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Leishmania infantum LeIF and its recombinant polypeptides induce the maturation of dendritic cells *in vitro*: An insight for dendritic cells based vaccine

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ABSTRACT

We previously showed that recombinant *Leishmania infantum* eukaryotic initiation factor (LieIF) was able to induce the secretion of cytokines IL-12, IL-10 and TNF-α by human monocytes. In this study, we explored *in vitro* the potential of LieIF to induce phenotypic maturation and functional differentiation of murine bone-marrow derived dendritic cells (BM-DCs). Moreover, in order to identify potential immunnomodulatory regions of LieIF, eight recombinant overlapping protein fragments covering the whole amino acid sequence of protein, were constructed and assessed *in vitro* for their ability to induce maturation of BM-DCs. Our data showed that LieIF and some of its recombinant polypeptides were able to induce elevated expression of CD40, CD80 and CD86 co-stimulatory molecules with concurrent IL-12 production. Moreover, we used an *in vivo* experimental model of cutaneous leishmaniasis consisted of susceptible *Leishmania major*-infected BALB/c mice and we demonstrated that LieIF-pulsed-BM-DCs adoptively transferred in mice were capable to confer protection against a high dose parasite challenge. This study further describes the immunomodulatory properties of LieIF and its polypeptides bringing relevant information for their exploitation as candidate molecules for vaccine development against leishmaniasis.

1. Introduction

Dendritic cells (DCs) consist an important tool for inducing cellular immunity since they are highly efficient antigen-presenting cells that initiate and direct adaptive immune responses and consequently they are considered as attractive targets for vaccine development [1]. In their immature state, DCs reside in the peripheral tissues where they are situated to recognize pathogen associated molecular patterns (PAMPs) with their pattern recognition receptors (PRRs) [2]. Upon receiving an activating stimulus, such as microbial products, proinflammatory cytokines and other endogenous signals, DCs migrate towards the draining lymph nodes whereby they undergo a maturation process that lead to an optimized ability to present processed peptides and initiate T-cell immunity [3]. This maturation process is associated with dramatic functional and morphological changes such as loss of phagocytic receptors, changes in morphology, increased expression of the surface molecules of the major histocompatibility complex (MHC) class I or II and of co-stimulatory molecules (*e.g.* CD80, CD86), increased antigen processing and induction of specific cytokine production [3,4].

Therefore, the pivotal role of DCs in priming immune responses makes them attractive target for vaccination and immunotherapy against intracellular pathogens and diseases for which cellular immunity seems to be a crucial part of the immune response [1]. Indeed, in recent years, the most extensively studied approach to induce both humoral and cellular immune responses is designing DCs targeted vaccines against infectious diseases [5,6], which induce the clonal expansion of T cells. Additionally, DCs-targeted vaccines are also used for the development of immunotherapeutics against cancer and

Abbreviations: BM-DCs, bone-marrow derived dendritic cells; CL, cutaneous leishmaniasis; TLRs, toll-like receptors; VL, visceral leishmaniasis; PAMPs, pathogen associated molecular patterns; PRRs, pattern recognition receptors; LeIF, *Leishmania* eukaryotic Initiation Factor; PBS, phosphate buffered saline; FACS, fluorescence-activated cell sorter

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autoimmunity which also require T cell immunity [7]. Characteristically, the first attempt utilizing DCs to induce protective immune responses in humans was used as immunotherapy against cancer and involved the adoptive transfer of *in vitro* cultured DCs loaded with antigens [8]. Moreover, it is noteworthy that nowadays the *ex vivo* antigen-loaded DCs-based vaccines are considered safe and effective in inducing tumor cellular immune responses in humans and DCs-based vaccines used as the tumor biological treatment scheme has been approved to enter III phase of clinical trials by the United States Food and Drug Administration [7].

Among infectious diseases for which no commercially available human prophylactic vaccine exists is leishmaniasis. Leishmaniasis constitute a complex set of diseases caused by obligatory intracellular protozoan parasites of the *Leishmania* genus, causing significant morbidity and mortality worldwide [9,10]. More than three hundred million people are at risk of infection and two million cases are reported each year [11]. It is characterized by a wide spectrum of disease manifestations ranging from the dermal lesions of cutaneous leishmaniasis (CL) to the deadly visceral leishmaniasis (VL), depending on the parasite species and on the host's immunological response [9,10]. The current chemotherapy involves limited number of drugs which present diverse disadvantages such as high toxicity and parasite resistance [12,13]. Therefore, the control of leishmaniasis is considered imperative and a prophylactic vaccine capable of inducing long-lasting protective cell-mediated immune responses is desired [14].

Many studies proved that CD4+ and CD8 + T cell-mediated responses are involved in susceptibility or resistance to *Leishmania* infection [15,16]. Thus, vaccine development efforts against *Leishmania* infection are focused on the identification and use of defined recombinant antigens able to elicit protective T cell responses and to generate long-lasting immunity [14]. Upon infection, *Leishmania* parasites are mainly detected in macrophages as well as in DCs [17]. Interactions between *Leishmania* parasites and DCs are complex and involve paradoxical functions that profoundly affect the adaptive immune response, leading to the control of infection or progression of the disease [18–20]. Activated DCs produce various cytokines, such as IL-1, IL-6 or IL-12, which regulate the type of T-cell responses [21]. It has been shown that *Leishmania*-infected DCs are able to produce the important for the development of the protective Th1-type immune response, IL-12 cytokine, in a CD40/CD40 ligand-dependent process [22] and the overall protective effect against leishmanniasis is reinforced by the production of IFN-γ [23,24]. Indeed, DCs based interventions were shown to mediate protection against intracellular pathogens, including *Leishmania* parasites [33]. The DCs-induced Th1 mediated protection has been associated with an increase in the level of IL-12 [25,26], generation of parasite-specific cytotoxic T lymphocytes [27] and suppression of T-cell derived IL-10 production [28]. In addition, it has been postulated that a DCs-targeted rather than a conventional *ex vivo* DCs-based vaccine formulation could result in better outcomes [29,30]. In this context, Matos *et al* have shown that direct targeting of a specific DC marker (DEC 205) results in durable protective immune response against *L major* infection of BALB/c mice [31]. Therefore, DCs must be educated in a specific manner to circumvent disease-promoting effects and drive an efficient immune response towards the resolution of the disease. Thus, the identification of *Leishmani*a antigens exhibiting immunomodulatory properties, capable to activate both innate and specific cellular responses, is imperative for the effective protection from *Leishmania* infection [14].

Among various *Leishmania* molecules that have been identified as immunogenic and potential protective antigens for second-generation vaccines against leishmaniasis, *Leishmania* eukaryotic Initiation Factor (LeIF) has attracted much attention because of its advantageous properties [32–34]. LeIF is highly conserved among *Leishmania spp*. and is expressed in all life stages of the parasite [33,35–38]. It is abundant in the secretome or the exosomes of *L. infantum* and *L. donovani* promastigotes [39–42]. LeIF also stimulates IL-12 and IFN-γ production by human peripheral mononuclear cells (PBMCs) from either leishmaniasis patients or normal individuals [32]. Moreover, it has the ability to skew an established Th2-type immune response into a protective Th1-type response in *L. major*-infected susceptible BALB/c mice with the production of IFN-γ [33], as well as the production of IL-12, IL-10 and TNF-α by human macrophages, DCs and monocytes [34,43,44]. Recently, it has been shown that co-delivery of a DNA vaccine encoding LeIF gene and IL-12 increases the protection against *L. major* infection in BALB/c mice [45]. Moreover, the NH_2- terminal part (1–226) of LeIF, known to retain the immunomodulatory properties [33,43], is part of the recombinant vaccine Leish-111f that is efficient in experimental mice and hamster models [46–48]. Additionally, we have recently shown that, in presence of IFN-γ, LieIF inhibits *L. donovani* growth in murine macrophages [49].

In the present study, we evaluated the potential of recombinant LieIF and selected recombinant overlapping polypeptides derived from LieIF, to induce phenotypic maturation and functional differentiation of murine bone-marrow derived DCs (BM-DCs). The potential of LieIF-pulsed and polypeptide-pulsed BM-DCs to prevent cutaneous leishmaniasis in an experimental murine model was also investigated as a DCs-based prototype vaccine against leishmaniasis.

2. Materials and methods

2.1. Experimental animals and parasites

Female BALB/c mice, 6–8 weeks of age, were obtained from the breeding unit of the Hellenic Pasteur Institute (HPI, Athens, Greece). Experimental animals were maintained under specific pathogen-free conditions, receiving a diet of commercial food pellets and water *ad libitum*. All animal procedures followed the ARRIVE guidelines and were approved by the Institutional Protocols Evaluation Committee according to PD 56/2013 as adoption of Directive 2010/63/EU. Protocol license was issued by the Official Veterinary Authorities of the Prefecture of Attiki in compliance with the above legislation in force.

The *Leishmania major* strain MRHO/SU/59/P (LV39) was used and its virulence was maintained by monthly passage in BALB/c mice as described previously [50]. LV39 promastigotes were grown in complete RPMI-1640 medium (Biochrom AG, Berlin, Germany) supplemented with 2 mM L-glutamine, 10 mM Hepes, 24 mM $NaHCO_3$, 0.05 mM of 2-mercaptoethanol, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% v/v heat-inactivated foetal bovine serum (FBS) (Gibco, Paisley, UK), as previously described [51].

2.2. Protein expression and purification

All constructs bearing the different protein coding genes: LieIF and eight recombinant polypeptides (1–226, 1–195, 129–226, 129–261, 196–403, D1 + 25 (1–238), D1 (25–238) and D2 (239–403)) were subcloned into the *NdeI* and *XhoI* or *EcoR1* sites of pET-22b or pET-17b expression vectors (Novagen) as previously reported [34] Expression and purification were performed as previously described [34] and their purity and concentration were verified on 12% coomassie-stained SDS PAGE gel. Recombinant proteins were tested for the absence of contaminating LPS by confirming that their IL-10 cytokine production activity was sensitive to proteinase K and resistant to polymixin B [34,44]. In addition all the recombinant proteins were tested for the amount of endotoxin levels using the *Limulus* amebocyte lysate (LAL) assay. LAL reagents, endotoxin-free water, endotoxin-free pipette tips, and endotoxin-free test tubes were purchased from Charles River (Charleston, USA). All recombinant constructs were characterized as free of residual endotoxin action (concentration of LPS ≤ 5 EU/mg).

2.3. Generation of murine bone-marrow-derived DCs (BM-DCs)

Bone marrow-derived DCs (BM-DCs) were generated as previously

described [52,53]. Briefly, bone marrow cells were isolated from femur and tibiae bones of naive BALB/c mice and depleted of red blood cells with ammonium chloride (ACK) lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM Na_2EDTA, pH 7.2). Isolated cells were cultured at a concentration of 3.5×10^5 cells/mL in 10 mL of RPMI-1640 medium (Biochrom AG, Berlin, Germany) supplemented with 10% heat-inactivated FBS (Gibco, Paisley, UK) (complete RPMI-1640 medium), in the presence of 20 ng/mL of recombinant mouse granulocyte-macrophage colony stimulating factor (rm-GM-CSF) (specific activity $\geq 2 \times 10^7$ units/mg; Peprotech, Cat. Number 315-03). At day 3, a further 10 mL of fresh complete RPMI-1640 medium containing 20 ng/mL of GM-CSF was added; while on day 6, 10 mL of the culture media were carefully aspirated and replaced with fresh medium also containing 20 ng/mL of GM-CSF. On day 9, non adherent cells were collected by gentle pipetting (cell viability was determined by trypan blue dye > 95%). Cells were stained with R-phycoerythrin-conjugated (R-PE) hamster anti-mouse CD11c (HL3 clone; BD Biosciences, Erembodegem, Belgium) or fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD8a (Lyt2 clone; BD Biosciences, Belgium) mAbs. The purity of myeloid DCs ($CD11c^+CD8a^-$) was > 75% and was determined by flow cytometry (Becton Dickinson & Co.) using the FlowJo V.10.0.8 software package (Tree Star, Inc., Ashland, OR, USA).

2.4. Pulsing of DCs

Non-adherent cells were harvested on day 9 and washed twice in complete RPMI-1640 medium. BM-DCs were placed in 24-well cell culture plates (Sarstedt, Germany) (10^6 cells/mL) and were pulsed with each one of the recombinant proteins (LieIF, 1–226, 1–195, 129–226, 129–261, 196–403, 1–238, 25–238, 239–403) at a final concentration of 10 μg/mL for 24 h, at 37 °C and 5% CO_2. Cells of the negative control group were cultured only with complete RPMI medium. Moreover, in order to compare LieIF protein to TLR ligands, cells were also treated with poly (I:C) (TLR3 agonist, 10 μg/mL, InvivoGen, US), LPS (TLR4 agonist, 1 μg/mL, *Escherichia coli*; Sigma, Deisenhofen, Germany) and the oligonucleotides CpG ODN 1826 (TLR9 agonist, 10 μg/mL, InvivoGen, US).

2.5. Phenotypic determination of maturation state of antigen-pulsed DCs

After 24 h, pulsed BM-DCs were collected and washed twice with sterile phosphate buffered saline (PBS). Single-cell suspensions were prepared and 5×10^5 cells were re-suspended in FACS buffer (FB) (PBS + 3% FBS). They were stained with anti-mouse PE-conjugated anti-CD40 (3/23 clone) and anti-MHCII (M5/114.15.2 clone) antibodies (BD Pharmingen) together with anti-mouse FITC-conjugated anti-CD80 (16-10A1 clone) and anti-CD86 (GL1 clone) (BD Pharmingen), for 30 min at 4 °C, according to manufacturer's instructions. Control unstained samples were similarly processed for all the above cases. 10,000 cells were acquired for each sample in a fluorescence-activated cell sorter analysis (FACS) (Becton-Dickinson, San Jose, CA, USA) by gating out the majority of non-viable cells based on low forward angle light scatter. Data were analyzed with FlowJo V.10.0.8 software.

2.6. Intracellular detection of cytokines

For intracellular cytokine staining, pulsed BM-DCs were incubated with Brefeldin A (2.5 μg/mL) for 5 h at 37 °C in 5% CO_2. Subsequently, cells were washed twice in staining buffer and fixed with 2% v/v paraformaldehyde (PF) for 20 min at 4 °C. Cells were then permeabilized at room temperature for 5 min in permeabilization wash buffer (PBS + 3% FBS + 0.1% saponin) and stained with anti-IL12-p40/p70-PE (C15.6 clone) and anti-IL4-PE (11B11 clone) together with anti-IFN-γ-FITC (XMG1.2 clone) and anti-IL-10-FITC (JES5-16E3 clone) (BD Pharmingen) for 30 min at 4 °C according to manufacturer's instructions. Thereafter, the cells were resuspended in PBS, and 10,000 cells per sample were acquired with FACS and data were analyzed with FlowJo V.10.0.8 software. Control unstained samples were also similarly processed for all the above cases.

2.7. Cellular vaccination and parasite challenge

Cellular vaccination protocol with BM-DCs pre-incubated with recombinant LieIF, fragment 1–226 and fragment 196–403; was designed and performed as previously described [53]. Briefly, BM-DCs (10^6 cells/mL) were placed in 24-well cell culture plates and were incubated with recombinant LieIF, polypeptide 1–226 or polypeptide 196–403 (10 μg/mL), for 24 h, at 37 °C and 5% CO_2. Three groups of BALB/c mice (n = 15/ group) were intravenously vaccinated in the lateral tail vein with 10^6 antigen-pulsed BM-DCs (LieIF, fragment 1–226 and fragment 196–403, respectively). Control mice received unpulsed BM-DCs (cells cultured with RPMI medium). 2 weeks later, the same mice were subcutaneously (s.c.) infected with 2×10^6 *L. major* stationary phase promastigotes [54]. The lesion progression was monitored weekly by measuring the thickness of the infected and contralateral footpad with a dial gauge caliper (Mitutoyo, Kanagawa, Japan), during 10 weeks after infection [50].

2.8. Statistical analysis

The *in vitro* experimental procedures were repeated at least three times, and each sample was placed in triplicate in any experimental procedure. In the *in vivo* procedures, we used five animals per group and the experiments were repeated three times. Data are expressed as the mean values ± standard deviation (SD). Statistical analysis was performed by the two-sided Mann-Whitney test using the IBM SPSS Statistics software (version 24). P values less than 0.05 were considered to indicate statistical significance.

3. Results

3.1. Recombinant LieIF and its polypeptides induce the up-regulation of co-stimulatory molecules on BM-DCs in vitro

DCs, depending on their degree of maturation, variously regulate antigen-specific T cell responses [55]. Low levels of expression of co-stimulatory surface molecules, particularly of CD80 and CD86, direct their activity towards the development of immunological tolerance while high levels of this expression direct the action of regulating the T cell mediated response [56]. Therefore, we examined whether recombinant LieIF and its polypeptides can modulate the expression of co-stimulatory molecules by BM-DCs. The used GM-CSF-generated BM-DCs phenotypically and functionally largely resemble the conventional or myeloid DCs which are present in the secondary lymphoid organs [52,57].

To achieve this objective, LieIF and its polypeptides were expressed and purified by Ni-affinity chromatography and the purity of the proteins was more than 90% (Fig. 1A). The purified recombinant proteins were used to sensitize BM-DCs and cells were labeled with antibodies directed against the surface markers CD40, CD80, CD86 and MHC class II. FACS analysis showed that stimulation of BM-DCs with LieIF induced a significant increase in the expression of CD40, CD80 and CD86 co-stimulatory molecules in terms of the percentage (%) of cells along with median fluorescent intensity (MFI) (Fig. 2A–C). Specifically, LieIF-stimulated BM-DCs expressing CD40 were significantly upregulated as compared to the negative control group of untreated BM-DCs (33.1 ± 13.9 vs 18.7 ± 3.8) (Fig. 2A,C). Similarly, it was observed a significant increase in the expression of CD80 and CD86 molecules (58.3 ± 15.3 and 17.8 ± 5.4, respectively) as compared to untreated BM-DCs (34.7 ± 8.3 and 11.2 ± 1.2, respectively) (Fig. 2A,C). Moreover, LieIF-stimulated BM-DCs exhibited a 1.5, 1.7 and 1.9-fold

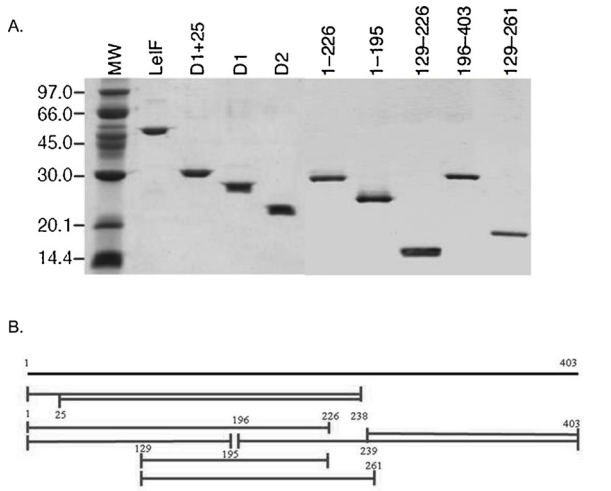

Fig. 1. (A) Expression and purification of the recombinant proteins. Aliquots of purified proteins were resolved by SDS-PAGE gel and stained with Coomassie brilliant blue. The positions of the Bio-RAD pre-stained markers (in KDa) are indicated at the left. **(B) LieIF recombinant polypeptides.** Eight recombinant polypeptides covering the whole length of the protein, were constructed based on the primary sequence of LieIF as well as on the solved crystal structure of other DEAD-box proteins. as described previously [34].

increase of MFI of cells expressing CD40, CD80 and CD86 respectively, as compared to untreated cells (Fig. 2B,C).The expression levels of MHC class II molecules in LieIF-treated BM-DCs and untreated cells were similar (data not shown) indicating the relative level of activation of the cultured and differentiated cells in the presence of recombinant GM-CSF [58].

Activation of TLRs induces maturation of DCs characterized by increased expression of co-stimulatory molecules and the production of cytokines (*e.g.* IL-12, IL-6) [59]. Furthermore, we investigated and subsequently compared the effect of known TLR ligands and recombinant LieIF on the phenotypic maturation of BM-DCs. In particular, LieIF caused a similar effect as TLR 3, 4 and 9 ligands, on the expression of CD40, CD80 and CD86 molecules also in terms of the percentage (%) of cells along with MFI (Fig. 2A–C). In more details, incubation of BM-DCs with LieIF caused higher or similar expression of CD40, CD80 and CD86 co-stimulatory molecules with each one of the used TLR ligands (p values ranging from 0.038 to 1.000), as indicated by the percentage of BM-DCs expressing the aforementioned molecules (Fig. 2A). It is noteworthy the fact that only poly(I:C) as compared to LieIF, induced higher expression of CD86 ($p = 0.023$) (Fig. 2A). In MFI analysis, LieIF also caused similar expression of all the three co-stimulatory molecules with the effect induced by LPS and CpG ligands (p values ranging from 0.513 to 0.827) (Fig. 2B). It is also remarkable that LieIF induced higher expression of all the three co-stimulatory molecules than poly(I:C) ($p = 0.050$) (Fig. 2B). Subsequently, in order to determine in which parts of LieIF immunomodulatory properties can be attributed, eight recombinant overlapping protein fragments covering the whole amino acid sequence of the protein, were constructed, as mentioned in Materials and Methods section. The recombinant polypeptides were designed to encode the regions 1–238 (D1 (+25)), 25–238 (D1), 1–226 (N-terminal region), 1–195, 129–226, 129–261, 196–403 (C-terminal region) and 239–403 (D2) (Fig. 1B). The purified polypeptides were also used to sensitize BM-DCs and cells were labeled with antibodies directed against the surface markers CD40, CD80, CD86 and MHC class II, as mentioned above. Untreated BM-DCs were the negative control group. FACS analysis was also performed in terms of the percentage (%) of cells along with MFI (Fig. 3A–C). Analysis of the percentage of BM-DCs expressing CD40, CD80 and CD86 co-stimulatory molecules revealed that all the polypeptides except the fragments

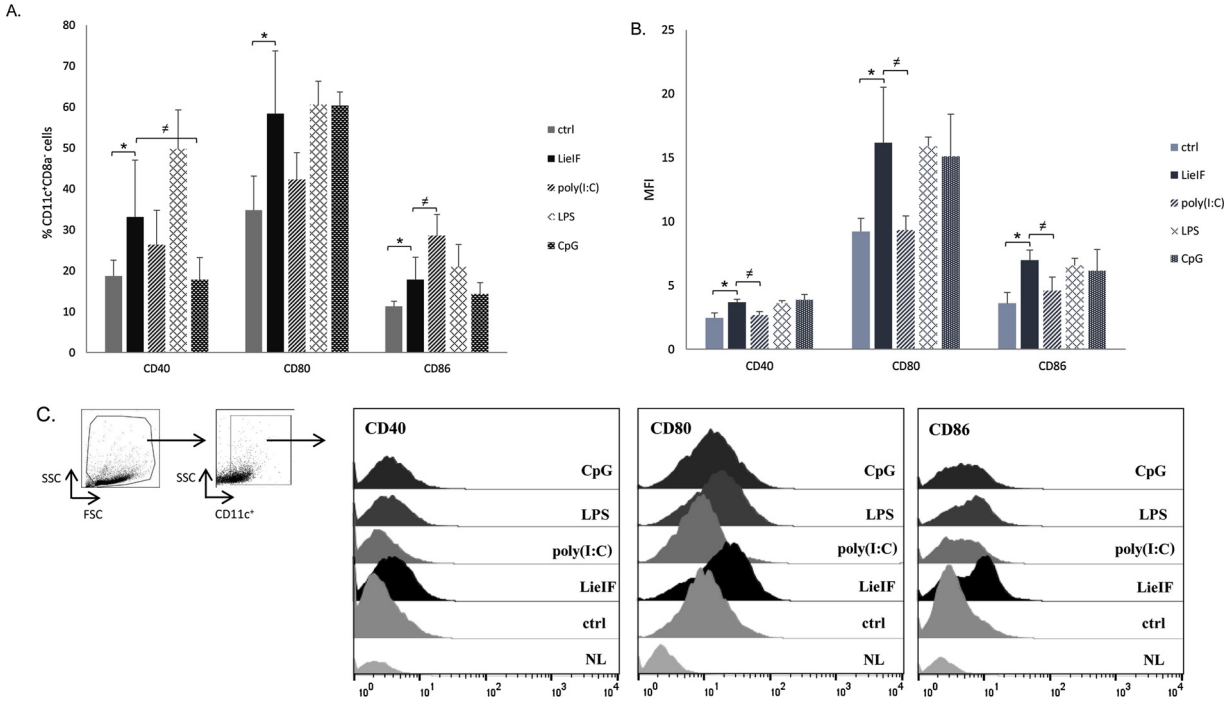

Fig. 2. *In vitro* comparative effect of recombinant LieIF protein and TLR ligands on the expression of co-stimulatory molecules by BM-DCs. BM-DCs were stimulated with recombinant LieIF (10 μg/mL), TLR3 ligand (poly(I:C), 10 μg/mL), TLR4 ligand (LPS, 1 μg/mL) and TLR9 ligand (CpG, 10 μg/mL), for 24 h. Expression of CD40, CD80 and CD86 co-stimulatory molecules was assessed by flow cytometry with the use of specific monoclonal fluorochrome-labelled antibodies. Unstimulated BM-DCs were used as the negative control. The results are expressed as mean values ± SD of three independent experiments and are presented as (A) bar diagram of percentage (%) of BM-DCs, (B) bar diagram of median fluorescent intensity (MFI) and (C) histogram overlays which are representative of one experiment. * indicates statistically significant differences as compared to the negative control and ≠ indicates statistically significant differences between LieIF and each one of the TLR ligands.

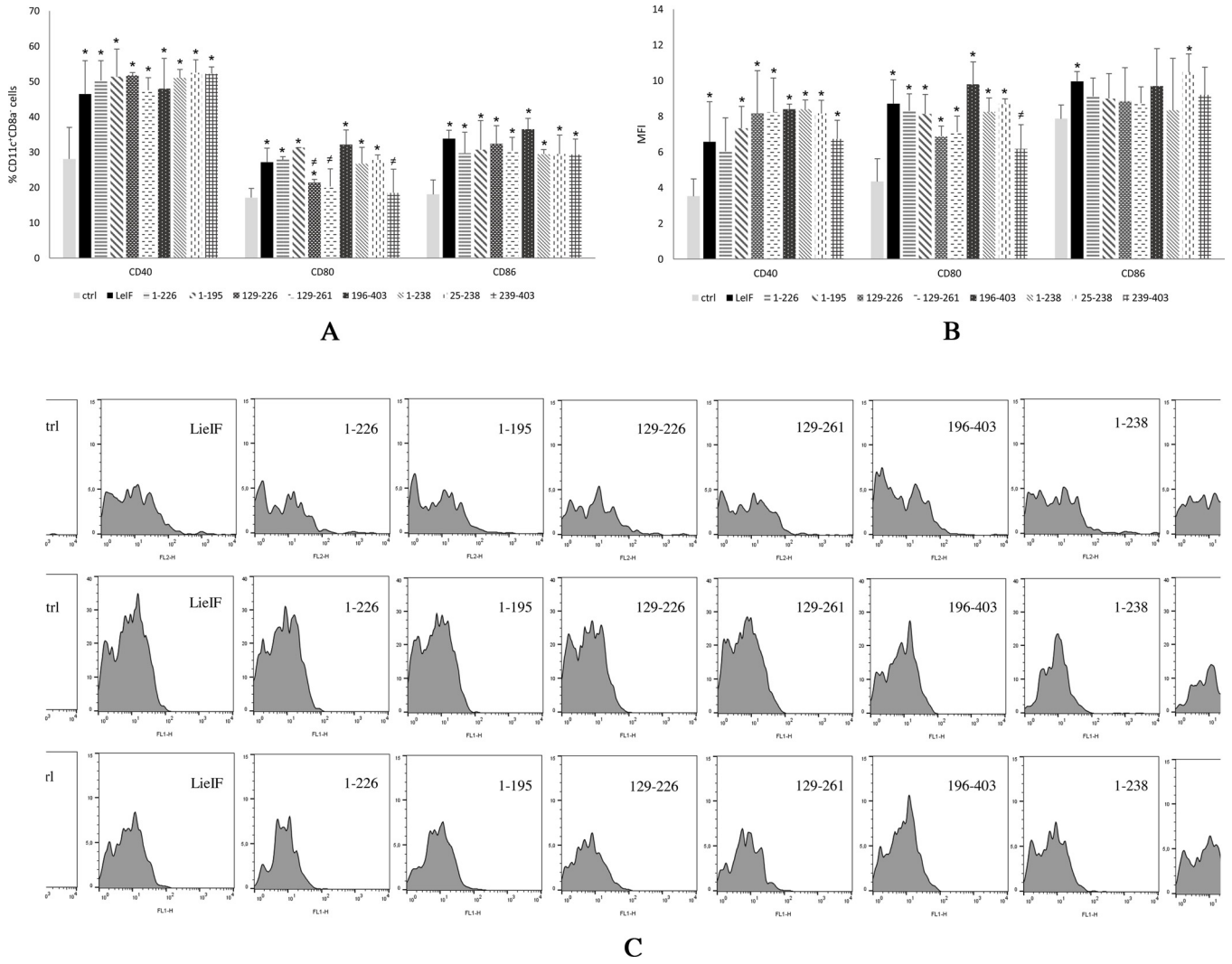

Fig. 3. *In vitro* effect of LieIF's recombinant polypeptides on the expression of co-stimulatory molecules by BM-DCs. BM-DCs were stimulated with the recombinant polypeptides 1–226, 1–195, 129–226, 129–261, 196–403, 1–238, 25–238 and 239–403 for 24 h. Expression of CD40, CD80 and CD86 co-stimulatory molecules was assessed by flow cytometry with the use of specific monoclonal fluorochrome-labelled antibodies. Unstimulated BM-DCs were used as the negative control while LieIF-stimulated BM-DCs were used as the positive control. The results are expressed as mean values ± SD of three independent experiments and are presented as (A) bar diagram of percentage (%) of BM-DCs, (B) bar diagram of median fluorescent intensity (MFI) and (C) histogram overlays which are representative of one experiment. * indicates statistically significant differences as compared to the negative control and ≠ indicates statistically significant differences as compared to the positive control.

129–261 and 239–403, are able to induce increased expression of all the three molecules as compared to untreated BM-DCs (Fig. 3A). Additionally, all the polypeptides except the fragments 129–226, 129–261 and 239–403, induced similar effect in the expression of all the three co-stimulatory molecules as compared to LieIF (p values ranging from 0.289 to 1.000) (Fig. 3A). Analysis of MFI revealed that the polypeptide 25–238 induced the increased expression of all the three co-stimulatory molecules, as compared to untreated DCs and this effect was similar to LieIF for all the co-stimulatory molecules (recorded p values are 0.480 for CD40, 0.827 for CD80 and 0.275 for CD86) (Fig. 3B). The polypeptides 1–195, 129–226, 129–261, 196–403 and 1–238 induced the increased expression of two co-stimulatory molecules, CD40 and CD80, as compared to untreated BM-DCs and these effects were also similar to LieIF ($p > 0.050$), while the polypeptides 239–403 and 1–226 induced the solely up-regulation of CD40 and CD80, respectively (Fig. 3B). The expression levels of MHC class II molecules in polypeptide-treated BM-DCs and untreated cells were similar (data not shown). All these results demonstrate that recombinant LieIF is able to induce the maturation of BM-DCs *in vitro* and that certain of its polypeptides retain this activity.

3.2. Recombinant LieIF and its polypeptides induce the production of IL-12 by BM-DCs

Antigen-sensitized DCs secrete a range of immunomodulatory/inflammatory cytokines (*e.g.* IL-12, IL-6, IL-1β, IL-8) or immunosuppressive cytokines (*e.g.* IL-10, TGF-β) [60], which also play a crucial role in priming T-cell responses [55]. Therefore, the analysis of whether the different recombinant proteins are able to induce the production of IL-12 cytokine by the sensitized BM-DCs was essential for their ability to induce a Th1-type immune response.

LieIF-pulsed BM-DCs showed a significant increased production of the Th1 polarizing IL-12 cytokine. LieIF induced a 7.5 fold increase in the production of IL-12 compared to the spontaneous production of the untreated BM-DCs (20.08 ± 8.9% vs 2.67 ± 0.4%, $p = 0.050$) (Fig. 4A-B). It is also noteworthy that LieIF-pulsed BM-DCs exhibited similar production of IL-12 with the LPS-treated cells ($p = 0.127$). When BM-DCs were sensitized with the recombinant polypeptides, a significant production of IL-12 was also obtained, which is consistent with the observed maturation of BM-DCs, mentioned above. More

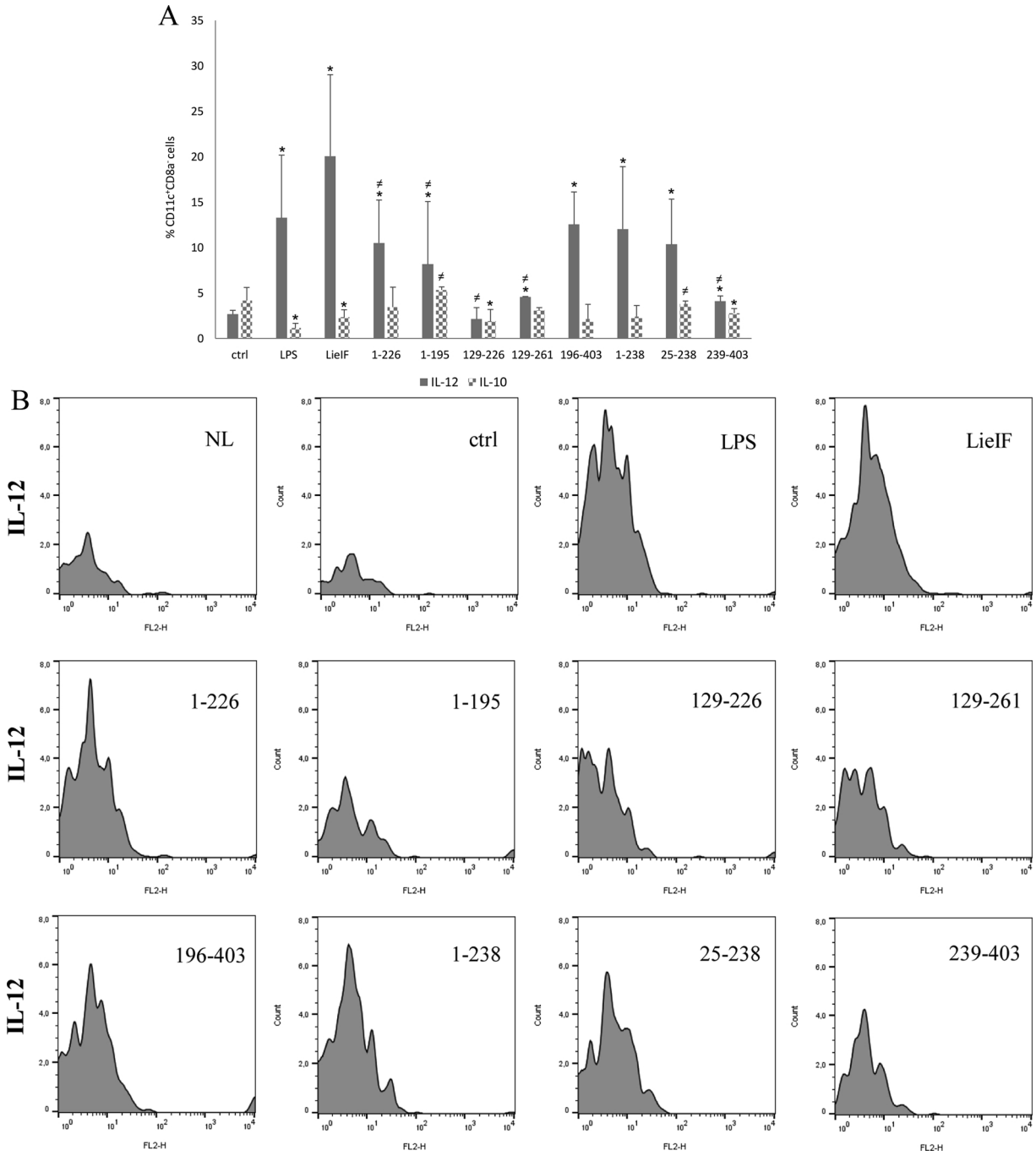

Fig. 4. *In vitro* effect of recombinant LieIF and its fragments on the production of IL-12 and IL-10 cytokines by BM-DCs. BM-DCs were incubated with LieIF and its overlapping polypeptides (10 μg/mL) for 24 h and the production of IL-12 and IL-10 was assessed by FACS. Unstimulated BM-DCs were used as the negative control while LPS-stimulated BM-DCs were used as the positive control group. The results are expressed as mean values ± SD of three independent experiments and are presented as (A) bar diagram of percentage (%) of BM-DCs and (B) representative of one experiment histogram plots for IL-12 production. * indicates statistically significant differences as compared to the negative control and ≠ indicates statistically significant differences as compared to LieIF-stimulated BM-DCs.

specifically, the effect of fragments 1–238, 25–238 and 196–403 which resulted in the most potent increased expression of all three surface markers, led also to a significant increase in IL-12 production compared to the spontaneous production of the untreated BM-DCs (12.05 ± 6.9, 10.4 ± 4.9% and 12.56 ± 3.5%, respectively, vs 2.67 ± 0.4%) (Fig. 4A-B). The fragments 1–226, 1–195 and 239–403 that also caused increased expression of co-stimulatory molecules but less potent according to MFI analysis, led also to increased expression of IL-12 (10.5 ± 4.7%, 8.2 ± 6.8% and 4.1 ± 0.6%, respectively) (Fig. 4A-B). The fragments of the central region 129–226 and 129–261 that exhibited the most moderate effect on the expression of co-stimulatory molecules, did not cause promising up-regulation in the expression of IL-12 (Fig. 4A-B). At the same time, we detected in smaller proportions, the suppressive IL-10 cytokine in all experimental groups (Fig. 4A), but clearly was not sufficient to inhibit the production of the Th1-type cytokine IL-12. Among the recombinant fragments, the fragments 1–195 and 25–238 led to a significant increase in the production of IL-10 compared to LieIF (5.35 ± 0.3% and 3.81 ± 0.3%, respectively vs 2.33 ± 0. 8%) (Fig. 4A). Moreover, we proceeded to the intracellular staining of IFN-γ and IL-4 cytokines and we did not observe significant

up-regulated production as compared to untreated BM-DCs (data not shown).

All these results indicate that recombinant LieIF and certain recombinant polypeptides induce the secretion of IL-12 by BM-DCs *in vitro*.

3.3. Vaccination with LieIF-pulsed BM-DCs induces protection against L. major infection in susceptible BALB/c mice

The intriguing properties of recombinant LieIF and its polypeptides described above, activation of BM-DCs to express co-stimulatory molecules and production of the IL-12 cytokine that determines the induction of the protective Th1-type response, prompted us to explore the potential of three recombinant proteins in inducing protective immunity to *L. major* infection upon delivery by protein-pulsed BM-DCs. To this end, we tested the entire LieIF protein, the recombinant polypeptide 1–226 that has been used in recombinant Leish-111f vaccine preparation [46–48] and the recombinant polypeptide 196–403 shown to induce both the up-regulation of all three co-stimulatory molecules tested above and the production of IL-12. Groups of BALB/c mice were immunized with adoptively transferred BM-DCs that had been pulsed with LieIF, polypeptide 1–226 and polypeptide 196–403. Control mice were treated with unpulsed BM-DCs. Two weeks later, animals were challenged with *L. major* stationary phase promastigotes, and the footpad swelling was measured weekly. The results demonstrated that BM-DCs pulsed with recombinant LieIF induced partial protection against *L. major* challenge reaching 56% reduction in footpad swelling at 6 weeks post-infection. All LieIF-vaccinated mice developed smaller footpad swelling during a monitoring period of 10 weeks (Fig. 5). In contrast, control mice developed progressive lesions and tissue destruction at 6 weeks post infection (Fig. 5). Mice that had been immunized with BM-DCs pulsed with the polypeptide 1–226 or 196–403 also developed smaller footpad swelling as compared to control group (22% and 34% reduction in footpad swelling respectively, at 6 weeks post-infection). However, mice immunized with BM-DCs pulsed with the polypeptides developed severe tissue destruction at 7 weeks post-infection (Fig. 5). Consequently, following the ARRIVE guidelines, mice of the control group as well as mice immunized with the polypeptides-pulsed BM-DCs, had to be sacrificed at 7 weeks post-infection.

4. Discussion

Leishmaniases are neglected, ill-controlled and emerging diseases [10]. One of the major obstacles in the design and formulation of an effective vaccine against leishmaniasis is the identification of appropriate antigens which exhibit immunomodulatory properties capable to activate both innate and specific cellular immune responses. Indeed, the effective protection against *Leishmania* requires the activation of T helper (Th) cells and cytotoxic T (CTL) cells by professional antigen presenting cells, in particular DCs [24].

In the present study, we evaluated the ability of recombinant LieIF to induce the maturation and activation of murine BM-DCs. The *ex vivo* effect of LieIF, led to a significant increase in the expression of co-stimulatory molecules CD40, CD80 and CD86. The novelty of this study is focused on the highlighted positive impact of recombinant LieIF especially in the expression of CD86 molecule. A similar study using the recombinant protein of *L. braziliensis* (LbeIF), having 98% identity with LieIF, showed that LbeIF induced a significant increase in the expression of CD80 in human macrophages and DCs, as well as the expression of CD40 in DCs, but not the expression of CD86 molecule [43]. This difference could be attributed to 8 amino acid substitutions between these two proteins. The relevance of CD40, CD80 and CD86 co-stimulatory molecules expression in anti-*Leishmania* immune responses was demonstrated in murine models [61–65] and in humans [66]. Thus, appropriate co-stimulation by CD40 is essential for resistance in *Leishmania*-infected mice, but an essential role for B7 co-stimulation is unclear [66]. The CD40–CD40L interaction is important for the induction of effective immune responses against infection with *L. major*. Indeed, CD40-deficient mice mounted an extremely polarized Th2 response to *L. major* and were unable to resolve the infection [62]. Moreover, blockade of CD86, but not CD80, has been shown to decrease parasite burden and production of Th2 cytokines, in *L. major*-infected susceptible mice [64]. Other studies have indicated that CD86 functions as the dominant co-stimulatory molecule in anti-*Leishmania* responses in both resistant and susceptible mice [65].

The potent immunomodulatory effect of LieIF on BM-DCs maturation was further confirmed by measuring the effect of known TLR agonists and comparing it to the one induced by LieIF. TLRs play important roles in the control of *Leishmania* infection [67]. Genetically resistant C57BL/6 mice deficient in the TLR adaptor molecule, myeloid differentiation factor 88 (MyD88), were highly susceptible to *L. major* infections [68]. Moreover, mice deficient in TLR4 were highly susceptible to *Leishmania* spp. infections and showed increased parasite load and footpad lesion as compared to wild type mice [69]. TLR9 is required for the induction of IL-12, a Th-1 type regulating cytokine, in BM-DCs infected by *L. major* parasites or exposed to genomic DNA prepared from *L. major* or *L. infantum*. It is also needed for the early IFN-γ expression and cytotoxicity of NK cells following infection with *L. major in vivo* [70]. Furthermore, myeloid DCs that rapidly internalized *in vitro Leishmania* parasite, produced IL-12 in the presence of TLR9

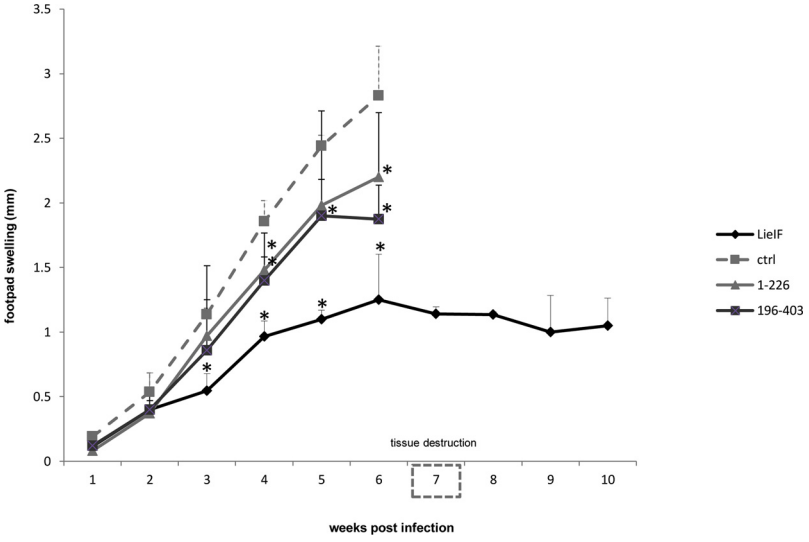

Fig. 5. Course of *L. major* infection in susceptible BALB/c vaccinated mice. Three groups of BALB/c mice were intravenously vaccinated with 10^6 antigen-pulsed BM-DCs (LieIF, polypeptide 1–226 and polypeptide 196–403). The negative control group was constituted of mice vaccinated with unpulsed BM-DCs. 2 weeks post vaccination, mice were subcutaneously infected with 2×10^6 *L. major* stationary phase promastigotes. Lesion progression was monitored weekly by measuring the footpad swelling. * indicates statistically significant differences as compared to the control group.

[71]. TLR3 is involved in the phagocytosis of *L. donovani* and in strengthening the leishmanicidal activity of macrophages [72]. Clearly, our results confirmed the immunomodulatory capacity of LieIF, since we have demonstrated that recombinant LieIF exhibited as potent effect on the maturation of DCs as the one observed by the ligands of TLR receptors 3, 4 and 9 (poly (I: C), LPS and CpG sequences, respectively).

Antigen-sensitized DCs secrete a range of cytokines [60], which also play a crucial role in priming T-cell responses [73]. Therefore, we tested the production of Th1-type (IL-12, IFN-γ) and Th2-type (IL-10 and IL-4) cytokines. It was shown that the production of IL-12 at the early stage of infections activates natural killer cells to produce IFN-γ and induced Th1 cell differentiation [21]. To our knowledge this is the first report describing IL-12 production by BM-DCs pulsed with LieIF. Studies confirmed the production of IL-12 by DCs or monocytes from healthy volunteers incubated with LbeIF or LieIF, respectively [34,43]. These two studies also showed that LbeIF and LieIF were able to induce the secretion of IL-10 by human dendritic cells and monocytes, respectively [34,43]. In our study, LieIF led to a moderate increase in the percentage of BM-DCs that produce IL-10. In addition, LieIF exhibited a very high IL-12/IL-10 ratio (as shown in Fig. 4A) supporting DCs differentiation into a DC1 subtype.

The determination of the smallest possible portion of a protein bearing immunomodulatory properties (such as expression of co-stimulatory molecules and induction of IL-12), is particularly important because it may mimic the natural course of infection with the subsequent degradation of the protein by intracellular pathways. Its use also excludes the sequences that can cause deterioration of the disease pathology [74]. Therefore, we tested the ability of eight overlapping recombinant polypeptides, covering the entire protein, to induce *in vitro* phenotypic maturation and functional modulation of BM-DCs. Our data showed that the majority of the recombinant polypeptides induced the increased expression of co-stimulatory molecules, as well as the increased production of IL-12. However, the fragments of the central region (129–226 and 129–261) did neither induce potent maturation of BM-DCs nor significant production of the Th1-type cytokine, IL-12. These data are consistent with what we have previously shown using monocytes from healthy subjects [34]. Indeed, we have previously reported that the fragments 1–195, 1–226 and 196–403, in contrast to the fragments of the central region 129–226 and 129–261, induced the production of IL-12p70 by human monocytes [34], suggesting that the smaller fragments 1–129 and 261–403 are important for the cytokine-inducing activity of LieIF. Furthermore, contrary to the results obtained with LbeIF [43], we demonstrated that LieIF does not contain polarity [34]. In argument with these results, our data showed that both the amino-terminal (1–226) and the carboxy-terminal regions (196–403) induce maturation of BM-DCs as well as significant production of IL-12.

Moreover, in the present study, we explored for the first time the *in vivo* efficacy of LieIF and its polypeptide-pulsed BM-DCs in a murine experimental model of cutaneous leishmaniasis. BM-DCs pulsed with the recombinant LieIF protein, but not with the fragments 1–226 or 196–403, induced partial protection against *L. major*. It is noteworthy that the protection level observed was obtained after a high challenge dose of parasites (2×10^6), while in general infectious challenges consisted in lower number of promastigote parasites (2×10^5) [53,75]. In a recent study, an alternative approach was used based on the use of monoclonal antibodies (mAbs) against receptors to deliver specific antigens to DCs *in situ*, within lymphoid tissues. It was shown that targeting the fragment 1–226 of LbeIF to DCs induced approximately 30–40% protection against infection with *L. major* [31]. The immunomodulatory properties of the LeIF protein were believed to be located in the N-terminal domain (1–226) [33]. The present study and our previous data [34] suggest that using the whole protein LieIF instead of the fragment 1–226 in such strategy could induce higher protection against *L. major* challenge.

5. Conclusions

In conclusion, all the results of the present study certify the potent immunomodulatory properties of LieIF protein and its poly-epitopic fragments, which led to further evaluation of their *in vivo* activity in an experimental mice model. Use of these molecularly defined antigens with strong immunomodulatory properties in DC-based interventions is a promising tool to induce effective immunity against leishmaniasis. The entire LieIF protein is a better vaccine candidate than the NH_2- or COOH- terminal parts of the protein.

Conflicts of interests

The authors declare that they have no competing interests.

Acknowledgments

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