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Optimized subunit vaccine protects against experimental leishmaniasis

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Abstract

Development of a protective subunit vaccine against *Leishmania* spp. depends on antigens and adjuvants that induce appropriate immune responses. We evaluated a second generation polypeptide antigen (Leish-110f) in different adjuvant formulations for immunogenicity and protective efficacy against *Leishmania* spp. challenges. Vaccine-induced protection was associated with antibody and T cell responses to Leish-110f. CD4 T cells were the source of IFN- γ , TNF, and IL-2 double and triple positive populations. This study establishes the immunogenicity and protective efficacy of the improved Leish-110f subunit vaccine antigen adjuvanted with natural (MPL-SE) or synthetic (EM005) Toll-like receptor 4 agonists.

Keywords

Leishmania; vaccination; MPL

1. Introduction

Leishmaniasis is a family of diseases caused by protozoan parasites of the genus *Leishmania*. Over 20 species and subspecies of *Leishmania* infect humans, each causing a different spectrum of diseases that can broadly be subdivided into cutaneous, mucosal, and visceral leishmaniasis. Infection occurs when the female sandfly vector, containing the flagellated metacyclic promastigote form of the parasite, delivers it into the dermis of the vertebrate host during ingestion of a blood-meal. *Leishmania* are obligatory intracellular parasites within the vertebrate host where they reside as non-motile amastigotes primarily within cells of the macrophage – dendritic cell lineage.

In human and experimental leishmaniasis, acquired resistance to *Leishmania* is mediated by T cells [1]. In experimental infection models of *L. major*, most mouse strains (C3H, C57BL/6, CBA/J, or B10D2) are resistant and normally develop a self-limiting skin ulcer, which heals spontaneously within 6–12 weeks post-infection with *L. major*. Mice of the resistant background typically develop a predominant T-helper type 1 (Th1) immune response. Acquired resistance in this model relies on activation of CD4 T cells, resulting in secretion of high levels of gamma interferon (IFN- γ) that induce nitric oxide-dependent parasite killing by infected macrophages [2–4]. CD8 T cells are also required for the control of primary infection

in the skin [5,6], and have been shown to confer resistance to secondary challenge [7–10]. By contrast, susceptible BALB/c mice develop a typical Th2 response associated with progressive local lesions and a systemic, visceralized disease [11]. The CD4 Th1/Th2 paradigm of resistance/susceptibility to intracellular infection is largely based on studies using *L. major* (reviewed by Sacks & Noben-Trauth [3]).

Susceptibility of BALB/c mice to *L. major* infection can be reversed by vaccination with crude preparations of leishmanial antigens (ALM, SLA), defined leishmanial molecules in the form of recombinant proteins (gp36, PSA-2, LACK, dp72, P0, CP, P4, P8, LCR1, A2, HASPB1, ORFF, Lip2a-Lip2b-P0-H2A, SMT, TSA, LmSTI1, LeIF, Leish-111f), or DNA (gp36, PSA-2, LACK, CP, A2, PapLe22, P0, P4, PRP-2, KMP-11, ORFF, NH36, LmSTI1, TSA), live vectors expressing leishmanial antigens (gp63, PSA-2, LACK, LCR1), or sandfly saliva components (maxadilan, SP15) [12–15]. Vaccines using attenuated parasites (infectious but not pathogenic), produced by long-term culture, irradiation, chemical mutagenesis, and more recently by gene deletion, were also shown to be protective in experimental leishmaniasis [12–15].

A practical vaccine for use in developing countries should be safe, effective, long lasting, and as inexpensive to produce as possible. A polyprotein vaccine consisting of a single fusion with multiple antigenic epitopes would be less costly to manufacture than a vaccine consisting of several recombinant proteins. For this reason, a polyprotein comprised of the three priority candidate antigens TSA, LmSTI1 and LeIF, fused in tandem, was made and referred to as Leish-111f. Recombinant Leish-111f adjuvanted with rIL-12 or with MPL[®], a detoxified 4'-monophosphoryl lipid A derivative of lipopolysaccharide (LPS) obtained from *Salmonella minnesota* in a stable oil-in-water emulsion (MPL-SE), was shown to be protective in mouse and hamster experimental models of leishmaniasis [16–18], and safe and immunogenic in humans (Piazza *et al.*, manuscript submitted for publication). In contrast, Leish-111f (also called MML) did not consistently protect dogs against visceral leishmaniasis [19–21]. For manufacturing and regulatory purposes, the Leish-111f polyprotein was modified in the following ways: The six-His sequence near the amino terminus was removed to eliminate a potential regulatory concern, and an apparent proteolytic hot spot was eliminated by replacing Lys₂₇₄ with Gln to potentially improve the manufacture of the fusion protein. The new 110kDa construct was named Leish-110f [22].

The other critical component of a subunit vaccine is the choice of adjuvant. Adjuvants are molecules, compounds, or macromolecular complexes that boost the potency and longevity of specific immune response to antigens, but cause minimal toxicity or long-lasting immune effects on their own [23–25]. Adjuvants can be used to enhance immunogenicity and modulate the type of immune responses. Many pathogen-derived molecules have pathogen-associated or danger-associated molecular patterns that stimulate innate immune responses via a Toll-like receptor (TLR) or NOD-like receptor (NLR) [26]. The TLR-4 ligand MPL[®] has been used as an adjuvant in several safety and immunogenicity human clinical trials, including vaccines for tuberculosis, malaria, hepatitis B, genital herpes, and allergy desensitization without any sign of systemic toxicity [19,27–45]. MPL[®]-SE is the current adjuvant used with the polyprotein Leish-111f and Leish-110f in clinical trials. MPL[®] is a natural product that requires rounds of purification and detoxification. Chemists at IDRI have developed defined formulations with a synthetic lipid A analog to MPL[®] that is more potent on human cells *in vitro* (Coler *et al.*, manuscript in preparation) and *in vivo* (Reed *et al.*, manuscript in preparation) and formulated the synthetic analog as a stable oil-in-water emulsion called EM005 [46].

In this study, the Leish-110f recombinant polyprotein was characterized by gel electrophoresis, reverse phase and LC-MS chromatography. The immunogenicity of Leish-110f alone or when used in combination with different adjuvants containing a natural or synthetic TLR-4 ligand

was characterized in BALB/c mice by following antigen-specific antibody and T cell responses. Five CD4 T cell-specific epitopes were identified in these analyses. Finally, the protective efficacy of the Leish-110f-containing vaccines was evaluated in mice against an *L. major* or an *L. infantum* challenge.

2. Materials and Methods

2.1. Gel electrophoresis, HPLC, LC-MS

The genes for each protein were isolated from *L. major* and fused together using recombinant DNA techniques [16]. Recombinant protein expression and purification were performed as described previously [22].

SDS-PAGE was performed according to the Laemmli method. Samples (5 µg) were diluted 1:1 in reducing 2× sample buffer, and boiled for 5 min. Proteins were then loaded onto a 4–20 % Tris-Glycine gel (Invitrogen) and run for 60 min at 30 mA. The gel was stained using GelCode® Blue Coomassie reagent (Pierce).

Aliquots of Leish-111f and Leish-110f were initially denatured with 4 M guanidine hydrochloride, followed by reduction using 25 mM dithiothreitol for 1 h at room temperature. Cysteine residues of the reduced proteins were alkylated with 10 mM iodoacetic acid for 1 h. The proteins were dialyzed into 1 M urea, 20 mM Tris pH 8 and digested for 4 h at 37° C with 1 µg of endoproteinase LysC per 250 µg of protein. The time course for this enzymatic digestion was determined in a prior experiment (data not shown). The peptide fragments produced with this digestion were separated by reversed phase chromatography using a 250 mm Vydac C18 column (W.R. Grace Corporation). Buffers were 0.5% TFA in water (buffer A) and 0.5% TFA in acetonitrile (buffer B). Digested protein (50 µg) was loaded on the column pre-equilibrated with 85% buffer A and 15% buffer B, and eluted with a 60 min linear gradient to 65% buffer B and 35% buffer A. Absorbance was measured at both 215 nm and 280 nm.

For peptide identification by Mass Spectroscopy (LC-MS), samples were run on an Agilent LC/MSD SL instrument. The HPLC gradient was the same as described above for the peptide mapping except that 0.1 % formic acid was substituted for the TFA. The ultraviolet signal was monitored at 280 and 215 nm. The peptides were identified by matching the ion mass/charge (m/Z) generated to predicted peptide fragment m/Z's using the protein sequence.

2.2. Immunization, challenge, & estimation of parasite loads

Female BALB/c and C57BL/6 mice, 5–7 week old, were purchased from Charles River (Wilmington, MA). All mice were maintained in the Infectious Disease Research Institute (IDRI) animal care facility under specific pathogen-free conditions and were treated in accordance with the regulations and guidelines of the IDRI Animal Care and Use Committee. Mice were immunized by the subcutaneous route (s.c.) three times, 2–3 wks apart with 0.5–10 µg protein in PBS and either 5 µg of EM005 (IDRI, Seattle) or 20 µg of MPL®-SE (GlaxoSmithKline Biologicals, Rixensart, Belgium) in a volume of 100 µl.

Three to five wks after the last immunization, groups of five BALB/c mice were inoculated with *L. major* metacyclic promastigotes (10^3) intradermally into the ear dermis using a 27.5-gauge needle in a volume of ~10 µl. *L. major* clone V1 (MHOM/IL/80/Friedlin) parasites were grown as previously described [47]. Infective-stage promastigotes (metacyclics) were isolated from stationary cultures (4–5 day-old) by negative selection using peanut agglutinin (Sigma). The evolution of the lesion was monitored by measuring the diameter of the ear lesion induration with direct-reading vernier calipers (Thomas Scientific, Swedesboro, NJ). Parasite titrations were performed on ear tissue homogenates obtained as previously described [5]. The number of viable parasites in each sample was determined by limiting dilution, using the

highest dilution at which promastigotes could be grown out after 7 days of incubation at 26° C.

C57BL/6 mice were challenged with 5×10^6 *L. infantum* promastigotes intravenously into the tail vein. Four weeks after the challenge, mice were sacrificed to harvest livers to determine the numbers of parasites in these tissues by limiting dilution, as previously described [48].

2.3. Antibody ELISA

Animals were bled 1 wk after each immunization, and serum IgG1 and IgG2a antibody titers were determined. Nunc-Immuno PolySorp plates were coated for 4 h at room temperature with 200 ng recombinant protein per well in 0.1 M bicarbonate buffer, blocked overnight at 4°C with PBS containing 0.05% Tween-20 and 1% BSA, washed with PBS Tween-20 (0.05%), incubated for 2 h at room temperature with sera at a 1:50 dilution and subsequent 5-fold serial dilutions, washed, and incubated for 1 h with HRP-conjugated goat anti-mouse IgG1 or anti-mouse IgG2a (1:2000, Southern Biotech) in PBS/Tween-20 0.05%/BSA 0.1%. Plates were washed and developed using SureBlue TMB substrate (KPL Inc., Gaithersburg, MD). The enzymatic reaction was stopped with 1N H₂SO₄, and plates were read within 30 min at 450 nm with a reference filter set at 650 nm using a microplate ELISA reader (Molecular Devices, Sunnyvale, CA) and SoftMax Pro5. Endpoint titers were determined with GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA) with a cutoff of 0.1.

2.4. Cytokine ELISA

One to three wks after the last immunization and 8 wks post-challenge, splenocytes from animals were plated in triplicate at $2\text{--}2.5 \times 10^5$ cells/well and cultured with medium, ConA (3 µg/ml), SLA (10 µg/ml), or each recombinant protein (10 µg/ml) for 72 h. Supernatants were harvested and analyzed for IFN-γ and IL-4 by a sandwich ELISA using specific mAb (eBioscience Inc., San Diego, CA) according to the manufacturer's protocol.

2.5. Flow cytometry

Splenocytes were isolated 1–3 wks after the last immunization or 8 wks post-challenge, plated at $1\text{--}2 \times 10^6$ cells/well in 96-well V bottom plates, and stimulated with anti-CD28/CD49d (1 µg/ml each, eBioscience) and recombinant proteins (20 µg/ml) for 6–12 h at 37°C in the presence of GolgiStop (eBioscience). The cells were then fixed for 10 min with cytofix/cytoperm (BD Biosciences, San Jose, CA), washed in PBS BSA 0.1%, incubated with Fc block (anti-CD16/CD32, eBioscience) for 15 min at 4°C, stained with fluorochrome-conjugated mAb anti-CD3, CD8, CD44, IFN-γ, TNF, IL-2, and CD4 (Caltag) in Perm/Wash buffer 1X (BD Biosciences) for 30 min at 4°C, washed twice in Perm/Wash buffer, suspended in PBS, and analyzed on a modified 3 laser LSRII flow cytometer (BD Biosciences). Viable lymphocytes were gated by forward and side scatter, and 50,000 CD3 events were acquired for each sample and analyzed with BD FACSDiva software v5.0.1 (BD Biosciences).

2.6. IFN-γ ELISPOT

MultiScreen 96-well filtration plates (Millipore, Bedford, MA) were coated with 10 µg/ml rat anti-mouse IFN-γ capture Ab (eBioscience) and incubated overnight at 4°C. Plates were washed with PBS, blocked with RPMI 1640 supplemented with 10% FBS for at least 1 h at room temperature, and washed again. Splenocytes or LN cell suspensions were plated at 2×10^5 cells/well and stimulated for 48 h at 37°C with medium, Con A (3 µg/ml), Leish-110f (10 µg/ml), or a peptide (1 µg/ml) from a library of 15-mer peptides overlapping by 10 aa and covering the entire sequence of Leish-111f (Mimotopes). The plates were subsequently washed with PBS and 0.1% Tween-20 and incubated for 2 h with a biotin-conjugated rat anti-mouse IFN-γ Ab (eBioscience) 5 µg/ml in PBS/0.5% BSA/0.1% Tween-20. The filters were

developed using the Vectastain ABC avidin peroxidase conjugate and Vectastain AEC substrate kits (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol. The reaction was stopped by washing the plates with deionized water, plates were dried in the dark, and the spots were counted on a automated ELISPOT reader (C.T.L. Serie3A Analyzer, Cellular Technology Ltd, Cleveland, OH) and analyzed with Immunospot® (CTL Analyzer LLC).

2.7. Statistical analysis

Student's *t*-test and standard 1-way ANOVA followed by Tukey's Multiple Comparison Test were used for statistical analysis; *P* values of 0.05 or less were considered significant.

3. Results

3.1. Optimized Leish-110f protein profile

A 111 kDa recombinant fusion protein (Leish-111f) consisting of three *Leishmania* proteins, thiol-specific antioxidant (TSA) [49], *L. major* stress-inducible protein 1 (LmSTI1) [50,51], and *Leishmania* eukaryotic initiation factor (LeIF) [52], was initially constructed [16]. Further modifications to the Leish-111f fusion protein were made to simplify manufacturing; process improvements included removal of the N-terminal six-His tag and the mutation of a proteolytic hot spot to reduce protein breakdown. The improved manufacturing method consisted of fermenting an engineered version of the HMS-174 strain of *E. coli* following current good manufacturing practice regulations at the 30-L scale. A purification scheme, combining anion exchange chromatography with hydroxyapatite chromatography, was developed that yielded purified protein product at greater than 100 mg/L [22]. The purified protein was formulated and lyophilized to yield a stable cake (> 3 years) that can safely be sent to clinical trial sites worldwide. The Leish-110f recombinant protein reported here is an improved version of the original leishmaniasis vaccine.

Recombinant Leish-110f protein was run in parallel with the original Leish-111f on a SDS-PAGE gel (Fig. 1A). Both Leish-110f (lanes 1, 2) and Leish-111f (lanes 3, 4) showed a major band above the 98 kDa standard marker (arrow). Western blot analysis confirmed that the major band and lower MW bands were product-related (data not shown). The removal of the six His from the amino terminus of the Leish-111f resulted in a new species (Leish-110f), which is about 1000 daltons less in molecular weight, and therefore runs slightly faster on the SDS-PAGE.

To confirm the identity of Leish-111f and Leish-110f, both proteins were denatured, reduced, and digested with endoproteinase LysC. Protein fragments were separated by reversed phase chromatography. Chromatograms of Leish-110f and Leish-111f showed similar profiles with many of the peaks co-eluting (Fig. 1B). The absence of the six-His repeat region in Leish-110f, and the loss of an endoproteinase LysC cleavage site due to the Lys→Gln mutation, account for the major differences between the Leish-110f and Leish-111f chromatograms as indicated by the arrows. The identity of the L110f protein was confirmed through the detection by LC-MS of six peptides from within the Leish-110f amino acid (aa) sequence (Fig. 1C, Table 1).

3.2. Humoral responses to Leish-110f

To identify possible differences in the immunogenicity of the two fusion proteins, experiments were conducted to compare antibody levels induced against Leish-110f and Leish-111f in the BALB/c mouse model. Mice were immunized three times 2 wks apart with saline, antigen alone, or antigen + MPL-SE. Antigen-specific IgG1 and IgG2a antibody endpoint titers were determined 1 wk after each immunization.

Three injections of 10 µg of fusion protein alone or of protein + MPL-SE induced measurable levels of IgG1 specific for the fusion protein and its three individual components in the pooled sera of three mice (Fig. 2A). In the absence of adjuvant, IgG1 endpoint titers after the third injection were $3.1 \log_{10}$ to LeIF, $5.0 \log_{10}$ to TSA, $6.7 \log_{10}$ to LmSTI1, and $6.8 \log_{10}$ to Leish-110f. IgG1 response patterns were similar between Leish-110f and Leish-111f-immunized mice with IgG1 titers to LeIF < TSA < LmSTI1 ≤ fusion protein. IgG1 titers to the fusion proteins and individual components were increased 10–1000-fold in mice injected with antigen + MPL-SE, with $6.4 \log_{10}$ to LeIF, $6.7 \log_{10}$ to TSA, $7.6 \log_{10}$ to LmSTI1, and $8.0 \log_{10}$ to Leish-110f. Adjuvanting the protein with MPL-SE was most effective at boosting IgG2a responses. IgG2a specific to the fusion proteins and the three components ranged from $5.3 - 10.2 \log_{10}$. Thus, adjuvanting the fusion protein with MPL-SE boosted IgG1 and IgG2a antigen-specific titers, and preserved the response hierarchy (LeIF ≤ TSA < LmSTI1 ≤ fusion protein). No IgG2a and background levels of IgG1 reactive with LeIF, TSA, and Leish-111f were observed in sera from mice injected with saline.

Kinetics of antibody responses to the immunizing antigens were determined by measuring IgG1 and IgG2a endpoint titers 1 wk after each injection. Mice receiving the protein alone showed titers still increasing after the third injection, with IgG1 predominating (Fig. 2B). In contrast, in the presence of MPL-SE, titers at day 7 were 2–4 $\log_{10}$ higher and started to plateau after the second injection, with similar levels of IgG2a and IgG1 produced. Leish-110f-specific IgG1 and IgG2a titers were significantly higher ($P < 0.01$) in mice immunized with Leish-110f + MPL-SE compared to Leish-110f at all time points examined, as determined with Student's *t*-Test. Antigen-specific IgG1 and IgG2a induced by immunizing mice with Leish-110f + MPL-SE and Leish-111f + MPL-SE were cross-reactive and equally recognized either protein (data not shown).

Finally, antibody responses induced by dose-ranging concentrations of Leish-110f + MPL-SE were determined. Both antigen-specific IgG1 and IgG2a titers were dose-dependent at all time points tested (Fig. 2C). At the highest antigen concentration (10 µg), two and three vaccine injections induced similar IgG1 and IgG2a titers. At lower protein concentrations (2, 0.5 µg), three vaccine injections induced higher antibody responses than two injections. In the presence of MPL-SE, IgG2a titers were higher at day 7, and similar to IgG1 titers at day 21 and 35 (Fig. 2D). In contrast, when Leish-110f was injected alone or with SE, two injections were required to detect antigen-specific IgG1 and IgG2a (Fig. 2C), and the response was strongly biased toward IgG1 (Fig. 2D). For any of the three antigen doses tested, Leish-110f adjuvanted with MPL-SE induced significantly higher ($P < 0.05$) Leish-110f-specific IgG2a titers two weeks after each injection compared to immunizing with Leish-110f or Leish-110f + SE, as determined with one-way ANOVA and Tukey's multiple comparison tests. Adjuvanting Leish-110f with SE or MPL-SE significantly increased IgG1 titers compared to Leish-110f alone at all time points tested, while MPL-SE conferred a significant advantage over SE after a single injection, but not at later time points.

3.3. T cell responses to Leish-110f

Experiments were conducted to compare T cell responses induced against Leish-110f and Leish-111f. BALB/c mice were immunized three times 2 wks apart with antigen alone, antigen + adjuvant, or saline as a negative control. Antigen-specific cytokine recall responses were determined seven days after the last injection.

Splenocytes from mice injected with antigen + MPL-SE showed high levels of IFN- γ . Responses to a fixed amount of stimulating antigen were dependent on the dose of immunizing antigen (Fig. 3A). In contrast, antigen alone and antigen + SE vaccination showed no IFN- γ recall responses, comparable to saline. Leish-110f and Leish-111f adjuvanted with MPL-SE showed similar responses ($P > 0.05$) at all concentrations tested. IFN- γ recall responses of

splenocytes from mice injected with Leish-110f + MPL-SE were further characterized by testing the individual polypeptide components of the polyprotein. High levels of IFN- γ were observed in response to the Leish-110f and Leish-111f fusion proteins and to LmSTI1 and low and no recall responses were seen to TSA and LeIF, respectively, at the antigen concentrations tested (Fig. 3B), suggesting that most of the response is directed at epitopes within LmSTI1. Leish-110f and Leish-111f were recognized similarly by vaccine-primed T cells.

To obtain a more detailed comparison of the cytokine expression profiles resulting from use of the two vaccines, CD4 T cell cytokine expression following antigenic stimulation was determined at the single cell level by intracellular cytokine staining (ICS) and flow cytometry using a modified 3-laser LSRII FACS analyzer. Expression of IFN- γ , TNF, and/or IL-2 by CD4/CD44^{high} T cells was determined seven days after the last immunization. Mice injected with saline, Leish-110f, or Leish-110f + SE showed low frequencies of TNF⁺, and no IL-2⁺ or IFN- γ ⁺ CD4 T cells in response to *in vitro* antigenic stimulation (Fig. 3C). In contrast, mice immunized with Leish-110f in MPL-SE showed a dose-dependent increase in the percent of CD4 T cells expressing any one of the three cytokines in the following order: TNF > IFN- γ > IL-2. The cytokine profile and relatively high % of CD4 T cells responding to Leish-110f stimulation suggest a Th1 effector phenotype, which can be expected one week post vaccination. The proportion of CD4/CD44^{high} T cells expressing one, two or the three cytokines was also determined (Fig. 3D). CD4/CD44^{high} T cells from mice injected with saline, Leish-110f in saline, or Leish-110f + SE were mainly 1⁺ (92%, 92%, 94%, respectively) with the majority of the cells expressing only TNF (81%, 84%, 90%, respectively; data not shown). In contrast, at equal dose of antigen (10 μ g), CD4 T cells from animals that received the MPL-SE containing vaccines were mainly 2⁺ (26%), with 69% being IFN- γ /TNF double positives, and 3⁺ (43%). The increase in frequencies of IFN- γ /TNF double-positives and IFN- γ /TNF/IL-2 triple-positive CD4 T cells was also antigen dose-dependent (0.5 < 2 < 10 μ g Leish-110f). Similar response profiles were observed for CD4/CD44^{high} T cells obtained from mice immunized with Leish-111f, Leish-111f + SE, and Leish-111f + MPL-SE (data not shown).

3.4. Leish-110f epitope mapping

We further characterized epitopes recognized by Leish-110f-specific T cells. Splenocytes from mice immunized with Leish-111f + MPL-SE were cultured with 243 15-mer overlapping peptides (offset by 5 aa) covering the entire Leish-111f sequence. The frequency of IFN- γ ⁺ cells was determined by ELISPOT, and those responses above media + 3 SD were considered positive. Nineteen groups of 1–4 overlapping peptides induced positive IFN- γ responses (data not shown). These peptides also induced IFN- γ recall responses by splenocytes from mice injected with Leish-110f + MPL-SE (data not shown). The five 15-mer peptides inducing the strongest IFN- γ recall responses were then assessed by flow cytometry for their recognition by CD4 and/or CD8 T cells from mice immunized with Leish-110f + MPL-SE (Table 2). All five peptides activated CD4, but not CD8 T cells, suggesting the presentation of MHC class II, but not MHC class I-restricted epitopes. In addition, 15-aa sequences associated with positive IFN- γ recall responses were analyzed using algorithms (SYFPEITHI, RANKPEP) predicting H-2^d (K^d, D^d, L^d) binding motifs, and corresponding 9-mer peptides were synthesized (ProImmune) and tested by ELISPOT. None of the predicted 9-mer peptides induced IFN- γ responses (data not shown). Peptides 14–28 and 94–108 were located within TSA, and peptides 262–276, 546–560, and 626–640 within LmSTI1. No T cell epitope was identified within LeIF.

3.5. Protection against *Leishmania* spp. challenge

To be considered equivalent vaccine antigens, it is essential that Leish-111f and Leish-110f-containing vaccines confer equivalent levels of protection against parasite challenge. Therefore, mice were vaccinated as described above with Leish-110f adjuvanted with MPL-

SE, Leish-111f + MPL-SE, or saline (control) and challenged with *L. major* 3 wks after the last injection with a low dose of metacyclic promastigotes in the pinna of the ear. The site of infection was monitored, and lesion diameters measured weekly. Four wks post-challenge, mice in the saline control group started to develop lesions that grew bigger over time (Fig. 4A). In contrast, mice injected with either Leish-110f + MPL-SE or Leish-111f + MPL-SE did not develop lesions at the site of infection. No differences in efficacy, as determined by prevention of lesion development, were observed between the two vaccines.

Eight wks post-challenge, parasite burdens were determined from 2-fold serial dilutions of ear homogenates. Mice in the saline control group showed $7.2 \pm 0.1 \log_{10}$ parasites in ear tissues, while both groups of mice vaccinated with Leish-110f + MPL-SE or Leish-111f + MPL-SE showed $1.9 \pm 0.4 \log_{10}$ parasites (Fig. 4B). Reduction in parasite burdens in the vaccinated groups were associated with high IFN- γ (antigen > ConA) and low IL-4 (antigen < ConA) antigen-specific recall responses by the in vitro stimulated post-infection splenocytes (data not shown). In addition, a higher frequency of Leish-110f-specific CD4 T cells expressing IFN- γ , TNF, and/or IL-2 was observed in the vaccinated group compared to saline controls (Fig. 4C). More CD4 T cells staining positive for the three cytokines were observed in the vaccinated groups compared to saline controls. Cytokine expression profiles were similar for Leish-110f and Leish-111f.

Leish-111f formulated with MPL-SE previously showed some protective efficacy in a mouse model of visceral leishmaniasis. Therefore, we also examined the efficacy of the new Leish-110f vaccine in this model and found that mice immunized with Leish-110f + MPL-SE also showed decreased liver parasite burden after challenge with *L. infantum*, compared to animals injected with MPL-SE alone, or saline (Fig. 4D). The differences in hepatic parasite burden between the vaccine group and the saline and adjuvant alone control groups were statistically significant ($P < 0.01$ and $P < 0.05$, respectively).

Protection against *L. major* infection with Leish-110f formulated in EM005—

EM005 is IDRI's proprietary oil-in-water stable emulsion containing a synthetic form of the TLR-4 ligand Lipid A [46]. The cell-free synthesis of Lipid A may provide alternative, lot-to-lot consistent and cost-effective means of manufacturing TLR-4 agonists for vaccines. Furthermore, Leish-110f + EM005 make a defined second generation leishmaniasis vaccine.

The protective efficacies of Leish-110f administered with EM005 or MPL-SE were compared in the mouse CL model. Mice were immunized three times 2 wks apart with saline, Leish-110f alone, or Leish-110f adjuvanted with SE, EM005, or MPL-SE in experiments designed to compare antigen-specific antibody titers and T cell responses.

Leish-110f-specific IgG1 and IgG2a antibody endpoint titers were determined one week after each immunization. Mice receiving protein alone or protein adjuvanted with SE showed antibody titers still increasing after the third injection (Fig. 5A). In the presence of EM005 or MPL-SE, titers at day 7 were $3 - 4 \log_{10}$ higher and reached a plateau after the second injection, with higher levels of IgG2a than IgG1 (Fig. 5B). In contrast, when Leish-110f was injected alone or with SE, the antibody response was strongly biased toward IgG1. Adjuvanting Leish-110f with either EM005 or MPL-SE resulted in increased frequencies of CD4 T cells positive with IL-2 > IFN- γ = TNF (Fig. 5C) when measured 3 wks after the last immunization. The cytokine profile and low % of CD4 T cells responding to Leish-110f stimulation at this time point suggest a Th1 memory phenotype. The proportion of CD4/CD44^{high} T cells expressing one, two or the three cytokines was further determined (Fig. 5D). CD4/CD44^{high} T cells from saline, Leish-110f, and Leish-110f + SE injected mice were mainly 1⁺ (93%, 89%, and 81%, respectively), while sizable frequencies of 2⁺ and 3⁺ CD4 T cells were observed in

animals that received Leish-110f + EM005 (26% and 21%, respectively) or Leish-110f + MPL-SE (27% and 10%, respectively).

Finally, Leish-110f adjuvanted with EM005 was tested for efficacy against an *L. major* challenge. Mice were vaccinated as described above and, 5 wks after the last injection, were infected with a low dose of metacyclic promastigotes in the pinna of the ear. Uncontrolled lesions developed rapidly in saline, antigen alone, and antigen + SE groups (Fig. 5E). By five weeks post-infection, mice injected with Leish-110f + EM005 or Leish-110f + MPL-SE had developed significantly smaller lesions ($P < 0.01$) and controlled the infection. The difference in lesion size in mice vaccinated with Leish-110f + MPL-SE in Fig. 4A and 5E is within experimental variation and is likely caused by a difference in parasite virulence.

4. Discussion

With the goal of producing a polyprotein vaccine that meets regulatory standards, we engineered an improved Leish-111f fusion protein, referred to as Leish-110f, by removing the six-His tag and mutating a proteolytic hotspot. Apart from these two changes, Leish-110f consists of the same amino acid sequence as Leish-111f. When adjuvanted with MPL-SE, Leish-110f induced antigen-specific IgG1, IgG2a and CD4 T cells comparable to Leish-111f, associated with a predominant elevation in IgG2a versus IgG1 antibodies, and expansion of TNF, IFN- γ , TNF/IFN- γ double-positive, and TNF/IFN- γ /IL-2 triple-positive CD4 T cells, characteristic of Th1 responses. Five 15-mer peptides, associated with the strongest IFN- γ recall responses were characterized and identified as CD4 T cell-specific. Finally, Leish-110f adjuvanted with oil-in-water stable emulsions containing a chemically synthesized (EM005) or detoxified natural (MPL) Lipid A retained protective efficacy against *Leishmania* spp. challenges. Leish-110f + EM005 is the first defined second generation vaccine against *Leishmania*.

Historically, both recovery from natural infection and leishmanization (deliberate injection of live parasites) have induced the best protection against leishmaniasis. Several problems, however, have precluded the widespread use of leishmanization to prevent cutaneous leishmaniasis, including difficulty in standardizing the virulence of the vaccine and occasional severe and persistent lesions resulting from the inoculum [12]. In contrast to leishmanization, the first generation vaccines using immunization with crude parasite preparations of killed *Leishmania* stocks resulted in variable protection ranging from good to no protection at all [12]. Second generation vaccines should be delivered as a single, defined molecule to facilitate compliance with regulatory and manufacturing standards and to lower the overall production costs. Ideally, the vaccine should protect against multiple forms of leishmaniasis. We developed a promising second generation vaccine, Leish-111f (TSA-LmSTI1-LeIF) and assessed its efficacy in preclinical [12,53], and multiple Phase Ia and Ib clinical trials for safety and immunogenicity responses (F. Piazza, manuscript submitted for publication). We further improved the Leish-111f manufacturing process [22] by removing the N-terminal six-His tag and inserting a point mutation in a proteolytic hotspot.

Amino acid modifications to Leish-111f did not alter its immunogenicity as shown by high antigen-specific humoral and cellular responses. In addition, Leish-110f-specific IgG1, IgG2a and CD4 T cells also recognized the single components, and were cross-reactive with Leish-111f, further confirming the similarity of the two fusion proteins. Altogether, the immunogenicity and protective efficacy of Leish-110f + MPL-SE were comparable to those obtained with Leish-111f + MPL-SE against *L. major* [16,17] and *L. infantum* [18] challenges, suggesting that Leish-110f might be broadly protective against both cutaneous and visceral leishmaniasis. Combination therapy with Glucantime and Leish-110f + MPL-SE has recently been shown to be effective for treating dogs against visceral leishmaniasis [21]. While in our

hands, both Leish-111f and Leish-110f in MPL-SE have shown efficacy as immunotherapeutics as adjunct to standard chemotherapy [21] and as standalone treatments (Trigo *et al.*, manuscript in preparation), others reported failure of the MML + MPL-SE vaccine to prevent disease progression in dogs [19,20]. Differences in dose, schedule of administration, and source of the vaccine might account for these discrepancies. Gradoni *et al.* used an antigen preparation from Novartis Animal Vaccines Ltd.; however, they did not show evidence of MML identity and integrity, which raises the possibility of antigen aggregation and/or degradation that is often seen with MML and that prompted us to develop Leish-110f. In addition, doses of MML (45 µg) and MPL-SE (50 µg) are much higher than what we typically use (10 µg of antigen in 25 µg of adjuvant). Their higher doses may have resulted in the induction of terminal effector T cells, as suggested by the weak PBMC proliferative response to MML observed two months after the last immunization [19,20]. It was recently reported by Aagaard *et al.* that lowering the dose of antigen resulted in enhanced protection in animal models of tuberculosis [54]. Finally, using a vaccine as a standalone immunotherapeutic might require a more aggressive regimen of weekly injections for prolonged periods, as indicated by our recent studies (Trigo *et al.*, manuscript in preparation).

Leish-110f in aqueous or stable oil emulsions (SE) lacking the TLR-4 ligand induced predominantly IgG1, little IgG2a, and little to no IFN-γ recall responses, consistent with a Th2 phenotype. There was a predictable lack of protection against *Leishmania* infection for these formulations. Inclusion of the TLR-4 ligand MPL in the adjuvant formulation resulted in a switch of Leish-110f-specific immune responses to a Th1 phenotype, characterized by 1000-fold more IgG2a and high levels of IFN-γ. We have previously reported that TLR4-containing adjuvant formulations tend to elicit comparable IgG2a and IgG1 titers after three injections [55], unlike such TLR9 agonists as CpG that favor greater IgG2a titers compared to IgG1 [55,56]. Leish-110f-specific pluripotent CD4 T cells were associated with the protective efficacy of the vaccines, as previously described for Leish-111f [18,57]. Interestingly, we could not detect a significant percentage of granzyme B, IFN-γ and/or TNF positive Leish-110f-specific CD8 T cells by ICS (data not shown), suggesting that the protection associated with Leish-110f + MPL-SE in BALB/c mice depends largely on the priming of a potent CD4 Th1 response. Interestingly, we observed that, post-challenge, protected mice harbored CD8 T cells that produced IFN-γ and/or TNF upon in vitro stimulation with soluble *Leishmania* antigen (data not shown). This suggests that vaccination with Leish-110f + MPL-SE or Leish-110f + EM005 elicited an immune response sufficient to control parasite multiplication upon primary infection and prevented disease, while natural priming of CD8 T cells occurred during infection. Therefore, we hypothesize that vaccine-induced CD4 and naturally acquired CD8 T cells will play a key role in long-term recall responses. Finally, the identification of five CD4 T cell-specific 15-mer peptides within the Leish-110f amino acid sequence provides an important tool to track and further characterize vaccine-induced T cells.

In this study, the EM005 formulation, which contains synthetic Lipid A, showed adjuvant activity similar to or better than MPL-SE. Enhanced immunogenicity of a protein antigen with EM005 and protection against pathogen infection extends recent observations made in mouse models of tuberculosis [55] and influenza [58]. IDRI's synthetic Lipid A is homogenous, well-characterized, and is cheaper to manufacture compared to available purified biological products.

In conclusion, our modifications to the original recombinant Leish-111f have made a more manufacturable product that has comparable immunogenicity and protective efficacy. By providing a synthetic adjuvant that also maintains high levels of protection, we are making a defined vaccine in the hope of creating a commercial product capable of protecting against numerous types of leishmaniasis.

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Abbreviations used in this paper

Lm	Leishmania major
MPL	monophosphoryl lipid A
SE	stable emulsion
TLR	Toll-like receptor

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Figure 1.

Leish-111f and Leish-110f protein profiles were compared. (A) SDS-PAGE gel of Leish-110f (lane 1–2) and Leish-111f (lane 3–4), 5 μ g of proteins were loaded in duplicates onto a 4 – 20 % Tris-Glycine gel. (B) Reverse phase chromatography profiles. Arrows indicate major differences between Leish-110f and Leish-111f profiles. (C) Schematic diagram of the Leish-110f fusion and location of the six peptides (I – VI) identified by LC-MS.

Figure 2.

Humoral responses elicited by the optimized antigen were determined. The immunogenicity of Leish-111f and Leish-110f adjuvanted with -SE and MPL-SE were compared. Mice were injected s.c. three times 2 wks apart with saline, antigen alone (10 μ g), or three different amounts of antigen (0.5, 2, 10 μ g) + MPL-SE (20 μ g MPL). IgG1 and IgG2a endpoint titers (Log₁₀) were determined on pooled sera from three animals. (A) Ab titers to the fusion protein and each of its components determined 1 wk after the third injection, (B) Ab titers to the corresponding antigen measured 1 wk after each injection, (C) Ab titers to Leish-110f induced by increasing concentrations of vaccines, and (D) IgG2a:IgG1 ratios (Log₁₀). ND, not detected. Data shown are representative of two independent experiments.

Figure 3.

T cell responses to the optimized antigen were determined seven days after the last immunization. The immunogenicity of different doses of Leish-110f and Leish-111f adjuvanted with -SE and MPL-SE was compared. Mice were injected s.c. three times 2 wks apart with saline, antigen alone (10 μ g), antigen (10 μ g) + SE, or antigen (0.5, 2, and 10 μ g) + MPL-SE (20 μ g MPL). (A) IFN- γ cytokine recall responses to the corresponding fusion protein. (B) IFN- γ recall responses to fusion proteins and individual components from mice injected with Leish-110f (10 μ g) + MPL-SE. Mean $\pm$ SEM (n = 3 mice) are shown. (C–D) CD4 T cells were identified by ICS based on CD3 and CD4 expression, and further gated as CD44^{high}. (C) Percent of cells expressing IFN- γ , TNF, and IL-2 in response to Leish-110f in vitro

stimulation. Mean $\pm$ SD (n = 3 mice) are shown. **(D)** Proportion of cells expressing one, two or the three cytokines. Data shown are representative of two independent experiments.

Figure 4.

Protection against *Leishmania* spp was evaluated in vaccinated mice. The efficacy of Leish-111f and Leish-110f adjuvanted with MPL-SE were compared. Mice were injected s.c. three times 3 wks apart with saline, adjuvant, and antigen + MPL-SE. Three wks after the last boost, mice were challenged i.d. with 10^3 *L. major* V1 (A–C) or i.v. with 5×10^6 *L. infantum* (D) promastigotes. (A) Lesion sizes $\pm$ SEM. (B) Parasite burden in the ear determined 8 wks post-infection with *L. major*. (C) Percent CD4 T cells expressing IFN- γ , TNF, and IL-2 in response to Leish-110f stimulation were determined. Data represent the Mean $\pm$ SEM (n = 5 mice) in a representative experiment. (D) Parasite burden in the liver determined 4 wks post-

infection with *L. infantum*. One way ANOVA followed by Tukey's Multiple Comparison Test were used for statistical analysis.

Figure 5.

The immunogenicity and protection efficacy of the novel adjuvant formulation EM005 were determined. Mice were injected s.c. three times 2 wks apart with saline, Leish-110f alone, or Leish-110f adjuvanted with SE, EM005, or MPL-SE. **(A)** IgG1 and IgG2a endpoint titers (Log₁₀) titers to Leish-110f were measured one wk after each injection. **(B)** IgG2a:IgG1 ratios (Log₁₀). ND, not detected. **(C)** Three wks after the last immunization, CD4 T cells were identified by ICS based on CD3 and CD4 expression, and further gated as CD44^{high}. Percent of cells expressing IFN-γ, TNF, and IL-2 in response to Leish-110f stimulation were determined. Mean ± SD (n = 3 mice) are shown for a representative experiment. **(D)** Proportion of cells expressing one, two or the three cytokines. **(E)** Five wks after the last boost, mice were challenged i.d. with 10³ *L. major* V1 promastigotes. Lesion sizes ± SEM are shown for a representative experiment. **P* < 0.01.

Table 1

Leish-110f peptides identified by LC-MS

Peptide#	Position	Size	Sequence
I	210	260.3	NK
II	414	405.2	EEK
III	435	622.0	LYLSK
IV	549	1447.6	AVEEAYIDPEIAK
V	616	461.5	DAEK
VI	735	374.4	INK

Table 2

Leish-110f T cell epitope mapping

Position		Sequence (aa)	IFN- γ^+ T cells (%)	
			CD4	CD8
14–28	TSA	SFEEVALMPNGSFKK	^a 2.8 $\pm$ 0.7	0.2 $\pm$ 0.1
94–108	TSA	KGGLGTMAIPMLADK	5.2 $\pm$ 0.3	0.2 $\pm$ 0.1
262–276	LmSTII	DKCISIKPNWAKGYV	3.5 $\pm$ 0.4	0.3 $\pm$ 0.2
546–560	LmSTII	QKAVEEAYIDPEIAK	5.1 $\pm$ 0.0	1.0 $\pm$ 0.8
626–640	LmSTII	FVKGYARKGHAYFWT	6.7 $\pm$ 0.1	0.8 $\pm$ 0.5
Leish-110f	-	-	13.0 $\pm$ 0.0	0.5 $\pm$ 0.2

Splenocytes from mice immunized with Leish-110f + MPL-SE were plated at 2×10^6 cells/well and cultured with the 15-mer peptides (1 μ g/ml), or Leish-110f (20 μ g/ml) for 12 h in the presence of GolgiStop. Activated T cells were identified by ICS based on CD3 expression, and further gated as CD4 or CD8. The percent of cells expressing IFN- γ was determined.

^aMean $\pm$ SD (n = 3 mice) are shown.