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REVIEW

Oxidative stress implications for therapeutic vaccine development against Chagas disease

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ABSTRACT

Introduction: Pathogenesis of Chagas disease (CD) caused by *the protozoan parasite Trypanosoma cruzi (T. cruzi)* involves chronic oxidative and inflammatory stress. In this review, we discuss the research efforts in therapeutic vaccine development to date and the potential challenges imposed by oxidative stress in achieving an efficient therapeutic vaccine against CD.

Areas covered: This review covers the immune and nonimmune mechanisms of reactive oxygen species production and immune response patterns during *T. cruzi infection* in CD. A discussion on immunotherapy development efforts, the efficacy of antigen-based immune therapies against *T. cruzi*, and the role of antioxidants as adjuvants is discussed to provide promising insights to developing future treatment strategies against CD.

Expert opinion: Administration of therapeutic vaccines can be a good option to confront persistent parasitemia in CD by achieving a rapid, short-lived stimulation of type 1 cell-mediated immunity. At the same time, adjunct therapies could play a critical role in the preservation of mitochondrial metabolism and cardiac muscle contractility in CD. We propose combined therapy with antigen-based vaccine and small molecules to control the pathological oxidative insult would be effective in the conservation of cardiac structure and function in CD.

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Chagas disease; immunity; oxidative stress; reactive oxygen species; *Trypanosoma cruzi*; therapeutic vaccine

1. Introduction

Trypanosoma cruzi (T. cruzi) is an intracellular kinetoplastid parasite that is the causative agent of Chagas disease (CD). *T. cruzi* experiences several biochemical and morphological modifications throughout its life cycle in insect vector and mammalian host, and it has tremendous adaptability to infect virtually all vertebrates. The parasite is primarily transmitted by triatomines, though other routes, e.g., transfusion of contaminated blood, consumption of contaminated food or infected triatomines, and congenital transmission to infants born to infected mothers, are also reported (reviewed in [1–2]). Autochthonous *T. cruzi* infection via vectorial transmission is noted in the southern parts of the US [3,4]. Because of large-scale migration of Latin Americans who may be exposed to the parasite in their native countries, CD is recognized as an important health problem in the US, Canada, Japan, Europe, and other countries [5,6].

Clinically, upon exposure to the parasite, flu-like symptoms associated with acute blood parasitemia are commonly noted. Parasites become practically undetectable in the blood in 2–4 months after infection, though infected individuals remain seropositive for *T. cruzi*-specific antibodies. Decades after initial parasite exposure, ~30% of the infected individuals eventually advance to the clinical phase of chronic CD that is presented with cardiac hypertrophy progressing to dilated cardiomyopathy and heart failure. Clinical evidence of digestive or neurological disorders may also be presented in Chagas patients (reviewed in [7]). Currently, CD is estimated to affect 6–8 million people that result in 10–12,000 deaths per year [8]. Approximately, 71 million people are exposed to risk of infection and ~28,000 new cases of *T. cruzi* infection occur every year [9,10].

Pathology of Chagas disease is multifaceted, heterogeneous, and relies on many host and parasite factors. In general, the research community believes that processes that depend, at least in part, on the few parasites that remain in the body during chronic phase, sustain the cardiac oxidative and inflammatory damage. Many intertwined processes contribute to degenerative, destructive, and reparative responses, that in-sum culminate in the varied outcomes of infection: from no disease to cardiac injury and remodeling that can ultimately lead to heart failure and/or stroke. Detailed review of multiple processes and mechanisms involved in pathology of acute and chronic Chagas heart disease can be found in recent review articles [7,11–13].

The currently available benznidazole and nifurtimox drug therapies are effective against acute *T. cruzi* infection [14–16], and are recommended for the treatment of all infected

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Article highlights

- *Trypanosoma cruzi* infection and Chagas disease remain major health concerns in the Americas.
- There is a critical lack of methods for prevention of infection or treatment of chronic Chagas disease.
- Immune therapies or therapeutic vaccines that can control *T. cruzi* persistence and associated disease pathology must be a public health priority.
- Multi-faceted nature of Chagas disease requires that immune therapies targeting the parasite persistence should be conjugated with adjuvants to address chronic inflammation and oxidative stress.

children under 15 years of age and adults with recent infection [17]. While effective in parasite clearance in children, these drugs exhibit therapeutic failure and/or adverse events in adults and are not always recommended for patients with chronic infection [18–20]. Thus, new therapies to cure, eliminate, and eradicate *T. cruzi* are needed. However, clinical trials testing new chemotherapeutics against *T. cruzi* have not been very successful in identifying replacements for benznidazole and nifurtimox. Several drugs, such as ravuconazole and posaconazole exhibited promising, parasite-specific effects in pre-clinical studies but failed to surpass the efficacy of benznidazole in clinical studies. Fexinidazole, a drug in the same class as benznidazole and nifurtimox, has completed phase II clinical trial (clinical trial identifier NCT03587766). This drug has shown promise for treating the indeterminate phase of CD in experimental studies but currently outcomes of the clinical trial are publicly documented [21–25].

Studies examining infection dynamics and parasite tropism failed to identify the organs, tissues, or cells that play a crucial role in the recrudescence of *T. cruzi* during chronic stage and may play an important role in clinical manifestations of CD. Yet, some studies showed that *T. cruzi* trypomastigotes can transition from an amastigote-like stage to an epimastigote-like morphological form that have the capability to initiate the recurrence of infection by invading the phagocytes and cardiac cells [26]. Others identified non-proliferative dormant amastigotes, which were resistant to anti-parasitic drugs and able to reestablish infection by converting to trypomastigotes even after 30 days of drug exposure [27]. These findings suggest that the dormancy state of *T. cruzi* accounts for the failure of potential therapeutic drugs and complete cure of infection.

Recent efforts are attentive to the development of therapeutic vaccines against *T. cruzi*. Besides modulating the host response to clear the low-grade parasite persistence, therapeutic vaccines also need to take into consideration to not trigger pathologic inflammation and fibrosis in the heart [13]. Studies demonstrating a mechanistic function of reactive oxygen species (ROS) in regulating the immune system vs. causing tissue damage during *T. cruzi* infection [28–32] suggest that balancing of oxidative stress may also be taken into consideration when designing immune therapies against CD. In this review, we will discuss the current efforts in the development of immune therapies against *T. cruzi* infection and the potential role of oxidative stress on efficacy of experimental therapeutic vaccines against CD.

2. Innate and nonimmune mechanisms of ROS production and role of ROS in *T. cruzi* infection and CD

The initial exposure to *T. cruzi* activates proinflammatory response of epithelial, macrophage, and dendritic cells. However, *T. cruzi* can infect a wide variety of cells, including cardiac myocytes, fibroblasts, and others. There are several reviews of the innate immune mechanisms in *T. cruzi* infection, including those relating to Toll-like receptors (TLRs), Nod-like receptors (NLRs), and DNA sensing receptors [33–35]. Herein, we focus on the mechanisms of ROS production and its role in shaping the disease outcome in CD.

In addition to producing cytokines and chemokines, macrophages and other innate immune cells exert cytotoxic effects against microbes by manufacturing ROS and reactive nitrogen species (RNS). NADPH oxidase (NOX2), a multimeric complex, uses NADPH as a substrate and reduces O_2 to manufacture superoxide ($O_2^{\bullet-}$) that is dismutated to stable pro-oxidant H_2O_2 [36]. Inducible nitric oxide synthase (iNOS) yields nitric oxide (NO) in a complex oxidoreductase reaction that utilizes L-arginine and O_2 as substrates [37]. Studies in human and mouse macrophages show that *T. cruzi* elicits very low levels of ROS/NO production and delayed inflammatory cytokines/chemokines response [38,39]. Yet, chemical or genetic inhibition of NOX2 or depletion of ROS by use of antioxidants arrested the phagocytes' production of inflammatory cytokines in mice and cultured cells, thus, indicating that low levels of ROS serve as signaling molecule in proinflammatory activation of macrophages infected by *T. cruzi* [40,41]. Further, reaction of NO with O_2•– produces a strong cytotoxic oxidant peroxynitrite. Some studies indicate that peroxynitrite and other cytotoxic effectors produced by innate immune cells are essential for parasite killing [42,43], while others suggest that macrophage oxidative environment acts as an enhancer of infection [44]. Readers interested in further details of the role of ROS in providing fuel for parasite growth or parasite killing are directed to recent reviews [45,46].

Nonimmune cells such as cardiac myocytes also respond to *T. cruzi* infection with ROS production, though mitochondria are the major source of O_2•– in cardiac myocytes [28,29]. Specifically, complex-I and complex-III of the electron transport chain were found to be the site of increased electron leakage to oxygen and O_2•– production in infected cardiomyocytes and Chagas murine hearts [28,47]. Mitochondrial dysfunction, while initiated in multiple tissues in response to acute infection [48–50], persists in the heart and contributes to high myocardial ROS levels [51,52]. Mitochondrial dysfunction as a source of ROS is well documented in chronically infected animals [48,53] and clinically symptomatic CD patients [54–57]. Regardless of the source, *T. cruzi* can control oxidative insult and survive in the host cells. For example, *T. cruzi*-specific Fe^{2+} superoxide dismutase (FeSOD) is shown to protect the parasite from ROS and other oxygen sensing metabolic responses in the

macrophages [43,58]. The parasite-specific antioxidant network is discussed in detail in recent reviews [59,60].

Besides influencing parasite, ROS also affect apurinic/apyrimidinic (AP) sites and cause DNA base modifications, which makes DNA susceptible to oxidative lesions. Indeed, 8-hydroxy-2-deoxyguanosine (8-OHdG) DNA lesions are routinely detected in cardiac biopsies of chronically infected experimental rodents as well as in CD patients [57,61,62]. Further, an increase in the expression of 8-oxoguanine glycosylase (OGG1) and poly(ADP-ribose) polymerase 1 (PARP1) was detected in infected cells and tissues, which indicates the activation of DNA repair process [29,31,32,61,63]. It is documented that PARP1, though a DNA repair enzyme, contributed to proinflammatory activation of cardiac myocytes and macrophages infected by *T. cruzi*. In cardiac myocytes and macrophages, *T. cruzi* elicited PARP1-mediated post-translational modifications of RelA (p65)-interacting nuclear proteins and facilitated the assembly and transcriptional activation of NF-κB-dependent cytokines' gene expression [29,31,32]. Further, macrophages exposed to *T. cruzi* or extracellular vesicles released in circulation of chronically infected mice and humans, also exhibited PARP1-dependent activation of cyclic GMP-AMP synthase/2′3′-cyclic GMP-AMP and downstream signaling of Stimulator of Interferon Genes (STING) that in synergy with c-Fos and Jun B (Activator Protein 1 family members) promoted profibrotic response [31,32].

A network of enzymatic and non-enzymatic antioxidants that control oxidative stress in the host is reviewed recently [64]. In the context of CD, an increase in mitochondrial ROS was correlated with a decline in mitochondrial Mn^{+2} superoxide dismutase (MnSOD) and cytosolic glutathione peroxidase (GPx) activities and reduced glutathione content in chronically infected rodents and human patients [55–57,65,66]. Nuclear Factor Erythroid 2 Like 2 (NFE2L2) is a transcription factor that regulates the expression of antioxidant proteins. The expression, nuclear translocation, and binding of NFE2L2 to cis-acting DNA regulatory antioxidant response elements (ARE) was significantly reduced and linked to deteriorating concentration of antioxidants, e.g., γ-glutamylcysteine synthetase, hemoxygenase 1, glutamate-cysteine ligase modifier subunit in *T. cruzi* infected murine cardiac myocytes and myocardium. Preservation of NFE2L2 transcriptional activity and antioxidant/oxidant balance occurred with the overexpression of MnSOD in murine cardiac myocytes that in turn improved the cardiac function in CD mice [67]. These findings indicated that the inhibition of NFE2L2/ARE pathway by mitochondrial ROS constitutes a key mechanism in signaling the inflammatory/fibrotic gene expression and evolution of chronic Chagas cardiomyopathy.

In summary, a balance between the levels of ROS that can induce parasite killing and the antioxidant machinery that the host requires to detoxify and keep a safe environment for cells exposed to infection is fundamental. The literature discussed above points to the possibility that in the initial phase of infection, *T. cruzi* suppresses the macrophages' ability to mount strong oxidative/nitrosative burst and instead utilizes macrophages to disseminate to different tissues. As parasite infects more cells, nonimmune cells also produce ROS, and mitochondrial dysfunction contributes to ROS production in the host. Further, the host antioxidant response is exhausted during chronic CD and increased ROS sustain inflammatory and profibrotic response contributing to evolution of chronic cardiomyopathy.

3. Immune responses in *T. cruzi* infection

Studies in susceptible and resistant experimental models and humans with and without clinical CD have contributed to our current understanding of the protective immune responses to *T. cruzi*. Besides the need for early and potent proinflammatory innate immune response against invading parasite, adaptive T cell immunity is paramount for intracellular control of *T. cruzi*. Adaptive immunity is provided by parasite-specific $CD4^+$ T cells that support macrophage phagocytosis function, B cell proliferation and antibody production, and differentiation and activation of $CD8^+$ T cells and secretion of T helper type 1 cytokines (e.g., interferon (IFN)-γ, interleukin (IL)-2) [68–70]. *T. cruzi* antigen specific $CD8^+$ T cells are frequently noted in infected host [71,72], and contribute to *T. cruzi* control. Antigen-specific $CD8^+$ T cells regulate *T. cruzi* infected cells by cytolysis or the release of cytokines (e.g., IFN-γ) that induce trypanocidal activity [73–75]. A robust lytic antibody reaction boosts the phagocytosis, opsonization and complement-dependent killing of parasites [76]. As in mice, type 1 B and T cell immunity is suggested to maintain the low levels of parasites in chronic human Chagas disease [77–79].

Immune responses are also documented to be harmful to the host, especially in chronic phase of infection when few parasites persist. For example, several studies indicate that excessive production of IFN-γ and tumor necrosis factor (TNF)-α cytokines correlate with tissue damage and clinical disease, while IL-27 controls proinflammatory IFN-γ and inhibits cardiac inflammation in CD [80–87]. Particularly, IL-17 plays a dual role in *T. cruzi* infection and CD. Earlier studies indicated that IL-17 elicits proinflammatory immune signature for the control of acute parasitemic infection [88,89]. IL-$17A^{-/-}$ mice, as compared to wild type mice, exhibited elevated mortality to acute *T. cruzi* infection, and treatment of infected, wild type mice with anti-IL-17A antibody resulted in increased myocarditis and mortality [88,89]. Others noted that *T. cruzi*-induced Th17 response caused severe multi-organ pathology despite reduced parasite burden [90]. Studies in humans documented high levels of IL-17A correlated with better left ventricular function in Chagas disease patients and suggested that IL-17A has an immune-modulatory role in controlling myocardial damage in CD [91]. Lower expression of IL-17 by total lymphocytes and lower frequency of Th17 cells was noted in Chagas patients with cardiac involvement, and treatment of these patients with benznidazole enhanced the plasma levels of IL-17, taken as an indicator of treatment success [85,92].

IL-17 RA is the common receptor for many IL-17 members and IL-$17RA^{-/-}$ mice lack responsiveness to several IL-17 cytokines. IL-$17RA^{-/-}$ mice infected with *T. cruzi* exhibited exacerbated IFN-γ and TNF- α production that promoted hepatic damage, tissue wasting, and mortality [93]. Authors noted that IL-17RA signaling was required for the recruitment of IL-10-producing neutrophils to regulate the damaging

proinflammatory responses [93], thus, providing a link between IL-17 and IL-10 induced protection from tissue damage in CD. Indeed, despite its immune-regulatory role that can potentially result in increase in parasitemia, IL-10 was required to prevent immune hyper-reactivity during *T. cruzi* infection and IL-10$^{-/-}$ mice exhibited increased mortality due to the development of pathologic immune response associated with CD4^{+} T cells and overproduction of IL-12 [94,95]. Increase in serum levels of IL-10 was also noted in CD patients in indeterminate phase of infection while patients with cardiac involvement primarily exhibited uncontrolled inflammatory response [96].

Summarizing, these studies indicate that effective immune response against *T. cruzi* would require elicitation of phagocytes, lytic antibodies, and the collaborative activities of Th1 cytokines, T helper cells, and cytotoxic T lymphocytes. However, a balance between proinflammatory and immune-regulatory cytokines is essential after the acute infection if the damage to host tissues is to be controlled to prevent the manifestation of clinically symptomatic cardiac Chagas disease.

4. Therapeutic vaccines against Chagas disease

History of attenuated *T. cruzi* and recombinant antigen-based subunit vaccines development and their efficacy as prophylactic experimental vaccines is discussed in excellent recent reviews. Initial efforts to vaccine development utilized live, killed, or attenuated parasite, cell fraction, purified protein, recombinant protein etc. (reviewed in [97]). Many investigators, including us, have demonstrated outstanding prophylactic efficacy of subunit vaccines in regulating infection and concomitant pathologies in murine models of *T. cruzi* infection (reviewed in [98,99]). For the delivery of subunit vaccines, most investigators have utilized DNA-based platform as DNA vaccines are cost effective, stable at room temperature, and have demonstrated clinical safety in animal models and early-phase clinical trials [100]. DNA vaccines were shown to provide antigenic peptides for MHC I and MHC II (major histocompatibility complex) presentation, and elicit antigen-specific antibodies, type I cytokines, and cytotoxic CD8^{+} T lymphocyte response to provide protection from *T. cruzi* infection [100,101]. Yet, there is a concern regarding the antibiotic resistance genes in the plasmid DNA backbone. Antibiotic resistance genes can potentially be taken up by bacteria and may also be expressed in mammalian host after insertion into the genome [102,103]. To alleviate this concern, the nanoplasmid DNA vaccine was developed. The prototype nanoplasmid utilizes an antibiotic-free selection method based on sucrose selection vector using a small antisense RNA known as RNA-OUT. Another advantage of the nanoplasmid DNA vaccine is the reduced plasmid size that improves in vivo level and duration of expression [104].

When developing a therapeutic vaccine, the purpose is to employ the biological response modifiers to control or improve the multiple effector mechanisms against *T. cruzi* while not having cytotoxic effects against self-cells and -tissues. Further, an immunotherapy is expected to target all circulating genotypes of the parasite to be potentially useful. Still, limited studies have tested the concept of immunotherapy for arresting the CD pathology. Some investigators have examined in acutely and chronically infected animals the therapeutic efficacy of select antigens, including trypomastigote surface antigen (TSA)-1, trans-sialidase (TS), amastigote surface protein (ASP)-2, glutathione *S*-transferase encoded by Tc52, a Ca^{2+} binding protein encoded by Tc24, a cathepsin I cysteine protease named cruzipain, and a cysteine protease inhibitor named Chagasin. Most of the antigens used in therapeutic vaccines were conserved within the TcI-TcVI lineages of *T. cruzi* (Table 1). Parasitemia and mortality in mice decreased when Tc52, TSA-1, and Tc24 based DNA therapies were delivered immediately after infection or two weeks post-infection [105,106], Delivery of Tc24 as a recombinant protein immune therapy also provided control of cardiac fibrosis in infected mice [107]. The protection provided by the Tc24 therapeutic vaccine was correlated with elevated CD4^{+} or CD8^{+} T cell proliferation and IFN-γ production [107–109]. Interestingly, Tc24, delivered with poly(lactic-co-glycolic acid) nanoparticle, provided a 3-fold increase in IL-4 production (Th2 immune response), while Tc24 delivered with adjuvant E6020 showed no significant change compared to that noted with Tc24 alone [107,109]. However, Tc52, TSA-1, and Tc24 DNA therapy did not regulate cardiomyopathy in the chronic murine or acute canine infection model [106,110]. Despite the known efficacy as a prophylactic vaccine, ASP2 and TS DNA-based immune therapy (individually or in combination) provided no defense against parasite load, mortality, and cardiomyopathy in infected mice [106,111]. Others identified that treatment of *T. cruzi* infected mice with TSA1 DNA resulted in an increase in myocarditis [108]. These studies did not extend to determine why the therapeutic treatment failed to arrest myocarditis and fibrosis, and how the immune system was (or was not) modulated.

Ribeiro et al [125] have tested therapeutic effects in mice concurrently infected with *T. cruzi* and immunized with type 5 adenovirus encoding ASP-2 (AdASP-2); and noted a significant decline in cardiac amastigote nests was associated with rapid increase in TNF-α, TLR-4, iNOS and IL-10 expression. Pereira et al [126] investigated recombinant adenovirus encoding ASP2 and TS as a therapeutic treatment in acutely and chronically infected mice. ASP2- and TS-based immune therapy in chronically infected mice improved survival rate, decreased electrocardiogram abnormalities, preserved IFN-γ levels, and reduced polyclonal stimuli, such as CD107a^{+}CD8^{+}T cells and peripheral nitric oxide concentrations. Results show ASP2-expressing recombinant adenovirus is a promising therapeutic against acute and chronic infection.

T. cruzi expresses cruzipain in all of its developmental stages, and cruzipain is shown to be essential for amastigote replication and parasite virulence [127,128]. *T. cruzi* also expresses a papain-like cysteine protease inhibitor, Chagasin, to fine-tune the proteolytic functions of cruzipain during parasite differentiation and invasion [129]. Cerny et al [130] showed the therapeutic potential of cruzipain encoding DNA, delivered with granulocyte macrophage colony stimulating factor (GM-CSF) intramuscularly or with *Salmonella*

Table 1. Summary of immunotherapies development efforts against *Trypanosoma cruzi*.

Antigen (found in *T. cruzi* lineages)	Adjuvant	Mice (infection phase)	*T. cruzi* strain used (genetic lineage)	Immune response (treated vs untreated) @ days post-infection (dpi)	Inflammatory cells per μm^2 (treated vs untreated) (Organ)	Parasite burden (location) [A]	% Survival (treated vs untreated)	Reference
TSA1 (TcI, TcII, TcVI)	NA	BALB/c, CD1 (acute and chronic)	H4 (TcI)	ND	Mild vs severe (heart) [B]	Decrease (blood)	>70% vs. 0% @ 45 dpi	[105,112,113]
TSA1	NA	ICR (acute and chronic)	H1 (TcI)	Acute: 1.5-fold & 2-fold ↑ $CD4^+$T and 2-fold & 2.5-fold ↑ $CD8^+$T cells @ 19 dpi & 33 dpi, respectively Chronic: 2.5-fold ↑ $CD4^+$T & $CD8^+$T cells @ 84 dpi (disappeared after 157 dpi); 2-fold ↑ $IFN\gamma^+CD4^+$T & 6-fold ↑ $IFN\gamma^+CD8^+$T cells @ 77 and 84 dpi, respectively.	Acute: ~1500 vs >4000 (heart) Chronic: ~200 vs >1200 (heart)	Decrease (blood & heart)	ND	[3,108,113]
[C] TSA1	Aluminum phosphate	ICR (acute)	H1	ND	~1000 vs >1500 (heart)	Decrease (blood & heart)	50% vs. 35% @ 70 dpi	[106]
Tc24 (TcI-TcVI)	NA	BALB/c (acute)	H1	ND	Mild vs severe (heart) [B]	Decrease (blood & heart)	100% vs. 0% @ 140 dpi	[105,114]
Tc24 (PLGA nanoparticle)	CpG-ODN	BALB/c (acute)	H1	9-fold ↑ IFN-γ secreting cells, 6-fold ↑ IFN-γ and ~3-fold increase in IL-4, $CD8^+$T cells, IgG1 and IgG2a levels	30% reduction compared to sham vaccine (heart)	Decrease (blood & heart)	ND	[109]
Tc24	E6020	ICR (acute)	H1	2-fold ↑ IFN-γ secreting cells, 6-fold ↑ IFN-γ, No change in IL-4, ↑ IgG1 and IgG2a titers	~1100 vs ~1200 (heart)	Decrease (blood)	ND	[107]
[C] Tc24	Aluminum phosphate	ICR (acute)	H1	ND	<500 vs >1500 (heart)	Decrease (blood & heart)	50% vs. 35% @ 85 dpi	[106,115,116]
TSA1 + Tc24	NA	Canine (acute)	H4	↓IgG levels	Higher inflammatory cell density (heart)	Not		quantifiable
ND	[110]							
[C] Tc52 (TcI, TcII)	Aluminum phosphate	ICR (acute)	H1	ND	~1250 vs >1500 (heart)	Decrease (blood & heart)	50% vs. 35% @ 75 dpi	[106,117]
[D] AdASP-2 (TcI, TcII, TcVI)	NA	A/Sn (acute)	Y (TcII)	ND	ND	Decrease (liver)	ND	[118,119,125]
[E] TS (TcI-TcVI)	Aluminum phosphate	ICR (acute)	H1	ND	ND	No change (blood)	50% vs. 35% @ 50 dpi	[106,120]
[F] TS + ASP-2-like clone 9 (TcI, TcII, TcVI)	Aluminum phosphate	ICR (acute)	H1	ND	>2000 vs >1500 (heart)	No change (blood & heart)	50% vs. 35% @ 60 dpi	[106,118,119]
[G] rAdVax (ASP-2 + TS)	NA	C57BL/6 (chronic)	Colombian (TcI)	Spleen: decline in IFN-γ, $CD8^+$T, and IFN-$\gamma^+CD107a^+$ $CD8^+$T cells, Heart: ↓PFN^+ T cells, NS change in IFN-γ^+ cells, ↑ IFN-γ^+ mRNA	ND	Decrease (heart)	87% vs. 0% @ 230 dpi	[121,126]
Cruzipain (TcI-TcVI)	GM-CSF or *Salmonella* + GM-CSF (SGM-CSF)	C3H/HeN (acute and chronic)	RA (TcVI)	Acute (+GM-CSF): 5-fold ↑ IgG titer and ↑ IgG2a/IgG1 ratio Acute (+SGM-CSF): 15-fold ↑ IgG titer and ↑ IgG2a/IgG1 ratio Chronic (+GM-CSF): 2-fold ↑ IgG titer and ↑ IgG2a/IgG1 ratio Chronic (+SGM-CSF): 6-fold ↑ IgG titer, ↑ IgG2a/IgG1 ratio	Chronic: scarce vs intense mononuclear cell infiltration (skeletal muscle)	Decrease in acute blood parasitemia	100% vs. 0% @ 100 dpi	[122,123,130,131]

(*Continued*)

Table 1. (Continued).

Antigen (found in *T. cruzi* lineages)	Adjuvant	Mice (infection phase)	*T. cruzi* strain used (genetic lineage)	Immune response (treated vs untreated) @ days post-infection (dpi)	Inflammatory cells per μm^2 (treated vs untreated) (Organ)	Parasite burden (location) [A]	% Survival (treated vs untreated)	Reference
Cruzipain (Cz, TcI-TcVI) and [H]Chagasin (Chg, TcI & TcII)	SGM-CSF	C3H/HeN (acute and chronic)	RA	Acute: 2-fold ↑ IgG titers (rChg & rCz) Chronic: 3-fold & 5-fold ↑ IgG titers for rChg & rCz respectively, ↑ splenic IFN-γ secreting cells	Acute: Few necrotic zones and nonspecific inflammatory foci vs necrosis and multifocal, diffuse inflammatory infiltrate [I] Chronic: Few necrotic zones and nonspecific inflammatory foci vs confluent inflammatory infiltrate foci with necrosis (skeletal muscle)	Decrease in acute blood parasitemia	75% vs. 0% @ 45 dpi	[129,131,132]

[A] = compared to that detected in infected mice that were not vaccinated or were injected with the plasmid vector only.
[B] = inflammation score – mild, moderate & severe.
[C] = Tc24 was most effective in reducing blood and heart parasite burden compared to TSA1 and Tc52.
[D] = AdASP-2 – Recombinant human type 5 replication-defective adenoviruses expressing ASP-2.
[E] = enzymatically active form conserved in the genetic lineages TcI-TcVI; [F] = *T. cruzi* antigens ASP-2-like clone 9 (ASP9) and trans-sialidase (TS) were delivered in recombinant plasmids pIgSPclone9 and P154/13, respectively.
[G] = rAdVax – recombinant adenovirus carrying sequences of ASP2 and TS (rAdASP-2+ rAdTS).
[H] = Chagasin was examined in the following *T. cruzi* strains (DTU): Dm28c (TcI [131]), G (TcI [131]), Y (TcII [131]), Sylvio X10/6 (TcI [131]), CL (TcII [124]) and Brazil (TcI [124]).
[I] = Low levels of pericardial infiltrates were present in the right ventricular area of both treated and untreated groups.
Abbreviations: ASP2 – amastigote surface protein 2; PLGA – poly(lactic-coglycolic acid); rChg – recombinant Chagasin; rCz – recombinant cruzipan; Tc24 – trypomastigote excretory–secretory protein 24; TS – trans-sialidase; TSA – trypomastigote surface antigen; NA – not applicable; ND: not determined; NS – no significance.

delivery system orally in mice infected with *T. cruzi*. Authors noted that cruzipain DNA vaccine adjuvanted with GM-CSF encoding plasmid or *Salmonella* reduced the acute parasite burden, mortality, and cardiac injury markers and enhanced the antigen-specific IgG response [130]. In another study, therapeutic potential of DNA combining cruzipain and Chagasin was tested. The DNAs of both antigens and GM-CSF adjuvant were orally administrated using an attenuated *Salmonella* strain in acutely infected mice. The bi-component therapy was found to be better than either of the mono-component therapy in eliciting antigen- and parasite-specific antibodies and IFN-γ secretion by lymphocytes and provided rapid control of acute parasitemia and decreased the tissue damage in chronic stage of the infection [129,131,132] (Table 1).

We have tested the protective efficacy of two antigens, named TcG2 and TcG4, as immune therapy (Table 2). TcG2 and TcG4 are expressed in all mammalian stages of *T. cruzi* [133,134] and consist of epitopes recognized by antibodies and T cells in mice, dogs, and humans (reviewed in [99]). Further, TcG2 and TcG4 were conserved in five of the six *T. cruzi* lineages with 80–96% homology, thus indicating that TcG2/TcG4-based therapeutics can extend protection against various *T. cruzi* genotypes circulating in the USA and Latin America. In all studies, where we tested therapeutic efficacy of TcG2 and TcG4, mice were given the immune therapy in indeterminate phase when natural immune response had controlled the acute parasitemia. In the first study, C57BL/6 mice with or without overexpression of glutathione peroxidase 1 (GPx1, detoxifies ROS) were infected with *T. cruzi* and 45 days later given TcG2/TcG4 as a DNA-prime/protein-boost therapy [135]. All mice receiving immune therapy exhibited >15-fold reduction in blood and tissue parasites, significant reduction of chronic inflammatory infiltrate in skeletal and cardiac tissues, and of hypertrophy (BNP and ANP) and fibrosis (collagens) markers in the heart. GPx1 transgenic mice were better equipped than the wild type mice in controlling the tissue pathological responses, including markers of inflammation and fibrosis [135].

We also tested the adjuvant properties of 7HP349, a small molecule agonist of αLβ2 and α4β1 integrins, in enhancing therapeutic efficacy of the subunit vaccine. 7HP349 is shown to enhance the αLβ2 and α4β1 dependent adhesion of immune cells and activation of adaptive immunity in an ovalbumin antigen model. When delivered systemically in a mouse model of Chagas disease, 7HP349 significantly enhanced the TcG2/TcG4-based DNA prime/DNA boost vaccine efficacy in therapeutic settings. Mice given 7HP349 adjuvanted (vs. non-adjuvanted) vaccine therapy exhibited better control of parasite persistence, as well as the tissue inflammatory infiltrate and fibrotic responses in skeletal muscle and heart tissue that otherwise were pronounced in non-treated, chronically infected mice (unpublished data).

Recently, we used a nanovector as a delivery vehicle. The composition of the nanovector for immunotherapy was designed with US Food and Drug Administration regulatory guidance, and it provides improved expression of target genes for enhanced immunogenicity [136]. TcG2/TcG4 were cloned

Table 2. Efficacy of TcG2- and TcG4-based immune therapy against *T. cruzi* and Chagas disease.

Antigen delivery	Adjuvant	Experimental Model, # of parasites/mouse, disease phase	Immune response (treated vs untreated)	Inflammation score – treated vs untreated, tissue, model	Oxidants & inflammation markers, tissue, model	Fibrosis, tissue, model	Parasite burden (treated vs non-treated), tissue, model	Ref
DNA prime in pCDNA3.1 & recombinant protein boost	IL-12 + GM-CSF	$GPx1^{Tg}$ and wild type (WT) CD1xC57 mice, 10,000 SylvioX10, chronic	ND	[A] Decrease: score 0–2 vs 1–4 (heart) and score 0–3 vs 1–3 (skeletal muscle), GPx^{Tg} > WT	Decrease of MPO and nitrite in heart, GPx^{Tg} > WT	Decrease, % fibrosis: 0.3–0.5 vs. 5.1–6.4 (heart), GPx^{Tg} > WT	Decrease by 15-fold in blood, skeletal muscle & heart, GPx^{Tg} = WT	[135]
DNA prime/DNA boost in pcDNA3.1 or nanovector	None	C57BL/6 mice, 10,000 SylvioX10, chronic	Increase: splenic IL-6 release, frequency of $CD4^+$ & $CD8^+$ T cells expressing IFN-γ, PFN, GZB. nanovector > pCDNA3.1	[A] Decrease: score 0–1 vs 0–3 (heart) & 0–1 vs 1–3 (skeletal muscle), nanovector > pCDNA3.1	Decrease of Trolox, LPO & MPO in serum; decrease of 4-HNE, protein carbonyls & 8-OHdG in heart, nanovector > pCDNA3.1	[B] 7–14-fold decline in heart & skeletal muscle, nanovector > pCDNA3.1	Decrease by 92.4–95.5% in heart & skeletal muscle, nanovector > pCDNA3.1	[137]
DNA prime/DNA boost in pCDNA3.1	7HP349	C57BL/6 mice, 10,000 SylvioX10, acute and chronic	ND	[A] Decrease by 62–85.6% in heart & skeletal muscle, adjuvanted > non-adjuvanted	ND	85.7–95% decline in heart & skeletal muscle, adjuvanted > non-adjuvanted	Decrease by 94.3–97.8% in heart & skeletal muscle, adjuvanted > non-adjuvanted	

[A] = H&E-stained tissue sections were scored for inflammatory infiltrate as 0 (absent), 1 (focal or mild, 0–1 foci), 2 (moderate, ≥2 foci), 3 (extensive inflammatory foci, minimal necrosis, and retention of tissue integrity), and 4 (diffused inflammation with severe tissue necrosis, interstitial edema, and loss of integrity).

[B] = Heart and skeletal muscle mRNA levels of collagens (Col1a1, Col3a1 & Col5a1), metalloproteinases (Mmp2, Mmp3, Mmp9, Mmp12 & Mmp13), hypertrophy markers and repair proteins (Nppa, Acta1 & Tagln) were monitored.

Abbreviations: GPx1 – glutathione peroxidase; 4-HNE – 4 hydroxynonenal; LPO – lipid hydroperoxides; MPO – myeloperoxidase; 8-OHdG – 8-Hydroxy-2′-deoxyguanosine; PFN – perforin; GZB – granzyme B; ND – not determined.

in nanovector (referred as nano2/4). Mice were infected with *T. cruzi*, given nano2/4 DNA vaccine at 21- and 42-days post-infection, and monitored at ~100 days pi [137]. The frequency of splenic, poly-functional $CD4^+$ and $CD8^+$ T cells expressing IFN-γ cytokine and cytotoxic molecules (perforin and granzyme B) that are required for intracellular parasite control were increased by nanoimmunotherapy. The nanotherapy-mediated increase in splenic T cells and immune components was associated with up to a 99.7% decline of the parasite burden in cardiac and skeletal tissue. Additionally, we identified a significant reduction of peripheral and tissue levels of oxidative stress markers (e.g. 4-HNE, protein carbonyls) and inflammatory infiltrate, that otherwise were prominent in *T. cruzi*-infected mice. Further, nano2/4 therapy efficiently regulated tissue infiltration of pro-fibrotic macrophages and provided a homeostatic environment managing the expression of collagens, metalloproteinases, and several markers of cardiomyopathy (e.g., ANP, BNP, β-MHC, SM22α, αsk-Actin). Moreover, nano2/4 improved the expression of Myh7 and GSK-3β necessary for preserving cardiac contractility in *T. cruzi*-infected murine hearts. The TcG2/TcG4 encoding nano-vaccine ultimately provided improved immune protection compared to the experimental vehicle (pCDNA3) in Chagasic mice [137].

5. Antioxidants as adjuvants to anti-parasite drug and immune therapies

In recent times, strong *T. cruzi* tropism is noted for cardiac myocytes [138], which induces oxidative stress-related pathology in Chagas myocardium (reviewed in [7]). Cardiac myocytes with dysfunctional mitochondria and cardiac resident and infiltrating macrophages cleaning up *T. cruzi* and *T. cruzi*-induced cellular debris are recognized as primary source of ROS in the chronic Chagas heart (reviewed in [139]). General antioxidants, including vitamin C, vitamin E, curcumin, resveratrol, melatonin, and mitochondria-targeted antioxidant, i.e. 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPOL) are discussed in detail for their beneficial effects in reducing plasma and cardiac levels of oxidative stress [64], and proposed to offer promising co-adjuvants with anti-parasite therapies. Briefly, co-administration of vitamin C with benznidazole did not affect the anti-parasite activity of benznidazole in vitro or in vivo, but vitamin C reduced the cytotoxicity of benznidazole on host cells and decreased the mortality and weight loss in infected mice [140]. Others showed low dose vitamin C enhanced the anti-parasitic effects of benznidazole and decreased the cardiac oxidative damage in infected mice [141]. Treatment with vitamins C and E increased the inflammatory infiltrate in skeletal muscle yet decreased the circulatory or cardiac levels of thiobarbituric acid reactive substances in acutely and chronically infected mice [142]. Likewise, treatment with curcumin enhanced the benznidazole efficacy in reducing the parasitemia, parasite load, and mortality [143]. Importantly, curcumin decreased the myocardial inflammatory infiltrate and oxidative stress and liver toxicity that were triggered by benznidazole [143]. A strong antioxidant and anti-inflammatory effect of resveratrol has also been documented in CD. Resveratrol enhanced the antioxidant enzymes activities and lowered the ROS and ROS-induced oxidative damage in acutely and chronically infected mice and improved the electrophysiological function of the heart in chronically infected mice [144,145]. Lastly, mitochondria-targeted antioxidant, TEMPOL was shown to decrease lipid peroxidation and improve heart function in mice infected with Colombian strain of *T. cruzi* [145].

Together, these studies suggest that antigen-based immune therapies can be effective for controlling parasite persistence and associated tissue injury in CD, and small molecules that enhance the protective immunity or control the pathological effects of oxidative stress can be used as adjuvant to gain better protection against chronic CD.

6. Expert opinion

Summarizing the findings discussed in this review, we believe that a therapeutic approach focused on only the control of the parasite is not sufficient to arrest the progression of chronic disease. Infected mice and rats treated with the anti-parasite drug (benznidazole) after immune control of acute parasitemia exhibited inhibition of parasite persistence. However, benznidazole treatment failed to inhibit deterioration of ventricular contractility and cardiac remodeling. Instead, maximal benefits were obtained when infected mice and rats were treated with the antioxidants in conjunction with benznidazole (discussed above). We noted the combination of phenyl-butyl-nitrone and benznidazole deterred free radical-mediated oxidative insult and mitochondrial deficiencies, resulting in the preservation of metabolic (mitochondrial) and contractile activity in Chagasic hearts [30]. Likewise, a better efficacy of a therapeutic DNA vaccine was noted in infected GPx transgenic mice with over-expression of antioxidant response compared to the infected/wild-type mice under similar conditions [135]. We, therefore, propose therapeutic vaccines could be designed to confront persistent parasites by achieving a rapid, short-lived stimulation of type 1, cellular immunity. Simultaneously, to prevent cellular injury, adjunct therapies could be given to inhibit the onset of oxidative insult and mitochondrial deficiencies. This combination of treatment would prove maximally advantageous in conserving cardiac structure and function in Chagas disease.

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Declaration of interest

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