Review: Clinical Trial Outcomes

CLINICAL INVESTIGATION

Leishmaniasis: recombinant DNA vaccination and different approaches for vaccine development

Clin. Invest. (2013) 3(11), 1023–1044

Designing an ideal vaccine against leishmaniasis using a suitable candidate antigen and appropriate antigen delivery system to induce the accurate type of immune response is a process still under investigation. There are some different strategies applied for vaccination against leishmaniasis including conventional and modern (genetically-modified vaccines) approaches. The type of induced immune response for each vaccine depends on the type of vaccine and route of administration, the amount and nature of the antigen/s, as well as adjuvant (if used). Recombinant DNA technology or genetic engineering has accelerated the identification of new types of prophylaxis approaches using crude lysates to single or multi-proteins, epitopes and DNA vaccines that are capable of creating less or more partial protection. At present, a few limited vaccine formulations are licensed in Brazil and Europe just for canines. Vaccine development for humans and even for canines is under serious study. In this review, we focus on current and suggested vaccination approaches based on recombinant DNA strategies against leishmaniasis.

Keywords: cocktail vaccines • DNA vaccine • leishmaniasis • prime–boost vaccination • recombinant DNA vaccines

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Infection by *Leishmania* protozoan parasites causes leishmaniasis, which is associated with relatively high rates of morbidity and mortality worldwide. It is still a global challenge and serious health problem for humans and some animals in many endemic countries [1]. There are different clinical forms of leishmaniasis including cutaneous leishmaniasis (CL), mucocutaneous leishamaniasis and visceral leishmaniasis (VL). It is known that the clinical manifestations of leishmaniasis are dependent on the interactions between the parasite species and host immune system [2]. Leishmaniasis creates more serious health problems in people suffering from other infectious diseases, such as HIV, and even noninfectious diseases, such as cancer.

According to a WHO report, leishmaniasis is endemic in 98 countries on five continents [3]. The estimated incidence is 0.2–0.4 million VL cases and 0.7–1.2 million CL cases [3]. This disease is one of the most important neglected diseases and is ranked the second in mortality rate and the fourth in morbidity rate among the tropical infections [4]. Due to infection of internal organs, symptoms of VL are more severe than CL. More than 95% of global VL cases occur in six countries: India, Bangladesh, Sudan, South Sudan, Brazil and Ethiopia. CL is more widely distributed in the Americas, the Mediterranean basin, and western Asia from the Middle East to central Asia. The ten countries with the highest estimated case counts are Afghanistan, Algeria, Colombia, Brazil, Iran, Syria, Ethiopia, Sudan, Costa Rica and Peru [3]. The people living in endemic areas, war zones or suffering from poverty, especially in third-world countries, account for the majority of the high-risk population who

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need an executive control program for prevention of leishmaniasis and other infectious diseases.

Annually, the emergence rate of new cases of leishmaniasis is widely increasing, because there is no successful program to control the disease. There are several factors that complicate the control of leishmaniasis, such as lack of an effective vaccine or safe therapy, resistance to current antileishmanials, probable transmission through tourists and change in geographic distribution of the insect habitats due to global warming [5].

In general, *Leishmania* has two different vertebrate hosts – humans and animals. In addition, the dimorphic life cycle in different species of parasite make *Leishmania* a very complex microorganism. Usually, the infection is transmitted from animal to animal/human by the bite of an infected female sandfly. However, some other rates of transmission are reported, such as human to human transmission of *Leishmania*, as is the case with *Leishmania donovani* [4,6] and *Leishmania tropica*.

L. tropica infection has been described as anthropozoonosis, but some cases of canine infection have been reported [6]. Actually, cell cycles of all species are not completely characterized. The human is generally considered as a second (or accidental) host [7] for some *Leishmania* species (e.g., *Leishmania major* and *Leishmania infantum*) and is the unique host for some other species, such as *L. donovani*.

Post-kala-azar dermal leishmaniasis is a second outcome of *L. donovani* infection in some individuals, which may occur either during or after treatment [8]. Hence, vaccination of both humans and animal reservoirs, especially dogs for VL, are the two main rational main missions for controlling the disease. Induction of stable immunological memory in humans after natural infection shows that antiparasitic vaccine development is possible. So far, various forms of vaccines have been developed and a few formulated vaccines are licensed for pets (Leishmune® vaccine [since 2004 in Brazil], LeishTec® [since 2008 in Brazil], CaniLeish® [since 2011 in Europe]), although there is still no licensed (approved) commercial vaccine for human use [1,9].

The existence of several infectious species of *Leishmania* and different forms of the disease has made it difficult to design a vaccine against *Leishmania* and more knowledge of the vaccine, further investigations and experiences are required. Another problematic issue in vaccine development is differences in known virulence factors between different species [10]. For example, *L. major* in the old world and *Leishmania mexicana*/*Leishmania amazonensis* in the new world are CL-causing species; however lipophosphoglycan (LPG) is a potent virulence factor for *L. major* but not for *L. mexicana* [11]. In addition, reservoirs of all *Leishmania* species are not the same. *L. donovani* and *L. infantum* are both causative agents for VL, but humans are the only known hosts for *L. donovani*; while *L. infantum* is primarily a zoonotic disease and canine species are the main animal reservoir [8]. Reservoir animals are usually used as animal models for experimental studies. *L. major* and *L. tropica* are causative agents for the cutaneous form of disease. For many years, BALB/c mice have been used as the best model for CL caused by *L. major*. However, some scientists believe that due to high susceptibility of BALB/c mice to the cutaneous form of leishmaniasis, they are not a suitable host to test the protective effect of antigens [12]. The animal reservoir for *L. tropica* is still unknown and debatable. Furthermore, human heterogeneity is another major obstacle. The severity and symptoms of disease are not the same in different infected individuals. However, there are still many known and unknown factors resulting in the lack of a good strategy to prevent this disease. There are some major questions that should be considered in order to select the best strategy, as demonstrated in Figure 1.

Due to the mentioned reasons, vaccine development for leishmaniasis is a more difficult task than for other organisms such as bacteria and viruses [10,13].

To design a potential vaccine, three main factors should be considered: identification of a suitable antigen/s; using appropriate antigen delivery route and a potential adjuvant. Nowadays, advances in molecular biology knowledge help us to identify some potential virulence factors for prophylactic or diagnostic use.

Recombinant DNA technology allows scientists to identify and transfer different genes to prokaryotic and/or eukaryotic hosts such as bacteria, yeast and even *Leishmania* parasites to express protein (transiently or permanent) and release it in different conditions, such as secretory form or inclusion bodies. Most of these antigens have conferred effective immune responses when delivered as a vaccine in different animal models. In clinical trials, however, the results showed weaker protection when the same antigens or vaccines are used. Development of molecular tools also allows manipulation of parasite genome, disruption and insertion of interested (target) genes to create new lines of parasites as live vaccines.

In this review, we will first provide a short history of vaccines, followed by new approaches to vaccine development for leishmaniasis.

Immune response of host against *leishmania*

During leishmaniasis a collection of immune cells and immunomodulators, cytokines and antibodies are involved in combating the parasite. Result of this fight is either removing the parasite and establishment of a long-lasting immunity or survival of parasite in the macrophages and disease progression. However, this

depends on the type of parasite, activation of $CD4^+$ and $CD8^+$ T cells and secreted cytokines. Immunity to leishmaniasis is mediated by both arms of the immune systems: innate (by neutrophils, macrophages, natural killer [NK] and dendritic cells) and adaptive (T cells) responses [14].

Neutrophils are the first line of host immune defense and primary effector cells of the innate immune system against entrance of *Leishmania* and induction of inflammatory reactions [15–17]. After infection, these cells produce higher levels of IL-8 that act as mediators to recruit more neutrophils.

Progression of disease is associated with inability of neutrophils to kill the parasite. NK cells are another component of the innate immune response and the primary source of IFN-γ and IL-12. In addition, IFN-γ, TNF and IL-12 are among other stimulator molecules for NK cells that migrate to the infection site [18]. The second group of cells involved in immunity are monocytes/macrophages. In leishmaniasis, the host immune system produces IL-12, which is essential for cellular immunity through macrophages and dendritic cells.

T-cell responses are very critical to protect against intracellular pathogens like *Leishmania,* which induce both innate and adaptive immune responses [19].

Two major, distinct $CD4^+$ T-cells, Th1 and Th2 are known. Many studies have shown that recovery and protection against most intracellular pathogens, such as leishmanial infection, directly depends on induction of a Th1 type of immune response, while Th2 responses are associated with nonprotective or nonhealing prognosis. Some cytokines, such as IFN-γ, TNF-α and IL-12, are hallmarks of the Th1 response, while IL-4, IL-5 and IL-10 are markers to identify Th2 response [20]. Most studies on vaccination against *Leishmania* have been done in the murine model. In mice models, development of an immune response depends on genetic background of animals and *Leishmania* species. Infection course and clinical signs in C57BL/6 mice are very mild because parasite replication is controlled efficiently, whereas BALB/c mice develop a severe course of disease [17,21]. In BALB/c mice, nonhealing and progressive infection is associated with lack or lower level of IFN-γ production and also increased production of IL-4, IL-5 and IL-10, and the level of IL-10 is critical in vaccine failure or success [22].

The other important issue in vaccination against leishmaniasis is maintenance of immune memory that is responsible for long-lasting immunity provided by T-memory cells. Several studies have shown that $CD8^+$ T cells are responsible for controlling leishmaniasis and inducing adaptive immunity, since depletion of $CD8^+$ T cells abolishes the generated protection in the vaccinated mice [23–25]. Therefore, a safe and effective vaccine should be able to increase significant levels of $CD8^+$ cytotoxic T lymphocytes [26].

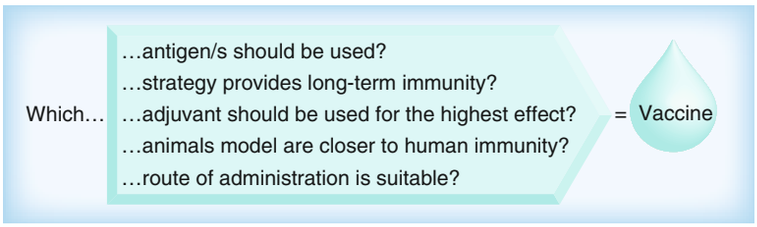

Figure 1. Major questions that should be considered in order to select the best strategy for vaccine design.

Short history of traditional & first-generation vaccines against leishmaniasis

Up to now, several attempts have been done with different vaccination approaches in human and animals, especially canines, including traditional live vaccines (leishmanization) and whole killed vaccines. Each approach has some benefits and risks (Table 1). One of the important advantages of live vaccines is generation and maintenance of the immunological memory during *Leishmania* infection [27], although it has some ethical issues for routine clinical use. Recently, live vaccines have been classified into two main groups: conventional live vaccine and genetically attenuated live vaccines. There is another new approach where nonpathogenic species of *Leishmania* are used. Leishmanization and live attenuated vaccines are examples of conventional vaccine.

■ Leishmanization (using live virulent promastigotes)

For many years, leishmanization as a vaccination method from the first-generation of live vaccines was the only way to control cutaneous form of disease. In this method, some covered parts of the body are inoculated with live virulent promastigotes. In spite of its dangers (Table 1), it is still used in humans only in urgent conditions in endemic areas such as Uzbekistan, Afghanistan, Iraq and Iran [28]. Leishmanization was used during the Iran–Iraq war but after that time it stopped due to probable dangers [29].

■ Live inactivated vaccines

Live attenuated vaccines are derived from a wild-type parasite grown in natural conditions, followed by some modifications to attenuate or weaken the parasites, usually by repeated culturing, chemical reagents, heat or radiation. These parasites are avirulent and cannot generate disease but retain the ability to grow, replicate, trigger the immune system and revert to virulent form. Within traditional methods, live vaccines were

Table 1. Advantages and disadvantages of conventional and modern vaccination strategies.

Type of vaccine	Advantages	Disadvantages
Live vaccines (leishmanization)	Single dose, long-term immunity Stimulates immune system naturally Produces strong immunity Elicits both humoral and cellular immune response Multiplication in the host Induces innate and adaptive immune responses Induces $CD4^+$ and $CD8^+$ T cells	Not safe Has side effects Uses whole pathogen Generation of unfavorable immune response
Killed vaccines	No risk and safer than live vaccines Inexpensive No amplification in the host	Less powerful than live vaccines Requires multiple doses to have significant reactogenicity Increased risk of allergic reactions
DNA vaccines	Safe to use, no adjuvant needed Stable at room temperature and shipment is inexpensive Easy for manipulation and production Elicits antigen-specific immune responses Induces both $CD4^+$ and $CD8^+$ T cells Elicits both humoral and cellular immune response Due to CpG motifs, are immunomodulators Prime antigen-specific memory T cells	Low potency in humans Often weak immunogenicity
Recombinant vaccines	No risk No interference with the maternal immunity and/or other vaccines Induces strong immune response	Needs cold chain for transportation Expensive, no biological activity No native conformation Needs adjuvant to enhance immunogenicity Contamination with bacteria substances Large-scale preparation is hard Needs a large amount of antigen to stimulate immunity
Prime–boost vaccine	Long-lasting response Antigen-specific memory T cells	Needs cold chain for transportation
Live genetically modified vaccines (avirulent)	Stable Safer than general live vaccines Stimulate immune system as in natural infection Multiplication in the host	Risk of reversion to virulent state Needs cold chain for transportation Unknown memory formation and duration Stimulates a weaker immune system response Generation of unfavorable immune response
Live non-pathogenic vaccines (avirulent)	Safe to use Life-long immunity No reversion to virulent state Stimulate immune system similar to natural infection Multiplication in the host	Unknown memory formation and duration Need cold chain for transportation

highly successful. These vaccines induce T-cell-mediated immune responses through intracellular processing and presentation of antigens with MHC class I and II antigens, which is critical to induce protection against intracellular pathogens [19]. The great advantage of this type of vaccine is the eliciting of a strong cellular and humoral response like a natural infection in disease, but milder than wild-type lines, and conferring life-long immunity. In spite of this advantage, this type of vaccine has many drawbacks (Table 1). They need a booster shot to maintain immunity in individuals. Moreover, not all people can receive live vaccines, such as HIV-positive patients due to the damage in their immune system.

■ Killed parasite vaccine

Since 1930, whole killed vaccines have been used as an effective approach in both therapeutic and prophylactic vaccines [8]. Killed vaccines with or without adjuvant are considered as an old method in the first-generation category of vaccines. Nevertheless, remarkable results of vaccination of dogs with killed parasite vaccines show that it seems, in spite of many risks, still one of the best strategies for vaccination [30,31]. Advantage of this group

of vaccines is induction of both $CD4^+$ and $CD8^+$ T-cell responses, which require both endogenous as well as exogenous antigen processing and presentation of antigens with MHC class I and II molecules [19]. In addition, due to stability of their biochemical composition and antigenicity, lower cost and higher safety compared with live vaccines, scientists pay more attention to these vaccines [30]. However, killed vaccines cannot stimulate the immune response as efficiently as live vaccines, so they need a booster to have a significant effect. These vaccines could increase the risk of allergic reactions due to large amounts of antigen involved (Table 1).

In Uzbekistan, to decrease the danger of using live vaccines, a mixture of live virulent *L. major* with killed parasites has been used as a prophylactic vaccine [29]. In Venezuela, autoclaved killed *L. mexicana* is used to treat patients with CL in specific condition [29]. This vaccine for CL has shown various results in different countries. However, in Phase III of clinical trials in Colombia [32,33], Ecuador [32,34] and Brazil [32], they showed low efficacies against *L. amazonensis*. Likewise, these kind of vaccines were not protective against *L. major* and *L. donovani* in Iran and Sudan, respectively [32].

Modern vaccination approaches against leishmaniasis

Recently, DNA sequence determination of the human genome as well as *Leishmania* provides new opportunities to identify novel approaches for vaccinology. Recombinant vaccines consist of recombinant protein vaccines, recombinant DNA vaccines, genetically modified live vaccines, recombinant viral-based vaccines, epitope-based or peptide vaccines and recombinant subunit vaccines that have been developed through recombinant DNA techniques. Table 1 shows a summary of advantages and disadvantages of different forms of vaccines.

Before emergence of recombinant vaccines, crude *Leishmania* antigens were used to immunize vulnerable individuals. Following development of recombinant DNA technology, different parasite components, especially essential genes for parasite survival, were focused on and their induced immune response in host has been characterized. In another approach, genetically attenuated live vaccines were made by knocking out of some main genes. Designing recombinant vaccines against *Leishmania* is based on fundamental information about genes involved in structure, metabolism and virulence. The main advantages of the recombinant vaccine technology are using just a single or a number of proteins (mixing or fusing together) from one or more species of an organism instead of the whole parasite cell antigens, so there is no risk of disease due to adverse virulent properties. There are reports suggesting that the use of several antigens is more effective than using a single antigen, due to possible synergistic effect of immune response. Therefore, these vaccines are safer than traditional vaccines. Another attractive aspect of modern vaccination is incorporation of immunologically stimulating proteins to increase the release of specific cytokines, though in some cases they have some limitations or disadvantages (Table 1).

Many antigens of different *Leishmania* species have been tested as recombinant proteins and DNA vaccine. However, none of them could elicit a perfect long-term protection and just generated a partial protection with different degree of immunity. Hence, researchers are using improved strategies to increase the duration of immune response in host, such as cocktail vaccines and heterologous prime–boost vaccination.

Importantly, other factors such as the type of selected animal with respect to high susceptibility/semisusceptibility/resistance to infection, the age of the animal, passage number of parasite in culture and type of infection are very critical. Lack of suitable animal model for some species, like *L. infantum* and *L. tropica*, do not allow precise studies with some antigens [14].

■ Recombinant protein vaccines (second-generation vaccines)

In recent decades, a large numbers of antigens from different strains of *Leishmania* have been identified and characterized. Some of these are considered to be an attractive vaccine candidate due to their critical role in entry and survival of the parasite. Among them, fucose/mannose ligand (FML; Leishmune vaccine) and A2 are successful examples from recombinant protein vaccines that achieved license for vaccination in dogs [5].

In spite of their limitations, proteins induce strong immune responses but shorter duration for protection against *Leishmania* infection. The recombinant proteins have no interference with the maternal immunity and/or other vaccines. These vaccines are safe due to their inability to replicate in the host; hence, a large amount of antigen is needed to stimulate immunity. Preparation of recombinant protein vaccines is very difficult and expensive since it needs purification systems and adjuvants or carriers to enhance immunogenicity [35]. The purified proteins are often associated with bacterial component after purification. In addition, these vaccines have no biological activity and native conformation, but some of the tested antigens showed a better ability to stimulate the immune system (Table 1).

As mentioned above, one of the major drawbacks of recombinant proteins is essentiality of using an adjuvant because the sole protein induces only Th2 response, while, coadministration of this type of

vaccines with an adjuvant shifts the immune response toward Th1. For example, although rLACK alone was not protective against *L. major*, rLACK plus rIL-12 as adjuvant was shown to induce partial protection in BALB/c mice [23]. In contrast, a similar immunization program (rLACK+rIL-12) could not generate protection against *L. amazonensis* infection [36]. This shows the species specificity of selected antigens in inducing immune response.

DNA vaccines (third-generation vaccines)

Parallel to development of protein vaccine, DNA vaccines encoding one or more immunogenic proteins have been introduced as a successful delivery system. In 1990, for the first time, RNA and DNA expression vectors containing reporter genes were introduced for injection into mouse skeletal muscle *in vivo* [37]. DNA vaccination holds considerable promise for vaccination against different diseases in which Th1 responses and cell-mediated immunity are responsible for generating protection, such as with leishmaniasis. During the last two decades, DNA vaccines have been developed against infectious microorganisms because this approach of immunization elicits both humoral and cellular immune response against native forms of protein, since antigens are produced in the host natural conformation due to post-translational modifications of the expressed proteins inside the cells. In addition, lower quantities of DNA should be used to induce protection. Of course, different elements could influence the magnitude of immune response generated, including immunogenicity of the desired expressed gene and synthetic oligodeoxynucleotides. The CpG motifs present in plasmid DNA are immunomodulators and by acting as an adjuvant switch the immune response toward Th1 [38]. In addition, immunization with DNA alone activates TLR9, whcih leads to high IL-12 production and finally a predominant Th1 immune response [39]. Another property of DNA vaccines is being more stable in shipping and transition. They are also cheaper than recombinant protein vaccines due to not needing any adjuvant and purification system or refolding procedures (Table 1).

In addition, a single multivalent DNA vaccine, in which a single plasmid would carry more than one gene encoding protective proteins, would be more cost-effective and easier to produce. On the other hand, naked DNA has a short lifetime within the cell since they get degraded by the enzymes. Some alternative methods such as coating DNA with nano- or micro- or gold-particles protect plasmids from degradation and increase phagocytic uptake by professional antigen-presenting cell [40]. A major drawback of DNA vaccines is their very low immunogenicity in humans when compared with animal models.

Recombinant protein vaccines versus DNA vaccines

There are a lot of debates over protein and DNA vaccination and their induction of natural immunity. Both DNA and protein vaccines have some advantages and limitations, as summarized in Table 1. There is a long list of candidate leishmanial molecules that were administrated as recombinant protein or DNA-based vaccines to evaluate prevention or reduction effect against CL and VL models with different adjuvants and routes of vaccination. However, none of them conferred complete long-term protection against leishmaniasis and often generated just partial protection.

Several reports with different antigens from different laboratories have confirmed that vaccination with DNA is more potent and induces a stronger immune response than recombinant protein alone or a combination of DNA and protein (prime–boost) [23,41–47]. However, there are some examples of failed DNA vaccinations using the *TRYP* gene alone [26] and γ-GCLC [48].

Many factors, such as nature [13], conformation [49] and dose of antigen, nature of used adjuvant, frequency and route of vaccine administration [13] and the time interval between prime–boost immunizations are very important and could have varying degress of influence on a protective level. Low immunogenicity of DNA vaccines is the main drawback of these vaccines when compared with protein vaccines [40]. To solve this problem, some strategies have been developed, such as cocktail vaccines, prime–boost vaccines and using novel adjuvant and delivery systems as good alternatives to increase the efficacy.

Some candidate molecules for vaccination

Many antigenic molecules as vaccine candidates have been prepared and tested by several groups against *Leishmania* infections and induced different levels of protection. Some of them were shown to induce protection against more than one *Leishmania* species. In addition, coadministration of different antigens in the same regime dramatically increased the potency of vaccine.

Leish-111f is a single polyprotein antigen composed of three fused components, *L. major* thiol-specific antioxidant (TSA), *L. major* stress-inducible protein 1 and *Leishmania braziliensis* elongation initiation factor [50–52]. These antigens are present in both amastigote and the promastigote forms and are highly conserved among different *Leishmania spp.* This was the first candidate for a subunit vaccine that was tested in Phase I and II clinical trials [45]. It has been shown that combined recombinant Leish111f with monophosphoryl lipid A-stable emulsion or IL-12 could protect various animals against *L. major*, *L. amazonensis* and *L. infantum* infection [51,53–55]. Furthermore, Leish-111f

vaccine has potential application in both prevention and treatment [56].

Leishmania homologue of receptors for activated C-kinase (LACK) is another attractive target that has been successfully used as an experimental vaccine against leishmaniasis. So far, this antigen is used frequently in vaccine studies against both CL and VL. Different studies have shown that LACK antigen is a protective antigen against *L. major* [23] and *L. amazonensis* [36] infection, but it did not confer significant protection against *L. donovani* infection [57].

Gp63 (leishmanolysin) is a zinc-metallo protease membrane surface glycoprotein expressed in promastigotes of different species of *Leishmania*. It has been successfully used as a candidate vaccine with different formulations in mouse models, and is a promising vaccine candidates against leishmaniasis.

FML is an antigenic glycoprotein complex from the promastigote form of *L. donovani*. This is the main antigen in Leishmune vaccine, the first commercial vaccine against VL. In several studies, immunization potential of FML was evaluated with different adjuvants, such as saponin and aluminium, and it demonstrated the ability to create strong protection and enhanced production of IgG in mice [58] and dogs [5,59,60]. In addition, the FML vaccine induced a significant long-lasting protective effect against canine kala-azar in the field [61,62]. Protection against canine kala-azar was also investigated in naturally exposed dogs vaccinated with the FML vaccine [59–60,63] and showed 95% seropositivity to FML and 100% intradermal reaction to *L. donovani* lysate 7 months after vaccination.

The nucleoside hydrolase (NH36) antigen is a 36-kDa surface glycoprotein complex and is the main antigen of the FML complex, which is identified in *L. donovani*, *L. chagasi*, *L. amazonensis* and *L. major* [64,65].

LPG is a dominant surface molecule expressed in all *Leishmania* species and is associated with several processes in promastigotes, such as parasite survival, interaction with host cell receptors, inhibition of midgut proteases, attachment and entry into the host macrophages and sand flies. Moreover, it also plays an important role in parasite resistance to complement molecules, manipulation of signal transduction pathways and gene expression in macrophages, and resistance to oxidative stress. Altogether, through all the mentioned pathways, this protein has a pivotal role in initiation and establishment of a durable infection [66–69]. In addition, it has been observed that LPG3 helps to synthesize the LPG, as the most important surface molecule in promastigotes [66,67]. LPG3 is the *Leishmania* homolog of the mammalian endoplasmic reticulum chaperone GRP94, a member of HSP90 family that is involved in assembly of LPG. LPG3 is highly immunogenic in BALB/c mice and can stimulate Th1 response against *L. major* in two regimens, DNA/DNA and prime–boost (DNA/protein) [70].

CPs are other vaccine candidates that belong to the group of papain-like enzymes and are expressed in the amastigote form [2,71]. CPs are among the important virulence factors that have several critical functions in establishment of infection [72,73]. There are different families of the CP group, including CPA, CPB and CPC in different species of *Leishmania*, which are characterized and the relation between their expression and parasite virulence is described [2]. Several early reports have highlighted the role of CPs in pathogenicity, invading the host cells, replication, and finally exiting the infected cells to establish further infections of *L. major* [24,74], *L. mexicana* [75,76], *L. chagasi* [77], *L. tropica* [78] and *L. (L.) amazonensis* [79] amastigote in mammalian host. Type I enzymes (CPB) are encoded by multi-copy genes (19 copies) and contain an unusual C-terminal extension about 110 amino acids, which is absent in other CPs of the papain superfamily [80]. The C-terminal extension has been postulated to be highly immunogenic and may play a role in the diversion of the host immune response [77,81–84].

The acidic ribosomal P0 protein of *L. infantum* is a structural component of the large ribosome subunit. Vaccination based on the ribosomal P0 protein-DNA or rLiP0 protein plus CpG oligodeoxynucleotides protected C57BL/6 mice from dermal pathology, accompanied by production of IFN-γ and reduced parasite load, but was not able to prevent the progressive disease in BALB/c mice, despite the induced Th1 immunological response in both models [12].

A2 was identified for the first time in *L. donovani* and is one of the excellent candidate antigens against VL identified so far and tested in different species of *L. donovani*, *L. infantum* and *L. chagasi* [85]. A2 genes are conserved in species of the *L. donovani* complex [36]. Attention to this antigen is due to its amastigote-specific expression in *L. donovani*. It is required for survival of VL-causing parasites in visceral organs, but it is present only in a truncated form in *L. major* [86] and is absent in *Leishmania tarentolae* [87], which is a member of nonpathogenic species. Immunization of mice with A2, in recombinant protein or DNA vaccination form, leads to protection against *L. donovani* infection [88,89]. At present, LeishTec® is one of the commercially available vaccines on the market that is based on A2 antigen. This vaccine is a recombinant A2-antigen of *Leishmania* amastigotes plus saponin as adjuvant [85].

Finally, kinetoplastid membrane protein-11 is a highly conserved membrane protein with high epitope density. This hydrophobic protein has been described to be associated with LPG. The protectivity effects of

this antigen as a DNA or protein alone or combined with some adjutants have been shown protection against two prevalent forms of leishmaniasis, CL and VL [90,91].

Cocktail vaccines

So far, various leishmanial antigens have been tested separately in order to induce protection with long-term immunity in animal models (Table 2) [12,23,24,36,41,43,44,47,48,51,53,54,70,74,88,89,92–106]. Although the responses are variable, one of the limitations on using a single antigen is their weak immune response. Thus, in order to achieve higher levels of protection, cocktail vaccines in form of combined recombinant proteins or genetically fused or even coadministrated with sand fly salivary molecules as a novel source of antigens have been suggested [107]. There are several examples, such as KSAC (which is a genetically fused protein comprised of KMP-1, SMT, A2 and CPB [108]) and recombinant antigens (*L. major* stress-inducible protein 1 and TSA) plus IL-12 [101]. Due to the high antigenic diversity of the *Leishmania* parasites, using more than one antigen could increase the chance of vaccine usability on a wide range of species [96].

The efficacy of DNA vaccinations as a cocktail of the two distinct plasmids encoding CPA and CPB was evaluated by their capability to induce protection and a specific immune response to *Leishmania* infection in the BALB/c mice as model. This vaccine induced a long-lasting protective response in immunized mice, whereas the separate injection of cysteine protease genes is not protective [74]. The next study showed no significant differences between the levels of induced protection in vaccination with bicistronic plasmid (encoding CPA and CPB separately) and monocistronic plasmid (encoding CPA/B hybrid protein) [102]. Cocktail vaccines have several advantages. Production of a vaccine composed of several antigens separately is more difficult to standardize and also more expensive to produce than a single-product vaccine [103]. In addition, multivalent vaccines containing a broader range of protective epitopes that would cover a wide range of MHC types in a heterogeneous outbreed population, such as humans and dogs [102], elicited a protective response stronger than when individually used [93,105]. This type of antigen delivery can induce cross-protection against different species of *Leishmania*.

The recombinant protein Q (a genetic fusion of five intracellular antigenic fragments, from the *L. infantum* acidic ribosomal proteins Lip2a, Lip2b, P0 and histone H2A) when mixed with BCG or CpG will be more protective than when administered alone against a *L. infantum* infection in mice [109] and dogs [110,111]. Recently, a cocktail vaccine containing four candidate antigens, KMPII, TRYP, LACK and PAPLE22, was introduced as a prime–boost vaccination that is more potent than DNA or protein alone [46].

Prime–boost vaccination

Early studies using different antigens with different conditons simultaneously suggested that induced protection after prime–boost vaccination is more stable and powerful. A number of scientists have compared the potency and efficacy of different strategies using DNA or protein alone (homologous prime–boost) and heterologous prime–boost (HPB)-based vaccination. Although there are different reports, most of them confirmed that the DNA-prime/boost-protein approach due to primary expression of antigen within cells is able to stimulate robust cellular and humoral responses. Of course, as mentioned above, type of antigens, rout of immunization and the type of adjuvant are critical factors that should be considered.

■ Homologous prime–boost vaccination

Homologous prime–boost strategy is a repeated vaccination program using the same antigens (boosting) [112]. Usually, protein or DNA vaccines (DNA/DNA or protein/protein) are applied for two or three steps of vaccination (or revaccination). One of the problems using this strategy is induction of antivector immunity encountered when using the same vector. Evaluation of protective efficacy and comparison between these two regimes revealed that in some cases DNA/DNA strategy is more potent than recombinant protein alone or combination with DNA in order to control infection [23,41,42,44].

■ HPB vaccination

As discussed above, while both recombinant protein and DNA vaccines are powerful approaches to induce a potent immune response, in many cases the generated immune responses are weak. In order to enhance the protective response, a HPB vaccination strategy encoding the same antigen is introduced as an alternative approach instead of homologous boosts. In this method, a particular antigen is administered in two rounds using more than one delivery method and two different vectors [112,113]. Usually, DNA vaccines are used for priming and recombinant proteins or live vectors are used for boosting. The first example of this approach was DNA vaccine for the priming and virus-based vaccine (attenuated avian poxvirus) for boosting; but now, different microorganisms could be used as delivery systems such as viruses, bacteria and parasites.

The major proof of using this approach is that it does not need any adjuvant. Due to carrying stimulatory unmethylated CpG motifs in their backbone, DNA plasmids induce cellular immunity via expression of

Table 2. Candidate antigens for immunization against *Leishmania*.

Protein or DNA	Antigen origin	Challenge strain	Adjuvant	Model	Results	Ref.
Single Ag						
cpa DNA	*Leishmania major*	*L. major*	–	BALB/c	↑ Lower level of IFN-γ, ↑ IL-5, ↑ IgG1, ↓ Swelling size, No protection	[74]
cpb DNA	*L. major*	*L. major*	–	BALB/c	↑ Th1 response, ↓ Swelling size, ↑ High production of IFN-γ, ↑ IgG2a, Partial protection	[74]
rCPA	*L. major*	*L. major*	Poloxamer 407	BALB/c	No protection, ↓ IFN-γ/IL-5	[24]
Rcpb	*L. major*	*L. major*	Poloxamer 407	BALB/c	Partial protection, ↓ Footpad swelling, ↑ IFN-γ/IL-5	[24]
CPB^{-CTE} Protein/protein	*L. major*	*L. major*	SLN	C57BL/6	↑ Protective response, ↓ IgG2a, ↑ IFN-γ/IL-4,↓ Parasite load	[92]
CPA/CPB/CPC DNA/DNA	*L. major*	*L. major*	SLN	BALB/c	↑ Protection level, ↓ parasite load, ↑ Th1 response, ↑ IgG2a/IgG1, ↑ IFN-γ/IL-5	[93]
LACK DNA	*L. major*	*L. major*	–	BALB/C	Protective response, ↑ IgG2a, ↑ Control of disease progression, ↓ Parasite burden, ↑ IFN-γ, CD8 T-cells-induced protection	[23]
rLACK	*L. major*	*L. major*	–	BALB/C	No protection	[23]
rLACK	*L. major*	*L. major*	rIL-12	BALB/C	Protective response, ↑ IgG2a, ↑ Control of disease progression, ↓ Parasite burden, ↑ IFN-γ,	[23]
rLACK	*Leishmania chagasi*	*Leishmania amazonensis*	rIL-12	BALB/C	No protection, ↑ Parasite load	[36]
Liposomal rLmSTI1	*L. major*	*L. major*	CpG ODN	BALB/c	↑ Th1 response, ↑ IgG2a/IgG1, ↓ Parasite load	[94]
rSPase	*L. major*	*L. major*	CpG + Montanide 720	BALB/C	↓ 66% parasite burden, ↑ IgG2a, ↑ IFN-γ, ↓ IL-5	[41]
SPase DNA	*L. major*	*L. major*	–	BALB/C	↓ 81% protection, ↑ IgG2a, ↑ IFN-γ, ↓ IL-5	[41]
Gp63 Liposomal	*L. major*	*L. major*	CpG ODN	BALB/C	Protection,↑ IFN-γ, ↓ Il-4, ↓ Parasite burden, ↑ IgG2a/IgG1	[95]
A2 DNA	*Leishmania donovani*	*L. chagasi* *Leishmania mexicana*	–	BALB/c	Significant protection, ↓ lesion size	[96]
rA2	*L. chagasi*	*L. chagasi*	Saponin	Canine	Partial protection, ↑ IFN-γ, ↓ IL-10 ↑ IgG, ↑ IgG2a, ↓ IgG1	[97]

Ab: Antibody; Ag: Antigen; CTE: C-terminal extension; DTH: delayed-type hypersensitivity; E6: Type 18 E6 protein human papillomavirus; LDU: Leishman–Donovan units; LN: Lipid nanoparticles; ODN: Oligodeoxynucleotide; SLN: Solid lipid nanoparticles.

Table 2. Candidate antigens for immunization against *Leishmania* (cont.).

Protein or DNA	Antigen origin	Challenge strain	Adjuvant	Model	Results	Ref.
Single Ag						
rA2	*L. donovani*	*L. amazonensis*	rIL-12	BALB/c	Protection, ↑ IFN-γ, ↓ parasite load, ↓ IL-4 and -10	[36]
rA2	*L. donovani*	*L. donovani*	Heat killed *Propianzibactrium acnes*	C57B/6	Protection, ↑ IFN-γ, ↑ titer Ab, ↑ Th1 and Th2 response	[89,98]
A2 DNA	*L. donovani*	*L. donovani*	pCDNA3	BALB/c	Protection, ↑ IFN-γ 65% reduction in LDU	[88]
Gp63 DNA	*L. major*	*L. major*	–	BALB/c	Partial protection 30% of mice	[47]
Gp63 DNA/DNA	*L. donovani*	*L. donovani*	CpG ODN	BALB/c	Protection, ↑ Th1 response, ↑ IgG2a/IgG1, ↑ IFN-γ, ↑ IL-12, ↓ IL-4	[44]
rgp63	*L. donovani*	*L. donovani*	CpG ODN	BALB/c	Partial protection, ↑ Th1 response, ↑ IgG2a/IgG1, ↑ IFN-γ, ↑ IL-12, ↓ IL-4	[44]
rLiP0	*Leishmania infantum*	*L. major*	CpG ODN	C57BL/6	Protection, Th1 response, ↓ parasite load, ↑ IFN-γ, ↑ IgG2a	[12]
rLiP0	*L. infantum*	*L. major*	CpG ODN	BALB/C	Partial protection, ↑ IgG2a, ↑ IFN-γ, Th1 and Th2 response Finally progressive disease	[12]
LiP0 DNA	*L. infantum*	*L. major*	CpG ODN	C57BL/6	Protection, Th1 response, ↓ parasite load, ↑ IFN-γ, ↑ IgG2a	[12]
NH DNA	*L. donovani*	*L. chagasi*	–	Canine	↓ DTH reactions, ↓ Ab, ↓ parasitem load, ↓ IL-10, ↑ IFN-γ, ↑ IL-2 and -4	[99]
NH DNA	*L. donovani*	*L. chagasi* *L. amazonensis*	–	BALB/c	No protection	[96]
NH DNA	*L. donovani*	*L. chagasi* *L. mexicana*	–	BALB/c	Partial protection ↓ 88% parasite load of *L. chagasi*, ↓ 65% parasite load of *L. mexicana*	[43]
rNH	*L. donovani*	*L. chagasi* *L. mexicana*	Saponin	BALB/c	Significant protection against *L. chagasi*, ↓ 70% parasite load Partial protection against *L. mexicana*	[43]
rLdγGCS	*L. donovani*	*L. mexicana*	Nonionic surfactant vesicles	BALB/C	Protect, ↑ IFN-γ, Delay in lesion growth, ↑ Ab titers	[48]

Ab: Antibody; Ag: Antigen; CTE: C-terminal extension; DTH: delayed-type hypersensitivity; E6: Type 18 E6 protein human papillomavirus; LDU: Leishman–Donovan units; LN: Lipid nanoparticles; ODN: Oligodeoxynucleotide; SLN: Solid lipid nanoparticles.

Table 2. Candidate antigens for immunization against *Leishmania* (cont.).

Protein or DNA	Antigen origin	Challenge strain	Adjuvant	Model	Results	Ref.
Single Ag						
rLdγGCS	*L. donovani*	*L. major*	Nonionic surfactant vesicles	BALB/C	Protection Delay in lesion growth ↑ Ab titers	[48]
DNA LdγGCS	*L. donovani*	*L. mexicana*	–	BALB/C	No protection ↑ Ab titers, ↑ IL-4	[48]
DNA LdγGCS	*L. donovani*	*L. major*	–	BALB/C	Transient protection ↓ Lesion size, ↑ Ab titers	[48]
Cocktail vaccine						
rLeish-111f	*L. major* *Leishmania braziliansis*	*L. major*	MPL-SE or Ribi 529-SE	BALB/c	High level protection, immunity for 3 months, ↑ IFN-γ, ↑ IgG2a, ↓ IL-4	[53–54]
rLeish-111f	*L. major* *L. braziliansis*	*L. major*	IL-12 or MPL-SE	BALB/c	Protection, ↓ lesion size, ↑ IFN-γ, ↓ IL-4	[100]
rLeish-111f	*L. major* *L. braziliansis*	*L. infantum*	MPL MPL-SE	BALB/c, C57BL/6C57BL/10	↓ Parasite loads of 91.7%, ↑ $CD4^+$ T cells, ↑ IFN-γ, ↑ IL-2, ↑ TNF, ↓ IgG	[54]
rLeish-111f	*L. major* *L. braziliansis*	*L. infantum*	MPL MPL-SE	Hamster	Protection: ↓ parasite loads of 99.6%, ↑ $CD4^+$ T cells, ↑ IFN-γ, ↑ IL-2	[74]
TSA/LmSTI1 DNA	*L. major*	*L. major*	–	BALB/c C57BL/6	↑ Humoral and cellular, ↓ lesion size	[101]
rTSA/LmSTI1	*L. major*	*L. major*	IL-12	BALB/c Rhesus monkey	Induce excellent protection	[51]
cpa + *cpb* DNA, monocistronic plasmid	*L. major*	*L. major*	–	BALB/c	71% reduction of infection, long lasting protection, ↑ IFN-γ	[74]
cpa + *cpb* DNA, bicistronic plasmid	*L. major*	*L. major*	–	BALB/c	Th1 phenotype, ↑ IFN-γ, 81% reduction of infection Partial protection	[102]
Hybrid rCPA/CPB	*L. major*	*L. major*	Poloxamer 407	BALB/c	Th1 response, ↑ IFN-γ, ↓ IL-5, ↑ IgG2a, partial protection	[103]
CPA/CPB^{-CTE} DNA/DNA	*L. major*	*L. major*	SLN	BALB/c	Significant protection, ↑ IFN-γ/IL-5, ↑ IgG2a/IgG1, ↓ parasite burden	[104]

Ab: Antibody; Ag: Antigen; CTE: C-terminal extension; DTH: delayed-type hypersensitivity; E6: Type 18 E6 protein human papillomavirus; LDU: Leishman–Donovan units; LN: Lipid nanoparticles; ODN: Oligodeoxynucleotide; SLN: Solid lipid nanoparticles.

Table 2. Candidate antigens for immunization against *Leishmania* (cont.).

Protein or DNA	Antigen origin	Challenge strain	Adjuvant	Model	Results	Ref.
Cocktail vaccine						
H2A, H2B, H3, H4 DNA	*Leishmania infantum chagasi*	*L. braziliensis*	–	Th1 response	Significant protection, ↑ Th1 response, IFN-γ, ↓ IL-5, ↓ parasite load in LN	[105]
A2 + E6 DNA	*L. donovani* HPV	*L. donovani*	–	BALB/c	High level of protection, 80% reduction in LDU, Th1 response, ↑ IFN-γ	[88]
rA2 + rLACK	*L. amazonensis*	*L. amazonensis*	–	BALB/c	No protection, ↑ parasite load	[36]
A2 + NH DNA	*L. donovani*	*L. amazonensis* *L. chagasi*	–	BALB/C	Significant protection, ↓ lesion size, ↑ IFN-γ, ↑ IL-4 and -10	[96]
NT + LPG3 DNA	*L. major*	*L. major*	Montanide 720	BALB/c	Significant protection, ↑ IgG2a, ↑ IFN-γ/IL-5, ↓ lesion size, ↓ parasite burden in spleen	[70]
rLmL3 or rLmL5	*L. major*	*L. major* *L. braziliensis*	CpG ODN	BALB/c	Protective responses, ↓ lesions size, ↑ IFN-γ, ↓ IL-10, ↓ parasite burden	[106]

Ab: Antibody; Ag: Antigen; CTE: C-terminal extension; DTH: delayed-type hypersensitivity; E6: Type 18 E6 protein human papillomavirus; LDU: Leishman–Donovan units; LN: Lipid nanoparticles; ODN: Oligodeoxynucleotide; SLN: Solid lipid nanoparticles.

cytokines related to Th1 response. Addition of CpG to vaccines enhances and increases the long-lasting protection and can efficiently shift the Th1/Th2 mixed response towards protective Th1-biased response [44]. Currently, there are four different regimes of HPB against leishmanial infection as described below (Table 3) [22,26,41,44,46,70,74,83,84,90,97,105,114–125].

DNA/protein strategy

So far, different candidate leishmanial antigens have been tested by this strategy (Table 3). Recently, in an interesting study, three vaccination strategies have been compared using kinetoplastid membrane protein-11, TRYP, LACK, and PAPLE22 vaccine candidate antigens against VL in the hamster model. The comparison between naked DNA, raw insect-derived recombinant protein and heterologous prime–boost strategies showed that the prime–boost strategy and raw insect-derived recombinant proteins elicited a stronger cell-mediated immune response and parasitological protection against *L. infantum* than naked DNA. This is while the recombinant proteins derived from baculovirus-infected insect cells was not protective [46].

DNA/virus strategy

In parallel to using DNA as a vehicle for vaccination, recombinant viral vectors were developed as powerful vaccine delivery systems. A major advantage of this approach is that it is suitable for use in humans [126]. Using recombinant viral vectors to deliver leishmanial antigens or peptides [127] is another new approach to vaccination. Vaccinia virus Ankara (MVA), recombinant Vaccinia virus, attenuated adenoviruses and attenuated pox viruses like fowl pox are some examples from modified viruses that are safe to use. These vectors lose replication properties after long serial passages into cells like chicken embryo fibroblasts, which is used for MVA [128]. However some inconsistent reports are published. As summarized in Table 3, immunization of BALB/c mice with DNA-LACK/MVA-LACK was successful and induced protection against *L. major* [128]; however, another group could not prove this vaccination regimen [22].

DNA/bacteria strategy

Live-attenuated *Salmonella enterica* serovar *Typhimurium* is another optimal vaccine vehicle for boosting strategy, since it stimulates both humoral and cellular immune responses. The main advantage of these systems is possibility for oral delivery. Vaccination with DNA-*Salmonella* as prime–boost regimen, while DNA encoded LACK antigen, expressing LACK antigen enhanced IFN-γ production and protection against *L. major* challenge in susceptible BALB/c mice [114].

Table 3. Examples of prime/boost vaccination against different species of *Leishmania*.

Ag prime/boost	Source of Ag	Challenge strain	Adjuvant	Animal model	Results	Ref.
DNA/protein						
ORFF DNA/protein	*Leishmania donovani*	*L. donovani*	–	BALB/C	↓ Parasite load (75%-80%) ↓ (not induce the typical T-helper response), ↓ IgG2a, ↓ IFN-γ	[116]
rA2	*Leishmania chagasi*	*L. chagasi*	Saponin	Canine	Partial protection	[97]
H2A, H2B, H3, H4 2 x DNA/protein	*Leishmania infantum chagasi*	*L. braziliensis*	CpG	BALB/c	Strong immune response, ↓ IFN-γ, ↓ IL-5, ↓ parasite load in LN, ↓ inhibition of disease	[105]
CPA + CPB DNA/protein	*Leishmania major*	*L. major*	IFA	BALB/c	↓ Th1 response, ↓ swelling size, ↓ high production of IFN-γ, ↓ IgG2a, partial protection	[74]
CPA + CPB DNA/protein	*L. infantum*	*L. infantum*	CpG ODN or Montanide 720	BALB/c	Th1 response	[84]
CPA + CPB DNA/protein	*L. infantum*	*L. infantum*	CpG ODN or Montanide 720	Canine	Protection	[83]
CTE (CPB) DNA/protein	*L. infantum*	*L. infantum*	CpG ODN or Montanide 720	BALB/c	Th1/Th2 response	[117]
CPC DNA/protein	*L. infantum*	*L. infantum*	CpG ODN or Montanide 720	BALB/c	Significant protection, ↓ IgG2a/IgG1, ↓ NO, ↓ IFN-γ, ↓ parasite burden	[118]
SPase DNA/protein	*L. major*	*L. major*	CpG ODN + Montanide 720	BALB/c	↓ 70% parasite burden ↓ IgG2a, ↓ IFN-γ, ↓ IL-5	[41]
gp63 DNA/protein	*L. donovani*	*L. donovani*	CpG	BALB/c	Induce Th1 responses, ↑ IFN-γ, ↑ IL-12, ↑ NO, ↓ IgG2a/IgG1, ↓ IL-4, IL-10, ↓ parasite load	[44]
LPG3 DNA/protein	*L. major*	*L. major*	Montanide 720	BALB/c	Significant protection, ↓ IgG2a, ↓ IFN-γ/IL-5, ↓ lesion size, ↓ parasite burden in spleen	[70]
KMPII, TRYP, LACK, PAPLE22 3 x DNA/ 2 x protein	*L. imfantum*	*L. infantum*	–	Hamster	↓ Parasite load, ↓ NO	[46]
DNA/virus						
LACK DNA/rVV	*L. infantum*	*L. infantum*	–	Canine	60% protection, ↑ IL-4, ↑ IFN-γ and ↑ IL-12 mRNA	[119]
LACK DNA/WR or DNA/ MVA	*L. infantum*	*L. infantum*	–	BALB/c	High levels of protection, ↓ parasite burden, ↑ IFN-γ, ↑ TNF	[120]
LACK DNA/MVA	*L. infantum*	*L. major*	–	BALB/c	Long lasting protection, ↑ IFN-γ and TNF-α secreting $CD8^+$ T cells, ↓ lesion size by 65–92%	[128]
LACK DNA/MVA	*L. infantum*	*L. infantum*	–	Canine	↓ VL symptoms ↓ T-cell activation, ↓ Ab	[121]

Ag: Antigen; CTE: C-terminal extension; IFA: Incomplete Freund's adjuvant; SLN: Lipid nanoparticles; MVA: Vaccinia virus Ankara; ODN: Oligodeoxynucleotide; ORFF: Open-reading frame gene from the LD1 locus; rVV: Recombinant Vaccinia virus; VL: Visceral leishmaniasis; WR: Vaccina Western Reserve virus.

Table 3. Examples of prime/boost vaccination against different species of *Leishmania* (cont.).

Ag prime/boost	Source of Ag	Challenge strain	Adjuvant	Animal model	Results	Ref.
DNA/virus						
LACK DNA	*L. infantum*	*L. major*	–	BALB/c	↑ IFN-γ, ↑ TNF-α and ↑ IL-2, ↑ $CD8^+$ and $CD4^+$ T cell	[122]
P36/LACK DNA/rVV	*L. infantum*	*L. major*	–	BALB/c	↓ lesion size, ↑IFN-γ, ↓ parasite load (1000 ×)	[123]
Influenza A viruses	LACK single peptide	*L. major*	–	BALB/c	↑ IFN-γ producing $CD4^+$ T cells, ↓ IL-4, IL-5, IL-10 and IL-13	[124]
LACK DNA/MVA	*L. major*	*L. major*	–	BALB/c	Not protective, Th2 response, IgG2a/IgG1 = 0.04	[22]
TRYP DNA/MVA	*L. major*	*L. major*	–	BALB/c	Protective, Th1 response IgG2a/IgG1 = 0.91	
TRYP DNA/MVA	*Leishmania (Viannia) panamensis*	*L. (V.) panamensis*	α-GalCer LPS, CpG Pam3CSK4 MALP-2	BALB/c	Induce protection but no protection	[26]
TRYP DNA/MVA	*L. infantum*	*L. infantum*	–	Out bred canine	Th1 response, ↑ IgG2, ↑ IFN-γ	[125]
KMP-11 DNA/rVV	*L. donovani*	*L. donovani*	–	Mice, hamster	↑ Activation $CD4^+$ and $CD8^+$ T cells, ↑ high-quality T cells ↑ IFN-γ, IL-2 and TNF-α	[90]
DNA/bacteria						
LACK DNA-Salmonella/ protein	*L. major*	*L. major*	–	BALB/c	Enhance protection, ↑ IFN-γ, ↓ IL-4 and IL-10, ↑ IgG2a, ↓ IgG1	[120]
DNA/*Leishmania*						
A2-CPA-CPB^{-CTE} DNA/*L. tarentolae*	*L. infantum*	*L. infantum*	SLN	BALB/c	Th1 response, protection ↑ IFN-γ, ↓ IL-10, ↑ IFN-γ/IL-10, ↑ NO, ↓ parasite burden	[115]

Ag: Antigen; CTE: C-terminal extension; IFA: Incomplete Freund's adjuvant; SLN: Lipid nanoparticles; MVA: Vaccinia virus Ankara; ODN: Oligodeoxynucleotide; ORFF: Open-reading frame gene from the LD1 locus; rVV: Recombinant Vaccinia virus; VL: Visceral leishmaniasis; WR: Vaccina Western Reserve virus.

DNA/parasite strategy

More recently, a new strategy using recombinant live nonpathogenic *Leishmania* species carrying antigens derived from pathogenic species are introduced as vectors to express that protein endogenously, present it to the immune system and elicit immune response [115,129–131]. *L. tarentolae* (isolated from lizard) is an example of the genus *Leishmania* that could not generate any signs of leishmaniasis in human, hence it is known as nonpathogenic for human [130]. Using *Leishmania* species has some advantages, such as its ability to grow in cell-free media condition within a cheap/easy media, with a mammalian-type *N*-glycosylation pattern [132]. Previous studies have shown that *L. tarentolae* can be used as a live vaccine against *L. donovani* and elicits a protective Th1 immune response [130].

Recently, the ability of recombinant *L. tarentolae* to induce protective immunity against leishmaniasis in mice model was evaluated using heterologous prime–boost immunization techniques, priming with plasmid DNA followed by a boost with the recombinant parasite [115,129].

In another study, BALB/c mice were immunized with a heterologous prime–boost regimen using DNA/live parasite carrying a trifusion gene (*A2-CPA-CPB-CTE*) against *L. infantum* challenge. For priming, the trifusion gene was formulated with cationic solid lipid nanoparticles acting as a delivery system. The protective immunity was associated with a Th1-type immune response with high levels of IFN-γ prior and after challenge and with lower levels of IL-10 production and parasite burden after challenge. Moreover, this immunization elicited high IgG1 and IgG2a humoral immune responses [115].

Nonpathogenic organism-based live vaccines

In spite of high challenges of live immunization, scientists still believe that live vaccines are the most powerful known vaccine to date. As summarized in Table 1, live vaccines induce T-cell mediated immune responses and mimic natural infection through correct processing and presentation of antigens in association with MHC class I and II antigens [19]. In vaccination with live parasites, both $CD4^+$ and $CD8^+$ T cells are induced to create protective immune responses and long-term immunity, hence, induce appropriate inflammatory and regulatory immune responses in the host animals [19]. However, the risk of reversion of the organism to its virulent state is the main obstacle when using these vaccines. Another approach to reach the best safe condition is using live vaccines through heterologous gene expression in a live nonpathogenic microorganism such as *L. tarentolae* [130]. This parasite does not survive in mice [130]. Table 4 shows a list of nonpathogenic microorganisms used as carrier to express heterologous proteins [115,124,129–131,133–135].

Recently, with development of transfection techniques, a number of genes (virulence or reporter genes) were transfected in *L. tarentolae* episomally or integrated into rRNA locus of the genome through homologous recombination to obtain knock-in recombinant parasites [115,133,136].

The A2 gene, which is expressed specifically by the *L. donovani* complex and promotes visceralization, is absent in *L. tarentolae* [87]. In 2010, effectiveness of A2-recombinant *L. tarentolae* as live vaccine was studied with two administration systems, intravenous and intraperitoneal injection. The results demonstrated that a single intraperitoneal vaccination of BALB/c mice with recombinant *L. tarentolae* expressing A2 antigens derived from *L. infantum* elicited a strong cell proliferation after challenging with *L. infantum* that was accompanied by reduced levels of IL-5 production after challenge, leading to a potent Th1 immune response. In contrast, intravenous injection elicited a Th2 type response, characterized by higher levels of IL-5 and high humoral immune response, resulting in a less efficient protection [133]. Moreover, vaccination of mice with live/live parasite carrying trifusion gene (*A2-CPA-CPB-CTE*) against *L. infantum* showed the same protective effect as of heterologous DNA/live injection [115].

In another study, three recombinant *Lactococcus lactis* strains were generated to express A2 and used as live vaccines in order to induce specific immune responses against *L. donovani* infection in BALB/c mice. *L. lactis* expressing *Leishmania* A2 protein at different subcellular locations (cytoplasm, secreted outside the cell and anchored to the cell wall) were tested as live bacterial vaccines against VL caused by *L. donovani* infection in BALB/c mice. Comparison between three different patterns of intracellular expression of the cell demonstrated that the subcellular localization of the antigen has more influence on the generated immune response [134].

Genetically attenuated live vaccines

Immunization using live parasites is stronger in inducing the immune system and eliciting powerful cytokines or antibodies than other vaccines, due to stability of the immune response and higher antigenicity [30]. Therefore, scientists are trying to generate genetically manipulated strains to stimulate the immune system without replication of parasite within the host and manifestation of the disease. In order to identify the parasites with the least virulence potential, essential gene/s for parasite growth and infectivity in amastigote form of the parasite are targeted for disruption. *DHFR-TS* was the first gene that was targeted as an essential metabolic gene from *L. major*'s genome to obtain genetically attenuated parasite vaccine [137]. After that, many target genes in *Leishmania* were disrupted and the knock-out strains were evaluated for infectivity potential and protectivity. However, the most important concern about safety and the possibility of reversion to virulence state [14] remained to be solved. For example *lpg2*-mutant *L. mexicana* maintain their ability to cause disease in the absence of the *lpg2* gene [138] through an unknown compensatory mechanism [139]. Consequently, to decrease infectivity potential of live attenuated parasite vaccines, it is likely that more than one gene should be disrupted [14].

Saliva-based vaccines

Leishmania parasite is transmitted by different species of infected female sand fly such as *Lutzomyia* (new world) and *Phlebotomus* (old world) species. In a natural pathway of infection, parasites are delivered into the skin of the host while accompanied by secretory saliva that helps induce protective immunity. Sand fly saliva contains immunogenic or immunomodulatory molecules, which are potential targets for development of vaccines to control *Leishmania* infection [140].

Many studies have shown that different sand fly proteins from different species allow progression of infection, increase in parasite numbers in the animal host [141,142], and development of protection against *Leishmania* infection. After identifying the effectiveness of the salivary gland of the sand fly and its components in 1998, many researchers have focused on further characterization of salivary proteins and used salivary gland homogenates in their experiments to stimulate the biological milieu of natural transmission. Different salivary proteins from *Phlebotomus papatasi*, the vector of *L. major,* have been characterized [143]. The role of these proteins in immunization are different; for example: SP44 caused exacerbation while SP15 led to protection against *L. major* infection [143,144]. Some of the salivary

Table 4. Nonpathogenic vectors used as live vaccine candidate against *Leishmania*.

Deliver host	Antigen	Infection agent	Immunization method	Animal model	Results	Ref.
Toxoplasma gondii mutant	KMP-11	*Lactococcus major*	ip.	BALB/c	Protection. Ultimately not control	[135]
Lactococcus tarentolae	*L. tarentolae*	*Lactococcus donovani*	ip.	BALB/c	↑ Activates the DC cell maturation, ↑ IFN-γ ↑ T-cell responses	[130]
L. tarentolae	HIV-1 Gag protein	HIV-1	ip.	BALB/c	↓ 75% virus replication, ↓ memory specific $CD4^+$ T lymphocytes, ↑ antibodies titers	[131]
L. tarentolae	A2	*Lactococcus infantum*	ip. iv.	BALB/c	↑ IFN-γ, ↓ IL-5	[133]
L. tarentolae	HPV type 16 *E7* gene	TC-1 tumor cells	sc.	C57BL/6	↑ IgG2a , ↑ IFN-γ, ↓ Tumor size	[129]
L. tarentolae	A2-CPA-CPB-CTE	*L. infantum*	sc. in footpad	BALB/c	↑ Th1 response, ↑ IFN-γ, ↓ IL-10, ↑ NO, ↑ IgG1, ↑ IgG2a, ↓ Parasite burden	[115]
Lactococcus lactis	A2	*L. donovani*	sc.	BALB/c	↑ Expression of A2 anchored to the cell wall, ↑ serum antibodies	[134]
Influenza A viruses	LACK single peptide	*L. major*	ip.	BALB/c	↑ Th1 response, ↑ IFN-γ ↑ $CD4^+$ T cell, ↓ Lesion size ↓ Parasite burden	[124]

DC: Dendritic cell; HPV: Human papillomavirus; ip.: Intraperitoneal; iv.: Intravenous; sc.: Subcutaneous.

proteins could be potential targets for *Leishmania* infection in animal models and vaccine development, either alone or in combination with other antigens. However, this potency is variable in different animals. For example, protective effect of PpSP15 is confirmed in mice [143], but not in Rhesus monkeys [140]. In addition, immunization of mice with LPG combined with salivary gland lysates failed to generate protection [145].

So far, two sand fly salivary proteins, maxadilan from *Lutzomyia longipalpis* and PpSP15 from *P. papatasi* [143,144], have been characterized as protective molecules against leishmaniasis. The maxadilan protein can exacerbate the infection with *L. major*, but vaccination against maxadilan could protect the mice against infection with *L. major* [146].

The first licensed *leishmania* vaccines (or first leishmaniasis vaccines approved)

To date, different vaccination strategies have failed to be effective in human and animals. However, there are some available licensed vaccines that are limited to be used only against VL in dogs.

Leishmune vaccine contains recombinant FML antigen isolated from *L. donovani* with saponin as adjuvant, which was the first licensed vaccine against canine visceral leishmaniasis in Brazil since 2004 [1,59,61,147]. FML contains an antigenic marker called NH36 (nucleoside hydrolase), which is a potential candidate for diagnosis and vaccine [64]. In 1993, FML was shown to be the most potent inhibitor of both promastigote and amastigote internalization, and to be present on the parasite surface during the vertebrate–host cycle. FML antigenic fraction was a potent immunogen in rabbits [148]. The protective effect of this vaccine has been confirmed in different projects in Phase I–III trials on small laboratory animals (mouse and hamster) and dogs in Brazil [64]. In Brazil, in Phase III trials it was shown that the number of deaths and symptoms derived from VL in dogs has decreased more than 92% with a long-lasting protection, followed by reduced transmission of disease to humans [61,147].

Leish-Tec® is the second commercial vaccine against VL in dogs that was licensed in Brazil in 2008 [1]. This vaccine contains recombinant A2 antigens from VL-causing *Leishmania* species that caused VL, plus saponin as an adjuvant [85].

The third vaccine licensed in Europe in 2011 is CaniLeish® [1]. It belongs to the second-generation of vaccines and is within the excreted–secreted proteins of the supernatant of cultures of *L. infantum* plus QA21 (highly purified fraction of the *Quilaja saponaria* saponin) as adjuvant (LiESP/QA-21) [5,9,149].

Future perspective

During the past few years, vaccine study has undergone great progression. Some vaccines developed are acceptable for vaccination in animal models (mice and dogs)

but could not help in the elimination and/or control of leishmnaiasis in humans or even dogs. The majority of known antigens have been checked in different modalities as a vaccine and shown contrastable protection.

The main challenge is the difficulty in creating substantial long-term immunity in the host. Selection of vaccine candidates is still problematic, although derived proteins from sand fly saliva could open a new therapeutic avenue for the development of effective vaccine.

The other main obstacle is to know and have deeper understanding of the host's genetic background, virulence factors and specific components of sand fly vectors for designing a preventable strategy against leishmaniasis. We need to know more information on how immune systems react with non pathogenic *Leishmania* such as *L. tarentolae* in comparison with pathogenic strains. We need to know and establish different criteria to increase the immunogenicity of live attenuated/nonpathogenic strains using different adjuvants. This strategy is highly important in HPB vaccination. One of the main crucial steps is how the innate immunity is stimulated and in fact how innate immunity can create a milieu for stronger and durable adaptive immune responses. It is highly important to manipulate the activation of T-regulatory cells in order to control as well as helping other T cells to act properly. The harmony between innate and adaptive immune response is highly important, although still more knowledge is needed for better shaping the vaccine development.

Acknowledgments

The authors thank A Mizbani for critically reading and editing the manuscript.

Financial & competing interested disclosure

This project was supported by Pasteur Institute of Iran (Grant number 653 to TT) and National Science Foundation of Iran (grant number 91053539 to SR). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Immunology focusing

- Vaccine delivery systems are critical for elicited immune response and protection provided by specific antigens.
- The $CD8^+$ T cells, due to their essential role in mediating host defense, should be considered as an important target for vaccine development against *Leishmania* parasites.
- Proper stimulation of innate immune responses are important for having proper milieu for adaptive immune response against *Leishmania* parasite infection.

Prime–boost strategy

- Administration of cocktail antigens through a suitable heterologous prime–boost system seems to be the best approach to create high-level protection.
- Prime–boost strategy using DNA and parasite could present a powerful vaccine approach against leishmaniasis.

Live vaccination

- Proteins expressed by live nonpathogenic *Leishmania* are biologically active and close to the native protein, hence they could be the most powerful approach for vaccination against leishmaniasis.
- Genetically attenuated or nonpathogenic *Leishmania* have gained further attention as an alternative in eukaryotic delivery systems.
- The species-specific genes and virulence factors should be determined.

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