Review

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Vaccines in leishmaniasis: advances in the last five years

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The leishmaniases are a group of diseases caused by protozoa of the genus *Leishmania* which affects millions of people worldwide. The leishmaniases are transmitted to the vertebrate hosts by phlebotomine sand flies. In this review, we focus on clinical aspects of the leishmaniases and on the immune response against the parasite, both in animal models and humans. These aspects are of key importance to understand the many attempts to obtain an effective vaccine against *Leishmania*. We considered the last advances in new generation vaccines, including the use of new adjuvants to improve the protective response against the parasite. Finally, the possibility to use components of the sand fly saliva as part of vaccines against the infection by *Leishmania* is mentioned.

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The leishmaniases represent endemic infections occurring in various regions of the world but predominantly in the tropical and subtropical regions. Outbreaks are often registered during deforestation or incursions into forests. Leishma niases are transmitted by different species of blood sucking phlebotomines (sand flies). Cur rently, the leishmaniases, prevalent in four conti nents, are considered to be endemic in 88 coun tries, 72 of which are developing countries: 90% of all visceral leishmaniasis cases occur in Bangla desh, Brazil, India, Nepal and Sudan; 90% of mucocutaneous leishmaniasis occur in Bolivia, Brazil and Peru; 90% of cutaneous leishmaniasis cases occur in Afghanistan, Brazil, Iran, Peru, Saudi Arabia and Syria

Leishmaniases present a wide spectrum of clinical manifestations, with either tegumentary or visceral involvement. The infection may be visceral, as in visceral leishmaniasis (VL) or kala azar, restricted to the skin, as with the chronic ulcer of Oriental sore, or may spread to mucosae to cause the disfiguring espundia. Tegumentary leishmaniasis ranges from localized cutaneous and mucocutaneous leishmaniases (CL and MCL), representing the responsive pole, to dif fuse cutaneous leishmaniasis (DCL), represent ing the unresponsive pole. VL ranges from sub clinical to fatal disease. All these different clinical presentations are caused by otherwise morphologically similar *Leishmania* species [1].

The parasites that cause New World CL and MCL are grouped under the *Leishmania bra-ziliensis* and *L. mexicana* complexes. *L. tropica, L. aethiopica* and *L. major* represent the etiological agents of the Old World CL.

Parasites from the *L. donovani* complex are responsible for VL cases in both the Old and New Worlds. *L. donovani* from the Americas is also designated *L. infantum*.

Transmission

The leishmaniases are transmitted by the bite of infected female phlebotomine sand flies, pri marily infected by animal reservoir hosts. Humans are also a reservoir for some forms of the disease. The life cycle begins when an infected female sand fly takes a blood meal from a human host. As the sand fly feeds, *Leish-mania* metacyclic promastigotes enter the vertebrate host via the proboscis.

Within the human host, the metacyclics are phagocytosed by macrophages where they differ entiate into amastigote forms, which reproduce by binary fission. They increase in number until the infected cell eventually bursts releasing para sites able to infect other phagocyctic cells. When the infected host is bitten by another female sand fly, parasites are picked up by the fly and the life cycle continues.

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Clinical manifestations

It is often assumed that the type of disease is determined by the species of parasite but actually this is an oversimplification. The genetics and immunocompetence of the host may be equally important for some parasite species.

Tegumentary leishmaniasis

Cutaneous lesions begin at the site of parasite entrance as a small papule which develops into a nodule that ulcerates in the center. The incubation period ranges from 2 weeks to several months (reviewed in [1]). The initial lesion generally transforms into an indolent ulcer. The most frequent aspect observed in CL cases is a single ulcer with elevated borders and a sharp crater. In around 1% of cases, due to hematogenous spread, a pattern of disseminated CL can be observed, sometimes with more than 700 small ulcer ated lesions in a patient. Disseminated CL is completely different from diffuse CL, a rare entity in which many lesions are also observed but in which the lesions are nodular and nonulcerated.

Mucosal involvement, a characteristic of MCL occurs in less than 3% of patients infected with *L. braziliensis* (reviewed in [1]. Patients with multiple or extensive skin lesions above the waistline and inadequate therapy of the primary lesion are at higher risk of developing MCL. Mucosal lesions occur a few weeks to many years after the onset of infection. Usually, this type of involvement begins in the nasal mucosa but the disease can spread to the hard and soft palate, uvula, pharynx, gums and upper lip and may cause extensive destruction of the nasal septum.

The cutaneous lesions of Old World leishmaniasis cause much less harm than those of the New World. Between 90 and 95% of these lesions heal spontane ously and only rarely cause a mutilated aspect. *L. tropica* is responsible for the 'dry' type of disease, characterized by lesions in exposed areas as single or mul tiple soft red papules. *L. major* produces the rural 'wet' type of disease. The lesions are larger, multiple in 80% of the cases and display a strong tendency towards central necrosis producing a hemorrhagic crust. The lesions show progressive flattening and complete spontaneous healing, frequently within a few months, leaving a fibrotic scar. Lesions caused by *L. aethiopica* are usu ally single, appearing as reddish plaques, generally with a shallow ulcer in the center and papular elements in the neighborhood. With few exceptions, the infection confers lifelong immunity.

Visceral leishmaniasis

The incubation period of VL is estimated to range from 2 to 4 months. The disease may present an acute, subacute or chronic evolution but most infected individuals remain completely asymptomatic (reviewed in [1]). The onset of disease is generally insidious but less frequently it manifests abruptly, with a fatal outcome within a few weeks. The subacute form of disease gen erally occurs in children as well as the very severe form, evolving to death. The chronic form has a prolonged course, frequently presenting remissions (reviewed in [1]).

The asymptomatic form (latent infection) is characterized by a positive serology and normal physical examination. Some of these infected individuals present a positive intradermal test. Infected individuals may evolve to a subclinical form of VL or directly to an overt form of disease (classical VL). The classical manifestations of VL are fever, cough, weight loss, weakness, diarrhea or dysentery and abdominal swelling. Patients also present with anemia, edema, bleeding episodes and huge hepat osplenomegaly. Association with conditions that cause imunos supression can lead to progression from an asymptomatic form to the classical disease.

Leishmania/*HIV coinfection*

Leishmania/HIV coinfection is emerging as an extremely seri ous, new disease and coinfections are being reported more fre quently in various parts of the world. It is anticipated that the number of *Leishmania*/HIV coinfections will continue to rise in the coming years and there are indications that cases are no longer restricted to endemic areas. The overlapping geographi cal distribution of VL and AIDS is increasing due to two main factors: the spread of the AIDS pandemic in suburban and rural areas of the world and the simultaneous spread of VL from rural to suburban areas.

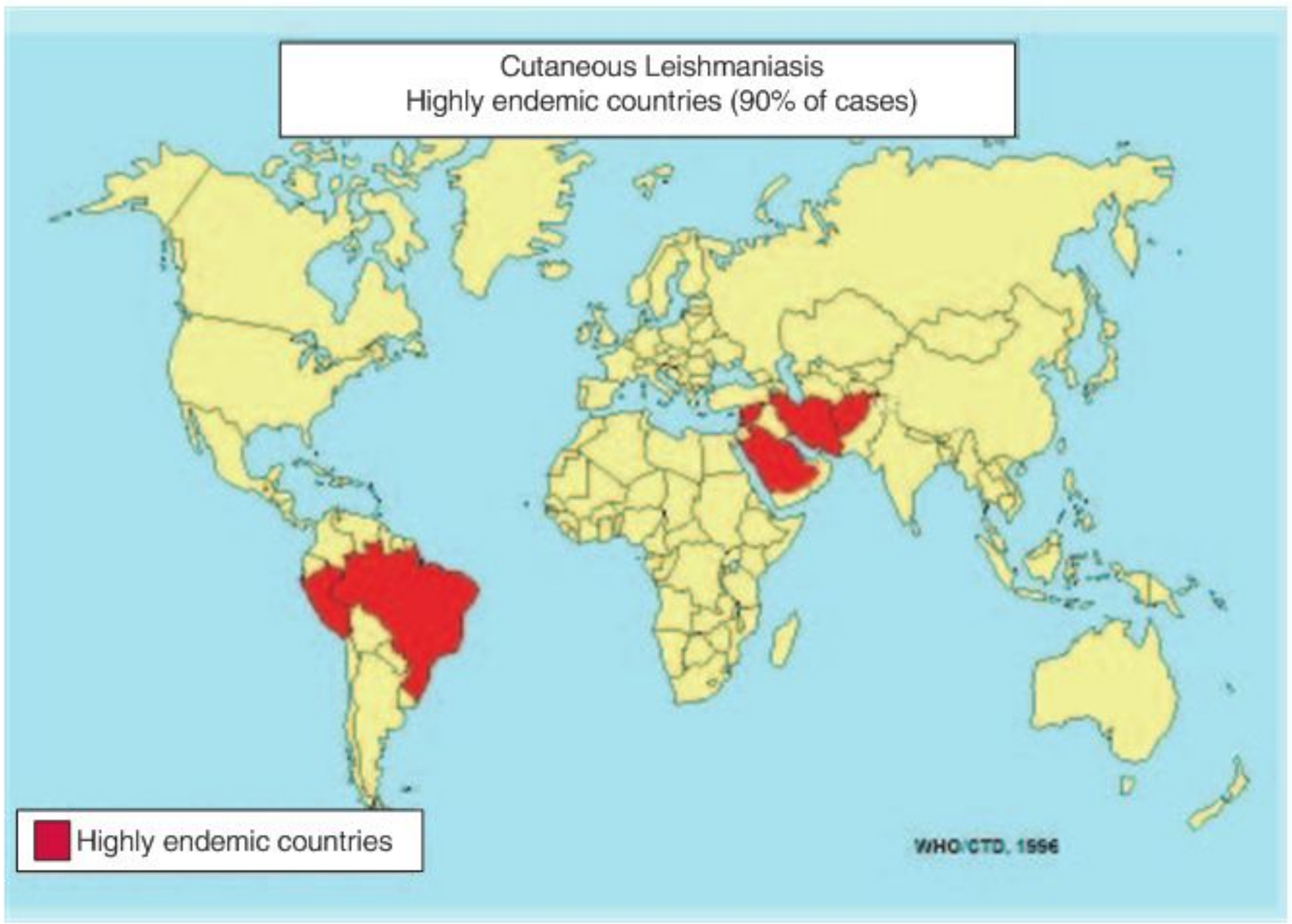

Figure 1. Global distribution of reported cases of cutaneous leishmaniasis, as examined by the world health organization (WHO) [101].

Table 1. Clinical characterisitics of human leishmaniasis.

Characteristic	Mucocutaneous	Cutaneous	Diffuse	Visceral
Lesions	Ulcerative destruction of the nasal septum	Single, occasionally a small number, ulcerated lesion with elevated borders and necrotic center	Nonulcerated nodules spread through different areas of the body	Internal organs
Histopathology and parasite numbers	Granulomatous reactions with very few parasites	Chronic inflammatory responses with moderate number of parasites	Monotonous macrophagic infiltration with abundant parasites	Marked macrophagic proliferation with heavy parasitism in the hematopoietic organs
Anti *Leishmania* antibody levels	Low	Low	Moderate to high	High
Anti *Leishmania* CMI (*in vitro* and *in vivo* tests)	Strongly positive	Positive	Negative	Negative

CMI: Cell mediated immunity.

AIDS and VL aggravate each other. VL accelerates the onset of AIDS and shortens the life expectancy of HIV infected people and AIDS increases the risk of VL by 100–1000 times in endemic areas. VL is considered a major contributor to a fatal outcome in co infected patients. Leish maniasis can be transmitted directly from person to person through the sharing of needles, and intravenous drug users are the main population at risk for coinfection.

Laboratory diagnosis

Routine diagnosis is made by direct examination of smears, his topathological examinations of lesions, serological tests or skin testing with *Leishmania* antigen (Montenegro's reaction). A definitive diagnosis depends on the identification of amastig otes in tissue or isolation of promastigote in culture. Antileish manial antibodies are present in the serum of patients with CL as detected by ELISA or immunofluorescence, direct agglutina tion or other assays [2]. The Montenegro skin test is positive in only 51.6% of cases of *L. amazonensis* infection, whereas a pos itivity of 87% is observed in infections with other species of *Leishmania* (reviewed in [1]).

The polymerase chain reaction (PCR) offers several advan tages over the previously described methods, in the sense that it can be more sensitive, more specific and, most importantly, more rapid than the above mentioned methods. A number of PCR assays have been described for the detection and charac terization of *Leishmania* parasites [3,4]. These assays target the amplification of kinetoplast DNA (kDNA), rRNA, miniexon genes and nuclear DNA sequences. These methods present varying specificities: some identify the parasite to the species complex whereas others will identify all *Leishmania* species. This information is sufficient for diagnostic purposes; how ever, higher resolution can contribute directly to understand ing the epidemiology of leishmaniasis. PCR is particularly useful in the case of CL because of the requirement of parasi tological confirmation for definite diagnosis and because of the low sensitivities of currently used methods. In the case of cutaneous leishmaniasis due to *L. braziliensis*, the scarcity of parasites in the lesion makes parasitological confirmation rather difficult. This may lead to misdiagnosis and a delay in starting the treatment, which is especially concerning since *L. braziliensis* parasites have a tendency to metastasize to the mucosal areas. Early detection can prevent these cases allow ing the implementation of the specific treatment to patients in areas where *L. braziliensis* is prevalent.

In this sense, we have investigated the use of the PCR as a diagnostic tool in an area endemic for *L. braziliensis*. We have found the PCR to be 100% sensitive since it was able to detect parasite DNA in all biopsies from patients infected with *Leish-mania*. Furthermore, samples from healthy individuals, residing at the endemic area with a positive Montenegro skin test were also negative when tested for the presence of parasite DNA (DE OLIVEIRA, IN PRESS). These results reinforce the specificity of the PCR since it only detects cases of active infection. These results are in agreement with other reports in the literature and, collec tively, suggest that the PCR could be employed as a routine diagnostic tool.

Treatment

Pentavalent antimony containing drugs (sodium stibogluco nate and meglumine antimoniate [Glucantime, Aventis, NJ, USA]) are the first choice for leishmaniasis treatment. Amphotericin B is an alternative for patients who fail to respond to pentavalent antimony.

Both antimonials and amphotericin exhibit frequent side effects, which should be taken in account by the physician responsible for the treatment.

In the mouse model, chemotherapy is ineffective in the absence of T cells responses [5,6]. These findings highlight the importance of searching effective immunopreventive an/or immunotherapeu tic approaches against leishmaniasis and that these approaches may find use even when combined to pharmacological products [7].

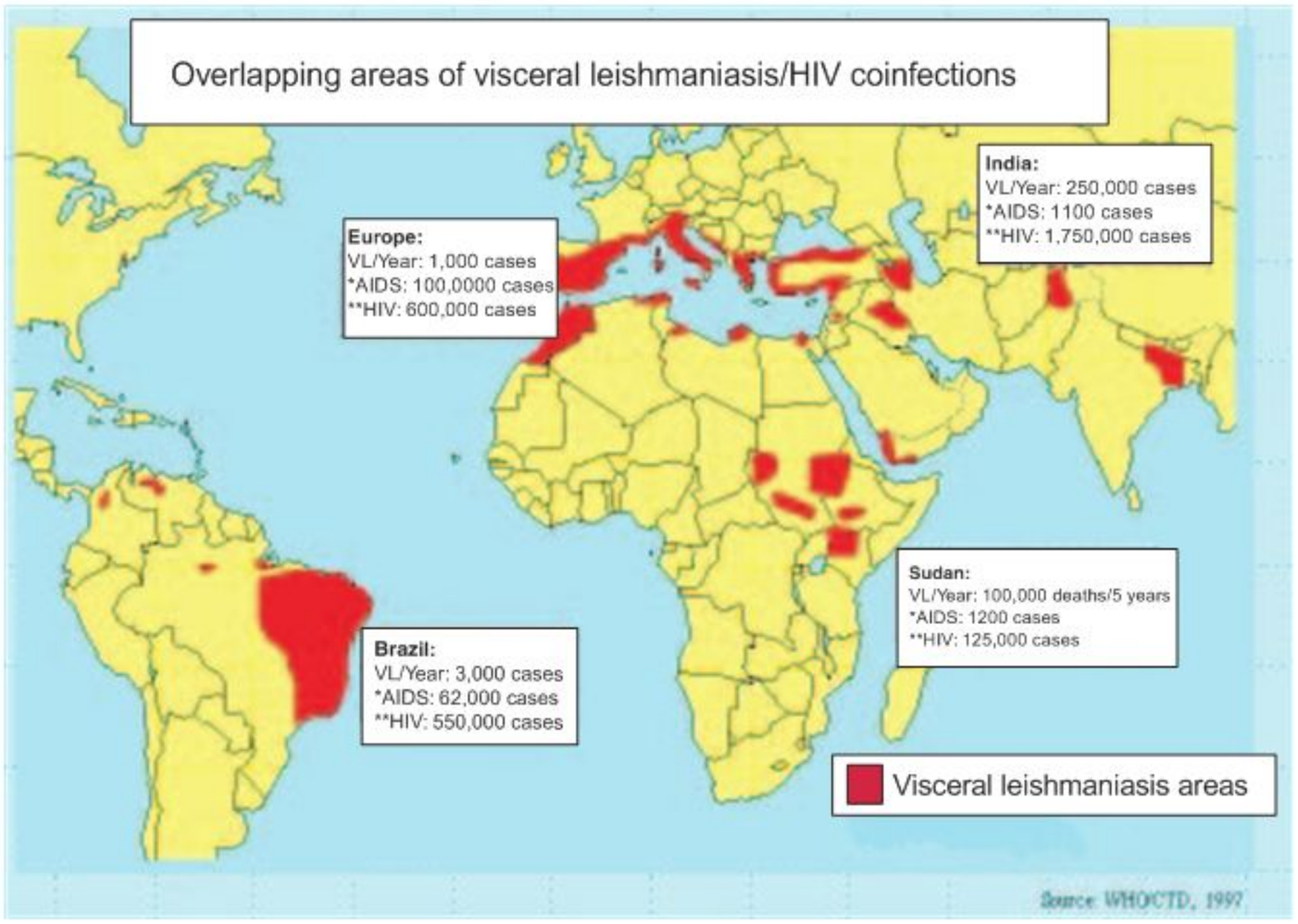

Figure 2. Global distribution areas of leishmaniasis and *Leishmania*/HIV coinfection, 1990–1998, as examined by the WHO [101].

Experimental leishmaniasis

Tegumentary leishmaniasis

Following inoculation into the skin, the flagellated promastigote penetrates into the macrophage, transforms into amastigotes and multiplies. The infected macrophage exhibits leishmanicidal activity as shown by its increased capacity to produce toxic oxygen and nitrogen radicals in response to interferon (IFN) γ.

Several experimental models of leishmaniasis have been developed and infection of mice by *L. major* is one of the best studied models. The mouse model reproduces many aspects of the human disease, including a range of susceptibility states depending on the strain of mouse used. BALB/c mice are highly susceptible, upon infection they develop large skin ulcers, which expand and metastasize, leading to death. On the other hand, C57BL/6 and CBA/N are resistant, they develop small lesions, which cure in 10 to 12 weeks and are resistant to reinfection. Most other strains show intermediate susceptibilities. The relevance of the T helper (Th) 1 or 2 balance to the regulation of outcome disease *in vivo* was demonstrated in several reports. Resistance has been thought to be linked to a Th1 response, with production of interleukin (IL) 12 and IFN γ, whereas susceptibility has been related to a predominant Th2 response determined by the presence of IL 4. Recent data, however, revealed a more complex picture.

The necessity to inhibit Th2 cytokine production in order to obtain a murine resistant genotype has been demonstrated using transgenic mice with a resistant background constitutively expressing IL 4 (for review [8]) or IL 10 [9]. These mice fail to control *L. major* infection, although they present a strong Th1 response. Therefore, the sustained production of Th2 cytokines leads to susceptibility, in spite of not precluding the development of a Th1 response. Th2 cytokines lead to the development of severe lesions in mice infected by *L. major*, probably by deactivating infected cells. The production of reactive nitrogen intermediates by IFN γ activated macrophages is inhibited by transforming growth factor (TGF) β, IL 4, IL 13, IL 10 [10]. The downregulation of Th2 cytokines might be crucial for the development of acquired resistance.

In correlation, there is also support for the need of IL 12 to redirect the early Th2 response. A downregulation of IL 12 receptor β2 chain (IL 12R) β2 may be responsible for the defective IL 12 response in BALB/c mice [11], which could explain the high susceptibility of BALB/c mice to *L. major*. The relevance of this process has been questioned, since BALB/c mice expressing an IL 12Rβ2 transgene maintain a susceptible phenotype, despite stable IL 12 signaling and activation of STAT4 [12]. Therefore, the susceptibility or resistance of different strains of mice to infection by *L. major* depends on multiple factors. Among them, the inflammatory or tissue environment in which the early Th2 response is induced might be important to the outcome of infection. For instance, the presence of neutrophils, present in the inflammatory infiltrate for several weeks in the BALB/c footpad, might contribute to the sustained induction of Th2 response because the depletion of neutrophils at the time of *L. major* inoculation in BALB/c inhibits IL 4 response and promotes partial resistance [13]. Another factor that contributes to the susceptibility of BALB/c mice is the existence of different populations of dendritic cells (DCs) with the capacity to induce preferentially Th1 or Th2 cells. Such populations are probably not distinct APC lineages but the result of distinct modulation by cytokines or chemokines present in specific tissue environment [14]. Skin DCs from both resistant C57BL/6 and susceptible BALB/c mice release IL 12 when infected with *L. major* [15]. Recently, it has been shown that IL 1α administration resulted in increased Th1 and decreased Th2 cytokine production. IL 1α and IL 12 treatments were similarly effective and the IL 1α efficacy was strictly IL 12 dependent. These data indicate that local administration of IL 1α acts in conjunction with IL 12 influencing Th development in cutaneous leishmaniasis [16, 17]. On the other hand, disease outcome may not be defined by cytokine production only. Although BALB/c and B10.D2 mice have the same major histocompatability complex (MHC) phenotype (H2 d), their $DC11b^{+}$ differ in the ability to polarize naive T cells into either Th1 or Th2 effector cells. This difference is cell intrinsic; is not restricted to MHC haplotype and is observed with both parasite specific and allospecific CD4 T cells [18].

Interactions between costimulatory molecules and their receptors (CTLA 4/CD28 B7 and OX 40 OX40L) may influ ence the development of Th2 responses to the parasite *in vivo* (for review, [8]) , suggesting the differential expression of these molecules on distinct subsets of APCs in different tissues.

Although an early Th2 response may not be a crucial event in the development of a nonhealing phenotype in BALB/c mice, there is little doubt that a sustained Th2 response is responsible for the outcome of infection. Recently, the importance of IL 13 in the mechanism of sustaining a Th2 response has been dem onstrated [19]. Some cytokines may alter the course of the disease without changing the Th1 or Th2 cytokine pattern.

Treatment of C57BL/6 crossed BALB/c F1 mice with anti bodies against TGF β enhanced the resistance to *L. major*. This treatment did not alter IL 4 or IFN γ production but increased the production of nitric oxide by macrophages in parasitized lesions [20].

There is also ample support for the concept of inflammatory type 1 cytokines as mediators of protection. Genetic ablation of molecules *in vivo* linked to Th1 development, such as IL 12, IFN γ and TNF α, IFN γR, transcription factors (T bet and STAT4) or costimulatory molecules (CD40 CD40L) leads to sus ceptibility to *L. major* [8]. IL 12 is considered a crucial cytokine to induce IFN γ production but in the absence of IL 12 other cytokines, such as IL 18, could contribute to resistance to *L. major*. IL 18 seems to be able to inhibit the Th2 default response [21]. However, in the absence of IL 12, IL 18 is not sufficient to drive immunity to *L. major* and IL 18 is not required in IL 12 competent mice [21]. These data indicate that alternative factors can co operate with IL 12 in redirecting the early Th2 response and can affect the early stages of *L. major* infection in resistant mice but IL 12–IL 12R signaling is essential in establishing and maintaining a protective Th1 response. Healed C57BL/6 mice when treated with anti IL 12 antibodies reactivate their infection [22]. Additionally, primed Th1 cells from healed mice cannot transfer immunity to IL 12 deficient mice [23]; IL 12 seems to be required to prevent the differentiation of newly emerging precur sors of Th2. As for the cell source of cytokines, $CD4^+$ T cells seem to be implicated in IL 10 production [8].

Mouse DCs, including epidermal Langerhans cells (LCs) take up *L. major* parasites, acquire a mature phenotype and release IL 12 p40 *in vitro* [24]. Their role in producing IL 12 *in vivo* has also been shown [15,25,26]. The ability of LCs to transport the parasites from infected skin to the draining lymph nodes seems to rely on the expression of CCR2. Mice of a resistant background, deficient in CCR2 exhibit deficient LC migration and are highly susceptible to *L. major* infection [27].

Natural killer (NK) cells have been related to Th1 develop ment through IFN γ production. However, they are not essen tial for resistance. Immune deficient T cell reconstituted mice, which selectively lack NK cells, have efficient IL 12 dependent IFN γ production by $CD4^+$ T cells and heal their lesions. [27, 28].

The crucial role of $CD4^+$ T cells to mediate protection against *L. major* infection is firmly established [8,25]. The protective role of $CD8^+$ T cells is more controversial [8].

Two key features of natural transmission (low parasite and intradermal inoculation) are important in order to obtain in the murine model, similar conditions to the disease in man. Using this approach, it has been shown that both $CD4^+$ and CD8 T cells are required for the control of primary *L. major* infection [29]. Recently, the outcome of infection in anti CD8 treated and CD8 deficient mice showed that these cells are required for the control of primary infection in the skin. These results are consistent with clinical studies reporting high numbers of antigen specific $CD8^+$ T cells in lesions and peripheral blood during acute stage of the lesions and during the healing process [30]. The existence of a latent infection in resistant mice after clinical cure has been demonstrated. Moreover, these healed mice are able to maintain life long immunity to reinfection. IL 10 has a crucial role in chronic ity, demonstrated by the inability of the parasite to establish a persistent infection after healing in IL 10 deficient mice and by the sterile immunity that was achieved in wild type mice treated during the chronic phase with antiIL 10R antibody. The source of IL 10 in C57BL/6 was found to be a popula tion of $CD4^+CD25^+$ $CD45RB^{low}$ T cell subset immunoregu latory T cells [31]. Importantly, the IL 10 deficient and IL 10R antibody treated mice that achieved sterile cure were no longer immune to reinfection [31], which indicated that the maintenance of effector T cells requires antigen persistence.

Visceral leishmaniasis

VL lacks a good animal model, making it difficult to deter mine the protective mechanisms against *L. donovani*. The golden hamster has been used for a long time, since it mim ics several aspects of human disease (anemia, hyperglob ulinemia and cachexia). However, the difficulty in exploring immunological responses in hamsters leads to a preferential use of the mouse models. Studies in mice have explored the immune mechanisms important for the development of organ specific immunity, which results into clearance of par asites from the liver but not from the spleen [32,33]. Recent studies indicate the importance of cytotoxic T lymphocyte associated antigen (CTLA) 4 engagement in T cells and of TGF β production in the immunopathogenesis of murine VL. Activation of $CD4^+$ T cells from *L. donovani* infected mice by antigen or anti CD3 leads to an intense CTLA4 mediated TGF β_1 production, which increases parasite rep lication and reciprocally regulates IFN γ production [34]. Blockade of CTLA 4 enhances host resistance, increasing the expression of inducible protein (IP) 10, a chemokine directly related to the frequency of IFN γ producing cells [35]. The course of infection with *L. donovani* was examined in mice lacking the gene for IL 10. BALB/c IL $10^{/}$, as well as C57BL/6 IL $10^{/}$ mice, were highly resistant to *L. dono- vani* infection, as evidenced by liver parasite burdens which were tenfold lower than those in control mice after 14 days of infection. Enhanced resistance was accompanied by increased production of IFN γ and nitric oxide in BALB/c IL $10^{/}$ mice [36].

DCs from mice with chronic *L. donovani* infection fail to migrate from the marginal zone to the periarteriolar region of the spleen. Defective localization was attributable to TNF α dependent IL 10 mediated inhibition of CCR7 expression. Effective immunotherapy was achieved with CCR7 expressing DCs, without the need of identifying protective *Leishmania* antigens [37].

Immunological aspects of human leishmaniasis

CL patients exhibit anti *Leishmania* cell mediated immunity (CMI). ML is considered to be the hyper responsive pole since these patients exhibit a potent anti *Leishmania* CMI. With disease progression, ML patients tend to develop larger intradermal skin test reactions and their lymphocytes exhibit higher proliferative responses and production of IFN γ than cells from CL patients. On the other hand, DCL patients have transient weak responses or do not exhibit any anti *Leishmania* CMI. Delayed type hypersensitivity (DTH) is negative and their lymphocytes do not respond to leishmanial antigen either by proliferation or cytokine production. DCL patients present an antigen specific immunosuppression, mounting normal responses to other antigens.

The clinical spectrum of leishmaniasis and control of the infection are influenced by the parasite–host relationship. Th1 crossed Th2 dichotomy is probably influenced by cytokine patterns present during the very early stages of *Leishmania* survival inside the macrophage. In humans, pro duction of IFN γ is associated with the control of infection in children infected by *Leishmania infantum*. In VL, impair ment of IFN γ production and high IL 4 and IL 10 levels (Th2 cytokines) are observed in antigen stimulated periph eral blood mononuclear cells (PBMCs). Moreover, IL 12 restores IFN γ production and enhances the cytotoxic response. In human leishmaniasis, IL 10 is the cytokine involved in downregulation of IFN γ production, since anti IL 10 mAb restores *in vitro* IFN γ production and lympho proliferative responses and IL 10 abrogates the effect of IL 12 (reviewed in [38]). Cure of VL is associated with the resto ration of IFN γ production. IL 12 plays an important role in the ability of treated VL patients to produce IFN γ, suggest ing the importance of this cytokine in maintaining a Th1 response in man. Interestingly, IL 10 but not IL 4 or TGF β, is able to inhibit human Th1 responses [39] In CL and MCL, high levels of IFN γ are found in *L. amazonensis* stimulated PBMC. However, low or absent IFN γ levels were observed in antigen stimulated PBMCs from 50% of subjects with less than 60 days of disease. This response was restored by IL 12 or anti IL 10 mAbs [40]. Later during disease, high levels of IFN γ and TNF α are found both in CL and MCL, with decreased TNF α levels after treatment. IFN γ and TNF α seem to be involved both in the control of parasite multipli cation in the early phases of *Leishmania* infection and in tissue damage seen in tegumentary leishmaniasis [40].

The role of cytotoxicity in the defense mechanisms or tissue damage of human CL is not yet well known. Machado and coworkers [41]. observed the presence of NK, $CD8^+$ and $CD45RO^+$ T cells, as well as the strong expression of a mole cule associated with cytotoxic properties (TIA 1) in the der mal cell infiltrate of lesions from CL patients. The presence of these cells with cytolytic capacity in CL argues in favor of an active participation of NK and $CD8^+$ T cells in the pathogen esis of disease. These cells may play a role in the parasite kill ing but also in ulcer development [41].

Vaccines

Human studies with first generation vaccines

Active immunization against leishmaniasis has been attempted for many years. The original version of a human vaccine consisted of inoculating material from lesions into naive individuals using a thorn since producing a mild disease precluded from developing a severe disfigurement after subsequent natural exposure.

The first generation of vaccines against leishmaniasis consisted of autoclaved *Leishmania* promastigotes with or without bacil lus of Calmette and Guerin (BCG) as adjuvant. This vaccine has been tested in Phase II field efficacy trials against CL and VL. In a randomized, BCG controlled, double blind trial in Sudan, against VL, no evidence was found that the two injections of the vaccine (autoclaved *L. major* [ALM]) resulted in significant pro tection against VL when compared with BCG alone [42]. How ever, the vaccine induced higher rates of leishmanin skin test (LST) conversion relative to BCG alone. Individuals who con verted LST had a significantly lower incidence of disease com pared to LST nonresponders. Similarly, in Iran, the single injec tions and triple injections of ALM^+BCG reduced the incidence of CL in LST converted individuals but not significant overall protection compared with BCG alone [43].

Vaccination trials in Brazil and Ecuador have demonstrated that a cocktail of five killed *Leishmania* stocks or a single strain of *L. amazonensis* induces significant protection from natural infection [44–46].

These studies indicated that DTH conversion could be used as a surrogate marker for protective immunity.In a monkey model of cutaneous leishmaniasis, protective immunity was achieved using killed *L. amazonensis* coadministered with recombinant IL 12 as adjuvant [47].

The human protective responses against *Leishmania* in humans are not entirely understood impairing rational approaches to vac cine development. Animal models currently available are perhaps somewhat, but not entirely, predictive of how effective a vaccine candidate will perform in man. It is also impossible to test several candidate antigens in vaccine trials due to economic constraints. In this sense, we have tested an *c* system to evaluate the response of naive individuals.

In vitro *priming assay*

It has been shown that an *in vitro* system using lymphoid cells from naive mice primed with *L. major* promastigotes mimics *in vivo* responses in murine leishmaniasis [48]. This system has also been used with human cells in order to explore early events of anti *Leishmania* responses.

Most individuals developed Th1 or Th0 anti *Leishmania* T cell lines and infected macrophages from Th1 responders produced IL 12. Th0 responders who produced little or no endogenous IL 12 could be converted to the Th1 phenotype by addition of IL 12 during priming [49].

Using IVP, our group evaluated a large number of normal volunteers both before and after receiving a *Leishmania* vaccine. In this way we could evaluate if *in vitro* primary responses were able to predict *in vivo* antileishmanial responses in man. PBMCs from volunteers who had negative responses against *Leishmania*, both cell mediated and antibodies, were used for *in vitro* stimulation with *Leishmania* antigen, IFN γ levels were then monitored. Volunteers fell into two different types of responders: those who produced low amounts of IFN γ (low responders) and those who produced high levels of this cytokine (high responders). Low responder volunteers remained low producers of IFN γ 40 days after vaccination, whereas high responders exhibited increased IFN γ production after vaccination. However, 6 months postvaccination, all individuals tested were producing similarly high levels of IFN γ upon stimulation of their PBMCs with *Leishmania* promastigotes, indicating that the low producers *in vitro* are slow *in vivo* responders to vaccination. High IFN γ producers exhibited an increased frequency of activated $CD8^+$ T lymphocytes both *in vitro* and *in vivo*, as compared with low/slow IFN γ producers [50]. Such findings suggest that the IVP response is able to predict the rate of postvaccination response and can contribute to a leishmanial antigen screening to test future potential candidates for vaccines. Although all vaccinated individuals eventually present a potent anti *Leishmania* CMI response, a delay in the rate of mounting the CMI response may influence resistance against leishmaniasis.

Live attenuated vaccines

In the same IVP system, we compared immune responses generated by PBMCs from normal volunteers stimulated with wild *L. major* or with a genetically manipulated parasite derived from *L. major, L. major dhfr-ts*$^-$. This mutant lacks a gene essential for long term survival in the mammalian host which codes for the enzyme dihydrofolate reductase–thymidylate synthetase (DHFR–TS). It has been shown that these organisms can invade and undergo a limited number of replications in macrophages without producing disease. In a mouse model, parasites lacking DHFR–TS induced protection against infection with *L. amazonensis* [51]. In our IVP system, we observed that cells from normal volunteers developed a preferential Th1 response and no significant differences were observed between *dhfr-ts*$^-$ and *L. major* concerning IFN γ production by *in vitro* stimulated PBMCs from normal volunteers [52]. An attenuated line of *L. mexicana* was also used successfully to protect against homologous infection. This mutant lacked two genes coding for the cysteine proteases *cpa* and *cpb* [53].

The use of attenuated organisms is very attractive because their use resembles the natural course of infection and may lead to similar immune responses. Attenuated organisms cause a limited infection, resulting in lower antigen load favoring a stronger Th1 response [54]. The disadvantages of such vaccines are the logistics of their large scale production and distribution in the field.

Vaccine approaches using defined antigens

Some of the target antigens are species and life cycle stage specific, while others are shared by promastigotes and amastigotes. The immune responses in leishmaniasis can range from protective to harmful. These differences in the quality of the response are at least partly due to predominance of Th1 or Th2 cytokines and may be greatly influenced by antigen dose [55]. This is a major concern in vaccine approaches with defined antigens.

Recombinant antigens can be delivered as purified proteins, as naked DNA or as bacteria manufacturing the proteins *in situ*. Manipulations now allow targeting the antigen to specific locations or to particular APCs, such as DCs or LCs, which are considered essential for the initiation of primary T cell responses. Injection of bacteria or naked DNA may have the added advantage of providing an adjuvant effect, which may activate these APCs.

Synthetic peptides have been in use for over 10 years. However, several considerations make the peptide antigens less attractive as potential candidate vaccines, such as the magnitude of T cell memory induced, the inability of all individuals in the population to respond to the peptide and the logistics of production. Since the antigenic peptide is processed and presented to T cells in the context of MHC class I or class II and since not all peptides associate with all MHC types, some peptides will not be recognized by all individuals in the population. There are additional problems in the ability to respond to individual peptides due to failure of processing, cleavage, transport or deletion of determined T cell specificities. Another reason for the low success rate of subunit vaccines is that some polypeptides may be a minor immunogens and so even though they may be excellent in a cocktail vaccine, individually they may provide only partial protection. Despite these problems, several *Leishmania* gp63 peptides have been tested successfully in animal models [56].

In general, the success of subunit vaccines based on recombinant proteins or peptides has been variable or poor. Mice immunized with killed promastigotes or recombinant proteins plus IL 12 as an adjuvant had a high level of protection when challenged 2–4 weeks after vaccination but they had already lost substantial protection when challenged after 12 weeks [57–59]. Immunity could be maintained by repeated administration of antigen or IL 12 [22] or by antigen and/or IL 12 delivered by plasmid DNA [57–59]. As mentioned before, there may be a need of persisting parasites in the host organism to maintain immunity [31]. Therefore, it may not be possible for subunit vaccine to induce long lasting immunity on its own.

One approach generating great interest is that of inducing protective immune responses by injecting engineered DNA sequences from infectious organisms against which protection is desired. The ability of plasmid DNA encoding

specific antigens to induce both $CD4^+$ and $CD8^+$ T cell responses could be of particular use for protection against diseases that require CMI, including leishmaniasis. The low production costs and high structural ability of DNA make DNA vaccination a very attractive tool for immunization. For instance, it has been shown that immunization with rVV (vaccinia virus) expressing the *L. amazonensis* gp46 antigen elicits significant protection and long term immunological memory in BALB/c mice [60]. Experiments in mice and other animal models have shown that a combination of priming with DNA and boosting with recombinant vaccinia virus expressing the same protein is associated with high immuno genicity and protective efficacy against several infectious dis eases. Using this approach, good protection against *L. major* in mice [55] and against *L. infantum* in dogs [61] has been obtained using this heterologous prime–boost immunization regime. Many different DNA vaccines, coding for different leishmanial antigens, have been tested in mouse models, showing good results [57,62,63] and therefore, this kind of approach should become a promise for leishmaniasis vac cines. More recently, it has been shown that the combination of antigens (cocktail) in DNA vaccines might be a more effective approach, providing stronger protection [64].

A recent and interesting approach is the development of minimalistic immunogenically defined gene expression (MIDGE) vectors [65]. The linear vectors contain only the minimum sequences required for gene expression and can be chemically modified to increase the immune responses. The authors demonstrated that MIDGE vectors coding for LACK antigen confer a highly effective protection against *Leishmania* infection in susceptible BALB/c mice. Protection could be achieved at lower doses of this vector when compared with conventional plasmids.

Adjuvants

Another issue concerning peptide vaccines is related to the use of different adjuvants. In most experimental systems, adju vants are essential to generate protective immunity. However, the most effective adjuvants generally cause strong inflamma tion, which may preclude their use in humans because of unacceptable side effects. Vaccination with either soluble leishmanial antigen or recombinant leishmanial protein plus IL 12 protein did not confer long term protection [59]. How ever, if leishmanial protein plus IL 12 protein vaccinated mice were boosted with IL 12 protein, control of infection was better sustained [22]. Furthermore, mice vaccinated with leishmanial antigen plus IL 12 DNA also showed long term Th1 immunity and protection [58].

Recently, some reports have shown the possibility of use CpG oligodeoxynucleotides (ODN) as potent adjuvants in different vaccine systems including diseases requiring Th1 immune responses. ODNs have been shown to stimulate macrophages and DC sto synthesize several cytokines, including IL 12, IL 18, TNF α, IFN α, β and γ and to upregulate costimulatory molecules, such as CD40 and MHC class II; [66,67]. The range and level of cytokine pro duction vary according to each ODN sequence and its par ticular modifications [68]. Moreover, CpG ODN have been shown to activate DCs, leading to the presentation of solu ble protein to class I restricted T cells and the induction of CTL responses [67,69]. In studying the role of CpG ODN as a prophylactic vaccine adjuvant, leishmanial protein plus CpG ODN was reported to confer some protection against a challenge with *L. major* [70]. More recently, it has been observed that the ability of CpG ODN to confer long term immunity and protection (up to 12 weeks after vaccination) when used as a vaccine adjuvant with autoclaved *L. major* or a recombinant leishmanial protein. These studies were per formed in susceptible and resistant mouse strains and the results showed that in C57BL/6 mice this protection was observed after 6 months of vaccination. These vaccines induced *L. major* Th1 and $CD8^+$ responses [71]. However, it is still unclear whether CpG ODN will be a feasible approach in humans, since no IL 12 inducing CpG sequences have been identified.

Vector directed vaccine approaches

The sand fly injects the mammalian host with *Leishmania* in the presence of its saliva. The saliva of blood sucking animals contains a varied repertoire of molecules that modulates their hosts' hemostatic, inflammatory and immune responses. In mice, these products seem to exacerbate the infection with *Leishmania* and may, in fact be mandatory for the establish ment of the parasite in the vertebrate host. It has been shown that components of *Lutzomyia longipalpis* or *Phlebotomus pap-atasi* salivary gland lysates mixed with *L. major* resulted in substantially larger lesions compared with controls [72,73]. Recent studies using the murine ear model of infection have shown it is possible to transmit *L. major* by the bite of its nat ural vector, *P. papatasi,* to BALB/c and C57Bl/6 mice. Inter estingly, for both BALB/c and C57Bl/6 mice, prior exposure to *P. papatasi* bites resulted in a striking reduction in the severity of the dermal lesions and the pre exposed mice had over 1000 fold reduction in the mean number of amastigotes per infected ears, transforming them in a bad source of trans mission to uninfected sand flies [74]. Whereas enhancement of *Leishmania* transmission by saliva is probably due to immu nomodulatory components of sand fly saliva, an explanation of the anti *Leishmania* effect resulting from host immuniza tion against salivary antigen is not straightforward. Immunity in this system could derive from neutralization of salivary immunomodulators, such as the peptide maxadilan from *L. longipalpis* [73]. Alternatively, immunity could derive from a DTH reaction at the site of the bite generated by a cellular response to salivary antigens injected by the fly [74,75]. This particular reaction could turn the lesion and is surroundings into an inhospitable site for the establishment of *Leishmania* infection in the new host, or it could modify the environ ment priming the initial events of the host immune reaction to *Leishmania*. A 15kDA component from *P. papatasi* saliva,

when extracted from sodium dodecyl sulfate (SDS) polyacry lamide gels, was able to protect mice challenged with para sites plus salivary gland homogenate (SGH) [76]. A DNA vac cine containing the cDNA for the 15 kDa protein provided the same protection, even in B cell knockout mice. The results indicate that a DTH response against saliva provides most or all the protective effects of this vaccine and that sali vary glands or their cDNAs are viable vaccine targets against leishmaniasis [76].

We have shown that serum samples from children living in an endemic area for visceral leishmaniasis have anti SGS immunoglobulin (Ig)G antibodies that differentially recog nize salivary gland antigens. Individuals with a positive anti *Leishmania* DTH response exhibited anti *Lu. longipalpis* saliva antibodies. A positive correlation was observed between anti *Lu. longipalpis* antibodies and anti *Leishmania* DTH response but no correlation was observed between anti saliva and anti *Leishmania* serologic status [77]. More recently, we observed that children who experience anti *Leishmania* DTH conversion have an increase in antisand fly saliva antibodies, showing that the development of antipara site DTH coincides temporally with the development of anti *Lu. longipalpis* saliva antibodies [78]. It is tempting to speculate that neutralization of sand fly salivary components by antibodies or cellular response to salivary proteins allows a more efficient mounting of an anti *Leishmania* CMI response, probably due to the development of a Th1 response against the parasite. The higher antibodies levels observed in individuals who converted their anti *Leishmania* DTH sug gest that mounting antisaliva antibody response is linked to developing CMI against *Leishmania*. We also observed that sera from these children recognized more frequently at least two salivary proteins, 45 and 35 kDa, which may, respec tively, be similar to the Yellow related protein and to the sali vary apyrase from the saliva of *Lu. longipalpis* [79]. Therefore, these proteins could be candidates to either study the expo sure to sand flies bites or as vaccine candidates to control infection with *L. chagasi*.

An effective leishmaniasis vaccine will be one that prevents the development of disease. The distinction between vaccines designed to prevent disease is an important one. A decrease in infection, such as was achieved through the prior exposure to sand fly bites or injection of purified protein, will not neces sarily confer immunity to the parasite itself or prevent subse quent disease progression. *Leishmania* has a tissue stage that is responsible for the pathology and is distinct from the promas tigotes transmitted by the sand fly. Thus, vaccines that only prevent infection and do not establish immunity to tissue par asites may be a risk. On the other hand, it is possible to immunize against sand fly components and produce a low grade infection, with a degree of immunity to the amastigote sufficient to prevent disease [80]. Probably, limiting the number of surviving parasites after exposure via a DTH reac tion caused by sand fly bites, is the reason that there are so many asymptomatic human infections in certain foci.

Ideally, through a combination of vaccine components directed against both vector and pathogen targets, safe and long lasting immunity can be achieved.

Expert opinion & five year view

Development of an anti *Leishmania* vaccine has proven to be a difficult endeavor. Use of several antigens, selected through diverse approaches, did not achieve an acceptable level of protection. It is possible that insufficient knowledge of *Leishmania* pathogenesis represents the main root of repeated insuccesses. There are numerous recent examples of problems revisited that have led to changes in our under standing of leishmaniasis. The use of subcutaneous route of infection, the fundamental role of sand fly saliva in the early steps of leishmanial infection, a regulatory role of $CD8^+$ T cells and the influence of DCs are some of these aspects. Another limiting issue in the development of a vaccine against leishmaniasis is the need for a protective cell medi ated immune response. A renewed effort in evaluating new adjuvants, some of them tailored for providing elements known to be important in building a protective Th1 immune response, offers hope.

After the hurdle of demonstrating effective antigens in experimental models, we will be faced with the challenge of testing them in man. Due to epidemiological considerations, a fluctuating incidence in most endemic areas among other aspects, vaccine assays may not be feasible for a large number of candidate antigens. Improving ways of selecting appropriate candidates for human protection in this disease may alleviate this problem pinpointing the best products for clinical assays. Another neglected area of considerable importance is the evaluation of reliable surrogate markers of protection. Development in this field directed to other infections, which are also dependent on CMI protection, may be of use in leishmaniasis.

A concerted effort in all these fields would be desirable and could offer some, albeit cautious, optimism.

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Key issues

Different clinical manifestations of leishmaniasis, caused by different species of *Leishmania*, complicate the development of a vaccine.

Infection by *Leishmania* results in a complex immune response in the vertebrate host, which hampers the design of an effective vaccine against the parasite.

The use of new technologies and experimental systems have allowed a better understanding of the mechanisms underlying the protective immune response against *Leishmania*.

First generation vaccines made use of inactivated parasites and many clinical trials have showed that a partial protection against the infection has been obtained.

Genetic manipulation of the parasite has been used in experimental models or *in vitro* priming (IVP) assays showing successful results due to, mainly, a lower antigen load and a stronger T helper response.

DNA vaccination using specific parasite antigens has proven to be the most promising approach, since high levels of protection were observed in different experimental models.

The use of sand fly saliva components in vaccine design is the newest vaccination approach. The studies in experimental models as well as of individuals in an endemic area for visceral leishmaniasis have showed the potential ability of these salivary gland products to protect against the disease rather than to protect against the parasite.

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