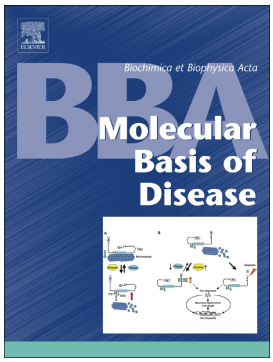


# Search for an efficient Trypanosoma cruzi immune-mediated control

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PII: S0925-4439(19)30389-8

DOI: https://doi.org/10.1016/j.bbadis.2019.165658

Reference: BBADIS 165658

To appear in: *BBA - Molecular Basis of Disease*

Received date: 30 August 2019

Accepted date: 20 December 2019

Please cite this article as: A.E. Bivona, A.S. Alberti, N. Cerny, et al., Search for an efficient Trypanosoma cruzi immune-mediated control, *BBA - Molecular Basis of Disease*(2019), https://doi.org/10.1016/j.bbadis.2019.165658

# Chagas disease vaccine design: the search for an efficient *Trypanosoma cruzi* immune-mediated control

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**Short title: Search for an efficient *Trypanosoma cruzi* immune-mediated control**

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**Abstract**

Chagas disease is currently endemic to 21 Latin-American countries and has also become a global concern because of globalization and mass migration of chronically infected individuals. Prophylactic and therapeutic vaccination might contribute to control the infection and the pathology, as complement of other strategies such as vector control and chemotherapy. Ideal prophylactic vaccine would produce sterilizing immunity; however, a reduction of the parasite burden would prevent progression from *Trypanosoma cruzi* infection to Chagas disease. A therapeutic vaccine for Chagas disease may improve or even replace the treatment with current drugs which have several side effects and require long term treatment that frequently leads to therapeutic withdrawal.

Here, we will review some aspects about sub-unit vaccines, the rationale behind the selection of the immunogen, the role of adjuvants, the advantages and limitations of DNA-based vaccines and the idea of therapeutic vaccines. One of the main limitations to advance vaccine development against Chagas disease is the high number of variables that must be considered and the lack of uniform criteria among research laboratories. To make possible comparisons, much of this review will be focused on experiments that kept many variables constant including antigen mass/doses, type of eukaryotic plasmid, DNA-delivery system, mice strain and sex, lethal and sublethal model infection, and similar immunogenicity and efficacy assessments.

**Keywords:** Neglected disease; Chagas disease; *Trypanosoma cruzi*; Vaccine; Recombinant antigen; DNA-delivery system

## Key issues for the search of Chagas disease vaccines

- Considering the large number of vertebrate reservoirs and triatomine vectors that participate in the life cycle of *Trypanosoma cruzi*, the eradication of Chagas disease seems to be an impossible mission.
- Chagas disease vaccine discovery received a strong boost from the evidence that parasite persistence is the main source of pathology, even though the release of DAMPs produced by parasite-damaged tissue or hidden self-antigens might contribute to the inflammatory process.
- Prophylactic and therapeutic vaccination might contribute to control the infection and pathology, as complement of other strategies such as vector control and chemotherapy.
- The immune response elicited by the natural infection includes several humoral and cellular effector components that are not enough to eliminate the parasite. Vaccine exploration must be focused on the search for unconventional rather than stronger immune responses.
- A deeper knowledge of the host-parasite relationship must be sought to improve prophylactic or therapeutic interventions.
- Ideal prophylactic vaccine would produce sterilizing immunity; however, a reduction of the parasite burden would prevent progression from *T. cruzi* infection to Chagas disease pathology.
- A therapeutic vaccine would be valuable for the treatment of individuals already infected with *T. cruzi*, since the drugs currently in use have significant drawbacks, such as adverse effects, prolonged therapeutic regimens and lack of efficacy in the chronic phase of the infection.

### 1. Introduction

Chagas disease, also known as American Trypanosomiasis, is a chronic parasitic disease caused by the flagellated protozoan *Trypanosoma cruzi*. It is a vector-borne disease, but the parasite can also be transmitted by congenital route, blood transfusions, organ transplantation or by ingesting contaminated food and beverages. It is recognized by the World Health Organization (WHO) as a neglected tropical infectious disease in Latin America where ~8,000,000 individuals are infected (56,000 new infections/year). Further estimates are that Chagas disease is responsible for 10,600 deaths per year in addition to 97,500 years lived with disability (YLDs) [1]. In total, combining years of life lost and YLDs, Chagas disease causes approximately 0.6 million disability adjusted life years (DALYs) [2,3]. Chagas disease is currently endemic to 21 Latin-American countries and has also become a global concern as a result of globalization and mass migration of chronically infected individuals. Thus, Chagas disease is now reported in 19 non-endemic areas including the European Union, United States, Canada, Japan and

Australia (*e.g*. 120,000 and 300,000 infected people are living in Europe and the United States, respectively) [4,5].

*T. cruzi* infection presents two main stages: acute and chronic. The 2-3 months acute phase is usually asymptomatic and goes usually unnoticed, being characterized by high level parasitemia. The chronic phase begins when parasitemia decreases and most of infected people remain asymptomatic throughout life. However, after 10-30 years, about 30-40% of infected individuals develop heart or gastro enteric manifestations that can lead to death. A major shortcoming is that available drugs for treatment (*e.g*. Nifurtimox and Benznidazole, Bz) are only effective in the acute or early infection phases. Furthermore, both drugs display a wide range of side effects (*e.g*. central nervous system toxicity, leukopenia), which lead to therapy discontinuation in 10-30% of treated patients. Efforts are focused on transmission control and the search for more efficient and less toxic drugs, as well as in the development of prophylactic and therapeutic vaccines. However, up to now, no vaccine has been advanced to clinical development [6,7].

During years vaccine discovery against Chagas disease was no promoted due to several reports suggesting that *T. cruzi* antigens would induce autoimmune reactions. Later, it became clear that autoimmune reaction, if any, is a consequence of parasite persistence and to avoid it, the parasite clearance is basic. However, the idea of vaccinating against *T. cruzi* arose almost simultaneously with the discovery of Chagas disease. In 1912, Blanchard showed that the animals that survived the acute phase were resistant to re-infections. Since then, different experimental vaccines have been evaluated: live attenuated parasites, non-pathogenic trypanosomes (i.e. *Trypanosoma rangeli*), non-infectious stages (epimastigotes), dead parasites by physical or chemical methods, subcellular fractions, purified native proteins, recombinant proteins and DNA vaccines [revised in 8,9].

Due to issues related to safety, large scale production or immuno-evasion mechanisms, vaccines based on whole microorganisms have been relegated and subunit vaccines have gained prominence. Here, we will review some aspects about sub-unit vaccines against *T. cruzi*: the rationale behind the selection of the immunogen, the role of the adjuvants, the advantages and limitations of DNA-based vaccines and the idea of therapeutic vaccines.

## 2. Immunogen discovery

An ideal anti-*T. cruzi* vaccine candidate should: a) be highly immunogenic; b) be expressed in all the parasite stages, mainly in those present in the host: amastigote and trypomastigote; c) be conserved and expressed in different *T. cruzi* strains; and d) be a crucial molecule for the pathogen which could constitute a molecular target for neutralizing antibodies. Our laboratory has studied different molecules that fulfill some of these properties. **Table 1** shows the results obtained with different *T. cruzi* antigens

as prophylactic vaccine candidates. Comparison can be made based on several elements that remained constant, including: antigen mass/doses, eukaryotic plasmid for genetic vaccination (pcDNA3.1); DNA-delivery system (attenuated *Salmonella enterica*); mice strain (C3H/HeN); lethal challenge (high virulence parasite strain RA); sublethal challenge (low virulence parasite clone K98 - strain CA1); immunogenicity parameters; and efficacy parameters.

Cruzipain (Cz), the major cysteine protease of *T. cruzi*, exhibits several attractive properties as a vaccine candidate: i. it is highly immunogenic in natural infection; ii. it is present in the three main developmental stages of the parasite in all tested strains; iii. it is a secreted antigen and its ability to cleave immunoglobulins has been proposed as an immunoescape mechanism; and iv. it plays an important role in the process of parasite internalization within mammalian cells [Revised in 10]. As **Table 1** shows, native or recombinant Cz have been successfully used in different vaccination strategies. Upon challenges with *T. cruzi*, mice previously immunized with Cz-based formulations presented a reduced parasitemia (67-99 % of reduction) and/or higher survival (60-100%) compared to non-immunized animals. Most importantly, Cz-based vaccines prevented chronic phase-related damages.

The immune response elicited by these successful vaccines was characterized by the presence of Cz-specific antibodies (IgG2a) which were able to block the infection of cell *in vitro* and also by the production of IFN-γ by T cells [11,12]. Cazorla et al. demonstrated cytotoxic T lymphocyte (CTL) activity against a antigenic peptide (H-2K$^k$ epitope) that belongs to the N-terminal region of Cz [12].

Another important vaccine candidate is Tc52, a highly-conserved glutathione S-transferase which is crucial for the survival of the parasite as the knockout of both alleles is lethal [13]. Tc52 is expressed in all parasite stages, however, the highest expression levels were found in the replicative forms of the parasite (epimastigotes and amastigotes) [14]. Ouaissi et al. [15] demonstrated that purified native Tc52 is able to activate TLR2 therefore this immunogen may present an auto-adjuvant effect. The same authors showed that this molecule formulated with *Bordetella pertussis* and alum was able to confer partial protection against *T. cruzi* infection. Our laboratory has produced recombinant Tc52 in different expression systems and evaluated its performance as vaccine candidate employing different immunization strategies (see **Table 1**). Immunization based on Tc52 also conferred protection against *T. cruzi* challenges. Antibodies anti-Tc52 were able to inhibit the infection of cells in vitro and cause trypomastigote lysis through complement activation. Matos et. al. [16] described different H-2K$^k$ epitopes within Tc52 molecule.

Recently, we have demonstrated that Tc80, a well-described 80 kDa prolyl oligopeptidase, is a potential immunogen to be considered as vaccine candidate. Tc80 is expressed in the extracellular blood trypomastigote and the replicative intracellular amastigote [17]. This secreted enzyme is able to degrade the major extracellular matrix components such as collagen and fibronectin, contributing to the invasion of the parasite to the mammalian cells [18]. Additionally, it was demonstrated that selective inhibitors

for Tc80 were able to block the parasite infection in vitro [19]. Bivona et. al. [20] evaluated Tc80 immunogenicity and its ability to confer protection against *T. cruzi* infection in a murine model. In spite of being little immunogenic in natural infection, the immunization with adjuvanted Tc80 elicited a strong humoral and cell-mediated immune response. Anti-Tc80 antibodies were able to carry out different functions such as: enzymatic inhibition, neutralization of parasite infectivity *in vitro* and complement-mediated lysis of trypomastigotes. Furthermore, spleen cells from immunized mice proliferated and secreted Th1 cytokines (IL-2, IFN-γ and TNF-α) upon re-stimulation with rTc80. Moreover, the stimulation of Tc80-specific polyfunctional CD4 T cells and cytotoxic T cells were also demonstrated. Tc80-based vaccines reduced parasite load in acute and chronic phases, increased mice survival, and prevented chronic phase-related damages.

In the genomic and post-genomic era, new tools for the discovery of vaccine candidates have emerged. Genomic and proteomic data together with bioinformatics tools can be used to find new immunogens. This approach is currently known as 'Reverse Vaccinology' and this terminology was coined and described by Dr. Rino Rappuoli [21]. By analyzing *T. cruzi* gene sequences, Bhatia et al. [22] identified potential immunogens using bioinformatics tools to find out putative new membrane-anchored or secreted proteins. In this way, 8 novel conserved candidates were selected (TcG1-TcG8) and their expression was demonstrated in different *T. cruzi* stages. Some of this immunogen were able to confer protection in different vaccination strategies. DNA vaccines based on plasmids encoding TcG1, TcG2 or TcG4 antigens administered with IL-12 and GMCSF-coding plasmid as adjuvants, induced Th1-skewed immune responses in C57BL6 mice that proved to be protective against a challenge with *T. cruzi* [23]. Moreover, DNA prime/protein boost protocols based on TcG2 and TcG4 induced long lived anti-*T. cruzi* cell immunity. Polyfunctional T effector cells were determined even 180 days post-vaccination [24].

| Immunogen | Adjuvant | N° of doses | Route | Acute phase efficacy | | Chronic damage prevention | | Immunogen-specific immune response | Comments | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Parasitemia reduction | Mice survival (25 dpi) | Serum CK level reduction | Muscle inflammatory infiltrate | | | |
| Purified natural Cz | CpG | 2 | i.m. | 99 % | 100 % | n.d. | n.d. | IgG; IgG2a > IgG1; IL-2; IFN-γ; IL-10; Proliferation | Lethal challenge | [11] |
| Cz DNA[a] | - | 4 | oral | 92 % | 100 % | 80 % | + | Proliferation; IFN-γ | Sub-lethal challenge | [25] |
| Cz DNA[a] | S-GM-CSF | 4 | oral | 84 % | 100 % | 76 % | + | Proliferation; IFN-γ | Sub-lethal challenge | |
| Cz DNA[a] + rCz | CpG | 4 (2 SCz + 2 rCz-CpG) | oral/i.m. | 76 % | 100 % | 78 % | ++ | Proliferation; IFN-γ; DTH | Sub-lethal challenge | |
| Cz DNA[a] + rCz | MALP | 4 (2 SCz + 2 rCz-MALP) | oral/i.m. | 74 % | 100 % | 78 % | ++ | - | Sub-lethal challenge | |
| rCz | MALP | 4 (2 rCz i.d. + 2 rCz-MALP i.n) | i.d./i.n. | 67 % | 60 % | n.d | n.d | IgG; IgG2a > IgG1; sIgA; IFN-γ; IL-10; Proliferation | Lethal challenge | [26] |
| rCz | MALP | 4 (2 rCz i.d. + 2 rCz-MALP i.n) | i.d./i.n. | 73 % | 100 % | 86 % | + | | Sub-lethal challenge | |
| rCzNT | CpG | 2 | i.m. | 72 % | 100 % | 86 % | + | IgG; IgG2a > IgG1; DTH; IFN-γ; IL-10; Proliferation; CTL activity; Antibody-mediated neutralization and complement activation | Sub-lethal challenge; Cz amino terminal relevance. | [12] |
| Tc52NT DNA[a] | - | 4 | oral | 87 % | 100 % | 44 % | + | IgG; IgG2a > IgG1; sIgA; DTH; IL-2; IFN-γ; IL-10; Proliferation; CTL activity; Antibody-mediated neutralization and complement activation | Sub-lethal challenge | [16] |
| Cz, Tc52 and Tc24 DNA[a] | - | 4 | oral | 73 % | 100 % | 81 % | -/+ | IgG; IgG2a > IgG1; sIgA; DTH; IL-12; IFN-γ; IL-10; Proliferation; Antibody-mediated neutralization and complement activation. | Sub-lethal challenge | [27] |
| Tc52NT DNA[a] + rTc52NT | CpG | 4 (2 SNTc52 + 2 rTc52NT-CpG) | oral/i.d. | 85 % | 80 % | n.d. (LDH level reduction 79%) | +/++ | IgG; IgG2a > IgG1; sIgA; DTH; IFN-γ; IL-10; Proliferation; Antibody-mediated neutralization and complement activation | Lethal and Sub-lethal challenge | [28] |
| rTc52 | c-di-AMP | 3 | i.n. | 85 % | 100 % | n.d. | n.d. | IgG; sIgA; DTH; IFN-γ; IL-17; Proliferation | Lethal challenge | [29] |
| rTc52NT | | | | 87 % | 80 % | | | | | |

| Immunogen | Adjuvant | N° of doses | Route | Acute phase efficacy | | Chronic damage prevention | | Immunogen-specific immune response | Comments | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Parasitemia reduction | Mice survival (25 dpi) | Serum CK level reduction | Muscle inflammatory infiltrate | | | |
| Traspain (Chimeric antigen) | c-di-AMP | 3 | i.n. | 86 % | 100 % | 92 % | -/+ | IgG; DTH; IFN-γ; IL-2; IL-17; Proliferation; Antibody-mediated neutralization; CTL activity | Lethal and Sub-lethal challenge | [30] |
| rTc80 | CpG | 4 | i.m. | 69 % | 33 % | n.d. | n.d. | gG; IgG2a > IgG1; DTH; IL-2; IFN-γ; TNF-α; Proliferation; CTL activity; Antibody-mediated neutralization and complement activation | Lethal and Sub-lethal challenge | [20] |
| Tc80 DNA[a] | | 4 | oral | 62 % (89% in sub-lethal challenge) | 80 % | 48 % | ++ | | | |
| Tc80 DNA[a] + rTc80 | | 4 (2 STc80 + 2 rTc80-CpG) | oral/i.m. | 62 % ( 97% in sub-lethal challenge) | 67 % | 59 % | ++ | | | |

**Table 1: Anti *T. cruzi* prophylactic vaccines. Cz**: natural cruzipain; **rCz**: recombinant Cz; **rCzNT**: Cz N-terminal domain; **rTc52**: recombinant *T. cruzi* 52 kDa glutathione S-transferase; **rTc52NT**: Tc52 N-terminal domain; **Tc24**: recombinant *T. cruzi* 24 kDa calcium binding protein; **rTc80**: recombinant *T. cruzi* 80 kDa prolyl oligopeptidase; **CpG**: CpG Oligodeoxynucletide 1826; **MALP-2**: syntetic macrophage-activating lipopeptide from *Mycoplasma fermentans*; **c-di-AMP**: Cyclic-di-AMP; **CK**: Creatin Kinase; **i.m.**: intramuscular; **i.d.**: intradermic; **i.n.**: intranasal; **dpi**: days post-infection; **DTH**: delayed type hypersensitivity reaction; **CTL**: cytotoxic T lymphocyte; **n.d.**: not determined.

[a] DNA-based vaccines were administered orally using an attenuated Salmonella strain as DNA delivery system.

[b] Inflammatory infiltrates were classified as: (-) absence; (+) isolated foci; (++) multiple non-confluent foci. Non-vaccinated mice usually develop multiple confluent foci (+++) or (++++) multiple diffuse foci.

## 3. Adjuvants paradigm

A successful sub-unit vaccine requires not only a good immunogen but also a proper adjuvant. The role of adjuvants has been crucial to increase the immunogenicity of the antigen and orchestrate an adequate adaptive immune response to counteract *T. cruzi* infection. In this regard, first attempts of immunization with Cz and Freund's adjuvant were really disappointing since immunized mice challenged with *T. cruzi* had higher parasitemia and died before control group [Malchiodi, unpublished results].

The discovery that CpG oligodeoxinucleotides (CpG-ODN) are immunoestimulatory motives, derived from the fact that bacterial or viral DNA are different from mammals and can be detected by Toll-like receptor 9 (TLR9). This opened a door for the induction of a Th1 immune response in subunit vaccines needed against many intracellular pathogens [31–33].

Natural Cz from lysed epimastigotes was purified using an immobilized monoclonal antibody [34] and inoculated in mice admixed with CpG-ODN. The elicited Th1 immune response in Cz+CpG-ODN group was characterized by a strong antibody response of IgG2a, in contraposition of the Cz+Alumn group that showed IgG1 antibody response. Cz+CpG-ODN group splenocytes showed strong proliferation after Cz stimulation with high IL-2 and IFN-γ secretion that clearly distinguished the Th1 immune reaction from the immune response elicited by Alumn, a known mostly Th2-inducer adjuvant. A lethal challenge with RA strain trypomastigotes made evident the importance of the Th1 immune response since hundred percent of mice immunized with Cz+CpG-ODN survived until the end of the experiment while 100% of Cz+Alumn immunized mice die by 17 days post infection (dpi), three days after all the control non-immunized mice had died [11]. The role of Cz as main candidate antigen for vaccine against Chagas disease was also indicated by its use in combination with IL-12 and a neutralizing IL-4 monoclonal antibody, that conferred protection when mice were challenged with *T. cruzi* trypomastigotes [35]. These results confirmed that protection require to modulate the profile induced by Cz from a predominantly Th2 to a Th1 profile.

Following experiments showed that the carbohydrates that heavily decorate Cz, are not crucial for the induction of a protective immune response since the recombinant Cz expressed in *E. coli* BL21 was as protective as natural Cz (Malchiodi, unpublished results). However, immune response against carbohydrates might not be disregarded since a recent article showed that an immunogen based on the trisaccharide Galα1,3Galβ1,4GlcNAc (Galα3LN) covalently linked to human serum albumin conferred full protection against a lethal *T. cruzi* challenge [36].

Before challenge, the immunization with Cz+CpG-ODN induced a strong IgG2a antibody response that recognized only Cz by immunoblot in a complex mixture of antigens. Naturally, the IgG1 antibody response induced by Cz+Alumn also recognized only Cz in the immunoblot [11]. After 15 days of the last immunization, all mice were challenged by RA trypomastigotes. Surprisingly, 70-100 dpi, the Cz+CpG-

ODN immunized and challenged mice responded to the rest of the *T. cruzi* antigens with IgG2a antibodies, which indicate that the immune response was Th1 oriented against other than the immunizing Cz, [11]. Similar results were later observed in vaccination protocols employing Traspain, a recombinant Nt-Cz chimeric molecule, formulated with a water/oil emulsion (Montanide ISA51), an adjuvant more associated with a Th2/Tfh response vs a Th1-skewed adjuvant [Sanchez Alberti, unpublished results]. These results rise the question whether a vaccine with an adjuvant able to induce a strong Th1 oriented immune response that would be very protective against an intracellular pathogen, will be dangerous in case of infection with a pathogen that needs a Th2 oriented immune response. These facts deserve to be further investigated.

The Th1 oriented immune response induced by CpG-ODN demonstrated to be protective not only with Cz. The amastigote surface protein 2 (ASP-2) with CpG-ODN provided remarkable immunity, consistently protecting 100% of the A/Sn mice [37]; the *T. cruzi* trans-sialidase (TS), an enzyme with neuraminidase and sialic acid transfer activities, combined with CpG-ODN can induced both mucosal and systemic protective immunity [38,39]; and more recently, Tc52, and Tc80, also protected when combined with ODN-CpG [14,15].

In addition to the stimulation of IFN-γ-producing T cells, the immunization with CpG-ODN induced IL-10 production [11,12,28]. The secretion of IL-10 in a strongly orientated Th1 immune response was surprising considering its anti-inflammatory properties. However, this regulatory component would prevent severe *T. cruzi*-induced disease and a dual-protective role has been described. Moreover, IL-10 limits the immunopathology triggered by the anti *T. cruzi* response [40]. On the other hand, IL-10 would stimulate some protection-related effector mechanisms such as CD8 T cell activation [41]. Additionally, the induction of IL-10 might have contributed to parasite clearance by favoring production of *T. cruzi*-neutralizing antibodies [42] and antibody-dependent cellular cytotoxicity [43], as well as by promoting CD14-mediated phagocytosis [44] and/or NK cell activity [45].

Recombinant Cz was also used in other formulation and inoculation routes, including prime/boost regimen. Cazorla [26] also assessed the synthetic derivative of the macrophage-activating lipopeptide *Mycoplasma fermentans* (MALP-2), an adjuvant able to improve humoral and cell mediated immunity by activation of TLR2/6. The administration of intradermal rCz plus intranasal rCz+MALP-2 proved to be effective to control a systemic intraperitoneal challenge of blood derived trypomastigotes. The elicited immune response in different regimens showed that the intradermal priming with rCz induce IgG1 specific antibodies that can be switched to IgG2a when Cz is combined with CpG-ODN. The switch can be improved by an intranasal boost of rCz +MALP-2, that also twist the immune response to Th1, as demonstrated by the release of IFN-γ, based on an increased number of IFN-γ-producing T cells [26]. The significance of intradermal immunization has been also assessed recently with the candidate vaccine TcVac1. Mice were immunized with TcVac1 through intradermal electroporation or intramuscular

injection and challenged with *T. cruzi*. TcVac1 intradermal induced significantly higher level of antigen-specific antibody response and lymphocyte proliferation after challenge [46].

Since *T. cruzi* natural infection transmitted by reduviid vector happens through skin or eye/lip mucosae when scratched or rubbed, the immune response elicited in mucosal tissues is important. By determining IgA in bronchial lavages, it was demonstrated that a stronger response can be obtained when priming with rCz i.d. and boosted with rCz-MALP-2 i.n., as compared to mice immunized with just rCz-MALP i.n. This difference could be due to the B cells intradermally primed with rCz, that switch from IgG to IgA producers when mice were boosted intranasally with rCz-MALP-2 [26]. The existence of B and T cell in skin and mucosa after i.d. immunization with Cz-MALP may also explain the stronger cellular response observed in the delayed-type hypersensitivity test since the experiment is conducted by i.d. inoculation of Cz in the footpads of the vaccinated mice [26].

Although Th1 responses have been strongly associated with protection against *T. cruzi* infection, last generation adjuvants that induce other immunological mechanisms/profiles have been also able to confer protection. In this sense, vaccines formulated with cyclic dinucleotides such as cyclic di AMP (CDA) were shown to induce protective immune responses with a mix Th1/Th17 profile. Matos et al. have demonstrated the high efficacy of a mucosal anti-*T. cruzi* vaccine based on Tc52 + CDA [29]. In that work, CDA-adjuvanted vaccine induced much more IL-17 secretion than the vaccine based on CpG-ODN. This IL-17 stimulation was correlated with the protective ability of the vaccine since the addition of a pegylated derivate of the α-galactosyl-ceramide, a Th17 cells inhibitor [47], decreases the vaccine efficacy [29]. A similar immunological profile was observed by Sanchez Alberti [30] with a vaccine based on a trivalent chimeric antigen. These results highlight the role of IL-17 during *T. cruzi* infection. In this regard, some authors have previously demonstrated that IL-17 is associated with protection during the acute phase of *T. cruzi* infection [48,49], and it has been recently found that Th17 cells may be more protective than Th1 cells [50].

**4. Antigen refinement**

*T. cruzi* has several escape mechanisms, among them it has decoy antigenic domains to circumvent the immune response against the portion of the antigens with important function for parasite survival. Cz is certainly a key parasite molecule that participate in both host cell invasion and parasite escape of the phagosome to the cytoplasm where it multiply. Cz has two domains: a catalytic N-terminal domain with papain-like activity which can cut the Fc portion of antibodies avoiding complement fixation and antibody dependent cytotoxicity; and a C-terminal extension with unknown function, but it is the major immunogenic domain of Cz in naturally infected humans. Surprisingly, expression of N- and C-terminal domains of Cz to dissect the immune response allowed to demonstrate that this phenomenon is not a property of the parasite but of the molecule itself, because inoculation of the full-length rCz was able to

produce a similar effect to the specific immune response against cruzipain in natural or experimental infection. This escape mechanism could be reversed using only the portion of the Cz with an essential function for parasite survival in the host. Thus, immunization with rCz N-terminal domain used as vaccine demonstrated to induce a low antibody titer but able to efficiently neutralize parasite infection of host cells. Similarly, the cellular immune response was also down-regulated by immunization with the full-length Cz, avoiding the reaction against the N-terminal domain. When the N-terminal portion was used, a stronger DTH response and proliferation index was induced. This N-terminal specific immune response confers better levels of protection in vaccinated mice against a lethal challenge [12].

Cz is not the only antigen with a highly immunogenic region of unknown function that somehow protects an essential domain for parasite survival. Trans-sialidase (TS) is a key *T. cruzi* enzyme since the parasite is unable to synthetize carbohydrates with sialic acid, as mammalian do. Thus, TS is responsible for transferring sialic acid from mammalian to parasite carbohydrates, assimilating them and masking as mammalian protein. This process in intended to avoid recognition and parasite lysis and actively participates in host cell invasion [51,52]. TS has also a decoy portion called SAPA (shed acute-phase antigen), which is made up of 12 tandemly repeated residues on the C-terminus of the molecule. These B cell epitopes act as a diversion for the immune system to concentrate the antibody response against it, thereby preserving the enzymatic activity of the N-terminal domain, which maintain the enzymatic activity in the presence of the strong antibody response against SAPA [53–55]. However, recent studies showed that these mechanisms can be reverted by using optimized immunogens able to redirect host responses to provide enhanced protection [38,56,57].

This “distracting immunodominance” was described not only for antibodies but also for T cell epitopes. In this sense, the highly immunogenic TSKb20 epitope (H2-Kb ANYKFTLV) can represent nearly 30% of $CD8^+$ T cell in *T. cruzi*-infected C57BL6 mice. Although this epitope contributes in some extent to the protection, its absence does not modify the course of the infection [58,59]. In addition, the TS super family is highly polymorphic and is constantly evolving in the parasite, generating antigenic diversity by recombination. The huge number of these proteins has been proposed to generate a “smoke screen” effect, compromising the priming of less dominant or abundant nonvariant epitopes [59]. All these reasons point out that TS might not be the best target for immune intervention.

Considering what has been mentioned so far, sub-dominant antigens or T/B cell epitopes should not be underestimated or neglected for the development of sub-unit vaccines.

Another example of immune evasion at the protein level is observed with the AgTc52. Tc52, which is a *T. cruzi* protein with glutathione transferase activity and immunomodulatory properties, has also two domains: The N-terminal domain that contains the enzyme active site, and a C-terminal domain, whose function is still unknown but could be responsible for some immunomodulatory activities. Immunization with Tc52N-terminal domain conferred greater protection than the C-terminal domain or full length

Tc52 in the acute and chronic stages of infection [16]. Similarly, vaccination with amastigotes surface protein-2 (ASP-2) protects ~65% of highly susceptible A/Sn mice against *T. cruzi* infection [60]. However, immunization with residues 261–500 of ASP-2 induced 100% protection [37].

## 5. Multiple antigens vaccine

The partial success of mono-component vaccines has led to the idea of combining vaccine candidates. Some attempts have been made to use more than one antigen with the aim ofincreasing the breadth of the immune response triggered. TSA-1 and Tc24 encoded in pcDNA3.1 plasmid vector has been combined in a naked DNA vaccine that partially protected against a *T. cruzi* challenge. Despite the use of alum as adjuvant, the authors reported that when administered as therapeutic vaccine, it induced an increase in parasite-specific IFN-γ producing $CD4^+$ and $CD8^+$ T cells in the spleen [61].

More recently, a tri-component vaccine candidate was assayed including Cz, Tc52 and Tc24 encoded individually in pcDNA3.1 plasmid but transported by attenuated *Salmonella enterica* [27]. The use of *Salmonella* has the advantage that can be orally administered, and it generates a strong mucosal immunity. A careful analysis of the sera antibody response against every single component showed that no immunogen precluded the antibody generation against any other. Similarly, intestinal lavages of immunized mice demonstrated the elicitation of specific IgA against every single antigen. As expected for a DNA vaccine, the titer of antibodies was modest, however, they induced significant complement-mediated killing of *T. cruzi* trypomastigotes in vitro and, more interesting, the antibodies were able to inhibit the in vitro invasion of trypomastigotes into mammalian Vero cells [27]. These results are significant because it clearly demonstrated that the quality of the antibody response is far more important than the titer, which correlated with the finding that infected humans who have lytic antibodies have better clinical outcome.

The comparison of antibody response in mice immunized with every single antigen showed that the major contribution to these effects in the tri-component vaccine, was induced by Cz and Tc52, with almost no contribution from Tc24. Similarly, cells from SCz- and STc52-immunized mice released higher levels of Th-1–associated cytokines than controls. In contrast, the level of cytokines produced by cells from STc24-immunized mice was not significantly different with respect to controls. In accordance with these results, the cytokine levels in the group receiving the multicomponent vaccine produced a strong release of IFN-γ and IL-12 upon Cz and Tc52 in vitro re-stimulation, whereas no differences were found upon Tc24 stimulation [27].

Both the low antibody title and the anti-inflammatory interleukins elicited by Tc24 immunization correlated with the low efficacy of this component against a sublethal challenge with *T. cruzi* trypomastigotes. On the contrary, the multicomponent vaccine as well as the single Tc52 and Cz

resulted in significantly decreased parasitemia, as compared with control. However, the analysis of the parasitemia and survival comparison of the tri-component vaccine with a bi-component excluding Tc24, showed no difference, which allow no conclusive decision whether Tc24 contribute to protection or not in this model [27].

Considering the complexity of *T. cruzi* infection and the immunogenicity profiles and efficacy of mono-, bi- and tri-component vaccines discussed, vaccines based on a single antigen are not likely to be able to confer an adequate level of protection. A multiantigen approach, which targets several metabolic and pathogenic mechanisms might result in better protection. In addition, a combination of domains from various antigens in a unique tailored molecule would result in a production costs reduction. In this sense, Traspain, a chimeric antigen including the N-terminal domain of Cruzipain (Cz), the central region of Amastigote surface protein 2 and a subdominant region of an inactive trans-sialidase was recently developed [30] This trivalent immunogen was designed considering a series of different criteria. First, Cz was chosen based on its protective capacity previously reported. It has several characteristics that make it appealing for incorporation in an anti-*T cruzi* multivalent vaccine. It is the major cysteine protease of the parasite and it is expressed in all stages. In the mammalian stages it has been shown to be located in the plasma membrane of amastigotes [62] and to be secreted by trypomastigotes forms. In spite of being part of a polymorphic gene family (~100 genes) its isoforms are highly similar at the amino acid levels [63]. Only the N-terminal domain was incorporated in the design whereas the highly immunodominant C-terminal domain was excluded as it was previously reported that distracts antibody responses [12].

Secondly, the sequence from iTS was selected based on the α-helix structure that it adopts in the native conformation of the enzyme. In Traspain, it was incorporated as a natural linker that may act as a molecular ruler in order to maintain both domain with spatial separation. This region did not include the highly dominant TS epitope TSkb20 (ANYKFTLV). Interestingly, it was shown to contain subdominant CTL epitopes that were primed upon Traspain vaccination. As previously stated, TS is part of one of the largest super families in *T. cruzi* with thousands of genes expressed at the same time on parasite surface that may generate a “smoke screen” effect in the immune response. Thus, only this small 25 amino acids region was incorporated to the final construction. Finally, the ASP2 central region was incorporated based on its protective properties, its location within the parasite membrane and expression pattern, a protein exclusive produced by the intracellular stage of the parasite [64,65]. It represents a potent target for the CTL response [66,67]. Vaccination with this chimeric antigen triggered an immune response that proved to be directed against both main domains in a similar fashion when T and B cell responses were analyzed. This fact highlights the lack of immunodominance by any of the regions that were included.

When Traspain was formulated with c-di-AMP (STING agonist) as a novel mucosal adjuvant a robust humoral and cellular immune response was obtained. Interestingly, the profile was associated with a TH1/TH17 skewed immune response with priming of CTL that showed degranulation and cytotoxicity activity in vivo upon antigen recall [30]. This immune response was later associated with protection at different time points of infection. In that way, vaccine efficacy was tested during the first days of infection upon challenge with tdTomato-expressing CL parasite in mice footpad. *In-vivo* animal imaging revealed a decrease in parasite replication in Traspain vaccinated mice in comparison with placebo groups. This prompt control was then associated with a decrease in blood parasites during the acute phase of infection and an increase in survival rate. Sub-lethal models were employed to assess chronic phase of infection and analyze tissue damage, a key outcome of interest for an anti-*T. cruzi* vaccine. These assays demonstrated the benefits of vaccine induced immunity in ameliorating pathology in target tissues like skeletal and cardiac muscle.

Traspain was recently employed in a mucosal prime-boost protocol employing different adjuvants and Ag formulations [Sanchez Alberti 2019, under revision]. Interestingly, CDA outperformed CpG in the subunit vaccine model administered for boosting a DNA-primed immunity. In correlation with previous results, the presence of Ag-specific Th17 immune response was associated with protection levels. Moreover, multiparametric flow cytometry analysis revealed key differences in the T cell response of vaccinated animals from groups with different levels of protection. Thus, highly polyfunctional $CD4^+$ and $CD8^+$ T cell responses were associated with a better outcome in terms of acute and chronic phase protection. Correlates of vaccine-induced protection are still missing in the field. However, measurement of T cell polyfunctionality appears to be a promising readout to be taking into account in the definition of this matter.

The major aim of a prophylactic vaccine is to prevent infection by conferring sterilizing immunity. However, parasites can still be detected in Traspain vaccinated mice as well as in all vaccines that have been proposed so far. This issue has raised questions from researchers of the field regarding the utility of a non-sterilizing immunity conferred by prophylactic vaccine. On the other hand, we and others believed that vaccine-induced immunity, if functional and robust enough, could be efficient to generate a prompt control of parasite replication. Thus, decreasing parasite load to a certain threshold might help to sustain an equilibrium between the infected host and the parasite so that the development of chronic disease would be finally prevented. This no-sterilizing immunity should be the goal for this kind of intervention.

## 6. Genetic vaccination

Recombinant proteins have revolutionized the vaccines history. However, soluble antigens need a very active cross presentation to generate a strong $CD8^+$ T cells response, a necessary effector mechanism against *T. cruzi* [68,69]. In the search for effective vaccines against Chagas disease, DNA vaccination appears as alternative to recombinant proteins. DNA vaccines are based on the administration of a plasmid containing an antigen-encoding sequence within an expression cassette. Therefore, once this DNA construction reaches the nucleus of an antigen presenting cells (APC), the own host cell can produce the immunogen. As proteins are synthesized in the cytosol, there is a greater probability of processing and presenting antigens through MHC class I molecules. Therefore, these kind of vaccines favors the activation of $CD8^+$ T cells. It has also been shown that DNA vaccines activate B and $CD4^+$ T cells.

Diverse approaches have been carried out to make it possible, such as immunizing directly with a plasmid containing the gene of interest in a eukaryotic expression cassette or delivering the transgene with a bacterial or viral vector.

**6.1. Naked DNA vaccines**

When a plasmid encoding TS catalytic domain (pTS) was used for immunizing BALB/c mice, it evoked humoral and T-cell mediated immune responses. Immunogen-specific antibodies were able to inhibit enzymatic activity of TS *in vitro*. This immunization protocol also generated a significant DTH response, and after a lethal challenge with *T. cruzi*, the immunized group presented a 100% reduction in the peak of parasitemia compared with the control group [70].

Afterwards, pursuing the development of central memory T cells (TCM cells) and the improvement in the duration of protection, the pTS was co-administrated with a plasmid encoding IL-15. Consistent with the known effect of IL-15 over T cell survival and homeostatic proliferation, a significant enhancement in the long-term protective immunity against *T. cruzi* was observed. Regarding to the immune response, significantly higher levels of $CD4^+$ Th1 cells and TS-specific IFN-γ producing $CD8^+$ T cells with improved proliferative capacitive after restimulation were detected when compared with the group vaccinated with pTS alone [71].

Many other attempts have been made including naked-DNA prime and recombinant protein boost regimes using different antigens and in therapeutic approaches that will be later described. However, it may be hard to implement the translation to human vaccine development because naked DNA vaccination is extremely inefficient since high dosages and multiple administrations are needed.

Bacterial or viral vectors as DNA-delivery system of foreign antigens have many advantages including that there is no need of DNA purification. In addition, the bacterial or viral carrier also acts as natural adjuvant by the presence of pathogen-associated molecular patterns (PAMPs) that recruit and

activate APC through the stimulation of pattern recognition receptors (PRRs). The advantage and disadvantages of genetic vaccination systems are depicted in **Table 2**.

**Table 2. Genetic vaccines comparison**

| | ADVANTAGES | DISADVANTAGES |
|---|---|---|
| **NAKED DNA** | High stability.<br>High MHC I presentation and $CD8^+$ response. | Limited to protein antigens.<br>Inefficient, multiple administrations and high doses are needed. |
| **BACTERIAL OR VIRAL DELIVERY SYSTEM** | High MHC class I presentation and $CD8^+$ response.<br>No need of DNA purification<br>Specific targeting of antigen presenting cells.<br>Active infection mechanism.<br>Adjuvant effect *per se* (i.e. PAMPs as CpG motifs naturally presented in bacteria genome activates TLR 9).<br>Needle-free immunization (in case of oral immunization). | Limited to protein antigens.<br>Development of immune response against vector.<br>Pre-existing immunity against vector.<br>Revertance risk. |

### 6.2. Bacterial delivery system

Prokaryotic vectors transformed with eukaryotic expression plasmids emerged as an interesting alternative to naked DNA immunization. Thus, attenuated *Salmonella enterica* serovar Typhimurium *aroA* SL7207 was used to carry Cz gene (SCz) [72]. Bacteria were orally administrated in C3H/HeN mice in combination with *Salmonella* carrying a plasmid encoding granulocyte-macrophage colony-stimulating factor (SGM-CSF). SCz oral doses generated a strong mucosal response characterized by Cz-specific sIgA and T cells in GALT, but a weaker systemic response when compared with SCz+rCz-CpG in terms of serum IgG levels, splenocyte proliferation, IFN-γ secretion and DTH. Surprisingly, the groups that had a weaker systemic response were also able to control the infection in a challenge with *T. cruzi,* presenting even greater parasitemia reduction than the heterologous prime/boost protocols [73].

The attenuated *Salmonella* was similarly tested carrying a plasmid encoding full-length Tc52 (STc52), its N-terminal or C-terminal domains. *Salmonella* carrying N-terminal (SNTc52) conferred the maximal protection of these three groups, showing similar results to SCz. However, the reduction in chronic tissue damage was not as promising as in SCz group. The immune response was characterized by sIgA, $CD4^+$ Th1 bias (high IgG2a titer and IFN-γ producing cells) and IFN-γ plus cytotoxic effect of $CD8^+$ T cells. Furthermore, IL-10 producing splenocytes were detected for this group, which could both down-regulate $CD8^+$ cytotoxic activity and/or prevent tissue damage. Considering the low Tc52 expression in

trypomastigotes, it was surprising the antibodies' ability to mediate complement-dependent cytotoxicity and inhibition of the invasion *in vitro* [16]. Later, a prime-boost scheme (Tc52PB) containing two SNTc52 oral doses and two rTc52 N-term intradermal doses (rNTc52) adjuvanted with CpG-ODN was compared with four doses of SNTc52 and with four doses of rNTc52. Even when using the same antigen, the immune response was diverse, finding the heterologous prime/boost as the best protocol due to its ability to induce strong humoral and cellular responses. This, plus the specific mucosal IgA produced, conferred good protection in acute and chronic phases of infection [28].

Encouraged by the results and benefits of the needle-free *Salmonella* DNA-delivery system, a vaccine candidate based on three components was assayed combining SCz, STc52 and *Salmonella* carrying a plasmid encoding Tc24 [27]. As described under the title **Multiple antigens vaccine**, the three components vaccine gave excellent results.

More recently, different immunization protocols with Tc80 were analyzed, including one with *Salmonella* carrying Tc80 gene (STc80), one with recombinant Tc80 (rTc80) adjuvanted with CpG-ODN and one combining both in a DNA prime/ protein boost regime (Tc80PB). Both protocols including the prokaryotic DNA delivery system overcame the subunit vaccine protocol in terms of acute mice survival. However, in Tc80PB group, halving the number of doses of both STc80 and rTc80 affected cellular and humoral response, respectively. It is remarkable the ability of STc80 to induce polyfunctional $CD4^+$ IFN-$\gamma^+$ TNF-$\alpha^+$ T cells. These polyfunctional cells presented significantly higher cytokine production than monofunctional cells [20].

Simultaneously, a different but interesting vaccine prototype has been designed and evaluated as a proof of concept. It consists of *Lactococcus lactis* bacteria with the ability to produce a specific fragment of *T. cruzi* TS and the cyclase enzyme CdaA, responsible to produce c-di-AMP [74], a previously proved adjuvant for Chagas disease vaccines [30].

### 6.3. Viral delivery system

Clinical trials of vaccines for tuberculosis, malaria, acquired immunodeficiency syndrome (AIDS) and others, have shown unprecedented cellular immune response against the recombinant proteins encoded by viral vectors [7,75]. Chagas disease has not been the exception and several antigens have been assessed as vaccine candidates being delivered by these kinds of system.

Considering the unsurpassed ability of replication-deficient human type 5 recombinant adenoviruses (rAd5) to generate Th1 biased immune response, Machado *et. al*. [75] developed two vectors encoding *T. cruzi* trans-sialidase (AdTS) and amastigote surface protein-2 (AdASP2). The second one, or a formulation combining both, was the best alternative assayed in terms of protection [75].

Five years later, the same group further characterized the protective long-lived $CD8^+$ T cells in a prime boost regime consisting in one dose of naked DNA encoding ASP-2 (pASP2) and one dose of rAdASP2 (ASP2PB). They found that the number of specific cells and *in vivo* cytotoxicity were similar at 14 or 98 days after immunization. Besides, the T cell phenotypes were quite similar, displaying a T effector (TEF)-like phenotype that subsequently changed to a TE memory (TEM)-like phenotype. On the other hand, they did not observe TCM-like cells, reflecting that ASP2PB delays or even blocks this T cell differentiation program, such as occurs after *T. cruzi* challenge [76]. It is not a minor aspect since TEF and TEM cells are considered the most protective phenotypes against this parasite [77,78].

Following this line, Barbosa *et. al.* [79] developed other prime-boost regime combining a first dose with a recombinant influenza virus encoding for either a polypeptide from the medial portion of ASP2 (TEWETGQI, immunodominant H-$2K^k$ epitope) with a rAdASP2 boost. It showed protection in C3H/He mice challenge, where most of $CD8^+$ T cells were polyfunctional (IFN-$\gamma^+$ $CD107a^+$ or IFN-$\gamma^+$ TNF-$\alpha^+$ $CD107a^+$) and immunized mice also presented a high frequency of TEWETGQI-specific $CD8^+$ T cells. Taken together, their results suggest that ASP2-specific subpopulations of $CD8^+$ T cells elicited after immunization could be directly related to the degree of protection generated [79]. Despite these promising results, viral vectors such as Ad5 could present a non-minor problem in the translation to human vaccine development due to the pre-existing immunity against the vector. This kind of issue should be considered and carefully select the vector.

Yellow fever (YF) 17D vaccine, based on an attenuated virus, is one of the most well-established human vaccines. Nogueira *et. al.* [80,81] used YF 17D backbone to express *in vivo* the immunodominant TS peptide TEWETGQI. Unfortunately, this single *T. cruzi* peptide immunization was not protective, however, the introduction of a larger fragment of ASP-2 containing TEWETGQI showed partial protection of challenged mice [81], supporting the idea of increasing the breadth of the immune response. This recombinant viral strategy deserves to be further explored. However, if we considered that there is an overlap between Chagas disease endemic area and America YF endemic region, where most people have already been YF17D-vaccinated, the above-mentioned preexisting anti-vector immunity problem might also tackle this platform in the field.

## 7. Therapeutics vaccines

A therapeutic vaccine for Chagas disease may improve or even replace the treatment with current drugs (Benznidazole or Nifurtimox) which have several side effects and require long term treatment that frequently leads to therapeutic withdrawal. Finding alternatives for controlling pathology could provide health benefits, socio-economic savings under a wide range of conditions, regardless of the impact of vaccination on reducing clinical progression of the disease [82].

To achieve improved control or parasite clearance, therapeutic strategies, as we saw in prophylactics vaccines, should focus upon mode of administration, antigen selection, adequate immune stimulation and the potential combination of therapeutic vaccines with antiparasitic drug therapies. Different antigens that were effective as prophylactics vaccines, were evaluated during the acute or chronic phases of experimental *T. cruzi* infection. That is the case of de la Cruz et. al. [57] that proved a recombinant protein-based vaccine with different adjuvants including monophosphoryl lipid A (MPLA), glucopyranosyl lipid A (GLA, IDRI) and E6020 (EISEI, Inc). TSA-1 with the TLR-4 agonists reduce parasitemia in BALB/c mice treated during acute phase, relative to rTSA-1 alone and with MPLA showed low parasite burden, high level of TSA-1-specific IFN-γ and IFN-γ/IL-4 ratios with an important reduction in cardiac tissue inflammation.

Similarly, Barry et. al [83] tested Tc24 in a mouse model of chronic *T. cruzi* infection and found that vaccinated mice had lower levels of parasites in their body and less damage to their hearts. Recombinant Tc24 vaccine with a stable emulsion containing E6020 as an immunomodulatory adjuvant showed a reduction in systemic parasitemia and a reduction in cardiac fibrosis and inflammation in vaccinated mice compared to control mice. The reduction observed in the Tc24+E6020-SE vaccinated group, in cardiac fibrosis is evidence of therapeutic efficacy in a mouse model of chronic *T. cruzi* infection [83].

Successfully therapeutic vaccines in mice are mostly based in DNA constructs expressing a relevant antigen, however, DNA vaccines need to be enhanced with a good adjuvant that promote Th1 response. A comparative evaluation of therapeutic DNA vaccines encoding various *T. cruzi* antigens was performed by Sanchez-Burgos [84]. Infected ICR mice were treated during acute phase of the infection with plasmid DNA encoding *T. cruzi* antigens TSA-1, TS, ASP-2-like, Tc52 or Tc24. Treatment with Tc52 DNA reduced parasitemia, as well as cardiac parasite burden and improved mice survival, although treatment with plasmid encoding TS and/or ASP-2-like antigens had no significant effect on parasitemia survival, or myocarditis. In the case of treatment with plasmids encoding Tc24 and TSA-1 presented the most protective efficacy.

A reduction in heart injury was also obtained with a therapeutic vaccination with the ASP2 sequences in Type 5 recombinant adenoviruses (rAdASP2) and transialidase (rAdTS) [85]. The adenovirus based vaccine rAdVax therapeutic vaccination in a homologous prime-boost protocol also preserved specific IFNγ-mediated immunity but reduced response to polyclonal stimuli, CD107a+ CD8+ Tcell frequency and plasma nitric oxide levels. The reprogramed immune responses, in heart tissue and systemically, induce IFNγ production and decrease cytotoxic activity, NOx production and iNOS/NOS2 expression in *T. cruzi* infection. These cytokine profiles contribute with the immunotherapy of C57BL/6 mice during chronic infection decreasing *T. cruzi* persistence in blood, not only delaying progression but also reversing

already established chronic cardiomyopathy. Increased levels of CK-MB activity in serum, was also reversed by rAdVax immunization of chronically infected mice.

Enhancement of antioxidant status was the alternative treatment evaluated by Gupta et. al. [86] to treat chronic Chagas disease. C57BL/6 infected mice were immunized with TcG2/TcG4 vaccine delivered by a DNA-prime/Protein-boost (D/P) approach. Parasite persistence was arrested by the therapeutic delivery of D/P vaccine; and glutathione peroxidase (GPx1) over-expression provided additive benefits in reducing the parasite burden, inflammatory/oxidative stress and cardiac pathology.

Parasite proteinases are interesting chemotherapeutic targets due to the possibility of selective blockage of key functions performed by these molecules in the parasite life cycle and in the host-parasite relationship. The major cysteine proteinase of *T. cruzi*, Cz, is a good example of it and after analyzing it as a prophylactic vaccine we investigated the therapeutic efficacy of a DNA vaccine based on Cz [87]. The treatment was not only evaluated during the acute phase of the infection but also in the advanced chronic phase. The administration of naked Cz DNA as therapeutic vaccine administered intramuscularly as well as the oral administration of attenuated *Salmonella* as Cz DNA-delivery system was evaluated. The treatment during both phases of *T. cruzi* infection were able to reduce blood parasites and prevents tissue pathology in C3H/HeN mice, as assessed by a reduced level of serum enzyme activity characteristic of tissue damage, and a normal target-tissue status (**Table 3**).

The coadministration of a plasmid encoding GM-CSF improved vaccine performance, indicating that the stimulation of innate immune cells is needed in the event of an ongoing infection. GM-CSF co-administered with Cz contributes to the early generation and persistence of Cz specific IgG antibodies, particularly IgG2a, in both naked and *Salmonella*-delivered DNA approaches, addressing the response to a protective and sustained Th1 biased profile not only against Cz but also against a variety of parasite antigens. These results suggest that immunotherapy with Cz and GM-CSF DNAs, either alone or in combination with other drug treatments, may represent a promising alternative for Chagas disease therapy [87].

| Immunogen | Adjuvant | N° of doses | Route | Acute phase efficacy | | Chronic damage prevention | | Immunogen-specific immune response | Challenge | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Parasitemia reduction | Mice survival (25dpi) | Serum CK level reduction | Muscle inflammatory infiltrate | | | |
| Cz nDNA | GM-CSF | 2 (in acute phase) | i.m. | 72% | 100% | 85% | + | IgG; IgG2a > IgG1; Proliferation; DTH; IFN-γ | Lethal | [87] |
| Cz nDNA | GM-CSF | 2 (90 y 100 dpi) | i.m. | n.d. | 100% | 76% | ++ | | Sub-lethal | |
| Cz DNA | S GM-CSF | 2 (in acute phase) | oral | 43% | 75% | 84% | + | | Lethal | |
| Cz DNA | S GM-CSF | 2 ( in chronic phase) | oral | n.d. | 75% | 73% | ++ | | Sub-lethal | |
| Cz + Chg DNA | S GM-CSF | 2 (in acute phase) | oral | 65% | 75% | 91% | + | IgG; IgG2a > IgG1; Proliferation; DTH; muscle qPCR | Sub-lethal | Cerny, unpublished |
| Cz + Chg DNA | S GM-CSF | 2 (in chronic phase) | oral | 65% | 75% | 85% | +/++ | | Sub-lethal | |

**Table 3: *Anti T. cruzi* therapeutic vaccines. Cz**: natural cruzipain; **nDNA**: naked DNA; **CK**: Creatin Kinase; **i.m.**: intramuscular; **i.d.**: intradermic; **i.n.**: intranasal; **dpi**: days post-infection; **DTH**: delayed type hypersensitivity reaction; **CTL**: cytotoxic T lymphocyte; **n.d.**: not determined.

[a] DNA-based vaccines were administered orally using an attenuated *Salmonella* strain as DNA delivery system.

[b] Inflammatory infiltrates were classified as: (-) absence; (+) isolated foci; (++) multiple non-confluent foci. Non-vaccinated mice usually develop multiple confluent foci (+++) or multiple diffuse foci (++++).

Considering the benefits of multiantigen vaccination described previously, as an alternative, Cz was evaluated coadministrated with chagasin (Chg), a natural Cz Inhibitor identified in a group of protein able to block the proliferation of both extracellular epimastigotes and intracellular amastigotes and also arrest metacyclogenesis [88,89]. A multicomponent therapy might induce a robust parasite-specific immune response even in late infection, controlling tissue damage and preventing Chagas disease progression. Effectively, DNA therapeutic vaccine based on the combination of Cz and Chg, induce a specific balanced immune response with a higher production of IFN-γ in the splenocytes, effective to decrease blood and tissue parasites and cardiac damage [Cerny et al. unpublished results]. An immunotherapeutic vaccine candidate based on *Salmonella* as delivery system of the DNAs encoding both parasite antigens (SCz+SChg+SGM-CSF) administrated during acute and chronic phase was able to improve the protection obtained by each antigen as mono-therapeutic vaccines in C3H/HeN mice.

With the aim to reduce the dose of Bz, is important the study of combined chemotherapy. In that sense, it was shown that monotherapy with Bz achieved RT-PCR conversion of *T. cruzi* in all subjects on treatment by 30 days, which was sustained for at least 1 year (STOP CHAGAS) [90]. It was the case of Jones [91] that analyzed the effect of combining a low-dose Bz with a recombinant vaccine candidate, Tc24 C4 + E6020. This immunotherapy regimen induced a robust parasite-specific balanced Th1/Th2 immune response and significantly reduce blood and tissue parasite burdens.

Instead of a multicomponent vaccine, the used of a chimeric antigen is a good alternative, as it was demonstrated for Traspain [30]. Immunotherapy with Traspain was also evaluated during the chronic phase of the disease followed by benznidazole treatment. The co-administration of Traspain with Bz elicited an increase in antigen-specific T cell-functionality which was later associated with a better outcome during the chronic phase of the infection [Sanchez Alberti, unpublished results]. Because *T. cruzi* has evolved many unique and clever mechanisms to evade, modulate and even exploit host immune responses, to develop a highly efficient and safe therapeutic vaccine remains a challenge. As in prophylactic vaccines, it is important to unify the procedures to have a collaborative growth in this area. The prominence of therapeutic vaccination, in conjunction with Bz treatment for indeterminate and chronic Chagas disease, is not only to reduce the number of patients experiencing worse cardiac outcomes but also would be an excellent tool to avoid congenital transmission.

## 8. Nanoparticles vaccines

Nanoparticles have several and relevant effects on the immune system like increasing dendritic cells uptake, MHC antigen presentation, Th1 oriented response with induction of potent CD8 T cells, plus an important depot adjuvant characteristic and slow antigen release. However, little has being explored the use of nanoparticles in the prevention or treatment of Chagas disease. The combination of poly (lactic-

co-glycolic acid) nanoparticles (PLGA) with encapsulated Tc24 and CpG-ODN as vaccine candidate, induced a Th1/Th2 balanced immune response when the IgG2a/IgG1 and IFN-γ/IL-4 relations were analyzed. However, it is surprising that when Tc24 specific $CD8^+$ T cells were analyzed, the Tc24 in PLGA particles without CpG-ODN induced 500% more response than when the adjuvant was present. The vaccine candidate group was the only one that partially controlled parasitemia when administrated 7 days after infection, which is a model of treatment while acute infection [92].

## 9. Economic impact of a vaccine for Chagas disease

Vaccines as control strategy for Chagas disease not only would have an important impact regarding to public health but also it would be advantageous in economic terms. It has been estimated that Chagas disease in Latin-American countries leads to a loss of about 752,000 working days per year due to premature deaths and it causes an annual 1.2-billion-dollar loss in productivity [93]. In addition to the productivity losses, it is important to consider the economic losses related to healthcare. In this regard, Lee et al. [94] have estimated that the annual global burden would be $627.46 million in healthcare costs. In this context, both prophylactic or therapeutic vaccines against *T. cruzi* would be cost-effective in a broad range of analyzed scenarios taking into account the risk of infection, as well as the price and the efficacy of the vaccine [82,95,96].

## 10. Issues to take care about

- Are the carbohydrates important in a protein vaccine candidate? Or recombinant proteins produced in bacteria will be enough to mount a protective immune response?
- May the use of a vaccine adjuvants able to induce a strong Th1 orientated immune reaction compromise the rejection of another pathogen that require an immune response orientated to Th2, and vice versa?
- A good vaccine candidate must promote an inflammatory reaction with IFN-γ and TNF-α, but it is also important to induce IL-10 to prevent tissue damage.
- A Th1-oriented immune reaction is needed for vaccine efficacy, in this sense CpG-ODN demonstrated to be an excellent adjuvant. However, the inclusion of cyclic-di-AMP as adjuvant induce a Th1/Th17 immune reaction that has proved to be more efficacious in at least 2 vaccination protocols.
- Immunogen must be engineered avoiding the antigen portions that act as decoy molecule attracting the immune reaction with the aim to redirect the immunity against the antigen domains that play fundamental roles for parasite survival.

- The real value of analyzing immune response in patients chronically infected to conclude about the usefulness of vaccine antigens must be revisited since the parasite persistence cast doubts about the usefulness of that immune reaction.
- One of the main limitations to advance vaccine development against Chagas disease is the high number of variables that must be considered, and the lack of uniform criteria among research laboratories. The **figure 1** depicts the topics for which the scientific community should establish a greater consensus, mainly in those related to the endpoints measurements to evaluate the success of a vaccine.

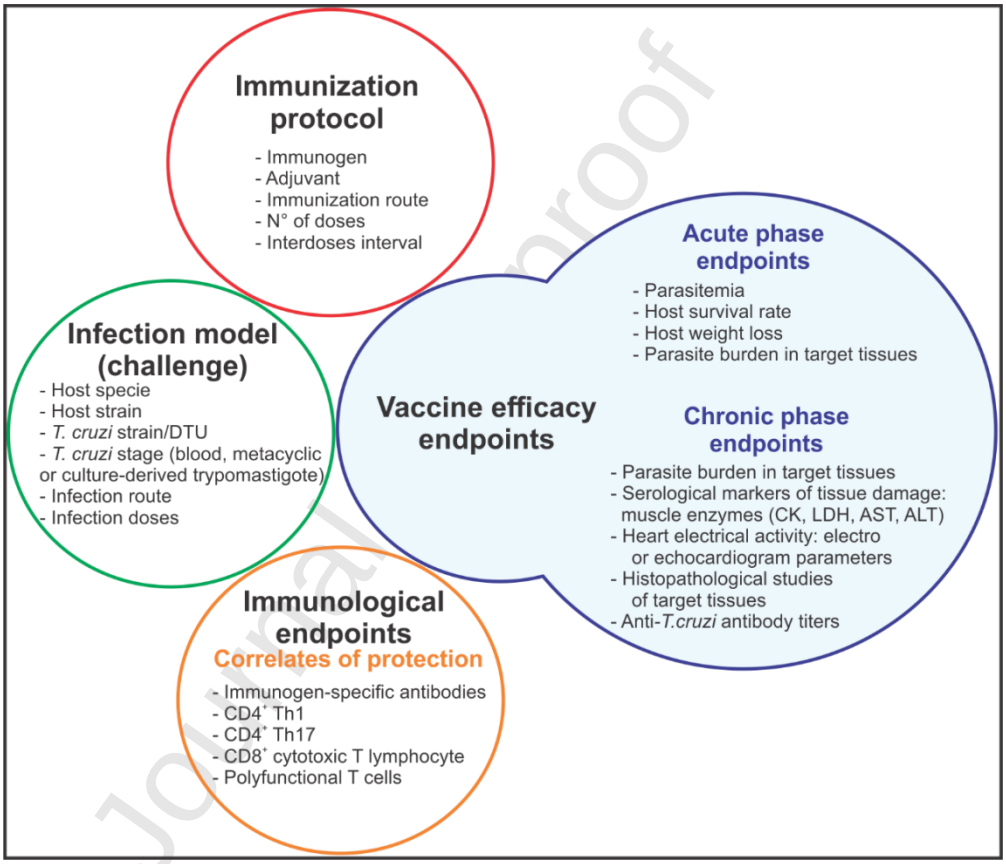


**Figure 1. Anti-*T. cruzi* vaccine parameters**.

**Funding**

Financial support was received from Universidad de Buenos Aires (20020130100788BA), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PUE-0017CO-2016), Agencia Nacional de Promoción Científica y Técnica (PICT-2010-0657 and PICT-2014-0854 to ELM), Argentina. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript

**Conflict of interest statement**

The authors declare no conflict of interest.

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