Expert Opinion

Recent developments in leishmaniasis vaccine delivery systems

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Background: The observation that recovery from infection with *Leishmania* confers immunity to reinfection suggests that control of leishmaniasis by vaccination may be possible. New generation vaccines, particularly those based on recombinant proteins and DNA, are found to be less immunogenic. *Objective*: There is an urgent need for the development of new and improved vaccine adjuvants. *Methods*: Based on their principal mechanisms of action, adjuvants can be broadly separated into two classes: immunostimulatory adjuvants and vaccine delivery systems. Vaccine delivery systems can carry both antigen and adjuvant for effective delivery to the antigen-presenting cells (APCs). In this article, we review the adjuvants, the delivery systems and their combinations used in the search of an effective vaccine against leishmaniasis. *Conclusion*: Based on current knowledge, cationic liposomes appear to have better prospects as effective delivery systems for developing a vaccine for leishmaniasis.

Keywords: adjuvant, cationic liposomes, *Leishmania*, vaccine, vaccine delivery systems

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1. Introduction

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 [1]. It is probably one of the few parasitic diseases that is most likely to be controlled by vaccines. The relatively uncomplicated leishmanial life cycle and the fact that recovery from a primary infection generally renders the host resistant to subsequent infections indicate that a successful vaccine is feasible. However, to develop vaccines against intracellular pathogens like *Leishmania*, the induction of cell-mediated immunity (CMI) is likely to be necessary. Unfortunately, non-living vaccines have generally proven ineffective at inducing CMI. In addition, although live vaccines may induce CMI, some live attenuated vaccines can cause disease in immunosuppressed individuals and some pathogens are difficult or impossible to grow in culture. Moreover, these vaccines also contain components that can cause undesirable effects and safety problems [2]. Efforts must therefore be made to produce one or more consistent, safe and effective vaccine against leishmaniasis. Several new approaches used in modern vaccinology are being investigated against leishmaniasis with molecularly defined subunit vaccines.

While these new approaches may offer important safety advantages, a general problem is that the vaccines alone are often poorly immunogenic. Effective protection against *Leishmania* infection has been largely attributed to the development of a potent $CD4^+$ mediated Th1 type immune response, characterized by the production of IL-12 and IFN-γ, which subsequently mediates macrophage

activation, nitric oxide production and parasite killing. Recently, the involvement of $CD8^+$ T cells has also been shown to play an important role in immunity against leishmaniasis [2]. Thus, appropriate antigen delivery to induce the right type of immune response against leishmaniasis is another crucial component of an effective vaccine. Unlike attenuated live vaccines, killed whole organism or subunit vaccines generally require the addition of an adjuvant to be effective. Adjuvants can be broadly separated into two classes based on their principal mechanisms of action: immunostimulatory adjuvants and vaccine delivery systems. Immunostimulatory adjuvants are predominantly derived from pathogens and often represent pathogen-associated molecular patterns (PAMPs), for example lipopolysaccharide (LPS), monophosphoryl lipid A (MPL), CpG DNA, which activate cells of the innate immune system. Once activated, cells of innate immunity drive and focus the acquired immune response [3]. In contrast, vaccine delivery systems are generally particulate, for example, emulsions, microparticles, iscoms and liposomes, and mainly function to target associated antigens into antigen-presenting cells (APCs) [4]. Nevertheless, these simple definitions become insufficient when immunostimulatory agents are included into delivery systems to focus their effects onto the APCs, to maximize their potency and to minimize their effects on non-immune cells. Hence, delivery systems can improve the therapeutic ratio of adjuvants, reduce the dose needed and improve their specificity and safety. Thus, optimal new generation vaccines are likely to comprise recombinant antigens used in conjunction with immunopotentiators and delivery systems for both antigens and adjuvants. In this review, we will discuss the role of different vaccine delivery systems, as well as immunostimulatory adjuvants used alone or in association with the vaccine development against leishmaniasis.

2. Immunostimulatory adjuvants

Vaccines that contain attenuated live or heat-killed viruses or bacteria include components that can engage Toll-like receptors (TLRs). These components therefore act as natural adjuvants via TLR signaling pathway, leading usually to improved Th1-related responses. In contrast, recombinant vaccines are highly purified, lack many of the features of the original pathogen and do not evoke strong immune responses. Hence, it can be argued that the role of adjuvants (e.g., saponins, cytokines, MPL, CpG) for recombinant vaccines is to initiate a potent innate immune response and control the type of acquired immune response induced. Some of these adjuvants are PAMPs, which are highly conserved in a broad range of pathogens. They are effective due to their ability to be recognized by and activate the pathogen recognition receptors (PRRs) which are present on innate immune cells, including the TLR family. Hence, they can activate cells of the innate immune system, including APCs such as dendritic cells (DCs). **Table 1** provides a summary of the types of adjuvants used in vaccines against leishmaniasis.

2.1 BCG and other microorganisms

Bacille Calmette-Guerin (BCG), a live attenuated bovine tubercle bacillus, is the most widely used vaccine in the world. It is the most heat stable of live vaccines and is inexpensive to produce. Although BCG alone could protect mice against leishmaniasis [5,6], its adjuvanticity was confirmed when in association with killed or autoclaved *Leishmania* promastigote antigens, it induced strong cellular immune responses. This was evidenced by a high index of conversion of the intradermal skin reaction to leishmanial antigen [7,8]. BCG, known to elicit long-lasting cellular responses, is the only adjuvant that has been widely used in vaccination trials against human tegumentary leishmaniasis and kala-azar. When comparing the incidence of disease between vaccines and control individuals, low or no vaccine efficacy was detected [8,9]. However, individuals whose leishmanin skin test converted after vaccination had a significantly lower frequency of visceral leishmaniasis than non-responders [9].

In contrast, the administration of killed or pasteurized *Leishmania* promastigotes with BCG demonstrated immunotherapeutic effects in American cutaneous leishmaniasis patients [10,11]. Immunization of dogs with quimeric multicomponent protein Q protein (formed by the genetic fusion of five antigenic determinants from four *Leishmania* proteins: acidic ribosomal proteins Lip2a, Lip2b, P0 and the histone H2A protein) formulated with live BCG as adjuvant conferred significant protection against *L. infantum* and prevented the development of clinical disease [12], giving an additional reason to continue investigation with this microorganism. Another bacteria, heat-killed *Propionibacterium acnes*, formulated with A2 antigen, an amastigote-specific protein, conferred significant protection against *L. donovani* [13]. It was postulated that reformulation of the vaccine mixture by adding another adjuvant (e.g., alum) would improve the immunogenic potential. Alum is widely used in human vaccines to elicit an early, high and long-lasting antibody response. Studies in monkeys showed that intradermal injections of alum-precipitated autoclaved *Leishmania* with BCG were highly protective against visceral leishmaniasis [14]. Moreover, in healthy individuals the rate of leishmanin skin test conversion was also higher in alum-precipitated autoclaved *Leishmania* and BCG vaccinated individuals than that induced by autoclaved *Leishmania* with BCG, making the formulation a potentially superior candidate vaccine for visceral leishmaniasis [15]. This formulation, when used in immunochemotherapy in association with antimony, increased the cure rate in post-kala-azar dermal leishmaniasis (PKDL) patients with minimal local adverse events [16]. But alum promotes a Th2-biased response with elevated titers of the Th2-dependent antibody isotypes IgG1 and IgE, rather

Table 1. Different vaccine delivery systems used in vaccines against leishmaniasis.

Type of adjuvant	Advantages
Immunostimulatory adjuvants	
BCG and other microorganisms BCG [5-12,14-15] *Propionibacterium acnes* [13]	Th1-stimulating adjuvant approved for human use
Saponin [17-22]	Functions mainly through the induction of Th1 cytokines
Recombinant IL-12 [23-27]	Has the ability to modify and redirect the immune response towards Th1
MPL [28-33]	T-cell adjuvant approved for human use, acts through TLR4 to activate innate and acquired responses
CpG [34-41]	TLR9 ligand induces innate and in turn Th1-biased adaptive immune response
Delivery systems for vaccine and/or immunostimulatory adjuvants	
Microorganisms BCG [42,43] *Salmonella typhimurium* [44] *Listeria monocytogenes* [45] *Toxoplasma gondii* [46]	Serve as an expression, carrier and adjuvant system for protein
Emulsions Freund's adjuvant (IFA) [47] Montanide ISA 720 [48]	Water-in-oil emulsion to deliver antigen to enhance the level of response or to focus the response through a desired pathway, for example Th1
Poloxamer Poloxamer 407 [49-51]	Non-ionic surfactant induces Th1 response
DNA vaccines [27,52-78]	Drives the expression of an antigen in eukaryotic cells, able to elicit humoral, $CD4^+$ and $CD8^+$ T-cell immune responses
Heterologous prime–boost strategy [79-89]	Involves priming with DNA and boosting with recombinant viral vectors or recombinant proteins, each encoding the same antigen to enhance the cellular immunity
Dendritic cells as delivery system [90-94]	Antigen carriers and professional APCs for the induction of cell-mediated immunity
Liposomes [95-113]	Act as both adjuvant and as delivery system for antigens and immunostimulatory adjuvant for the presentation to APCs

than Th1 response [4]. Thus, this aspect of alum needs to be reconsidered prior to its routine use against leishmaniasis.

2.2 Saponin

Saponins, derived from the bark of a Chilean tree, *Quillaja saponaria*, are considered to be one of the choices of adjuvants for different experimental models in leishmaniasis where cell-mediated immunity is required. Santos *et al.* showed that saponin in combination with fucose-mannose ligand (FML) antigen of *L. donovani* was found to be more effective than either incomplete Freund's adjuvant (IFA) or aluminium hydroxide in murine model of experimental visceral leishmaniasis [17]. The FML–saponin vaccine formulation also promotes significant, specific and strong protective effects against murine visceral leishmaniasis compared to BCG and IL-12 as adjuvant [18]. This Riedel de Haen saponin contains the QS21 and deacylated saponins of *Quillaja saponaria* as the main adjuvant components [19]. In a field trial against canine visceral leishmaniasis, the FML–QuilA vaccine also induced a significant, long-lasting and strong protective effect, suggesting its strong potentiality as adjuvant [20]. The possible use of a protective vaccine in the immunotherapy of human kala-azar and different experimental models of visceral leishmaniasis is highly encouraging and would have broader acceptance to control the disease. Interestingly, FML–saponin vaccine has been shown to be immunotherapeutic in mice [21] and seropositive in asymptomatic dogs from Brazilian endemic areas [22]. Although the adjuvant activity of saponins has been extensively demonstrated, an undesirable hemolytic effect has restricted their use with human vaccines [4].

2.3 Recombinant IL-12

As an alternative to the use of cytokine-inducing adjuvants, cytokines may also be used directly. Most cytokines have the ability to modify and redirect the immune response. IL-12 is the cytokine that has been used most extensively in vaccines against *Leishmania*.

In a study carried out by Aebischer *et al.*, in which six adjuvants were assayed, it was found that the best protection against *L. amazonensis* infection was obtained when IL-12 was injected with gp63 [23]. The combination of IL-12 with recombinant *L. major* TSA protein (homolog to eukaryotic thiol-specific antioxidant proteins) was also effective to

confer protective immunity against infection with *L. major* [24]. This antigen, together with another *L. major* recombinant protein, LmSTI1, administered in combination with human IL-12 and alum as adjuvants, elicited protective immunity in rhesus monkeys against the development of cutaneous lesions after a *L. major* challenge [25]. However, IL-12 is presently not considered to be a safe adjuvant for use in humans because of observations indicating that under certain circumstances IL-12 may be detrimental to resistance to infectious agents, and overproduction during autoimmune diseases can lead to an exacerbation of disease [26]. Most importantly, one drawback of IL-12 is its inability to stimulate strong immunological memory to the immunizing antigen and failure to elicit long-term protective immunity [27].

2.4 Monophosphoryl lipid A

Given that IL-12 does not provide long-term immunity and is not being developed as an adjuvant for use in human vaccines, MPL might be an alternative for human use as a T cell adjuvant. MPL-SE is a detoxified derivative of 40-monophosphoryl lipid A of LPS obtained from *Salmonella minnesota* formulated with squalene and has been approved for human use. Several studies demonstrate that it is a potent immunostimulant that lacks the toxic properties of LPS and acts through activation of TLR4, a receptor that contributes to the control of parasite growth in both the innate and acquired immune response to *Leishmania* infection [28]. To develop an effective vaccine against leishmaniasis, a multivalent cocktail of several antigens containing a broader range of protective epitopes that can cover a wide range of major histocompatibility complex (MHC) types in a heterogeneous population is required. For this reason, a single recombinant polyprotein comprising the sequences of open reading frames of three priority candidate *Leishmania* antigens – TSA, LmSTI1 and LeIF – were genetically linked in tandem. The resulting molecule, Leish-111f, comprises an open reading frame that codes for a 111 kDa polypeptide. Initial immunization trials in mice demonstrated that Leish-111f was able to protect mice against *L. major* and *L. amazonensis* infection [29,30]. Moreover, in contrast to other protein-based vaccines, Leish-111f formulated with MPL-SE conferred durable immunity to experimental leishmaniasis for at least 3 months [29]. This vaccine formulation is also effective in inducing partial protection against visceral leishmaniasis in animal models [31], however, it could not prevent disease development in a recent Phase III vaccine trial in dogs [32]. In a human Phase I, double-blind, dose-escalation trial in normal volunteers performed in the USA, the vaccine was found to be safe and immunogenic. Therapeutic trials in mucosal leishmaniasis (Peru) and cutaneous leishmaniasis (Brazil) were both completed successfully in 2006. Considering the safety profile of Leish-111f + MPL-SE in healthy and active cases, now the vaccine trial will be performed in Sudan to evaluate the safety and immunogenicity in patients with PKDL [33]. With these clinical trials against different species of *Leishmania*, there is hope for this adjuvant in human use against leishmaniasis.

2.5 CpG

Stimulation of the innate immune system by determinants expressed by infectious microorganisms serves to limit the early spread of a pathogen while promoting the development of antigen-specific immunity. TLRs, which recognize conserved microbial determinants, activate the cells of the innate immune system to limit the early spread of pathogens while promoting the development of antigen-specific immunity. Unmethylated CpG motifs that present at high frequency in bacterial but not vertebrate DNA are recognized by TLR9 expressed by B cells and plasmacytoid DCs. The interaction of TLR9 with CpG motifs triggers an immune cascade, resulting in improved Ag uptake/presentation by APCs and the secretion of polyreactive Ig, chemokines and cytokines by B cells, NK cells, DCs and monocytes. Synthetic oligodeoxynucleotides (ODNs) expressing CpG motifs mimic the immunostimulatory activity of bacterial DNA. CpG DNA, acting as a TLR9 ligand, induces protective immunity as well as curative response in murine leishmaniasis [34,35]. These effects are strongly dependent on IL-12 and mechanistically mediated by a TLR9-dependent activation of DCs, which in turn enables the adaptive immune system to mount a protective Th1-biased immune response. Moreover, CpG ODN, when used as a vaccine adjuvant with either a recombinant protein or heat-killed leishmanial antigen, can induce long-term protection against an intracellular infection in a $CD8^+$ dependent manner [36]. Two types of CpG ODNs that activate PBMCs from human and nonhuman primates have been identified. 'D' type ODNs (also known as type A), which has a single PuPyCpGPuPy motif, a mixed phosphorothioate–phosphodiester backbone, and a poly(G) tail on the 3′ end. Type D/A ODN induce human and nonhuman primate plasmacytoid DCs to secrete alpha interferon (IFN-α), monocytes to mature into functionally active DCs and NK cells to secrete IFN-γ. D/A ODN do not activate B cells directly. This distinguishes them from CpG ODN type K (also known as type B) and type C, which induce polyclonal B cell activation, higher levels of IL-6 and IL-10, and lower secretion of IFN-γ [37]. While all CpG ODN types have demonstrated some adjuvant activity in primates [38], the protective effects of CpG ODN administered alone have so far been demonstrated only in a macaque model of cutaneous leishmaniasis using CpG ODN type D/A [39]. Again, administration of CpG ODN D/A was effective in the prevention and treatment against *L. major* infection, suggesting its use as an immunoprotective and therapeutic agent [37]. CpG DNA has also been proven effective in the model of leishmaniasis for live vaccines [40]. The addition of CpG to the live vaccine resulted in early activation of dermal DCs and increased IL-6 production, as well as in a reduction

in the accumulation of $Foxp3^+CD4^+CD25^+$ regulatory T (Treg) cells that naturally occurs in the skin following *Leishmania* infection, causing limited pathology at the vaccination site [41]. Thus, CpG DNA-powered vaccines might be an effective tool to combat leishmaniasis in the future.

3. Vaccine delivery systems

Vaccine delivery systems (e.g., emulsions, poloxamer particles, microorganisms, viruses and DCs as delivery vehicles and liposomes) function mainly to promote the uptake of associated antigens into the APCs (**Table 1**). Immunostimulatory adjuvants may also be included in the delivery systems to enhance the level of response or to focus the response through a desired pathway, for example Th1, by targeting APCs. In addition, formulating potent immunostimulatory adjuvants into delivery systems may limit adverse events through restricting the systemic circulation of the adjuvant.

3.1 Microorganisms as delivery systems

An approach to vaccine delivery systems using BCG and other microorganisms is the recombinant form that expresses parasite antigens. A promising vaccination result was observed with cloned *L. major* gp63 [42] and *L. chagasi* LCR1 [43] genes in a BCG strain. Significant protection was also observed after delivering the *Salmonella typhimurium* mutant orally, transformed with the same *L. major* gp63 gene [44]. *Listeria monocytogenes* is a short-lived delivery system and a known inducer of IL-12 production [45]. The stimulation of the endogenous production of IL-12 at the time of immunization seems to be a promising way of optimizing the efficiency of a protective anti-*Leishmania* vaccine. Thus, a vaccination trial with recombinant attenuated *Listeria* strain expressing the *L. major* LACK (*Leishmania* homolog of receptors for activated C kinase) protein induced Th1 responses and partial protection against *L. major* [45]. As an alternative to prokaryotic vaccine carriers, the use of attenuated *Toxoplasma gondii* strain expressing the *L. major* KMP-11 gene elicited a protective response against *L. major* infection [46]. The use of microorganisms as live recombinant vectors has clear advantages as they simultaneously serve as an expression, carrier and adjuvant system, however this probably has limited practical application.

3.2 Emulsions

This class includes oil-in-water or water-in-oil emulsions such as the incomplete Freund's adjuvant (IFA), Montanide, etc. The mechanism of action of adjuvant emulsions includes the formation of a depot at the injection site, enabling the slow release of antigen and the stimulation of antibody-producing plasma cells. Inoculation of histone H1 as a perchloric acid extract, as a recombinant protein, or as synthetic peptide emulsified in IFA, followed by a *L. major* challenge, elicited partial protection in mice [47]. Montanide ISA 720 has been previously shown to be safe and to confer immunogenicity (antibody, T cell proliferation and IFN-γ production) in human trials. The vaccine formulation of recombinantly produced H1 antigen and MISA720 adjuvant promoted a reduced development of lesion size in vervet monkeys after challenge with *L. major* [48]. Although they may be used to enhance immunogenicity of antigen, in general these adjuvants, with frequent side effects of inflammatory reactions, granulomas and ulcers at the injection site, are too toxic for routine human prophylactic vaccine use.

3.3 Poloxamer

The adjuvant poloxamer 407 is a polyoxypropylene/polyoxyethylene copolymer with temperature-dependent sol/gel transition characteristics ideal for the slow release of antigen. Also known as pluronic F127, poloxamer 407 is a mild surface active agent which is known to induce IgG2a and a Th1 response in BALB/c mice. This Th1 stimulator has been successfully assessed in vaccine formulations against cutaneous leishmaniasis. A single immunization with a combination of poloxamer-407 and a synthetic T cell epitope derived from the *L. major* gp63 was sufficient to provide long-lasting protection against *L. major* [49]. This adjuvant was also chosen to assay the vaccine potential of cysteine proteases CPA and CPB of *L. major* as recombinant protein [50]. Interestingly, CPB but not CPA could elicit partial protection mainly dependent on IFN-γ producing $CD8^+$ T cells. Additionally, immunization with a hybrid protein of CPA/B in combination with the poloxamer was also able to induce a protective immune response in mice against *L. major* [51].

3.4 DNA vaccines

DNA-based vaccination consists of the direct injection of the DNA corresponding to a eukaryotic expression vector, which drives the expression of an antigen of interest.

The development of the simple and potentially powerful technology of DNA vaccination relies on two fundamental principles. The first is that DNA is not simply a vehicle to ensure protein production in transfected cells, but also has intrinsic adjuvant properties due to the presence of immunostimulatory sequences in the backbone of bacterial DNA. These sequences are composed of unmethylated CpG dinucleotides, with particular surrounding flanking nucleotides, found in the bacterial genome at 20-fold greater frequency than in vertebrates. The second principle to emerge was that the transfected cell did not directly prime T cells, but that transfer from cell depots to professional APCs was a prerequisite. The process of transfer, termed 'cross-presentation', may be the route by which antigens from intracellular pathogens elicit MHC class I-dependent $CD8^+$T-cell responses [52]. DNA vaccines are relatively simple to produce and administer; they are often very

immunogenic and offer a protein that is usually correctly folded and may be post-translationally modified in a fashion similar to the native protein. Such vaccines are able to elicit humoral, $CD4^+$ and $CD8^+$ T cell immune responses, which can be further modulated by the addition of cytokines and/or CpG oligonucleotides [53]. They can also be modulated by prime–boost strategies that involve priming with DNA and boosting with protein [54].

Most nucleic acid vaccination efforts have been directed against viral infections, which require the induction of CTL responses, a major feature of DNA vaccines. This method of immunization is also attractive for leishmaniasis since the induction of Th1 responses is also a general property of DNA vaccines [55]. In addition, a growing body of evidence implicates $CD8^+$ T cells in anti-leishmanial immunity [55,56]. The gene encoding gp63 was the first to be used as a DNA vaccine, and immunized mice developed strong Th1 responses as well as significant resistance to infection with *L. major* [57]. More recently, a comparative study evaluating *Leishmania* vaccines with different DNA vaccine candidates including gp63 showed that protection was transient, and eventually the immunized mice developed lesions similar to those observed in controls [58]. The same study also included PSA-2, which did not confer protection. This is in contrast with previous studies using PSA-2 DNA immunization as either prophylactic [59] or therapeutic vaccines [60], which showed protection associated with strong Th1 responses. The difference in outcome between the two studies could be due to the use of susceptible BALB/c mice in the first and resistant C3H/He mice in the second. Another comparative study demonstrated that gp63 DNA immunization was able to reduce lesion size as well as parasite burden, while gp46/PSA-2 DNA vaccination led only to a reduction in lesion size without reduction of parasite burden [61].

LACK is the most extensively studied DNA vaccine against both cutaneous and visceral leishmaniasis. DNA vaccination with a plasmid harboring the LACK gene induced robust, long-lasting protection against *L. major* challenge in mice, dependent on the immunoregulatory role of $CD8^+$ T cells [27,55,62]. Protective vaccination against *L. major* was also achieved following delivery of LACK in a minimalistic, immunogenically defined gene expression (MIDGE) vector with lower doses of plasmids required for protection [63]. The intranasal delivery of LACK DNA also protected mice against *L. amazonensis* challenge [64]. These positive outcomes are overshadowed by several studies where immunization with LACK DNA offered no protection. These reports are mainly restricted to visceral leishmaniasis, but there are also reports in the *L. major* [58] and *L. mexicana* models of disease [61]. Melby and colleagues reported that despite triggering strong Th1 responses, the LACK DNA vaccine did not induce protection in mice against *L. donovani* challenge [65]. Moreover, the co-administration of IL-12 did not improve the protective outcome. A recent study in the *L. chagasi* model confirmed that LACK DNA vaccination does not confer protection against visceral leishmaniasis, despite the presence of Th1 responses [66].

Besides these, several other antigens have also been successfully tested as DNA vaccines against cutaneous and visceral infection. Protection was achieved when acidic ribosomal protein P0 was administered as DNA vaccine in mice against *L. major* infection [67]. DNA immunization with the gene encoding *L. amazonensis* P4 nuclease protects mice against two forms of cutaneous leishmaniasis [68]. The protection against *L. amazonensis* infection was improved when P4 vaccine was administered together with a construct expressing IL-12, whereas the best protection against *L. major* was achieved by co-administration of the *L. amazonensis* P4 and hsp70 genes. Co-injection of the plasmids expressing cysteine proteases *cpa* and *cpb* induced a protective response, whereas separate injections were not protective [69]. Similarly, co-administration of four plasmids expressing the *Leishmania* core histones (H2A, H2B, H3 and H4) induced solid immunity that protected mice against *L. major* infection [70]. Moreover, genetic immunization of BALB/c mice with the individual histones only resulted in a delay in lesion development, whereas immunization with any one of the plasmids encoding a pair of histones provided stronger, although still partial protection against *L. major* infection compared to the combination of the four histones [71].

DNA vaccination has proven to be a successful vehicle for protection against viscerotropic species. The DNA vaccine based on the *Leishmania* A2 gene has been found to provide significant protection in mice not only against *L. donovani* infection [72] but also *L. chagasi* and *L. amazonensis* [73]. It has been also shown that BALB/c mice injected intramuscularly with ORFF DNA and challenged with *L. donovani* resulted in a decreased parasite burden in the liver and spleen [74]. Moreover, administration of IL-12 DNA as an adjuvant with recombinant ORFF protein induced a significant protection in the same mice model [75]. DNA vaccines encoding KMP-11 gene induced protective immunity against *L. donovani* in a hamster model [76]. Another DNA vaccine based on nucleoside hydrolase (NH) antigen, initially identified in *L. donovani* as a major fraction of the FML antigenic complex, showed strong immuno-protection against visceral and cutaneous leishmaniasis, suggesting the cross-protective efficacy of this vaccine [77]. The NH36 DNA vaccine was also highly effective in immunotherapy against *L. chagasi* and provided a new tool for the control of visceral leishmaniasis [78].

DNA vaccination against *Leishmania* is considered a promising technology, but no development of such a vaccine for use in humans has been reported so far. Conflicting reports as to the protective efficacy of the antigens delivered through this mode add to the confusion in the field. To complicate issues further, protective outcomes seem to be influenced by many factors, including plasmid backbone, number of injections, challenge dose and virulence of the

leishmanial strain, developmental stage of the parasite (promastigote vs. amastigote), experimental protocol employed, immunomodulators and type of animal model. Therefore, it is not surprising that the initial enthusiasm has been tempered by the complexities and difficulties that have surfaced.

3.5 Heterologous prime–boost strategy

DNA vaccines, recombinant viral vectors and recombinant proteins are all effective antigen delivery systems for inducing cellular immunity; however, when used alone, the levels of specific responses they induce are low. Prime–boost immunization strategies involve priming with DNA and boosting with recombinant viral vectors or recombinant proteins, each encoding the same antigen, some weeks apart. Such strategies have been shown to enhance cellular immunity in several different animal and disease models [54].

Recombinant vaccinia virus recombinants (rVV) expressing different foreign antigens have been used successfully to elicit protective immunity to a variety of pathogens. The strategy based on DNA priming followed by a booster with a rVV expressing the same parasitic antigen has been evaluated in animal model systems against parasitic infections, such as malaria, which markedly enhances specific cellular immune responses and leads to protection [79]. Priming with a DNA vector expressing LACK followed by boost with a replication competent rVV expressing the same antigen is effective in triggering a Th1 response and protection against cutaneous leishmaniasis in the murine model [80]. These results can be improved by the co-delivery of IL-12 and IL-18 in the priming [81]. A heterologous prime–boost regimen using DNA and the replication-competent Western Reserve (WR) strain of vaccinia virus expressing the LACK antigen was recently explored in canine visceral leishmaniasis and found to be protective [82]. Due to safety concerns surrounding the use of replicating live virus-based vaccines, different vectors based on attenuated viruses have been developed. Modified vaccinia virus Ankara (MVA) is considered to be the current VV strain of choice for clinical research. MVA is unable to replicate productively in human cell lines and primary human cell cultures. Despite this growth deficiency, MVA can efficiently express viral and recombinant proteins as efficiently as more virulent VV strains and has been found to be as an efficient vector for vaccination in a murine model of cutaneous leishmaniasis [83]. The efficacy of a heterologous prime–boost vaccination using DNA and two types of vaccinia viruses WR and MVA expressing the LACK antigen was compared in an intradermal murine infection model employing *L. infantum*. Comparable levels of protection were found for mice boosted with either LACK–WR or LACK–MVA, supporting the use of an attenuated vaccinia virus-based vaccine against visceral leishmaniasis [84]. In contrast, a clear advantage was shown for MVA–LACK as a vaccination vector against canine visceral leishmaniasis [85]. Boosting with recombinant *Salmonella* expressing LACK following a priming injection with DNA also conferred protection against infection and skewed responses towards Th1, thus enhancing the protection observed upon immunization with DNA or *Salmonella* alone [86]. In another strategy, immunization with cocktail of plasmids DNA encoding type I (*cpb*) and II (*cpa*) cysteine proteases followed by a boost with rCPA/rCPB protein, in addition to CpG ODN and Montanide720 as adjuvants, conferred protective immunity in murine and canine visceral leishmaniasis challenged with *L. infantum* [87,88]. This prime–boost vaccination was also found to be effective against *L. donovani* infection in BALB/c mice model immunized with the plasmid carrying the gene for ORFF (F/pcDNA) and given a booster dose of the rORFF protein vaccine. However, the protective response induced in the prime–boost group was not more than that elicited in the DNA vaccine group [89]. Thus, the prime–boost immunization strategies are an effective way to induce high levels of cellular immunity against a number of pathogens, including *Leishmania*. Further work on the mechanism of action of this promising strategy will allow optimization of this vaccination strategy, and ultimately the development of an improved vaccine against leishmaniasis.

3.6 Dendritic cells as a delivery system

Recent studies have begun to uncover the importance of DCs in the initiation and regulation of antimicrobial immune response. DCs secrete different cytokines (IL-1, -6 or -12) which attract $CD4^+$ T cells and modulate the type of developing T cell response. In addition, to gain effector functions, Th cells and cytotoxic T lymphocytes need to be activated by professional APCs, in particular DCs. Thus, the central role of DCs in orchestrating cell-mediated immunity converts these cells as vaccine carriers and natural adjuvants for the induction of protective immune response against leishmaniasis. It has been found that Langerhans cells (LCs), a member of the DC family, pulsed *in vitro* with a *L. major* crude extract, induced long-lasting protection of BALB/c mice against subsequent challenge with *L. major* [90]. Similarly, adoptive transfer of DCs pulsed with soluble *L. donovani* antigens (SLDA) to naïve mice induced protection in the mouse model of visceral leishmaniasis [91]. In addition, SLDA-pulsed DCs were also effective in treating an established visceral infection. Remarkably, total protection was observed engaging antigen-pulsed DCs previously engineered to secrete murine IL-12 [91]. The protective effect of LCs loaded with a set of *Leishmania* antigens – LACK, gp63, PSA, KMP-11 and LeIF – was tested following adoptive transfer into naïve mice, and these cells were shown to mediate significant protection against challenge with *L. major* [92]. The type of DC stimulus is a key factor in determining the capacity of DCs to promote an effective Th1 response and protection against *Leishmania*. Thus, antigen-pulsed bone marrow-derived DC (BMDC) stimulated

by prior *in vitro* exposure to CpG-containing oligodeoxynucleotides (ODN) developed an antigen-specific Th1 response and solid and long-lasting protection against *L. major* [93]. Surprisingly, IL-12 expression by the immunizing BMDC was not required for the induction of host resistance. In contrast, availability of IL-12 derived from recipient cells was essential for the initial triggering of protective immunity by transferred BMDC [93]. In a new approach DCs loaded with a mixture of the *L. infantum* histone proteins pulsed with CpG motifs conferred control to *L. major* infection in BALB/c mice. Importantly, the protection was dependent on the ability to induce a low frequency of Foxp3$^+$ regulatory T cells at the site of infection [94]. Thus, DC vaccination is a promising tool for inducing anti-leishmanial immunity, provided that the feasibility of large-scale delivery of such a vaccine can be addressed. If a widely accepted standard protocol for the production and preparation of DC-loaded vaccines can be developed, DC immunization could become a useful tool for the preclinical evaluation of vaccine candidates.

3.7 Liposomes

Traditionally, liposomes are composed of mainly phospholipids with an aqueous phase inside the particle made up of a lipid bilayer. For vaccine delivery, an antigen (or adjuvant) may be either encapsulated in the core of the liposomes, buried within the lipid bilayer or adsorbed on the surface for presentation to APCs. The advantage of the use of liposomes as delivery vesicles includes their ease of preparation, low toxicity and biodegradability. In addition to mediating efficient delivery, liposomes have also been shown to have immunogenic properties [95] and found to be protective when used as adjuvant with *Leishmania* antigens [96,97]. Their efficacy, however, depends on several physical parameters, including size, surface charge of the liposomes, phospholipid composition and stability of the vesicles. Liposome stability can be improved by the substitution of the basic lipid, underivatized L-α-phosphatidyl choline (egg lecithin, liquid crystalline transition temperature [Tc] = -10°C) with phospholipids having high Tc such as distearyol phosphatidyl choline (DSPC; Tc = 54°C), dimyristoyl phosphatidyl choline (DMPC; Tc = 23°C) or dipalmityol phosphatidyl choline (DPPC; Tc = 41.5°C). Liposome vesicles prepared with DSPC were as efficient as DPPC and DMPC derivatives in the entrapment of antigens and in their ability to stimulate a humoral response [98]. However, the superiority of DSPC liposomes in comparison with DPPC and DMPC was observed in their ability to potentiate cell-mediated immune responses which can be correlated with low level of parasitemia in the spleens of hamsters immunized with antigens of *L. donovani* promastigotes (LAg) in DSPC liposomes [98]. Further, LAg entrapped in DSPC liposomes stimulated a mixed Th1/Th2 response causing resistance in BALB/c mice against a progressive infection by *L. donovani* [99]. Non-phosphatidylcholine (non-PC) liposomes (escheriosomes) prepared from *E. coli* lipids are also useful to entrap *L. donovani* promastigote soluble antigens (sLAg) and have the potential to deliver the antigen to APCs. Thus, the delivery of sLAg via escheriosomes elicited parasite-specific CD8$^+$ and CD4$^+$ T cell immune response and protected hamsters against visceral leishmaniasis [100].

Other than the composition, the surface charge has a major influence on the adjuvant effect of the liposomes. Importantly, we have reported that that cationic liposomes formulated with stearylamine are superior as adjuvants as they induced greater extent of protection against *L. donovani* compared to anionic and neutral liposomes [101-104]. However, there was no preferential entrapment of antigens when soluble leishmanial antigens (SLA) from *L. donovani* were used. Although the total amount of associated antigen per mg egg lecithin was highest in cationic liposomes (35 μg), followed by neutral (30 μg) and anionic liposomes (25 μg), the antigens entrapped within the liposomes were highest in negatively charged liposomes (81%) followed by positive (75%) and neutral liposomes (65%) [104]. These cationic liposomal formulations with SLA were also effective when used in immunotherapy in BALB/c mice with established visceral infection [104]. Formulated with DSPC, cationic liposomes entrapped gp63 could induce a protective response to visceral disease in BALB/c mice not only after a short vaccination protocol but also 12 weeks after immunization by generating a solid and durable immunity [105]. It is well established that liposomes channel protein and peptide antigens into the major histocompatibility complex class II pathways of APCs, resulting in enhanced antibody and antigen-specific T cell proliferative responses [106]. Cationic liposomes target antigens for endocytosis by APCs more efficiently as the cationic entities interact with the negatively charged molecules on the surface of APCs. Thus, anionic liposomes interact with a very low percentage of human and murine DCs *in vitro*, whereas the opposite is the case for cationic liposomes leading to dense intracellular localization of the liposomes [107]. In addition, there have been reports of the use of cationic liposomes for the generation of CD8$^+$ T cell response, which requires antigen presentation in the context of the major histocompatibility complex class I pathway [108,109]. Stable liposomes formulated with DSPC, a saturated phospholipid with a high transition temperature, reduces clearance from blood and enhanced cationic lipid-mediated endosomal membrane destabilization [110]. Thus, gp63 containing cationic liposomes formulated with DSPC may serve to protect the antigen further and thereby improve antigen-delivering capacity, resulting in the generation of both CD4$^+$ and CD8$^+$ response [105].

The immunopotentiating activity of liposomes also depends on the method of preparation. A study was carried out in our laboratory to compare the adjuvanticity of different types of DSPC cationic liposomes prepared by different methods. It was found that the entrapment efficiency was

highest in dehydration–rehydration vesicles (DRVs), compared to conventional or reverse phase vesicles (REVs). These vesicles with entrapped antigens were also efficient in protecting BALB/c mice against challenge infection, which was comparable to conventional liposomes (unpublished data). Thus, DRV vesicles may be the best choice for the incorporation of purified proteins as they cause the minimum loss of protein.

Cationic liposomes are commonly combined with immunomodulators to enhance and bias the immune response in the desired direction. Immunomodulators may be lipid structures, MPL or CpG. In GlaxoSmithKline's adjuvants AS01, MPL is incorporated in liposomes as an immunomodulating agent. However, the combination of MPL with liposomes has not yet been used against leishmaniasis. Recently, studies from our laboratory revealed that MPL mixed with cationic liposomal SLA induced significant protection when a subcutaneous route of immunization was used (unpublished data). Earlier we observed that liposomal SLA failed to elicit protection through the subcutaneous route. Thus, the use of MPL can help to overcome the need for the protective intraperitoneal route of immunization with subcutaneous route, which is preferable for clinical trials. Non-coding plasmid DNA (pDNA) bearing immunostimulatory sequences (ISS) are a potent activator of innate immunity, and can thus act as an adjuvant with vaccine antigen. Presumably, CpG motifs that are present in the plasmid backbone act as adjuvants in a fashion similar to CpG–ODNs. The adjuvant activity of pDNA can be augmented by using liposomes as a delivery system. As nucleic acids are negatively charged, the inclusion of cationic lipids in the liposome formulation results in a strong association of the nucleic acids with the liposomal carrier, facilitating humoral and cell-mediated immunity [111]. Thus, SLA in association with cationic liposomes and non-coding pDNA bearing ISS elicited impressive protective responses over the cationic liposomal SLA against visceral leishmaniasis [112]. As a vehicle, cationic liposomes with pDNA either complexed or entrapped within, significantly increased the potentiating effect of pDNA. Further, comparison of the two vaccine formulations demonstrated an impressive increase in the protective efficacy up to twofold when both antigen and pDNA were within the vehicle [112]. Other than non-coding plasmid DNA co-encapsulation of CpG ODN with recombinant gp63 in cationic liposomes formulated with dimethyldiocta-decylammonium bromide (DDAB) enhanced immune response and protection in immunized BALB/c mice against cutaneous leishmaniasis [113].

From all these findings it appears that stable cationic liposomes formulated with DSPC and combined with different immunomodulators appear to be the best delivery vehicle to initiate protective immunity against leishmaniasis. Further improvements in the design of vaccine delivery vehicles based on cationic liposomes will require understanding of the effects of size, charge and lipid formulation on the generated immune response.

4. Conclusions

Vaccines are recognized as the best and most cost-effective protection measure against pathogens, and *Leishmania* is no exception. *Leishmania* vaccine development has proven to be a difficult and challenging task, one of the major factors being the search for a delivery system that can promote Th1-related protective responses. MPL, the T cell stimulating adjuvant, is already in human trials against leishmaniasis and may be a practical solution for the search of a vaccine adjuvant. In addition, CpG, which is in clinical vaccine trials for HIV and cancer patients [114], may also be an option for leishmaniasis. Interestingly, liposomes are the most versatile delivery vehicles and by changing the different parameters, such as size, charge and composition, the quality of the generated immune responses can be changed. More specifically, cationic liposomes formulated with DSPC were the most useful delivery vehicle to carry not only the antigens but also a variety of immunostimulating adjuvants. These cationic liposomes target the DCs and other APCs to stimulate a protective immune response against *Leishmania*. Thus, it is likely that liposomes will play an important role in the development of a new generation of vaccine adjuvants against leishmaniasis.

5. Expert opinion

Vaccines are considered by many to be one of most successful medical interventions against infectious diseases, including *Leishmania*. In designing effective vaccines an antigen is required, against which a memory response is targeted. But antigens are seldom sufficiently immunogenic by themselves. Hence coadministration of an effective adjuvant is crucial to activate the innate immune responses to aid in the generation of robust and long-lasting adaptive immune responses. One role of the antigen delivery systems is to recruit and present antigens to APCs and improve their ability to activate, expand and drive the differentiation of effector T and B cells. Liposomes have been extensively used as delivery vesicles and liposomes, in particular cationic liposomes, by themselves have immunological properties. Some adjuvant activity can be accounted for by the fact that positively charged liposomes can directly activate DCs [115]. Cationic liposomes also affect the route of antigen uptake by APCs, inducing efficient uptake via endocytosis. Charged liposomes can also serve another important function when used as adjuvants in vaccines intended for immunization against *Leishmania* infections, where $CD8^+$ T cells may be critically important. Cationic liposomes promote the entry of proteins into the cytoplasm of APCs, which results in cross-priming, or the induction of $CD8^+$ T cell responses against exogenous protein antigens [109,115]. The quality of

Table 2. The advantage of cationic liposomes as a future vaccine delivery system.

Cost-effective and biodegradable
Stability can be improved by using phospholipids having high Tc such as DSPC and apolar lipid analog TDB
Quality of the innate immune response and, subsequently, the adaptive response, can be modulated by targeting PRRs, mostly endosomally located TLRs like TLR9, TLR3 and TLR7/8 by using their agonists
Can be developed as a needle-free vaccine delivery system for topical and mucosal routes of immunization

the innate immune response, and thus the ensuing adaptive response, can be modulated if PRRs on most innate immune cells are triggered. The PRRs include TLRs, C-type lectin receptors, mannose receptors and nucleotide binding oligomerization domain (NOD) proteins. These receptors recognize PAMPs, which are conserved molecular structures expressed by the pathogens. The new generation of adjuvant systems may be composed of combinations of agonists for PRRs, including the TLRs and NOD, expressed on most innate immune cells. For certain PRR agonists, particularly those whose receptors are located intracellulary, liposomal delivery offers attractive advantages in terms of efficiency and selectivity of targeting. At present, 13 different TLRs have been described in humans and mice. In addition, natural (microbe-derived) or synthetic agonists have been identified for nearly all of the known TLRs [3]. In *Leishmania* this has particularly been investigated with CpG, TLR9 agonist. Cationic liposomes encapsulating CpG potentiated the activation of protective immunity against *Leishmania* [113]. Presently, a TLR4-based vaccine adjuvant (MPL) is in clinical trials for vaccines against *Leishmnia* [33]. Very recently, imiquimod, a TLR7/8 agonist approved for topical treatment in humans, has represented a potential Th1 promoting vaccine adjuvant when delivered topically with autoclaved *L. major* (ALM) [116]. Cationic liposomes can be used to potentiate immune activation by targeting mainly endosomally located TLRs, TLR9, TLR 7/8 by using their agonists, including the TLR3 agonist polyI:C [3]. It can be also speculated that agonists and/or ligands for innate immune receptors other that TLRs are important targets for immune activation and can constitute an interesting new area in adjuvant research.

In the development of adjuvants for tropical diseases, including *Leishmania*, cost, stability and dependence on a cold chain are the key issues for consideration. Thus, despite the good immunological prospects of liposomes as adjuvant against leishmaniasis, for their human use the above factors, along with the regulatory hurdles, need to be overcome. Preparation of liposomes can be cost-effective when large-scale production is considered. The stability of the liposomes can be improved by formulation with phospholipids having a high Tc, such as DSPC (Tc = 54°C). Therefore, it is encouraging to find that cationic liposomes formulated with DSPC could increase the protective immune response against *L. donovani* infection, suggesting its superiority over other formulations [105]. Incorporations of apolar lipid trehalose 6,6′-dimycolate (TDM) or its synthetic analog TDB in the cationic liposomal formulation can enhance immunogenicity by potentiating both cell-mediated and humoral responses. Moreover, the use of TDB can stabilize the liposomes and prevent aggregation during prolonged storage at 4°C. These TDB-formulated liposomes can be freeze-dried using cryo-protectors like trehalose [115]. Additionally, the development of vaccines for needle-free immunization will probably be a high priority area in the near future. Liposomal vaccines can be used in topical [117] and mucosal immunization [118] mainly administered orally or nasally without a needle and syringe. Therefore, cationic liposomes can be formulated to suit all these requirements for new vaccines against leishmaniasis (**Table 2**) and therefore can be a powerful tool for enhancing the effect of antigens, as well as immunomodulators. Liposomal drugs like Ambisome are already on the market for human use against leishmaniasis. Thus, the application of liposomal vaccines in veterinary trials will open the door and subsequently include these formulations for human trials.

An increasing knowledge of cell receptor biology including agonists and/or ligands for different TLRs and other PRRs has paved the way for a new generation of adjuvants. Moreover, stimulation of distinct immunological profiles with different balances of Th1, Th2 and/or cytotoxic T lymphocyte stimulation are required for effective vaccine against different species of *Leishmania*. Thus, an efficient delivery system is a crucial tool for targeting specific receptors by designing their agonists for increasing the uptake of vaccine antigen by the relevant cells and thus enhancing the effect of specific immune modulators. Therefore, in the struggle to design these smart new generation adjuvants, cationic liposomes may serve as the most efficient, by fulfilling many of these requirements.

Declaration of interest

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