The Emergence of Defined Subunit Vaccines for the Prevention of Leishmaniasis

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Abstract Infection with *Leishmania* parasites can cause disease across a range of clinical manifestations and outcomes. Given the practical difficulties in maintaining vector control programs, vaccination is likely the only way in which the sustained reduction or elimination of the leishmaniasis can be achieved. In this review, we focus on the development of second-generation vaccines with emphasis on defined subunit vaccines using recombinant expression techniques. We highlight particular antigens that have provided protection in animal models, and discuss methods, such as inclusion in chimeric fusion proteins and use of appropriate adjuvants, that are likely required to transition these into clinically-relevant candidates. We also outline the alternative strategies, such as attenuated parasites, DNA vaccination, and use of sand fly proteins, that are being proposed.

Keywords *Leishmania* · Protein · Adjuvant · Protozoa · Vaccine · Protozoa

Introduction

The leishmaniasis are a family of diseases, caused by infection with various *Leishmania* species parasites, that cause human suffering on a global scale. Estimates are that there are 12 million current cases, with an additional 2 million cases each year and a population of approximately 350 million people at risk of infection in endemic areas. The geographic distribution of each *Leishmania* species determines the disease presentation in each region. Cutaneous leishmaniasis (CL) can be caused by several *Leishmania* species that are

endemic in the tropics and neotropics, where it manifests as a spectrum of clinical presentations ranging from small cutaneous nodules to gross mucosal tissue destruction (mucocutaneous leishmaniasis; ML) [1]. Visceral leishmaniasis (VL) is the most severe form and is fatal if left untreated. VL cases in South Asia and Africa are caused by infection with *L. donovani*, while in the Mediterranean, the Middle East, Latin America and other parts of Asia it results from infection with *L. infantum* [2]. Mainly observed in Sudan and India, post-kala-azar dermal leishmaniasis (PKDL) is a post-treatment complication in a subset of recovered VL patients that is characterized by a macular, maculopapular, and nodular rash [3]. Although improved drug regimens for the treatment of VL are becoming available [4–8], this requires the diagnosis and prompt treatment of active cases. Vaccination could provide not only long-term protection against infection/disease but also impact potential reservoirs to reduce transmission. Thus, widespread vaccination is likely the only way in which elimination of leishmaniasis can be achieved.

Historical Vaccines for the Prevention of Leishmaniasis

Vaccination against *Leishmania* species was occurring empirically for centuries in the form of leishmanization, a practice that involved the inoculation of virulent *L. major* parasites into hidden areas of the skin. Leishmanization, which continued until relatively recently in many regions, typically induced mild infection and subsequent immunity against further naturally occurring infection [9]. However, the occasional development of progressive infection and disease has led to leishmanization being abandoned as unsafe. The practice did, however, demonstrate the potential for successful vaccination against *Leishmania* parasites if responses can be harnessed appropriately. Even as recently as the last decade, vaccine trials have been conducted using whole, killed or

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inactivated parasites [10–18]. Probably because this strategy involves the use of crude preparations that are very difficult to standardize, clinical results have generally been inconsistent and these vaccines were typically not protective.

Understanding how responses that can clear or restrict the parasites are initiated and evolve during the protective immune response against *Leishmania* parasites is highly informative in the rationale design and development of more refined vaccines capable of promoting protection without detrimental side effects. Basic research has provided significant insight as to how to induce protective immune responses against *Leishmania* species [19]. *Leishmania* parasites reside within antigen-presenting cells, mainly macrophages, and these cells play a pivotal role in determining the quality of the adaptive immune response. The seminal discovery of the T helper (Th)1/Th2 paradigm of CD4 T-cell response was aided by studies using CL-resistant and CL-susceptible inbred mouse strains [20–22], and protection studies have accordingly demonstrated the Th1-dependence of effective immunity against *Leishmania*, particularly against CL. In turn, the *Leishmania*-infected cells require and can respond to T-cell-derived cytokines to activate anti-parasitic mechanisms such as inducible nitric oxide synthase, nitric oxide and interferon gamma (IFN γ) production. Importantly, appropriate antigen-specific Th1 responses also correlate with protection against CL in humans. Thus, despite there being many more complexities in the mechanisms responsible for acquired immunity, a core hypothesis across *Leishmania* vaccine development programs is that a strong, long-lasting Th1 cell response is necessary to reduce parasite replication/survival and preventing disease.

The Here and Now of Vaccines for the Prevention of Leishmaniasis

Toward Defined Subunit Vaccines

Antigen Candidates

While crude extraction or purification of proteins from pathogens was used for a long time to derive potential vaccine candidates, the ready availability of genetic data over the last two decades has led to the evolution of candidate identification through bioinformatic selection and recombinant production methods Fig. 1. Molecular biology tools are now routinely using to select candidates with perceived desirable features (i.e. membrane-spanning domains versus secreted proteins) and anticipated acceptable safety profiles (i.e. low homology to human genes).

A plethora of recombinant proteins have been investigated as *Leishmania* vaccine antigen candidates in animal models and new candidates are regularly being proposed (Table 1).

How these antigens are prepared and/or formulated can dramatically impact their use. For example, immunization with only native Parasite Surface Antigen (PSA)-2 polypeptides, but not other forms, protected mice against infection [46–50]. Among the many recombinant proteins that have elicited protection, the surface expressed glycoprotein leishmaniolyisin (gp63) generated promising findings from animal models following delivery by numerous immunization regimens; however, those results were overshadowed by mostly negative T-cell responses in humans [51, 52]. Although protective efficacy of *Leishmania* homolog for receptors of activated C kinase (LACK) has been demonstrated mainly in the *L. major* model, LACK has failed to protect against experimental VL [23, 24]. In contrast, recombinant nucleoside hydrolase (NH) vaccinated BALB/c mice demonstrate a significant decrease in parasite burden following challenge with *L. infantum*, *L. mexicana* and *L. major* [25, 26]. We identified that the sterol 24-c-methyltransferase (SMT) protein is similarly capable of protecting against challenge with both CL- and VL-causing parasites [27, 28]. Despite the large number of recombinant proteins that have been suggested as vaccine antigen candidates, to date very few have advanced to clinical trials.

Chimeric Fusion Proteins

Analogous to the use of whole parasites, the current state-of-the-art in vaccinology is to simultaneously provide multiple antigen candidates to expand the likelihood that an immunized individual will recognize and respond to at least some of the included epitopes. Recombinant proteins have been used for years in standard immunization schedules (e.g. hepatitis B surface antigen [HBsAg] for hepatitis B virus) and chimeric fusion proteins representing many antigens in a single product have advanced to clinical trials for several indications [53–57]. Using genetic information on sequence conservation among *Leishmania* species, selection criteria for the inclusion of genes within fusion constructs can also consider the potential for cross-species protection and even the ability to protect against the various clinical presentations of leishmaniasis. For practical considerations, such as the ability to be expressed and purified as a single product (rather than multiple individual proteins to be combined later, all of which would require full GMP production and evaluation), and based on the design of the construct, expression can be driven to high levels even for components that are difficult to produce as single, non-fused proteins, fusion proteins have great appeal Fig. 1.

Fusion proteins may also enhance the levels of protection that can be elicited. As an example, when used alone the *L. major* cysteine proteinases (Type I [CPB] and Type II [CPA]) induce only partial protection against *L. major* infection in BALB/c mice [58]. However, relative to the single proteins, greater protection was afforded when a cocktail of

Table 1 A representative sampling of antigens that have been proposed as vaccine antigen candidates for leishmaniasis

Antigen	Full given name	Parent <i>Leishmania</i> species	References
LACK	<i>Leishmania</i> homologue for receptors of activated C kinase	<i>major</i>	[23, 24]
NH	Nucleoside hydrolase	<i>infantum</i>	[25, 26]
SMT	Sterol 24-c-methyltransferase	<i>infantum</i>	[27, 28]
A2	Amastigote-specific A2 proteins	<i>donovani</i>	[29]
ORFF	Open-reading frame gene from the LD1 locus	<i>donovani</i>	[30]
LiP0	Acidic ribosomal protein P0	<i>infantum</i>	[31]
PFR-2	Paraflagellar rod protein 2	<i>mexicana</i>	[32]
PPG	N-terminal domain of proteophosphoglycan	<i>donovani</i>	[33]
MAPK10	MAP kinase 10	<i>major</i>	[34]
LeIF-2	Elongation factor-2 protein	<i>donovani</i>	[35]
TDR1	Thiol-dependent reductase 1	<i>infantum</i>	[36]
115 kDa	115 kDa serine protease	<i>donovani</i>	[37]
LmL5	Ribosomal protein	<i>major</i>	[38]
rLdAld	Aldolase	<i>donovani</i>	[39]
rLdEno	Enolase	<i>donovani</i>	[39]
Cyp1	Cyclophilin protein 1	<i>infantum</i>	[40]
LPG3	Lipophosphoglycan	<i>major</i>	[41•]
HbR	Hemoglobin receptor	<i>donovani</i>	[42•]
gGCS	Gamma-glutamyl cysteine synthetase	<i>donovani</i>	[43, 44•]
TPR	Trypanothione reductase	<i>donovani</i>	[45]

CPA and CPB containing DNA constructs was used. Following fusion of the genes and expression of the CPA/B hybrid protein, mice vaccinated with CPA/B exhibited a delay in the expansion of *L. major*-induced lesion size compared with control groups [59].

Fig. 1 Schematic representation of the evolution of vaccine strategies for leishmaniasis. Each arrow is sized with the intent of reflecting the degree of transition that has occurred between the respective areas

We have generated multiple fusion proteins and evaluated their protective efficacy in experimental models. Of these, to date three have advanced to clinical trials. The first to progress was a recombinant tri-fusion polypeptide comprising antigens thiol-specific antioxidant (TSA), *L. major* stress-inducible protein 1 (LmSTI1), and *Leishmania* elongation initiation factor (LeIF) that was generated and named L111f. Immunization of BALB/c mice elicited a predominant Th1 immune response characterized by in vitro lymphocyte proliferation, IFN γ production, and IgG2a antibodies with little, if any, interleukin (IL)-4. Moreover, L111f formulated with monophosphoryl lipid A-stable emulsion (MPL-SE) conferred immunity to leishmaniasis for at least 3 months. The L111f polypeptide has been slightly modified, by removing the His tag, to render L110f which retains all of the antigenic and protective activity [60]. The L111f and L110f antigens have been further developed as therapeutic candidates [61] and have been used in numerous clinical trials for various presentations of leishmaniasis [62].

The second fusion protein that we have advanced is a tetra-fusion named KSAC. KMP-11, SMT, A2, and CPB have all been demonstrated to individually confer protection in different disease models of VL, and each of these components is represented in KSAC [27–29, 63–66]. When the KSAC polypeptide was generated and delivered with adjuvant, we discovered that in our VL model, two of the individual antigens, SMT and CPB, stimulated the majority of the protective

response. However, because KMP-11 was shown to be an effective antigen when delivered as DNA, and because A2 has shown some degree of efficacy in dogs, these antigens were retained [64, 67]. KSAC induced protection equal to L110f in our mouse protection models of CL [68•] and, more recently, was demonstrated to be effective in protecting mice against *L. major* using a sand fly challenge [69•].

The most recent polyprotein we have advanced is a diffusion pairing NH with SMT. This fusion protects against experimental VL and its safety was recently evaluated in a phase I trial (unpublished data).

Emerging Alternatives

Live-Attenuated Vaccines

Building on the experience with leishmanization and the prior use of killed parasites, a variety of studies have now evaluated the ability of *Leishmania* attenuated strains to protect against subsequent challenge with virulent parasites. Defined genetic alteration to generate avirulent *Leishmania* species theoretically permits mimicry of natural infection and delivery of a complete spectrum of antigens to the antigen-presenting cells to provide a broad protective response.

L. major phosphomannomutase (PMM)-deficient mutants were able to protect *L. major*-susceptible BALB/c mice against infection, most likely due to the suppressed IL-10 and IL-13 production early during infection, and increased magnitude of T-cell responses [70]. Inoculation with dihydrofolate reductase thymidylate synthase (dhfr-ts) knock-out parasites was also able to protect I a mouse, but not in a monkey, model of *L. major* infection [71–73]. *L. major* parasites lacking the *lpg2* gene were able to confer protection against homologous infection and established a chronic presence in mice without causing pathology [74]. Cross-species protection has been demonstrated in the case of an *L. infantum* deletion mutant lacking HSP70 type II gene that was evaluated for protective efficacy against challenge with *L. major* [75•]. Administration of the mutant parasite by numerous routes to BALB/c mice promoted the production of high levels of nitric oxide and the development of Th1 responses that were protective against *L. major* challenge. Deletion of serine protease (CP) in *L. mexicana* led to an attenuated strain that established only a temporary infection in mice but was capable of triggering partial protection against homologous challenge [76]. While *L. donovani* centrin-deleted mutants (LdCEN-/-) were viable in culture as promastigotes, they demonstrated a selective growth arrest in the amastigote stage of development and, in vitro, could not survive in human macrophages [77]. Animals inoculated with LdCEN-/- mutants were protected against challenge with *L. donovani* as well as other *Leishmania* species [78, 79]. *L. tarentolae* parasites expressing the A2 virulence gene have similarly been

demonstrated to protect against *L. infantum* infection of mice [80].

Based on similar principles to the gene knockout-based attenuation of *Leishmania* parasites, another approach is the addition of suicide cassettes that lead to inducible parasite death following inoculation. It has been suggested that parasites carrying drug-sensitive cassettes could provide suitable candidates for leishmanization, with an example being the introduction of drug-sensitive genes such as *Saccharomyces cerevisiae* cytosine deaminase gene, which is sensitive to 5-fluorocytosine [81]. Alternatively, parasites can be modified to produce biological substances that activate immune attack, such as granulocyte macrophage colony-stimulating factor [82].

While attenuated or genetically altered whole parasite vaccines offer an intriguing approach to immunization against leishmaniasis, fears that the parasite may revert to a virulent form remain. Of particular note, mutants lacking the *lpg2* gene developed an unknown compensatory mechanism over time that allowed them to regain their ability to cause disease [83]. Thus, persistence may not be a desirable feature of a live-attenuated vaccine, and the potential emergence of compensatory mechanisms will likely be an ongoing concern of regulatory bodies that may preclude their widespread use.

Naked DNA

Immunization with naked DNA is a relatively new approach in which single or multiple genes encoding various antigens are cloned into a mammalian expression vector. Administration is through simple intramuscular injection and multiple plasmids can be combined to yield multivalent vaccines [84]. The mechanism by which DNA vaccination generates potent immune responses appears to be through the inherent adjuvant activity of the non-methylated DNA sequences that stimulate innate responses, then by replication within the host leading to the in situ expression of the encoded proteins. Compared with the more typical vaccines, it is believed that cold chain storage, delivery, and distribution in settings with limited infrastructure should be facilitated by the stable nature of the DNA molecule.

Several studies have examined the use of experimental DNA vaccines against *Leishmania* species. Vaccinations with DNA encoding gp63, LACK, or PSA-2 elicited antigen-specific Th1 responses and reduced lesion development in *L. major*-infected mice [48, 85, 86], while DNA vaccination against the parasite enzyme gamma glutamylcysteine synthetase protected mice against *L. donovani* infection [87]. However, a multi-antigenic DNA vaccine encoding KMP11, TRYP, LACK and gp63 did not protect dogs against *L. infantum* challenge [88].

Sand Fly Salivary Proteins

Female phlebotomine sand flies are critically required for the transmission of *Leishmania* in the natural setting (i.e. excluding transfer of infected blood products, etc.), with two genera transmitting *Leishmania* parasites to humans (*Lutzomyia* in the New World and *Phlebotomus* in the Old World). Although the saliva of sand flies and other blood-feeding insects contain potent pharmacologic components that facilitate blood meals, the immune response to saliva proteins can preclude the parasites from establishing themselves in the vertebrate host [89, 90]. It may be that *Leishmania* infection persists due to the counteraction of protective versus aggravating proteins, and it has been proposed that recognition of proteins that are part of the protective response could be harnessed as vaccine candidates to limit *Leishmania* infection during the sand fly's blood meal.

DNA immunization with distinct salivary proteins from *P. papatasi* modulates *L. major* infection: while PpSP44-immunized mice aggravate the infection, PpSP15-immunized mice demonstrate protection [91]. Several reports have indicated the possibility that some recombinant salivary proteins from sand flies could serve as targets for vaccine development to control *Leishmania* infection [92–94]. *Lu. longipalpis* salivary recombinant protein LJM11 (rLJM11) induces long-lasting immunity when administered to mice that results in ulcer-free protection against *L. major* delivered by vector bites [69]. Interestingly, this protection is antibody independent and abrogated by depletion of CD4 T cells, indicating that protection is actively mediated by cells but not by antibody-mediated phagocytosis. Mice immunized with DNA plasmid coding LJM19, a *Lutzomyia longipalpis* salivary protein, were also protected against *L. major* infection [95]. Hamsters similarly immunized with LJM19 were protected from *L. brasiliensis* infection and cutaneous lesion development [96], as well as against disease development and the fatal outcome of experimental VL caused by *L. infantum* [97, 98].

While some sand fly salivary proteins have demonstrated good vaccine potential in preclinical settings, it is noteworthy that populations living in *Leishmania*-endemic regions are regularly exposed to the vector over an extended period of time and over periods of intense biting/feeding. A study examining varying exposure periods to *Phlebotomus dubosqi* bites followed by *L. major* infection, indicated that protection was limited to only short-term exposure to sand flies immediately before infection [99]. Alternatively, colonization of *P. papatasi* appears to modulate the outcome of *L. major* infection from lack of protection to protection [100, 101]. Thus, inferences based on long-term colonized populations of sand flies should be considered with caution. It is also unclear if sensitization to the sand fly proteins and boosting and presentation of delayed type hypersensitivity responses

through regular exposure to bites is an acceptable byproduct of this strategy.

Importance of Adjuvants and Formulation

One caveat regarding the use of recombinant proteins within vaccines is that, alone, they generally induce only weak T-cell responses. Adjuvants are compounds that enhance the potency of the antigen-specific immune response and can be an essential component of an efficacious vaccine. A primary function of adjuvant is to appropriately bias the antigen-specific response to elicit effective and durable T-cell responses. The discovery that properly formulated toll-like receptor (TLR) agonists can stimulate Th1 immune responses has profoundly impacted vaccine development against intracellular pathogens such as *Leishmania*. The clinical importance of adding a TLR4 agonist to vaccine formulations, as well as the safety and efficacy of engaging TLR4, has been demonstrated during the development of MPL. Indeed, MPL is now approved and used extensively in vaccines for other indications [102]. Our first defined vaccine against leishmaniasis used L111f together with MPL formulated in an oil-in-water emulsion (MPL-SE). The L111f+MPL-SE vaccine protects mice, hamsters, and rhesus macaques, and was the first defined *Leishmania* vaccine to enter clinical trials [103–106].

Inclusion of adjuvant can also potentially broaden the antigen-specific responses that are elicited. As a pertinent and clinically relevant example, Cervarix is an FDA-approved prophylactic vaccine against human papillomavirus (HPV) types 16 and 18 that is formulated with MPL (a defined mixture of TLR4 agonists and antagonists obtained from the cell wall of *Salmonella*). The approval of Cervarix was granted in part due to the protective immunity it conferred against oncogenic HPV strains that were not contained in the vaccine. In addition, adjuvants can reduce the cost to the end-user by allowing the use of much lower quantities of protein. The appropriate selection and use of adjuvants will likely remain an important consideration for the advancement of additional leishmaniasis vaccines.

Conclusions

With the potential to generate long-term protection and interrupt transmission, vaccination holds significant promise, and may be critically required, for sustained control of the leishmaniasis. However, while both protein- and DNA-based vaccines have advanced as veterinary products, only recombinant proteins have advanced as far as licensure in human vaccines. With the large number of defined antigens that have been, and continue to be, demonstrated to afford protection in animal

models of leishmaniasis, and the continued refinement of adjuvant components and delivery systems of these vaccines, there is a strong probability that a vaccine (or even multiple vaccines) for the leishmaniasis will emerge in the coming years.

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Compliance with Ethics Guidelines

Conflict of Interest Malcolm S. Duthie and Steven G. Reed declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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