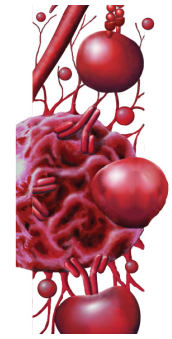
Research Article

Immunotherapy



Trans-sialidase overcomes many antigens to be used as a vaccine candidate against *Trypanosoma cruzi*

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Aim: The development of vaccines against *Trypanosoma cruzi* remains in an exploratory stage. Despite several antigen candidates have been evaluated, a comparison among the performance of the immunogens cannot be carried out because the available reports differ in formulations and infection model. In this work, we compared the protective capacity of seven *T. cruzi* antigens in the same model of five new antigens and two well-established candidates. **Materials & methods:** We evaluated highly immunogenic proteins that contain tandem repeats (FRA [flagelar repetitive protein], Tc3, Tc6); enzymes involved in metabolic pathways critical for parasite survival (cytosolic tryparedoxin peroxidase and tryparedoxin peroxidase); and enzymes involved in parasite invasion (trans-sialidase [TS] and cruzipain). All these antigens were formulated with Freund's adjuvant and protection against the parasite infection was assessed in BALB/c mice. **Results:** Tc3, cytosolic tryparedoxin peroxidase, cruzipain and TS showed the best outcome after infection in survival level and parasitemia. According to these data, these groups were also assessed using the ISCOMATRIX™ adjuvant which is being used in clinical trials. **Conclusion:** Taken together, our results showed that the TS overcomes the performance of other antigens when the same model is employed, confirming that TS is a promising antigen that could be used as a vaccine against *T. cruzi*.

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American trypanosomiasis (Chagas disease) is one of the major health problems in Latin America and is a worldwide public health problem that will remain so for the foreseeable future [1]. Currently, it is estimated that about 7.5 million people are infected [2] and in multicenter studies, the available antiparasite drugs have disappointingly shown to have a very limited efficacy in these patients [3,4]. Then vaccination approaches increasingly arise as an alternative for the prevention of Chagas disease through prophylactic control of infection [5].

Up to now, several antigens have been assessed as vaccine candidates against Chagas disease [6]. However, the use of different experimental models by different authors make it difficult to compare the performance of the antigens that could be used in vaccine design. Herein, we compare the protection capacity of different kinds of antigens, using the same murine model in order to determine which antigen has better performance. As a first approach, seven antigens were formulated with Freund's adjuvant (FA) and the protection capacity of each formulation was compared. Then, four antigens that showed better performance were selected and assessed using ISCOMATRIX™ (IMX), which is a new-generation adjuvant that has shown very promising results in several settings [7]. IMX is composed of cage-like particles that trigger

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a balanced Th1 and Th2 response, stimulating a cytotoxic cellular response that is hardly obtained with other adjuvants [7,8] and that is critical against *Trypanosoma cruzi* infection [9]. Moreover, the safety and tolerability of IMX for human application have been confirmed in a recent meta-analysis [10].

Identification and isolation of *T. cruzi* proteins that may represent targets of the immune system has been an important goal in order to obtain useful antigens for diagnosis [11]. Many of the proteins that have been analyzed contain tandem repeats (TR), which are composed of a basic structural element (the repeating unit) that ranges from 5 to 68 amino acids [12,13]. TR antigens are recognized by sera from almost every individual from Latin America [14]. This fact indicates that TR present low variability among different *T. cruzi* strains and that they are recognized by the human immune system. These are important behaviors for vaccine antigens but the use of proteins with TR has been scarcely explored in the field of vaccine development against *T. cruzi*. Notably, a repetitive antigen has been used in the formulation of RTS,S/AS01 vaccine against malaria, being the only vaccine against a human parasite that has overcome Phase III assay [15]. FRA is a flagellar antigen that is present in both epimastigotes and trypomastigotes [16,17]. It has been shown that mice immunized with FRA develop a poor humoral response [18]. However, a partial survival was obtained after challenge in a different model of *T. cruzi* infection [19], suggesting that FRA could be used in a subunit vaccine.

On the other hand, the analysis of the *T. cruzi* genome using bioinformatics allowed the identification of several proteins with TR [20]. For instance, Tc3 and Tc6 are proteins with amino-terminal TR with unknown function that have been shown to react strongly and specifically against sera from chagasic patients [20]. These antigens have not been previously assessed as vaccine candidates. The Tc3 gene (Tc00.1047053511821.179) encodes a 114-kDa polypeptide that includes 21 repeats of a 35 amino acid motif. The Tc6 gene (Tc00.1047053508119.200) encodes a 103-kDa protein that includes a repeating region composed of 14 TR of a 39 amino acid unit.

Redox enzymes of *T. cruzi* represent a group of proteins that have never been assessed as vaccine candidates. These enzymes may be of interest because they have high levels of expression in the parasite [21] and could therefore be subject to high antigen presentation in the context of MHC I and II [22]. Once *T. cruzi* has infected a mammal, it invades different cell types including macrophages, muscle cells (smooth and striated) and fibroblasts. Macrophages are one of the first cellular defenses of the innate immune response in vertebrates that play a central role in the control and evasion of the parasite [23]. Redox homeostasis is efficiently regulated in *T. cruzi*. These parasites can resist the oxidative environment during host infection using trypanothione-dependent peroxidases [24]. Cytosolic tryparedoxin peroxidase (CPX) enzymes and tryparedoxin peroxidase (TXN) enzymes play a particular role in the infectious process of *T. cruzi*, since both participate in the metabolic pathway that confers resistance to peroxides and peroxynitrites that are present in the phagosome during macrophage infection [24]. It has been shown that parasites that overexpress CPX and TXN are able to infect and multiply more efficiently in both phagocytic and nonphagocytic cells [25]. In addition, it has been shown that virulent strains have high levels of CPX and TXN as compared with attenuated strains [25].

Finally, enzymes that are secreted by the parasite and are involved in cell invasion and immune evasion represent another group of antigens that could be used as vaccine candidates. In particular, trans-sialidase (TS) and cruzipain (CZ) have achieved a high percentage of protection using different platforms and *T. cruzi* infection models [6,26]. Furthermore, we have previously reported that mice immunized with TS formulated with IMX have approximately 50-times less parasitemia and a decreased parasite load in heart in a chronic infection model [27].

To compare the protection capacity of several antigens in the same murine model is an approach that has not been performed previously in studies of *T. cruzi* vaccine development. Thus, the aim of this work was to compare the protection efficiency of different kinds of antigens in the same setting, analyzing not only proteins that have been already assessed, such as FRA, TS and CZ, but also antigens that have not been evaluated before, such as Tc3 and Tc6, CPX and TXN. By using this strategy, one antigen clearly overcomes the performance of the others, supporting its potential as a vaccine candidate against *T. cruzi* infection.

Materials & methods

Recombinant antigen & adjuvants

In this work, we used the following seven proteins: FRA, Tc3, Tc6, TPX, TXN, TS and CZ. Inactive TS was selected from a collection of mutants that was created via random mutagenesis and subsequently expressed in *Pichia pastoris* strain GS115 (*his4*) as described in Bontempi *et al.* (2015) [27]. We previously reported expression of the antigen FRA [28], TXN (TccTXNPx) and CPX (TccPx) [29], and CZ [21]. Briefly, FRA was cloned as a single repetition of nucleotides coding for 63 amino acids in pET32 plasmid (Novagen, Argentina) that also expresses a His tag and

the fusion protein, thioredoxin (TRX). TXN and CPX were cloned in the plasmid pET 28 (Novagen) as the whole proteins with a His tag. Proteins were purified from supernatant with a nickel pseudo-affinity IDA-Sepharose column (Novagen). In the case of FRA, to remove the fusion protein TRX, the recombinant protein was incubated with 2 ng of enterokinase per milligram of protein (New England Biolabs, USA) followed by purification by pseudo-affinity with nickel.

Tc3 and Tc6 expression and purification were not previously described. We cloned Tc3 and Tc6 sequences using pET-32a. Genomic DNA from CL Brener strain epimastigotes was used as a template for amplification of the selected encoded antigens, by means of standard PCR. Sequences of the primers used were as follows:

- Tc3f (5′-GAATTCGAGATCGAGCAGCTGC-GTGTG-3′);
- Tc3r (5′-AAGCTTCGCACGAAGCTCCTCCAG-3′);
- Tc6f (5′-GAATTCATGGCAACGGACGAGTTG-3′);
- Tc6r (5′-AAGCTTGAGCGCAGTCGCATCCCT-3′).

We confirmed 100% of identity of each nucleotide sequence obtained with the sequence indexed in GenBank*:* Tc00.1047053511821.179 for Tc3 and Tc00.1047053508119.200 for Tc6 by automatic sequencing in each cloning step (Sequencing Service, INTA Castelar, Argentina). Plasmidic DNA minipreparations were performed according to the procedure described by the manufacturer (Promega, USA). We harvested *Escherichia coli* cells BL21 (DE3) bearing the plasmids of interest overnight in Luria-Bertani (LB) medium with 0.1 mg ml^{-1} ampicillin at 37°C. Competent bacteria were transformed by one-pulse electroporation (2.5 kV, 25 µF) using a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Inc. USA), under the conditions specified by the manufacturer. *Escherichia coli* BL21(DE3) cells bearing the different plasmidic constructions, pET-32a/Tc3, pEt-32a/Tc6, were grown overnight in LB medium, supplemented with 0.1 mg ml^{-1} ampicillin at 37°C, with agitation. Protein expression was induced for 3 h with isopropyl-β-D-thiogalactopyranoside, washed with phosphate-buffered saline (PBS), centrifuged and re-suspended in 50 mM NaH_2PO_4 (pH 8), 300 mM NaCl, 10 mM imidazole buffer. We purified the respective Tc3 and Tc6 with an Ni-nitrilotriacetic acid column (GE, USA), as described elsewhere [28]. Briefly, once we applied supernatants into the columns, they were washed with the same buffer and eluted into different fractions, using the mentioned buffer plus 50, 100 and 250 mM imidazole, consecutively. The fusion protein TRX of Tc3 and Tc6 was removed as described previously for FRA antigen. Purity of the recombinant proteins was analyzed by 15% SDS-PAGE and staining with Coomassie brilliant blue, according to the method described by Laemmli [30]. Quantification was performed by densitometry using Gel Doc XR System (Bio-Rad Life Science, USA) and the software, Quantity One (Bio-Rad, Inc. USA) and bovine serum albumin as calibration standard being estimated to be above 95%. The presence of lipopolysaccharide (LPS) in *E. coli* expressed proteins was determined to be within 0.1–0.5 EU/10 µg of protein (the dose inoculated) determined by Limulus Amebocyte Lysate assay (Pierce™, Thermo Fisher Scientific, USA).

In this project, we used two different adjuvants, IMX (ISCONOVA, Sweden) and the complete and incomplete FA (Sigma-Aldrich, USA).

Mice

BALB/c female mice (6–8 weeks old), used in all experiment procedures, were obtained from the Centro de medicina comprada, ICIVET Litoral. Animals had free access to food and water, and were handled according to institutional guidelines.

Immunization schedules & infection protocol

Four groups of BALB/c mice (5 animals per group) were used for these experiments. We immunized them subcutaneously with the following formulations: immunization using FA, one dose with 10 µg of protein per mouse of each group (FRA, Tc3, Tc6, CPX, TXN, TS and CZ) emulsified with equal volume of complete FA and two more doses at day 14 and 28 using incomplete FA; and immunization using IMX adjuvant, three doses each 14 days with 10 µg of protein per mouse of each group (Tc3, CPX, TS and CZ) with 5 µg of IMX. In both cases we immunized two control groups, one with PBS formulated with the adjuvant and the other with PBS alone. Blood was collected on days 7, 21 and 35 postimmunization and the sera were analyzed for the presence of specific antibodies. Two weeks after the last immunization, animals were challenged intraperitoneally with 1000 bloodstream trypomastigotes of Tulahuen strain. Parasitemia was monitored by counting peripheral parasites every 7 days for 35 days in 5 µl of blood by direct microscopy. Survival was recorded daily for 100 days postinfection (dpi).

Antibody determination

Microtiter plates (Greiner Bio-One, Austria) were coated overnight at 4°C with 0.5 µg TS 0.05 M carbonate–bicarbonate buffer, pH 9.6, and incubated

overnight at 4°C. Plates were washed three-times with PBS (pH 7.4) containing 0.05% Tween-20 and then incubated with PBS with 5% bovine serum albumin for 1 h at room temperature (RT). Mouse polyclonal sera were diluted 1/1000 in PBS with 1% bovine serum albumin, and incubated for 1 h at RT. Wells were washed three-times with PBS (pH 7.4) containing 0.05% Tween-20, and then 100 µl of peroxidase-conjugated goat antimouse total IgG (1:10,000, Sigma-Aldrich, USA) was added. Optical density was read at 450 nm in an ELISA reader (Bio-Tek Instruments, USA) after 10 min incubation with 50 µl of tetramethylbenzidine in H_2O_2. For antibody subclass determination, peroxidase-conjugated second antibodies against IgG1 (diluted 1:10,000), IgG2a (1:5000, Southern Biotechnology, USA) were added after incubation with polyclonal mouse sera and then incubated for 1 h at RT. Plates were washed three-times, and the coloration was revealed as described above. Positive levels of all specific antibodies were considered with a value equivalent to at least the mean from normal control sera plus three-times the standard deviation.

Delayed-type hypersensitivity reactions

The delayed-type hypersensitivity test was performed 12 days after the last immunization by intradermal challenge. This assay was performed for the IMX formulations. Each group of mice was challenged with 5 µg of the respective protein that has been previously immunized (TS or CZ or Tc3 or CPX). Four control groups were challenged with 5 µg of TS, CZ, Tc3 and CPX protein, respectively. The thickness of hind footpads was measured before and 48 h after the injection of the antigen with a vernier caliper (Stronger, Argentina). Results were expressed as the difference in thickness of footpads after and before the inoculation.

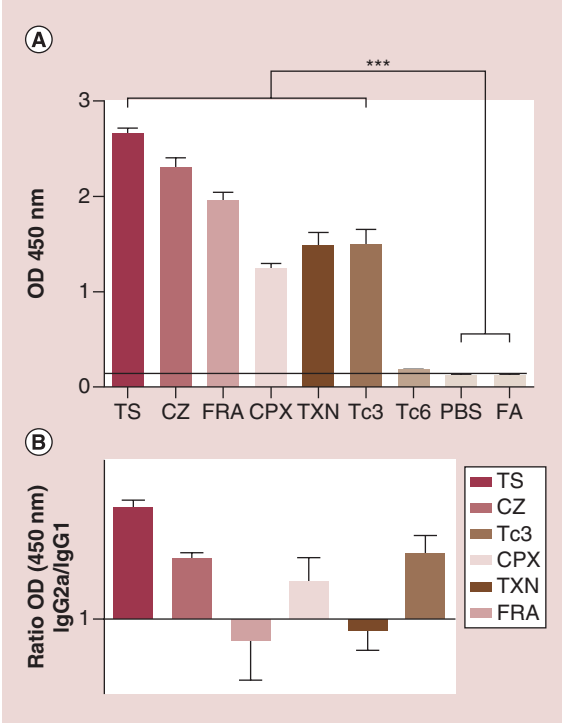

Figure 1. Humoral immune response in vaccinated mice of immunogens formulated with Freund's adjuvant.

Statistics

Data were expressed as mean ± standard deviation (n = 5 per group, representing 1 of the 3 independent experiments). Data were analyzed using nonparametric tests (Kruskal–Wallis test for k samples followed by Mann–Whitney U-test for comparisons between the two samples). Parasitemia was calculated at day 21 postinfection. Mantel–Cox log-rank test was used to evaluate survival curves. All analyses were performed using GraphPad Instat 4.0 software (GraphPad). All the comparisons were referred to control group immunized with PBS. Significance is indicated with (*) when $p < 0.05$ and with (**) when $p < 0.01$ compared between the indicated groups.

Results

Humoral response from immunized candidates

In the first round of assessment, immunogens were compared using FA. The seven immunogens tested (FRA, Tc3, Tc6, CPX, TXN, TS and CZ) elicited specific total IgG antibodies except for the Tc6 antigen, which induced a level of response comparable with the control groups FA and PBS (Figure 1A). The Tc3, CPX, TS and CZ groups of mice showed a ratio IgG2a/IgG1 higher than 1 (Figure 1B), indicating the presence of antibodies compatible with a proinflammatory immune response ($p < 0.05$). The FRA and TXN groups showed a ratio lower than 1, presenting antibodies of a regulatory type immune response.

Challenge with *T. cruzi*

Fourteen days after the last immunization, all mice were challenged with 1000 parasites of *T. cruzi*. Parasitemia and survival were measured (Table 1). The Tc3, CPX, TS and CZ groups showed a tendency to have lower parasitemia with respect to the control group. In contrast, the FRA, Tc6 and TXN groups presented higher parasitemia in comparison with control groups. Regarding survival, the Tc3, CPX, TS and CZ groups had survivals between 50 and 66%, showing a tendency to be higher than the control groups, which had a survival between 17 (FA) and 33% (PBS). However, the survivals of the FRA, Tc6 and TXN groups were between 0 and 17%, similar to the control groups.

Table 1. Parasitemia and survival in immunized BALB/c mice.

Immunization groups	Parasitemia			Survival 100 dpi (%)
	14 dpi	*21 dpi*	*28 dpi*	
FRA + *Trypanosoma cruzi*	43 (30–57)	113 (15–235)	198 (38–378)	1/6 (17)
Tc3 + *T. cruzi*	14 (4–40)	35 (7–70)	8 (0–20)	3/6 (50)
Tc6 + *T. cruzi*	60 (30–89)	202 (89–322)	45 (20–100)	1/6 (17)
CPX + *T.c ruzi*	47 (11–93)	54 (8–136)	24 (6–65)	3/6 (50)
TXN + *T. cruzi*	65 (51–92)	278 (94–394)	202 (120–402)	0/6 (0)
CZ + *T. cruzi*	10 (3–25)	37 (1–96)	5 (0–34)	3/6 (50)
TS + *T. cruzi*	8 (0–23)*	14 (0–40)*	1 (0–3)*	4/6 (67)
FA + *T. cruzi*	58 (18–98)	134 (32–322)	100 (5–187)	1/6 (17)
PBS + *T. cruzi*	68 (18–48)	131 (24–310)	60 (12–182)	2/6 (33)

Data correspond to one representative immunization and challenge experiment. FA or PBS (negative controls). Two weeks after the last injection, mice were challenged with 1000 bloodstream trypomastigotes of *Trypanosomacruzi* (n = 5 per group). Parasitemias were studied by direct microscopic observation in standard conditions. At 14, 21 and 28 dpi, 5 µl of blood obtained from the tail of infected mice were analyzed. Results were expressed as median (rank) of parasites/50 microscopic fields (400×). *$p < 0.05$.
CPX: Cytosolic tryparedoxin peroxidase; CZ: Cruzipain; dpi: Day postinfection; FA: Freund's adjuvant; FRA: Flagelar repetitive protein; PBS: Phosphate-buffered saline; TS: Trans-sialidase; TXN: Tryparedoxin peroxidase.

Re-evaluation of the selected immunogens with the IMX adjuvant

The antigens that showed the better results using the FA adjuvant were also assessed with IMX. The criteria to select the antigens were based on the following principles: to include at least one antigen of each group of proteins; and that the antigens selected elicited lower parasitemia and higher survival rate (≥50%). Thus, the Tc3 antigen was selected from the group of proteins with TR, the CPX enzyme from the group of metabolic pathway enzymes, and the TS and CZ enzymes from the group of vaccine candidates previously evaluated by several authors.

Mice were immunized with antigens with and without the IMX (Figure 2). The evaluation of the antibodies showed significant differences in all groups with respect to the control groups ($p < 0.01$). Immunogens with adjuvant triggered higher levels of antibodies in mice in comparison with the immunization with the respective antigens without using adjuvant (Figure 2, $p < 0.01$). The Tc3 immunogen showed lower level of total IgG antibody response in relation to the CPX, TS and CZ immunogens ($p < 0.05$).

The cellular response *in vivo* was assessed using the delayed hypersensitivity reaction technique (Figure 3). The groups immunized with antigens and IMX produced greater inflammation response as compared with the groups immunized without IMX or immunized with PBS ($p < 0.05$). Mice immunized with TS–IMX, CPX–IMX and CZ–IMX showed higher response as compared Tc3–IMX ($p < 0.05$, Figure 3).

Challenge of mice immunized with the selected immunogens

Parasitemia was evaluated after challenge (Figure 4). At day 21 of infection, parasitemia reached the highest values in all groups. Mice immunized with Tc3–IMX, CPX–IMX and antigens that were not formulated with the adjuvant showed higher parasitemia in comparison to the control groups (IMX and PBS, Figure 4). In contrast, TS and CZ groups had significantly lower parasitemia as compared with control groups (IMX and PBS).

Concerning the survival, an improvement was generally observed when mice were immunized with the IMX adjuvant as compared with mice immunized without the adjuvant (Figure 5). In particular, TS–IMX group showed a significant increase in survival as compared

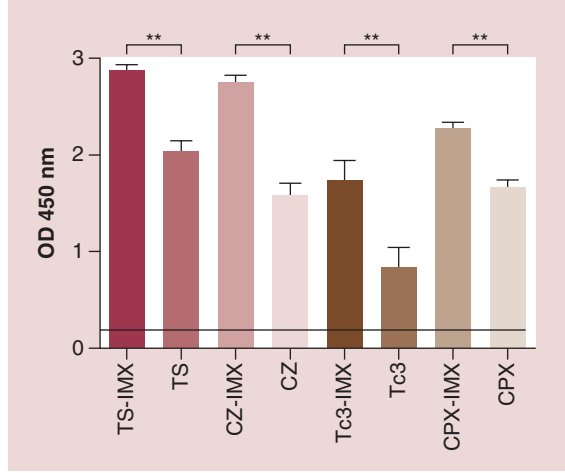

Figure 2. Humoral immune response in vaccinated mice of immunogens formulated with ISCOMATRIX™.

with TS alone and control groups ($p < 0.05$). The CZ–IMX also showed an increased survival of 80% relative to 20% of the CZ alone or PBS. The Tc3–IMX group presented a survival of 60% that was superior to the Tc3 and PBS groups, but the increase was not significant ($p = 0.333$). Survival of CPX–IMX showed no difference between the control groups. However, this group showed an increase in the time of survival rate (Figure 5).

Discussion

The selection of defined antigens for vaccine development has the advantage to avoid adverse responses to the host [31]. Furthermore, when antigens that are able to elicit protective immune responses are found, the vaccine research for a given pathogen is strongly encouraged. In this context, our purpose was to compare the protection capacity of several antigens in the same model of *T. cruzi* infection, which is an approach that has been scarcely used in previous studies. Three groups of antigens were selected for assessment: proteins with TR (FRA, Tc3 and Tc6), redox enzymes (CPX and TXN) and secreted enzymes (TS and CZ). All groups of proteins have at least one previous study that highlights the usefulness of each kind of antigen. A TR protein has been successfully used in the development of a vaccine against the parasite *Plasmodium* spp. [32]; redox enzymes have been assessed as vaccine candidates against *Fasciola hepatica* [33]; and secreted proteins have been particularly employed for the development of a vaccine candidate against *T. cruzi* [6,27,34].

Since a favorable immune response do not always translate in better protection, as has been shown, for instance, for HIV infection [35], herein the evaluation of new candidate antigens against *T. cruzi* was mainly based on survival rates and parasitemia, which are reliable indicators of protection that integrate the whole immune response when an adequate infection model is available. The subclass of specific antibodies was analyzed as a complement to infer the profile of the triggered immune response.

The initial screening of the seven antigens selected was carried out using the FA as adjuvant. TS, CZ, Tc3 and CPX groups presented lower parasitemia and higher survival rates compared with the FRA, Tc6 and TXN groups. Instead, the survival rates of FRA, Tc6 and TXN groups were similar to the control groups

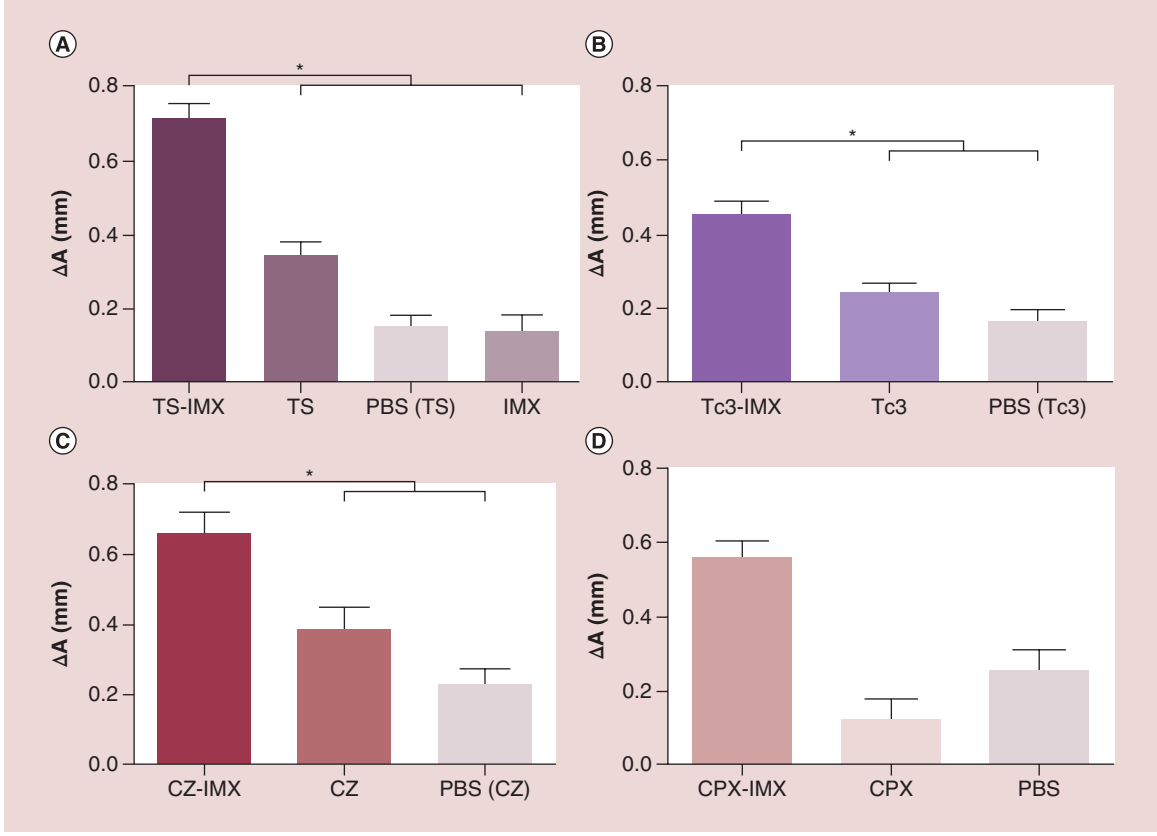

Figure 3. Delayed-type hypersensitivity test. Footpad thickness was measured before and 48 h after inoculation of 5 µg of each antigen. Results are expressed as 'delta A': the difference between the values obtained after and before inoculation. **(A)** DTH of the TS antigen. **(B)** DTH of the Tc3 antigen. **(C)** DTH of the CZ antigen. **(D)** DTH of the CPX antigen. An extra control group (IMX) were added in TS, to show the DTH from the adjuvant. Data (mean ± SD) are represent one of three independent experiments (n = 5/group). *$p < 0.05$.

(Table 1). Humoral evaluation showed that sera from all groups of mice increased the levels of total specific IgG antibodies after immunization, excepting for the Tc6-vaccinated group. Tc6 is a protein of 8 KDa which turned out to be very low immunogenic. This antigen was also evaluated fused to TRX, presenting the same results (data not shown). Subclasses of antibodies allow to infer the response profile generated by the immunogens [36]. Evaluations of the ratio IgG2a/IgG1 showed that the TS, CZ, Tc3 and CPX groups had ratios higher than 1. The IgG2a/IgG1 ratio of FRA and TXN were lower than 1. Although a previous study reported that FRA immunization elicited poor antibody response and protection capacity, another study showed that FRA immunization can induce an important increase of antibody levels. Results reported herein showed that the increase of antibodies against FRA were mainly of IgG1 isotype. Since IgG1 antibodies correlate with a Th2 profile, while a Th1 response has been shown to be necessary to cope *T. cruzi* infection, our results

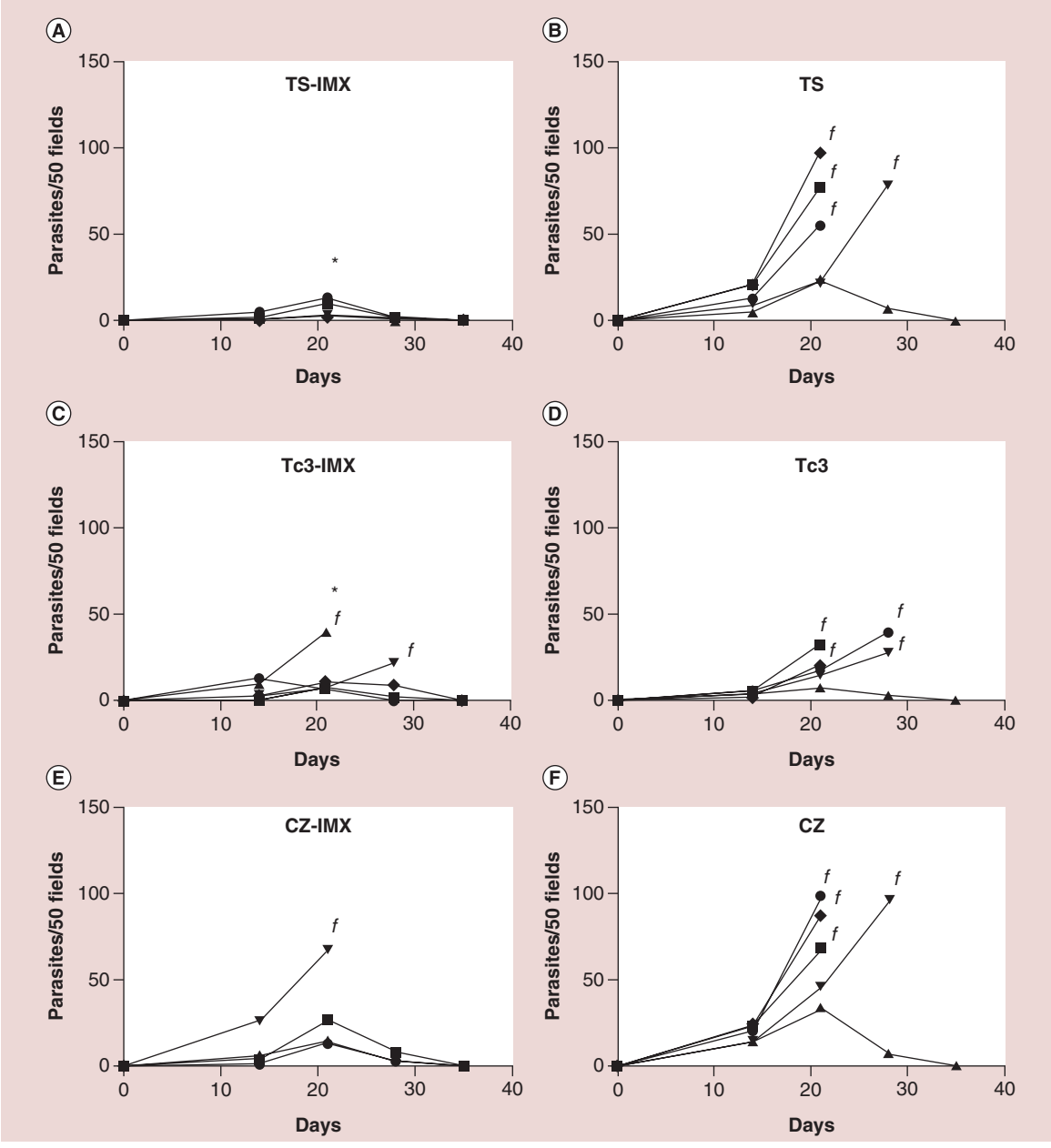

Figure 4. Parasitemiain immunized mice after *Trypanosoma cruzi* challenge. Mice immunized (n = 5/group) were challenged with 1000 trypomastigotes of the Tulahuen strain 14 days after the last immunization. Individual parasitemias from mice immunized with **(A)** TS-IMX, **(B)** TS, **(C)** TC3-IMX, **(D)** Tc3, **(E)** CZ-IMX, **(F)** CZ, **(G)** CPX-IMX, **(H)** CPX, **(I)** IMX, **(J)** PBS. f indicates animal death. The results represent one from three independent experiments. *$p < 0.05$ at 21 dpi.

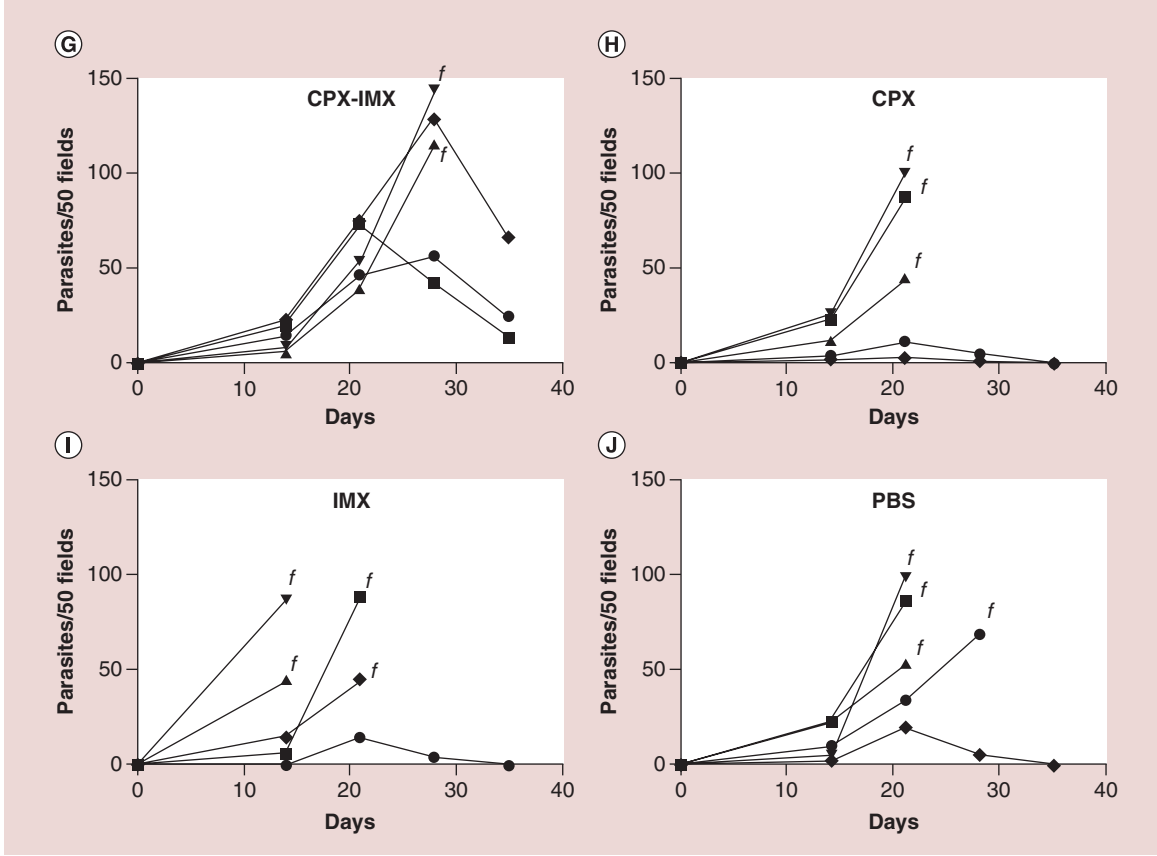

Figure 4 (cont.). Parasitemiain immunized mice after *Trypanosoma cruzi* challenge.

might explain why the increased humoral response elicited by FRA is not enough for improving protection against *T. cruzi* [19].

It is important to note that although immunization with TS formulated with FA adjuvant elicited one of the highest values of survival in our model (60%). This value was notably lower as compared with the 100% protection that was obtained by other group using a similar protocol and infection model [37]. This fact particularly shows the variability that might exist among different laboratory studies and further highlight the need of comparing protective antigen capacity in the same setting or under the most similar conditions.

It is noteworthy that TS antigen was the only one that was expressed in *P. pastoris*, while all the other antigens were expressed in *E. coli*. Taking this into account, LPS was quantified being the residual LPS amounts in the prepared doses similar or lower than 0.5 ng. This value was lower than the 2.5 ng dose that is considered low in mice [38]. Interestingly, LPS is usually used as an adjuvant enhancer of the immune response since this molecule stimulates the toll-like receptor 4 receptor [39]. In our experiments, the fact that the TS protein (without LPS) elicited higher humoral responses than the antigens with residual LPS suggests that the responses obtained were not influenced by the presence of residual LPS. In the same sense, different proteins had similar amounts of LPS per dose but have triggered different immune response.

As a strategy for vaccine design, the use of recombinant subunits formulated with suitable adjuvants is considered the safest class of vaccines by regulatory agencies and therefore this approach would greatly decrease the assessments that are required for a human vaccine candidate against *T. cruzi* [40]. In this work, subunit vaccine candidates against *T. cruzi* were formulated with an adjuvant whose use has already been approved for clinical trials. CZ, TS, Tc3 and CPX immunogens that gave the best results were formulated with the IMX adjuvant. This adjuvant is able to elicit a balanced cellular/humoral response [7], showing no toxicity in the tests performed [41]. All groups of immunized mice showed specific IgG antibodies when IMX was used. This result is consistent with the ability of IMX to elicit high levels of antibodies, even when the immunogen dose is reduced (up to 100-times) [42]. In addition, the results of delayed-type hypersensitivity experiments showed that an increase in inflammation is elicited in all groups of mice that were immunized with the antigen and IMX as compared with

the PBS group. This confirms the ability of this adjuvant to generate a specific cellular immune response with different antigens [7]. To assess the protection reached with different formulations, the lethal dose of parasites previously described was carried out and a significant decrease of the parasitemia was observed in the case of the TS–IMX and CZ–IMX groups as compared with the groups without adjuvant ($p < 0.05$ at 21 dpi). The survivals were 100 and 80% for the TS and CZ, respectively, the difference being significant only for the TS-IMX in relation to the control group ($p < 0.05$). On the other hand, mice immunized with Tc3 showed high parasitemia and low survival rates, as in the case of the evaluation with the FA (Table 1). It appears that immunization with Tc3 renders mice still vulnerable to infection. In the case of CPX, although the immune parameters were highly favorable, the survival results showed a lack of protection as compared with the TS and CZ immunogens. Furthermore, CPX presented a shift in the survival rate with respect to the control group, increasing survival for about 10 days. This difference was significant at 32 dpi ($p < 0.05$). CPX-immunized mice showed 100% survival when the control group began to die (between 15 and 30 dpi). However, the survival rates were equal after two more weeks of infection. Although a similar observation has been reported in a study that evaluated the adjuvant capacity of the Omp19 protein in a *T. cruzi* infection model [43], we cannot conclude that this type of performance generates any protection. In our experiments, the PBS and IMX control groups as well as the immunized groups without adjuvant showed high parasitemia and survival of 20%.

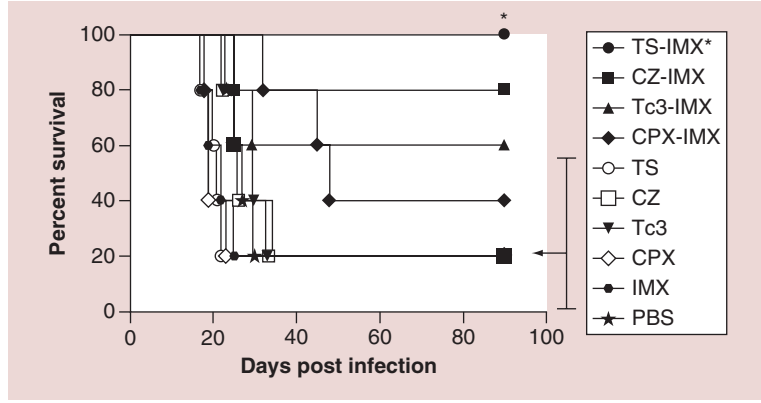

Figure 5. Survival rates in immunized mice after *Trypanosoma cruzi* challenge.

Conclusion

Based on the presented results, we conclude that the best vaccine candidate that protected against *T. cruzi* infection is the TS enzyme formulated with the adjuvant IMX. In line with other vaccine designs that also employed IMX [7], our results demonstrate that the TS–IMX formulation induces strong humoral and cellular immune responses. TS was the only one of the seven antigens evaluated that always correlated with slight parasitemia and strong protection. We have previously reported that the TS–IMX formulation elicits beneficial immune parameters during the acute and chronic stages of *T. cruzi* infection [27]. In the present work, we show that this formulation overcomes the performance of other antigens when the same model of *T. cruzi* infection is used. Further research will now be conducted to get insight into the immunological mechanism that allows for such a high protection to be reached.

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Summary points

- The protective performances of seven recombinant antigens formulated with Freund's adjuvant were compared in the same murine – *Trypanosoma cruzi* infection model. Trans-sialidase (TS), cruzipain (CZ), Tc3 and cytosolic tryparedoxin peroxidase (CPX) groups presented lower parasitemia and higher survival rates compared with the FRA, Tc6, tryparedoxin peroxidase and controls groups.
- Before *T. cruzi* challenge, the humoral evaluation showed that sera from all groups increased the levels of total specific IgG antibodies after immunization, excepting for the Tc6-vaccinated group that turned out to be very weakly immunogenic. Evaluations of the ratio IgG2a/IgG1 showed that the TS, CZ, Tc3 and CPX groups had ratios compatible with a Th1 profile.
- CZ, TS, Tc3 and CPX immunogens formulated with the ISCOMATRIX™ (IMX) adjuvant, triggered high levels of IgG antibodies and delayed-type hypersensitivity responses to the each respective antigen.
- After *T. cruzi* challenge to the groups formulated with IMX, a significant decrease of parasitemia was observed in the case of the TS–IMX and CZ–IMX as compared with the controls groups ($p < 0.05$). The survivals rates were of 100 and 80% for the TS and CZ, respectively, the difference being significant only for the TS–IMX in relation to the control group ($p < 0.05$).
- Based on the obtained results, we conclude that the best vaccine candidate that protected against *T. cruzi* infection is the TS enzyme formulated with the adjuvant IMX.

Financial & competing interests disclosure

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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