Immunization with the recombinant surface protein rTcSP2 alone or fused to the CHP or ATPase domain of TcHSP70 induces protection against acute Trypanosoma cruzi infection

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Introduction

Chagas disease or American trypanosomiasis is a neglected disease that affects more than 16 million people in Latin America. Over 300,000 patients become newly infected every year, and approximately 21,000 individuals die annually [1]. In the United States [2] and several European countries, Chagas disease is becoming an emerging public health problem as a consequence of the immigration of infected people from endemic countries [3]. Chagas disease is caused by the hemoflagellate protozoan parasite Trypanosoma cruzi, which infects a wide variety of mammals, including humans [4]. T. cruzi infection is transmitted mainly by blood-feeding triatomine bugs, but it can also be transmitted from mother to child as well as through blood transfusions, organ transplants and the ingestion of contaminated food or fluids [Reviewed in 5]. Initially, the infection caused by T. cruzi presents with a short acute phase with nonspecific clinical symptoms that may last between eight and sixteen 8 and 16 weeks; this phase is characterized by an elevated burden of parasites in the bloodstream and tissues. This is followed by a relatively long asymptomatic phase known as the indeterminate phase. The disease becomes clinically evident decades after the initial infection, with 30-40% of infected people developing a chronic chagasic cardiomyopathy and, to a lesser degree, complications of the digestive system known as mega-colon and mega-esophagus [6,7].

Currently, Benznidazole and Nifurtimox are considered the drugs of choice to treat this illness. However, these drugs are not effective in chronically infected individuals and parasites that are naturally resistant to chemotherapy have been reported in several regions of Latin America [1,8]. Therefore, the lack of effective chemotherapy has prompted the development of new strategies to prevent infection; one such alternative approach is the development of protective vaccines. Although the immune response associated with protection in Chagas disease is complex and not fully understood, experimental evidence has indicated that the activation of CD4+ and CD8+ T cells during infection and the presence of specific IgG2a antibodies are required to confer protection [9-11].

Several research groups have used various antigens, such as immunogens, to immunize animals and study the development of experimental Chagas disease. These immunizations were carried out at the level of DNA or recombinant proteins, showing varying levels of protection (parasitemia) and survival, and in most cases a Th1-type immune response was observed. In experimental models, parasite-derived antigens such as ASP-1, ASP-2, CRP, cruzipain, Tc52, PFR, ToA-like surface protein, trans-sialidase, TSA-1 and LYT have been shown to induce effective protective immunity against T. cruzi infection [12-24]. DNA immunization is effective in priming antigen-specific memory B cell but the delivery of vaccine candidates as recombinant proteins is generally more effective at eliciting antibody responses and may directly stimulate antigen-specific memory B cells to differentiate into antibody-secreting cells, resulting in high titers of antigen-specific antibodies [25,26].

Keywords: Chagas disease; Trypanosoma cruzi; rTcSP2; Recombinant proteins; Vaccine

Abstract

A surface protein (TcSP2) of Trypanosoma cruzi was evaluated alone or fused to the chaperone (CHP) or ATPase (ATP) domains of heat shock protein 70 (TcHSP70) as a vaccine candidate in a murine model for experimental acute T. cruzi infection. BALB/c mice were immunized with the recombinant proteins rTcSP2, rTcSP2-CHP, or rTcSP2-ATP and infected with blood trypomastigotes. rTcSP2 and rTcSP2-ATP induced IgG1, IgG2a, and IgG2b isotypes (mixed Th1-Th2), and rTcSP2-CHP induced IgG2b>IgG2a>IgG1 isotypes, with an IgG2b/IgG1 ratio > 1 (Th1). After immunization and parasite challenge, a 75% decrease in parasitemia was detected in mice immunized with rTcSP2 or rTcSP2-CHP, and a 50% decrease was observed with rTcSP2-ATP. Survival was 100% for animals immunized with rTcSP2 and 75% for those immunized with rTcSP2-CHP or rTcSP2-ATP. Before the parasite challenge, the recombinant proteins promoted increased serum levels of cytokines interleukin (IL)-2, IL-10 and interferon (IFN)-γ but not IL-4, indicating a Th1-type cellular immune response; these levels increased after the challenge. Histological staining revealed decreased heart tissue damage and little inflammatory cell infiltrate in animals immunized with rTcSP2. The above result indicates that rTcSP2, alone or fused to the TcHSP70 domains, is a potential candidate for the development of a vaccine against Chagas disease.
A strategy currently being investigated for vaccine development is the use of fusion proteins using the 70-kDa heat shock protein (HSP70) as the carrier. HSPs are chaperone proteins that assist in protein folding and translocation and are induced under stress conditions [27]. Certain HSPs are potent inducers of innate and antigen-specific immunity. They activate dendritic cells and macrophages to produce proinflammatory cytokines and chemokines [28,29], activate natural killer cells, increase presentation of antigens to effector cells and augment T cell and humoral immune responses against their associated antigens [30]. In this regard, it has been found that immunization with antigens genetically fused to HSP70 induces long-lasting humoral and cellular immune responses in the absence of adjuvants [31,32]. It has been shown that antigen-HSP70 fusion proteins induce antigen-specific CD8+ cytotoxic T lymphocytes (CTLs) that protect mice against tumor development [33,34]. This ability to elicit CD8+ CTLs is conferred by a 200-amino acid protein domain (within the ATPase domain) of HSP70 and is independent of CD4+ cell response [35].

Moreover, several reports using different parasite antigens such as Knp11 and Pfr-2 fused to full-length or specific domains of \( T. cruzi \) HSP70 (TcHSP70) have demonstrated that TcHSP70 behaves as an immunomodulator capable of directing the immune response towards a Th1 response and of inducing antigen-specific. This response has been reported to improve the elimination of \( T. cruzi \) [36-38].

Previous results in our lab have shown that mice immunized with a 5315-bp DNA molecule (GenBank: HQ642765) selected from a \( T. cruzi \) genomic expression library were partially protected against parasite challenge, with a 75% reduction in parasitemia and 100% survival (unpublished results). The DNA sequence has two open reading frames (ORFs) that code for surface proteins (SP) that are highly homologous to parasite surface proteins TcSP1 and TcSP2 respectively. TcSP1 shows 91% identity with the surface antigen PHGST#5 (GenBank: AAC79848.2), and TcSP2 has 81% identity with the surface glycoprotein GP90 (GenBank: AAM47176.1).

For the present work, we have investigated the humoral and cellular immune response generated after mouse immunization with the recombinant protein TcSP2 either with adjuvant or fused to the Chaperone (CHP) or ATPase (ATP) domains of TcHSP70, and its ability to confer protection against acute \( T. cruzi \) infection.

Materials and Methods

Recombinant Proteins: Cloning and purification

The TcSP2 gene was amplified by PCR using genomic DNA clone A83 as the template and the primers SP2BglII-5’-CCACACAGATCTATTATGCCGCCC-3’ and SP2PstI-5’-GCAAAAGAATTCTGGACCTGGACACC-3’. The PCR reaction (25µl) contained 1µg of template, 2 mM MgCl2, 0.2 mM of each dNTP, 1X PCR buffer and 2.5 U of Taq DNA polymerase (NEB, MA, USA). PCR conditions involved 30 cycles of denaturing at 95°C for 30 s, primer annealing at 60°C for 30 s and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min. The purified PCR product was digested with the restriction enzymes Bgl II and Pst I and cloned in frame into a pRSET-B plasmid digested with the same restriction enzymes to generate the pRSETB::TcSP2 vector. DNA fragments coding for the CHP and ATP domains of the \( T. cruzi \) HSP70 gene were obtained by Pst I digestion of the pBSTcHSP70 plasmid, purified and were subsequently cloned in frame at the 3’-end of the TcSP2 gene into the Pst I-digested pRSETB::TcSP2 vector to finally generate the pRSETB::TcSP2-CHP and pRSETB::TcSP2-ATP vectors. The DH5α \( E. coli \) strain was used as a host during the cloning experiments and to propagate plasmids.

To obtain the recombinant proteins TcSP2, TcSP2-CHP and TcSP2-ATP (rTcSP2, rTcSP2-CHP and rTcSP2-ATP), \( E. coli \) BL21 (DE3) cells were transformed with the recombinant plasmids and grown in LB media to an optical density of 0.6 at 540 nm. The cells were then induced by incubation with IPTG at 25°C overnight. The cells were then harvested by centrifugation, washed in ice-cold 50 mM Tris/HCl-buffer (pH 7.5), and suspended in extraction buffer (50 mM Tris/HCl-buffer (pH 7.5), 150 mM NaCl, 10 mM MgCl2, 5 mM β-mercaptoethanol, 5 mM EDTA). After disruption by sonication, the crude extract was clarified by centrifugation at 30,000 × g for 30 min, and the His-tagged fusion proteins were purified by affinity chromatography using Ni-NTA-agarose (Quigen, Hilden, Germany) according to the manufacturer’s instructions. The collected proteins were dialyzed for 48 h at 4°C against PBS. The recombinant proteins did not contain detectable levels of endotoxin contamination as measured by the E-Toxate assay (Sigma, St. Louis, MO, USA).

TcHSP70 antibodies

The recombinant protein MBP-TcHSP70 was obtained as described previously [39], and male BALB/c mice (six to eight weeks old) were immunized with 10µg/dose. Each animal received two or three intraperitoneal (i.p.) doses of antigen every 7 days; the first immunization dose was administered in complete Freund’s adjuvant and the following immunizations were administered in incomplete Freund’s adjuvant. At the end of the immunization scheme, animals were bled to obtain immune sera.

SDS-PAGE and immunoblotting

Proteins were resolved on 12% SDS-PAGE [40], and either visualized by staining with Coomasie blue or electrophoretically transferred onto nitrocellulose membrane for immunoblotting [41,42]. Anti-Histidine antibodies (Santa Cruz Biotechnology, Inc., CA, USA) at 1:1000 dilution and anti-rTcHSP70 antibodies at 1:2000 dilution were used as primary antibodies in TBS-T (150 mM NaCl, 0.05% Tween 20, 2% skim milk, and 10 mM Tris-HCl (pH 7.4)). Bound antibodies were detected using alkaline phosphatase-conjugated goat anti-mouse IgG (Zymed Labs, San Francisco, CA) diluted at 1:5000 and the blot was developed with NBT and BCIP (Sigma, St. Louis, MO, USA).

Immunization and parasite challenge

All mice (female BALB/c mice 6-8 weeks old) were randomly assigned into control or vaccinated groups of four mice each in two independent experiments (n=8). The mice were immunized with rTcSP2 (10µg/dose) using 20µl of Titer-Max® adjuvant (1:1 mixture) (Sigma, St. Louis, MO, USA), or with either rTcSP2-CHP or rTcSP2-ATP (20µg/dose) without adjuvant. Each animal received four intraperitoneal (i.p.) doses of antigen every seven days, respectively, and control animals were administered with Titer-Max® or PBS. At the end of the immunization scheme, animals were bled to obtain immune sera. Both immunized and non-immunized mice had access to food and water \textit{ad libitum}, and two weeks after the last immunization they underwent i.p. injection of 8 x 10^7 bloodstream trypomastigotes of the RH Yucatan strain of \( T. cruzi \). Parasitemia was monitored every three days. Blood samples were collected from the tail vein to determine the number of parasites in the bloodstream, and survival rates were monitored daily. Mice were housed in a controlled environment and managed according to the
The levels of Th1- and Th2-type serum cytokines (INF-γ, IL-2, IL-10, IL-4) were analyzed in duplicate using a fluorescent bead immunoassay (Mouse Th1/Th2 cytokine FlowCytomix; Bender MedSystems, Vienna, Austria), according to the manufacturer’s instructions. Briefly, incubation in the assay buffer of the beads coated with antibodies against specific cytokines and specific anti-cytokine biotinylated antibodies was followed by washing and centrifugation. Samples were incubated with conjugated streptavidin-phycocerythrin and analyzed in a FACScalibur Flow Cytometer (BD Biosciences, CA, USA), and fluorescent intensity was calculated using the Flow CytomixPro Software (Bender MedSystem, Vienna, Austria). The concentration of each cytokine was determined by comparison with a standard curve and expressed in pg/ml. Cytokine concentrations were determined in sera from immunized mice (two weeks after the last immunization), and in sera from both immunized and non-immunized challenged mice (42 days after parasite challenge).

Histology

Hearts were isolated aseptically, rinsed with PBS, and fixed for 24 h in 4% formaldehyde in PBS (pH 7.4). Fixed hearts were embedded in paraffin, sectioned (5 µm), stained with hematoxylin-eosin, and examined by light microscopy (Nikon Eclipse E600, Nikon, Japan).

Statistical analysis

Results were expressed as means ± SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's test. The survival time was calculated by the Kaplan-Meier method. Differences were considered to be statistically significant when the p value was <0.01 or <0.05.

Results

Construction of recombinant proteins

With the aim of finding an antigen that could be used as an immunogen against Chagas disease, it was decided to consider whether the recombinant protein rTcSP2 could induce an immune response capable of controlling the acute phase of T. cruzi infection in a murine model. This 247-aa protein is encoded by the second ORF of clone A83 and has homology of 47-86% with proteins of the trans-sialidase family and 70-80% with the 85-kDa surface glycoprotein (Tc-85) of T. cruzi, and shows 81% identity with the surface glycoprotein GP90 (TriTrypDB, Kinetoplastid Genomic Resources). The DNA fragment (790 bp) that encodes rTcSP2 was obtained by PCR from clone A83 and cloned in frame in the expression vector pRSETB to generate the pRSETB:rTcSP2 plasmid. This plasmid was used as a template to construct two rTcSP2 proteins fused with the Chapereone (CHP) or the ATPase domain of TcHSP70 (Figure 1A).

The affinity-purified recombinant proteins showed a single band with the expected size when analyzed by SDS-PAGE, with greater than 95% purity (Figure 1B). The identity of the fusion proteins was confirmed by immunoblotting using anti-histidine and anti-rTcHSP70 antibodies for rTcSP2 and rTcSP2-CHP/rTcSP2-ATP, respectively (Figure 1C).

Immunoglobulin isotype

To determine the isotypes of antibodies induced by immunization with rTcSP2 alone or fused to TcHSP70 domains, as an alternative to rTcSP2, anti-rTcSP2 and anti-TcHSP70 monoclonal antibodies (1:1000) were used to detect rTcSP2 and anti-rTcHSP70 polyclonal antibodies (1:2000) were used to detect rTcSP2-CHP and rTcSP2-ATP.

Figure 1: Purification and analysis of TcSP2 and recombinant proteins. A) The TcSP2 gene was amplified by PCR and cloned in frame in the pRSETB plasmid. DNA fragments coding for the CHP and ATPase domains were obtained from the TcHSP70 gene and cloned in frame at the 3’ end of TcSP2. The expected sizes of the recombinant proteins rTcSP2, rTcSP2-CHP and rTcSP2-ATP are 30.1, 45.0 and 66.5 kDa, respectively. B) Purified recombinant proteins were analyzed by 12% SDS-PAGE and visualized by staining with Coomassie blue. The arrows indicate the location of the recombinant proteins: MW, molecular weight marker. C) Immunoblotting of recombinant proteins. Anti-histidine monoclonal antibodies (1:1000) were used to detect rTcSP2 and anti-TcHSP70 polyclonal antibodies (1:2000) were used to detect rTcSP2-CHP and rTcSP2-ATP.
Vaccination with rTcSP2, rTcSP2-CHP and rTcSP2-ATP provides protection against *T. cruzi* challenge

To assess whether recombinant protein immunization might induce protective immunity against *T. cruzi* infection, two weeks after the last immunization mice were infected with bloodstream trypomastigotes, and parasitemia and survival rates were recorded for 42 days post-infection. When mice were immunized with rTcSP2 in the presence of adjuvant, the parasitism profile showed a 70% decrease compared to the control group (p<0.05) (Figure 3).

It has been reported that the adjuvant effect of Hsp70 results from the relatively complete, rapid, and durable activation of Ag-specific CD8+ T cells [43], and that this adjuvant effect depends on a discrete 200-amino acid sequence within the ATP domain [35]. In this context, we studied the immunomodulatory capacity of rTcSP2 fused to the Chaperone or the ATPase domain of *T. cruzi* TcHSP70. Mice immunized with the recombinant proteins rTcSP2-CHP and rTcSP2-ATP showed a 72% and a 50% reduction in parasite burden, respectively, compared to non-immunized animals (p<0.05) (Figure 4). These results point out that the Chaperone domain fused to rTcSP2 exhibited protective properties when mice were challenged with a lethal dose of trypomastigotes. This protective effect was not detected with the ATPase domain, which showed less reduction in parasitemia.

Likewise, survival rates were recorded by daily observation of mice challenged with bloodstream trypomastigotes. Mice immunized with rTcSP2 showed 100% survival compared to the control group, which had a survival of 25% (p<0.001) at the end of the experiment, and mice immunized with the fusion proteins showed 75% survival (p<0.05) (Figure 5).

**Cytokine profile before and after parasite challenge**

To ascertain whether there was a particular pattern of cytokine response, we studied the serum levels of IL-2 (Figure 6A), IFN-γ (Figure 6B), IL-10 (Figure 6C) and IL-4 (Figure 6D) before and after parasite infection, two weeks after the last immunization, four mice per group were challenged with blood trypomastigotes, and parasitemia levels were evaluated every three days. The values plotted are the mean ± standard deviation for four mice per group and are representative of two independent experiments. At the peak of infection, parasitemia levels were compared using one-way analysis of variance and Tukey's honestly significant difference test. A significant difference was detected when we compared rTcSP2 to PBS (*p<0.05).

Figure 2: Antibody isotype generated against rTcSP2, rTcSP2-CHP, and rTcSP2-ATP in immunized mice. Antibody isotypes were determined by the ELISA method with sera obtained from mice seven days after the last immunization with recombinant proteins. Immune serum dilution was 1:500, and nonspecific adsorption to plates without antigen was measured using normal serum at the same dilution. Plotted data show optical density (OD) values for four mice per group. These data are representative of at least three independent experiments.

Figure 3: Parasitemia in mice immunized with the recombinant protein rTcSP2. BALB/c mice were immunized with PBS / TiterMax or rTcSP2/Titer-Max. Two weeks after the last immunization, four mice per group were challenged with blood trypomastigotes, and parasitemia was evaluated every three days. The values plotted are the mean ± standard deviation for four mice per group and are representative of two independent experiments. At the peak of infection, parasitemia levels were compared using one-way analysis of variance and Tukey's honestly significant difference test. A significant difference was detected when we compared rTcSP2 to PBS (*p<0.05).

Figure 4: Parasitemia in mice immunized with rTcSP2-CHP or rTcSP2-ATP. BALB/c mice were immunized i.p. with either rTcSP2-CHP or rTcSP2-ATP without adjuvant, and control mice received only PBS. Two weeks after the last immunization, four mice per group were challenged with blood trypomastigotes, and parasitemia levels were evaluated every three days. The values plotted are the mean ± standard deviation for four mice per group and are representative of two independent experiments. At the peak of infection, parasitemia levels were compared using one-way analysis of variance and Tukey's honestly significant difference test. A significant difference was detected when we compared rTcSP2-CHP and rTcSP2-ATP vs. PBS (*p<0.05).
immunization) showed high serum levels of IL-2 and IL-10, low levels of IFN-γ before parasite challenge. Results before parasite challenge (two weeks after the last immunization) revealed a significant increase in serum levels of IFN-γ (p<0.05). However, the IL-2 level was detected (p<0.01) post-challenge. A large increase in IL-2 was observed in animals immunized with rTcSP2, rTcSP2-CHP or rTcSP2-ATP, which was lower compared to that in the rTcSP2 and rTcSP2-CHP groups. IL-10, a cytokine with an important role in regulating the immune response, was significantly increased in animals immunized with rTcSP2, rTcSP2-CHP or rTcSP2-ATP pre-challenge compared to non-immunized mice (p<0.01); a significant increase was detected in both rTcSP2- and rTcSP2-ATP-immunized mice post-challenge (p<0.05), but not in mice immunized with rTcSP2-CHP.

The post-challenge cytokine profile showed the presence of IL-2, IL-10 and INF-γ in immunized and infected mice, whereas a significant increase in IL-4 was detected in non-immunized and infected mice (p<0.05). These results indicate that a polarized Th1-type immune response was maintained even after parasite challenge.

**rTcSP2 immunization confers cardiac protection**

Several research groups have studied the cellular inflammatory response and cardiac tissue damage induced by *T. cruzi* infection to demonstrate the protection conferred by immunization with different antigens in murine and canine models [37,44,45]. With this aim, we evaluated tissue damage and the presence of inflammatory cells in histological sections of hearts from both immunized and non-immunized mice. rTcSP2-immunized mice showed decreased parasitemia and higher survival than control non-immunized mice. Analysis of the tissue damage caused by *T. cruzi* in animals immunized with rTcSP2 revealed slight damage and scarce inflammatory cell infiltrate (Figure 7B). Moreover, there was no observed inflammatory cell infiltrate in 50% of the immunized animals, and the tissue structure and morphology were similar to those observed in hearts from healthy animals (Figure 7A).

In non-immunized animals however, there was widespread damage and inflammatory cell infiltrate, as well as detectable inflammatory foci in some areas (Figure 7C, D). We could not detect any parasite nests, which have been described up to 15-30 days post-infection [46], although we continued observation for 42 days post-infection.

The minor damage and low inflammatory cell infiltrate in the immunized mice indicate the protective role of rTcSP2 against the acute phase of Chagas disease, which primarily affects cardiac tissue.

**Discussion**

Chagasic Cardiopathy (CC) is one of the most frequent heart conditions in the world [47,48]. CC occurs as result of a progressive inflammatory process characterized by the presence of a large number of inflammatory cells in the myocardium, where infiltration may be promoted by the cardiac tropism of the parasite or because of compromised immune tolerance [Reviewed in 49].

A pronounced immunological response in the myocardium has commonly been observed during the acute phase, causing additional damage and death in some cases [50]. Experimental studies have found that NK cells, CD8+ Th1 cells, INF-γ, macrophages, and antibodies are important in controlling parasite replication during the acute phase of infection [51-53]. In the indeterminate and chronic phases, complement fixing anti-trypomastigote clearance antibodies are hypothesized to play a protective role in maintaining latency of infection [53,54].

To date, there is no protective vaccine available against Chagas disease, although several research groups have made constant efforts to find antigens that, in the form of DNA or recombinant proteins, could be used to prevent infection [55,56]. Thus, the aim of this study was to determine the potential utility of surface protein-2 (TcSP2) as a possible vaccine antigen. Our initial hypothesis was that the immunization of mice with TcSP2 would generate a pronounced antibody response and a cytokine profile typical of a Th1-type immune response, reported as being essential for the elimination of the parasite [57,58].

The generation of different immune response isotypes is controlled by different cytokines. Th1 cytokines (IL-12, INF-γ, TNF-α) are responsible for the production of IgG2a and IgG3, whereas Th2 cytokines (IL-4 and IL-10) stimulate the production of IgG1 [59-61].

It has been previously reported that, during the subacute phase of experimental *T. cruzi* infection, polyclonal proliferation is characterized by a typical profile of IgG2a and IgG2b production in the spleen and lymph nodes of infected individuals [62]. For this reason, after mouse immunization with rTcSP2 in the presence of the adjuvant TiterMax, we determined the serum profile of generated antibodies; this profile showed high titers of IgG1, IgG2b and IgG2a (Figure 2), as previously reported with a variety of *T. cruzi* antigens used for immunization [36,39,53,63]. We selected TiterMax because it is a Th1-type modulator [64] that can induce antibody titers comparable to those induced by Freund’s complete adjuvant in rabbits, mice, and goats with minimal inflammatory responses [65].

Recently, various studies have shown that the fusion of proteins with highly immunogenic antigens such as HSPs, can modulate the
immune response towards a Th1-type response, and prospective *T. cruzi* vaccines using such fusion proteins have resulted in the induction of a strong Th1 immune response in the absence of adjuvant [36,37]. Moreover, *T. cruzi* HSP70 (TcHSP70) has proven to be highly immunogenic and is present in the sera of chagasic patients [66].

It has also been demonstrated that TcHSP70, as well as an internal fragment of 242 amino acids within the ATPase domain, can induce the maturation of dendritic cells in a mouse model [67]. Based on the immunomodulatory properties of TcHSP70 [32], we decided to fuse rTcSP2 with either the Chaperone (CHP) or the ATPase (ATP) domain of TcHSP70 to yield fusion proteins for immunization without adjuvant and determine which domain is more effective in modulating the immune response.

Our results showed that rTcSP2, as well as rTcSP2-CHP and rTcSP2-ATP, induce high antibody titers, with an IgG2a/IgG1 ratio value around 1, which suggests a mixed Th1-Th2 T cell immune response. Although the generation of IgG1 as a marker for Th2 cells is less definitive than IgG2a as a marker of a Th1-type response, the IgG2a/IgG1 ratio provides an indication of immune bias [68-71]. No such bias was detected when comparing the IgG2a/IgG1 ratio values for rTcSP2 with those obtained with rTcSP2-CHP or rTcSP2-ATP, although all three proteins induce high levels of IgG2a which were not statistical different between them.

In order to determine whether the induced T cell immune response was polarized toward a Th1-type response in animals immunized with the recombinant proteins, we quantified Th1 (IL-2) and Th2 regulatory cytokines (IL-4 and IL-10). After assessing the presence of cytokines in sera from animals immunized with rTcSP2, rTcSP2-CHP or rTcSP2-ATP we found a higher expression of IL-2, IFN-γ and IL-10, but not IL-4, with respect to control sera. Similar to these results, increased levels of INF-γ, IL-2, and IL-10 were produced by spleen cells from mice immunized with Ag163B6/cruzipain and protected against parasite challenge [72]. Previous studies have demonstrated an association between antigen-specific induction of IFN-γ responses and resistance to *T. cruzi* infection using various parasite antigens, such as paraflagellar rod protein-2, fused to the HSP70 [37], cruzipain [73], and amastigote surface protein-2 [74]. Our findings of low levels of IL-4 and increased levels of INF-γ and IL-2 indicate that immunization with the recombinant proteins induces a Th1-type cellular immune response.

We also evaluated the cytokine profile after the parasite challenge, to observe whether there was a change in the expression of cytokines in favor of a Th2 immune response. Surprisingly, we confirmed that, not only was the Th1 cytokine expression profile maintained, but there was an increase TH1 cytokine expression compared to pre-challenge levels.

Among the cytokines whose expression increased significantly after the parasite challenge were IL-10, IFN-γ, and IL-2. IL-10 is a cytokine that stimulates NK cells and promotes the recruitment of macrophages and neutrophils [Reviewed in 75], and IFN-γ is required to activate macrophages that can kill intracellular parasites like *T. cruzi* using nitric oxide (NO)-dependent mechanisms [57,76,77]. IL-2 is the major cytokine that is produced during the primary response of Th cells. Upon differentiation into one of the two types of Th effector cells, Th1 and Th2, IL-2 production declines and is replaced by production of Th1-like (IFN-γ) or Th2-like (IL-4) cytokines [78].
The crucial role of CD8\(^+\) T cells in the control of \textit{T. cruzi} infection is evident from multiple studies demonstrating the inability of mice lacking CD8\(^+\) T cells to survive infection and the ability to increase the level of resistance to infection by boosting CD8\(^+\) T cell responses [7]. Moreover, \textit{T. cruzi} is one of a number of pathogens that establish persistent infections that are controlled, but not eliminated by the action of pathogen-specific CD8\(^+\) T cells [83]. CD4\(^+\) and CD8\(^+\) T cells also secrete IFN-\(\gamma\), which is crucial for activating macrophages that exert trypanocidal activity via nitric oxide [84].

On the other hand, it is known that antibodies are very important in the control of the chronic phase of infection. Protection induced by adoptive transfer of spleen cells from chronically infected mice is prevented by depletion of B cells, and passive transfer of serum or IgG antibodies from chronically infected mice protects naive recipients [85]. To this regard, anti-rTcSP2, anti-TcSP2-CHP and anti-rTcSP2-ATP antibodies could also participate in the clearance parasites in the infected mice, which suggest that the recombinant proteins can induce lytic antibodies in naive mice. Additional experiments are needed to determine whether the recombinant proteins induced CD8\(^+\) T cell-dependent cytotoxic activity and lytic antibodies in immunized mice.

The invasion and destruction of host cells represent the initial manifestations of the damage associated with infection by \textit{T. cruzi}, and inflammation is a hallmark of the pathology of Chagas disease. The extent of inflammation in vital organs such as the heart varies during the course of Chagas disease; it tends to be mild/moderate and transient in most acute patients, and it is occasionally detected in patients in the indeterminate phase [86,87].

In order to determine the presence or absence of inflammatory cells in the heart tissue, we obtained histological sections of heart muscle from healthy mice and infected mice that were either immunized with rTcSP2-immunized or not immunized. There was an increased presence of inflammatory cells in the infected control animals, but not in animals immunized with rTcSP2; immunized animals showed minimal increase in inflammatory cells, almost similar to healthy tissue. Moreover, the structural appearance of the tissue showed no widespread damage of the infected tissue (Figure 7).

These findings certainly demonstrate the protection induced by immunization with rTcSP2, including a decrease in parasite load, increased survival and the prevention of widespread damage to heart muscle, events that are associated with the observed Th1 immune response; this response was maintained throughout the course of the infection and was probably self-regulated by controlled IL-10 production. Several cytokines, especially IL-10 and IL-4, have an important role in controlling the T helper (Th)1-predominant immune response against \textit{T. cruzi}. IL-10 may participate as an immunoregulatory cytokine in the Th1 response [88] to prevent the collateral damage generated by a strong immune response against the parasite, thus preventing unwanted excessive tissue inflammation that would otherwise be exacerbated. This has been also suggested to occur when heterologous DNA prime/protein-boost TcVac2 immunization against \textit{T. cruzi} provides immunity in a mouse model [89].

However, the precise mechanism underlying the activation of the immune system during acute infection with \textit{T. cruzi} and the ability to clear the parasite without causing collateral damage to the host is not yet fully understood, so further studies are needed to understand the precise mechanism of immune regulation.

Previous studies have established that decreases in the parasite burden and increases in survival can be considered good parameters of evaluation for potential vaccine antigens [37,55,74]. To this regard, we observed a significant reduction in the mean parasite burden (70% with rTcSP2 and 72% with rTcSP2-CHP) after the parasite challenge, whereas only a 50% decrease was observed in the group immunized with rTcSP2-ATP (Figure 3 and 4). In accordance with these results, a 100% survival rate was observed with rTcSP2 immunization, whereas both fusion proteins showed similar survival rates of 75%. Reduced parasitemia and enhanced survival have been reported after immunization with various recombinant proteins: paraflagellar rod protein [80], trans-sialidase [81], and cruzipain [82]. However, these studies used alun, CpG-ODN, and macrophage activating lipopeptide from \textit{M. fermentans} (MALP-2), respectively, as adjuvants. In this work, we did not use a non-protein adjuvant.

When proteins fused to HSP70 are used as immunogens to study antigen-specific CD8\(^+\) cytotoxic T cell responses [34,35], the effective immunization route is intraperitoneal (i.p)/subcutaneous (s.c.). In the present work we used the i.p. route to deliver the recombinant proteins, so experiments using the i.p./s.c. immunization route are needed to determine whether rTcSP2-ATP and rTcSP2-CHP are more effective in protecting mice from acute Chagas disease.

IL-2 acts through its receptor (IL-2R) to activate signaling molecules involved in cell proliferation. Although IL-2 has been characterized as a Th1-like cytokine, increasing evidence indicates that IL-2 and its downstream signaling molecule, Stat5, are also vital for the induction of anti-inflammatory Th2 cytokines [79]. Contrary to our results, when the native protein Ag163B6/cruzipain was used as an immunogen, lower levels of IL-2 were observed in immunized and challenged mice compared to animals that were only immunized, and similar amounts of IFN-\(\gamma\) and IL-10 were found in both groups of mice [72]. Our results suggest that immunization with recombinant proteins induces memory cells that are activated by the native antigen after parasite challenge.

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The use of rTcSP2 as a vaccine could be a promising immune intervention against Chagas disease. Fusing rTcSP2 with the Chaperone or the ATPase domain offers the advantage of direct use in immunization without the use of an adjuvant and polarization of the immune response towards a Th1 response.

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References


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