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Second-generation vaccines against leishmaniasis

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Several species of *Leishmania* cause human diseases that range from self-healing cutaneous lesions to fatal visceral leishmaniasis, mucosal leishmaniasis and diffuse cutaneous leishmaniasis. Drug resistance and toxicities associated with chemotherapy emphasize the need for a safe, effective vaccine. Studies of the immunopathogenesis and mechanisms of protective immunity define several features that should be met by an effective vaccine. The leishmaniases are unique among parasitic diseases because a single vaccine has the potential to protect against more than one species (disease) and be successful at both treating and preventing disease. In addition, several antigens have been identified and characterized that might be potential vaccine candidates. In this article, we focus on advances made with second-generation vaccines against leishmaniasis.

Immunology of leishmaniasis

Leishmaniasis is caused by several species of protozoan parasites that are transmitted by the bite of the female phlebotomine sand fly. Leishmaniasis is currently endemic in 88 countries, and is a threat to 350 million people with a worldwide prevalence of 12 million cases.

Acquired resistance to leishmaniasis is mediated by T cells [1]. T-cell-deficient mice succumb rapidly after infection with *Leishmania* and adoptive transfer of normal T cells confers resistance to these animals. Moreover, patients with AIDS are highly susceptible to leishmaniasis either as a result of concurrent infection or reactivation of older, sub-clinical infection [2]. Among T cells, CD4+ T cells are crucial for resistance, whereas CD8+ T cells participate more in the memory events of the immune response than as effector cells involved in parasite elimination. More recently, a role for CD4+ CD25+ regulatory T cells has been identified in the persistence of *Leishmania major* infections [3].

In humans, there is a good correlation between T helper 1 cell (Th1) responses and resistance to cutaneous leishmaniasis (CL). Generally, a predominance of cells that produce interferon γ (IFN-γ) occurs in healing, cutaneous lesions, whereas in chronic cutaneous and mucosal lesions there is a mixture of Th1 and Th2 cytokines with an abundance of interleukin 4 (IL-4) and IL-10 [4]. In visceral leishmaniasis (VL) however, no association between increased IL-4 and active disease has been identified. In the spleen, mRNAs that encode IFN-γ and IL-4 are elevated during active disease and decline significantly after cure. The same cytokine profile occurs after antigenic stimulation of peripheral blood monocytes from patients [5]. However, a direct correlation between production of IL-10 and active disease is reported in patients with VL [6].

Innate immunity has an important role in the control of *Leishmania* infections. To respond rapidly to organisms that have not been encountered previously, a highly effective system of innate immunity has developed that enables pattern-recognition receptors to respond to conserved, pathogen-associated molecular patterns. Toll-like receptors (TLRs) have a central role in orchestrating the type and strength of this response. Recently, important immunomodulatory roles of natural killer cells [7,8], IL-1α and myeloid differentiation factor 88 [9] have been identified in early resistance to infection, development of acquired immunity and the pathology observed during infection with *Leishmania* parasites.

Vaccine studies

Vaccination against human cutaneous leishmaniasis has been practiced for centuries. Deliberate inoculation of virulent organisms from the pus of an active lesion is an ancient practice [10]. Promastigotes of *L. major* grown in culture were first used in Russia in 1937 to induce protection against natural infection [11]. More recently, standardized inoculums of culture promastigotes have been developed by Israeli scientists and used in several trials [11]. This process, known as 'leishmanization', is still used in some countries, notably Uzbekistan [12]. Leishmanization been is efficacious against Old World CL [13]. However, several basic, logistical problems preclude the widespread use of this procedure to prevent CL, including difficulty in standardizing the virulence of the vaccine and occasional severe, persistent lesions resulting from the innoculum [14]. Vaccination using a crude antigen preparation obtained from promastigote forms of various species of *Leishmania*, either with or without BCG (bacillus of Calmette and Guerin) as adjuvant, has been tested against CL and VL in human clinical trials in both the Old and New World. Generally, there is no evidence

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that two injections of autoclaved *L. major* results in significant protection compared with BCG alone [15].

Vaccination trials that demonstrate that either a cocktail of five, killed *Leishmania* stocks or a single strain of *Leishmania amazonensis* induces significant protection from disease caused by natural infection [16], has led to the registration in Brazil of a vaccine for leishmaniasis. These studies also indicate that delayed-type hypersensitivity (DTH) conversion might be used as a surrogate marker for protective immunity.

Overall, the results from several clinical trials using whole parasite antigens vary from 0–75% efficacy against CL and little (<6%) or no protection against VL [13–15,17–19]. Although these crude vaccine approaches are not ideal, they confirm some of the animal data that indicates that induction of protection against leishmaniasis is feasible and can be achieved with either a viable vaccine or with parasite components.

It is certain that a consistently effective, stable and reliable source of vaccine against leishmaniasis is unlikely to arise from whole-parasite approaches. The manufacturing, quality control, potency and stability issues are likely to be insurmountable for any but extremely local situations with relaxed regulatory requirements. Indeed, the variability seen with the whole-parasite-vaccine trials is reminiscent of the experience with BCG. Efforts must be made to produce one or more consistent, safe and effective vaccines against leishmaniasis. Although several new approaches used in modern vaccinology are being investigated in models of experimental leishmaniasis, only one defined second-generation vaccine has reached clinical trials in humans [20].

Second-generation vaccine development

An ideal vaccine against leishmaniasis should have several properties, including (i) it must be safe; (ii) a minimum number of immunizations must induce long-term protection against most or all human pathogens that cause leishmaniasis; (iii) it must be free of animal products that are used to manufacture the product; (iv) it must be produced as cost-effectively as possible; and (v) it should be effective in both treating and preventing leishmaniasis. Other attributes (e.g. a needle-free, single-dose vaccine) could also be listed. To develop such a vaccine, it is essential to characterize protective antigens and to deliver them in creative systems that are optimized to meet both scientific and regulatory standards.

Candidates for second-generation vaccines

From experimental vaccine studies in the mouse model it is evident that solid protection against either syringe or sand-fly challenge can be achieved with defined proteins. It is also clear that not all antigens protect against leishmaniasis in the mouse model, which has enabled the selection of a few antigens as candidate vaccines. Several *Leishmania* proteins have been identified, based on abundance and surface localization [21], T-cell clones, screening of antigen pools [22] and screening expression libraries with sera from infected animals and humans [23–30]. These include glycoprotein 63 (gp63) [31], membrane glycoprotein 46 (gp46, also known as M-2) [32], *Leishmania* homolog of receptors activated for C kinase (p36/LACK) [33], cysteine proteinase (CP)B and CPA [34], LD1 antigens [35], hydrophilic acylated surface protein B1 (HASPB1) [36], LCR1 [37], salivary protein 15 (SP15) [38], M-2 [39], promastigote surface antigen 2 (PSA-2) [40], histone H1 [41], *Leishmania* elongation and initiation factor (LeIF) [42], *L. major* homolog of the eukaryotic stress-inducible protein-1 (LmSTI1) [30], and the *L. major* homologue of the eukaryotic thiol-specific-antioxidant (TSA) [43].

The ideal vaccine is a pan-*Leishmania* vaccine that includes several molecules that are, preferably, conserved among different species and expressed abundantly on the tissue amastigote stage. Many of the antigens mentioned above meet these criteria, but few protect against more than one species in animal models. Apart from protection, another important consideration is safety. Many of the protective antigens of *Leishmania* are highly conserved with mammalian proteins, so steps must be taken to ensure that anti-host responses are not induced, particularly with prophylactic application.

Adjuvant and delivery selection

Most vaccine studies aim to limit parasite replication in the vertebrate host. Over the past decade several investigators have searched for genes encoding leishmanial proteins that induce protection against CL and VL in experimental models [22,29,33,35,36,42–47]. However, identifying candidate antigens is not enough. Appropriate antigen delivery to induce the right type of immune response against leishmaniasis (i.e. induction of a strong antigen-specific Th1 response) is another crucial component of an effective vaccine. The two adjuvants that are approved in human vaccines, alum and squalene, induce potent antibody responses but are poor inducers of antigen-specific Th1 responses. Several strategies, including IL-12, live vectors, naked DNA, DNA vaccines encapsulated in microspheres and oligonucleotides (CpG sequences), have been evaluated extensively in preclinical models.

Subcutaneous injection of IL-12 and soluble leishmanial antigens (SLAs), induces a strong, anti-SLA Th1 response with no detectable Th2 response to this antigen mix [48]. IL-12 has been used successfully as a Th1 adjuvant for antigens in both murine and non-human-primate models of several infectious diseases, including leishmaniasis [49–51]. One drawback of IL-12 is its inability to stimulate strong immunological memory to the immunizing antigen. Thus, vaccination of BALB/c mice with the leishmanial homologue of receptors for activated C kinase (LACK) protein mixed with IL-12 as adjuvant results in short-term protection against challenge with *L. major* [50]. By contrast, vaccination with either LACK DNA or LACK protein and IL-12 DNA induces long-term protection [52].

Vaccines that encode gp63 and gp46 have also been tested as DNA vaccines, either alone or in combination, in BALB/c mice challenged with *Leishmania mexicana* [53]. These studies demonstrate that mice immunized with a combination of high-dose plasmids (50 μg of each antigen) have higher levels of protection than mice immunized

with either individual plasmids or low-dose plasmids (20 μg of each plasmid) [53].

The A2 genes are amastigote-stage-specific, form part of a multigene family of at least 11 genes and are considered to be virulence factors that are required for the survival of *Leishmania* parasites in the mammalian host. A2 has been tested both as a recombinant protein (with IL-12 as adjuvant) and as a DNA vaccine in the murine model of VL. These studies demonstrate that immunization of mice with A2 confers significant protection against challenge infection with *L. Donovani* [54].

DNA vaccines that encode histone H1, CPB and CPA, either alone or in combination, encapsulated in microspheres, have been also tested in mice. These studies demonstrate that CPA DNA is not protective, and that CPB DNA and a combination of both DNA vaccines are only partially protective for up to 14 weeks after challenge with *L. major* [53]. The addition of alum and IL-12 as adjuvant did not increase either the immunogenicity or protection of these DNA vaccines. Recombinant CPA and CPB proteins mixed with poloxamer as adjuvant have also been tested in the *L. major* mouse model of CL, demonstrating that CPA is not protective and that CPB elicits only partial protection. More recently, a construct has been developed in which the CPA and CPB genes are fused to give rise to a single hybrid protein. Protection studies in BALB/c mice indicate that the hybrid CPA/B elicits a protective immune response against *L. major* challenge. Similar studies using a cocktail of *Leishmania infantum* type I and II cysteine proteinases have been performed in mixed-breed dogs.

Vaccination studies using either recombinant histone H1 antigen or a long synthetic peptide representing the complete *L. major* histone H1 sequence, each formulated with Montanide ISA 721 as adjuvant, have also been performed in an African green monkey model of CL [53].

The fucose mannose ligand (FML) is a complex glycoprotein fraction that has been used to immunize both mice and dogs against *L. donovani* and *L. infantum*. Adjuvants used in combination with FML or the gp36 component of the FML antigen complex include alum, BCG, IL-12, saponin and QuilA. These studies indicate that vaccines that contain FML/gp36 provide significant protection (reduction in parasite burden and increased survival rates) in the murine model of *L. donovani*. Another study that has monitored naturally exposed dogs for 2 years demonstrates that this antigen induces significant protection against canine VL [53].

Several other adjuvant and delivery systems deserve attention. These include methods to deliver antigen such as vectored DNA (adenovirus and pox virus), peptides that target APC, water-in-oil emulsions (mineral oil and squalene), oil-in-water emulsions (MF59) and TLR agonists including monophosphoryl lipid A (MPL®) and CpG.

Until recently, a major hurdle in the development of vaccines against infectious diseases was the availability of safe, effective, T-cell adjuvants. Previously, only IL-12 with antigen afforded consistent prophylactic efficacy in animal models of CL. Given that IL-12 does not provide long-term immunity and is not being developed as an adjuvant for use in human vaccines, we have evaluated candidate leishmanial antigens in the presence of several adjuvants, including those that contain MPL® and might be more suitable for human use.

MPL® is a detoxified derivative of 4′-monophosphoryl lipid A of lipopolysaccharide (LPS) obtained from *Salmonella minnesota*. Several studies demonstrate that it is a potent immunostimulant through activation of TLR4 [55] that lacks the toxic properties of LPS. MPL® has been used as an adjuvant in several safety and immunogenicity human clinical trials, including vaccines for malaria, hepatitis B, genital herpes, allergy desensitization and human papilloma virus. These studies find that MPL® is well tolerated with no evidence of systemic toxicity and is likely to be included in vaccines for several human indications. MPL® is likely to be the first T-cell adjuvant to be approved for human use.

Thus, the use of MPL® as a vaccine adjuvant for leishmaniasis is a practical solution. In addition, MPL® is a logical adjuvant because it activates antigen-presenting cells through TLR-4, a receptor that contributes to the control of parasite growth in both the innate and acquired immune response to *Leishmania* infection [56].

Development of Leish-111f as a vaccine candidate

An ideal vaccine against leishmaniasis is unlikely to consist of a single antigen. Our approach (research and preclinical validation) is to select antigens with demonstrated ability to protect mice and non-human primates, and to test antigen combinations in both prophylactic and therapeutic models (Figure 1).

TSA

The *L. major* homologue of eukaryotic TSA was discovered by screening expression libraries [43] to characterize the immune responses elicited by proteins isolated from filtrates of *L. major* promastigote cultures. Immunizing BALB/c mice with recombinant TSA protein formulated with either IL-12 or TSA DNA results in the development of strong cellular immune responses and confers protective immune responses against infection with *L. major* [43,49,57].

LmSTI1

LmSTI1 was identified by screening an *L. major* amastigote cDNA library with sera from BALB/c mice infected with *L. major* [29]. Vaccination experiments with recombinant LmSTI1 protein plus either IL-12 or LmSTI1 DNA elicit a mixed cellular response that is skewed toward a Th1 phenotype, and protects BALB/c mice [29,30,49,57].

LeIF

LeIF was identified by screening a *Leishmania braziliensis* genomic library with sera from a patient with mucosal leishmaniasis (ML). LeIF stimulates the innate immune system to produce IL-12, IL-18 and IFN-γ, and, therefore, is a Th1 inducer. LeIF has immuno-therapeutic properties in mice [8,27,42,58].

The protective efficacy of LmSTI1 and TSA has also been tested in rhesus monkeys [49]. Although used less than the mouse, this model is accepted as a system that mirrors human immunity more closely [51,59]. Monkeys

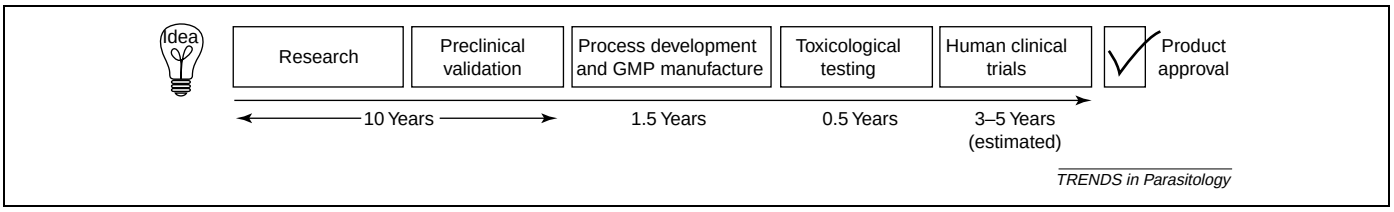

Figure 1. Proposed timelines for the development of a defined vaccine against human leishmaniasis.

immunized with a preparation containing LmSTI1 and TSA with the recombinant human IL-12 and alum as adjuvant mount excellent protection against challenge with *L. major* [49].

Recent studies using the mouse model point to high immunogenicity and protective efficacy of the antigens LmSTI1, TSA and LeIF formulated with the adjuvant MPL®-SE.

Constructing Leish-111f

The concept of producing a polyprotein containing several *Leishmania* antigens is based on the practical consideration of producing a multi-antigen vaccine as inexpensively as possible. For this reason, a polyprotein was made that comprises the three priority candidate antigens, TSA, LmSTI1 and LeIF, fused in tandem. This polyprotein is called Leish-111f (Figure 2). In prophylactic immunization studies, long-term protection in BALB/c mice is achieved using 2 μg of Leish-1111f. The most effective combination of Leish-111f, which is formulated with 20 μg of MPL®-SE, affords protection for >14 weeks [55,60]. No decrease in immunogenicity, diversity of epitope recognition and protection is noted with Leish-111f compared with protein mixtures. The Leish-111f– MPL®-SE vaccine has been evaluated extensively in preclinical safety and toxicology studies in five animal species, with no adverse effects observed.

Post-infection vaccine studies

Whole-parasite vaccines have been used therapeutically in leishmaniasis for decades, with mixed results. In general, this approach is applied to cases of drug-refractory disease. Overall, post-infection immunization is safe. We have reported that immunotherapy with recombinant leishmanial antigens can have therapeutic effects in humans with chemotherapy-refractive ML [61]. In these studies, patients were treated with a mixture of antigens including TSA, LmSTI1 and LeIF. The results of the study are impressive, with 10 out of 11 patients cured following re-treatment courses of immunotherapy.

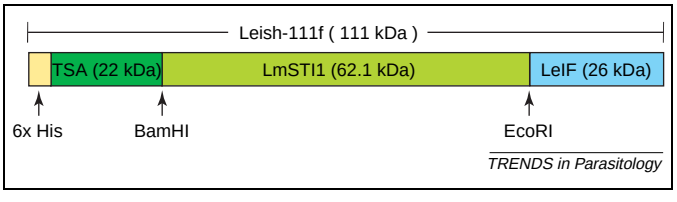

Figure 2. Generation of the recombinant Leish-111f construct. Leish-111f was generated by the sequential linkage in tandem of the open reading frames (ORFs) of the full-length sequences of TSA and LmSTI1, and the 26-kDa N-terminal portion of LeIF. This was accomplished using sequence-specific oligonucleotides that include unique restriction sites (BamHI and EcoRI) and that (in the case of TSA and LmSTI1) lack stop codons at the C termini to PCR amplify and link the ORFs. The final construct encodes a single polyprotein, Leish-111f. The size of the predicted ORF is 2946 nucleotides, which encodes 982 amino acids, with a predicted molecular weight of ~111 kDa.

Clinical development of Leish-111f in MPL®-SE

It is important to obtain safety and immunogenicity data in healthy volunteers who react positively and negatively in skin tests for *Leishmania* as a prelude to developing a prophylactic vaccine. Information on both antigen and adjuvant dose that induce optimal responses are important. The overall clinical development plan for Leish-111f–MPL®-SE is to perform safety and efficacy studies in therapeutic and prophylactic applications in parallel. Detailed analyses of the immune response will be performed in the context of these trials, which should enable us to predict vaccine formulations that have the best chance of success in efficacy trials. These trials will be performed against multiple species of *Leishmania* in several countries. It is hoped that a comprehensive approach will be the fastest path to product approval. As a first step, a Phase I, double-blind, dose-escalation trial in normal volunteers has been performed in the USA (BB-IND 10116.0037). Safety and immunogenicity has been demonstrated at each dose of protein (10, 20 and 40 μg) and the results are being evaluated currently. Therapeutic trials in ML (Peru) and CL (Brazil) (BB-IND-11505) are ongoing to evaluate safety and efficacy of the candidate vaccine used in combination with standard chemotherapy. The goal of these studies is to develop safer therapeutic regimens of shorter duration than those used currently for these diseases.

Concluding remarks

Major progress has been made in defining the major T-cell antigens of *Leishmania* spp. that have desirable properties as vaccine candidates. From the ongoing identification and characterization of ~30 vaccine-candidate antigens, three have been selected and fused to develop a vaccine against CL and mucocutaneous leishmaniasis. Each of these three T-cell antigens, LmSTI1, TSA and LeIF, are present in both amastigote and promastigote forms of the parasite, conserved among most *Leishmania* species that cause human disease (a requisite for ensuring cross-species protection), and elicit primarily a Th1-type immune response in murine and human cells [27,29,42,43]. Two of the antigens, rLmSTI1 and rTSA, protect mice and non-human primates against fatal *Leishmania* infections [49]. The rLeIF antigen is therapeutically effective against leishmaniasis in mice. It also has potent Th1-adjuvant properties, including eliciting IL-12 and IL-18 [8,27,42,58], both of which are potent inducers of IFN-γ, maintain memory–effector Th1 cells and are required for primary immunity to leishmaniasis.

The combination of these antigens has been used successfully to treat patients with drug-resistant ML [61]. These results in patients give us confidence in the ability of animal models to predict successful candidates for prophylactic and therapeutic vaccines in humans. The vaccine formulation that contains Leish-111f and MPL®-SE confers protection in animal models against leishmaniasis caused by *L. major*, *L. amazonensis* and *L. infantum*. Although completion of a Phase I study in the USA is an important milestone in developing a second-generation leishmaniasis vaccine, it is only the beginning of a long process and we hope that there will be clinical trials of other candidates during the coming years.

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