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REVIEW

# Vaccines for leishmaniasis in the fore coming 25 years

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**Summary** Human vaccination against leishmaniasis using live *Leishmania* was used in Middle East and Russia (1941–1980). First-generation vaccines, composed by killed parasites induce low efficacies (54%) and were tested in humans and dogs Phase III trials in Asia and South America since 1940. Second-generation vaccines using live genetically modified parasites, or bacteria or viruses containing *Leishmania* genes, recombinant or native fractions are known since the 1990s. Due to the loss of PAMPs, the use of adjuvants increased vaccine efficacies of the purified antigens to 82%, in Phase III dog trials. Recombinant second-generation vaccines and third-generation DNA vaccines showed average values of parasite load reduction of 68% and 59% in laboratory animal models, respectively, but their success in field trials had not yet been reported. This review is focused on vaccine candidates that show any efficacy against leishmaniasis and that are already in different phase trials. A lot of interest though was generated in recent years, by the studies going on in experimental models. The promising candidates may find a place in the forth coming years. Among them most probably are the multiple-gene DNA vaccines that are stable and do not require cold-chain transportation. In the mean time, second-generation vaccines with native antigens and effective adjuvants are likely to be licensed and used in Public Health control programs in the fore coming 25 years. To date, only three vaccines have been licensed for use: one live vaccine for humans in Uzbekistan, one killed vaccine for human immunotherapy in Brazil and a second-generation vaccine for dog prophylaxis in Brazil.  
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**Contents**

Introduction .....	1710
Immunology of leishmaniasis .....	1710
First-generation vaccines that arrived to clinical assays .....	1712

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Candidates for second-generation vaccines .....	1712
Live vaccines.....	1712
Vaccines using recombinant viruses and bacteria as delivery vehicles .....	1713
Vaccines based on purified <i>Leishmania</i> antigens.....	1713
Recombinant antigens.....	1714
Candidates for third-generation vaccines .....	1717
Vaccines based on sand fly salivary antigens .....	1718
Synthetic vaccines.....	1718
Conclusions and perspectives .....	1718
Acknowledgements.....	1719
References.....	1719

## Introduction

Leishmaniasis is caused by parasitic protozoa transmitted by the bite of female sand fly and is currently endemic in 88 countries, affecting 12 million people worldwide and threatening 350 million more. Several species of *Leishmania* caused human diseases that range from self-healing cutaneous lesions to fatal visceral leishmaniasis (VL), mucosal leishmaniasis and diffuse cutaneous leishmaniasis. Cutaneous leishmaniasis (CL) is an antroponotic or zoonotic disease with wild rodent, canids and marsupials as reservoirs. The difficulties in control of the wild reservoir population led to the development of human vaccines only. On the other hand, VL, the most severe disease, is an antroponoose in India and Central Africa, and a zoonosis transmitted by domestic dogs in the Mediterranean and America [1]; leading to the search of both, human and dog vaccines. The need for safe prophylactic vaccines for human and dogs is made greater by the drug resistance and toxicity of chemotherapy, the increase of the disease incidence in immunocompromised subjects, and the difficulties of epidemiological control based upon sacrifice of seropositive dogs [2]. VL is caused by *Leishmania donovani* (Africa, India, Asia), *Leishmania chagasi* (America) and *Leishmania infantum* (Mediterranean basin). CL is produced by *L. mexicana* and *L. braziliensis* complexes in Americas and *L. major*, *L. tropica* and *L. aethiopica* in the Old World [1,3]. As all leishmaniasis are caused by closely phylogenetically related *Leishmanias* species, the development of a unique polyvalent vaccine is expected and will be very valuable not only for prophylaxis but for treatment as well.

The first vaccine against leishmaniasis was developed by Professor Adler at the Hebrew University of Jerusalem, Israel, who had observed that mothers of Lebanon exposed their children's arms to the bite of sand flies because they intuitively knew that the development of a self-healing single first lesion would protect them from the severe disease in future [4]. Therefore, the ancient practice was to inoculate uninfected individuals with infectious material from lesions, in regions of the body where the scar would be hidden [4]. After a method for axenic culture of the parasites was established, "leishmanization" became usual in Israel and Russia and further evolved to the use of first-generation vaccines composed of whole killed parasites or crude extracts. Leishmanization process was discontinued due to uncontrolled long-lasting skin lesions, the spread of HIV and the use of immunosuppressive drugs, ethical rea-

sons, parasite persistence, and difficulties in the quality inoculum control. Its use at present is limited to one vaccine registered in Uzbekistan and to live challenge in vaccine efficacy trials in humans in Iran [5]. First-generation *Leishmania* vaccines composed of killed parasites [6] have gradually replaced leishmanization. Second-generation vaccines thus far have been based on the following: live, genetically modified *Leishmania* spp. designed to cause abortive infection in man, recombinant bacteria or viruses carrying *Leishmania* antigen genes, defined synthetic or recombinant subunits [7–9] and native fractions purified from parasites [10–12]. The use of third-generation vaccines [6] that include genes coding for a protective antigen, cloned into a vector containing eukaryotic promoter, is the more recent approach [13–16].

According to the WHO recommendations, Phase I trials are designed to compare vaccinated to placebo treated subjects to assess the immunogenicity and safety of the vaccine [17]. Phase IIa trials are designed to check the vaccine-induced protection against an experimental challenge. Although they are needed in order to standardize the vaccine dosage, route and schedule prior to the field test, they are limited by the questionable value of artificial challenge. Phase III trials analyse the vaccine efficacy against the natural infection. All these assays are performed as double-blind randomized control trials with small amounts of individuals (dozens to thousand). If the vaccine has successfully passed these steps, the formulation can be forwarded to registration and industrialization and used in Phase IV trials larger populations (10,000–100,000 individuals) as part of national immunization campaigns. For ethical reasons, given the benefit of the vaccine, no placebo treated controls are used and the vaccine efficacy is measured by comparing the incidence of the disease before and after the vaccine treatment [17].

## Immunology of leishmaniasis

Studies of anti-*Leishmania* vaccine candidates have advanced in recent years due to the understanding of cell-mediated immunological mechanisms for controlling infection. Most current knowledge, however, is based on experimental mouse models and cannot be extrapolated to dogs or humans.

Acquired resistance to leishmaniasis is mediated by T cells [18]. CD4+ lymphocytes are crucial for resistance and CD8+ are more involved in memory than as effector cells.

CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells are involved in persistence of *L. major* infection [19].

In humans, a correlation between TH1 cell responses and resistance and healing of cutaneous leishmaniasis was described with a predominance of cells producing IFN- $\gamma$ , while a mixed TH1/TH2 response with IL-4 and IL-10 characterized mucocutaneous and chronic cutaneous lesions [20]. Other studies show this mixed cytokine profile in cutaneous leishmaniasis and in natural resistance, with IL-4 and IL-10 predominating in early infection and a Type-1 immune profile in patients with older lesions [21], or a main exacerbated TH1 response with high levels of IFN- $\gamma$  and TNF- $\alpha$  in mucosal patients [22]. In VL, no association of IL-4 with active disease was described; however sustained levels of IFN- $\gamma$  and a direct correlation between increase in IL-10 and active disease by *Leishmania donovani* were detected [23,24] while TNF- $\alpha$  was significantly elevated in cases of PDKL [25]. The mixed cytokine pattern has been confirmed in active disease by *L. chagasi* [26], though this study used non-adequate naïve and asymptomatic controls selected by a Montenegro skin test that could itself determine an increase in IFN- $\gamma$  levels [27] or, together with the immunofluorescent assay, lead to misdiagnosis of CL. The problem with this approach is illustrated by the identification of higher levels of IL-4, the hallmark of CL [28], in control individuals [26]. Furthermore, no skin test was performed on the patient group [26].

Innate immunity, toll like receptors, as well as natural killer cells, IL-1 $\alpha$  and myeloid differentiation factor 88 [29] have been identified in early resistance to the infection and acquired immunity [30]. The contribution of innate immunity in human visceral leishmaniasis was also recently described [31]. However, this study also selected uninfected and asymptomatic controls by Montenegro skin test.

In the mouse model for VL, *Leishmania* specific TH2 cells and antigen presenting cells are involved in suppression of T cell responses [32]. The DTH response is suppressed in mice, dogs, and humans and is recovered after cure. Macrophage mediated suppression leads to the increase in parasite burden and antigen energy, and is linked to either defective antigen presentation or inhibition of expression of class I and class II major histocompatibility complex molecules [28]. Immunosuppression in mice is related to enhanced TGF- $\beta$  expression [33], IL-10, and the possible participation of CD4<sup>+</sup> CD25<sup>+</sup> regulatory cells. There are two receptors for B-7 molecules: the CD28 for T cell activation (TH1) and the CTLA-4 for termination of T cell activation. Blockade of CTLA-4 leads to resistance to infection, suggesting that the expression of CTLA-4 plays an important role in maintaining unresponsiveness in CD4<sup>+</sup> T cells during chronic VL in the mouse model [34]. The expression of CTLA-4 results in the secretion of TGF- $\beta$ , which promotes the growing of the parasites inside the macrophage. In tegumentar leishmaniasis, the role of CTLA-4 is paradoxical, dual [35]. In VL, apoptosis of T CD4<sup>+</sup> cells is accompanied by a decrease in IL-2 and IFN- $\gamma$ . In addition, apoptosis is detected in inflammatory cells in liver and spleen during infection with *L. donovani* [36]. Soluble factors in serum, such as triglycerides, may also be involved in immunosuppression. These disappeared if serum is delipidated [37].

In mice infected with *L. donovani*, the number of DC increases during infection but their distribution is altered

because they fail to localize the periarteriolar lymphoid sheath (PALS) [38]. CCL21 and CCL19 expression by gp38<sup>+</sup> PALS stromal cells is decreased during infection. For these reasons DC do not migrate properly in spleen nor do they destroy the amastigotes. The major mechanism underlying defective localization of DCs is TNF- $\alpha$  dependent, IL-10-mediated inhibition of CCR7 expression [38].

The murine model for CL is considered one of the best for the study of the mechanisms controlling the T helper TH1 and TH2 cell balance [39–41]. In the resistant strains (C57Bl/6, C3H, CBA) resolution of the limited infection is mediated by TH1 cells secreting IFN- $\gamma$  in response to IL-12. IFN- $\gamma$  activates the parasiticide activity of macrophages through the synthesis of inducible nitric oxide synthase (iNOS), which leads to production of nitrogen radicals. In contrast, the susceptible Balb/c mice develop a main TH2 response that results in progression of lesions and systemic disease. In draining lymph nodes, CD4<sup>+</sup> cells secrete IL-4 [42,43].

Human kala-azar is characterized by high titers of *Leishmania*-specific antibodies appearing soon after infection and before the development of cellular immunological abnormalities. The role of these antibodies in disease resolution or protection is largely unknown. The TH1 cytokine IFN- $\gamma$  probably upregulates IgG1 and IgG3 in humans, while the TH2 cytokines IL-4 and IL-5 stimulate the production of high levels of IgM, IgE, and IgG isotypes such as IgG4. Analysis of the *Leishmania* specific Ig isotypes in and IgG subclasses in VL patient sera revealed elevated levels of IgG, IgM, IgE and IgG subclasses during disease [44–47]. Drug resistance was associated with a reduction in IgG2 and IgG3. A marked elevation of IgG1, however, was observed in all these patients [48,49]. A successful cure corresponded with a decline, most significantly, in the levels of IgE, IgG4, and IgG1 [45,46,48].

Canine visceral leishmaniasis (CVL), on the other hand, shows very similar characteristics to the human disease [50]. IgA, IgE, and IgM responses have been shown to be markers of the disease [51–53]. IgG antibody increase is also observed and correlated with symptomatology [50,54,55] with IgG1 subtype associated to susceptibility and severe disease and IgG2 increased in natural resistant or vaccinated dogs [15,56–66]. IgG2 subtype also predominates in the canine response to vaccination against Lyme disease [67] or *Echinococcus granulosus* infection [68]. However, conflicting results have been reported, with the predominance of the IgG2 subtype observed in symptomatic dogs [53,69–72]. Since all investigations used the same manufactured anti-dog conjugates, the discrepancy in results could be due to difference in titration mainly of the IgG2 conjugate, which systematically shows significantly higher titres than the IgG1 sera [59]. On the other hand, while most studies defending the IgG2 predominance deal with vaccinated dogs and used purified [59,63,66] or recombinant vaccine antigens for diagnosis [15,57,62,64,65], the investigations that associate IgG2 to symptomatology and disease used promastigote crude antigens [15,69–71]. The differential affinity of the antigen could be another factor of the discrepancy. In dogs vaccinated with the CPa and CPb cystein proteinases, the work of Rafati et al. [15] show that higher IgG2 than IgG1 titers are detected against the recombinant antigens but not against the *Leishmania infantum* lysate. While the recombinant antigen interacts

with a defined fraction of sera antibodies, the total lysate interacts with the whole plethora of antibodies directed against the total parasite, masking or diluting the response against the recombinant protein, which is not a major *Leishmania* antigen. Another factor of discrepancy could be the use of different batches of the polyclonal anti-IgG2 and anti-IgG1 antisera, which show low repeatability (our experience and Dr MJ Day personal communication). These difficulties stimulated Dr MJ Day to obtain dog monoclonal antibodies that recognize four different IgG subfractions [72]. Unfortunately, these antibodies are not commercially available.

When cellular immune response is present, dogs appear asymptomatic and IDR positive, with higher levels of IL-2 and TNF- $\alpha$  and a mixed TH1/TH2 response involving primarily TH1 mediated by IL-12, IL-18 and IFN- $\gamma$  [73]. In asymptomatic dogs, macrophages are capable of killing amastigotes through the nitric oxide route [74], and a CD8<sup>+</sup> increase in peripheral lymphocytes, MHC class II molecules and receptors CD45RA e CD45RB are also found [75]. In contrast, symptomatic dogs show a failed cellular response, with no IDR or lymphocyte proliferation and the following additional characteristics: decrease in monocytes; decrease in CD8<sup>+</sup> [75] CD4<sup>+</sup> and CD21<sup>+</sup> B lymphocytes, either *Leishmania*-specific or not; antibody increase; deficiency of the co-stimulatory response; decrease of IFN- $\gamma$ ; and reduced expression of MHC class II molecules, which renders the animal more susceptible [66,75–80]. The involvement of IL-10 and IL-4 has been shown in both symptomatic [81,82] and asymptomatic dogs [73]. The susceptibility of the canine disease is related to a genetic polymorphism. Mutations on the gene NRAMP1, which controls the intralysosomal replication of *Leishmania*, determine the susceptibility or resistance to the infection [83]. Moreover, a relationship between the MHCII alleles DLA (DRB1, DQA1, DQB1) and the course of infection was described with the genotype DLA-DRB1 associated to severe disease [84].

## First-generation vaccines that arrived to clinical assays

The human efficacy trials involving the so called “killed-vaccines”, composed of crude total parasite antigens, started in Brazil in the 1940s [85] and are summarized in Table 1. These Phase III efficacy trials were performed with no previous Phase I or Phase IIa assays. Most trials were developed against CL [86–90] while only one [91] was directed against VL.

Since the average vaccine efficacy value (VE) was relatively low (54.38%), the number of individuals tested was large. In America, most vaccines used the *L. amazonensis* autoclaved lysate (ALA) [87,88] or a mixture of autochthonous species [86], while all studies in the Old World used the autoclaved *L. major* antigen (ALM) (Table 1). Autoclaving of the killed parasite vaccine was introduced [89–92] as the best form of sterilization and preservation of vaccines in countries that have a rudimentary Biotechnology industry and where a cold-chain for distribution is not feasible. The work of de Luca et al. [93] proved however, that, as expected for a protozoa parasite, autoclaving destroys most of the proteins of the parasite and the vaccine loose immunogenicity. Nev-

ertheless, the LPG complex resists autoclaving [94] and has been implicated in immunogenicity and immunosuppression [95] in the mice model.

While some protocols have used no adjuvant [86,87,96] (Table 1), most prophylactic vaccines used BCG for human [88–91] and dog assays [97,98] (Table 1).

The most striking aspect of the first-generation human vaccines is that a leishmanin skin test (LST) is used for candidate selection and for confirmation of immunogenicity [86,89–91]. Whenever the LST is performed, VE is obtained among the individuals whose skin tested positive [86,89–91], whereas no efficacy is detected in assays that did not include an LST [87]. One exception is a study done in Ecuador [88] that showed positive LST but not efficacy, probably due to the use of a lower number of vaccine doses (Table 1). On the other hand, a study in Sudan that achieved 43.3% of VE against VL is impressive considering the high mortality and virulence of kala-azar there [91].

The first-generation vaccines were also used against canine visceral leishmaniasis (CVL) inducing protection in Iran [97] but failing to do so in Brazil [98] (Table 1). Results of Phase I trials of first-generation human vaccines are also available [92,99], and they show that immunogenicity is obtained after a single vaccine dose [92] (Table 1). Furthermore, these vaccines have been used for immunotherapy against human CL with success in Brazil [96] and Venezuela [100] (Table 1). Although differing in the use of BCG adjuvant and in the number of doses, the authors of both studies agree that the vaccine should not be used as monotherapy but instead, combined with the usual chemotherapy, in order to reduce toxic and very painful antimonial treatment [96,100]. Based on these results, a first-generation vaccine has been registered as adjunct to antimony therapy in Brazil [96].

## Candidates for second-generation vaccines

### Live vaccines

This category includes vaccines made of genetically modified, “knock-out” *Leishmania* spp., which lack essential genes, such as dihydrofolate-reductase thymidilate synthase [101], cystein-proteinase [102,103] or bipterin transporter [104]. These parasites undergo a short life cycle, enough to generate a specific immune response causing abortive infection and no disease in man. Another approach is to introduce in the *Leishmania* genome “suicidal cassettes” including drug sensitive genes, such as *L. major* expressing the ganciclovir-sensitive thymidine kinase gene of Herpes I virus [105] or the *Saccharomyces cerevisiae* cytosine deaminase gene sensitive to 5-fluorocytosine [106]. The authors of these studies have suggested that in Iran, where “leishmanization” is used as vaccine challenge to ensure consistency of infection rates and reduce the number of participants and duration of the assay [5,106], the use of parasites with “suicidal cassettes” would guarantee effective treatment of non-resolving lesions or of infections resistant to the usual chemotherapy [106]. However, the use of live challenge for humans is considered ethically unacceptable [107], and an artificial challenge cannot provide the valuable information obtained by exposure to natural

**Table 1** Vaccine efficacies of first-generation vaccines (since 1941)

Disease	N	<i>Leishmania</i>	Country	Adjuvant	Positive LST (%)	VE (%)	Treatment	Doses	Ref.
CL	1312	Five killed	Brazil	none	51.5	67.3	Prophylaxis	3	[86]
CL	2597	ALA	Columbia	none	Nd	0	Prophylaxis	3	[87]
CL	1506	ALA	Ecuador	BCG	74.4	0	Prophylaxis	2	[88]
CL	3637	ALM	Iran	BCG	9.85	56.7	Prophylaxis	1	[89]
CL	2543	ALM	Iran	BCG	36.2	35.5	Prophylaxis	1	[90]
VL	2306	ALM	Sudan	BCG	30	43.3	Prophylaxis	2	[91]
CVL	349	ALM	Iran	ALOH + BCG	Nd	69.3	Prophylaxis	1	[97]
CVL	1763	Lb	Brazil	BCG	Nd	0	Prophylaxis	3	[98]
CL	900	ALA	Venezuela	BCG +/-	36.5	nd	Phase I	3	[99]
CL	36	ALM	Sudan	BCG	61.6	nd	Phase I	1	[92]
CL	542	Five killed	Brazil	—	Nd	76.0	Immunotherapy	Monthly	[96]
CL	94	Live and killed	Venezuela	BCG	Nd	95.7	Immunotherapy	3	[100]

infection, which is modulated by components of sand fly saliva [108]. On the other hand, the large number of participants needed for a field trial, due to the low expected vaccine efficacy [17], can be reduced only by the use of vaccines with higher efficacies [17], such as second-generation ones formulated with potent adjuvants.

### Vaccines using recombinant viruses and bacteria as delivery vehicles

Another approach to second-generation vaccines is to use live recombinant bacteria or virus expressing *Leishmania* parasite antigen; the bacteria and virus serve as expression carrier and adjuvant system. These vaccines have limited practical application. Examples of bacteria vaccines are: *L. major* GP63 surface protease, a major *Leishmania* antigen, cloned in *Salmonella thymurium* mutant [7,109] or in BCG [110]; the LCR1 *L. chagasi* antigen (similar to a *T. cruzi* flagellar protein) in BCG [111] and the KMP-11 (kinetoplastid) antigen in attenuated tachyzoites of *Toxoplasma gondii* [112]. Examples of vaccines based on virus are Vaccinia virus expressing the G46/M-2/PSA-2 promastigote surface protein which protects against *L. amazonensis* [113]; or Vaccinia expressing the *L. infantum* LACK antigen (parasite analogue to the receptor for activated mammalian kinase C) which in prime boost vaccination, protects mice against *L. major* [114] and dogs against *L. infantum* infection [64].

### Vaccines based on purified *Leishmania* antigens

A further approach to second-generation vaccines includes the purified *Leishmania* sub-fractions. Proteins or lipophoglycan have been used to assess their immunogenicity, but because of difficulties in their mass production, they never advanced to Phase IIa or Phase III trials. In the first Phase III trial with a second-generation dog vaccine, Dunan et al. [115], using a semi-purified lyophilized protein preparation from *L. infantum* (94–67 kDa), paradoxically achieved a significantly higher rate of infection in the vaccinated group than in the control group. This vaccine then,

while effective in murine models, did not induce protection against canine kala-azar in the field [115].

Two dog vaccines achieved successful results in Phase III trials: the FML-saponin [116–118] and the LiESAp-MDP [119,120] vaccines. The glycoproteic enriched preparation of *L. donovani* promastigotes, named FML (Fucose–Mannose ligand) [12], antigenic for human [121] and dogs [122], was formulated with *Quillaja saponaria* saponin and passed Phase I–III trials to become the Leishmune® licensed vaccine in Brazil [123]. FML was immunogenic, immunoprophylactic and immunotherapeutic, in mice and hamsters, and dogs field trials [116–118,124–126]. In the first Phase III assay, 4 obits and 6 symptomatic cases among 30 placebo treated dogs (33%) were detected and confirmed by parasite analysis and PCR. No obits were detected among vaccines ( $n = 36$ ) and infection was confirmed in 3 oligosymptomatic dogs (8.33%) corresponding to 92% of protection and 76% vaccine efficacy [11]. In the second assay, the infective pressure was higher and 2 years after vaccination, obits were detected in 8/33 (25%) placebo treated and 1/20 (5%) vaccinated dogs, corresponding to 95% of protective effect and 80% vaccine efficacy [118]. This protection lasted for at least 3.5 years and was concomitant with the reduction of the human incidence of the disease in the area [118]. Noteworthy, the VE values for the FML-saponin vaccine showed protection against severe disease and obits due to visceral leishmaniasis.

The other second-generation vaccine, LiESAp, composed of the 54 kDa excreted protein of *L. infantum* plus MDP, protected dogs in a kennel assay against *L. infantum* infection. Parasites were detected in the bone marrow of 3/3 placebo treated controls, while they were absent in 0/3 vaccinated dogs [119]. A double-blind randomized trial was further performed with LiESAp+MDP in naturally exposed dogs in Southern France [120]. After 2 years, the incidence of infection was 0.61% (1/165) in vaccinees versus 6.86% (12/175) in control dogs, corresponding to a 92% VE. In any dog showing clinical and/or serological evidence, infection was confirmed by the presence of parasites in bone marrow aspirates cultured in NNN media and also by PCR analysis [120]. In contrast to results of the FML-saponin vaccine [117,118], LiESAp vaccine induced protection against infection [120], but not against severe disease or death by VL.

No obits at all were described in the 2 years *LiESAp* assay [120], reflecting the lower infective pressure of the endemic region. As explained by WHO guidelines [17], confirmation of infection by very sensitive method such as PCR or culture [119,120] represents a very early end-point of infection, while kala-azar obits and severe clinical cases [117,118] are distant end-points of infection. A comparison between efficacies of two vaccines should only be made using the same infective pressure and the same end-point targets [17]. In spite of those differences, it is worth to note that the average of VE of second-generation vaccines together is 82.67% (IC 95%, 71.13–94.21).

The FML-vaccine is considered a second-generation vaccine candidate and it was featured at the fourth Meeting on Second-Generation *Leishmania* vaccines held in Mérida in May 2001 [127]. When used at double adjuvant concentration, it is also immunotherapeutic for dogs naturally [66] or experimentally infected [63] with *Leishmania chagasi*. The sustained proportions of CD4 and CD21 lymphocyte levels in blood of vaccinated animals [63,66] indicate that the FML-vaccine reduces dog infectivity to sand flies. The adjuvants used in the FML-vaccine are the QS21 and aldehyde-containing deacylated saponins of *Quillaja saponaria* [128,129]. In 2004, the FML-vaccine became the first second-generation vaccine licensed for prophylactic veterinary use, under the name of Leishmune® [55]. Exposed dogs vaccinated with Leishmune® proved to be not infectious, as indicated by the complete absence of clinical signs, of skin-parasites based on the negative PCR results of blood and lymph node samples [55]. Leishmune® is a transmission blocking vaccine [130] for the IgG2 antibodies associated to protection, generated in vaccinated dogs, [59] caused an 80% inhibition of the *in vitro* *L. donovani* and *L. chagasi* promastigote binding to sand fly midguts and a 79.3% inhibition of *in vivo* sand fly infection, helping in the interruption of the epidemics [130]. Leishmune® vaccine proved to be tolerable, safe, and highly immunogenic [123] in a recent 600 dogs assays in the field.

Recently, the mechanism of action of the QS21 saponin was elucidated. QS21 contains two carbohydrate chains attached to a triterpene nucleus C3 and C28, besides a hydrophobic moiety which is acylated to a C28 sugar attached residue. The QS21 hydrophobic moiety is related to the induction of the CTL CD8+ protective lymphocyte response, while the aldehyde group present in triterpene C4 is involved in direct T lymphocyte stimulation, mimicking the B7-1 co-stimulatory molecule to induce the TH1 protective response [131]. Preliminary results suggest that protection induced by the FML-QS21 vaccine is also related to the activation of a bradikinin mediated inflammatory response at the site of injection, which stimulates immature dendritic cells through their B1R and B2R surface receptors thereby triggering a TH1 response against *Leishmania chagasi* [132].

## Recombinant antigens

The last approach in second-generation vaccines is the use of recombinant proteins that were intensively tested since the 1990s (Table 2). The *Leishmania* recombinant vaccine candidates were assayed alone

[7,16,109,110,133–142], in combination [143–145], or as polyproteins or chimeras [65,144,146–151]. In order to develop protection, most of them needed to be formulated with adjuvants [17,65,135,137–141,143–145,147–151], or delivered by bacteria [7,109,110,142,146], with the exception of LeIF [134] and HASPB1 proteins [133]. While most recombinant proteins were assayed for their immunogenicity and protective potential in mice models [7,17,109,110,133–137,139–143,149,150], only a few of them advanced to monkey trials for CL [143,138], to kennel assays in dog model against VL [65,144–146,151], or to pre-clinical studies in humans [147,148]. None has advanced to dog's Phase III trials (Table 2). The recombinant proteins were used against all forms of the disease and all parasite species (Table 2). In contrast to the results for first (Table 1) and second-generation vaccines with native antigens, the results of protection developed by each recombinant formulation in mice do not allow determination of VE values, since no exposure to natural infection occurred. In this review, we considered for comparison of protection due to recombinant or DNA vaccines, the reported reduction of LDU or of limited dilution values in vaccinated mice compared to untreated controls. In the specific case of CL mouse models, the results were obtained from each report as the percent of the size of the footpad lesion of the vaccinated animals compared to that of the untreated saline control, at the latest time point when data for the saline control was still available. The mean average of parasite reduction for all the assays with recombinant antigens (Table 2) is 68.02% (IC 95%, 58.32–77.71).

An interesting approach is the induction of protective immunity by a polyprotein vaccine formulation (Table 2). The TSA (thiol-specific antioxidant) and LmSTI1 (*L. major* stress inducible protein 1) are protective for mice and monkeys against CL [143], although the use of recombinant adjuvant IL-12 is not recommended at present because it may promote immune disorders [152] and fail to induce long-term immunity [135]. The multicomponent Leish-111f fusion protein containing the antigens TSA, LmSTI1 and LeIF (*Leishmania* elongation initiation factor), in formulation with MPL-SE9 and squalene, protect mice against CL and VL [149,150,153] but, in combination with MPL-SE or AdjuPrime, was only immunogenic in dogs challenged with *L. chagasi* [65] and *L. infantum* (MML) [144], and failed to prevent *Leishmania infantum* natural infection, or disease progression in dogs in an open kennel trial [151] (Table 2). These dogs [151] received two courses of three-dose vaccine with 1 year interval. Nevertheless, Leish111f + MPL-SE showed to be safe, immunogenic, and reactogenic in healthy volunteers in the US and in patients of CL and ML in Brazil and in Peru, respectively [148], while vaccination with the Leish111f components, *Leishmania* heat shock protein 83 (Lbhsp83) and GM-CSF, combined to chemotherapy, led to clinical improvement and complete cure of six human patients with MCL [147]. Furthermore, the H1 histone that protected mice [137] and monkeys against CL [138], the HASPB1 (hydrophilic acylated surface protein B1) or both in combination with Montanide [144], and the protein Q, a chimeric antigen composed of the genetic fusion of five fragments of the acidic ribosomal protein Lip2a, Lip2b, P0 and the histone H2A used with BCG [146] developed partial protection against CVL in dogs against infection and at

**Table 2** Second-generation recombinant antigens (1990s)

Antigen	Adj./delivery system	Model	Disease	<i>Leishmania</i>	Parasite reduction (%)	Phase	Ref.
GP63	<i>S. thyphimurium</i>	Mice	CL	<i>L. major</i>	67–78	I–IIa	[7,109]
GP63	BCG	Mice	CL	<i>L. mexicana</i>	68–83	I–IIa	[110]
GP63	BCG	Mice	CL	<i>L. major</i>	42	I–IIa	[110]
HASPB1	–	Mice	CL	<i>L. donovani</i>	60	I–IIa	[133]
LeIF	–	Mice	CL	<i>L. major</i>	57	I–IIa	[134]
TSA and LmSTI1	IL12	Mice	CL	<i>L. major</i>	98–99	I–IIa	[143]
	IL12 and alum	Monkey	CL	<i>L. major</i>	97	IIa	[143]
Leish 111 = LeIF + TSA + LmSTI1	MPL-SE/RIBI 529-SE	Mice	CL	<i>L. major</i>	53–67	I–IIa	[149]
Leish 111 = LeIF + TSA + LmSTI1	MPL-SE/Adjuprime	Mice	VL	<i>L. infantum</i>	99.6	I–IIa	[150]
Leish 111 = LeIF + TSA + LmSTI1	MPL-SE/Adjuprime	Dog	CVL	<i>L. chagasi</i>	–	I	[65]
Leish 111 = LeIF + TSA + LmSTI1	MPL-SE/Adjuprime	Dog	CVL	<i>L. infantum</i>	–	Open kennel	[151]
MML	MPL-SE	Dog	CVL	<i>L. infantum</i>	–	IIa	[144]
LeIF + TSA + LmSTI1 + Lbhsp83	GM-CSF	Human	MCL	<i>L. brasiliensis</i>	Cure	it <sup>a</sup>	[147]
Leish 111 = LeIF + TSA + LmSTI1	MPL-SE	Human	CL	–	Safe	I	[148]
LACK	IL-12	Mice	CL	<i>L. major</i>	53	I–IIa	[135]
LACK	–	Mice	CL	<i>L. amazonensis</i>	0	I–IIa	[136]
H1	IFA and IL-12	Mice	CL	<i>L. major</i>	64–53	I–IIa	[137]
H1	Montanide	Monkey	CL	<i>L. major</i>	37.5	IIa	[138]
H1, HASPB1, H1 +HASPB1	Montanide	Dog	CVL	<i>L. infantum</i>	5/8–4/8	IIa	[144]
CPb	Poloxamer 407	Mice	CL	<i>L. major.</i>	41	I–IIa	[139]
CPb + CPa	QuilA + IL-12	Dog	CVL	<i>L. infantum</i>	0	I–IIa	[145]
PSA-2	<i>C. parvum</i> /ISCOM <sup>is</sup>	Mice	CL	<i>L. major</i>	0	I–IIa	[140]
LCR1	CFA	Mice	VL	<i>L. chagasi</i>	67	I–IIa	[141]
NH36	<i>Q. saponaria</i> saponin	Mice	VL	<i>L. chagasi</i>	79	I–IIa	[17]
A2	<i>P. acnes</i>	Mice	VL	<i>L. donovani</i>	88	I–IIa	[142]
Protein Q chimera	BCG	Dog	CVL	<i>L. infantum</i>	50–90	I–IIa	[146]

<sup>a</sup> it = Immunotherapy.

**Table 3** Candidates for third-generation-DNA vaccines against leishmaniasis (1990s)

Antigen	Adj./delivery system	Model	Disease	<i>Leishmania</i>	µg	Virulence	Challenge	Parasite reduction (%)	Phase	Ref.
GP63	pCMV	Mice	CL	<i>L. major</i>	100	NV	1 × 10 <sup>6</sup> pro	57	I–IIa	[14]
LACK	pcDNA3	Mice	CL	<i>L. major</i>	100	NV	1 × 10 <sup>5</sup> pro	42	I–IIa	[135]
LACK	pcDNA3	Mice	CL	<i>L. major</i>	100	NV	1 × 10 <sup>5</sup> pro	56	I–IIa	[9]
LACK	pcDNA3 ± IL-12	Mice	VL	<i>L. donovani</i>	100	NV	1 × 10 <sup>6</sup> pro	0	I–IIa	[162]
HPB-LACK <sup>a</sup>	pcDNA3-Vaccinia Virus	Mice	CL	<i>L. major</i>	100	NV	5 × 10 <sup>4</sup> pro	68	I–IIa	[114]
HPB-LACK <sup>a</sup>	pCIneo-Vaccinia Virus	Dogs	CVL	<i>L. infantum</i>	100	V	1 × 10 <sup>8</sup> pro	3/5	I–IIa	[64]
LACK	pCMV3ISS	Mice	CL	<i>L. major</i>	50	V	5 × 10 <sup>3</sup> ama	26	I–IIa	[156]
GP63	pCMV3ISS	Mice	CL	<i>L. major</i>	50	V	5 × 10 <sup>3</sup> ama	21	I–IIa	
P20	pCMV3ISS	Mice	CL	<i>L. major</i>	50	V	5 × 10 <sup>3</sup> ama	20	I–IIa	
PSA2	pCMV3ISS	Mice	CL	<i>L. major</i>	50	V	5 × 10 <sup>3</sup> ama	26	I–IIa	
TSA	pcDNA3	Mice	CL	<i>L. major</i>	100	V	1 × 10 <sup>4</sup> ama	84	I–IIa	[157]
LmST11	pcDNA3	Mice	CL	<i>L. major</i>	100	V	1 × 10 <sup>4</sup> ama	32	I–IIa	
TSA + LmST11	pcDNA3	Mice	CL	<i>L. major</i>	100	V	1 × 10 <sup>4</sup> ama	90	I–IIa	
H2A + H2B + H3 + H4	pcDNA3	Mice	CL	<i>L. major</i>	200	NV	5 × 10 <sup>4</sup> pro	74	I–IIa	[158]
Cpa + CPb	pCB6	Mice	CL	<i>L. major</i>	200	NV	1 × 10 <sup>6</sup> pro	38	I–IIa	[159]
HPB CPa and CPb <sup>a</sup>	pCB6+ Montanide 720 and CPG	Dog	CVL	<i>L. infantum</i>	200	NV	5 × 10 <sup>6</sup> pro	8/10	I–IIa	[15]
KMP11, TRYP, LACK and GP63	pMOK	Dog	CVL	<i>L. infantum</i>	800	V	5 × 10 <sup>7</sup> pro	0/12	I–IIa	[51]
P4	pcDNA3	Mice	CL	<i>L. amazonensis</i>	100	V	2 × 10 <sup>5</sup> pro	25	I–IIa	[168]
P4	pcDNA3 + HSP70	Mice	CL	<i>L. amazonensis</i>	100	V	2 × 10 <sup>5</sup> pro	60	I–IIa	
P4	pcDNA3 + IL-12	Mice	CL	<i>L. amazonensis</i>	100	V	2 × 10 <sup>5</sup> pro	99	I–IIa	
A2	pcDNA3	Mice	VL	<i>L. donovani</i>	100	NV	2 × 10 <sup>8</sup> pro	97	I–IIa	[160]
ORFF	pcDNA3	Mice	VL	<i>L. donovani</i>	100	NV	2 × 10 <sup>8</sup> pro	59	I–IIa	[163]
NH36	VR1012	Mice	VL	<i>L. chagasi</i>	100	V	2 × 10 <sup>8</sup> ama	88	I–IIa	[16]
NH36	VR1012	Mice	CL	<i>L. mexicana</i>	100	V	1 × 10 <sup>6</sup> pro	65	I–IIa	[16]
NH36	VR1012	Mice	CL	<i>L. amazonensis</i>	100	V	1 × 10 <sup>6</sup> pro	81	I–IIa	[164]
NH36	VR1012	Dog	VL	<i>L. chagasi</i>	750	CVL	7 × 10 <sup>8</sup> ama	4/6	I–IIa	[165]
KMP11	pCMV-LICK	Hamster	VL	<i>L. donovani</i>	100	V	1 × 10 <sup>6</sup> pro	85	I–IIa	[166]
papLe22	pcDNA3.1	Hamster	VL	<i>L. infantum</i>	100	V	1 × 10 <sup>7</sup> ama	50	I–IIa	[161]
NH36	VR1012	Mice	VL	<i>L. chagasi</i>	100	V	2 × 10 <sup>8</sup> ama	91	I–IIa	[167]

<sup>a</sup> HPB = Heterologous prime-boost.

the clinical level. Finally, poloxamer 407 adjuvant CPb, but not CPa was able to protect mice against CL [139] whereas both in combination with IL-12 and QuilA, failed to protect dogs from *L. infantum* infection, probably due to the low concentration of the adjuvants (50 µg in each vaccine dose) [145].

As single candidate recombinant vaccines tested in mice, the *L. major* LACK with IL-12 was less efficient than the plasmid DNA encoding LACK [135,136,154], the recombinant PSA2 antigen (parasite surface antigen 2) in ISCOMs or with *C. parvum*, induced a TH1 response but not protection against CL [136,140], while the LCR1 protein [141] and the amastigote-specific A2 antigen [142] conferred protection to VL. The nucleoside hydrolase of *L. donovani* (NH36) is an essential enzyme that releases DNA bases from imported nucleosides, allowing the parasite to construct its own DNA [16], since *Leishmania* parasites lack the *de novo* purine biosynthesis pathway. NH36 is the main antigen of the FML complex [155] that constitutes the Leishmune® vaccine for CVL [55]. The recombinant NH36 in combination with *Quilaja saponaria* Molina saponin protected mice against *L. chagasi* and *L. mexicana* infection (Table 2) through a TH1 response [16].

In 1996, the WHO-TDR program organized comparative studies of several leading recombinant proteins known at that time. In 2001, the fourth Meeting on Second-Generation *Leishmania* vaccines evaluated these studies and discussed trends in *Leishmania* vaccine development [127]. While most antigens induced lymphocyte proliferation and IFN $\gamma$  secretion in naïve mice and human patients, the only antigens that protected mice against CL were the MIX, LACK, 4H6 and FPA. No assays were performed against VL and no quantification of reduction of parasitic load was done. The main detected problem was the lack of stability and potency during the transportation of the antigens [127], suggesting potentially severe problems with future scale-up of recombinant vaccine production.

## Candidates for third-generation vaccines

Compared to recombinant protein vaccines, DNA vaccines are much more stable and have the advantage of their low cost of production, no need of cold chain for distribution, and flexibility of combining multiple genes in a simple construct. The mechanism by which DNA vaccination generates potent immune responses appears to be through the activation of innate immune responses by the non-methylated CpG sequences of bacteria and to the intense replication within the host, leading to the expression of the recombinant proteins for longer periods.

The most-studied antigens (Table 3) were those previously assayed as recombinant proteins [14,16,51,135,137,156–161]. Most of them were tested as single vaccines [9,14,16,135,156,157,160–167], and some, as combination of genes [156–159,168] or as heterologous prime-boost (HPB), which involves an injection of the DNA vaccine followed by an injection of the recombinant protein [15] or a Vaccinia virus expressing the recombinant protein [64,114]. Adjuvants were added to formulations in only two studies [15,162] (Table 3). Protection was observed in vaccines using all the tested plasmids (Table 3)

[14,16,64,135,156,159,161,164–167], with the exception of pMOK in a dog assay [51], and of pcDNA3 in a mice assay [162]. Most trials were performed in mice against CL and VL [9,14,16,114,135,156–160,162–168], and some in the hamster against VL [166,161] and in dogs against CVL [15,51,64,165]. All the studies involved artificial challenge (Phase I–IIa) with agents of the New and Old World leishmaniasis (Table 3). The most striking characteristic of the DNA vaccine studies is that about a half of them used for challenge non-virulent (NV) *Leishmania* strains, grown in liquid culture media [9,14,15,114,135,158–160,162,163] and at a low number of parasites [9,114,135,158], while the rest used virulent [V] parasites isolated from infected animals [16,51,64,156,157,161,164–168] (Table 3). This heterogeneity of protocols determined a high variation of percents of reduction of parasite load, with a mean average of 59.24% (IC 95% 47.75–70.73).

The different codified antigens could be the responsible for the different degrees of protection. However, controversial results for the same antigen [9,64,114,135,156,162] refute this idea (Table 3). Protection did not correlate to the type of expression vector ( $p > 0.05$ ), or to the addition of IL-12 [162,168]. It seems to be easier to achieve protection in isogenic mice than in dogs [15,51,64,165]. Most of the mouse investigations used 100 µg of plasmid, while protection due to CP proteinase vaccine required 200 µg [159], and protection of dogs against CVL was achieved by using 750 µg of NH36 plasmid DNA vaccine [165]. These results suggest the need for higher plasmid concentrations to achieve efficacy.

DNA vaccines are indeed protective, although to date no Phase III trial data are available. Although this review is focused on vaccine candidates that already show any efficacy, a lot of interest was though generated in vaccine development against leishmaniasis in recent years, with studies going on in the labs on experimental models. The LACK, LeIF, TSA, LmSTI1, H1, CpA + CpB, KMP11 and NH36 are the most promising candidates that may find a place in the forth coming years (Table 4), since they have already been tested in more animal models.

In mice, LACK DNA induced a TH1 response [9,156] that protected against infection by *L. major* [9,135], but not *L. donovani* [162]. Even truncated portions of the LACK gene and PSA2 gene were superior to GP63 and p20 against *L. major* infection [156], and immunization with HPB-LACK (Table 3) protected mice against VL [114] and dogs from CVL [64]. The immunization resulted in an increase in IFN- $\gamma$  and IL-12 expression, lymphocyte proliferative response, IgG2 to IgG1 ratio while it led to decreases in clinical

**Table 4** Promising candidates for third-generation vaccines

Candidate antigen	Tested animal model
LACK	Mice and dogs
LeIF, TSA, LmSTI1	Mice, monkey, human
H1	Mice, monkey
Cpa + CpB	Mice, dogs
KMP11	Mice, hamsters, dogs
NH36	Mice, dogs

**Table 5** Efficacy of vaccines for leishmaniasis developed so far (1941–2007)

Generation	Adjuvant	Phase	Average protection	IC 95%	Evaluation	Model
1st	Yes/no	III	54.38	39.84–68.92	VE	Human, dog
2nd native	Yes	III	82.66	71.12–94.20	VE	Dog
2nd recombinant	Yes	Ila	68.02	58.32–77.71	Parasite load	Mice, monkey, dog
3rd	No	Ila	59.24	47.75–70.73	Parasite load	Mice, dog

symptoms, number of parasites in target tissues, and IL-4 expression.

Vaccination of mice either with the TSA or the LmSTI1 DNA vaccines, or with both as a tandem digene construct [157], protected against CL through a CD4+ TH1 response. The digene and the TSA gene were the most protective, with the latter involving a CD8+ response [157].

Injection of a mixture of four histone plasmids (H2A, H2B, H3 and H4) in Balb/c mice also protected against *L. major* infection through a TH1 response [158].

While plasmid CPa and CPb on their own did not, the combination of both conferred protection on mice against *L. major* [159], and a prime boost vaccination with Montanide 720 and CPG protected dogs against CVL, as evidenced by the increase in IgG2 specific antibody synthesis, lymphocyte proliferation, IFN $\gamma$ /IL10 secretion, and DTH response. No death or clinical signs were reported [15], probably due to the very low infective challenge ( $5 \times 10^6$  promastigotes). Eight of 10 vaccinated dogs were considered protected based on their PCR negative results [15]; however, no significant conclusion could be drawn from the study, because it used only two untreated control dogs.

Vaccination with 200  $\mu$ g of KMP11 protected hamsters against VL through a mixed cytokine TH1/TH2 response [166], while a cocktail of plasmid DNA encoding KMP11, TRYP, LACK, and GP63 (200  $\mu$ g of each plasmid) did not protect dogs against *L. infantum* virulent challenge [51].

The NH36 DNA vaccine protected mice against infection by *L. chagasi*, *L. mexicana* [16], and *L. amazonensis* [164], indicating its potential usefulness in a bivalent immunoprophylactic vaccine for the control of both endemics. Protection by the DNA vaccine was higher than that induced by the recombinant NH36 or the FML antigen plus saponin, and it is related to IFN- $\gamma$ -producing CD4+ T cells, which are characteristic of a TH1 type immune response [16]. A dog Phase I-IIa study on the immunoprophylactic effect of the NH36 vaccine is in progress, and protection has been observed so far in 4/6 vaccinated dogs [165]. While the therapeutic PSA/GP46 DNA vaccine caused reduction of *L. major* skin lesions in mice [169], the NH36 DNA vaccine reduces parasite load and increases survival of mice infected with *L. chagasi* [167].

The comparison of the efficacies achieved by the different generations of vaccines against leishmaniasis is summarized in Table 5. While the first-generation vaccines, with or without adjuvant, display a relatively low VE, the second-generation vaccines with native antigens show a significant increase in VE. The VE results derived from these two groups of vaccines are very robust, since they arise from field trials with exposure to natural challenge in the target species (human and dogs). On the other hand,

while the protection induced by the recombinant protein second-generation vaccines are slightly greater, neither the recombinant nor the DNA vaccines are significantly different from the first-generation ones. It is worth noting that the results of these last two groups are calculated based on reduction of parasite load achieved mainly in mice laboratory studies.

### Vaccines based on sand fly salivary antigens

Based on the observations that the saliva of sand flies enhances the infectivity of pathogens [170], vaccines have been designed against components of saliva or insect gut antigens that can protect from infection [171] and decrease the viability and reproducibility of the insect [172, 173]. This is the case of the protein MAX or MAXADILAN [174] and of the SP15 antigen obtained from *Phlebotomus papatasi* that induced substantial resistance in mice to infection by *L. major* [175].

### Synthetic vaccines

Recent reports suggest that CD8+ in addition to CD4+ T cells might play a role in defence and cure of leishmaniasis. To identify epitopes recognized by CD8+ T cells to be used in future synthetic vaccines, the sequence of the KMP11 protein was scanned and thirty nonapeptides were identified which specifically trigger IFN- $\gamma$  secretion by human CD8+ cells through the MHC class system [176]. In the future, this approach will allow the development of synthetic T cell vaccines against leishmaniasis.

### Conclusions and perspectives

In spite of the many genes identified as vaccine candidates, the slow knowledge transfer from the laboratory to industry, the GMP regulations that dampen down the industrial interest, the poorly developed biotechnology industry and the lack of scientists in regulatory agencies of underdeveloped countries, the ethical constraints on research in animals and the increasing dog-chemotherapy in Europe, where human leishmaniasis is less frequent, contribute to the continuous use of first-generation and live vaccines, and to the delay in the arrival of combined DNA vaccines to Public Health. The prediction is that vaccines for leishmaniasis in the fore coming 25 years will be of second-generation type, composed of complex native antigens and well developed adjuvants.

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