Chagas disease is a neglected tropical disease caused by the flagellate protozoan parasite Trypanosoma cruzi. This illness is now becoming global, mainly due to congenital transmission, and so far, there are no prophylactic or therapeutic vaccines available to either prevent or treat Chagas disease. Therefore, different approaches aimed at identifying new protective immunogens are urgently needed. Live vaccines are likely to be more efficient in inducing protection, but safety issues linked with their use have been raised. The development of improved protozoan genetic manipulation tools and genomic and biological information has helped to increase the safety of live vaccines. These advances have generated a renewed interest in the use of genetically attenuated parasites as vaccines against Chagas disease. This review discusses the protective capacity of genetically attenuated parasite vaccines and the challenges and perspectives for the development of an effective whole-parasite Chagas disease vaccine.

KEYWORDS: Chagas disease • immune response • infectivity • live vaccine • parasite knock-out • safety • Trypanosoma cruzi

Chagas disease, also known as American trypanosomiasis, is a zoonosis caused by the flagellate protozoan Trypanosoma cruzi and is transmitted to humans by reduviid blood-sucking insects. Infection typically occurs when infected hematophagous insects deposit their feces on the skin during their blood meal [1]. Other routes of transmission, increasingly important in developed countries, include transfusion and organ transplantation, mother-to-child transmission and ingestion of contaminated food [2].

Two or three weeks after infection, the parasite proliferates and invades the circulatory system. A 1–2 month acute phase is then initiated, accompanied by a potent activation of the immune response. At this point, most infected patients experience fever, with swollen lymph nodes and inflammation at the biting site. The acute phase is followed by development of acquired immunity, which usually controls parasite growth, reaching a state of concomitant immunity with subclinical infection that persists through the life of the host. Progress to an intermediate, asymptomatic phase occurs in almost all patients. Only 30–40% of them develop overt disease at a later stage with variable symptoms, characterized by heart damage associated with intense myocarditis, leading to progressive heart failure and death. The gastrointestinal form is characterized by digestive tract pathology [1,3,4].

A number of relevant features of the disease encourage the research and development of vaccines for its management, such as: drawbacks of current Chagas chemotherapy; ineffective vector-borne control programs; parasite persistence, rather than immunopathology, as a pathogenic factor; and the fact that Chagas is a neglected tropical disease affecting low-income individuals of underdeveloped countries.

Drawbacks of current chemotherapy
Currently, and for more than 40 years, the only available treatment against Chagas disease is based on two drugs, benznidazole (N-benzyl-2-nitroimidazolylacetamide) and nifurtimox.
(R,S)-3-methyl-N-[(1E)-(5-nitro-2-furyl)methylene]thiomorpholin-4-amine-1,1-dioxide [2]. Drug treatments are prolonged (60–90 days), expensive and have undesirable side effects in about 30–50% of patients, including digestive intolerance, peripheral neuropathy, bone marrow suppression, urticarial rashes and hepatitis [5–7]. The efficacy of these drugs has been fairly described and its use is mandatory for the treatment of children during the acute stage of the disease. However, the action of these drugs during the indeterminate and determinate stages is still under study [7]. Two long-term follow-up clinical trials of benznidazole in adults (TRAENA and BENEFIT) are currently being analyzed and the results of these studies will contribute to elucidate this controversy [8,9]. Despite the associated risk of congenital transmission of the disease, both drugs are contraindicated for pregnant women [2].

More recently, inhibitors of the ergosterol biosynthesis have been tested as trypanocidal drugs because it has been demonstrated that ergosterol is an important component of the parasite plasma membrane that is essential for parasite viability and proliferation during its entire life cycle [10,11]. Unfortunately, recent human clinical trials have evidence that two ergosterol inhibitors (posaconazole [Merck and Co., New Jersey, USA] and ravuconazole [Eisai Co., Tokyo, Japan]) are ineffective for Chagas disease treatment [12–14].

Vaccination, as an alternative to chemotherapy, would offer several advantages. Vaccination requires a limited number of doses/boosters, while chemotherapy involves the prolonged administration of drugs during 60–90 days. The affected patients live in areas with accessibility difficulties and therefore the expenses for the management of the patients could be reduced substantially.

A recent modeling of the advantages that a vaccine against Chagas disease would bring to Mexico demonstrated that the development of such an intervention would be highly beneficial not only in terms of health but also from the economic point of view [15]. Moreover, vaccination may eliminate problems related to drug toxicity and prevent the development of heart disease in patients who are in the indeterminate or determinate phase. In addition, a vaccine would also have the potential to be used in pregnant women to prevent congenital transmission [16].

Ineffective vector-borne transmission control

The ‘kissing bug’ T. cruzi vector has adapted to live in low-quality housing conditions; therefore, Chagas disease primarily affects people living in poverty [17]. Vector control of the disease has been successful in several Latin American regions, substantially reducing the risk of infection [18]. However, sometimes the large distances from the control disease centers and the limited accessibility to endemic areas lower the success rates of vector control programs. Furthermore, only a limited number of domestic triatomine species have been adequately controlled. Autochthonous, sylvatic and insecticide-resistant Triatoma species have emerged, which causes difficulties in controlling them [19,20].

Parasite persistence as a pathological factor

Several researchers have proposed that vaccination may induce immune responses that may increase the severity of the disease through autoimmunity stimulation [21,22]. However, there is a broad consensus that parasite persistence has a role in the pathogenesis of Chagas disease. In this regard, several studies have provided evidences for the following:

- T. cruzi is present in inflammatory lesions [11,23–25];
- there is a lack of correlation between the level of ‘autoantibodies’ and the severity of the disease in both chronically infected humans and animal models [22,26,27];
- there is a direct correlation between the parasite load, the intensity of the inflammatory process and the severity of the disease in experimental animals and humans [22–24,28,29];
- there is a positive effect of both specific chemotherapy and immunization in the evolution of human and experimental Chagas disease [130–33]; and
- an exacerbation of human and experimental T. cruzi infection occurs in immune-suppressed hosts [34–38].

Chagas disease: a neglected disease

Chagas disease affects approximately 10% of the population of Latin America [17,39,40]. About 10 million people are infected worldwide, 99% belonging to the poorest countries of Latin America, causing 10,000–50,000 deaths per year [17,40]. In spite of the existence of control programs, transmission of the disease has increased in several regions of Latin America [41]. Although the illness causes economic losses of approximately US$1.2 billion annually [42], a prophylactic vaccine to prevent the infection or a therapeutic vaccine to reduce the consequences of the infection is still absent from the market. Probably, this is because the disease affects mainly low-income individuals belonging to underdeveloped countries or immigrants who carried the disease to non-endemic areas of North America, Europe, Japan and Australia [43,44]. Because of these reasons, and historically, pharmaceutical companies have not been involved in the development of interventions against the disease, including vaccines [45]. This situation has changed during the last years and several important pharmaceutical companies are now interested in the development of new drugs and clinical trials.

Immune protection against T. cruzi & protective mechanisms

The innate immune system is fundamental in the pathogenesis of Chagas disease because parasite replication, migration and spread into host tissues, as well as the inflammatory response in the infected host are under its control. The understanding of immune mechanisms associated with protection will orient us in the design of different immunization approaches and formulations, with ameliorated side effects. Toll-like receptors (TLRs) have been the center of many studies in T. cruzi since their activation leads to a productive stimulation of B and T cells [46–48].

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Since the activation of CD4+ T cells against *T. cruzi* is reduced in the absence of TLR signals, a connection between the innate and adaptive immune responses against this parasite became evident [49]. Thus, the strength of the protective response generated is related to the activation of antigen-presenting cells during *T. cruzi* infection. Therefore, vaccines should incorporate immune-activating molecules, in addition to those specific to the parasites. Some TLR ligands, Glycophosphatidylinositol anchors and DNA induce proinflammatory cytokine production and host defense mechanisms [50-52]. After infection, Natural Killer cells are activated via IL-12 and TNF-α stimulation to produce IFN-γ, a key molecule controlling *T. cruzi* infection as it activates the macrophages that release effector molecules capable of controlling parasite replication and clearance [53]. IL-12 is also necessary for differentiation and clonal expansion of Th1 CD4+ and CD8+ T and B cells. CD4+ T cells orchestrate the immune response via IL-12 and IFN-γ production, stimulating the expansion of CD8+ T cells that are essential for systemic protection against *T. cruzi* [54]. CD8+ T cells destroy the nucleated cells containing intracellular amastigotes. On the other hand, although extracellular trypomastigotes are detected by antibodies, their subsequent lysis by complement is subverted by this parasite stage. CD8+ T cells are thus crucial for *T. cruzi* regulation [55-60]. The adaptive immune response generated, despite being strong and efficient in controlling parasite proliferation, is sometimes not enough to mediate sterile immunity. Rather, an immune state concomitant with infection is thus achieved. The reasons why this phenomenon occurs are not fully understood and represent an important feature to be considered in the efforts to develop an effective vaccine.

### Vaccines against Chagas disease

For more than 60 years, several strategies have been developed for the production of an effective vaccine against Chagas disease (reviewed in [61-65]). The first-generation vaccines were prepared with the whole parasite, alive or killed by different methods. Following pioneer studies by Pizzi and Prager [64] and Menezes [65], Brazilian researchers described, in the 90s, the first *T. cruzi* strain utilized as live, experimental vaccine. Experimental vaccination trials revealed that despite the apparent absence of infectivity, inoculated trypomastigotes protected mice against a lethal challenge. All vaccinated mice survived and showed negative parasitemia [66].

Another live experimental vaccine, extensively characterized by our group, was the *T. cruzi* TCC strain. This attenuated parasites were generated by prolonged and uninterrupted in vitro culture [67]. An early immunological characterization of this strain revealed that TCC induced in mice a Th2-type response profile with detectable levels of IL-10 and IL-4 [68]. This clone neither produced detectable parasitemia nor induced immunopathological effects in immunocompetent animal models. Moreover, the parasites did not revert to a virulent phenotype after an in vivo selection [69,70]. Further experiments demonstrated that the TCC clone did not elicit lytic antibodies, as shown by complement-mediated lysis tests [71]. Thus, the detection of TCC infections required the use of more sensitive techniques and immunodeficient animals. Recent experiments conducted in our laboratory demonstrated the persistence of TCC parasite by hemoculture after immunosuppression of mice infected with TCC trypomastigotes. Immunization of BALB/c mice with TCC epimastigotes induced a strong and long-lasting protective immune response after challenge with Tulahuen strain blood trypomastigotes, as evidenced by a decrease in parasitemia and mortality in both mice and guinea pigs [70,72]. TCC immunization induced long-term protection since the control of parasitemia and tissue damage was still effective in animals challenged at least a year after the immunizing inoculum [73]. Also, the TCC protective response was successfully extended to field experiments using natural, vector-delivered infections in guinea pigs [72] and dogs [74].

Second-generation vaccines employed native or recombinant immunogenic *T. cruzi* proteins. Several native antigens were used as vaccines in mice, such as the paraflagellar rod protein and the trypomastigote excretory–secretory antigens [75-77]. The KMP11 recombinant protein, associated with parasite microtubule scaffold, the GP82 membrane glycoprotein of metacyclic trypomastigote, the trans-sialidase proteins and the amastigote surface protein, all showed strong immunoprotective responses [78-82]. In addition to the above-mentioned proteins, immunization with cruzipain, a protease highly immunogenic in *T. cruzi*, regulates the expression of TLR, inducing the production of nitric oxide, IL-12 and IFN-γ [83]. The vaccination with recombinant cruzipain co-administered with MALP-2, a macrophage-activating lipopeptide, can enhance the protective response [84]. Furthermore, vaccination assays using a genetically modified transsialidase enzyme demonstrated that it is highly protective against subsequent infections by *T. cruzi* [85]. Even though recombinant protein technology allowed testing of several well-established antigens, the principal immune response generated by this approach is the production of specific antibodies but limited cellular immunity. As mentioned, antibodies are not enough to effectively control *T. cruzi* infection as parasites can persist within the host cells, avoiding direct contact with antibodies. In this scenario, a cellular response detecting and eliminating the infected cells seems more appropriate to control *T. cruzi* proliferation.

In recent years, the so-called third-generation vaccines, which include DNA vaccines, live non-pathogenic modified organisms (viral vectors and *Salmonella*) and *T. cruzi* genetically attenuated parasites (GAPs), have attracted particular attention [32,86-89]. DNA vaccines have several advantages such as easy manufacturing, broad administration and not requiring a cold chain for their distribution, features of particular interest in poor countries where Chagas disease is endemic. Several antigen candidates have been evaluated in DNA encoding vaccines, such as ASP-1/2, TSA-1, Tc24, TeVac-1/2 and cruzipain [32,84,90,91]. Besides developing a strong cellular Th1 response, sterile immunity after challenge has not been reported for DNA immunization approaches.
As novel strategies, heterologous approaches using DNA prime/modified vaccinia Ankara boost regimens and recombinant yellow fever viruses have elicited promising results [92]. Recently, Salmonella carrying DNA encoding the full-length T. cruzi polypeptide, Tc52, or its amino- or carboxy-terminal domain resulted in low antibody titers and a predominantly Th1 response [89]. Even more, a multicomponent oral DNA vaccine carried by Salmonella and targeting invasion and metabolic pathways has resulted in an improved protection compared to that afforded by one-component DNA vaccine [93].

Live attenuated parasite vaccines

The above-mentioned first-generation live vaccines generally use different ways to induce the attenuation of the parasites. For example, the parasites can be subjected to prolonged in vitro cultures or treated with chemical or physical agents to induce the attenuation. However, in this kind of live vaccines, the molecular basis of the attenuation is not fully understood and they must meet specific safety conditions that prevent reversion to the virulent phenotype. Also, they must have persistence at low level in the vaccinated individual, thus establishing a subclinical infection without detectable pathology. Several commercial live vaccines such as those against poliomyelitis, tuberculosis, rubella, yellow fever, measles and ovine toxoplasmosis fulfill these conditions. Furthermore, these live attenuated vaccines must be able to induce a long-term immunoprotective response against a subsequent parasite challenge. In this regard, many effective vaccines show their weaknesses when the immunization challenge time is increased, revealing their short-term protection, or when the challenging parasite dose is augmented.

Live attenuated vaccines offer several advantages:

- They mimic the typical course of infection and provide an approximation of the microenvironment of antigen processing and presentation, thus creating a subclinical infection that ensures antigen persistence and thus multiple immunogenic stimuli.
- They induce a strong, long-lasting protective response, compared to the responses induced by dead parasites [70,94].
- They provide the full spectrum of native antigenic epitopes and immune stimulating molecules, such as pathogen-associated molecular patterns that may interact with TLRs or other innate immunity stimulating receptors (pattern recognition receptors, in general) present in various immune competent cells, including antigen-presenting ones. Thus, potent cooperative innate and adaptive immune responses may be generated.
- They induce a transient infection that delivers a small, self-controlled antigenic burden so that the immune response would be more inclined toward a Th1 protective one [95,96].
- They have lower production costs with respect to other types of vaccines, since parasites can be produced in low-cost axenic conditions.
- These vaccines are relatively easy to modify genetically in order to increase their safety and immunogenicity.

Despite the advantages offered by first-generation live vaccines, uncertainties persist with regard to safety in parasite attenuation. This feature is not genetically determined, and the reversal of an attenuated to a virulent phenotype was observed by inoculating immunodeficient mice with culture-attenuated strains [97]. On the other hand, excessive attenuation may lead to a weak protective response by failing to establish a subclinical infection. Moreover, parasite persistence in the host should be limited, mainly to avoid a possible reactivation in immunosuppressed hosts, as occurs in HIV carriers, patients subjected to organ transplants or patients under cancer therapy.

Gene targeting as a tool for the development of attenuated parasites

In the last few years, and with the advances in DNA recombinant technology, alternative strategies have emerged. The use of parasites with an attenuated phenotype induced by precise, defined and stable genetic manipulations is now possible. The techniques of stable transfection in trypanosomatids [98,99] allowed the characterization of genes responsible for parasite development and survival. Transfectant organisms provide new insights into the mechanisms of gene expression, parasite–host interactions and the identification of targets for chemotherapy. Electroporation has been the most efficient transfection tool, allowing the expression of foreign genes, as well as gene-targeted deletion/disruption in many organisms, including parasite trypanosomatids. This method involves the use of electrical pulses that permeabilize the parasite membrane and permit the entry of exogenous DNA [100]. Then, the integration of the DNA into the parasite genomic locus takes place exclusively by homologous recombination. For gene-targeted replacement, recombinant fragments utilize the 3' and 5' untranslated regions of the target gene, which are arranged on either side of a selectable marker gene providing resistance to a particular antibiotic, usually hygromycin or neomycin. Thus, both untranslated regions act as 'recombination arms' recognizing their homologous sequence genome and facilitating the transcript processing. Later, the cloning of the transfected population allowed the inheritance of the deletion to the next generation. This technique presents some difficulties due to the long time required to perform two rounds of transfection necessary to eliminate both allelic copies using two different antibiotic resistance genes. Furthermore, the use of RNA interference technology has not been effective in achieving gene replacement in T. cruzi [101], leaving homologous recombination as the only effective method.

In summary, gene deletion, by homologous recombination, allowed the use of mutant parasites for the functional analysis of specific genes and for their alternative use as GAP vaccines. The introduced genetic modification generates a homogeneous population of parasites with a built-in safety mechanism, such as a stable modification at the genomic level, which is thus transmissible to the progeny.
**T. cruzi GAPs as vaccines against Chagas disease**

Numerous evidences argue for the effectiveness of GAP vaccines against malaria and leishmaniasis (reviewed in [102-106]). Recently, *Plasmodium falciparum* mutants were generated by deletion of two pre-erythrocytic-specific-stage genes (*P52* and *P36*) [107]. The first-in-human clinical trial is presently under development and is based on the infection of human volunteers with these GAPs by *Anopheles* mosquito bites [108]. Numerous genetically attenuated *Leishmania* parasites have been used as vaccines. Gene targeting was directly mated with two genes related to polylamine, purine and pyrimidine metabolism, cytoskeletal structure, chaperones and other specific genes of the amastigote stage of *Leishmania*. Among them, the development of stage-specific attenuated parasites emerged as an interesting approach. Deletion of the centrin gene of *L. donovani* arrested the growth of amastigotes and was found to be safe and efficacious against virulent challenges in mice, hamsters [109] and dogs [110]. Similar results were obtained using GAP for the genes P27 (a cytochrome c oxidase complex component) [111], UFM1 (a ubiquitin fold modifier-1) [112] and UFSP (involved in fatty acid metabolism) [113].

To date, approximately a dozen *T. cruzi* genes have been deleted for several purposes [114-126] and the biological characteristics of a few *T. cruzi* mutants have been evaluated in *in vivo* models. Moreover, only a limited number of these mutants have been evaluated as experimental vaccines against Chagas disease; most of them were totally or partially characterized in our laboratory (Table 1).

**gp72**

Pioneering work on biallelic deletion of the *gp72* gene in the *T. cruzi* Y strain was performed in the laboratory of George Cross. Western blot analysis of these null mutants demonstrated the complete absence of the *Gp72* glycoprotein. This resulted in the modification of the normal adhesion of flagellum to the parasite cell membrane [118]. The mutants infected and proliferated in murine macrophages and in non-phagocytic cells, but showed a decreased ability to colonize the insect vector *Triatoma infestans*. These data indicate that *gp72* expression or the normal position of the flagellum is essential for propagation in the vector [127]. In addition, synthesis of *Gp72* and the infective capacity were restored after episomal transfection [128]. Similar experiments were conducted in our laboratory; our results about reduced ability of this mutant to proliferate in the insect vector were consistent with those described by De Jesús *et al.* [127]. Furthermore, we found that these parasites do not produce sustained infection in immunocompetent and immunodeficient mice or strong tissue inflammatory and anti-*T. cruzi* antibody responses. In addition, the stability of the engineered locus was verified by amplifying the genomic regions corresponding to the antibiotic resistance gene coding sequences, demonstrating that the mutation was stable after 5 years of propagation in axenic cultures [129].

We also evaluated the protective capacity of this mutant strain by inoculating adult Swiss mice with a single dose of 10⁶ mutant or wild-type epimastigotes, and 10 days later, the mice were challenged with 10⁷ blood trypomastigotes of the Tulahuen strain. The animals vaccinated with mutant as well as wild-type *T. cruzi* showed a highly significant reduction of the parasitemia levels, compared to non-vaccinated mice. Interestingly, despite the low infectivity of the mutant, no difference in terms of protective capacity was observed with respect to the wild-type strain, indicating that in this experimental setup, the level of infectivity was independent of the induction of the protective response [129].

**Calmodulin-ubiquitin B**

Calmodulin-ubiquitin B (*cub*) is a single copy gene actively expressed in *T. cruzi*. Although its function is unknown, targeted deletion experiments determined that *cub* is an essential gene for parasite viability because both alleles can be deleted only when an additional copy is episomally expressed [114]. The monoallelic deletion of this gene in the Tulahuen strain did not affect epimastigote growth in axenic culture; however, the mutants showed significant infectivity attenuation in murine models and the parasites could only be isolated from mice by sensitive techniques. Furthermore, the restitution of *cub* function, through episomal transfection, increased its infectivity [130].

In order to evaluate the protective capacity, adult Swiss mice were inoculated with 10⁵ mutant or wild-type epimastigotes, followed with a boost similar to the initial inoculation on day 7. After 38 days of inoculation, the animals were challenged with 10⁶ Tulahuen strain blood trypomastigotes. This study showed that mice inoculated with both mutant and wild-type parasites were strongly protected, as indicated by significantly reduced parasitemia [130]. As in the *gp72* mutants previously described, *cub* mutants did not show differences in protection compared to the wild type, so once again, the level of infectivity was independent of the induced protective response.

**lyt1**

The *lyt1* gene functions were identified by comparing null biallelic mutant parasites from the *T. cruzi* CL strain (clone L16) with the wild type. The deletion did not affect the growth capacity of epimastigotes in axenic culture, but the parasites presented a substantial reduction of the *in vitro* infectivity and hemolytic activity at low pH conditions [122]. In our laboratory, the mutants exhibited a reduced infectivity in mice, and serial *in vivo* passages did not restore the virulent phenotype. Histopathological studies showed a substantial decrease in tissue inflammatory response, consistent with the low parasite load [131].

Mice immunized with 10⁵ mutant or wild-type epimastigotes presented, after a challenge with 10⁴ trypomastigotes of the Tulahuen strain, an active protective response, evidenced by low parasitemia during 60 days. In mice, splenomegaly is a macroscopic manifestation of the increase in B and T lymphoid cell populations caused by *T. cruzi* infection. Thus, the spleen weight index represents an indirect effect of
Table 1. Genetically attenuated parasite vaccines as candidates against Chagas disease.

<table>
<thead>
<tr>
<th>Deleted gene</th>
<th>Parasite strain</th>
<th>Biological characterization</th>
<th>Mouse strain</th>
<th>Prime/boost</th>
<th>Challenge (parasites/days after prime)</th>
<th>Results of vaccination</th>
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<td>Atypical flagellum insertion.</td>
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<td>Development in <em>Triatoma infestans</em>.</td>
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<td>↓ In vivo infectivity.</td>
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<td>↓ Tissue inflammatory response.</td>
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<td>↓ Anti-<em>T. cruzi</em> antibodies.</td>
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<td>Stability of the engineered locus.</td>
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<td>cub</td>
<td>Tulahuen</td>
<td>Monoallelic deletion.</td>
<td>Swiss</td>
<td>$10^6$ epis/10^6 epis.</td>
<td>$10^6$ blood tryps/38.</td>
<td>Protective response against a virulent challenge with Tulahuen strain: ↓ parasitemia.</td>
<td>[114,130]</td>
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<td>Normal in vitro growth.</td>
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<td>cub episomal restitution.</td>
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<td>↓ In vivo infectivity.</td>
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<td>LYT1</td>
<td>CL</td>
<td>Biallelic deletion.</td>
<td>Swiss</td>
<td>$10^3$ epis/ND.</td>
<td>$10^4$ blood tryps/420.</td>
<td>Protective response against a virulent challenge with Tulahuen strain: ↓ parasitemia, ↓ spleen index, ↓ tissue inflammatory response, long-term protection.</td>
<td>[122,131]</td>
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<td>↓ In vivo and in vitro infectivity.</td>
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<td>↓ Hemolytic activity.</td>
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<td>Stability of the engineered locus.</td>
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<td>ECH1 and 2</td>
<td>CL</td>
<td>Monoallelic deletion.</td>
<td>C57BL/6</td>
<td>$5 \times 10^5$ meta tryps/1.35 $\times 10^5$ and $5 \times 10^5$ meta tryps.</td>
<td>$2.5 \times 10^5$ meta tryps/45.</td>
<td>Protective response against a virulent challenge with fluorescent CL strain: tracking through in vivo imaging; ↓ parasite load in infection site; ↓ protection levels in mice with ↓T. cruzi specific CD8+ T cells.</td>
<td>[117]</td>
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<td>Biallelic deletion ECH2+/—-.</td>
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<td>↓ Amastigotes in VERO cells.</td>
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<td>↓ In vivo infectivity.</td>
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<td>oral gavage: ↑ T. cruzi specific CD8+ T cell response.</td>
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<td>DHFR-TS</td>
<td>TCC</td>
<td>Monoallelic deletion.</td>
<td>C57BL/6</td>
<td>$5 \times 10^5$ meta tryps/5 $\times 10^5$ meta tryps.</td>
<td>$10^4$ blood tryps and $2.5 \times 10^5$ meta tryps/30 and 360.</td>
<td>Protective response against a virulent challenge with Tulahuen and CL fluorescent strain: ↓ parasitemia, ↓ T. cruzi specific CD8+ T cell response, long-term protection.</td>
<td>[123,136]</td>
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<td>↓ In vitro growth.</td>
<td>Balb/c</td>
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<td>↓ In vivo infectivity.</td>
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<td>↓ T. cruzi specific CD8+ T cell response.</td>
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: Reduction, inhibition. ↑: Increase, augment; Blood tryps: Blood trypomastigotes; ECH: Enoyl-coenzyme A hydratase; Epis: Culture epimastigotes; Meta tryps: Metacyclic trypomastigotes; ND: Not done.
### Table 1. Genetically attenuated parasite vaccines as candidates against Chagas disease (cont.)

<table>
<thead>
<tr>
<th>Deleted gene</th>
<th>Parasite strain</th>
<th>Mouse strain</th>
<th>Biological characterization</th>
<th>Challenge (parasites/days after prime)</th>
<th>Mouse Prime/boost</th>
<th>Ref.</th>
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<td>TcCRT</td>
<td>TCC</td>
<td>Balb/c nude</td>
<td>Monoaallelic deletion.</td>
<td>10⁵ blood tryps/120</td>
<td>TcCRT TCC Monoallelic deletion.</td>
<td>[124,145]</td>
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<td>In vitro growth.</td>
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<td>Stability of the engineered locus.</td>
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<td>Metacyclogenesis in vitro.</td>
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<td>Susceptibility to ergosterol biosynthesis inhibitors.</td>
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</table>

**Results of vaccination**

- **Under progress.**

- **T. cruzi gene-deleted live-attenuated strain**: Balanced: 10⁴ blood tryps/120. Protective response against a nude complement susceptibility.

**Parasite load at the infection site was monitored by an in vitro imaging system that allowed rapid assessment of the infection success by determining the fluorescence levels.** Immunized mice presented, at the infection site, a fluorescence reduction consistent with a lower parasite load and strong fluorescence signals. It is noteworthy that before the last immunization, a group of mice showed a high number of activated CD8⁺ T cells in peripheral blood. After challenge, this group showed a strong protective response compared to mice with a reduced number of CD8⁺ T cells, thus showing that T cell proliferation could be a good indicator of the effectiveness of the vaccine [117].

**Enoyl-coenzyme A hydratase**

Enoyl-coenzyme A hydratase 1 and 2 genes (ech1/2) encode the putative enoyl-coenzyme A hydratase/isomerase protein family, enzymatically involved in fatty acid oxidation, a process essential in amastigote energy metabolism [132]. The MultiSite Gateway cloning system has emerged as an efficient way to accelerate the time-consuming multiple gene cloning and to generate efficient constructions able to delete genes in the *T. cruzi* genome [133]. Mutants carrying only one copy of the *ech1* gene (ECH1⁺/-) and none of the *ech2* genes (ECH2⁻/⁻) were generated using Gateway constructions. These mutant epimastigotes grew slightly slower than their wild-type counterparts and did not grow as efficiently as amastigotes in cultured cells. The *ech* mutant parasites also failed to establish persistent infections in mice, as evidenced by negative direct parasitological tests, inclusive after subjecting the animal to immunosuppression to reveal parasites. However, oral gavage of *ech* mutants in mice induced a systemic muscle tissue infection and a potent *T. cruzi* specific CD8⁺ T cell response [117].

To determine the protective response induced by *ech* mutants, three doses of 1.3–5 × 10⁵ trypomastigotes, 2 weeks apart, were administered to mice by oral gavages. After 45 days, footpad challenge was performed using 2.5 × 10⁵ CL strain fluorescent trypomastigotes transfected with a gene encoding tdTomato protein [134]. The parasite load at the infection site was monitored by an in vivo imaging system that allowed rapid assessment of the infection success by determining the fluorescence levels. Immunized mice presented, at the infection site, a fluorescence reduction consistent with a lower parasite load and a substantial protection at all evaluated times. Control and naïve mice displayed high parasite load and strong fluorescence signals. It is noteworthy that before the last immunization, a group of mice showed a high number of activated CD8⁺ T cells in peripheral blood. After challenge, this group showed a strong protective response compared to mice with a reduced number of CD8⁺ T cells, thus showing that T cell proliferation could be a good indicator of the effectiveness of the vaccine [117].

**Dihydrofolate reductase–thymidylate synthase**

In trypanosomatids, dihydrofolate reductase–thymidylate synthase (*dhfr-ts*) is a single-copy gene encoding an important enzyme involved in the thymidine biosynthesis pathway and, therefore, in the DNA synthesis. Inhibition of *dhfr-ts* prevents thymidine biosynthesis, thereby causing cell death. In *L. major*, deletion of both *dhfr-ts* alleles caused a reduced infectivity in infection severity. Sixty days after the challenge, spleen index was significantly lower in mice immunized with mutant parasites. The mutant parasites induced a long-lasting protective immunity since protection was evident up to 14 months after challenge. The necropsies of mice at day 60 after challenge revealed a reduction of tissue damage and a significant decrease in the presence of lymphocytic infiltrates and calcium deposits [131].
both susceptible and immunodeficient mice and a highly protective response after challenges with virulent parasites [135].

Using the Gateway technology, our team generated a clonal strain with a monoallelic deletion of the \textit{dbhfr-ts} gene in the attenuated TCC strain. The deletion of both copies was not possible, probably because the gene is essential for the life cycle of \textit{T. cruzi}. The monoallelic deletion was sufficient to reduce the parasite growth in axenic cultures. Mouse infectivity assays demonstrated that mutant parasites produced a low percentage of specific CD8+ T cells and a reduced parasite load, compared to wild-type TCC parasites [123]. However, both wild-type and mutant parasites activated a protective immune response against a second virulent infection. This protective function was evaluated using different mouse strains injected intraperitoneally with \(5 \times 10^5\) mutants and wild-type metacyclic trypomastigotes. On day 15, a boost similar to the initial inoculation was administered and then after 30 days from the last inoculation, the animals were challenged with CL or Tulahuen metacyclic trypanosomes. The study revealed a decrease in the load of peripheral blood circulating parasites in mice inoculated with mutant as well as wild-type parasites. In all experiments performed, the protection induced by mutant parasites was similar to that obtained with wild-type parasites, suggesting that the deletion of one \textit{dbhfr-ts} allele did not modify the protective capacity of the original wild-type live immunogen. Also, these mutant parasites retained their protective effect against a virulent challenge with CL strain fluorescent trypomastigotes even 1 year after inoculation [123,136].

The expansion of specific CD8+ T cells is defined by both the kind and quantity of accessible antigen. The lower proportion of \textit{T. cruzi} specific CD8+ T cells in mice infected with \textit{dbhfr-ts} mutant parasites was probably associated with the slower propagation rate observed for these parasites and, therefore, with a late or lower availability of parasite antigens presented by dendritic cells. Interestingly, a proportion of mice immunized with \textit{ech} mutant parasites showed a high number of activated CD8+ T cells in peripheral blood, a fact that correlated with improved protection. The opposite was observed with \textit{dbhfr-ts} gene mutants. These last results are in agreement with previous studies showing that antigen-specific CD8+ cells contribute to control the acute infection, but are not essential for the evolution of immune resistance [137]. Perhaps other immune mechanisms or cell populations targeted against alternative, yet undefined, epitopes induced by the vaccination with GAP could be playing an important role in the elicited protection.

**Calreticulin**

Trypanosoma cruzi calreticulin (TcCRT) is a pleiotropic and multifunctional calcium-binding chaperone involved in the quality control of endoplasmic reticulum nascent proteins [138]. After being translocated to the flagellum pocket, TcCRT hijacks C1 and L-ficolin molecules, thus inhibiting the activation of the classical and lectin complement pathways, respectively. Thus, \textit{T. cruzi} has developed through TcCRT, a mechanism for evading the lytic action of the complement in the host blood system, now considered an important virulence factor [139–141].

We generated, using the Gateway technology, mutant parasites with a monoallelic deletion of the TcCRT gene (TcCRT+/-) in the attenuated TCC strain. Western blot assays using TcCRT+/- protein extracts showed a considerable reduction of TcCRT expression levels. The polypeptide TcCRT is most likely essential for parasite viability, as we could not rescue TcCRT–/- null mutants. The monoallelic modification did not alter the morphology of the parasites, but TcCRT+/- epimastigotes showed a significant decrease in the growth capacity in axenic cultures. Moreover, complement-mediated lysis assays using normal human serum revealed that the mutant epimastigotes have a high susceptibility to the lytic action of the complement system, compared with the wild-type strain [124]. We showed that TcCRT+/- mutant parasites displayed a stable loss of virulence and that the presence of parasites could not be demonstrated by molecular methods after a 6-month follow-up period posterior of the infection of BALB/c mice, even after immune suppression of the animals. In contrast, mice infected with the wild-type TCC strain showed evidences of an attenuated, but detectable infection with \textit{T. cruzi} at all times points of the study. Ramirez et al. proposed that \textit{T. cruzi} TcCRT expression promotes C1q coating [142], phagocytic cell chemotaxis and parasite infectivity in the early stages of infection [143]. Thus, the TcCRT monoallelical deletion and, consequently, the reduced expression possibly causes a lower hijack of C1q, thereby inducing decreased pro-phagocytic signals, and diminishes the infectivity of phagocytic cells. Most likely, these properties contributed to the remarkable attenuation of infectivity observed in TcCRT+/- mutants.

To evaluate the protective response of TcCRT mutant parasites, adult BALB/c mice were inoculated intraperitoneally with \(5 \times 10^5\) mutant or wild-type TCC metacyclic trypomastigotes. On day 15, a boost similar to the initial inoculation was delivered. After 120 days, all groups were challenged with \(10^9\) blood trypanosomes of a virulent \textit{T. cruzi} field isolate, recently characterized in our laboratory [144]. Mice immunized with TcCRT+/- and TCC wild-type parasites displayed, after challenge, a significantly lowered parasite density in peripheral blood. Parasitemia curves between wild-type and TcCRT+/- immunized groups were not significantly different. Non-immunized control mice presented high parasitemia and 50% mortality. In the remaining experimental groups, no mortality was recorded. Necropsies at day 60 post infection revealed that mice immunized with mutant parasites showed reduced inflammatory response in heart and muscle tissues, compared to controls. Also, the spleen index was significantly reduced in mutant and wild-type TCC immunized mice [145]. Taken together, these results showed that TcCRT+/- parasites were limited in two major properties conferred by TcCRT and indirectly by C1q: the capacity to evade the host immune response and their virulence status. Consequently, deletion of one copy

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of the TcCRT gene in the attenuated TCC strain resulted in the production of a GAP with strong immunoprotective properties. These results reinforce the possibility of generating transgenic experimental vaccines that combine the immunogenicity of live vaccines and a genetically supported built-in safety modification, currently absent in naturally attenuated parasites.

3-hydroxy-3-methylglutaryl coenzyme A reductase

As mentioned above, the ergosterol biosynthesis pathway is essential for the integrity of the plasma membrane and parasite viability [11]. 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) is a single-copy gene that encodes a mitochondrial key enzyme of the ergosterol pathway. HMGR catalyzes the reduction of 3-hydroxy-3-methylglutaryl-CoA to mevalonate, which turns into precursors useful for the final biosynthesis of ergosterol and essential derivatives [146]. We generated, using the Gateway cloning technology, a mutant strain carrying a monoallelic deletion of the hmgr gene (HMGR+/−) from the virulent Tulahuen strain. Mutant epitopes exhibited lower growth capacity in axenic culture than their wild-type counterpart and also an increased sensitivity to ergosterol biosynthesis inhibitors such as mevinolin. Since mevinolin (and other ergosterol/cholesterol biosynthesis inhibitors) is commonly used as a safe hypcholesterolemic agent in humans, the hyper-susceptibility of hmgr mutants to these drugs constitutes an interesting strategy to eliminate the possible persistence of the GAP without affecting the vaccinated host. Presently, we are characterizing the infectivity and immunoprotective properties of HMGR+/− mutants in murine models [Sanchez-Valdez, et al., Unpublished Results].

Perspectives & challenges for the development of GAP T. cruzi vaccines

Immunization route & vaccine delivery

T. cruzi is not capable of infecting the mammalian host through intact skin, but can invade through lesions in the dermis or mucosal surfaces. The first contact between the parasite and the host triggers the immunological response, and the different ways of infection (cutaneous, enteral or parenteral) will affect antigen capture and presentation to the immune system determining the direction of the protective response. Oral infection has gained renewed interest since the microepidemic outbreak in the Amazon region in Brazil due to consumption of contaminated palm fruit and sugarcane juice [147,148]. Except for the immunization with the ech mutant parasites performed by oral gavage in mice, all the immunization procedures with mutant T. cruzi parasites reviewed here were done by intraperitoneal inoculation. In the particular case of immunization with ech mutants, Collins et al. demonstrated that regardless of the inoculation route (oral or intraperitoneal), the animals develop an extended systemic infection characterized by a strong CD8+ T cell response [117]. A recent study comparing the intraperitoneal versus oral route of infection with the Y T. cruzi strain indicated that the former renders in a higher parasitemia, an increased number of peritoneal macrophages, nitric oxide/IL-12 concentrations and cardiac inflammatory foci. However, no differences in the proliferation of CD4+ and CD8+ T cells were found between both immunization paths [149].

An important issue to consider in the immunization with live attenuated parasites is the effectiveness of the delivery strategies. The prime–boost strategies influence the resulting response dramatically [150,151]. In the last few years, heterologous regimens consisting of priming and boosting with immunogens delivered in different platforms have been successfully utilized. In fact, those heterologous protocols that combine the efficacy of DNA vaccines with recombinant viral or bacterial vectors were successfully used [87,92,150]. So far, we have not found reports of heterologous regimens with live attenuated parasites for T. cruzi. If the protective capacity of a GAP is taken as a gold standard, it would be interesting to combine with DNA vaccine and prime–boost regimens to test whether that standard can be improved.

Inter-specific cross-protection: a pan-T. cruzi GAP vaccine

An important challenge that faces the production of vaccines, in particular, those produced by GAP, is the generation of strain-specific immunity. These vaccines should be able to provide cross-protection against a large number of strains and to people living in different geographic regions, where there is a high genetic heterogeneity among circulating T. cruzi strains. In this context, 17 T. cruzi isolates were collected from insect vectors within a 57,000 km² endemic area of Argentina and used to challenge 17 groups of TCC-immunized or naïve mice. This experiment showed that TCC-immunized mice developed protection against each isolate compared to naïve control, measured by parasitemia in fresh blood mounts, xenodiagnoses and histopathological exam of muscle, heart and urinary bladder tissues. These results demonstrated that at least in this geographic region, immunity induced by TCC parasites is not strain- or isolate-specific [152].

Genome plasticity, stability & safety of the engineered locus

Obtaining parasites with the deletion of a single allele has been, in our experience, a relatively easy task. However, obtaining an absolute double knock-out parasite with both allelic copies deleted and the selection with two different antibiotics is quite laborious. This is possibly due to the essential character of these single copy genes. TcCRT, dhfr-ts and ech1 mutants have experienced, after the deletion of one allele, only a partial reduction in infectivity and growth, indicating that these properties are probably dependent on the remnant allele.

The main objective of the targeted-gene deletion on parasites intended to be used as live vaccines is to provide a defined and stable system in terms of security to avoid the possibility of reversion to the virulent phenotype. However, during the genetic manipulations, our group evidenced some unexpected results, such as possible duplications of the target gene and presence of trisomy, probably related to the genome plasticity of T. cruzi. dhfr-ts mutants showed, after the proper
substitution of both allelic copies, an endogenous extra copy in the parasite genome [123]. Duplication and gene amplification are common phenomena in Leishmania parasites; this may be a compensatory mechanism to prevent the deletion of essential genes [153–155]. However, in T. cruzi, these mechanisms have not been described so far. It would be interesting to learn whether a similar mechanism operates also in T. cruzi and if so, to determine its frequency. In this context, the stability of the engineered locus was studied in TcCRT mutants after a long-term infection in mice. The attenuated TcCRT+/− mutants were re-isolated, 90 days post infection, from immunodeficient mice and the status of the TcCRT coding sequence as well as the antibiotic resistance gene were determined by PCR analyses. This study showed that the mutants not only retained the deletion of one allele but also remained in the original locus. Thus, the mutation was stable, without demonstrable reversion to the wild-type genotype, even after long-term infection cycle in the mammalian host [145]. Similar results using this approach were obtained by Zago et al. for lyt1 mutants [131] and by Basombrio et al. [129] for a gp72 mutant.

A veterinary vaccine

The interruption of Chagas disease transmission could be achieved by a strategy based on the elimination or reduction of the capacity of natural reservoirs to transmit the parasite to the insect vectors, mainly in the endemic areas of disease transmission [156–158]. In this regard, a few protozoan diseases of dogs, such as giardiasis, babesiosis, leishmaniasis and rabies, have been importantly reduced through vaccination [159]. The GAP vaccination would offer a safe and effective alternative for the interruption of the disease transmission by reservoir species. The vaccination of dogs with live attenuated TCC wild-type strain in an endemic area of Argentina reduced the rate of natural T. cruzi infection from 26.7 to 12.3% after 1 year [74]; however, there have been no reported field trial of vaccination with genetically modified T. cruzi. Vaccination of domestic canines has also an importance on its own, as a veterinary tool to protect these animals against a potentially serious pathogenic infection.

GAP transmission to triatomine vector

A GAP vaccine directed at domestic reservoirs may prevent transmission through the triatomine vector. It is important to study the ability of GAP to divide and differentiate into infective forms in the insect gut. In this setting, GAP will probably increase their potential of genetic recombination due to the selection pressure in new hosts. We have evaluated the differentiation capacity of TcCRT+/− mutants by feeding T. infestans with blood artificially infected with mutant trypanostigotes. A clear reduction in the ability to multiply inside the vector was detected for mutants. The proportion of infected bugs was significantly lower in the group of insects fed on blood infected with TcCRT+/− parasites in comparison to insects fed on wild-type parasites. Moreover, the concentration of parasites in feces of each group was different [124]. The TcCRT+/− impaired differentiation in insect vectors, together with the undetectable parasitemia in the mouse blood system could contribute to the reduction of the strain transmission [145]. Interestingly, the differentiation capacity was also evaluated for gp72 mutant parasites, and the same evidence was obtained for this gene. As described above, mutants presented a marked decrease in their ability to multiply in the vector compared to the wild-type strain, as indicated by the proportions of infected bugs and the concentrations of parasites in feces [129].

Expert commentary

T. cruzi is a complex parasite with a life cycle including three stages in animals, humans and in the insect vector. Furthermore, according to paleoparasitological evidence, the parasite has co-evolved with humans since prehistoric times. Attempts to intervene in this strong adaptation with new vaccines and drugs against T. cruzi, without affecting the host, may face unsuspected difficulties. The present lack of effective vaccines, the limitations of chemotherapy treatments and the recent failure of two human clinical trials with ergosterol inhibitors are a clear evidence of this situation.

Obviously, given the large array of vertebrate species susceptible to this infection, eradication of the parasite with immunoprophylactic or pharmacologic tools is extremely difficult, if not impossible. In endemic areas of the Chagas disease, the parasite will be maintained in the wild transmission cycle. Thus, it is important to combine vaccination campaigns with efficient vector control systems due to the possibility that wild triatomines may be incorporated into the domestic transmission cycle [20].

Only seven T. cruzi GAPs have been reported to date as experimental vaccines against Chagas disease. Meanwhile, there have been major developments in the field for leishmaniasis and malaria. The cause of this disparity is probably related to the efficacy of the genetic manipulation causing difficulties for the generation of absolute knock-out T. cruzi in contrast with Leishmania, T. brucei and Plasmodium. This situation is reflected by the presently available large number of T. cruzi monoallelic mutants used as vaccines (5/77). For these reasons, new methods as well as a better understanding of the mechanisms of parasite genetic regulation are necessary. Such progress should provide us with information as to why RNA interference strategies do not work in T. cruzi, leaving gene replacement by homologous recombination as the only method available to achieve the inhibition of protein synthesis. In this regard, the Gateway technology recently used to produce the ech, dhfr-ts, TcCRT and hmgr GAP has proven effective to reduce the time required for gene cloning and has shown to be effective for gene replacement in T. cruzi.

In most of the studies reviewed here, no experimental approaches evaluating the protective immune response (CD8+ T cell, cytokines and lytic antibodies) generated by T. cruzi GAP vaccines were achieved, except for ECH and DHFR-TS−/+ mutant parasites. Thus, a more detailed knowledge is required about the immune mechanisms by which GAP
vaccines, compared to wild-type parasites, induce a protective response and can achieve long-term and sustained protective immunity. An ideal vaccine should elicit and maintain an immune response as robust as the one induced by a *T. cruzi* virulent infection. In addition, this live vaccine should not induce the pathogenic effects caused by persistent parasites. In this sense, the attenuation induced should be carefully analyzed considering that the gene deletion could lead to a loss of the ‘so-desired’ protective immunity since such genetically modified parasites could not be expressing epitopes essential for inducing an efficient immune response. The maintenance of the protective effect is associated with the persistence of the vaccinating parasites. In this context, GAP vaccines should have the capability of surviving long enough in the immunized host to completely activate the immune system. In this regard, it is important to evaluate the time required for the development of a potent protective response. The intervals between immunization and challenge events, as well as the booster doses should be evaluated.

The development of *T. cruzi* GAP vaccines must overcome several challenges before being allowed to be used in clinical trials. Thus, clear definitions must be provided with regard to the following: safety issues related to the risk of reversion to wild-type phenotype or expression of alternatives genes; development of a rapid test to check the engineered locus stability and the possibility of virulence reversion; increased efficacy of production of double or multiple knock-out parasites; suitable inoculation route and prime–boost regimens for induction of a strong and long-term protection; disruption of transmissibility of mutants to vectors; and production of large-scale parasite cultures in serum-free non-toxic culture media.

Field trials of vaccination in dogs are likely to precede human trials due to the high infection risk of these animals and their role as domestic reservoirs of *T. cruzi*.

**Five-year view**

Despite the fact that the GAP genetic mutation can lead to apparently safe immunization methods the possibility of reversion to virulence in the host should not be ruled out. Thus, in the coming years, GAP improvement and optimization will be necessary. Progress in *T. cruzi* genomics will have a significant role in this area. The continued availability of species-specific *T. cruzi* genome sequences and the development of proteomics, transcriptomics and metabolomics research will offer tools for the identification of novel metabolic pathways. Deletion of the suitable gene will produce a next generation of GAP with strengthened safety, virulence attenuation and immune protection. In addition, the production of stage-specific GAP will permit, for instance, the normal development of epimastigote forms (allowing large-scale production of the vaccine), but arrest the intracellular amastigotes or insect trypomastigote development (increasing mutant safety).

Contrary to *Leishmania* centrin mutant, in *T. cruzi*, there are no RNA or protein biomarkers useful to monitor the safety of a GAP vaccine. Using microarray technology to monitor the gene expression in the *Leishmania* mutant, two additional genes, different from the deleted genes, have been found to be down-regulated. The same expression profile was identified in these mutants after infection of mice. The expression level of these two genes can now be used as a biomarker to monitor GAP vaccine safety [160]. Also, in the near future, biomarkers useful to monitor the induction of an effective immune response could be identified by analyzing the expression patterns of peripheral blood mononuclear cells from GAP vaccinated humans [104].

Multi-targeted deletion of genes leading independent biological processes in a single *T. cruzi* parasite will allow the generation of non-virulent parasites bearing multiple defects. This would reduce the potential for reversion to virulent phenotypes, thus increasing vaccine safety. A similar approach was intended for TcCRT and dhfr-ts GAPs. These mutants were developed on the basis of an already attenuated strain in order to reinforce their safety character. In *Leishmania*, the heterologous co-expression of suicide genes has been used to eliminate the parasite from the host and to reinitiate the safety of the immunizing parasites. This is the case for parasites co-expressing the cytosine deaminase and thymidine kinase genes conferring susceptibility for 5-fluorocytosine and ganciclovir [161,162]. These parasites were able to confer protection against a subsequent challenge, with the advantage that they could be eliminated from the host by specific drug treatment. This kind of strategy has not been reported in *T. cruzi*; however, and as mentioned before, we are assessing the use of hmgr *T. cruzi* mutants that could potentially be eliminated from the host by ergosterol biosynthesis inhibitors.

Alternatively, parasites could be manipulated to overexpress immunogenic molecules or adjuvants capable of modulating the immune response. Further, the recent generation of novel genetic manipulation tools, such as improved and easy-to-use *T. cruzi* expression vectors [163], *T. cruzi* artificial chromosomes [164] and the nuclease-mediated gene-targeting technology, CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats) [165] will contribute significantly to the development of this area in the next 5 years.

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Chagas disease is a neglected tropical disease affecting more than 10 million people in the poorest regions of Latin America. For more than 40 years, Chagas disease treatment was based on the use of relatively toxic drugs that produce serious side effects and have restricted indications in adult patients and pregnant women. Chagas vaccine development is being justified by a progressive replacement of the autoimmune hypothesis by the notion that parasite persistence is the main inductor of pathogenesis. Live vaccines are likely to be more efficient in inducing long-lasting protection, but safety issues linked with their use must be addressed. Genetic manipulation techniques, through gene deletion by homologous recombination, have increased the safety of live vaccines. Only seven genetically modified *Trypanosoma cruzi* parasites have been used as immunogens in experimental models of Chagas disease, demonstrating the limited development of this area compared to leishmaniasis and malaria. All *T. cruzi* mutants tested so far (either mono- or biallelic) have displayed some degree of attenuation in their infective capacity. Before undergoing clinical trials, gene-deleted live attenuated *T. cruzi* vaccines will have to overcome several challenges. Prior immunization, with these tools, of intra- or peri-domiciliary mammal reservoirs (i.e., canines) may hinder transmission of the parasite to humans, besides its intrinsic veterinary therapeutic value.

**Key issues**

- Chagas disease is a neglected tropical disease affecting more than 10 million people in the poorest regions of Latin America.
- For more than 40 years, Chagas disease treatment was based on the use of relatively toxic drugs that produce serious side effects and have restricted indications in adult patients and pregnant women.
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Review

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• This paper describes the generation and the phenotypic alterations induced by the deletion of the dhfr-ts gene in a T. cruzi clone.
• This paper describes the biological characterization of a T. cruzi mutant carrying the mononallelic deletion of the calreticulin gene.
This paper describes improved molecular


• This paper describes improved molecular techniques used to generate GAPs.


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