LIVE ATTENUATED VACCINES, A FAVORABLE STRATEGY TO PROVIDE LONG-TERM IMMUNITY AGAINST PROTOZOAN DISEASES

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

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## Abstract

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.

## 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 to be 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 reproducible. Some such 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 confers protection in experimental models [12]. However, biosafety concerns, and production and administration issues led to live vaccines falling out of favor.

Although each of aforementioned diseases has its own peculiarities due to the different interaction of the immune system with the respective parasite genus, 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. On the other hand, 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 live attenuated vaccines (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 of 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 co-workers [23] showed that immunity to malaria can be experimentally induced by immunization with **radiation attenuated sporozoites (RAS)**. 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: RAS, 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 absence of pathology, the inoculated RAS 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, non-replicating RAS 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*

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 anti-malarial 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 dependend on both dosage and 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, either drugs are administered *in vivo* after a few rounds of replication or delayed death antimalarial drugs that affect the progeny of treated parasites (doxycycline and azithromycin) are used [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 cellular 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 genetically attenuated parasites (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 intra-erythrocytic parasite forms can be synthesized under the control of promoters engeenered 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 RAS (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 sub stages, 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 a 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 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 RAS, 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 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 *Pf*Δ*b9*Δ*slarp* sporozoites (known as PfSPZ-GA1 vaccine) have the advantage that attenuation is linked to a precise genetic alteration; consequently, the resultant vaccine produced are 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-naive Dutch volunteers, and the results achieved are similar to those provided by the PfSPZ vaccine as determined via controlled human malaria infection [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 erytrhocytic 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 only been tested 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) to 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 also able to down-modulate 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 controlled parasitemia after a subsequent challenge with blood trypomastigotes of the highly virulent Tulahuen strain. It also reduced the transmissibily 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 *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].

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 live attenuated vaccines 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 for 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 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 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

292 infection with different *Leishmania* species, either causing cutaneous leishmaniasis (CL)
293 or visceral leishmaniasis (VL), depends on the generation of a moderate Th1 response
294 capable of activating amastigote-infected innate immune cell phagocytes, including
295 monocyte derived macrophages / dendritic cells, to destroy the parasite. In contrast,
296 susceptibility is associated with the induction of an anti-inflammatory or regulatory
297 response that impedes the full generation of a Th1 response, favoring the multiplication
298 of the parasite. Additionally, modulation of this cellular response mediated by
299 **regulatory T cells (TREG)** is essential to prevent problems caused by an uncontrolled
300 inflammatory response (tissue destruction of skin and mucosae), or to avoid an
301 inefficient immune function of the lymphoid organs. After a secondary challenge,
302 protected individuals (who are cured or properly immunized) develop a rapid cellular
303 response at the site of infection, that being somehow different from that elicited during
304 the primary infection, is capable of controling parasite multiplication while avoiding the
305 exacerbated or ineffective inflammatory immune responses that contribute to
306 progression of the disease (reviewed in [67]).

307 The high efficacy of leishmanization in humans and live chemically attenuated
308 vaccines in dogs [68] indicate that LAV strategy results in robust protection against
309 natural infection. Most of the attenuated *Leishmania* vaccines have been obtained by
310 genetic manipulation, which allows precise elimination of target genes [20]. *Leishmania*
311 GAPs have been used in vaccination experiments in animal models in which robust
312 immunity was achieved (**Table 3**). An *L. donovani* line lacking the gene for centrin (*LdCen*$^{-/-}$),
313 a microtubule-related protein, is the most advanced vaccine candidate. Inoculation
314 of this cell-line proved to be safe and effective in protecting mice, hamsters and dogs
315 [69-71], and able to produce proinflammatory protective responses in human peripheral

316 blood mononuclear cells (PBMCs) [72]. Such genetically manipulated parasites can
317 usually be cultivated as promastigotes *in vitro*, but they have problems transforming into
318 amastigotes inside macrophages [20]. In mice, the *L. infantum* knockout for the *HSP70*
319 type II gene (*LiΔhsp70-II* line), coding for the heat shock protein of 70 kDa, affects its
320 capacity to multiply as amastigote forms [73], but its inoculation induces a protective
321 cellular response that involves the rapid migration of IFN-γ-producing $CD4^+$ T cells to the
322 site of challenge [74]. This rapid protective response resembles that seen after cure from
323 natural CL and VL [75]. This would explain why live vaccines are able to generate an
324 effective immune response against natural infection, unlike many subunit vaccines that
325 are only effective against needle-inoculated parasites [76].

326 The species used for vaccine development and the route of vaccination often
327 condition the capacity of *Leishmania* attenuated vaccines to elicit effective protection.
328 Also, the number of parasites may affect the immune response elicited (as seen in PfSPZ
329 vaccines), although comparisons of different doses are not usually included in preclinical
330 studies. Normally, vaccines based on viscerotropic species (*L. infantum* or *L. donovani*)
331 are intravenously or intraperitoneally administered, leading to dispersion of the
332 parasites to the internal organs (**Table 3**). On the contrary, vaccines based on lines that
333 cause CL, such as *L. major Lmlpg2*$^-$ (deficient in phosphoglycan synthesis) [77] or the
334 auxotrophic *Lmdhfr-ts*$^{-/-}$ [78] line, are subcutaneously or intradermally administered.
335 Interestingly, these routes have also been studied for vaccines based on *L. infantum* and
336 *L. donovani.* For example, subcutaneous vaccination with the *LiΔhsp70-II* line, results in
337 persistence of very low numbers of parasites in the draining lymph node (not in the
338 internal organs) and to a protection level similar to that achieved by intravenous
339 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 *Li*Δ*hsp70-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 of

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 causes 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 into 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 into 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

388 steps under Good Laboratory Practice (GLP) conditions. It has been shown to be safe in
389 immunosuppressed hamsters and C57BL/6 and BALB/c mice, and is just as effective as
390 leishmanization against *L. major,* even upon natural infection caused by sand fly bites
391 [88, 89]. It is now set to be trialed in dogs naturally exposed to *L. infantum*.
392
393 **Concluding remarks**
394 Protozoan parasites remain leading causes of disease in tropical / subtropical
395 regions. Although control measures and treatments have improved, it remains a priority
396 to produce vaccines against these diseases of poverty. This would be the easiest way to
397 stop their spread and reduce their socioeconomic impact [94]. Vaccination is also the
398 most egalitarian way of protecting the population and solves the problem of people that
399 remain asymptomatic and could act as a reservoir from which new outbreaks could stem
400 [95]. In the era of vaccines based on parasite fractions, recombinant proteins or genes
401 (DNA/RNA), there has been renewed interest in the use of live attenuated vaccines.
402 In general, live vaccines are thought to produce an infection similar to that
403 caused by the real pathogen, and thus induce an equivalent immune response.
404 Nevertheless, LAVs do not generate any pathology and cannot manipulate and pervert
405 the host immune system, as occurs with virulent infections, but they elicit similar
406 responses to those seen in survivors who become protected from reinfection. This
407 protective response relies on the maintenance of either rapidly recruited or tissue-
408 resident effector T cells [96], in addition to the coordinated functions of other immune
409 cell types (reviewed in [22, 67, 97, 98]). Evidence suggests that persistence of LAVs in
410 very low numbers is needed to maintain these protective populations in the long-term
411 [1, 14]. If parasites disapear the protective response relies only on non-effector memory

412 cells, which confer limited or delayed protection as occurs with both killed-parasite and
413 subunit vaccines (**Figure 1, Key Figure**). However, the persistence of live parasites, even
414 attenuated ones, is of concern in patients with Chagas disease, as the continuous
415 presence of the parasite could lead to undesirable autoimmune responses [59].

416 The persistence of live attenuated parasites is plagued by concerns over their
417 biosafety, if undesirable reactivation occurs. These concerns have created a bottleneck
418 in the transit from preclinical to clinical trials. Other alternatives have been proposed to
419 generate antigen-depot vaccines that provide a source of persistent stimuli, but whole
420 live vaccines may induce superior and wider immunogenicity for the complete
421 repertoire of antigens presented to APCs. Thus, many efforts have to be made to
422 underwrite the safety and also the challenges for manufacturing and distributing
423 attenuated vaccines (see **Outstanding Questions**). These problems can be overcome, as
424 demonstrated by several whole parasite live attenuated vaccines for malaria (irradiated
425 sporozoites and - chemically or genetically - attenuated sporozoites and asexual blood-
426 stage parasites) that meet all regulatory standards and that are being tested in clinical
427 trials [10, 34]. Moreover, several candidates for *Leishmania* LAVs are under scrutiny [99],
428 highlighting the *LmCen*$^{-/-}$ vaccine [88, 89], and an *L. major* line (also produced under GMP
429 conditions) that is ready for controlled infection in humans [100]. The emerging
430 potential of live, attenuated vaccines warrants funding to test them beyond the pre-
431 clinical phase.

432

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## Declaration of interest

The authors declare no competing interests.

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## Glossary

**Adjuvant:** 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.

**Antigen:** 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 cell (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 (GAP)**: protozoan parasites lacking one or more genes that encode for 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 in 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 cure, 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 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 (RAS)**: *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 (Treg):** 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:** are 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. Major role is cell-mediated immunity.

**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]. The population most susceptible to severe malaria is children under the age of 5 who have experienced few parasitic infections; in contrast, after repeated exposures to the parasite, individuals living in endemic areas develop clinical immunity against the blood-stage of the

820 infection, showing low parasitemia levels rather than a sterilising immunity. Sporozoites
821 are injected into the skin by female *Anopheles* mosquitoes, travel to the liver and initiate
822 a clinically silent expansion in hepatocytes and mature into schizonts. Afterwards,
823 merozoites are formed by cytokinesis and then released from the host cell to initiate
824 asexual multiplication in erytrhrocytes. Disease symptoms are fever, anemia, organ
825 failure, and coma [101].
826 *Trypanosoma cruzi* is the causal agent of Chagas disease affecting 7 million people,
827 especially in Latin America, where it is endemic. It is mainly transmitted via the feces of
828 triatomines, but also by the consumption of contaminated food, blood transfusions,
829 organ transplantation or vertically during pregnancy [102]. After cell invasion,
830 trypomastigotes transform into amastigotes, and multiply in the cytosol. Later
831 amastigotes differentiate back into highly motile trypomastigotes that are released
832 upon cell lysis. Then they can infect neighboring cells and migrate to different tissues
833 [102]. In the acute phase that lasts for a few weeks after infection, the parasite is
834 detectable in the blood and causes mild, few specific symptoms or infection may be
835 asymptomatic. During the chronic phase (lasting 20 years or more from the first
836 infection), the parasites are detected mainly in cardiac muscles and those of the
837 intestinal tract. Over the years some 30% of patients may develop megacolon and
838 experience the destruction of cardiac muscle leading to heart disease [102].
839 The leishmaniases include several pathologies associated with different species of the
840 *Leishmania* genus. There are three main forms of the disease: cutaneous (CL; the most
841 common), mucocutaneous (MCL; causes destruction of oronasal mucosa and cartilages)
842 and visceral (VL; fatal systemic infection, if untreated). VL in South America, the
843 Mediterranean Basin and China is caused by *Leishmania infantum*, while in Africa and

844 Asia the causal agent is *Leishmania donovani*. The parasites invade tissues rich in
845 phagocytes, such as the liver, spleen and bone marrow, leading to organ dysfunction.
846 Leishmaniasis affects around 12 million people in 98 tropical and subtropical countries
847 wherever the vectors (sand flies) are distributed. Every year, 1 million new cases are
848 estimated, 100,000 of which are of VL, which annually causes 20,000-50,000 deaths
849 [103]. It is likely that there are also many undeclared cases. The *Leishmania*
850 promastigotes (extracellular form of the parasite) are inoculated into the vertebrate
851 host's skin by female phlebotomine sand flies during a blood meal. The promastigotes
852 are captured by phagocytes (neutrophils, DCs and macrophages) and differentiate into
853 the amastigote form (the replicative intracellular stage). Then the amastigotes lyse the
854 infected cells and infect new phagocytes.
855
856 **Box 2. The immune correlates of protection against protozoan parasitic tropical**
857 **diseases**
858 Common to *Plasmodium, Trypanosoma* and *Leishmania* infections is that immunity is
859 associated with the generation of specific cellular responses [22, 58, 67]. These cells are
860 characterized by their functions when stimulated, persistence in the body over time and
861 the ability to migrate to different tissues (**Figure I**). Effector $CD4^+$ and $CD8^+$ T cells (TEFF)
862 do not proliferate but instead produce the effector cytokines that determine the
863 immune response against the parasite. Since these cells survive for a short time in the
864 absence of antigen, the persistence of a small number of parasites sustains a continuous
865 antigen presentation by **antigen presenting cells (APCs)** maintaining effector T-cell
866 populations for the long-term, which is known as **concomitant immunity** [1]. Different
867 **memory lymphocyte** populations capable of maintaining themselves in the absence of

868 live parasites are also generated during the infection, including central memory (TCM),
869 effector memory (TEM) and resident memory T cells (TRM) [13, 67, 104]. TCM are only
870 present in lymphoid tissues, maintain high proliferating capacity after re-stimulation;
871 these cells generate delayed protection because of the time they take to proliferate and
872 differentiate to TEFF, while TEM can be found in non-lymphoid tissues and can rapidly
873 migrate into infected tissues to contribute to cellular immunity [13, 104]. In these
874 pathologies TRM cells have an important role, since they can be found in the tissues
875 where the infection occurs including the skin where many insect-transmitted parasites
876 enter the vertebrate host [105] and the liver [106], where the first stage of malaria
877 replication occurs in their mammalian host. These TRM are long term persistent and can
878 rapidly secrete cytokines after parasite encounter, in addition to their involvement in
879 the recruitment of effector cells as well as pro-inflammatory monocytes (**Figure I**).
880 Evidence support that, for a long-term protective response to be achieved, the target
881 antigen has to persist in the vertebrate host [75]. If it disappears, the host immunity
882 seems to depend on memory T lymphocytes, which are neither fast enough nor
883 powerful enough to efficiently ward off reinfection [1, 13, 18].
884

**Figure I (in Box 2). 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 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 non-lymphoid tissues and play a limited effector role in the site of infection after re-stimulation. 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 after re-stimulation to reinforce the primary immune response.

**Table 1**. **Live attenuated vaccines (LAV) against malaria.**

| LAV candidate | Attenuation | | Model | Immunization[a] Dose of sporozoites Prime/boost (days of boost) | Protection[b] Challenge time and dose Protected/total | Ref. |
|---|---|---|---|---|---|---|
| | Liver/ blood-stage | Parasite in blood | | | | |
| ***Pf* γ-SPZ** | Early | No | Non-human primates<br>Healthy and malaria- exposed humans | iv<br>sc<br>mosquito bites<br>variable doses | 10% -70% - 100%<br>Variable: dose, homologous challenge, heterologous challenge | [26-31, 107, 108] |

| | | | | | | |
|---|---|---|---|---|---|---|
| ***Pf* SPZ-CVac** | Liver & blood stages | No | Malaria-naïve human | iv variable doses + chloroquine, pyrimethamine, and others (*in vivo*) | 50-100% (28d) 9/9 (28mo) 4/6 (105d) 8/14 | [31, 32, 35, 109, 110] |
| ***Pb/Py* SPZ-CVac** | Liver & blood stages | No | C57BL/6 BALB/c | 50k/20k/20k + Centamycin (*in vitro*) (74d, 141d) | (10-21d) 5-10k *Pb* 4/4 (10-21d) 100 *Py* 4/4 | [111] |
| ***Pc* pRBCs + centanamycin/TF-A CVac** | Blood-stage | Yes | A/J mice | $10^6$ + Centanamycin / tafuramycin A (*in vitro*) | (180d) $10^5$ *Pc* pRBC homologous & heterologous | [112] |
| ***Py* pRBCs + centanamycin CVac** | Blood stage | Self resolving | C57BL/6 BALB/c | $10^6/10^6/10^6$ + centanamycin (*in vitro*) | (28d) $10^5$ *Py* pRBCs 9/9 or sporozoites (mosquito bite) 5/5 | [113] |
| **** Pf* pRBCs + TF-A CVac** | Blood stage | Self resolving | Malaria-naïve human | $3x10^7$ + tafuramycin-A (*in vitro*) | ND | [34] |
| ***Pc/Py* pRBCs + Doxycyclin/ Azithromycin CVac** | Blood stage | Self-resolving | BALB/c C57BL/6 | $10^7/10^7/10^7$ (7,28d) + Doxycyclin/ azithromycin/ others (*in vivo*) | (28d) $10^5$ Pc/Py pRBCs Homologous (100%), heterologous (60-100%) | [33] |
| **** Pf* pRBCs + Doxycyclin CVac** | Blood stage | Yes (rescue treatment in 2/4) | Malaria-naïve human | $3x10^6$ + Doxycyclin (*in vivo*) | ND | [33] |
| ***Pb uis3(-)*** | Liver (Early) | No Yes | C57BL/6 | 50k/25k (14d,21d) | (30d) 10k 5/5 | [114] |
| ***Pb uis4*$^-$** | Liver (Early) | Yes | C57BL/6 | 50k/25k (14d,28d) | (38d) 50k 8/8 | [115] |
| ***Py uis3*$^-$** | Liver (Early) | No | BALB/c | 10k/10k (14d,28d) | (60d) 10k 4/4 (180d) 10k 8/12 | [39, 116] |
| ***Py uis4*$^-$** | Liver (Early) | No | BALB/c | 10k/10k (14d,28d) | (180d) 10k 8/8 | [39, 116] |
| ***Pb uis3*$^-$*uis4*$^-$** | Liver (Early) | No | C57BL/6 | 10k/10k (7d,14d) | (118d) 10k 14/14 | [46] |
| ***Pb p36p*$^-$** | Liver (Early) | Yes | BALB/c | 50k | (120d) 10k 5/5 | [38] |
| | | | C57BL/6 | 50k/20k (7d,14d) | (30d) 10k 5/5 | |
| ***Pf Δp52*** | Liver (Early) | Return to wt | Primary human hepatocytes | ND | ND | [40] |
| ***Py Δp52Δp36*** | Liver (Early) | No | BALB/c | 10k/10k (7d,14d) | (30d) 10k 7/7 | [117] |
| ***Pb Δp52Δp36*** | Liver (Early) | No | BALB/c | 10k | (180d) 10k 10/10 | [41, 118] |
| | | Yes | C57BL/6 | 50k/20k (7d,14d) | (180d) 10k 6/7 | |
| ****Pf Δp52Δp36* (*Pf* GAP2KO)** | Liver (Early) | Yes | Hu-hepatocytes Humanized SCID Alb-uPA Mice | ND | ND | [48] |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | Malaria-naïve humans | Mosquito bites | (90d) Cellular immunity | [49, 118] |
| ***Py sap1*$^{-}$** | Liver (Early) | No | BALB/c | 10k/10k (14d,28d) | (210d) 10k 15/15 | [37, 45] |
| ***Pb Δslarp*** | Liver (Early) | No | C57BL/6 | 50k/25k (14,28) | (42d) 10k 5/5<br>(98d) 10k 2/5 | [37] |
| ***Py Δb9*** | Liver (Early) | Yes | BALB/C | ND | ND | [41] |
| ***Pb Δb9*** | Liver (Early) | No | BALB/C | 10k | (10d) 10k 10/10 | [41, 47] |
| | | Yes | C57BL/6 | 50k/20k (7d,14d) | (180d) 10k 9/9<br>(365d) 10k 5/11 | |
| ***Pb Δb9Δp52Δp36*** | Liver (Early) | Yes | C57BL/6 | ND | ND | [41] |
| ***Pb Δb9Δslarp*** | Liver (Early) | No | BALB/C | 10k<br>1.2k | (10d) 10k 20/20<br>(28d) 10k 6/6 | [43, 47] |
| | | | C57BL/6 | 50k/20k (7d,14d)<br>10k/10k (7d,14d) | (180d) 10k 6/6<br>(14d) 10k 10/10 | |
| ****Pf* Δb9Δslarp (PfSPZ-GA1)** | Liver (Early) | No | Human primary hepatocytes<br>Human liver-uPA-SCID | ND | ND | [47] |
| | | | Malaria-naïve human volunteers | 900k (56/112d/168d) | (21d) mosquito bite 3/25 (sterile protection) | [52] |
| ***Pb Δmrp2*** | Liver (mid-to late) | No | BALB/c | 1.2k | (21d) 10k 5/5 | [43] |
| | | | C57BL/6 | 10k/10k (7d,14d) | (14d) 10k 9/10 | |
| ***Pb Δfabb/f*** | Liver (Late) | Yes | BALB/c<br>C57BL/6 | ND | ND | [118] |
| ***Py fabb/f*$^{-}$** | Liver (Late) | No | Swiss | 20k/20k (91d) | (60d) 1k 18/20 | [45, 119] |
| | | | BALB/c | 10k/10k (14d,28d) | (210d)10k 8/8<br>(150d) 10k 5/5 *P. berghei* | |
| | | | C57BL/6 | 20k/20k (111d) | (60d) 1k 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 | 10k/10k (35d) | (110d) 5 bites 20/20<br>(35d) 10k 20/20<br>(110d) 10k 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 | 50K/20k (14d,28d) | (42d) 10k 4/4<br>(102d) 10k 2/2 | [114] |
| ***Pb LISP2*$^{-}$*uis3*$^{-}$** | Liver (Late) | No | C57BL/6 | 50K/20k (14d,28d) | (102d) 10k 8/8 | [114] |
| ***Py plasmei2*$^{-}$ *lisp2*$^{-}$** | Liver (Late) | No | BALB/c | 10k/10k (90d) | (40d) 10k 14/14 | [119] |
| | | | Swiss | 50k/50k (30d,60d) | (30d) 15 bites 9/10 | |
| | | | C57BL/6 | 50k/50k (28d) | (30d) 10k *Py* pRBC | |

| | | | | | | |
|---|---|---|---|---|---|---|
| ***Pf mei2*⁻** | Liver (Late) | No | Human liver-chimeric FRG-HuHep mice | $1x10^6$ | ND | [52] |
| ***Py p52⁻/p36⁻/sap1⁻* (*Py* GAP3KO)** | Liver (Early) | No | BALB/c | 10k/10k (14d) | (180d) 10k 5/5 | [51] |
| ***Pf p52⁻/p36⁻/sap1⁻* (*Pf* GAP3KO)** | Iver (Early) | No | FRG-HuHep mice Human healthy volunteers | 200 mosquito bites | ND | [50, 51] |
| ***Pb* ΔPDH-E1-PFO$_{LS}$ (GAP$^2$)** | Liver (Late PVM) | Yes | BALB/c | 5k single-mutant *Pb*PFO$_{LS}$ | (27d) 5k 10/10 | [122] |
| | | | C57BL/6 | 5k/5k (25d,50d) | (30d) 5k 10/10 | |
| ***Py PNP*-INT pRBCs** | Blood stage | Self-resolving | BALB/c | 200k | (56d) 200k *Py* pRBC Homologous % heterologous challenge 10/10 | [53] |
| ***Py nt1⁻* pRBCs** | Blood stage | Self-resolving | BALB/c C57BL/6 | 100 | (90d) 100k *Py* pRBC 5/5 (90d) 1000 *Pb* pRBC 2/5 | [54] |
| ***Pb* Δ*pm4/msp7* pRBCs** | Blood stage | Self-resolving | BALB/c | $10^7$ | (140d) $10^7$ *Pb* pRBC 14/14 | [55] |
| | | | C57BL/6 CD1 | | (30d) $10^4$ *Py* pRBC 10/10 | |
| | | | BALB/c | | (190d) $10^7$ *Pb* pRBC 10/10 | |
| ***Pb*NK65-*hrf*Δ1 pRBCs** | Blood stage | Self-resolving | C57BL/6 | $10^5$ | (396d) $10^7$ *Pb* pRBC (23d) $10^7$ *Py* pRBC 25d) $10^4$ *Pb*/*Py* sporozoites | [56] |
| ***Δhmgb2PbNK65* pRBCs** | Blood stage | Self-resolving | C57BL/6 | 100k | (160d) 100k *Pb* pRBCs (100%) | [57] |

[a] Immunization with sporozoites ($x10^3$); prime/boost (days of boost).

[b] Sterile protection (not considering pre-patency period); (time after last immunization); challenge ($k=10^3$ parasites), protected/total.

* Vaccine candidate evaluated in humans.

ND: Not determined.

iv: intravenous inoculation; sc: subcutaneous inoculation

pRBCs: parasitized red blood cells.

cVac: chemoattenuated vaccine

SPZ: sporozoites.

CVac: chemically attenuated vaccine.

*Pf: P. falciparum; Pb*: *P. berghei* b; *Py*: *P. yoelii; Pc*: *Plasmodium chanaudi.*

**Table 2. Live attenuated vaccines (LAV) against *T. cruzi.***

| ***T. cruzi* strain / LAV** | **Model** | **Immunization[a]** | **Protection[b]** | | | | **Ref.** |
|---|---|---|---|---|---|---|---|
| | | | **Challenge** | **Blood** | **Tissue** | **Trans.** | |
| **TCC** | Swiss | $10^6/10^6$ (15d/30d) ip | (19d) $10^2$ Tul | Yes | Yes | Yes | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Guinea pig | 28 x $10^6$/kg id | Natural | Yes | ND | Yes | [12, 15, 60] |
| | Dog | $10^7$/$10^7$ (2mo/14mo) | Natural | Yes | ND | Yes | |
| | BALB/c | $10^6$/$10^6$ (7d/14d) sc | (361d) $10^3$ Tul | Yes | ND | ND | |
| **Tul TulCub8** | Swiss | $10^3$/$10^3$(7d) ip | (30d) $10^6$ Tul | Yes | ND | ND | [61] |
| **CL *LYT1*$^{-/-}$ (L16)** | Swiss | $10^3$ ip | (14mo) $10^4$ Tul | Yes | Yes | ND | [62] |
| **Y *gp72*$^{-/-}$** | BALB/c | $10^6$ sc | (10d) $10^3$ Tul | Yes | ND | ND | [63] |
| **TCC** *dhfr-ts*$^{+/-}$ | C57BL/6 | 5×$10^5$/5×$10^5$ (15d) ip | (15d) $10^4$ CL<br>(370d) 2×$10^5$ CL | Yes | ND | ND | [64] |
| | BALB/c | | (15d) 5×$10^3$ Tu | | | | |
| **TCC TcCRT**$^{+/-}$ | BALB/c | 5×$10^5$/5×$10^5$ (15d) ip | (120d) 5×$10^4$ TcV1 | Yes | Yes | Yes | [65] |
| **CL ECH1**$^{+/-}$ **ECH2**$^{-/-}$ | C57BL/6 | 5×$10^5$/1.35×$10^5$ (14d) /5×$10^5$(14d) oral | (14d) 2.5×$10^3$ CL | ND | Yes | ND | [66] |
| **Tu DDDHA** | C3H | 5×$10^3$ ip | (42d) 4.6 × $10^5$ Tu | Yes | Yes | ND | [123] |
| | C57BL/6 | | (42d) 5 × $10^5$ Brazil<br>(42d) 5 × $10^5$ Tu | | | | |
| ***T. rangeli*** | Swiss | 3x$10^5$ ip | (30d) $10^4$ Y | Yes | Yes | ND | [124] |

[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).

ND: Not determined.

**Table 3. Live attenuated parasites used against *Leishmania.***

| GAP / LAV | Model | Immunization | | Challenge | | | Ref. |
|---|---|---|---|---|---|---|---|
| | | Via | Persistence | Species | Wk[a] | Protection | |
| ***L. major dhfr-ts*$^{-/-}$** | BALB/c | iv | ND | *L. major* | 4 | Yes | [78] [82] |
| | | sc | 95 weeks in skin<br>9 weeks in LN | *L. major* | 1 | No | |
| | | | | *L. chagasi* | 4 | No | |
| | CBA | iv<br>sc<br>im | ND | *L. major* | 4 | Yes | |
| ***L. chagasi dhfr-ts*$^{-/-}$** | BALB/c | sc | ND | *L. chagasi* | 4 | No | [82] |
| | | sc | ND | *L. major* | 4 | No | |
| | | iv | ≥ 4 weeks in spleen, liver | ND | ND | ND | |
| ***L. donovani dhfr-ts*$^{-/-}$** | BALB/c | sc | ND | *L. chagasi* | 4 | No | |
| ***L. mexicana* Δ*cpa/cpb*** | BALB/c | sc | ND | *L. mexicana* | 16 | Yes | [125] |
| | C57BL/6 | | | | 8 | Yes | |
| | CBA | | | | 8 | Yes | |
| | Hamster | id | ≥ 12 weeks in skin, LN<br>Pathology develops | *L. mexicana* | 12 | No | [90] |
| ***L. donovani BT1*$^{-/-}$** | BALB/c | iv | ≥ 12 weeks in spleen, liver | *L. donovani* | 6 | Yes | [126] |
| ***L. major lpg2*$^{-/-}$** Compensatory mutant | BALB/c | sc | ≥ 10 weeks in skin, LN | *L. major* | 10 | Yes | [77] |
| | SCID | sc | ≥ 16 weeks in skin, LN | ND | ND | ND | |
| | C57BL/6 | sc | ≥ 10 weeks in skin | *L. major* | 10 | No (Yes with CpG) | [127] |
| ***L. mexicana* ΔGDP-MP** | BALB/c | id | ≤ 5 h in skin, LNs, spleen, liver | ND | ND | ND | [128] |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | ip | ND | *L. mexicana* | 3 | Yes | |
| | | sc | | | | No | |
| ***L. infantum SIR2*$^{-/+}$** | BALB/c | ip | ≤ 8 weeks in spleen, liver, LN | *L. infantum* | 6 | Yes | [91] |
| | SCID | ip | ≥ 8 weeks spleen, liver | ND | ND | ND | |
| ***L. donovani Cen*$^{-/-}$** | BALB/c | iv | ≤ 12 weeks in spleen, liver | *L. donovani* | 24 | Yes | [69] [83] |
| | | | | *L. braziliensis* | 5 | Yes | |
| | | | | *L. major* | 5 | Yes | |
| | | | | *L. mexicana* | 30 | Yes | |
| | SCID | iv | ≤ 12 weeks in spleen, liver | ND | ND | Yes | |
| | Hamster | ic | ≤ 10 weeks in spleen, liver | *L. donovani* | 5 | Yes | |
| | Dog | sc | ND | *L. infantum* | 8 | Yes | [70] |
| ***L. donovani Cen*$^{-/-}$ + LJM19** | Hamster | id | ≥ 5 weeks in skin, LN<br>≤ 5 weeks in spleen, liver | *L. donovani* | 5 | Yes | [71] |
| ***L. major Cen*$^{-/-}$** | C57BL/6 | id | ≥ 6 weeks in skin | *L. major*<br>*L. major*-infected sand flies | 7 | Yes | [96] [88, 89] |
| | BALB/c | id | ND | *L. major* | 7 | Yes | |
| | | sc | ≥ 20 wk in skin | ND | ND | ND | |
| | STAT-1 KO | sc | ≥ 7 weeks in skin | | | | |
| | IFN-γ KO | sc | ≤ 20 weeks in skin | | | | |
| | Rag2 KO | sc | ≤ 15 weeks in skin | | | | |
| | Hamster | id | ≥ 7 weeks in skin, LN | *L. donovani* | 56 | Yes | |
| | | | | *L. donovani*-infected sand flies | 7 | | |
| ***L. donovani p27*$^{-/-}$** | BALB/c | iv | < 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 | sc | < 12 weeks in spleen, liver | *L. major*<br>*L. infantum* | 4 | Yes | [85] |
| ***L. donovani* Δ*ALO*** | BALB/c | iv | < 16 weeks in spleen, liver | *L. donovani* | 20 | Yes | [129] |
| ***L. infantum hsp70-II*$^{-/-}$** | BALB/c | iv | < 12 weeks in spleen, liver, BM | *L. major*<br>*L. infantum*<br>*L. amazonensis* | 12 | Yes | [73, 74, 86, 87] |
| | | sc | > 20 weeks in LN | | | | |
| | C57BL/6 | sc | > 25 weeks in LN | *L. major* | | | |
| | SCID | iv | < 8 week in liver | ND | ND | ND | |
| | Hamster | ic | < 9 months in spleen, liver | | | | |
| ***L. donovani* Δ*Fbpase*** | BALB/c | ip | < 14 weeks in spleen, liver | *L. donovani* | 24 | Yes | [130] |
| ***L. infantum ΔKHARON1*** | BALB/c | iv | < 15 days in spleen, liver | *L. infantum* | 5 | Yes | [131] |
| | | sc | | | | No | |
| ***L. donovani Hel67*$^{-/-}$** | Hamster | im | ≥ 90 days in spleen | *L. donovani* | 3 | Yes | [132] |
| ***L. infantum* + Gentamicin CVac** | Dog (clinical trial) | sc | ND | Natural infection (endemic area) | 168 | Yes | [68] |

[a] Weeks after immunization.

ND: Not Determined.

iv: intravenous inoculation; id: intradermal inoculation; sc: subcutaneous inoculation; im: intramuscular inoculation
LN: lymph node
CVac: chemically attenuated vaccine

**Figure 1 (Key Figure). Protection elicited by Live Attenuated Vaccines.** Vaccination strategies against malaria, Chagas disease and leishmaniasis aim to generate of 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 $T_{EFF}$ are ephemeral and depend on antigen presence, subunit vaccine-mediated protection depends solely on $T_M$. On the other hand, the protective responses mediated by live attenuated vaccines are based on the maintenance of $T_{EFF}$ by reactivation of $T_M$ and/or new generation of T lymphocytes from $T_N$. 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 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: $T_N$, T naïve precursors; $T_{EFF}$, effector memory T cells; $T_M$, memory T cells.