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Review Article

Strategy for the Development of Vaccines Against Chagas Disease

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Abstract

Chagas disease caused by *Trypanosoma cruzi*, is a major public health issue, particularly in the Americas. There are approximately 10 million infected people and more than ten thousand annual deaths due to this parasite. *T. cruzi* infection usually results in parasite persistence in the infected host. The available therapeutic agents have limited efficacy in these chronic infections and also have significant side effects which limit their usefulness. There are limitations to the innate and acquired immune responses to the infection in a non-immunized host. Currently, no vaccine is available for protecting against *T. cruzi* infection, despite considerable research in this area. A safe, effective and reliable prophylactic vaccine should significantly reduce the threat of *T. cruzi* infections and prevent Chagas disease. In addition, an efficient therapeutic vaccine should improve the outcome of treatments. Research indicates that the development of vaccines against *T. cruzi* infection is feasible and highly significant. This article reviews efforts to develop vaccines against Chagas disease. In addition, the authors discuss a new strategy to develop safe and effective vaccines against *T. cruzi* infection.

Introduction

Chagas disease or American trypanosomiasis, is a neglected tropical disease caused by the protozoan *Trypanosoma cruzi* (*T. cruzi*) [1]. Currently, this disease affects about 10 million people in endemic Latin American country and is becoming a significant health issue globally due to immigration to many developed countries [1]. Every year, more than forty thousand new cases occur in endemic areas and about fourteen thousand infants are born with congenital infection [2-3]. Chagas disease causes more than ten thousand deaths and results in over $7 billion in lost productivity and health care costs annually [4].

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The major mode of transmission of *T. cruzi* in endemic countries is through infected insect vectors called triatomine or Reduviid bugs (i.e., *Rhodinius*, and *Panstrongylus*) [2]. These infected bugs usually take blood meals from people at night and then defecate while feeding. Their feces contain metacyclic trypomastigotes. These metacyclic trypomastigotes enter the host through the bite wound or through intact mucosal membranes. Inside the host, the trypomastigotes invade cells, where they differentiate into intracellular amastigotes. Amastigotes multiply by binary fission and differentiate into trypomastigotes, which are then released into the circulation as bloodstream trypomastigotes. When the bugs take blood meals containing trypomastigotes from infected people or animals, the parasites transform to a dividing form called epimastigotes in the insect midgut. Epimastigotes then differentiate into infective metacyclic trypomastigotes in the insect hindgut. *T. cruzi* can also be transmitted through blood transfusions, organ transplantation, oral intake of contaminated foods [5], mother-to-child and laboratory accidents [6-7]. *T. cruzi* infection can occur in more than 150 other animals [2].

In the early stage of *T. cruzi* infection, many infected patients are asymptomatic or have mild symptoms such as fever, swollen lymph nodes, headaches, or local swelling at the site of the bite [7]. After 2 to 3 months, individuals enter the chronic stage of *T. cruzi* infection, where the majority of individuals remain asymptomatic while about 30% develop chronic cardiomyopathy, leading to heart failure or sudden death and about 10% develop an enlarged esophagus or enlarged colon [7]. Current pharmacological treatments are depended on benznidazole or nifurtimox [8]. Antiparasitic treatment is effective if given very early in the course of infection, but has limited effect on chronic infection. Since many patients are asymptomatic after infection, these patients usually miss the window for early treatment. Resistance to these drugs has been reported in treated patients [9]. Recently, a prospective, multicenter, randomized study has been completed. Trypanocidal therapy with benznidazole in patients with established Chagasic cardiomyopathy significantly reduced serum parasite detection but did not significantly reduce cardiac clinical deterioration through 5 years of follow-up [10].

Currently, prevention focuses on vector control by decreasing the numbers of the triatomine bugs that transmit the infection using insecticides and by preventing the contact of these bugs with humans using mosquito nets and improving housing conditions. These measures significantly reduce disease incidence and prevalence in endemic countries [11]. However, in spite of the vector control interventions, a number of triatomine bugs have proven to be difficult to control and a low level of transmission to humans continues. Therefore, a safe, effective and reliable vaccine should significantly reduce the threat of *T. cruzi* infections and prevent the development of Chagas disease. Studies highlight the significant cost-effectiveness of prophylactic vaccine for humans [12]. A therapeutic vaccine that boosts immune responses to combat chronic infection may greatly improve the prognosis for Chagasic patients by increasing treatment efficacy, reducing its duration and cost, or delaying disease progression to advanced stages and heart failure. In addition, an effective veterinary vaccine should provide control of the domestic

reservoir of *T. cruzi* (dogs) in specific geographical regions, contributing to the effectiveness of Chagas disease control programs.

Currently, there is no suitable vaccine against Chagas disease despite considerable research in this area. In this article, we review efforts to develop vaccines against *T. cruzi* infection. Moreover, we also discuss some new techniques and novel strategies to design vaccines protecting against Chagas disease based on work in our laboratory group.

Protective Immunity Against *Trypanosoma Cruzi* Infection

Our knowledge of protective immunity against *T. cruzi* infection is still incomplete. We do not understand the mechanisms why the immune system fails to totally eradicate the parasite once *T. cruzi* infection is established. An improved understanding of the interactions between host immune response and *T. cruzi* infection will provide rational rules for vaccine design and development. In recent years, the research community has made progress in understanding both innate and adaptive immune responses against *T. cruzi* infection.

One striking feature of *T. cruzi* infection is that *T. cruzi* infection usually results in parasite persistence in the infected host. This phenomenon indicates that there are limitations to the innate and acquired immune responses to the infection. Pattern Recognition Receptors (PRR) which recognize Pathogen-Associated Molecular Patterns (PAMPs) such as Toll-Like Receptors (TLRs) 2, 4, 7, and 9, Nucleotide-binding Oligomerization Domain-like receptor (NOD) 1 have been shown to participate in *T. cruzi* recognition by macrophages and dendritic cells [13-18]. In the initial period after infection (days 7 to 10 following infection) innate responses play a key role in containing parasitemia, through the activation of innate immune response pathways leading to the activation of NFκ-B/AP-1 and the productions of microbicidal mediators such as Reactive Nitrogen Intermediates (RNI) and Reactive Oxygen Species (ROS). The production of these microbicidal mediators are enhanced by the action of proinflammatory cytokines (IL-12, TNF-α, and IFN-γ) released by macrophages, Natural Killer (NK), and γδ T cells [19-20].

Following this innate response, acquired immunity mediated by the T-helper 1 (Th1) cell response plays a key role in controlling parasitemia and host survival. IFN- γ secreted by Th1 $CD4^+$ cells induces the activation of phagocytic cells for parasite killing. Th1 lymphocytes also stimulate the appropriate production of antibodies by B cells and the activation of cytotoxic $CD8^+$ T cells. However, the development of adaptive immunity to *T. cruzi* infection is relatively slow with delayed development of *T. cruzi*-specific $CD8^+$ T effector cells [21] which may be explained by diverse factors, including the postulated poor PAMP activity of *T. cruzi* [22]. *T. cruzi* is able to suppress the immune system through the release of several molecules and can promote IL-10 production by dendritic cells favoring its persistence [23]. *T. cruzi* also induces death signaling molecules such as Fas on *T. cruzi* specific $CD8^+$ T cells [24] as well as PD-1 and PD-L1 on $CD4^+$ and $CD8^+$ T cells. The persistent infection causes the senescence of $CD4^+$ T cells [25] and exhaustion of $CD8^+$ T cells [26-27]. Therefore, a prophylactic vaccine should be aiming at early recognition and killing of *T. cruzi* and a therapeutic vaccine should be aiming at counteracting the immune suppressive mechanisms mediated by the parasites and boosting the host Th1 immune response for parasite killings.

Subunit Experimental Vaccines

The early efforts in developing subunit vaccines against *T. cruzi* tried to identify molecules that provided protective immunity against *T. cruzi* infection using sub cellular fractions of *T. cruzi* or purification of *T. cruzi* proteins as experimental vaccines. In sub cellular fractions from epimastigotes (Tulahuén strain), the flagellar fraction gave a partial protection in a challenge experiment [28-29]. The Paraflagellar rod proteins (PAR) purified from epimastigotes were shown to protect mice against a lethal challenge. The protective immunity in mice induced by PAR with adjuvants was associated Th1 response and cell mediated responses induced by PAR was more important than humoral responses in immunoprotection against *T. cruzi* infection [30- 31]. Two cell surface glycoproteins (90 kDa and 72 kDa) were found to have the ability to protect mice against an acute lethal infection from both bloodstream and metacyclic trypomastigotes [32]. Afterwards, several *T. cruzi* proteins were purified by different methods and these molecules demonstrated some levels of protective activities against *T. cruzi* lethal infection [33-36]. The role of IFN-γ in controlling parasite replications and against lethal infection in mice was reported with a *T. cruzi* soluble extract antigen treatment in mice [37].

The application of native antigens purified from *T. cruzi* as experimental vaccines has limitations because it is difficult to obtain sufficient amounts of these antigens to perform the studies. With the availability of *T. cruzi* genome and the modern biotechnology for protein expression, many *T. cruzi* genes can be used to produce a large amount of recombinant proteins which can be further tested for the ability to protect animals against *T. cruzi* infection.

Recombinant proteins of Cytoplasmic Repetitive Antigen (CRA), and Flagellar Repetitive Antigen (FRA) were used to immunize Balb/c mice and the levels of IgG1, IgG2a, IgG2b and IgG3 isotypes were analyzed. CRA-immunized mice produced high levels of all IgG isotypes, mainly IgG3 and IgG1. FRA-immunization elicited only high levels of IgG1 [38]. With co-adsorbed to alum and recombinant murine IL-12, recombinant Paraflagellar Rod proteins (PFR), which are conserved among seven highly diverse strain of *T. cruzi*, induced protective immunity against *T. cruzi* [39].

Immunization with the recombinant trans-sialidase generated a Th2-type immune response while using a plasmid, p154/13 which encodes the same *T. cruzi* trans- sialidase, induced a Th1-type immune response in mice. Simultaneous administration of both p154/13 and recombinant *T. cruzi* trans-sialidase protein also led to a predominant type 2 immune response. Two priming doses of p154/13 harboring the gene followed by booster injections with recombinant *T. cruzi* trans-sialidase protein significantly improved specific type 1 immune response, as revealed by a drastic reduction of the serum IgG1/IgG2a ratio and by an increase in the *in vitro* IFN-γ secretion by CD4 T cells. However, mice immunized with the DNA-priming protein-boosting regimen did not have better survival rate with the challenge as compared to mice with DNA alone, indicating that the boost did not contribute protective immunity against *T. cruzi* infection [40]. Different regions of recombinant proteins from a major *T. cruzi* cystein proteinase, cruzipain, were used to investigate the protective immunity in mice. The N- terminal domain as an immunogen with CpG-ODN as an adjuvant was able to direct the host with better protective immunity [41].

Recombinant protein vaccines have some disadvantages against *T. cruzi* infection. The protection is usually insufficient because

T. cruzi can still establish persistent infection in the immunized animals. *T. cruzi* strains are highly diverse and it is difficult to have a protein vaccine that can confer broad protections cross different strains. In addition, protein vaccines are more suitable for inducing antibody responses and are not efficient in eliciting the cell-mediated immunity that is essential for controlling the intracellular stage of a pathogen. In their favor, recombinant protein vaccines are safe and have been licensed for hepatitis B and human papillomavirus. With some types of adjuvant, some *T. cruzi* protein vaccines have been demonstrated to induce a degree of protective immunity. Even with a moderate protection, immunization with a protein vaccine may still change the outcome of morbidity and mortality of *T. cruzi* infection. Recombinant protein vaccines are more feasible for the population in poor areas to gain some immunizations. Therefore, recombinant protein vaccines Tc24 and TSA-1 formulated on alum together with the Toll-like receptor 4 agonist, E6020, are being tested in patients [42]. Techniques to produce large amount of Tc24 recombinant proteins in *Escherichia coli* or in yeast were reported and the recombinant proteins were tested as experimental vaccines that demonstrated partial protective efficacy in murine models [43].

Another approach termed DNA vaccines has also been developed, using a vector to express the antigen(s) in hosts [44]. The direct introduction of a plasmid containing a DNA sequence encoding *T. cruzi* antigen(s) into appropriate tissues has been tested. An immune response relies on the *in situ* production of the target antigen. This approach offers a number of potential advantages over traditional approaches, including the stimulation of both B- and T-cell responses and improving long-term persistence of immunogen. Moreover, these types of vaccines are easy to produce, store and ship and therefore, the approach is cost effective. However, there are disadvantages of using this approach. It is limited to protein antigens with the possibility that tolerance to the antigens by the hosts may occur because of the persistence of immunogen. In addition, there is a risk of affecting host genes controlling cell growth, leading to the development of malignancy. Autoimmunity is still considered as one of the factors contributing to Chagas disease and the persistent presentation of *T. cruzi* antigens in host tissues may be a concern for possible development of pathology. In spite of these concerns, experimental DNA vaccines against Chagas disease are progressing.

Murine immunization with DNA vaccine using a chimeric gene of *T. cruzi* kinetoplastid-specific KMP11 Protein and Heat-Shock Protein-70 (HSP70), but not the *KMP11* gene alone, induced both IgG2 long-lasting humoral immune response against KMP11 protein and activation of $CD8^+$ cytotoxic T lymphocytes specific for two KMP11 peptides. Mice immunized with the chimeric gene also gained protection against parasite challenge. This study indicates that immunization of animals with antigens fused to heat shock proteins in the absence of an adjuvant elicits antigen- specific immune responses [45]. In addition, HSP70 alone or fused to the KMP11 antigen, as well as a HSP70 fragment, were capable of stimulating the maturation of murine dendritic cells, suggesting that HSP70 may be used as a candidate or as an adjuvant for vaccination [46]. The immunization of mice with the DNA vectors containing the Paraflagellar Rod proteins (PFRs) genes or PFRs-HSP70 fused genes induced high level of IgG2 anti-PFRs. The immunization with the PFR2-HSP70 fused genes triggered a Th1 response profile and protection against *T. cruzi* infection [47]. *T. cruzi* HSP70 was subsequently studied for its role in inducing immunity against *T. cruzi* infection [48-50]. A cDNA clone that codes for an amastigote-Specific Surface Protein (TcSSP4) was used as a candidate to develop a DNA vaccine. Mice immunized with a eukaryotic expression plasmid containing the TcSSP4 cDNA were able to reduce the acute phase of infection and induce a Th1 response, resulting in a better immune protection than that of using a recombinant protein vaccine from the same gene [51]. Similarly, the TcSP gene with DNA-based immunization provided a better protective immunity than that of using recombinant protein [52]. Both DNA vaccines were then further tested in a canine model of Chagas disease. Immunizations with DNA vaccines coding both genes in Beagle dogs reduced the severity of clinical signs of acute infection including electrocardiographic abnormalities [53]. Immunizations with both genes also induced IgG2 immunoglobulin's and IFN-γ productions as well as lymph proliferation, indicating cell mediated immunity. In chronic stage, immunized dogs with DNA vaccines coding both genes challenged with *T. cruzi* still developed myocarditis with a partial protection from the experimental vaccines [54].

The cDNAs for TcG2 and TcG4, which code for membrane-associated GPI proteins of *T. cruzi,* were cloned into eukaryotic expression plasmid pCDNA3.1 to make the experimental vaccine, TcVac3. A DNA-prime/MVA-boost approach in C57BL/6 mice against *T. cruzi* challenge was reported. Vaccination with TcVac3 elicited the production of lytic antibodies against *T. cruzi* and antigen- and parasite-specific CD4/CD8 T cell proliferation. The $CD8^+$ T cells were cytolytic effector cells with predominantly IFN-γ +. In the chronic stage of infection, immunized/infected mice exhibited a significant decline in IFN-γ^+CD8^+T cells with a predominance of immunoregulatory IL-10^+/$CD4^+$T and IL10^+/$CD8^+$T cells, indicating a shift to type 2 cytokine and T cell response which reduce the development of cardiomyopathy [55]. Subsequently, TcVac3 was tested as a therapeutic vaccine in mice infected with *T. cruzi* and demonstrated some therapeutic efficacy in reducing parasite burden and cardiac damages in chronic stage [56].

DNA prime/protein boost vaccine (TcVac2) based on a eukaryotic expression plasmid pCDNA3.1 coding for the cDNAs for TcG1, TcG2, and TcG4 was tested in C57BL/6 mice. Immunized mice demonstrated a significant reduction of parasite burden and myocarditis with the production of lytic antibodies against *T. cruzi* and generation of type 1 $CD8^+$ T cells. The TcVac2 vaccine reduced chronic myocarditis due to the anti-proliferative and anti-inflammatory responses of macrophages [57].

A fragment of *T. cruzi* Amastigote Surface Protein-2 (ASP-2) was cloned into the backbone of Yellow Fever (YF) 17D virus as an experimental DNA vaccine against *T. cruzi*. YF 17D virus has been established as a human vaccine and therefore, using this system to express *T. cruzi* antigen(s) may facilitate the development of *T. cruzi* DNA vaccine for human. A/J mice were immunized subcutaneously with two doses of YF 17D recombinant virus and four weeks after the last dose they were challenged with *T. cruzi*. Mice immunized with recombinant YF 17D viruses expressing *T. cruzi* antigens resulted in a reduction in peak parasitemia, an increase in IFN-γ - secreting splenocytes as well as a reduction in mortality in challenged mice [58].

DNA vaccines based on the pcDNA3.1 plasmid vector and encoding for TSA-1 and Tc24 *T. cruzi* antigens was tested in mongrel dogs. As a preventive vaccine, mongrel dogs were immunized with two doses of 500 g of DNA vaccine with aluminum phosphate as an adjuvant intramuscularly and infected with SylvioX10/4 strain of *T. cruzi* two weeks after the second vaccine dose. As a therapeutic

vaccine, dogs was infected first and then treated with the vaccine. Both approaches of vaccinations did not improve survival rate in dog with *T. cruzi* challenge, indicating that the protective or therapeutic effects by the DNA vaccine were not sufficient. The effect of vaccination on the immune response was weak because it did not observe the induction of a humoral response although an increased level of IFN-γ in immunized dogs was detected. Parasitemia, cardiac inflammation and cardiac parasite burden were reduced by preventive or therapeutic vaccine treatment [59], indicating that the DNA vaccination may be used to reduce *T. cruzi* transmission as veterinary vaccine.

Heterologous prime-boost vaccination using plasmid DNA followed by replication-defective adenovirus vector generates a large number of specific $CD8^+$ T effect or memory cells that provide long-term immunity against a variety of pathogens. Plasmid pIgSPclone9 and human replication-deficient adenovirus type 5 expressing the amastigote surface protein, ASP-2 gene were generated and used to immunize mice. After *T. cruzi* challenge in the immunized mice, these mice showed the increase in the frequency of specific $CD8^+$ T cells and these specific $CD8^+$ T cells were capable of producing simultaneously the antiphrastic mediators IFN-γ and TNF. Mice immunized with this *T. cruzi* virus vaccine had a significant increase in survival rate with *T. cruzi* challenge [60].

Attenuated *Salmonella* carrying plasmids coding for a *T. cruzi* cysteine protease, cruzipain, a *T. cruzi* -released protein related to thiol-disulfide oxidoreductase family, Tc52 and a 24 kDa *T. cruzi* flagellar calcium-binding protein, Tc24 were generated and these bacterial strains bearing the DNA vaccines were administrated to mice orally. Immunization with either individual *T. cruzi* antigen or triple-antigen demonstrated lytic antibodies against trypomastigotes. Mice with the triple-antigen vaccination demonstrated better protection against *T. cruzi* infection [61]. Recently, it was reported that co-administration of cruzipain and GM-CSF DNAs as a new immunotherapeutic vaccine against *Trypanosoma cruzi* infection by intramuscular administration or by attenuated *Salmonella.* Both approaches showed some efficacy in preventing tissue damages in mice [62].

Replication-defective human Type 5 recombinant adenoviruses carrying sequences of amastigote surface protein-2 and trans-sialidase *T. cruzi* antigens were used to immunize mice. Both vaccines were tested as prophylactic and therapeutic vaccines. Prophylactic immunization with both vaccines induced antibodies and specific cytotoxic and Interferon (IFN) γ-producing $CD8^+$ T-cells, reduced acute heart parasitism and electrical abnormalities in the chronic phase. Therapeutic vaccination with both vaccines increased survival and reduced electrical abnormalities [63]. A DNA vaccine termed TcVac4, which includes pcDNA3.1 encoding TcG1, TcG2, and TcG4 antigens plus IL-12- and GM-CSF-encoding plasmids, and glutaraldehyde-inactivated *T. rangeli* epimastigotes were tested in mongrel dogs. Dogs were immunized with two-doses of DNA vaccine followed by two doses of fixed parasites and then challenged with highly pathogenic *T. cruzi*. TcVac4 induced a strong IgG response which was expanded post-infection. Blood parasitemia, tissue parasite burden, electrocardiographic and histological abnormalities were lower in DNA-prime (TcVac4)/*T. rangeli*-boost dogs as compared to dogs given *T. rangeli* or empty plasmid DNA only [64].

In summary DNA vaccines have demonstrated some protection against *T. cruzi* infection in animals; however, the data from dog models indicate that the immune protection from current DNA vaccines is modest. With improvements in *T. cruzi* antigen selection and in expression system constructions, it is possible that DNA vaccine against Chagas disease may eventually become clinically feasible.

Attenuated *Trypanosoma Cruzi* Experimental Vaccines

Early efforts in developing vaccines against *T. cruzi* used heat-killed parasites. Unfortunately, these killed parasites did not elicit protective immunity [28]. A general principle for vaccines is that the more similar a vaccine is to the natural disease, the better the protective immunity that is seen. The success of live-attenuated microorganisms as vaccines against yellow fever, smallpox, measles, rubella, tuberculosis, and other pathogens supports this principle. The efficacy of these vaccines depends on the use of live naturally attenuated organisms and a truly self-limited infection is necessary to attain a strong and long lasting protection. Recently, *Plasmodium* was genetically attenuated and tested as a vaccine and this approach has been successful with this pathogen [65]. In *T. cruzi,* several naturally attenuated strains have been used in immunization-infection experiments in animal models and have provided strong protection [66-67]. In addition, single or double gene deletion to remove virulence or metabolic factors has been used to attenuate parasites and obtain attenuated strains of *T. cruzi*. A range of these genetically altered parasites has been evaluated as experimental vaccines.

One of the two alleles of the calmodulin-ubiquitin gene was disrupted in Tulahuen strain and the mutant strain demonstrated much lower parasitemia in mice as compared to the wild type. A clone of mutant epimastigotes was used to immunize mice and then challenge with wild type Tulahuen strain. The inoculation of mutant parasites in mice demonstrated a strong protection [68].

The deletion of both LYT1 alleles in the CL Brenner strain was achieved and a null mutant *T. cruzi* clone (L16) that showed an attenuated phenotype in culture and in mice. L16 blood counts were almost undetectable but blood-based PCRs indicated the presence of latent and persistent infection during all of the study period. The recovered parasites from infected mice showed that the parasites were lacking the LYT1 gene and still bearing the antibiotic resistance genes indicating the stability of the genetic manipulation. Inoculation with L16 provided a strong protection against a virulent challenge with a cogenic strain [69].

A targeted deletion of the gp72 gene in Y strain was performed and infection with this mutant in highly susceptible BALB suckling mice and immunodeficient athymic mice did not detect blood parasitemia. Only hemocultures from athymic mice in acute infection were able to isolate the mutant parasites. Inoculation with the mutant parasites in mice was able to induce long lasting protection against a subsequent infection by virulent *T. cruzi* [70].

To test the possibility of using attenuated *T. cruzi* for oral vaccination, C57BL/6 mice were given mutant parasites lacking enoyl co-A hydratase gene orally. Oral inoculation of *T. cruzi* established infection in muscles and induced a robust $CD8^+$ T cell response. Moreover, oral vaccination with the attenuated parasites provided protection against wild type *T. cruzi* challenge. Therefore, it is possible to develop live parasite oral vaccines, especially veterinary vaccine for the immunization of reservoir hosts [71]. Monoallelic mutant parasites for the *dhfr-ts* gene in both a naturally attenuated

TCC *T. cruzi* strain and Tulahuen strain were generated. The mutant Tulahuen strain showed significant attenuation with much lower parasitemia in mice as compared to the wild type. Inoculation with mutant Tulahuen provided strong protection against lethal challenge. No obvious differences in the protective effect of TCC wild type versus TCC mutant parasites were observed. Mice challenged with virulent parasites a year after the original infection with the mutant parasites still displayed a significant control over the secondary infection, indicating that immunization with the attenuated *T. cruzi* confer a strong protective immunity with a prolong immune memory [72].

However, despite the promising data of the attenuated live *T. cruzi* vaccines mentioned above, these attenuated strains are still not safe human vaccines because of the danger of reversion of an attenuated strain to a virulent form and the increased virulence of attenuated strains in immune compromised individuals. For example, TCC is a naturally attenuated strain of *T. cruzi* which that was initially thought to be unable to persistently infect immune competent mice was found to cause persist infection in animal models [72]. Therefore, it is important to develop an attenuated vaccine which can provide sterile immunity for the hosts. Also, attenuated vaccines should be evaluated in different animal models to overcome the limitation of murine models.

Novel Strategy to Develop Safe and Reliable Vaccines Against Chagas Disease

Genetic engineering approaches have provided powerful tools for elucidating unknown gene functions and factors involved in gene regulation. Gene targeted replacement by homologous recombination has been one of the most powerful methods for many organisms. This method leads to a potent, safe and well-tolerated *Plasmodium falciparum* live attenuated vaccine [65]. In *T. cruzi,* this method is laborious and time-consuming. Homologous recombination in *T. cruzi* has a very low efficiency and the drug selection process is slow as compared to other kinetoplastids. As a result, only a few mutant strains of *T. cruzi* have been evaluated as live attenuated vaccines.

Recently, the CRISPR-Cas9 system has been established in *T. cruzi* [73]. This system has enabled rapid, targeted modifications of wide range of genomes [73]. The system has proven to be relative ease and high efficiency, and has the ability to achieve multiple modifications in *T. cruzi*. With this system, more genes will be disrupted in *T. cruzi* and genes that mediate stage differentiation will be discovered, which can facilitate the development of vaccine strains. For example, a strain of *T. cruzi,* which can only infect a host but cannot differentiate to another form, will not be able to cause persistent infection in a host and could be used as a live attenuated vaccine to induce protective immunity. To obtain such a strain, new techniques for inducible gene expression in *T. cruzi* are necessary. Our laboratory has recently established techniques that should prove useful for these studies.

Rapid regulation of protein levels can be readily achieved in *T. cruzi*, by expression of a target protein as a fusion with the destabilization domain (ddFKBP) of the "rapamycin binding protein". In the absence of the synthetic legend, Shield-1, proteins bearing this domain are rapidly degraded by the cytoplasmic proteosome. However, protein stabilization can be rapidly achieved by treatment with and reversible binding of Shield-1 to ddFKBP [74]. This method can be used to make essential gene disruption in *T. cruzi* feasible by expressing a copy of a same gene with an inducible form by adding a ddFKBP domain. For example, if an essential gene in *T. cruzi* is knocked out in presence of inducible copy of the same gene, withdrawing the expression of the inducible expression will cause the mutant *T. cruzi* to die. This type of mutant can be used to immunize the animals and induce sufficient immunity without posing a risk.

We have also established another effective inducible system for *T. cruzi* employing the degradation domain based on the *escherichia coli* Dihydrofolate Reductase (ecDHFR). The DHFR Degradation Domain (DDD) can be stabilized by trimethoprim-lactate and can be used to express detrimental or toxic proteins. *T. cruzi* lines with Alpha-toxin, Cecropin A and GFP under the control of DDD with a Hem Agglutinin tag (HA) were developed. Interestingly, amastigotes bearing GFP- DDDHA, Alpha-toxin-DDDHA, Cecropin A-DDDHA and DDDHA all resulted in inducible cell death with these fusions, indicating that DDDHA protein is also detrimental to amastigotes [75]. These strains were found to be attenuated in mouse experiments and infection of mice with these strains provided significant protection against lethal infection with wild type strains. Potentially, these strains may be used as vaccine strains against *T. cruzi* infections and the DDD technique can serve as a bio-safety device to eliminate parasitism. The combination of this approach with other methods such as gene deletion should significantly improve the safety of an attenuated live *T. cruzi* vaccine because we can terminate parasitism in the immunized host by turning on the bio-safety device.

Conclusion

Development of a vaccine against Chagas disease has been delayed due to controversy about the pathogenic mechanisms that underlie disease progression and concerns about the role of autoimmunity in the development of chronic Chagas disease. The genetic complexity of *T. cruzi* and the lack for efficient engineering techniques for gene disruption have also hindered progress in the generation of suitable strains for vaccines. It is clear, however, that prophylactic vaccines will be cost effective strategies for preventing the disease and for controlling the domestic reservoir of *T. cruzi.* Therapeutic vaccines could also play a significant role in increasing treatment efficacy or delaying disease progression.

Recombinant subunit vaccines based on *T. cruzi* antigens are easy to produce and safe. It is convenient to have a variety of combinations of these antigens to improve the stimulation of immune system. However, this type of experimental vaccine has not demonstrated robust protection against *T. cruzi* infection. The history of the development of Malaria subunit vaccines may serve a guide in the outcome of *T. cruzi* subunit vaccines. The most advanced subunit malaria vaccine, RTS, S/AS01 only reduced the rates of clinical and severe malaria acquired over a 12-month period by 31.3 and 36.6%, respectively [65]. However, *T. cruzi* recombinant subunit vaccines may be used as therapeutic vaccines to improve drug treatment outcome due to stimulation of host immune responses. DNA vaccines expressing *T. cruzi* antigens are more likely to stimulate Th1 helper T cells and IFN-γ secretion which are necessary to confer protection against *T. cruzi* infection. However, the protection from this approach is still not robust and the persistent of *T. cruzi* infection in tissue has not been prevented with the DNA subunit vaccines. In addition, there are concerns about the safety with this approach. With the development of new vector system and *T. cruzi* antigens, one day this approach may be useful.

Recently, the success of a Malaria sporozoite vaccine demonstrated a novel approach to produce an attenuated live vaccine [65]. With the advent of inducible protein expression methods and inducible suicide vector systems as well as the CRISPR-Cas9 system, live attenuated

T. cruzi strains as veterinary and human vaccines will be possible in the near future and hold significant promise as vaccines for both prophylaxis and therapy.

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