Immunity and vaccine development efforts against *Trypanosoma cruzi*

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ABSTRACT

Trypanosoma cruzi (*T. cruzi*) is the causative agent for Chagas disease (CD). There is a critical lack of methods for prevention of infection or treatment of acute infection and chronic disease. Studies in experimental models have suggested that the protective immunity against *T. cruzi* infection requires the elicitation of Th1 cytokines, lytic antibodies and the concerted activities of macrophages, T helper cells, and cytotoxic T lymphocytes (CTLs). In this review, we summarize the research efforts in vaccine development to date and the challenges faced in achieving an efficient prophylactic or therapeutic vaccine against human CD.

1. Introduction

Chagas disease is a chronic degenerative illness inflicted by the protozoan *Trypanosoma cruzi*. This infectious agent is prevalent in South Central America and Mexico, and the disease is continually spreading due to worldwide human migration efforts. An estimated 6–7 million people, globally, are infected with *T. cruzi* and many more are exposed to risk of infection. Routes of primary infection consist of direct transmission by triatomine vector, consumption of food contaminated with feces of infected triatomines, transfusion of blood from infected donors, and vertical transmission from infected mothers to their newborns (WHO: World Health Organization, 2019; Coura, 2015). Other routes of transmission, e.g., transplantation of organs from infected individuals, sexual transmission, laboratory accidents, are also described (Coura, 2015). The oral route of transmission is noted to result in a higher mortality rate of 8–35% than is generally observed by vector transmission which is <5–10% (Antunes et al., 2019). *T. cruzi* infected mothers can vertically transmit the parasite to the fetus in 5–10% of pregnancies (Messenger and Bern, 2018). Benznidazole and nifurtimox are the currently available chemotherapeutic treatment options (Meymandi et al., 2018). These treatments were developed over 40 years ago and benznidazole is the only drug that is approved by US Food and Drug Administration to treat *T. cruzi* infection (CDC, 2019). These anti-parasitic drugs are most effective in treating the disease in children and acutely infected adults. However, it is common for patients to suffer from unspecific symptoms, or be asymptomatic, leading to lack of treatment. Patients will encounter a high parasitic load within blood circulation during acute stage and a low to intermittent parasite load during the chronic phase of the disease (D'Ávila et al., 2018). Studies in mice infected with 10^5 trypomastigotes of *T. cruzi* (Y strain) showed peak parasitemia developed by 5 days post infection (dpi) with a median range of 9.3×10^4 parasite equivalent/mL (par. eq/mL); and parasite levels fell below 10^{-1} par eq/mL at 100- and 260-dpi in infected mice (Mateus et al., 2019). Acutely infected patients from Colombia had median parasite loads of 6.68 par eq/mL (Hernández et al., 2018). Chronic patients from Southern Brazil, tested for parasite load via blood culture and analyzed using quantitative real-time PCR (qPCR), had a median parasite load of 1.18 par eq/mL of blood (D'Ávila et al., 2018). Another study of chronic patients from various locations of Brazil determined a median load of 2.54 par eq/mL (Rodrigues-dos-Santos et al., 2018). Chronic disease progression occurs due to *T. cruzi* amastigote infiltration into organs, specifically cardiac and digestive tissues. Approximately, 30–40% of chronically affected Chagas patients will experience cardiomyopathies, and to a lesser extent, gastroenterological and neurologic disorders. Chagas disease can ultimately cause death due to heart failure (Meymandi et al., 2018).

Although the immune system is required to control acute infection (discussed below), low-grade systemic infection that persists during

intermediate-to-chronic phase supports consistent activation of pathologic immune reactions and contribute to the pathogenesis of Chagas heart disease (reviewed in (Marin-Neto et al., 2007; Gutierrez et al., 2009; Rassi and Marin, 2017)). Indeed, with the advent of modern techniques, e.g., PCR and qPCR, parasite DNA is detected in blood and heart tissue biopsies of chronically infected animals and human patients (D'Ávila et al., 2018; Gutierrez et al., 2009). Others have reported transmission of *T. cruzi* via blood transfusion and transplantation of infected organs obtained from asymptomatic individuals (Leiby et al., 2000; Schmunis and Cruz, 2005; CDC, 2007; Angheben et al., 2015). The reactivation of acute parasitemia in chronic individuals, following immunosuppression after heart transplantation (Campos et al., 2008; Gray et al., 2018), AIDS (Cordova et al., 2008; Lattes and Lasala, 2014) or drug therapy (D'Almeida et al., 1996; Ribas et al., 2016), illustrates that parasites persist for years after initial infection. It is, thus, accepted by the research community that parasite persistence provides consistent antigens that continue to trigger immune responses, cause pathologic tissue injury leading to cardiac inflammation and fibrosis, and, subsequently, cardiac insufficiency (Fonseca et al., 2018).

Our work suggests that Chagas myocardium sustains oxidative stress associated with mitochondrial dysfunction. A functional decline in the respiratory chain and increased generation of reactive oxygen species (ROS), coupled with an inability to efficiently scavenge mitochondrial free radicals, predisposed the heart to oxidative insult during infection and disease development (Garg, 2005; Zacks et al., 2005; Wen and Garg, 2008; Wen and Garg, 2010; Lopez et al., 2018). The sustained occurrence of oxidative adducts was shown in the myocardium of experimental models of Chagas disease (Wen et al., 2008; Wen et al., 2004; Dhiman et al., 2012; Paiva et al., 2018) and in peripheral blood of rodents and human Chagas subjects (Wen et al., 2006; de Oliveira et al., 2007; Dhiman et al., 2008; Dhiman et al., 2009) that was exacerbated by inefficient antioxidant capacity (Wen et al., 2004; Wen et al., 2006; Wen et al., 2006; Perez-Fuentes et al., 2003; Wen et al., 2017; Wan et al., 2016; Dhiman et al., 2013). Recent studies demonstrating ROS signaling of cytokine and chemokine production in infected cardiomyocytes and murine hearts (Gupta et al., 2009; Ba et al., 2010; Wen et al., 2010; Barroso et al., 2016) have provided a potential mechanistic link between ROS generation and chronic inflammation in Chagas cardiomyopathy.

Readers interested in further understanding the complexity of Chagas pathogenesis for all stages of infection and disease are referred to excellent recent reviews (Pinazo et al., 2015; Nunes et al., 2018).

2. Immunity to *Trypanosoma cruzi* infection

The innate immune system is the first to actively elicit a defensive role followed by a cascade of components from the adaptive immune response. Briefly, innate immunity is comprised of an anatomic barrier, physiologic boundary, phagocytic cells and inflammatory components. Skin and mucosal surfaces are anatomic barriers that prevent foreign materials initial entry, while the complement system provides a physiologic defense. Macrophages and neutrophils are the primary phagocytic cells which consume pathogenic organisms. Dendritic cells are also capable of phagocytosis and operate as antigen presenting cells during an adaptive immune response. Basophils and mast cells are responsible for initiating an inflammatory response due to damage or infection. Eosinophils have both inflammatory and phagocytic properties specific to the removal of parasites. Natural killer cells are important for the destruction of infected cells and provide the cytokine interferon-gamma (IFN-γ). The adaptive immune response utilizes two cell types: antigen specific T cells and B cells. Antigen presenting cells signal the B cells differentiation into plasma cells that generate antibodies. Dendritic cells, macrophages, and B cells are able to present antigens to CD4^{+} and CD8^{+} T cells. CD8^{+} T cells directly destroy infected cells, while CD4^{+} help amplify the immune response by releasing cytokines. Cytokines and chemokines secreted by innate and adaptive immune cells are responsible for cell recruitment, cell-to-cell communication, and facilitating pathogen clearance by various mechanisms (Marshall et al., 2018).

T. cruzi interacts with different arms of the host immune defense in a complex manner and is capable of establishing a persistent infection. Recent reviews have discussed how parasite evades classical, lectin and alternative complement pathways to establish infection (Acevedo et al., 2018; Ferreira et al., 2014; Lidani et al., 2017). Studies in a variety of experimental models showed that *T. cruzi* surface glycoproteins (mucins) and/or glycophospholipids (GIPLs) are recognized by innate immune cells and stimulate the production of multiple cytokines, e.g., IFN-γ, TNF-α, IL-1β, and IL-6; and chemokines, e.g., MCP-1, RANTES/CCL5, and IP-10 (interferon gamma-induced protein 10) in macrophages (Almeida et al., 2000; Aliberti et al., 2001; Almeida and Gazzinelli, 2001; Buscaglia et al., 2004; Fonseca et al., 2019). These cytokines/chemokines induce macrophage production of superoxide ($O_2^{\bullet-}$) and nitric oxide (NO) that are important free radicals for direct killing of *T. cruzi* (Martins et al., 1998). However, these free radicals are produced at low levels, and not sufficient to clear the infection (Koo and Garg, 2019). Others showed that IFN-γ promoted infection of astrocytes by *T. cruzi* Colombian strain (Silva et al., 2015).

In mice acutely infected with *T. cruzi* Y strain, IFN-γ of NK origin and IL-12 of macrophage origin supported the protective adaptive immunity constituted of parasite-specific type 1 T cells (Abrahamsohn and Coffman, 1996). Parasite-specific CD4^{+} T cells assist in the control of *T. cruzi* through secretion of Th1 cytokines (IFN-γ, IL-2), amplification of phagocytic activity of macrophages, stimulation of B cell proliferation and antibody production, and differentiation and activation of CD8^{+} T cells (Brener and Gazzinelli, 1997). A strong lytic antibody response by activated B cells enhances the opsonization, phagocytosis, and complement-dependent killing of the parasites (Lidani et al., 2017; Krautz et al., 2000). Yet, B cells also manage the inflammatory/anti-inflammatory balance. Mice deficient in B cells responded to *T. cruzi* (Y strain) with an increased frequency of TNF-α producing CD4^{+} T cells and a decline in anti-inflammatory factors, including IL-10^{+}, Fox3^{+}, and IL-17^{+} CD4^{+} T cells, which ultimately led to early death instigated by toxic levels of inflammation (Gorosito Serrán et al., 2017). Humans infected with *T. cruzi* display protection through antibodies that target the parasites α-Gal epitope containing the Galα1,3-Galβ1,4-GlcNAc. This epitope is present in a majority of the mucin glycoproteins expressed on the surface of *T. cruzi* trypomastigotes. Antibodies that target the α-Gal epitope are expressed in high concentrations during acute infection, detectable during chronic phase, and are capable of killing the parasite independently of the presence of complement or immune cells (dos Santos Virgilio et al., 2014). *T. cruzi* antigen-specific CD8^{+} T cells are frequently present in infected mice and humans (Wizel et al., 1997; Wizel et al., 1998), and suggested to contribute to *T. cruzi* control through cytolysis of the infected cells and secretion of Th1 cytokines (IFN-γ) that induce trypanocidal activity (DosReis, 1997).

Some studies in chronically infected mice and patients suggest that immune exhaustion of CD4^{+} and CD8^{+} T cells contribute to reduced cytokine production and parasite persistence (Mateus et al., 2019; Pérez-Antón et al., 2018). Conversely, others used mice chronically infected with Brazil, TCC or Colombian strains of *T. cruzi* to show that CD8^{+} T cells in infected organs were immunologically non-exhaustive; and concluded that CD8^{+} T cell exhaustion was the unlikely culprit for long-term parasite persistence (Pack et al., 2018). Other studies indicate that too much of IFN-γ, IL-17, TNF-α cytokines correlates with tissue damage and clinical disease in chronically infected patients (Chevillard et al., 2018). Recent studies have also identified a role for interleukin 27 (IL-27) in Chagas disease. IL-27, composed of epstein-Barr virus-induced gene 3 (EBI3) and IL-27p28, is a member of the IL-12 cytokine family, expressed by antigen presenting cells, and it induces differentiation of diverse T cell populations and also upregulates IL-10. In human Chagas patients, IL-27 expression was significantly associated with cardiac protection. In mice chronically infected with *T.*

cruzi Y strain, IL-27 regulated the proinflammatory IFN-γ and inhibited cardiac muscle inflammation (Medina et al., 2017).

To sum up, these studies indicate that an efficient protective response to *T. cruzi* infection requires elicitation of Th1 cytokines, lytic antibodies, and the concerted activities of phagocytes, T helper cells, and cytotoxic T lymphocytes; and an absence of either of these components can result in parasite persistence associated pathologic events contributing to cardiomyopathy and heart failure in the host.

3. Vaccine development against *T. cruzi* and Chagas disease

Given the detailed knowledge about the status of anti-*T. cruzi* protective immunity discussed above, a successful vaccine against the parasite is envisioned to elicit lytic antibody response, type 1 cytokines, and cytotoxic T lymphocytes (dos Santos Virgilio et al., 2014; Gupta et al., 2019). It is generally accepted by the research community that a vaccine should include antigens targeting all stages of the parasite and should be useful as a prophylactic and therapeutic vaccine (Dumonteil et al., 2019; Bustos et al., 2019; Quijano-Hernandez and Dumonteil, 2011). Computational modeling studies show that a vaccine against *T. cruzi* would be economically viable and provide net cost savings, even with stringent simulation parameters of 1% infection risk, treatment efficacy of 25%, and a cost of $20/dose (Lee et al., 2010; Lee et al., 2012). With an efficacy of >50% and infection risk of 20%, vaccine costing even >$200 would also be economically advantageous (Lee et al., 2010; Lee et al., 2013). A safe, effective, and reliable vaccine will also alleviate the costs for vector control and drugs used to dispel this infection in endemic countries (Ma et al., 2015).

3.1. Live and attenuated whole organism vaccines

The proponents of the live and attenuated vaccine believe that such a vaccine will provide a full spectrum of antigenic epitopes for stimulation of protective immunological responses. Early efforts utilized whole parasites killed by chemical, physical or irradiation methods or sub-cellular fractions (e.g., flagellar, soluble, or parasite membrane fractions) for their prophylactic vaccine potential in various animal models, including mice, guinea pigs, dogs, and monkeys. While chemically treated *T. cruzi* provided no protection from lethal challenge infection, other approaches generated a degree of resistance to *T. cruzi* and a large percentage of immunized animals survived from acute infection (reviewed in Bhatia et al., 2009; Vazquez-Chagoyan et al., 2011; Rodriguez-Morales et al., 2015). Live vaccines, constituted of *T. cruzi* strains attenuated by treatment with pharmacological agents or by serial passage in cultures have also been tested for their potency in experimental animals (reviewed in Sanchez-Valdez et al., 2015). With the advancement of CRISPR and other technologies to knockdown genes in trypanosomes, recent efforts have also focused on utilizing the genetically modified *T. cruzi* knocked down in one or more virulence genes as live vaccines. These live vaccines were largely effective in controlling challenge infection; vaccinated animals exhibited decreased parasitemia and increased survival rates compared to the unimmunized animals (Quijano-Hernandez and Dumonteil, 2011). Further efforts will determine if a live vaccine protective against one lineage of *T. cruzi* would be efficacious in providing protection against infection from the other five lineages of *T. cruzi*. Some researchers also remain concerned that an attenuated live vaccine may trigger a full-blown infection and disease in immunocompromised individuals (Sanchez-Valdez et al., 2015).

Phytomonas serpens, a tomato parasite that shares antigens with *T. cruzi* but is non-pathogenic to humans, has also been used as a potential prophylactic vaccine. Authors noted that mice immunized with *P. serpens* followed by challenge infection with lethal dose of *T. cruzi* Y strain (5×10^3 or 10^5 trypomastigotes) exhibited a significant decrease in parasitemia and mortality (Bregano et al., 2003). Basso and colleagues have promoted the use of *Trypanosoma rangeli,* which shares significant sequence homology with *T. cruzi* and infects triatomines and mammals but does not cause disease in humans, as a prophylactic vaccine (Basso and Marini, 2015). Indeed, mice immunized with glutaraldehyde-fixed *T. rangeli* epimastigotes were protected from challenge infection with a virulent isolate (Tulahuen) of *T. cruzi* (10^2 trypomastigote inoculum) (Introini et al., 1998). In our studies, we found that glutaraldehyde fixed *T. rangeli* itself did not offer protection from challenge infection in mice and dogs. Including fixed *T. rangeli* with a subunit DNA vaccine consisting TcG2 and TcG4 did not enhance the vaccine's efficacy in mice (Gupta et al., 2019). However, DNA vaccine (consisting TcG1, TcG2 and TcG4 subunits) adjuvanted with fixed *T. rangeli* provided significant protection against Sylvio X10/4 strain of *T. cruzi* that is highly pathogenic in dogs (Aparicio-Burgos et al., 2015).

Overall, we believe that the concerns in the use of *T. rangeli* or *T. cruzi* based attenuated vaccines appear to outweigh the benefits offered by whole organism vaccine in the context of Chagas disease.

3.2. Selection of T. cruzi antigens for subunit vaccines

Early studies utilized *T. cruzi* cDNA expression libraries and performed immune screening with sera from infected animals or human patients to detect the antigenic candidates (referenced below). This approach identified the candidates that were recognized by anti-parasite antibodies but may or may not consist of epitopes for T cell activation. Subsequently, Garg et al. (1997) utilized recombinant *T. cruzi* expressing a model antigen (chicken ovalbumin) in different cellular compartments to demonstrate that parasite GPI- anchored proteins (glycosylphosphatidylinositol), that are abundantly expressed in infective and intracellular stages of *T. cruzi* and released in host cell cytoplasm during parasite differentiation, are the most likely source of peptides for immune activation of B and T cells. Accordingly, several surface proteins of *T. cruzi* that belong to the abundantly expressed surface antigens of the large families, e.g., trans-sialidase (TS) super family (737 genes), mucins (662 genes), mucin-associated surface proteins (MASPs, 944 genes), and glycoprotein 63s (GP63s, 174 genes) (El-Sayed et al., 2005) have been selected as vaccine candidates. For example, among the members of the TS superfamily, trypomastigote surface antigen (TSA1, 85 kDa), TS, and amastigote surface proteins (ASP1, ASP2, ASP9 (ASP-2 like clone 9)) were shown to be recognized by antibody response and CD8^{+} T lymphocytes in infected mice and humans (Wizel et al., 1997; Wizel et al., 1998; Low and Tarleton, 1997; Pereira-Chioccola et al., 1994; Ribeirao et al., 2000; Santos et al., 1997; Boscardin et al., 2003; Low et al., 1998). Mucins contain a large proportion (85%) of O-glycosidic-linked carbohydrates (Schenkman et al., 1993), and GPI component of GP35/50, SSP3 and other mucins was noted to be a powerful inducer of polyclonal B cells, inflammatory cytokines and macrophages (Bhatia et al., 2009; Acosta-Serrano et al., 2001; Cardoso et al., 2013; de Paulo Martins et al., 2010). Other antigens that do not belong to large families, but nevertheless were tested for their antigenic potential include complement regulatory protein (CRP or gp160) (Norris et al., 1994), a lysosomal cysteine proteinase (cruzipain, 60 kDa) (Fonseca et al., 2005), flagellar calcium binding protein (FCaBP or Tc24, 24 kDa) (Godsel and Engman, 1999, Krautz et al., 1998), GP90 (90 kDa) and GP82 (82 kDa) (Yoshida et al., 1993), kinetoplastid membrane protein (KMP-11, 11 kDa) (Trujillo et al., 1999; Maranon et al., 2001), LYT1 (61 kDa) (Fralish and Tarleton, 2003), paraflagellar rod proteins (70–86 kDa) (Wrightsman et al., 2002; Michailowsky et al., 2003), and TC52 (52 kDa) (Fernandez-Gomez et al., 1998). These antigens were shown to be recognized by antibodies and IFN-γ-producing CD8^{+} T cells in experimental models of *T. cruzi* infection and/or Chagas subjects. These *T. cruzi* surface membrane proteins are discussed in recent reviews (Pech-Canul et al., 2017; De Pablos and Osuna, 2012).

We have employed a computational algorithm to screen the *T. cruzi* sequence database for desirable B and T epitopes (Bhatia et al., 2004; Bhatia and Garg, 2008), and then performed a biological screen to

Table 1

Properties of some of the *T. cruzi* antigenic targets.

Antigen	Model	Recognition by immune components	References
TSA-1	C57BL/6 mice, Human (PBMC's)	MHC-I, CD8^{+} T cell, HLA-A2	Wizel et al. (1997), Wizel et al. (1998)
ASP1	C57BL/6, C3H/HeSnJ mice, Human (PBMC's)	MHC-I, CD8^{+} T cell, HLA-A2	Wizel et al. (1998), Santos et al. (1997), Low et al. (1998)
ASP2 (ASP-2-like clone 9)	BALB/c, C3H/HeJ, C57BL/6 mice, Human (PBMC's)	CD4^{+} Th1, CD8^{+} CTL, HLA-A2	Wizel et al. (1998), Boscardin et al. (2003), Barbosa et al. (2013), Ribeiro et al. (2019), Pereira et al. (2015)
MASP	BALB/c mice, Human (sera)	B cells, CD8^{+} T cell, IgM, IgG2A, IgA	Serna et al. (2014), Durante et al. (2017)
Tc24 (FCaBP)	BALB/c, ICR mice	Th1, IgG2b	Krautz et al. (1998), Sanchez-Burgos et al. (2007), Dumonteil et al. (2004), Taibi et al. (1993), Martinez-Campos et al. (2015)
TSA-1 & Tc24	Human (PBMC's)	HLA-A, HLA-B	Villanueva-Lizama et al. (2018)
CRP (gp160)	BALB/c, C3H/HeJ mice, Human (sera)	Th1, IgG, Complement mediated lysis	Norris et al. (1994), Sepulveda et al. (2000)
Cruzipain	Human (PBMC's)	HLA-A2, CD8^{+} T cell	Fonseca et al. (2005)
Cruzipain	BALB/c mice	Th2, CD19^{+}	Guiñazú et al. (2004)
Cruzipain	C57BL/6 mice	Th1, CD4^{+} TGFβ^{+}	Guiñazú et al. (2004)
TcG1, TcG2, & TcG4	C57BL/6 mice	Th1, IgG2b/ IgG1	Bhatia and Garg (2008)
Gp63	NA	NA	Cuevas et al. (2003)
Gp35/50	BALB/c mice anti-sera	No response	Yoshida et al. (1993)
Gp82 & gp90	BALB/c mice anti-sera	Complement mediated lysis, CD4^{+} T cell	Yoshida et al. (1993)
Ssp3	NA	NA	Frevert et al. (1992)
KMP-11	Human (sera)	IgG1, IgG3, IgG4	Flechas et al. (2009)
LYT1	C57BL/6 mice	CD8^{+} CTL	Fralish and Tarleton (2003)
PFR1, PFR2	C57BL/6, BALB/c mice	CD8^{+} CTL	Wrightsman et al. (2002)
PFR2 or PFR3	BALB/c mice, Human (PBMC's)	CD8^{+} CTL, HLA-A	Egui et al. (2012)
Tc52	ICR mice	NA	Sanchez-Burgos et al. (2007)

Abbreviations: ASP2 - amastigote surface protein 2; CRP - complement regulatory protein; CTL - cytotoxic T lymphocyte; HLA - human leukocyte antigen (type A and type B); Ig - immunoglobulin isotypes; MASP – mucin-associated surface protein; MHC-1 - major histocompatibility complex 1; PBMCs - peripheral blood mononuclear cells; Tc24 - trypomastigote excretory–secretory protein 24; TSA - trypomastigote surface antigen; Th1 - T helper type 1; Ssp3 - stage-specific antigen 3; KMP - kinetoplastid membrane protein; PFR (1, 2 or 3) - paraflagellar rod protein (1, 2 or 3); NA - not available.

identify those that were recognized by IgGs and elicited type 1 CD8^{+} T cell response in infected mice, dogs, and humans (Bhatia et al., 2004; Bhatia and Garg, 2008; Gupta et al., 2013; Aparicio-Burgos et al., 2011; Bhatia et al., 2004; Garg and Bhatia, 2005). Of the 11 antigens thus selected, TcG1, TcG2, and TcG4 passed additional criteria for vaccine design. The three candidate antigens were (a) expressed (mRNA/protein) in mammalian stages of *T. cruzi* (Bhatia et al., 2004; Bhatia and Garg, 2008), (b) released in host cell cytoplasm during parasite differentiation (Bhatia et al., 2004; Bhatia and Garg, 2008), and (c) consisted of epitopes presented by MHC alleles of mice, dogs (Aparicio-Burgos et al., 2015; Aparicio-Burgos et al., 2011), and humans (Gupta et al., 2013). Notably, TcG2 and TcG4 were conserved in five of the six *T. cruzi* lineages (80–96% homology), providing confidence that TcG2/TcG4-based vaccine would provide protection against diverse *Tc* isolates in the US and Latin America (Gupta et al., 2019; Bhatia et al., 2004). Immune properties of some of the antigenic targets selected for vaccine efficacy studies are listed in Table 1.

3.3. Subunit vaccines

Several investigators have utilized a protein immunization approach to test the vaccine potential of *T. cruzi* antigens (Wizel et al., 1997; Low et al., 1998; Serna et al., 2014). Others favored the DNA immunization approach due to the ease of construction and production of the vectors, the stability of DNA and the ability to enhance the immune response by the co-delivery of genes encoding cytokines (Gupta et al., 2019; Bhatia and Garg, 2008; Aparicio-Burgos et al., 2011; Vasconcelos et al., 2003). Most importantly, antigen delivery by DNA vaccination has proved to be efficient in eliciting antibodies, Th1 cytokines and CD8^{+} T cell immune responses to encoded antigens (Aparicio-Burgos et al., 2011; Gupta and Garg, 2015). Efforts were also made to enhance the protective efficacy of recombinant protein and DNA vaccines by use of alternative routes of antigen delivery, by

increasing the amount or the number of doses, and heterologous prime-boost strategies (Gupta and Garg, 2015). In parallel with the efforts towards testing of vaccine candidates, adjuvants were tested to enhance or skew the immune responses toward desirable Th1 type. Accordingly, studies have examined the utility of IL-12, GM-CSF, CD40, HSP70, and CpG oligodeoxynucleotides (ODN-CpG), among others, in enhancing the Th1 responses to defined antigen vaccines (Corral and Petray, 2000). Several reviews have discussed the vaccine potential of antigenic candidates in experimental models (Vazquez-Chagoyan et al., 2011; Rodriguez-Morales et al., 2015; Arce-Fonseca et al., 2015). Herein, we discuss in brief the past literature and add the updates as relevant.

A majority of studies have tested the efficacy of genes/proteins as prophylactic vaccine in murine models (summarized in Table 2). A few studies that have tested the therapeutic immunization are discussed where relevant, and also mentioned in Table 2. Some investigators tested immunization with a single gene/protein, and others have utilized multi-component candidate vaccines. The ability of the individual or mixed genes to elicit protection from challenge infection depended upon the amount of the given recombinant protein or antigen-encoding plasmid sufficient to elicit protective B and T cell responses. For example, we have tested various dilutions of *ASP1, ASP2,* and *TSA1* (0.001-33 μg/mouse), and showed that the level of resistance to *T. cruzi* infection correlated with the amount of DNA delivered, the maximal protection being obtained with 1 μg (or higher) amount of each candidate with IL-12 and GM-CSF cytokine adjuvants (Garg and Tarleton, 1998). Yet, the level of protection from *T. cruzi* infection induced in mice immunized with these antigens (individually or in combination) (Fralish and Tarleton, 2003, Garg and Tarleton, 2002) was not sufficient to offer sterile immunity and no protection was observed in mice immunized with a pool of genes encoding mucin family members (Fralish and Tarleton, 2003). Researchers postulated that genes of large families express shared epitopes that when delivered

Table 2
Subunit vaccines' efficacy against *T. cruzi* and Chagas disease (modified and adapted from Vazquez-Chagoyan et al., 2011).

Antigens	Prophylactic or Therapeutic	Adjuvant	Experimental Model (*T. cruzi* strain), acute, intermediate or chronic	Parasite burden (location)	% Survival (days post-infection)	References
Recombinant DNA or recombinant protein						
PFR1, PFR3	Prophylactic	iFreund	C57, BALB/c mice (Peru & Y), chronic	Decrease[B] (blood)	100 (17[A])	Wrightsman et al. (2002)
TSA1	Prophylactic	IL-12 / GM-CSF	C3H, C57 mice (Brazil), acute & chronic	Decrease[B] (heart, skm)	60 (140)	Wizel et al.(1998, Garg and Tarleton (2002)
ASP1	Prophylactic	IL-12 / GM-CSF	C3H, C57 mice (Brazil), acute & chronic	Decrease[B] (heart, skm)	60 (140)	Garg and Tarleton (2002)
ASP2	Prophylactic	IL-12 / GM-CSF	C3H, C57 mice (Brazil), acute & chronic	Decrease[B] (heart, skm)	80 (140)	(Garg and Tarleton, 2002)
ASP1, ASP2, TSA1	Prophylactic	IL-12 / GM-CSF	C3H, C57 mice (Brazil) acute & chronic	Decrease[B] (heart, skm)	80 (126)	Garg and Tarleton (2002)
ASP2 (ASP-2-like clone 9)	Prophylactic	Complete Freund	BALB/c, C57 mice (Y)	Decrease[B] (blood)	100 (60)	Boscardin et al. (2003)
TcG2, TcG4	Prophylactic	IL-12 / GM-CSF	BALB/c mice (Sylvio), acute	Decrease[B] (heart, skm)	66.7 (60)	Hegazy-Hassan et al. (2019)
LYT1	Prophylactic	IL-12 / GM-CSF	C3H, C57 mice (Brazil), chronic	NA	80 (75)	Fralish and Tarleton (2003)
TCβ3	Prophylactic	IL-12 / GM-CSF	C3H, C57 mice (Brazil), chronic	NA	40 (75)	Fralish and Tarleton (2003)
FCaBP (Tc24)	Prophylactic	IL-12 / GM-CSF	C3H, C57 mice (Brazil), chronic	NA	0 (65)	Fralish and Tarleton (2003)
PFR2, PFR3	Prophylactic	HSP70	BALB/c, C57 mice (Y), chronic	NA	100 (40)	Morell et al. (2006)
TcG1	Prophylactic	IL-12 / GM-CSF	C57 mice (Sylvio), acute, intermediate, chronic	66% Decrease[B] (heart)	100 (30-120)	Bhatia et al. (2004), Bhatia and Garg (2008)
TcG2	Prophylactic	IL-12 / GM-CSF	C57 mice (Sylvio), acute, intermediate chronic	50% Decrease[B] (heart)	100 (30-120)	Bhatia et al. (2004), Bhatia and Garg (2008)
TcG4	Prophylactic	IL-12 / GM-CSF	C57mice (Sylvio), acute, intermediate, chronic	90% Decrease[B] (heart)	100 (30-120)	Bhatia et al. (2004), Bhatia and Garg (2008)
CRP (gp160)	Prophylactic	iFreund	BALB/c, C3H mice (Y) acute, chronic	Decrease[B] (blood)	100 (40)	Sepulveda et al. (2000)
Tc24	Prophylactic	MPLA	BALB/c mice (H1), acute	Decrease[B] (blood & heart)	50 (50)	Martinez-Campos et al. (2015)
SCz DNA prime / rCz boost[C]	Prophylactic	CpG-ODN, GM-CSF, MALP-2	C3H mice (RA), acute, chronic	Decrease[B] (blood)	100 (30)[D]	Cazorla et al. (2008)
Galα3LN-HSA[E]	Prophylactic	LMPLA	C57 mice (Y), acute	Decrease[B] (heart, lung, spleen, skm)	80, 100 (32)[F]	Portillo et al. (2019)
TcG1, TcG2, TcG4 (DNA prime / DNA boost)	Prophylactic	IL-12 / GM-CSF	Canine (Sylvio), acute, chronic	No change[B] in acute heart, Decrease[B] in chronic heart	67 (60-365)	Aparicio-Burgos et al. (2011)
TcG1, TcG2, TcG4 (DNA prime / protein boost)	Prophylactic	IL-12 / GM-CSF	C57 mice (Sylvio), acute, chronic	Decrease[B] (heart, spleen, liver, kidney, skm)	NA	Gupta and Garg (2010)
TcG2, TcG4 (DNA prime / protein boost)	Prophylactic	IL-12 / GM-CSF	C57 mice (Sylvio), acute, chronic	Decrease[B] (blood, spleen, heart, skm)	NA	Gupta and Garg (2015)
TcG1, TcG2, TcG4 (DNA prime / T. rangeli boost)	Prophylactic	IL-12 / GM-CSF	Canine (Sylvio), acute, chronic	Decrease[B] (blood)	100 (60-365)	Aparicio-Burgos et al. (2015)
TcSP, TcSSP4	Prophylactic		Canine (Ninoa), acute, chronic	Decrease[B] (blood)	100 (65)	Arce-Fonseca et al. (2013)
TcG2, TcG4 (DNA prime/T. rangeli-boost)	Prophylactic		C57 mice (Sylvio), acute	Decrease[B] (heart, skm)	NA	Gupta et al. (2019)
Recombinant virus						
rAd5-Tc80	Prophylactic	CpG-ODN	BALB/c & C3H mice (Tulahuen, RA, K98), acute, chronic	Decrease[B] (heart, skm)	80-STc80, 67-Pboost (23)	Bivona et al. (2018)
Flu-ASP2 (DNA prime / Ad5 boost)[G]	Prophylactic		C57, C3H mice (Y)	Decrease[B] (blood)	80, 0 (50)[H]	Barbosa et al. (2013)
rAdVax[I]	Therapeutic & Prophylactic		C57 mice (Colombian, CL-Brenner), acute, chronic	Decrease[B] (heart)	87 (230)	Pereira et al. (2015)
Ad5ASP-2[J]	Therapeutic		A/Sn mice (Y), acute	Decrease[B] (liver)	NA	Ribeiro et al. (2019)
Immunotherapeutic vaccine						
TSA1	Therapeutic		BALB/c mice (H4), acute	Decrease[B] (blood)	>70 (45)	Dumonteil et al. (2004)
TSA1	Therapeutic		ICR mice (H1), acute, chronic	Decrease[B] (blood, heart)	100 (20-80)	Zapata-Estrella et al. (2006)
Tc24 (FCaBP)	Therapeutic		CD1 mice (H1), chronic	Decrease[B] (blood, heart)	100 (140)	Dumonteil et al. (2004)
TSA1[K]	Therapeutic	Alum	ICR mice (H1), acute	Decrease[B] (blood, heart)	70 (50)	Sanchez-Burgos et al. (2007)
Tc24 (FCaBP)[K]	Therapeutic	Alum	ICR mice (H1), acut	Decrease[B] (blood, heart)	85 (50)	Sanchez-Burgos et al. (2007)
Tc52[K]	Therapeutic	Alum	ICR mice (H1), acute	Decrease[B] (blood, heart)	75 (50)	Sanchez-Burgos et al. (2007)

Table 2 (continued)

Antigens	Adjuvant	Prophylactic or Therapeutic	Experimental Model (*T. cruzi* strain), acute, intermediate or chronic	Parasite burden (location)	% Survival (days post-infection)	References
TcG2, TcG4 (DNA prime / protein boost)	IL-12 / GM-CSF	Therapeutic	CD1xC57 mice (Sylvio), chronic	Decrease[B] (blood, skm, heart)	NA	Gupta et al. (2015)
Tc24	CpG-ODN	Therapeutic	BALB/c mice (H1), acute	Decrease[B] (blood, heart)	NA	Barry et al. (2016)
Tc24	E6020	Therapeutic	ICR mice (H1), chronic	Decrease[B] (blood)	NA	Barry et al. (2019)

Abbreviations: ASP2 - amastigote surface protein 2; CRP - complement regulatory protein; Tc24 - trypomastigote excretory–secretory protein 24; TSA - trypomastigote surface antigen; PFR (1, 2 or 3) - paraflagellar rod protein (1, 2 or 3); MPLA - monophosphoryl-lipid A; LMPLA - liposomal-monophosphoryl lipid A; TcSSP4 - gene encoding an amastigote-specific surface protein in *T. cruzi*; TcSP - gene encoding a member of the trans-sialidase family in *T. cruzi*; C57 – C57BL/6; C3H – C3H/HeN; skm – skeletal muscle; NA - not available.

Key:

A = mean survival time >120 days

B = compared to that detected in infected mice that were not vaccinated or were injected with the plasmid vector only

C = *Salmonella* carrying Cz-DNA priming and recombinant cruzipain (rCz) boosting

D = *Salmonella* carrying Cz DNA (SCz) only, no adjuvants used

E = Galα1,3Galβ1,4GlcNAc (Galα3LN), also known as the α-Gal epitope, coupled to the carrier protein human serum albumin (Galα3LN-HSA)

F = Galα3LN-HSA + LMPLA (80% survival), Galα3LN-HSA (100% survival)

G = ASP2 medial (Flu-M-ASP2) and carboxi-terminal (Flu-C-ASP2) segments

H = Flu-M-ASP2 (80% survival), Flu-C-ASP2 (0% survival)

I = rAdVax - recombinant adenovirus serotype 5 carrying sequences of ASP2 and TS *T. cruzi* antigens (rAdASP2 + rAdTS)

J = AdASP-2 - recombinant human type 5 replication-defective adenoviruses expressing *T. cruzi* Amastigote Surface Protein-2

K = Tc24 was most effective in reducing blood and heart parasite burden compared to TSA1 and Tc52

together may not offer additive protective benefits in in-bred mice. Yet, it was predicted that the potential synergistic immunologic benefit of a combination of epitopes from many genes would stimulate a larger frequency of immune effectors in heterogeneous host populations and provide efficient immunity against diverse parasite strains (Vazquez-Chagoyan et al., 2011), to be verified in future studies.

Other investigators have tested the vaccine efficacy of non-family proteins in a variety of in-bred mouse models. For example, Cazorla et al. used a *Salmonella* carrying Cz-DNA (SCz) and adjuvants (CpG-ODN, GM-CSF, MALP-2) in mice. SCz oral doses induced a mucosal immune response (IgA) but weak systemic immune response, while including a boost with recombinant CZ protein and CpG-ODN produced a strong systemic immune response (i.e., antigen-specific IgG, splenic lymphocyte proliferation and IFN-γ production) that provided significant protection from challenge infection and tissue damage (Cazorla et al., 2008).

Immunization of mice with PFR2 and PFR3 genes (encode for paraflagellar rod proteins) alone or fused to the 70 kilodalton heat shock protein (HSP70) induced high levels of anti-PFRs IgG2a; however, only the PFR2-HSP70 immunization induced a significant increase in the IL-12 and IFN-γ expression and a degree of protection against *T. cruzi* experimental infection (Egui et al., 2012; Morell et al., 2006).

Recently, prophylactic vaccine efficacy of α-Gal epitope, Galα13Galβ14GlcNAc bound to human serum albumin (HSA) was investigated. C57BL/6 mice immunized with this vaccine elicited anti-α-Gal antibody-mediated humoral reaction and were protected from lethal challenge infection with *T. cruzi* Y strain (1×10^5 parasite inoculum). Authors also observed a decline in parasite burden, T cell permeation in tissues, necrotic myocytes and cardiac inflammation in the heart of vaccinated (vs. non-vaccinated) mice (Portillo et al., 2019).

Screening of a cDNA expression library with anti-amastigote monoclonal antibodies identified FCaBP (Tc24), Tcb3 and LYT1 genes as potential antigens for vaccine design. The three gene peptides induced a response of cytotoxic T cells in chronically infected mice; however, the immunization with only *LYT1* gene protected 80% of mice from *T. cruzi* lethal challenge (Fralish and Tarleton, 2003), thus, suggesting that proteins that may be recognized by immune responses in an infected host may not always be the best candidates for vaccine development.

Other groups used recombinant adenovirus serotype 5 as vector and evaluated the protection raised by immunization of mice (C57BL/6 and C3H/HeJ) with recombinant Ad5 encoding amastigote surface protein 2 (ASP2). This immunization protocol elicited a robust expansion of CD8^{+} effector T cells producing TNF-α, IFN-γ, and a higher mobilization of surface marker CD107a that is indicative of cytolytic activity and provided significant protection against challenge infection with *T. cruzi* Y strain (Barbosa et al., 2013). Afterwards, the therapeutic effects were evaluated in mice simultaneously infected with *T. cruzi* (Y strain) and immunized by AdASP-2. A significant control of tissue amastigote nests associated with a rapid immune response characterized by the expression of inflammatory genes, such as iNOS, TNF-α, TLR-4, and IL-10 was noted in mice therapeutically vaccinated with Ad-ASP2 (Ribeiro et al., 2019). Pereira et al. (2015) also used recombinant Ad5 encoding ASP2 and TS sequences and tested their prophylactic and therapeutic efficacy in C57BL/6 mice that were acutely or chronically infected with *T. cruzi* Colombian strain. These authors found there was an increase in CD8^{+} T cells and production of IFN-γ, accompanied by a decrease in cardiac electrical alterations and cardiac tissue parasite burden in mice given prophylactic vaccine. Therapeutic treatment of chronic mice with Ad5-ASP2 / Ad5-TS enhanced survival, reduced electrocardiogram abnormalities, maintained IFN-γ concentrations, and decreased CD107a^{+} CD8^{+} T cells and plasma nitric oxide production (Pereira et al., 2015). Taken together, these results suggest promising application of ASP2-expressing recombinant virus as a prophylactic and therapeutic vaccine against acute and chronic infection.

In a recent study, Bivona et al. examined the 80 kDa prolyl

oligopeptidase (Tc80) as a novel immunogen for Chagas vaccine. Authors immunized mice with recombinant Tc80 protein and recombinant *Salmonella* encoding Tc80 (STc80) in a prime-boost approach, and showed that immunized mice elicited Tc80-specific, complement-dependent trypanolytic antibodies, splenic production of Th1 cytokines, such as IFN-γ, IL-2 and TNF-α, as well as polyfunctional CD4$^+$ T cells and cytotoxic T lymphocytes associated with significant protection from challenge infection and chronic pathology (Bivona et al., 2018).

We have followed the protective efficacy of three antigens TcG1, TcG2, TcG4 in two experimental models (mice and dogs). Studies in mice were conducted with delivery of the three antigens (individually or in combination) by several prime / boost approaches (e.g., DNA/ DNA, DNA/protein, or DNA/ Modified Vaccinia Ankara (MVA)). In all cases, we showed that vaccine provided 80-90% protection from acute parasite burden and chronic myocarditis in mice (Bhatia et al., 2004; Bhatia and Garg, 2008; Gupta and Garg, 2010). The overall extent of protection was associated with elicitation of trypanolytic antibodies, Th1 cytokine response, and CD8$^+$ T cells cytolytic activity. In further studies, we also found that TcG1 is dispensable, and TcG2/TcG4-based two component candidate vaccine was highly effective (Gupta and Garg, 2015). Importantly, these studies showed an increase in inflammatory responses in the vaccinated mice immediately after challenge infection; however, chronic inflammatory infiltrate was decreased in the vaccinated mice. Each of the delivery modes had several advantages and drawbacks. For example, DNA vaccine was the simplest in design, and delivery of DNA vaccine via intradermal/electroporation route (vs. intramuscular route) was most effective in generating protective immunity to challenge infection (Hegazy-Hassan et al., 2019). Further, Hegazy-Hassan et al found that the use of a single intradermal electroporation dose of 10 μg of TcG2 and TcG4 DNA vaccine without adjuvants, induced a protective immune response reducing the amount of DNA and eliminating the need for boost immunizations. DNA-protein vaccine was very complex-requiring two doses of six DNA-encoding plasmids and two doses of three recombinant proteins (Gupta and Garg, 2015). In contrast, MVA can accommodate multiple foreign genes in its genome, can be administered by a variety of routes, has an excellent safety record, and was shown to generate immune responses to a variety of foreign antigens (Meseda et al., 2002). MVA itself can act as an adjuvant to signal the innate immune system and boost T cells (Climent et al., 2011).

As in mice, dogs immunized with TcG1, TcG2, and TcG4- based vaccine also showed significant protection from challenge infection and acute myocarditis (Bhatia et al., 2004; Bhatia and Garg, 2008; Aparicio-Burgos et al., 2011). Others have shown the TcSP and TcSSP4 encoding DNA vaccine induced anti-parasite IgG2 and IFN-γ-producing lymphocytic cell proliferation in dogs. Upon challenge infection, *TcSP-* and *TcSSP4*-immuninzed dogs exhibited a moderate control of tissue parasites and electrocardiographic aberrations (Arce-Fonseca et al., 2013).

3.4. Therapeutic vaccines against Chagas disease

The overall basis for therapeutic vaccines is to modulate or enhance the multiple effector mechanisms against *T. cruzi* so as to clear the parasite's persistence in the infected host. Several candidate antigens (e.g. TSA-1, TS, Tc52, Tc24 and ASP-2) all belonging to trans-sialidase family, have been tested for therapeutic efficacy in acutely or chronically infected mice. When tested in mice immediately after infection or within two weeks post-infection, *Tc52, TSA-1 and Tc24* DNA vaccine decreased the parasitemia and mortality from infection (Sanchez-Burgos et al., 2007; Dumonteil et al., 2004) that was associated with a vaccine- induced rapid increase in the number of CD4$^+$ and CD8$^+$ T cells (Zapata-Estrella et al., 2006). However these same candidates did not arrest cardiomyopathy in chronically infected mice or infected dogs

(reviewed in Quijano-Hernandez et al., 2008). Others showed that therapeutic delivery of ASP2 and TS (individually or in combination as DNA vaccine) did not limit parasitemia or improve the survival rate in infected mice (Sanchez-Burgos et al., 2007; Dumonteil, 2007) despite the fact that these antigens have exhibited excellent efficacy as a prophylactic vaccine. Some studies also observed an increase in myocarditis in infected mice inoculated with TSA1 DNA vaccine (Zapata-Estrella et al., 2006).

In a recent study, Barry et al. (2016) have explored the potential for a nanoparticle, therapeutic vaccine. Authors encapsulated Tc24 protein in poly(lactic-co-glycolic acid) nanoparticle and used the nanoparticles to immunize mice that were previously infected with a highly lethal *T. cruzi* H1 strain (5 × 10^4 trypomastigotes inoculum). Mice immunized with Tc24 nanoparticles exhibited a Th1 immune response with an increase in splenic antigen-specific IFN-γ, IgG2A, and CD8$^+$ T cells. There was also a visible reduction in inflammatory cell infiltrate and parasite load in tissues (Barry et al., 2016). In another study, the same group utilized Tc24 recombinant protein along with E6020 adjuvant to immunize mice chronically infected with *T. cruzi* H1 strain (500 trypomastigotes inoculum) and showed that 60% of therapeutically vaccinated mice had untraceable parasitic levels, accompanied with a decrease in cardiac fibrosis (Barry et al., 2019).

In our experience, a therapeutic approach focused on the control of the parasite burden is not sufficient to arrest the progression of chronic disease, and immune therapy against parasite persistence should be adjuvanted with other agents to prevent cardiac damage. It is shown that repeat challenge infection of mice enhanced the immune responses but did not provide protection from parasite persistence and cardiac damage in chronically infected mice (Rosas-Jorquera et al., 2013). We noted that infected mice and rats, treated with chemotherapeutic drug (benznidazole) during the indeterminate phase-controlled parasite persistence; however, failed to avert cardiac remodeling and deterioration of ventricular contractility. Instead, maximal benefits were obtained when infected mice and rats were treated with benznidazole, in conjunction with PBN (phenyl-alpha-tert-butyl nitrone) antioxidant to prevent free radical-mediated oxidative insult and mitochondrial deficiencies. This resulted in the preservation of oxidative phosphorylation and left ventricular contractile activity in Chagas hearts (Wen et al., 2010). Likewise, we noted a better efficacy of a TG2/TcG4-based therapeutic vaccine in infected mice that overexpressed glutathione peroxidase and were capable of controlling the oxidative damage caused by mitochondrial deficiencies of electron transport chain of antioxidant response (Gupta et al., 2015). In comparison, wild type infected mice continued to mount cardiac damage after TG2/TcG4 therapeutic vaccine (Gupta et al., 2015). We, therefore, propose that therapeutic vaccines designed to achieve a rapid, short-lived stimulation of type 1 cellular immunity to attack persistent parasites, but prevent cellular injury, along with adjunct therapies capable of controlling the onset of oxidative insult and mitochondrial deficiencies, would prove to be maximally beneficial in preserving cardiac structure and function in Chagas disease.

4. Summary and future directions

An immune therapy that will control the *T. cruzi* transmission and chronic Chagas cardiomyopathy is urgently needed. In last few decades, significant research efforts have led to the development of several experimental vaccines that have shown promising results in small animal models of *T. cruzi* infection and Chagas disease. We call for significant level of academic-public-private partnership to support future scientists who will test the current and new vaccines against *T. cruzi* in large animal models and seek further development of the best possible immune therapy to prevent human Chagas cardiomyopathy.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2019.105168.

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