Trends in Parasitology

CellPress

Review

# Live attenuated vaccines, a favorable strategy to provide long-term immunity against protozoan diseases

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**The control of diseases caused by protozoan parasites is one of the United Nations' Sustainable Development Goals. In recent years much research effort has gone into developing a new generation of live attenuated vaccines (LAVs) against malaria, Chagas disease and leishmaniasis. However, there is a bottleneck related to their biosafety, production, and distribution that slows downs further development. The success of irradiated or genetically attenuated sporozoites against malaria, added to the first LAV against leishmaniasis to be evaluated in clinical trials, is indicative that the drawbacks of LAVs are gradually being overcome. However, whether persistence of LAVs is a prerequisite for sustained long-term immunity remains to be clarified, and the procedures necessary for clinical evaluation of vaccine candidates need to be standardized.**

## Highlights

Malaria, Chagas' disease, and leishmaniasis are the parasitic diseases causing the highest morbidity/mortality rates and have a worldwide distribution. Efforts to develop effective vaccines are urgent.

The insufficient efficacy of subunit vaccines has led to renewed interest in vaccination strategies based on live parasites.

Natural immunization after infection is more the norm than the exception, as shown by the higher percentage of asymptomatic individuals observed in endemic areas.

The persistence of the parasite allows an effective immune response in the long term (concomitant immunity). This involves important biosafety issues that must be evaluated.

The current genetic manipulation tools have allowed the creation of promising live attenuated vaccines, which, together with advances in manufacturing and distribution of live organisms, suggest that clinical evaluation is timely.

## An old approach for confronting persistent problems

Despite efforts to develop and implement surveillance systems and eliminate parasitic diseases, such as leishmaniasis, Chagas disease, and malaria, they remain major public health challenges in tropical and subtropical regions of the world (Box 1). Since recovered patients develop immunity to new infections, vaccination seems a feasible prevention strategy [1–3].

Parasite subunits or recombinant proteins have been the most prolific way of producing vaccines (**subunit vaccines**) (see Glossary) because they are usually simple to obtain and are reproducible. Some of these vaccines are now in clinical trials or have even been approved for veterinary use [4,5]. In general, protozoan parasites themselves efficiently avoid the immune response elicited by these vaccines [6], which is further impacted by immunomodulators released by the invertebrate vectors (components of mosquito or sand fly saliva, and triatomine feces) [7]. In addition, the response is usually not sufficiently strong or long-lasting, and booster doses are necessary. There is, therefore, an urgent need to develop more effective vaccines [8].

An old principle of vaccination postulates that the more similar a vaccine is to the natural disease the better the protective immune response obtained. The use of whole parasites, especially in the form of live vaccines, was one of the early focuses of vaccine development. Controlled infection with *Plasmodium falciparum* whole parasites, for example, induces a level of immune protection against clinical malaria similar or superior to that seen in naturally resistant adults living in high-exposure areas [9,10], while **leishmanization** remains the only effective method of vaccination against leishmaniasis in humans to date [11], and naturally attenuated *Trypanosoma cruzi* parasites confer protection in experimental models [12]. However, biosafety concerns, and production and administration issues, led to live vaccines falling out of favor.

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### Box 1. Malaria, Chagas disease, and leishmaniasis: key facts

Malaria occurs in tropical and subtropical areas; over 200 million cases are recorded annually due to the infection of four *Plasmodium* species. The disease kills 400 000 people every year, with African nations the most affected [101]. Children under the age of 5, who have experienced few parasitic infections, are the most susceptible to severe malaria. In contrast, after repeated exposures to the parasite, individuals living in endemic areas develop clinical immunity against the blood stage of the infection, showing low parasitemia levels rather than a sterilising immunity. Sporozoites, injected into the skin by female *Anopheles* mosquitoes, travel to the liver and initiate a clinically silent expansion in hepatocytes and mature into schizonts. Afterwards, merozoites are formed by cytokinesis and then released from the host cell to initiate asexual multiplication in erythrocytes. Disease symptoms are fever, anemia, organ failure, and coma [101].

*Trypanosoma cruzi* is the causal agent of Chagas disease which affects 7 million people, especially in Latin America where it is endemic. It is transmitted mainly via the feces of triatomines but also by the consumption of contaminated food, blood transfusions, organ transplantation, or vertically during pregnancy [102]. After cell invasion, trypomastigotes transform into amastigotes and multiply in the cytosol. Later, amastigotes differentiate back into highly motile trypomastigotes that are released upon cell lysis. Then they can infect neighboring cells and migrate to different tissues [102]. In the acute phase, that lasts for a few weeks after infection, the parasite is detectable in the blood and causes mild, few specific symptoms or infection may be asymptomatic. During the chronic phase (lasting 20 years or more from the first infection), the parasites are detected mainly in cardiac muscles and those of the intestinal tract. Over the years, some 30% of patients may develop megacolon and experience the destruction of cardiac muscle, leading to heart disease [102].

The leishmaniases include several pathologies associated with different species of the genus *Leishmania*. There are three main forms of the disease: cutaneous (CL; the most common), mucocutaneous (MCL; which causes destruction of oronasal mucosa and cartilages), and visceral (VL; fatal systemic infection, if untreated). VL in South America, the Mediterranean Basin, and China is caused by *Leishmania infantum*, while in Africa and Asia the causal agent is *Leishmania donovani*. The parasites invade tissues rich in phagocytes, such as the liver, spleen, and bone marrow, leading to organ dysfunction. Leishmaniasis affects around 12 million people in 98 tropical and subtropical countries wherever the vectors (sand flies) are distributed. Every year, 1 million new cases are estimated, 100 000 of which are of VL, which annually causes 20 000–50 000 deaths [103]. It is likely that there are also many undeclared cases. The *Leishmania* promastigotes (extracellular form of the parasite) are inoculated into the vertebrate host's skin by female phlebotomine sand flies during a blood meal. The promastigotes are captured by phagocytes (neutrophils, dendritic cells (DCs) and macrophages) and differentiate into the amastigote form (the replicative intracellular stage). Then the amastigotes lyse the infected cells and infect new phagocytes.

Although each of aforementioned diseases has its own peculiarities due to the different interaction of the immune system with the respective parasite, they all have in common that protective immunity is related to the generation of specific cellular responses against the pathogens (Box 2). The use of live parasite-based vaccines ensures immunization with the complete repertoire of pathogen **antigens** for the generation of varied and complete effector and memory $CD4^+$ and $CD8^+$ **T cells**. Of note, terminally differentiated T cells with a protective profile and high effector properties are capable of producing a rapid response (in hours) to challenge infection. In contrast, long-lived memory T cells need first to differentiate to attain effector functions. Hence, these cells generate a delayed cellular response that would be insufficient to hold parasites at the inoculation site. Conversely, as rapid effector T cells are short-lived, in order to achieve a long-term protective immunity the presence of small populations of live persistent parasites would be necessary to generate continuous waves of circulating effector T cells and then the maintenance of quick protective responses [1,13,14]. Accordingly, the elimination of persistent parasites by chemotherapy causes a loss of long-term immunity [15–17]. Thus, live vaccines, in which the parasite persists for at least some time, would appear to have a much greater chance of being effective given the wider range of antigens presented and would be more likely to provide long-lasting protection [1,18].

Recently, improved knowledge of the biology of protozoan pathogens and the availability of new genetic engineering tools have resurrected the interest in LAVs [19–21]. With some of these 'new generation' vaccines, very high levels of protection have been achieved in animal models and they have been shown to be sufficiently safe to allow clinical testing. This review examines the present situation with regard to the development of live attenuated vaccines against malaria, Chagas disease, and leishmaniasis.

## LAVs for preventing malaria

Because an effective malaria vaccine requires both arms of the immune system to elicit an effective response [2], and the complexity of the *Plasmodium* life cycle might preclude the successful development of subunit-based vaccines (Box 1), there has been renewed interest in vaccines based on attenuated live organisms. Thus, sporozoites and the hepatic stages of the cycle are ideal targets for the design of attenuated vaccines because they are the bottleneck to the erythrocytic stage (responsible for all disease symptoms); thus, clinical symptoms might be stopped if the liver phase can be arrested. Sporozoite-based LAVs would trigger both production of antibodies that could prevent parasites from infecting hepatocytes and T cell responses eliminating parasites that had invaded hepatocytes [22]. A seminal study by Nussenzweig and coworkers [23] showed that immunity to malaria can be experimentally induced by immunization with **radiation-attenuated sporozoites** (**RASs**). Nevertheless, more reproducible methods and the possibility of introducing defined genetic modifications in the *Plasmodium* genome are contributing to the development of safe live-attenuated parasites, including blood-stage forms. Here, we summarize recent advances in three strategies for producing live-attenuated vaccines against malaria: RASs, chemically attenuated parasites (CAPs), (also called chemoprophylaxis vaccination (CVac), and **genetically attenuated parasites** (**GAPs**). Table 1 lists the candidate LAVs against malaria.

### Irradiated *P. falciparum* sporozoites

In the absence of pathology, the inoculated RASs remain metabolically active inside hepatocytes, helping to induce and maintain an effective immune response, as complete elimination of the parasites from the liver by chemotherapy may cause a loss of immunity [16,24]. The main hurdle to translating RAS vaccination to the clinic involves the necessity to balance the dose of radiation (DNA damage) in a manner that prevents parasite progression to the symptomatic erythrocyte stage while maintaining sporozoite infectivity and **immunogenicity**.

The Sanaria company was founded with the objective of establishing a production system using mosquitoes that carried metabolically active, nonreplicating RASs to produce a vaccine called PfSPZ [25]. In a clinical trial with human volunteers, protection of over 90% against a controlled *P. falciparum* challenge was attained by the natural administration route (i.e., infected mosquito bite) [26]. The effective immune response consisted of the production of antibodies, effector and memory $CD4^+$ and $CD8^+$ T cells, and multifunctional Th1 cytokine-producing $CD4^+$ T cells. These results, together with the finding that the vaccine provides protection against different strains of *P. falciparum* [27], led to the launch of clinical studies in endemic areas [28,29]. A phase III clinical trial, including over 2000 people aged 2–50 years, is now underway on the island of Bioko (Equatorial Guinea), and the preliminary results indicate that PfSPZ vaccines are well tolerated, immunogenic, and able to impede parasitemia or prolong prepatency, although vaccine doses need to be adjusted [30,31].

### Chemically attenuated parasites

CAPs have also been evaluated for eliciting protective immunity (Table 1). In this approach, attenuation of fully infectious parasites can occur *in vivo* when administered in conjunction with antimalarial drugs (often chloroquine or atovaquone/proguanil), which eliminates blood-stage forms and blocks disease progression. Alternatively, parasites can be previously attenuated *in vitro* (reviewed in [9]). In recent studies, using **controlled human malaria infection** (**CHMI**), chemically attenuated sporozoites (PfSPZ-CVac) were found to be highly effective *in vivo*, but dependent on both dosage and the vaccine schedule [32]. Also, chemically attenuated blood-stage forms have been used in CHMI trials [9]. In this case, to allow the development of immunity, drugs are either administered *in vivo* after a few rounds of replication or use is made of delayed death antimalarial drugs that affect the progeny of treated parasites (doxycycline and azithromycin)

## Glossary

**Adjuvants:** essential vaccine components that enhance the magnitude, breadth, and durability of the immune response. They can also polarize and modulate the type of immune response elicited by an immunogen.
**Antigens:** molecule that serves as a target for antibodies or the T cell receptors. The epitope is the fragment of a pathogen-derived antigen that is recognized by particular components of the host immune system (antibodies, B and T lymphocytes).
**Antigen-presenting cells (APCs):** cells that display antigen-derived peptides associated with major histocompatibility complex (MHC) on their surface. T cells will recognize this complex using their T cell receptor (TCR) and be consequently activated against that antigen/pathogen. Common APCs include dendritic cells, macrophages, and B lymphocytes.
**Concomitant immunity:** immune status in which the protective immune response against the newly entering infective stages of a parasite coexists with the persistence of the primary infection. Concomitant immunity has been demonstrated in infections with helminth and protozoan parasites.
**Controlled human malaria infection (CHMI):** deliberate infection with infectious *Plasmodium* parasites either by mosquito bite or by direct injection of sporozoites or parasitized erythrocytes. When required, the resulting erythrocyte phase of parasite multiplication is curtailed by the administration of antimalarial drugs.
**Genetically attenuated parasites (GAPs):** protozoan parasites lacking one or more genes that encode essential functions, resulting in impaired parasite growth.
**Immunogenicity:** the ability to induce complete humoral and/or cell-mediated immune responses. Antigens are usually immunogenic.
**Leishmanization:** ancient practice consisting of the inoculation of *Leishmania* parasites derived from an active cutaneous leishmaniasis (CL) lesion into hidden skin areas of the body. This induces a lesion that spontaneously cures, leaving the patient protected against new infections from natural sources. This strategy was used during much of the 20th century to control CL in highly endemic areas, such

[33]. The first clinical trial evaluating the safety and immunogenicity of blood-stage CAPs (attenuated *in vitro* with the DNA-binding drug tafuramycin-A, TF-A) showed that the vaccine is safe and induces a cell-specific response against *Plasmodium* [34]. Therefore, the objective for CAPs is to match the infective doses, drug treatments, and the intervals between them to elicit a durable, reproducible, and robust immunity [35,36].

### Genetically attenuated parasites

A more reproducible strategy for live vaccines against malaria is the use of GAPs. In this strategy, specific genes are altered in order to arrest pathogen development at specific points during the hepatic stage or the blood stage. Additionally, antigens specific to the intraerythrocytic parasite forms can be synthesized under the control of promoters engineered to activate those genes in the hepatic phase, contributing to the induction of immunity against the parasite also in the erythrocytic phase [19].

The protective mechanism elicited by genetically attenuated sporozoites would be the same as that associated with RASs (i.e., liver-stage arrest). However, not all genetic alterations affect the parasite in the same way, nor do they confer the same level of protection. The liver stage consists of several substages, and developmental arrest can be induced by different genetic alterations at different times. Thus, GAPs can be classified according to whether they affect early or late liver-stage differentiation (Table 1).

On the one hand, the *uis* genes 3 and 4 (which are upregulated in infective sporozoites) code for small membrane proteins that belong to the early transcribed membrane protein (ETRAMP) family. The P52, P36, or B9 proteins, belonging to the *Plasmodium*-specific 6-Cys family, are also important during the early hepatic stage and have been the target for creating attenuated vaccines. Elimination of these genes, or their expression via the transcription factor SAP1 [37], leads to the formation of an impaired parasitophorous vacuole, blocking liver-stage development but able to elicit protective immunity against a challenge with wild-type parasites [38–41]. On the other hand, achieving arrest in a more advanced liver stage might confer the same protection as achieved with early phase arrest, or even greater protection due to the wider spread of antigens being presented. These are vaccines based on the deletion of genes important for merozoite formation [42] or schizogony development [43]. For example, a GAP vaccine based on the deletion of *FabB/F*, which is involved in the fatty acid synthesis pathway II (FASII) that is essential in late liver-stage development [44], confers better protection than that achieved with RASs, eliciting more intense $CD8^+$ T cell and memory cell responses [45]. Table 1 summarizes these and other candidate GAP vaccines, and relevant features are briefly described here.

Deletion of two or more genes increases the safety of GAP vaccines [46,47]. For example, *P. berghei* cell lines that are either LISP2 or *uis3* (–) occasionally cause breakthrough infections, but mutants lacking both genes are completely attenuated. Another remarkable case is found in a *P. falciparum* line generated by the double deletion of the genes for the P52 and P36 proteins. Although safe in humanized mice [48], the first evaluation of this GAP vaccine in humans indicated that these mutants cause peripheral parasitemia after high-dose exposure [49]. As a follow-up on this strategy, a *P. falciparum* GAP was developed based on a triple deletion of *p52*, *p36*, and *sap1* genes [50]. This triple knockout line (Pf GAP3KO) proved to be fully attenuated in humanized mouse and human red blood cells, safe in human volunteers, and to confer complete protection against infectious sporozoite challenge in mice [51].

In a rodent malaria model, *PbΔb9Δslarp* parasites were completely attenuated, showing no breakthrough infections while efficiently inducing high-level protection [47]. These

as Israel, Iran, and several ex-Soviet republics.

**Memory cells:** T or B lymphocytes that have previously encountered a pathogen and are able to induce a stronger response upon subsequent encounter with the same pathogen.

**Prime-boost strategy:** a consecutive immunization with a vaccine in several stages. Usually one stage involves injecting DNA coding for the immunogenic protein. Later, the immunogen is administered directly in the protein form. This approach may be better than a single vaccine for protection against infectious diseases.

**Radiation-attenuated sporozoites (RASs):** *Plasmodium* sporozoites that have been treated with a radiation source (such as gamma rays or X-rays) so that they can invade the host hepatocyte but do not fully develop.

**Regulatory T cells (Tregs):** a specialized subpopulation of T cells which suppress activation of the immune system, maintaining immune system homeostasis and tolerance to self-antigens. Also known as suppressor T cells.

**Subunit vaccines:** vaccines prepared using one or more components from the parasite (usually proteins that best stimulate the immune system), but not the whole live organism.

**T cells:** lymphocytes with several functions in the immune system, such as cytotoxic (expressing CD8 on their surface), helper (expressing CD4 on the surface), regulatory, and memory. Their major role is cell-mediated immunity.

## Box 2. The immune correlates of protection against protozoan parasitic tropical diseases

Common to *Plasmodium, Trypanosoma*, and *Leishmania* infections is that immunity is associated with the generation of specific cellular responses [22,58,67]. These cells are characterized by their functions when stimulated, persistence in the body over time, and the ability to migrate to different tissues (Figure I). Effector $CD4^+$ and $CD8^+$ T cells (TEFF) do not proliferate but instead produce the effector cytokines that determine the immune response against the parasite. Since these cells survive for a short time in the absence of antigen, the persistence of a small number of parasites sustains a continuous antigen presentation by **antigen-presenting cells** (**APCs**) maintaining effector T cell populations for the long term, which is known as **concomitant immunity** [1]. Different **memory** lymphocyte populations capable of maintaining themselves in the absence of live parasites are also generated during the infection, including central memory (TCM), effector memory (TEM), and resident memory T cells (TRM) [13,67,104]. TCM cells are present only in lymphoid tissues and maintain high proliferating capacity after restimulation; these cells generate delayed protection because of the time they take to proliferate and differentiate to TEFF cells, while TEM cells can be found in nonlymphoid tissues and can rapidly migrate into infected tissues to contribute to cellular immunity [13,104]. In these pathologies, TRM cells have an important role since they can be found in the tissues where infection occurs, including the skin, where many insect-transmitted parasites enter the vertebrate host [105], and the liver [106], where the first stage of malaria replication occurs in the mammalian host. These TRM cells are long-term persistent cells and they can rapidly secrete cytokines after an encounter with the parasite, in addition to their involvement in the recruitment of effector cells as well as proinflammatory monocytes (Figure I). Evidence supports the notion that, for a long-term protective response to be achieved, the target antigen has to persist in the vertebrate host [75]. If it disappears, the host immunity then seems to depend on memory T lymphocytes, which are neither fast enough nor powerful enough to efficiently ward off reinfection [1,13,18].

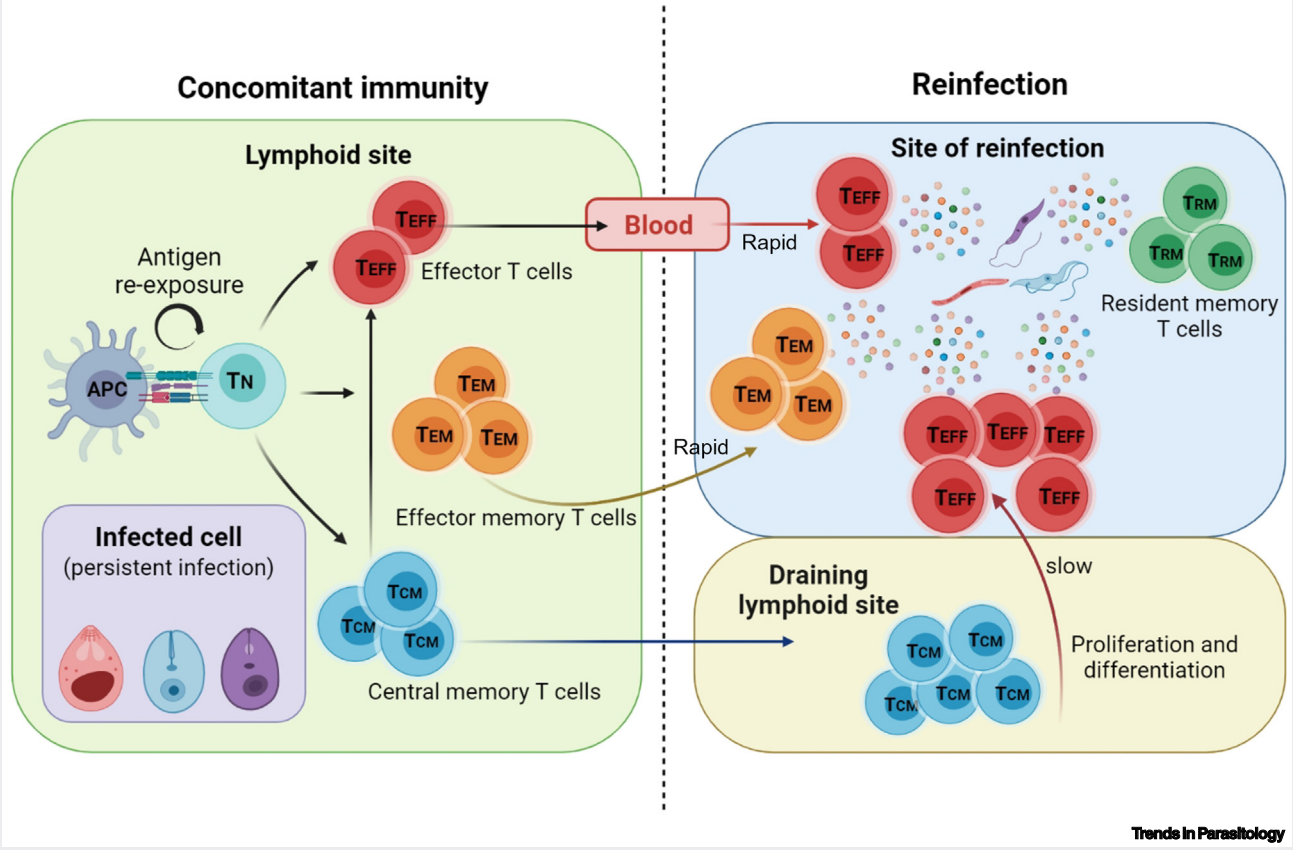


Figure I. Concomitant immunity provides protection against protozoan parasitic diseases. Individuals cured of malaria, Chagas disease, or leishmaniasis generate different and specific effector and memory T cell populations that provide resistance to new infections. After reinfection, resident memory T cells (TRM), which persist in the absence of antigen in some tissues (lung, kidney, brain, and skin), maintain their effector properties and quickly initiate the immune response against the pathogen. Effector T cells (TEFF) are rapidly recruited from the blood to the site of infection and initiate the immunological control of parasite multiplication. TEFF cells are short-lived; therefore, their existence depends on the presence of persistent parasites from the first infection (concomitant immunity). Additionally, persistent parasites constitute a constant source of antigen to be presented to naïve T cells (TN) by antigen-presenting cells (APCs) for the differentiation towards different antigen-specific T cells. Effector memory T cells (TEM) have a mixed phenotype: they can persist for some time in the absence of antigen but also migrate to nonlymphoid tissues and play a limited effector role at the site of infection after restimulation. Finally, long-lived central memory T cells (TCM) migrate to the draining lymph node of the reinfection site where they proliferate and differentiate into TEFF cells after restimulation to reinforce the primary immune response.

Table 1. Live attenuated vaccines (LAVs) against malaria

| LAV candidate | Attenuation | | Model | Immunization[a] Dose of sporozoites Prime/boost (days of boost) | Protection[b] Challenge time and dose Protected/total | Refs |
|---|---|---|---|---|---|---|
| | Liver/blood stage | Parasite in blood | | | | |
| *Pf* γ-SPZ | Early | No | Nonhuman primates Healthy and malaria-exposed humans | i.v. s.c. mosquito bites variable doses | 10%–70% –100% Variable: dose, homologous challenge, heterologous challenge | [26–31,107,108] |
| *Pf* SPZ-CVac | Liver and blood stages | No | Malaria-naïve human | i.v. variable doses + chloroquine, pyrimethamine, and others (*in vivo*) | 50–100% (28 d) 9/9 (28 mo) 4/6 (105 d) 8/14 | [31,32,35,109,110] |
| *Pb/Py* SPZ-CVac | Liver and blood stages | No | C57BL/6 BALB/c | 50 k/20 k/20 k + centanamycin (*in vitro*) (74 d, 141 d) | (10–21 d) 5–10 k *Pb* 4/4 (10–21 d) 100 *Py* 4/4 | [111] |
| *Pc* pRBCs + centanamycin/TF-A CVac | Bloodstage | Yes | A/J mice | $10^6$ + centanamycin/ tafuramycin A (*in vitro*) | (180 d) $10^5$ *Pc* pRBC homologous and heterologous | [112] |
| *Py* pRBCs + centanamycin CVac | Blood stage | Self resolving | C57BL/6 BALB/c | $10^6/10^6/10^6$ + centanamycin (*in vitro*) | (28 d) $10^5$ *Py* pRBCs 9/9 or sporozoites (mosquito bite) 5/5 | [113] |
| *Pf* pRBCs + TF-A CVac | Blood stage | Self resolving | Malaria-naïve human | 3 x $10^7$ + tafuramycin-A (*in vitro*) | ND | [34] |
| *Pc/Py* pRBCs + Doxycyclin/ Azithromycin CVac | Blood stage | Self-resolving | BALB/c C57BL/6 | $10^7/10^7/10^7$ (7,28 d) + doxycyclin/ azithromycin/ others (*in vivo*) | (28 d) $10^5$ Pc/Py pRBCs homologous (100%), heterologous (60–100%) | [33] |
| *Pf* pRBCs + Doxycyclin CVac | Blood stage | Yes (rescue treatment in 2/4) | Malaria-naïve human | 3 x $10^6$ + doxycyclin (*in vivo*) | ND | [33] |
| *Pb uis3(-)* | Liver (early) | No Yes | C57BL/6 | 50 k/25 k (14 d, 21 d) | (30 d) 10 k 5/5 | [114] |
| *Pb uis4*$^-$ | Liver (early) | Yes | C57BL/6 | 50 k/25 k (14 d, 28 d) | (38 d) 50 k 8/8 | [115] |
| *Py uis3*$^-$ | Liver (early) | No | BALB/c | 10 k/10 k (14 d, 28 d) | (60 d) 10 k 4/4 (180 d) 10 k 8/12 | [39,116] |
| *Py uis4*$^-$ | Liver (early) | No | BALB/c | 10 k/10 k (14 d, 28 d) | (180 d) 10 k 8/8 | [39,116] |
| *Pb uis3*$^-$*uis4*$^-$ | Liver (early) | No | C57BL/6 | 10 k/10 k (7 d, 14 d) | (118 d) 10 k 14/14 | [46] |
| *Pb p36p*$^-$ | Liver (early) | Yes | BALB/c | 50 k | (120 d) 10 k 5/5 | [38] |
| | | | C57BL/6 | 50 k/20 k (7 d, 14 d) | (30 d) 10 k 5/5 | |
| *Pf Δp52* | Liver (early) | Return to wt | Primary human hepatocytes | ND | ND | [40] |
| *Py Δp52Δp36* | Liver (early) | No | BALB/c | 10 k/10 k (7 d, 14 d) | (30 d) 10 k 7/7 | [117] |
| *Pb Δp52Δp36* | Liver (early) | No | BALB/c | 10 k | (180 d) 10 k 10/10 | [41,118] |
| | | Yes | C57BL/6 | 50 k/20 k (7 d, 14 d) | (180 d) 10 k 6/7 | |
| *Pf Δp52Δp36* (*Pf* GAP2KO) | Liver (early) | Yes | Hu-hepatocytes humanized SCID Alb-uPA Mice | ND | ND | [48] |
| | | | Malaria-naïve humans | Mosquito bites | (90 d) Cellular immunity | [49,118] |
| *Py sap1*$^-$ | Liver (early) | No | BALB/c | 10 k/10 k (14 d, 28 d) | (210 d) 10 k 15/15 | [37,45] |

Table 1. (continued)

| LAV candidate | Attenuation | | Model | Immunization[a] Dose of sporozoites Prime/boost (days of boost) | Protection[b] Challenge time and dose Protected/total | Refs |
|---|---|---|---|---|---|---|
| | Liver/blood stage | Parasite in blood | | | | |
| *Pb Δslarp* | Liver (early) | No | C57BL/6 | 50 k/25 k (14,28) | (42 d) 10 k 5/5<br>(98 d) 10 k 2/5 | [37] |
| *Py Δb9* | Liver (early) | Yes | BALB/C | ND | ND | [41] |
| *Pb Δb9* | Liver (early) | No | BALB/C | 10 k | (10 d) 10 k 10/10 | [41,47] |
| | | Yes | C57BL/6 | 50 k/20 k (7 d, 14 d) | (180 d) 10 k 9/9<br>(365 d) 10 k 5/11 | |
| *Pb Δb9Δp52Δp36* | Liver (early) | Yes | C57BL/6 | ND | ND | [41] |
| *Pb Δb9Δslarp* | Liver (early) | No | BALB/C | 10 k<br>1.2 k | (10 d) 10 k 20/20<br>(28 d) 10 k 6/6 | [43,47] |
| | | | C57BL/6 | 50 k/20 k (7 d, 14 d)<br>10 k/10 k (7 d, 14 d) | (180 d) 10 k 6/6<br>(14 d) 10 k 10/10 | |
| *Pf Δb9Δslarp* (PfSPZ-GA1) | Liver (early) | No | Human primary hepatocytes Human liver-uPA-SCID | ND | ND | [47] |
| | | | Malaria-naïve human volunteers | 900 k (56/112 d/168 d) | (21 d) mosquito bite 3/25 (sterile protection) | [52] |
| *Pb Δmrp2* | Liver (mid- to late) | No | BALB/c | 1.2 k | (21 d) 10 k 5/5 | [43] |
| | | | C57BL/6 | 10 k/10 k (7 d, 14 d) | (14 d) 10 k 9/10 | |
| *Pb Δfabb/f* | Liver (late) | Yes | BALB/c C57BL/6 | ND | ND | [118] |
| *Py fabb/f*$^{-}$ | Liver (late) | No | Swiss | 20 k/20 k (91 d) | (60 d) 1 k 18/20 | [45,119] |
| | | | BALB/c | 10 k/10 k (14 d, 28 d) | (210 d)10 k 8/8<br>(150 d) 10 k 5/5 *P. berghei* | |
| | | | C57BL/6 | 20 k/20 k (111 d) | (60 d) 1 k 20/20 | |
| *Pb ΔLipB* | Liver (late) | Yes | C57BL/6 | ND | ND | [120] |
| *Py e1α*$^{-}$ or *e3*$^{-}$ | Liver (late) | No | BALB/c | ND | ND | [44] |
| *Pf lsa-1*$^{-}$ | Liver (late) | No | SCID/Alb-uPA humanized liver | ND | ND | [121] |
| *Pb PALM*$^{-}$ | Liver (late) | Yes | C57BL/6 | 10 k/10 k (35 d) | (110 d) 5 bites 20/20<br>(35 d) 10 k 20/20<br>(110 d) 10 k 6/7 | [42] |
| *Py PlasMei2*$^{-}$ | Liver (late) | Yes | BALB/c | ND | ND | [119] |
| *Py lisp2*$^{-}$ | Liver (late) | Yes | BALB/c | ND | ND | [114,119] |
| *Pb LISP2*$^{-}$ | Liver (late) | Yes | C57BL/6 | 50 k/20 k (14 d, 28 d) | (42 d) 10 k 4/4<br>(102 d) 10 k 2/2 | [114] |
| *Pb LISP2*$^{-}$*uis3*$^{-}$ | Liver (late) | No | C57BL/6 | 50 k/20 k (14 d, 28 d) | (102 d) 10 k 8/8 | [114] |
| *Py plasmei2*$^{-}$ *lisp2*$^{-}$ | Liver (late) | No | BALB/c | 10 k/10 k (90 d) | (40 d) 10 k 14/14 | [119] |
| | | | Swiss | 50 k/50 k (30 d, 60 d) | (30 d) 15 bites 9/10 | |
| | | | C57BL/6 | 50 k/50 k (28 d) | (30 d) 10 k *Py* pRBC | |
| *Pf mei2*$^{-}$ | Liver (late) | No | Human liver-chimeric FRG-HuHep mice | 1 x $10^6$ | ND | [52] |

(continued on next page)

Table 1. (continued)

| LAV candidate | Attenuation | | Model | Immunization[a] Dose of sporozoites Prime/boost (days of boost) | Protection[b] Challenge time and dose Protected/total | Refs |
|---|---|---|---|---|---|---|
| | Liver/blood stage | Parasite in blood | | | | |
| *Py p52⁻/p36⁻/sap1⁻* (*Py* GAP3KO) | Liver (early) | No | BALB/c | 10 k/10 k (14 d) | (180 d) 10 k 5/5 | [51] |
| *Pf p52⁻/p36⁻/sap1⁻* (*Pf* GAP3KO) | Liver (early) | No | FRG-HuHep mice<br>Human healthy volunteers | 200 mosquito bites | ND | [50,51] |
| *Pb* Δ*PDH-E1-PFO*$_{LS}$ (GAP$^2$) | Liver (late PVM) | Yes | BALB/c | 5 k single-mutant *Pb*PFO$_{LS}$ | (27 d) 5 k 10/10 | [122] |
| | | | C57BL/6 | 5 k/5 k (25 d, 50 d) | (30 d) 5 k 10/10 | |
| *Py PNP*-INT pRBCs | Blood stage | Self-resolving | BALB/c | 200 k | (56 d) 200 k *Py* pRBC Homologous % heterologous challenge 10/10 | [53] |
| *Py nt1*$^-$ pRBCs | Blood stage | Self-resolving | BALB/c<br>C57BL/6 | 100 | (90 d) 100 k *Py* pRBC 5/5<br>(90 d) 1000 *Pb* pRBC 2/5 | [54] |
| *Pb* Δ*pm4/msp7* pRBCs | Blood stage | Self-resolving | BALB/c | $10^7$ | (140 d) $10^7$ *Pb* pRBC 14/14 | [55] |
| | | | C57BL/6<br>CD1 | | (30 d) $10^4$ *Py* pRBC | |
| | | | | | 10/10 | |
| | | | BALB/c | | (190 d) $10^7$ *Pb* pRBC 10/10 | |
| *Pb*NK65-*hrf*Δ1 pRBCs | Blood stage | Self-resolving | C57BL/6 | $10^5$ | (396 d) $10^7$ *Pb* pRBC<br>(23 d) $10^7$ *Py* pRBC<br>25 d) $10^4$ *Pb*/*Py* sporozoites | [56] |
| *Δhmgb2PbNK65* pRBCs | Blood stage | Self-resolving | C57BL/6 | 100 k | (160 d) 100 k *Pb* pRBCs (100%) | [57] |

ND, not determined.
i.v., intravenous inoculation; s.c., subcutaneous inoculation.
pRBCs, parasitized red blood cells.
CVac, chemoattenuated vaccine.
SPZ, sporozoites.
*Pf*, *Plasmodium falciparum*; *Pb*, *Plasmodium berghei* b; *Py*, *Plasmodium yoelii*; *Pc*, *Plasmodium chabaudi*.
[a]Immunization with sporozoites (x$10^3$); prime/boost (days of boost).
[b]Sterile protection (not considering prepatency period); (time after last immunization); challenge (k = $10^3$ parasites), protected/total.
*Vaccine candidate evaluated in humans.

results led to the development of a *P. falciparum* GAP vaccine based on the deletion of the *b9* and *slarp* genes. These *PfΔb9Δslarp* mutant parasites, which did not incorporate drug-resistance markers, infected human hepatocytes but failed to fully develop. Importantly, purified, aseptic cryopreserved *PfΔb9Δslarp* sporozoites (known as PfSPZ-GA1 vaccine) have the advantage that attenuation is linked to a precise genetic alteration; consequently, the resultant vaccine produced is both safer and more reproducible than those vaccines based on irradiation or chemotherapy attenuation. PfSPZ-Ga1 has been tested for safety, immunogenicity, and preliminary efficacy in malaria-naïve Dutch volunteers, and the results achieved are similar to those provided by the PfSPZ vaccine as determined via CHMI [52].

Compared to live sporozoite vaccines, whole-parasite blood-stage vaccines induce a distinct immune response, similar to the naturally acquired immunity after multiple infections against the erythrocytic phase, and are easier to produce. A *P. yoelii* deficient for the purine nucleoside phosphorylase (PNP) gene first demonstrated the feasibility of this approach [53]. Later, infection with an auxotrophic *P. yoelii* lacking nucleoside transporter NT1 resulted in the impossibility of normal replication in the host, although it is easy to grow *in vitro* for vaccine production in the presence of purines [54]. A *P. berghei* line lacking the protease plasmepsin 4 (PM4), which is involved in hemoglobin digestion, results in attenuated parasites that do not induce cerebral malaria but do stimulate a protective immune response. Interestingly, a double-null mutant for PM4 and Merozoite Surface Protein-7 genes is more attenuated than the single-knockout strains [55]. These and other blood-stage candidates (reviewed in [36]) have been shown to be highly immunogenic and protective against both homologous and heterologous challenge with different parasite strains (Table 1), although the persistence of high levels of parasitemia after immunization raises some concerns about their biosafety. Further GAP strategies and recent vaccine candidates intend to improve the attenuated phenotype of whole blood-stage parasites, boost T and B cell responses, and induce cross-stage, cross-species and long-lasting immunity [56,57].

Most GAPs have been tested only in murine models of infection with *P. berghei* or *P. yoelii*. Whilst these models are useful as platforms for exploring safety and protection, phenotypic differences may exist when such mutations are introduced into the *P. falciparum* genome. Another relevant question for malaria vaccines (not only when GAPs are used) is the translation of the experimentally achieved efficacy (usually against homologous challenge) in clinical trials. Thus, including heterologous challenges during evaluation of candidate vaccines should be considered as efficacy results differ when either homologous or heterologous challenges are used [22,35,36].

## Live attenuated parasites against Chagas disease

Early studies of experimental *T. cruzi* infections, performed mainly by the use of mouse models, showed that survival after acute infection provided resistance to reinfection. This immunity relied on a parasite-specific Th1 response accompanied by a humoral anti-*Trypanosoma* response, as well as the action of cytotoxic $CD8^+$ T cells. The latter lymphocytes are key in developing an effective response and in its maintenance during the chronic phase of infection [58]. However, a sustained immune response during the chronic phase would also be responsible for myocardial damage [59] (Box 1). This pathology has an autoimmune component that might be provoked by the persistence of the parasite in the tissues and its capacity to manipulate the immune system [7]. Given these peculiarities of Chagas disease, it is desirable to produce a vaccine that acts rapidly to control the acute phase but which is also able to downmodulate the aberrant immune response associated with the presence of parasites during the chronic phase [3].

As the first proof of concept, immunization with a *T. cruzi* strain (TCC), attenuated by culture passage, proved to be safe and it controlled parasitemia after a subsequent challenge with blood trypomastigotes of the highly virulent Tulahuen strain. It also reduced the transmissibility and tissue damage in mice [60] and dogs [12]. The increasing number of tools available for genetic manipulation (including CRISPR technology) has allowed the creation of several GAP lines that have been tested in mice (Table 2). For example, the elimination of an allele for the calmodulin–ubiquitin gene in the Tulahuen strain gave rise to an attenuated vaccine – TulCub8 – that reduced parasite load after infection with the wild-type strain [61]. Also, the attenuated L16 line, generated by eliminating the *lyt-1* gene (coding for a parasite virulence factor) conferred resistance to parasitemia for at least 14 months after vaccination [62]. Similarly, immunization with the $gp72^{-/-}$ knockout of the *T. cruzi* Y strain (that lacks a glycoprotein interacting with the C3 complement protein) led to reduced parasitemia after challenge with the Tulahuen strain [63].

Table 2. Live attenuated vaccines (LAVs) against *Trypanosoma cruzi*

| *T. cruzi* strain/LAV | Model | Immunization[a] | Protection[b] | | | | Refs |
|---|---|---|---|---|---|---|---|
| | | | Challenge | Blood | Tissue | Transmission | |
| TCC | Swiss | $10^6/10^6$ (15 d/30 d) i.p. | (19 d) $10^2$ Tul | Yes | Yes | Yes | [12,15,60] |
| | Guinea pig | 28 x $10^6$/kg i.d. | Natural | Yes | ND | Yes | |
| | Dog | $10^7/10^7$ (2 mo/14 mo) | Natural | Yes | ND | Yes | |
| | BALB/c | $10^6/10^6$ (7 d/14 d) s.c. | (361 d) $10^3$ Tul | Yes | ND | ND | |
| Tul TulCub8 | Swiss | $10^3/10^3$(7 d) i.p. | (30 d) $10^6$ Tul | Yes | ND | ND | [61] |
| CL *LYT1*$^{-/-}$ (L16) | Swiss | $10^3$ i.p. | (14 mo) $10^4$ Tul | Yes | Yes | ND | [62] |
| Y *gp72*$^{-/-}$ | BALB/c | $10^6$ s.c. | (10 d) $10^3$ Tul | Yes | ND | ND | [63] |
| TCC *dhfr-ts*$^{+/-}$ | C57BL/6 | $5\times10^5/5\times10^5$ (15 d) i.p. | (15 d) $10^4$ CL<br>(370 d) $2\times10^5$ CL | Yes | ND | ND | [64] |
| | BALB/c | | (15 d) $5\times10^3$ Tu | | | | |
| TCC TcCRT$^{+/-}$ | BALB/c | $5\times10^5/5\times10^5$ (15 d) i.p. | (120 d) $5\times10^4$ TcV1 | Yes | Yes | Yes | [65] |
| CL ECH1$^{+/-}$ ECH2$^{-/-}$ | C57BL/6 | $5\times10^5/1.35\times10^5$ (14 d) /$5\times10^5$(14 d) oral | (14 d) $2.5\times10^3$ CL | ND | Yes | ND | [66] |
| Tu DDDHA | C3H | $5\times10^3$ i.p | (42 d) $4.6\times10^5$ Tu | Yes | Yes | ND | [123] |
| | C57BL/6 | | (42 d) $5\times10^5$ Brazil<br>(42 d) $5\times10^5$ Tu | | | | |
| *T. rangeli* | Swiss | $3\times10^5$ i.p. | (30 d) $10^4$ Y | Yes | Yes | ND | [124] |

ND, not determined.
i.d., intradermal inoculation.
i.p., intraperitoneal inoculation.
s.c., subcutaneous inoculation.
[a]Immunization dose. Prime/boost (time of boost); route of immunization.
[b]Protection. Time after last immunization; challenge with a *T. cruzi* strain; observed reduction in parasitemia, tissue damage and/or transmission to the vector (xenodiagnosis).

Another set of GAPs have been obtained from the attenuated TCC strain of *T. cruzi* (Table 2). This strategy has different advantages, including the increased attenuated state of the GAP line and the fact that, if reversion occurs, the TCC strain itself is avirulent. Further, these mutants are rapidly eliminated, or at least left in undetectable numbers, impeding their transmission by the insect vector [21]. Of note, it was not possible to eliminate both alleles in any of these mutant lines (nor in many others), an indication that many of these genes are essential. One example of such cell lines is the auxotrophic *dhfr-ts*$^{+/-}$ mutant that provides the same protection as the TCC strain, with the advantage of having a better characterized attenuation [64]. The highly attenuated TcCRT$^{+/-}$ line, also obtained from the TCC strain, lacks calreticulin and is very susceptible to the action of complement. Nevertheless, its inoculation conferred protection against a challenge with virulent *T. cruzi* trypomastigotes. Thus, in vaccinated animals, parasite loads were scarce and tissues showed lower inflammatory responses [65].

Remarkably, some LAVs against *T. cruzi* can be administered orally, due to the parasite's capacity of transversing mucosa. Thus, an attenuated *T. cruzi* CL line, generated by deletion of one allele of the gene encoding enoyl-CoA-hydratase-1 (ECH1) and both alleles of the ECH2 gene (*ech1*$^{+/-}$ *ech2*$^{-/-}$), has been tested as an oral vaccine in mice. It provides protection against a challenge with infectious *T. cruzi* parasites, which correlates with the presence of antigen-specific CD8$^+$ T cells [66].

All of these advances have renewed interest in generating GAPs that could be tested as attenuated vaccines against Chagas disease. However, despite these good results, the risk of reversion to a virulent phenotype, plus the idea that cardiopathy in the chronic phase could be related to parasite

persistence, may limit the use of these vaccines against this disease. Full characterization of the effector and/or memory responses generated by these genetically modified vaccines will help to determine their future usefulness.

## Genetically attenuated parasites against *Leishmania*

Extensive studies on experimental models and data from patients have served to establish many of the mechanisms related to the immunopathology of the different forms of leishmaniasis (Box 1). In general, the protective response developed after infection with different *Leishmania* species, causing either CL or VL, depends on the generation of a moderate Th1 response capable of activating amastigote-infected innate immune cell phagocytes, including monocyte-derived macrophages/dendritic cells, to destroy the parasite. In contrast, susceptibility is associated with the induction of an anti-inflammatory or regulatory response that impedes the full generation of a Th1 response, favoring the multiplication of the parasite. Additionally, modulation of this cellular response mediated by **regulatory T cells** (**Tregs**) is essential to prevent problems caused by an uncontrolled inflammatory response (tissue destruction of skin and mucosae), or to avoid an inefficient immune function of the lymphoid organs. After a secondary challenge, protected individuals (who are cured or properly immunized) develop a rapid cellular response at the site of infection; being somehow different from the response elicited during the primary infection, this response is capable of controlling parasite multiplication while avoiding the exacerbated or ineffective inflammatory immune responses that contribute to progression of the disease (reviewed in [67]).

The high efficacy of leishmanization in humans, and live chemically attenuated vaccines in dogs [68], indicates that LAV strategy results in robust protection against natural infection. Most of the attenuated *Leishmania* vaccines have been obtained by genetic manipulation, which allows precise elimination of target genes [20]. *Leishmania* GAPs have been used in vaccination experiments in animal models in which robust immunity was achieved (Table 3). An *L. donovani* line lacking the gene for centrin (*LdCen*$^{-/-}$), a microtubule-related protein, is the most advanced vaccine candidate. Inoculation of this cell line proved to be safe and effective in protecting mice, hamsters, and dogs [69–71] and was able to produce proinflammatory protective responses in human peripheral blood mononuclear cells (PBMCs) [72]. Such genetically manipulated parasites can usually be cultivated as promastigotes *in vitro*, but they have problems transforming into amastigotes inside macrophages [20]. In mice, the *L. infantum* knockout for the *HSP70* type II gene (*LiΔhsp70-II* line), coding for the heat-shock protein of 70 kDa, affects its capacity to multiply as amastigote forms [73] but its inoculation induces a protective cellular response that involves the rapid migration of IFN-γ-producing CD4$^{+}$ T cells to the site of challenge [74]. This rapid protective response resembles that seen after cure from natural CL and VL [75]. This would explain why live vaccines are able to generate an effective immune response against natural infection, unlike many subunit vaccines that are only effective against needle-inoculated parasites [76].

The species used for vaccine development, and the route of vaccination, often condition the capacity of *Leishmania* attenuated vaccines to elicit effective protection. Also, the number of parasites may affect the immune response elicited (as seen in PfSPZ vaccines), although comparisons of different doses are not usually included in preclinical studies. Normally, vaccines based on viscerotropic species (*L. infantum* or *L. donovani*) are intravenously or intraperitoneally administered, leading to dispersion of the parasites to the internal organs (Table 3). On the contrary, vaccines based on lines that cause CL, such as *L. major Lmlpg2*$^{-}$ (deficient in phosphoglycan synthesis) [77] or the auxotrophic *Lmdhfr-ts*$^{-/-}$ [78] line, are subcutaneously or intradermally administered. Interestingly, these routes have also been studied for vaccines based on *L. infantum* and *L. donovani.* For example, subcutaneous vaccination with the *LiΔhsp70-II* line results in persistence of very low numbers of parasites in the draining lymph node (not in the internal organs)

Table 3. Live attenuated parasites used against *Leishmania*

| GAP/LAV | Model | Immunization | | Challenge | | | Refs |
|---|---|---|---|---|---|---|---|
| | | Via | Persistence | Species | Weeks[a] | Protection | |
| *L. major dhfr-ts*$^{-/-}$ | BALB/c | i.v. | ND | *L. major* | 4 | Yes | [78] [82] |
| | | s.c. | 95 weeks in skin 9 weeks in LN | *L. major* | 1 | No | |
| | | | | *L. chagasi* | 4 | No | |
| | CBA | i.v. s.c. i.m. | ND | *L. major* | 4 | Yes | |
| *L. chagasi dhfr-ts*$^{-/-}$ | BALB/c | s.c. | ND | *L. chagasi* | 4 | No | [82] |
| | | s.c. | ND | *L. major* | 4 | No | |
| | | i.v. | ≥ 4 weeks in spleen, liver | ND | ND | ND | |
| *L. donovani dhfr-ts*$^{-/-}$ | BALB/c | s.c. | ND | *L. chagasi* | 4 | No | |
| *L. mexicana Δcpa/cpb* | BALB/c | s.c. | ND | *L. mexicana* | 16 | Yes | [125] |
| | C57BL/6 | | | | 8 | Yes | |
| | CBA | | | | 8 | Yes | |
| | Hamster | i.d. | ≥ 12 weeks in skin, LN Pathology develops | *L. mexicana* | 12 | No | [90] |
| *L. donovani BT1*$^{-/-}$ | BALB/c | i.v. | ≥ 12 weeks in spleen, liver | *L. donovani* | 6 | Yes | [126] |
| *L. major lpg2*$^{-/-}$ Compensatory mutant | BALB/c | s.c. | ≥ 10 weeks in skin, LN | *L. major* | 10 | Yes | [77] |
| | SCID | s.c. | ≥ 16 weeks in skin, LN | ND | ND | ND | |
| | C57BL/6 | s.c. | ≥ 10 weeks in skin | *L. major* | 10 | No (Yes with CpG) | [127] |
| *L. mexicana ΔGDP-MP* | BALB/c | i.d. | ≤ 5 h in skin, LNs, spleen, liver | ND | ND | ND | [128] |
| | | i.p. | ND | *L. mexicana* | 3 | Yes | |
| | | s.c. | | | | No | |
| *L. infantum SIR2*$^{-/+}$ | BALB/c | i.p. | ≤ 8 weeks in spleen, liver, LN | *L. infantum* | 6 | Yes | [91] |
| | SCID | i.p. | ≥ 8 weeks spleen, liver | ND | ND | ND | |
| *L. donovani Cen*$^{-/-}$ | BALB/c | i.v. | ≤ 12 weeks in spleen, liver | *L. donovani* | 24 | Yes | [69] [83] |
| | | | | *L. braziliensis* | 5 | Yes | |
| | | | | *L. major* | 5 | Yes | |
| | | | | *L. mexicana* | 30 | Yes | |
| | SCID | i.v. | ≤ 12 weeks in spleen, liver | ND | ND | Yes | |
| | Hamster | i.c. | ≤ 10 weeks in spleen, liver | *L. donovani* | 5 | Yes | |
| | Dog | s.c. | ND | *L. infantum* | 8 | Yes | [70] |
| *L. donovani Cen*$^{-/-}$ + LJM19 | Hamster | i.d. | ≥ 5 weeks in skin, LN ≤ 5 weeks in spleen, liver | *L. donovani* | 5 | Yes | [71] |
| *L. major Cen*$^{-/-}$ | C57BL/6 | i.d. | ≥ 6 weeks in skin | *L. major* *L. major*-infected sand flies | 7 | Yes | [88,89,96] |
| | BALB/c | i.d. | ND | *L. major* | 7 | Yes | |
| | | s.c. | ≥ 20 weeks in skin | ND | ND | ND | |
| | STAT-1 KO | s.c. | ≥ 7 weeks in skin | | | | |
| | IFN-γ KO | s.c. | ≤ 20 weeks in skin | | | | |
| | Rag2 KO | s.c. | ≤ 15 weeks in skin | | | | |
| | Hamster | i.d. | ≥ 7 weeks in skin, LN | *L. donovani* | 56 | Yes | |
| | | | | *L. donovani*-infected sand flies | 7 | | |

Table 3. (continued)

| GAP/LAV | Model | Immunization | | Challenge | | | Refs |
|---|---|---|---|---|---|---|---|
| | | Via | Persistence | Species | Weeks[a] | Protection | |
| *L. donovani p27*$^{-/-}$ | BALB/c | i.v. | < 16 weeks in spleen, liver | *L. donovani* | 20 | Yes | [84] [83] |
| | | | | *L. major* | 12 | Yes | |
| | | | | *L. braziliensis* | 12 | Yes | |
| | | | | *L. mexicana* | 30 | Yes | |
| *L. major p27*$^{-/-}$ | BALB/c | s.c. | < 12 weeks in spleen, liver | *L. major* *L. infantum* | 4 | Yes | [85] |
| *L. donovani ΔALO* | BALB/c | i.v. | < 16 weeks in spleen, liver | *L. donovani* | 20 | Yes | [129] |
| *L. infantum hsp70-II*$^{-/-}$ | BALB/c | i.v. | < 12 weeks in spleen, liver, BM | *L. major* *L. infantum* *L. amazonensis* | 12 | Yes | [73,74,86,87] |
| | | s.c. | > 20 weeks in LN | | | | |
| | C57BL/6 | s.c. | > 25 weeks in LN | *L. major* | | | |
| | SCID | i.v. | < 8 weeks in liver | ND | ND | ND | |
| | Hamster | i.c. | < 9 months in spleen, liver | | | | |
| *L. donovani ΔFbpase* | BALB/c | i.p. | < 14 weeks in spleen, liver | *L. donovani* | 24 | Yes | [130] |
| *L. infantum ΔKHARON1* | BALB/c | i.v. | < 15 days in spleen, liver | *L. infantum* | 5 | Yes | [131] |
| | | s.c. | | | | No | |
| *L. donovani Hel67*$^{-/-}$ | Hamster | i.m. | ≥ 90 days in spleen | *L. donovani* | 3 | Yes | [132] |
| *L. infantum* + Gentamicin CVac | Dog (clinical trial) | s.c. | ND | Natural infection (endemic area) | 168 | Yes | [68] |

ND, not determined.
i.v., intravenous inoculation; i.d., intradermal inoculation; s.c., subcutaneous inoculation; i.m., intramuscular inoculation; i.c., intracardiac inoculation; i.p., intraperitoneal inoculation.
LN, lymph node.
CVac, chemically attenuated vaccine.
[a]Weeks after immunization.

and to a protection level similar to that achieved by intravenous infection [74]. Similarly, in hamsters, intradermal inoculation with the *LdCen*$^{-/-}$ line, along with the sand fly salivary protein LMJ19, confers a level of protection equivalent to that associated with intracardiac vaccination [71].

Since leishmaniasis is caused by different species and presents in different clinical forms, one might think that a one-size-fits-all vaccine is unattainable. However, the large number of antigens shared by the different species, and the considerable evidence of protective cross-reactions [79–81], indicate that a universal vaccine could be possible. In fact, attenuated vaccines based on a given *Leishmania* species are able to elicit different degrees of protection against other species (Table 3). For example, the *L. major dhfr-ts*$^{-/-}$ line induces protection against *L. major* [78] although not against *L. infantum* [82]. Similarly, vaccination with the *L. chagasi dhfr*$^{-}$ line confers no protection against infection by *L. major* [82]. The *LdCen*$^{-/-}$ line, which offers robust protection against *L. donovani*, only provides limited cover against *L. mexicana* and *L. major* [69,83]. Interestingly, vaccination with an *L. donovani* line lacking a cytochrome *c* oxidase component (*Ldp27*$^{-/-}$) protects against infection by *L. donovani, L. major*, and *L. braziliensis* [84]. The elimination of the same gene in *L. major* (*Lmp27*$^{-/-}$) also provides protection against *L. infantum* when administered subcutaneously [85]. Finally, subcutaneous vaccination with the *LiΔhsp70-II* line prevents disease being caused by *L. major* [74] in murine self-healing CL and a CL progressing to VL models. It also protects against *L. infantum* [86] and *L. amazonensis* [87]. In sum, the idea that a single vaccine

involving one type of attenuated parasite could provide durable protection against CL and VL caused by other species is very encouraging from both healthcare and economic standpoints.

Apart from the efficacy and long-term protection observed in experimental models (Table 3), there are some additional aspects to consider when developing LAVs against leishmaniasis. Most importantly, it must be investigated whether attenuated strains can induce pathological processes in immunosuppressed individuals. In this sense, vaccines based on *Ldp27*$^{-/-}$ and *LmCen*$^{-/-}$ cause no disease in immunosuppressed mice [84,88,89], and also the inoculation of the *LiΔhsp70-II* vaccine does not cause disease in immunodeficient SCID mice [73]. However, *L. mexicana Δcpa/cpb,* which lacks two genes encoding cysteine proteases, causes persistent infection in hamsters and eventually disease [90], and infection based on strain *LiSIR2*$^{+/-}$ (which lacks an allele of the gene that codes for sirtuin) causes persistent infection in SCID mice [91]. One way of improving the safety of attenuated vaccines is to include suicide genes in the parasite genome (the gene for herpesvirus thymidine kinase), making the parasite drug-sensitive to gancyclovir [92].

It is also relevant to know whether the multiplication dynamics of the vaccine parasite are influenced by infection with virulent *Leishmania* parasites. In this regard, assays performed with the *LiΔhsp70-II* vaccine have shown that the persistence and replicative capacity of these attenuated parasites are not altered by a subsequent infection with virulent forms of either *L. major*, *L. infantum*, or *L. amazonensis* [74,86,87]. Another safety issue is whether attenuated parasites can multiply and complete their cycle in the vector. It is expected that attenuated parasites should not be able to survive or differentiate in the sand fly vector, thus avoiding an uncontrolled spread of the parasite in the wild. Studies performed with the attenuated *LdCen*$^{-/-}$ and *Ldp27*$^{-/-}$ strains have shown that, at least, these do not establish in the vector [93].

The most advanced attenuated vaccine ever made against leishmaniasis was recently developed using CRISPR technology. The antibiotic-selection-marker-free, centrin-gene-deficient *L. major* (*LmCen*$^{-/-}$) has overcome all biosafety and production steps under Good Laboratory Practice (GLP) conditions. It has been shown to be safe in immunosuppressed hamsters and C57BL/6 and BALB/c mice, and is just as effective as leishmanization against *L. major,* even upon natural infection caused by sand fly bites [88,89]. It is now set to be trialed in dogs naturally exposed to *L. infantum*.

## Concluding remarks

Protozoan parasites remain leading causes of disease in tropical/subtropical regions. Although control measures and treatments have improved, it remains a priority to produce vaccines against these diseases of poverty. This would be the easiest way to stop their spread and reduce their socioeconomic impact [94]. Vaccination is also the most egalitarian way of protecting the population and solves the problem of people who remain asymptomatic and could act as a reservoir from which new outbreaks could stem [95]. In the era of vaccines based on parasite fractions, recombinant proteins, or genes (DNA/RNA), there has been renewed interest in the use of live attenuated vaccines.

In general, live vaccines are thought to produce an infection similar to that caused by the real pathogen and thus induce an equivalent immune response. Nevertheless, LAVs do not generate any pathology and cannot manipulate and pervert the host immune system, as occurs with virulent infections, but they elicit similar responses to those seen in survivors who become protected from reinfection. This protective response relies on the maintenance of either rapidly recruited or tissue-resident effector T cells [96], in addition to the coordinated functions of other immune cell types (reviewed in [22,67,97,98]). Evidence suggests that persistence of LAVs in very low numbers is needed to maintain these protective populations in the long term [1,14]. If parasites

## Outstanding questions

Can we clearly determine if only concomitant immunity works in the most promising LAVs against protozoan diseases? Can elicited tissue resident memory (TRM) cells maintain the same degree of protection if the attenuated parasites eventually disappear?

May the attenuated phenotype of the vaccine parasites be influenced by infection with virulent pathogen? Could attenuated parasites elicit sterilizing immunity against virulent infection and/or prevent parasite transmission?

Reversion to the virulent phenotype has occurred due to the overexpression of another gene (compensatory mechanisms). Do we have standardized protocols to meet the demands of biosafety and good manufacturing practices? What would be the behavior of LAVs in patients with either complete or transient immunosuppression? May the parasites in LAVs be transmitted by their insect vectors?

Could it be that, although LAVs have safety drawbacks, these would be less serious than the adverse effects caused by adjuvants used for inactivated vaccines?

Could we count on a distribution method less dependent on cryopreservation at ultralow temperatures in order to reduce costs and facilitate distribution to areas where these parasites are endemic?

Is subcutaneous administration of *Leishmania* LAVs required for their biosafety? This administration route seems to elicit a global protective response, avoiding the spread of the attenuated parasites to internal organs.

Could the long-term persistence of attenuated *T. cruzi* parasites be a risk or does it prevent the chronic phase of Chagas disease? Can they be used as therapeutic vaccines for the chronic phase?

disappear, the protective response relies only on non-effector memory cells, which confer limited or delayed protection – as occurs with both killed-parasite and subunit vaccines (Figure 1, Key figure). However, the persistence of live parasites, even attenuated ones, is of concern in patients with Chagas disease as the continuous presence of the parasite could lead to undesirable autoimmune responses [59].

The persistence of live attenuated parasites is plagued by concerns over their biosafety, if undesirable reactivation occurs. These concerns have created a bottleneck in the transit from preclinical to clinical trials. Other alternatives have been proposed to generate antigen-depot vaccines that provide a source of persistent stimuli, but whole live vaccines may induce superior

## Key figure

### Protection elicited by live attenuated vaccines

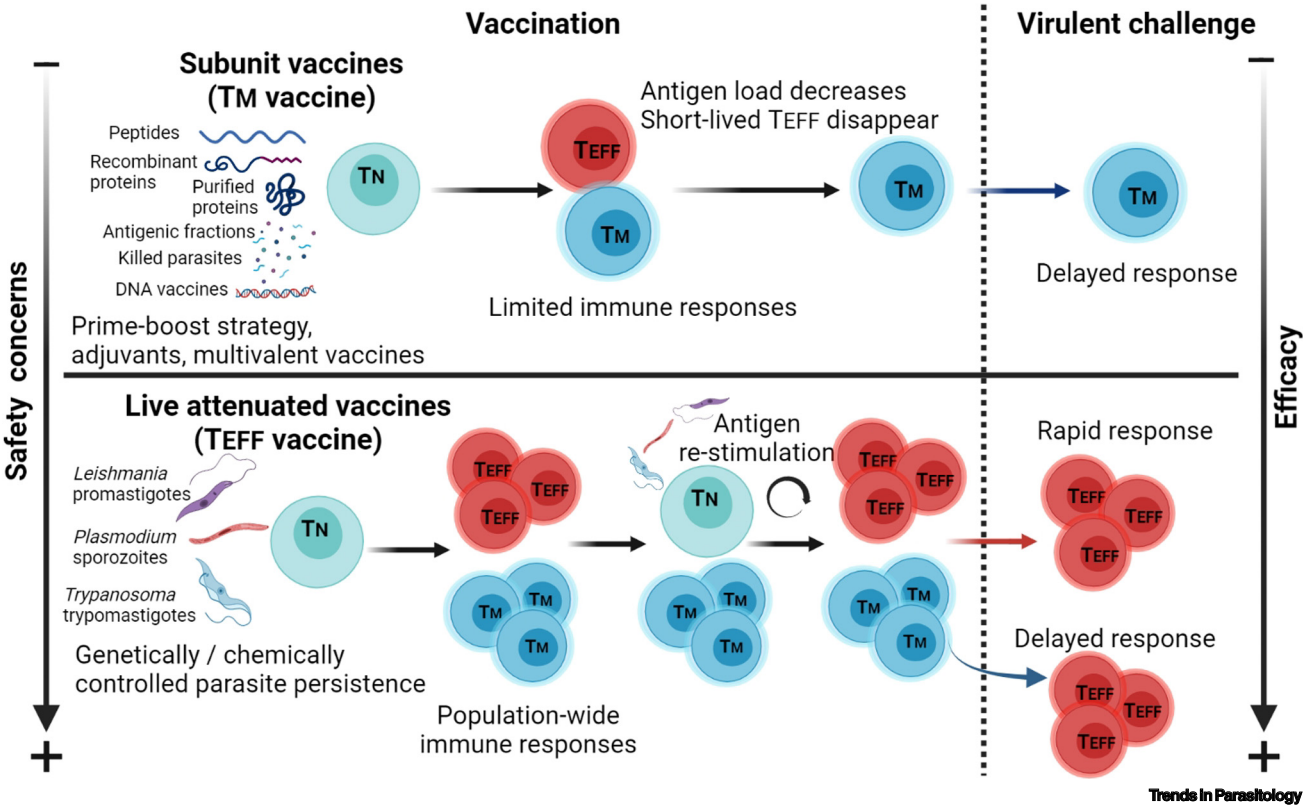


Figure 1. Vaccination strategies against malaria, Chagas disease, and leishmaniasis aim to generate specific memory and/or effector T lymphocytes able to protect against a virulent challenge. Subunit vaccines produce limited responses against the selected antigens, while the use of whole-parasite approaches present the entire repertoire of pathogenic antigens for the generation of varied $CD4^+$ and $CD8^+$ T cells. Furthermore, since TEFF cells are ephemeral, and depend on antigen presence, subunit-vaccine-mediated protection depends solely on TM. Conversely, the protective responses mediated by live attenuated vaccines are based on the maintenance of TEFF cells by reactivation of TM and/or new generation of T lymphocytes from TN. When infection occurs, only the rapid recruitment of T cells with effector properties confers adequate protection and prevents the establishment of infectious parasites. Vaccination against protozoan diseases has classically faced the dichotomy of having to choose between the safety of subunit vaccines and the immunogenicity of live vaccines. However, improvements in both approaches are overcoming these drawbacks. The use of **adjuvants**, **prime-boost strategies**, and the rising number of recognized antigens increase the immunogenicity of subunit vaccines, while new genetic tools are greatly improving the safety of live vaccines. We should combine the highest safety (and ease of production) with the highest immunogenicity and efficacy. Abbreviations: TN, T naïve precursors; TEFF, effector memory T cells; TM, memory T cells.

and wider immunogenicity for the complete repertoire of antigens presented to APCs. Thus, many efforts have to be made to underwrite the safety and also the challenges of manufacturing and distributing attenuated vaccines (see Outstanding questions). These problems can be overcome, as demonstrated by several whole-parasite LAVs for malaria (irradiated sporozoites and – chemically or genetically – attenuated sporozoites and asexual blood-stage parasites) that meet all regulatory standards and that are being tested in clinical trials [10,34]. Moreover, several candidates for *Leishmania* LAVs are under scrutiny [99], highlighting the *LmCen*$^{-/-}$ vaccine [88,89], and an *L. major* line (also produced under GMP conditions) that is ready for controlled infection in humans [100]. The emerging potential of LAVs warrants funding to test them beyond the preclinical phase.

## Acknowledgments

J.C.S. holds a research contract from the Universidad Autonoma de Madrid (Ref. UAM/133). S.I. is funded by the Spanish Ministerio de Ciencia, Innovación (MICINN), Agencia Estatal de Investigación (AEI) and Fondo Europeo de Desarrollo Regional (FEDER), RTI2018-094484-B-I00, and RYC-2016-19463. Parts of the research described in this review were supported by a grant from the Instituto de Salud Carlos III (Network of Tropical Diseases Research, RD16/0027/0008), co-funded with FEDER funds, to J.M.R. and M.S.

## Declaration of interests

The authors declare no competing interests.

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