did not induce significant production of the protective IL-12 cytokine, which was compatible with the observed modest phenotypic maturation of BM-DCs. Nevertheless, the effect of the LieIF_2 peptide resulted in significant increase of production of the Th1-type cytokines; IL-12 (65% vs 44%, $p = 0.021$) and IFN- γ (40% vs 20%, $p = 0.021$), compared to the negative control group (Fig. 5). However, the LieIF_2 peptide resulted in enhanced production of IL-4 and IL-10

(64% vs 48% and 43% vs 27%, $p < 0.05$) (Fig. 5). Interestingly, the LieIF_2 peptide caused the same pattern of expression of IL-12 and IL-10 cytokines in BM-DCs derived from resistant C57BL/6 mice (data not shown).

Moreover, the LieIF_2 peptide which was revealed as the most promising peptide among the five synthetic peptides, also induced significant up-regulation of TNF- α mRNA transcripts in BM-DCs, compared to the negative control group, as was revealed by semi-quantitative PCR analysis (Fig. 6).

Table 5

Synthetic peptides of LielF. The selected peptides (16–18 amino acid long) met the following criteria: a) belonging to the N-terminal region of the protein (1–226 aa), b) showed high absolute scores and c) contained both 9-mer and 15-mer epitopes. Summary of the candidate epitope characteristics. The charge of the peptides was calculated using the program <http://www.designerbioscience.com/Calculator.asp>.

peptides	amino acid sequences	residues	MW	peptide charge	status	9-mer	15-mer
LielF_1	KPSSIQQRAIAPFTRGG	48–64	2,086.36	+3	basic	57-IAPFTRGGD-65	48-KPSSIQQRAIAPFTR-62 49-PSSIQQRAIAPFTRG-63 50-SSIQQRAIAPFTRGG-64
LielF_2	IQQRAIAPFTRGGDII	52–68	1,970.26	+1	basic	57-IAPFTRGGD-65	
LielF_3	TRELALQTAEIVSRIGE	103–119	2,075.38	–1	acidic	100-LSPTRELAL-108	103-TRELALQTAEIVSR-117
LielF_4	FLDDQPGVVRPIPSFDDM	15–31	2,030.40	–3	acidic	17-DDQPGVVRPI-25 22-VRPIPSFDD-30 23-RPIPSFDDM-31	16-LDDQPGVVRPIPSFDD-30
LielF_5	PKDIQVALFSATMPPEEVL	200–216	2,186.5	–2	acidic	199-LPKDIQVAL-207	202-DIQVALFSATMPPEE-216 199-LPKDIQVALFSATMP- 213

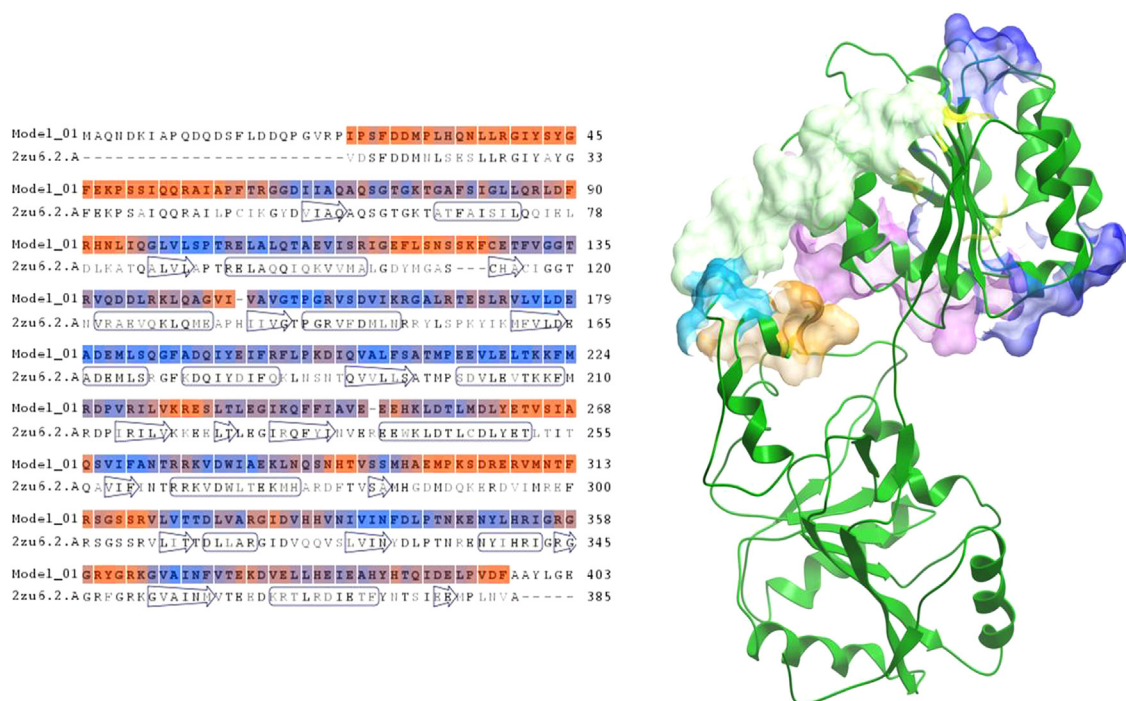


Fig. 2. LielF structure and localization of LielF peptides. Model of LielF structure was based on template structure of Eukaryotic Initiation Factor 4A-I (EIF4A-I, PDB: 2zu6) that shares 60.65% homology with LielF. The model was built using Promod-II version 3.70. The localizations of LielF_1, LielF_2, LielF_3, LielF_4 and LielF_5 peptides are represented as surface meshes with different colors (LielF_1 in cyan, LielF_2 in yellow, LielF_3 in purple, LielF_4 in orange and LielF_5 in blue). The LielF_1 and LielF_2 peptides are overlapped and their common region is represented in light green.

4. Discussion

Although leishmaniasis is a serious protozoan disease with high prevalence in the tropic regions, it also appears with growing incidence in the developed western countries. The ability of *Leishmania* to evade the immune mechanisms of the host, has rendered the efforts to design protective vaccines so far; as unsuccessful.⁴² In order to develop successful protective approaches against leishmaniasis, the scientific interest has been focused on *Leishmania* antigens which exhibit immunomodulatory properties, capable to activate both innate and specific cellular immune responses.

It is important to determine the minimum region of a protein that bears immunogenic properties because it imitates the natural process of infection and the subsequent degradation of the protein by the intracellular pathway, while excluding all the sequences that can cause deterioration of the disease pathology. Epitope mapping is an essential step in the procedure of designing an effective subunit vaccine.^{43,44} In our study, the objective was to use current immunoinformatics resources in order to design peptides

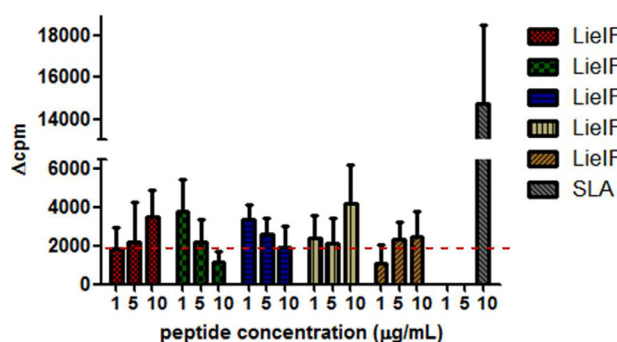
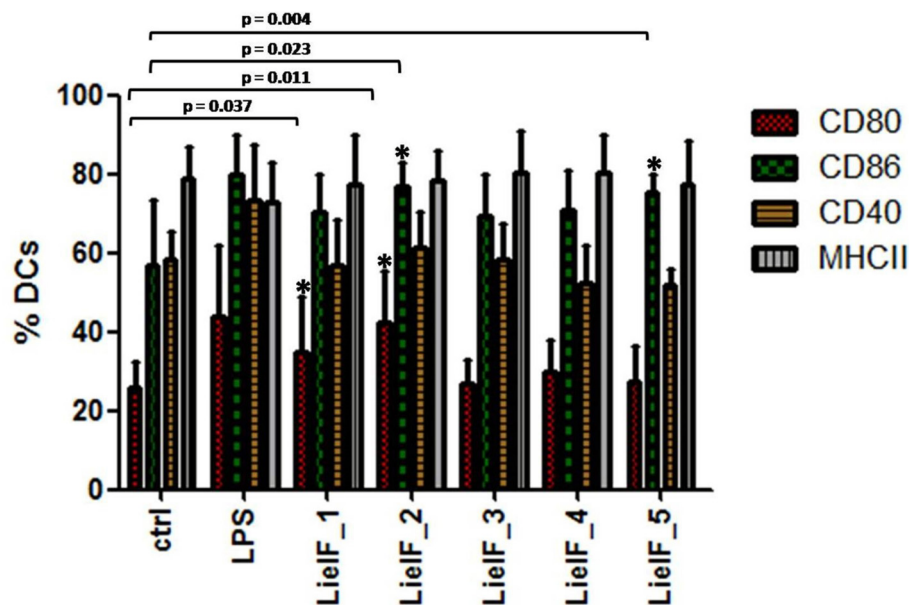


Fig. 3. Proliferation of splenocytes against LielF peptides. Splenocytes were isolated from *L. major*-infected BALB/c mice, at 30 days post infection and tested in triplicate for reactivity to synthetic peptides of LielF over a dose-response of 1–10 $\mu\text{g/mL}$ of each peptide. Over the last 18 h, [³H]thymidine (2 $\mu\text{Ci/mL}$) was added and the results are presented as $\Delta\text{cpm} \pm \text{SD}$, where $\Delta\text{cpm} = \text{mean}_{\text{cpm}}$ of cells stimulated with the peptide – mean_{cpm} of unstimulated cells, as described in Section 2.6. Splenocytes were also stimulated *in vitro* with ConA ($\Delta\text{cpm} = 100882 \pm 9231$) for comparison objectives. The experimental procedure was repeated three times.

(a)



(b)

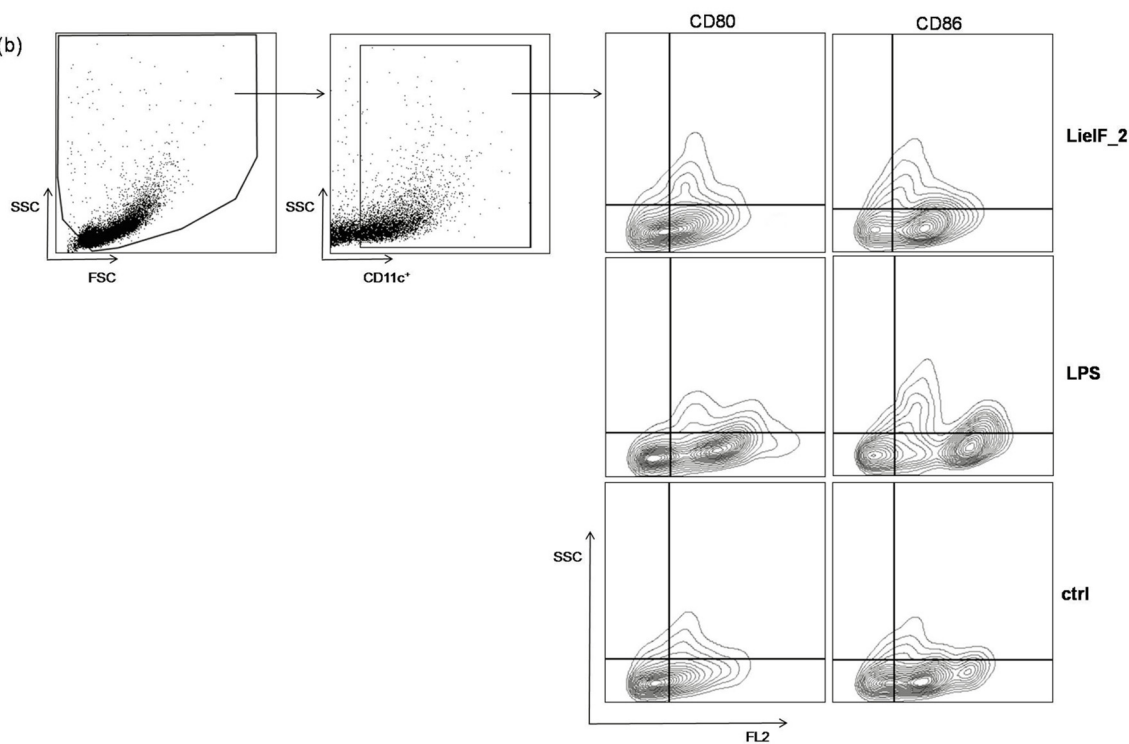


Fig. 4. *In vitro* effect of LielF synthetic peptides on maturation of BM-DCs. BM-DCs were incubated with the synthetic peptides of LielF (10 μ g/mL) and the expression of CD80, CD86, CD40) and MHC class II molecules was assessed by FACS. (a) Bar diagram representing the percentage (%) of CD80⁺, CD86⁺, CD40⁺ and MHCII⁺ DCs, (b) facs plots representative of one experiment. The results are presented as the mean value of each cellular group from three independent experiments \pm SD. * indicates statistically significant difference compared to the negative control group of unstimulated cells.

considered as immunodominant and conserved antigens among *Leishmania* parasites. For that reason, the above mentioned tools were used to model the MHC:epitope interface and to predict accurately the immunogenic peptides.⁴⁵ Currently, exist more than 20 T-cell epitope mapping tools⁴⁵ and a series of World Wide Web algorithms which distinguish binders from nonbinding peptides, based on the amino acid sequence, are available.⁴⁶ We proceeded to the *in silico* analysis of LielF by using antigenicity prediction

algorithms. Our choice for H2 MHC class I and MHC class II epitope prediction algorithms was made in terms of ISI citation indexes and regarding their availability for download and local machine implementation.¹¹ This analysis revealed a significant number of possible epitopes; sixty-one (61) 15-mer peptides, and forty-one (41) 9-mer peptides, belonging to the N- and C-terminal portion. Even though, only a small number of the predicted epitopes was provided by all the used algorithms with binding capacity to

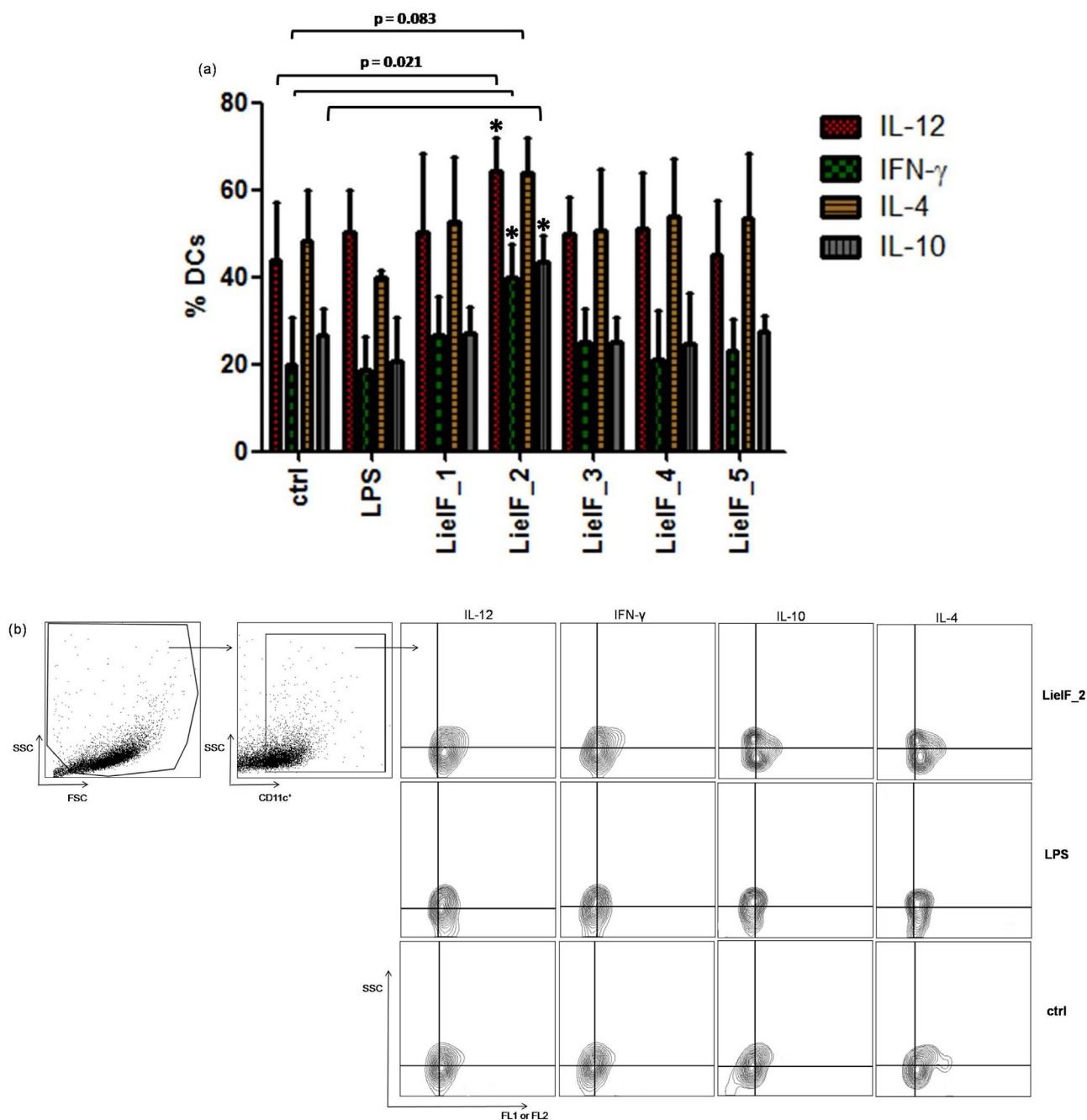


Fig. 5. *In vitro* effect of LiEF synthetic peptides on the production of cytokines. BM-DCs were incubated with the synthetic peptides of LiEF (10 μ g/mL) and the production of cytokines characteristic of the protective Th1 (IL-12, IFN- γ) or non-protective Th2 (IL-4, IL-10) immune response was assessed by FACS. (a) Bar diagram representing the percentage (%) of DCs positive for intracellular cytokines, (b) histogram facs plots representative of one experiment. The results are presented as the mean value of each cellular group from three independent experiments \pm SD. * indicates statistically significant difference compared to the negative control group of unstimulated cells.

multiple alleles. More specifically, only three out of sixty-one (3/61) 15-mer nominated peptides (199–213, 138–152 and 376–390 aa) and none of the 9-mer peptides; were predicted to bind to alleles of susceptible, as well as of resistant mice. Our results are partially consistent with the unique to our knowledge published analysis of LiEF, which concerns the prediction of binding epitopes to H2^d MHC class I and II molecules.³⁴ Our approach revealed totally sixty-one (61) 15-mer and forty-one (41) 9-mer peptides for three alleles (H2^d, H2^b and H2^k) versus thirty-nine (39) 15-mer and twenty (20) 9-mer for H2^d allele.

Synthetic peptides have attracted considerable attention as a basis for subunit vaccine design due to their stability and ease of synthesis and characterization.⁴⁷ Towards this direction, we finally

focused on five peptides, 16–18 amino acids long; that met the following criteria: were part of the N-terminal portion of the protein (1–226 aa) due to previous published data that claim that the immunomodulatory ability of LiEF is located in that region,⁴¹ exhibited high binding scores and contained 9-mer and 15-mer epitopes.

Nevertheless, all the above mentioned algorithms are indirect methods for the prediction of binding T-cell epitopes, even those with high sensitivity and specificity, and for that reason it is necessary to use these *in silico* tools in conjunction with *in vitro* and *in vivo* validation. Thereafter, the *in vitro* validation of the *in silico* predictions by using T-cell assays, was followed. The monitoring of antigen-specific T-cell responses *in vitro* involves measurement of their proliferation by radiolabeled thymidine incorporation.⁴⁵

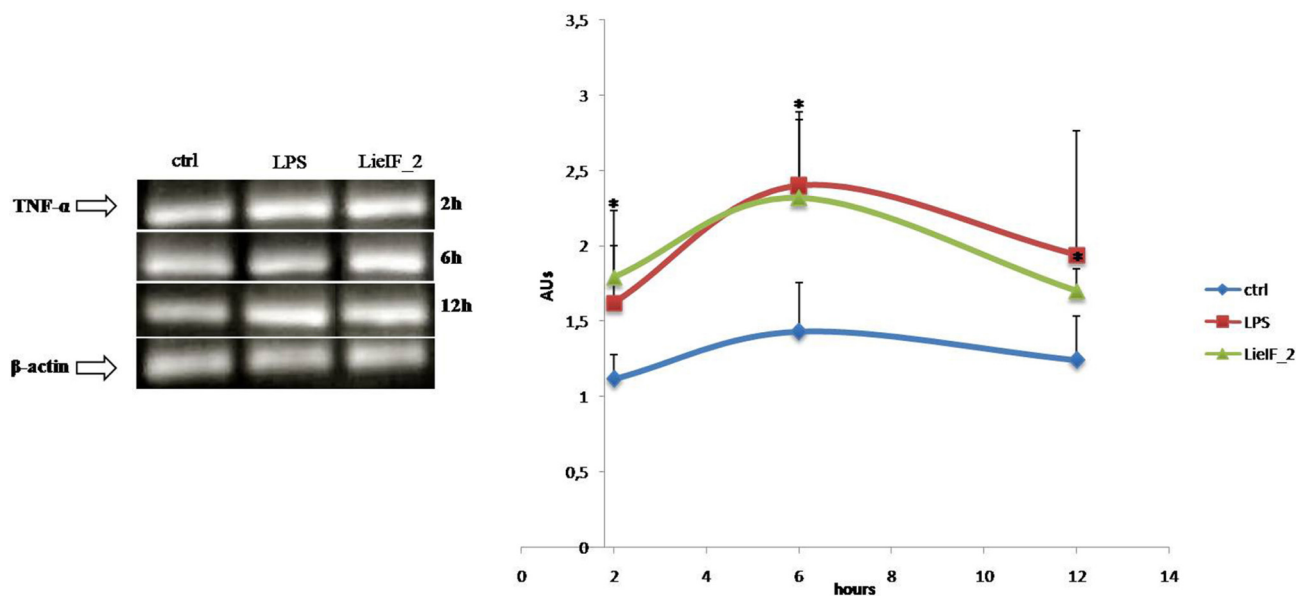


Fig. 6. TNF- α mRNA levels in BM-DCs pulsed with the LielF_2 peptide. BM-DCs were co-cultured *in vitro* with the LielF_2 peptide (10 μ g/mL) for 2, 6 and 12 h. TNF- α mRNA levels were determined and β -actin was used as an internal control. Representative ethidium-bromide stained gel is shown on the left and the diagram that displays TNF- α transcript levels at the designated timepoints is shown on the right. Results are presented as mean arbitrary units (AUs) \pm S.D. of three independent experiments. * indicates statistically significant difference compared to the negative control group of unstimulated cells.

We assessed the re-stimulation of T-cells derived from *Leishmania*-infected mice in order to activate and expand relevant T-cells. We noticed that the immunoinformatics analysis described above led to an accurate prediction of immunogenic and conserved peptides in *Leishmania* species, since the synthetic peptides exhibited positive proliferative responses.

The development of a prophylactic vaccine against infectious diseases often requires the *in vitro* screening of candidate antigens for their ability to activate innate immune cells, as a predictive tool for their *in vivo* immunogenicity. DCs play an important role in the initiation and regulation of antimicrobial immune responses, since co-stimulation of T-cells by DCs influence their activation. Mature DCs, characterized by high levels of the surface co-stimulatory molecules, stimulate positive responses by T-cells whose TCR recognize the displayed antigens. In contrast, low levels of co-stimulatory surface molecules, especially B7 (CD80 and CD86), characterize immature DCs and enhance tolerogenicity.⁴⁸ The importance of CD86, CD80 and CD40 co-stimulatory molecules in anti-*Leishmania* immune responses has been investigated in both murine and human models. Consequently, we evaluated *in vitro* the immunomodulatory properties of the synthetic peptides. More specifically, the peptides were evaluated for their capacity to induce BM-DCs maturation and functional differentiation. Four out of five synthetic peptides did not induce the maturation and functional differentiation of BM-DCs. The only exception was the LielF_2 peptide (52–68 aa), which induced an increased expression of B7 molecules (CD80 and CD86) and production of Th1- and Th2-type cytokines. The regulation of the expression of co-stimulatory molecules CD80 and CD86 is influenced by many cytokines, such as IFN- γ , IL-4 and IL-10. The IL-4 cytokine, which is a characteristic of Th2 immune response, acts as a potent inducer of CD86 and less of CD80, on B lymphocytes.⁴⁹ Moreover, the LielF_2 peptide caused significant production of IL-12, IFN- γ and IL-10 cytokines by BM-DCs, indicating the induction of a mixed Th1/Th2 immune response. Additionally, the IL-12/IL-10 cytokine ratio was determined in order to further characterize the cytokine-induction activity of the LielF_2 peptide and the calculated ratio (1.5 > 1) suggested the predominance of IL-12 induction. The LielF_2 peptide also modulated the TNF- α production by BM-DCs. TNF- α secretion

has been demonstrated to play an important role in the control of leishmaniasis.⁵⁰ In TNF-deficient mice *L. major*-specific T-cells failed to achieve control of the parasite *in vivo*.⁵¹ Moreover, a recent study demonstrated that the TNF- $^{-/-}$ and TNFR1 $^{-/-}$ mice lack of resistance to *L. major* infection despite the increased production of IFN- γ , pointing out the central role of TNF/TNFR1 signaling in linking innate leishmanicidal effector mechanisms with the adaptive immune response.⁵² A recent study partially confirmed the inability of the LielF_4 peptide (15–31 aa) to induce the production of cytokines. In that study, the effect of the protein region LielFD25 (not containing the first 25 amino acid residues of the protein) in human monocytes, led to a similar production of IL-12p70, IL-10 and TNF- α with the corresponding effect of the entire molecule of LielF, indicating that the part 1–25 is not necessary for the production of these cytokines.²⁸ It is also noteworthy that the LielF_2 peptide which exhibited the most prominent immunomodulatory properties seems to be located away from EIF4A-I's interaction sites with either Eukaryotic Initiation Factor 4G (EIF4G) or Programmed Cell Death Protein 4 (PDCD4).

The current study followed the concept of the promising strategy for developing a peptide-based vaccine against *Leishmania* parasites, based on the identification of immunogenic and concurrently conserved peptides of LielF with high affinity to MHC class I and II molecules, presented by antigen presenting cells. The determination of antigens with immunomodulatory properties and the ability to use chemically defined antigens; strongly suggest that vaccination with DC vehicles is a promising tool to induce effective immunity mediated by MHC class I-restricted as well as class II-restricted T-cell responses to microorganisms. The aforementioned results certify the immunogenic and immunomodulatory properties of synthetic peptides of LielF and can be further used in DCs-based vaccines as a potential tool for the immunointervention in leishmaniasis. Nevertheless, even if *in silico* modeling to predict antigenicity in microorganisms, helps to make large microbial genomes more tractable and focus efforts prior to embarking on *in vivo* models, animal immunogenicity and efficacy studies constitute an important part of pre-clinical development for novel vaccines.⁵³ Moreover, although the extrapolation of such results to human studies is important to evaluate the potential

development of protective vaccines, it contains a degree of judgment and assumption.⁵³ This can be partially overcome with the use of HLA (human leukocyte antigen) transgenic mice; a new tool in vaccine development closer to the human system. Expression of human transgenes (e.g. HLA-DR or DQ molecules) and targeted inactivation of mouse genes encoding factors controlling the development and function of innate immune system (e.g. lack expression of endogenous mouse MHC class I and/or II molecules) have recently addressed many limitations, allowing an evaluation of human immune responses in a murine framework.^{54–56} Towards this direction, we also conducted a preliminary *in silico* analysis of the Lief protein for specific HLA epitopes and revealed that the aforementioned peptides are promising binding epitopes to multiple HLA alleles and this would be interesting to be further evaluated in HLA-transgenic mice.

5. Conclusions

In the current study, we followed the key modules of a modern subunit vaccine development pipeline by using antigenicity prediction algorithms in order to reveal possible Lief epitopes that could be presented via H-2 class I and II alleles. Subsequently, we synthesized five promising antigenic peptides of Lief and we further validated their immunogenicity *in vitro*. Furthermore, we evaluated their immunomodulatory properties, since *Leishmania* antigens that exhibit that kind of properties are imperative in the development of successful protective approaches against leishmaniasis. Their immunomodulatory properties were tested in an *in vitro* system of bone marrow derived DCs, since the use of DCs as vehicles is a promising tool to induce effective immunity mediated by MHC class I- and class II-restricted T-cell responses. The 52–68 aa peptide induced high expression of B7 molecules and production of Th1- (IL-12, IFN- γ , TNF- α) and Th2- (IL-10) type cytokines by DCs. These data indicate that the 52–68 aa peptide is a candidate tool for vaccine development against leishmaniasis.

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Conflict of interest

Authors declare no conflict of interest.

Acknowledgements and author's contribution

The experiments were conceived and designed by OK, JR and ED. The experiments were performed by OK, JR and IK. The data were analyzed by OK, JR, MB, IG and ED. AT critically revised the manuscript. The paper was written by OK, JR and ED.

A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmc.2017.07.013>.

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