



Efficacy of a trans-sialidase-ISCOMATRIX subunit vaccine candidate to protect against experimental Chagas disease



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ABSTRACT

Recombinant protein vaccines are safe but elicit low immunological responses. The new generation of adjuvants is currently reversing this situation. Here, a new antigen–adjuvant combination for protection against experimental Chagas disease was assessed. The antigen used in the formulation was a glycosylated mutant inactive trans-sialidase (mTS) that was previously proven to be highly protective against *Trypanosoma cruzi* infection; here, we show that it can be produced in large quantities and high quality using *Pichia pastoris*. The adjuvant used in the formulation was ISCOMATRIX (IMX), which was found to be effective and safe in human clinical trials of vaccines designed to control other intracellular infections. Fifteen days after the third immunization, mice immunized with mTS-IMX showed a TS-specific IgG response with titers $>10^6$ and high avidity, an increased IgG_{2a}/IgG₁ ratio, significant delayed-type hypersensitivity (DTH) reactivity, a balanced production of IFN- γ and IL-10 by splenocytes and a strong IFN- γ secretion by CD8 $^{+}$ T lymphocytes. When these mice were challenged with 1000 trypomastigotes of *T. cruzi*, all mTS-IMX immunized mice survived, whereas mice immunized with mTS alone, IMX or PBS exhibited high mortality. Remarkably, during acute infection, when the parasitemia is highest in this infection model (day 21), mTS-IMX immunized mice had \sim 50 times less parasitemia than non-immunized mice. At this moment and also in the chronic phase, 100 days after infection, tissue presented \sim 4.5 times lower parasite load and associated inflammatory infiltrate and lesions. These results indicate that protection against Chagas disease can be achieved by a protein antigen–adjuvant mTS formulation that is compatible with human medicine. Therefore, the current formulation is a highly promising *T. cruzi* vaccine candidate to be tested in clinical trials.

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1. Introduction

Chagas disease, caused by the protozoan parasite *Trypanosoma cruzi* (*T. cruzi*), is a serious health problem in Latin America. About 8 million people are estimated to be infected, causing 11,000 deaths per year. Although Chagas disease can be treated with benznidazole or nifurtimox, both drugs have very limited efficiency in chronic patients, who may suffer some adverse reactions (occurring in up to 40% of treated patients [1]). Thus, prophylactic and therapeutic

vaccines would be suitable alternatives for preventing and treating Chagas disease.

An overall trend in *T. cruzi* vaccine research is to use delivery systems that allow MHC-I antigen presentation to trigger cellular response. Therefore, platforms using DNA delivery [2–4] or adenovirus [5] have been mainly used up to now. All of these platforms still require several years of assessment to be incorporated in human vaccines. Conversely, recombinant protein vaccines formulated with adjuvants are less objected with regard to safety, being widely accepted for use in humans by regulatory agencies [6].

Trans-sialidase (TS) family members are surface proteins encoded by more than 1400 genes, and have been classified into 8 groups. Only proteins in group 1 have shown trans-sialidase activity [7]. TSs are recognized as a key virulence factors from *T. cruzi*, and

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members of this group have shown protective properties against *T. cruzi* infection in mice (reviewed in [8]). In the field of preclinical recombinant protein-based vaccines, this antigen has presented outstanding levels of protection when administered to mice formulated with the TLR9 mouse agonist ODN 1826 [9,10]. However, the usefulness of these proteins as immunogen still raises some concerns. On the one hand, TSs have a very long immunodominant tail of Shed Acute Phase Antigen (SAPA) repeats that act as a decoy for the immune system, successfully reducing the antibody response to the catalytic site of the enzyme [11]. On the other hand, it has been described that active forms of TS may cause several deleterious effects [12–14]. To overcome these constraints, a mutant TS containing an enzymatically inactive catalytic domain, also lacking the immunodominant SAPA repeats (mTS), has been proposed as an immunogen candidate [15]. This antigen has been expressed in yeast, which results in high-mannose type glycosylation and has rendered promising results in a previous preclinical assessment. Based on these previous results, in the present work attempts have been made to enhance the yield of the mTS antigen. Furthermore, mTS purification was redesigned to be compatible with standard downstream processing (DSP), as used in industrial biopharmaceutical manufacturing, rather than the non-scalable epitope-tag affinity purification that was used in the above mentioned work [13].

A safe adjuvant for human use was selected for the present study because the previous promising study with the mTS antigen involved the non-human use complete Freund's adjuvant (CFA) [15]. Immune-stimulating complexes like ISCOMATRIX are able to produce a balanced humoral/cellular response by inducing multiple innate and adaptive mediators (reviewed in [16]). ISCOMATRIX differs from most adjuvants currently proposed for human use in that it yields particularly robust CD8⁺ T responses together with enhanced CD4⁺ T and B cell response [17]. Furthermore, ISCOMATRIX formulated vaccines were found to be safe, well tolerated, and highly immunogenic in humans [18].

Here we evaluated the protection effectiveness and safety of a new generation of vaccine candidate formulated with mTS and ISCOMATRIX in a murine model of infection with *T. cruzi*.

2. Materials and methods

2.1. Recombinant antigen and adjuvants

GlycoSwitch M5 *Pichia pastoris* strain [19] were used to obtain wild type (active) TS and mTS proteins. Yeasts were transformed with the pPICZTSjE TS expression vector or with a point-mutated version of this vector by introducing a D98E mutation in the encoded TS protein sequence, as described previously [15]. These vectors were designed for the expression of secreted C-terminally E-tagged TS protein without the C-term SAPA-repeats. The TS sequence was previously amplified from genomic DNA of Y-strain of *T. cruzi* (Genbank accession number CAC34453.1). The TS sequence and the pPICZTSjE vector were described previously [19]. Furthermore, GlycoSwitchM5 *P. pastoris* strains were also transformed with the pBLHisHAC1-AOX plasmid to complement the his4 auxotrophy, simultaneously introducing a methanol-inducible expression cassette for the HAC1p transcription factor that induces the Unfolded Protein Response [12].

For protein expression, transformed strains were grown in BMGY medium (1% yeast extract, 2% peptone, 1% glycerol, 400 µg/L biotin, and 0.1 M potassium phosphate, pH 6.0) for 48 h. Then, yeasts were induced in BMMY medium (1% yeast extract, 2% peptone, 400 µg/L biotin, 1% methanol, and 0.1 M potassium phosphate, pH 6.0), for 48 h, by adding 0.5% methanol every 12 h and subsequently collected and used for TS purification. The filtered

medium was passed over a DEAE-Sepharose column (equilibrated in 50 mM PBS pH 6.0, 2.5 mM EDTA) and the flow-through containing mTS was diafiltrated and concentrated at 40 mM NaAc pH 5.2, 2.5 mM EDTA (1/10 original volume) and subsequently allowed to bind on a S-Source column equilibrated in the same buffer. After elution using a NaCl gradient, TS-containing fractions were dialysed against 30 mM Tris-HCl pH 8.4, bound on a Q-Source column, equilibrated in the same buffer, and eluted with a NaCl gradient. Collected fractions enriched in mTS and TS were analyzed using SDS-PAGE and staining with Coomassie Brilliant Blue as previously described [20]. The wild type form of TS was used to perform ELISAs and splenocyte stimulation, because the active TS reflect better the protein expressed by the parasite. Two different adjuvants were used in this in the present work, ISCOMATRIX (Isconova, Sweden) and CFA (Sigma-Aldrich).

2.2. Mice

BALB/c female mice (6–8 weeks old) used in all experiment procedures were obtained from the Centro de Medicina Comparada, ICIVET-CONICET UNL, Argentina. All protocols for animal studies were approved by the Animal Care & Use Committee, according to the Institutional guidelines.

2.3. Immunization schedules and infection protocol

BALB/c mice ($n = 5$ /group) were used to evaluate the mTS plus ISCOMATRIX effect on the specific anti-TS antibody production. Briefly, mice were immunized with subcutaneous doses (three, one every two weeks) containing: (a) 10 µg of mTS formulated with 5 µg of ISCOMATRIX (mTS-ISMX), (b) 10 µg of mTS emulsified with CFA (mTS-CFA), (c) 10 µg of mTS without any adjuvant (mTS), (d) 5 µg of ISCOMATRIX without antigen (IMX), (e) vehicle (PBS). Blood was collected on days 7, 21 and 35 post-immunization to analyze titres of specific anti-TS antibodies.

In another series of experiments, animals were infected intraperitoneally with 1000 bloodstream trypanosomes of Tulahuen strain two weeks after the last immunization. In this case, a new control group was added (non-immunized and non-infected mice, -PBS ni-). Parasitemia was monitored every 7 days by examining 5 µl of blood by direct microscopic examination, as previously described [15]. Mice were weighed weekly and survival was recorded daily until day 100 post-infection.

2.4. Total IgG, subclass profile and avidity determination

Microtiter plates (Greiner Bio One) were coated with active TS (0.5 µg) in carbonate-bicarbonate buffer (0.05 M; pH 9.6) and blocked with PBS/5% bovine serum albumin (BSA). Half-serial dilutions in PBS/1% BSA of serum samples obtained after immunization protocol were incubated by duplicate in TS-coated wells. TS-specific antibodies were detected by incubation with goat anti-mouse IgG (1:10,000, Jackson Immunoresearch) or gG1 and IgG_{2a} (1:10,000, Southern Biotechnology). Samples were read at 450 nm in an ELISA reader (Bio-Tek Instruments) after incubation with 50 µl of ready to use trimethylbenzidine (Invitrogen). The titer for each TS-specific Ig was defined as the end-point dilution that yielded an optical density (O.D.) higher than the 1/100 preimmune serum dilution.

To measure antibody avidity, the same ELISA was also performed using six-technical replicates for each sample, diluted 1/1000. After incubation, half of the replicates were treated with urea (6 M) for 30 min and further steps were performed as described above. Antibody avidity was calculated as follows: [O.D. with urea/O.D. without urea treatment] × 100) [21].

2.5. Delayed-type hypersensitivity (DTH) test

DTH test was performed by intradermal challenge with 5 µg of mTS in the left hind footpad 12 days after the last immunization. Hind footpad thickness was measured before antigen injection and after 48 h with a Vernier caliper (Stronger). Results were expressed as the millimeters increased in footpad thickness induced by inoculation.

2.6. Spleen cell culture and cytokine determination

Groups of mice were immunized with mTS-IMX, mTS and PBS ($n=5$ /group) as described above. Fifteen days after the end of the immunization protocol, mice were sacrificed to analyze ex vivo cytokine production of splenocytes stimulated with active TS. Additionally, other groups of mice were immunized as previously described and then infected with *T. cruzi*. In this case, mice were sacrificed 15 days after infection in order to analyze ex vivo cytokine production. Briefly, spleens were aseptically harvested and homogenized. Red blood cells were lysed by addition of Lysis buffer (Sigma), and splenocytes were then re-suspended in RPMI 1640 medium (Gibco) supplemented with 10%BFS, 2% penicillin (100 µg/mL), streptomycin (100 U/mL) and 0.4 mM 2-mercaptoethanol. Thus, splenocytes (1×10^6 cells/mL/well) were cultured in 48-well plates (Nunc) in complete RPMI alone or simultaneously stimulated with active TS (10 µg/mL) or Concanavaline A (2.5-µg/mL) as positive control of stimulation. Interferon-gamma (IFN- γ), interleukin(IL)-10 and IL-4 levels in supernatants after 72 h of stimulation were determined by ELISA according to the manufacturer's instructions, (BD Biosciences). Detection limits were 10 pg/mL for IFN- γ and IL-10 and 1 pg/mL for IL-4.

2.7. IFN- γ production by CD8 $^{+}$ and CD4 $^{+}$ T cells upon ex vivo stimulation with mTS

For IFN- γ detection upon ex vivo stimulation, spleen cell culture was performed in complete RPMI 1640 (Gibco) at 1.10^6 cell/mL in a 48-well plate and stimulated with active TS (10 µg/mL) during 24 h. Subsequently, cells were incubated with 1 µg/mL of 12-myristate 13-acetate (PMA, Sigma-Aldrich), 0.5 µg/mL of ionomycin (Sigma-Aldrich) and brefeldin A (GolgiPlug, BD Biosciences) at 37 °C and 5% CO₂. After 4 h, cells were washed twice with PBS, incubated with anti-FcγIII/II receptor antibody for 30 min and stained with anti-CD8-APC-Cy7 and anti-CD4 FITC during 30 min. Then, cells were washed and resuspended in fixation/permeabilization solution (eBiosciences) during 1 h, according to the manufacturer's instructions, and subsequently stained with PE-Cy7-conjugated anti-IFN- γ Ab (eBiosciences) in permeabilization buffer. Cell acquisition was performed on FACS ARIA II flow cytometer (Becton Dickinson, San Jose, CA, USA) operating FACS-DiVa software (BD Biosciences). Living cells were gated on the basis of forward- and side cell- scatter. Data were analyzed using FlowJo Version 9.4.4 (TreeStar) software.

2.8. Tissue parasite burden and histopathology

Hearts from immunized and *T. cruzi* infected mice were removed on day 14 (acute infection) or 100 post-infection (chronic infection) and transversally sectioned in two pieces. One portion of each heart was fixed in buffered formalin and paraffin-embedded 5-µm sections were then stained with hematoxylin and eosin for evaluation of tissue parasitism and myocarditis, as previously reported [22,23]. Afterwards, an experienced pathologist (E.A.R.) blinded to experimental groups examined all sections. Briefly, myocarditis was scored as follows: normal tissue (score 0); mild foci: slight infiltration with damage of one or two myocardial fibers (score 1);

moderate-sized foci: aggregated infiltrates compromising three to five muscle fibers (score 2); and intense foci: heavy accumulation of mononuclear cells with destruction of more than five muscle fibers (score 3). In addition, hearts from chronic mice were also stained with picrosirius red staining to evaluate fibrosis, and were classified as follows: normal tissue (score 0); slight myocardial fibers (score 1); moderate myocardial fibers (score 2); intense myocardial fibers (score 3). Chronic myocarditis was scored as follows: [score of hematoxylin and eosin staining] × [score of picrosirius red staining]. The other heart portion was used for determining heart parasite burden. Briefly, total DNA (50 ng) was isolated from tissue and used as a template to perform real-time PCR on a StepOne™ thermal cycler using SYBR Green Supermix (Solis BioDyne) and TCZ-specific oligonucleotides. Data were normalized to murine-TNF- α [24].

2.9. Statistical analyses

Data were analyzed using nonparametric tests (Kruskall-Wallis test for k samples followed by Mann-Whitney U -test for comparisons between two samples). Mantel-Cox Long rank test was used to evaluate survival curves. All analyses were performed using GraphPad Instat 4.0 software (GraphPad, California, USA). Significance is indicated with (*) when $p < 0.05$ and with (**) when $p < 0.01$ compared between the indicated groups.

3. Results

3.1. Optimization of mTS antigen production

We developed an improved expression of mTS in *P. pastoris* strain and optimized an industrial manufacturing-compliant downstream processing protocol for the antigen. First, we expressed the proteins in the GlycoSwitchM5 background [19], which reduces the N-glycosylation heterogeneity as compared to the GS115 strain (glycosylation wild type) used for expression of mTS in a previous work [15]. Furthermore, the obtained expression strains were transformed with a plasmid that complemented the his4 auxotrophy and which, at the same time, introduced an expression cassette for methanol-inducible overexpression of the Unfolded Protein Response-driving *Pichia* HAC1p transcription factor [19]. Second, to overcome proteolytic degradation of the antigen, which occurred during ammonium sulfate precipitation [12], we introduced an ion exchange chromatography-based downstream process that involved bulk medium contaminant removal on DEAE-Sepharose followed by a diafiltration and two high-resolution ion exchange purification steps. All of these steps that are potentially scalable yielded a high quality protein (Fig. 1). Using this protocol we usually obtained 15–20 mg of TS per liter of culture medium.

3.2. mTS-IMX immunization elicited a strong TS-specific IgG response and an increased IgG_{2a}/IgG₁ ratio

First, we analyzed the quantitative and qualitative characteristics of the TS-specific humoral immune response elicited after three successive immunizations with mTS-IMX. Since earlier studies showed high titers of specific anti-TS after mTS-CFA immunization [15], to compare the performance of our formulation, we simultaneously performed immunizations with mTS-CFA and mTS alone. mTS formulated with IMX improved the titers of specific anti-TS antibodies ~4 times compared to mTS alone (Fig. 2A, $p < 0.01$). In addition, titers of TS-specific IgG antibodies tend to be higher in mTS-IMX group than those obtained for the group inoculated with CFA as adjuvant ($p = 0.07$). As expected, TS-specific antibodies were

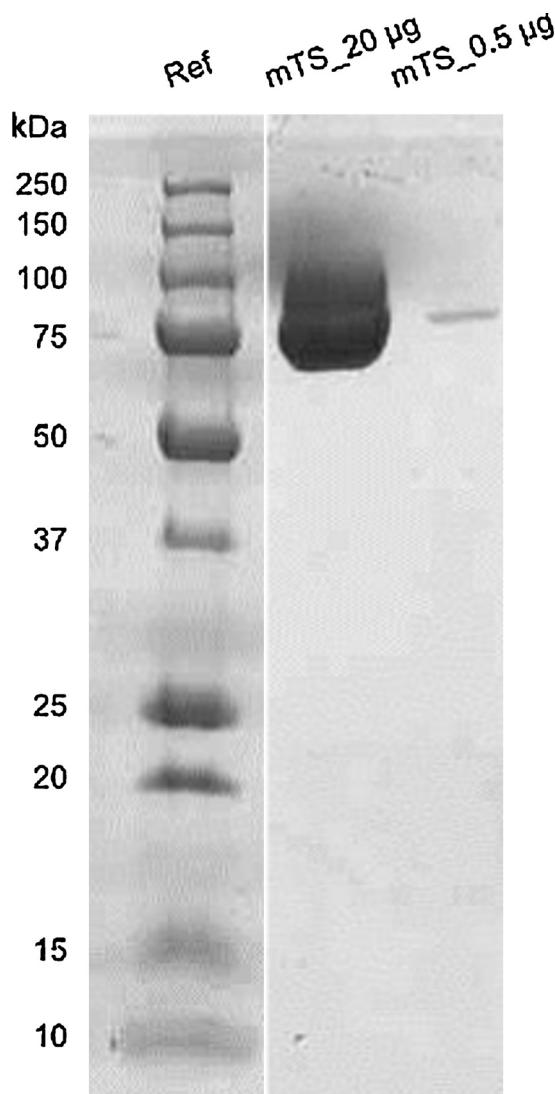


Fig. 1. mTS antigen characterization. Purified mTS (20 µg and 0.5 µg) was assessed by SDS-PAGE and staining with Coomassie Brilliant Blue. Precision Plus Protein All Blue standards (BIO-RAD) was used as reference.

not detected in IMX or PBS control groups. Interestingly, the avidity of anti-TS specific IgG antibodies was increased in mTS-IMX mice compared to mTS-CFA and mTS mice (~12% and ~50%, respectively), reaching high statistical significance in the latter ($p < 0.01$). Moreover, while mTS immunization alone caused a Th2-biased response, as evidenced by a ~2.5 times increase of IgG1 titers than of IgG2a titers (Fig. 2C, $p < 0.05$); immunization with mTS formulated with IMX caused a ~3 times increase in the titers of IgG2a compared with IgG1 titers (Fig. 2C, $p < 0.01$), indicating that mTS-IMX immunization induces a clearly Th1-biased response. Similarly to mTS-IMX, mTS-CFA caused a ~3 times increase in the titers of IgG2a compared with IgG1, however not reaching statistical significance (Fig. 2C, $p = 0.14$).

3.3. mTS-IMX immunization induced a TS-specific cell-mediated immune response and a skewed Th1-related cytokine profile

Footpad testing for DTH was performed to analyze the in vivo cell-mediated immune response elicited by mTS-IMX formulation. As shown in Fig. 3A, 12 days after the last immunization the increase in footpad thickness showed a significant cellular DTH response in mTS-CFA and mTS compare with PBS group ($p < 0.05$) being this

difference more significant for mTS-IMX group ($p < 0.01$). To confirm that mTS-IMX immunization caused a Th1-biased response, splenocytes obtained 15 days after the last immunization were ex vivo challenged with TS to evaluate IFN-γ, IL-4 and IL-10 production. Cultured splenocytes from mTS-IMX-immunized mice produced increased levels of IFN-γ and IL-10 after TS challenge as compared to splenocytes from mTS-immunized mice or PBS mice (Fig. 3B-D, $p < 0.01$). In contrast, mTS immunized mice exhibited lower levels of IFN-γ accompanied by noticeable concentrations of IL-10. As results an increased IFN-γ/IL-10 ratio was observed in the mTS-IMX compared to mTS group (~4.64 vs. ~0.39 times, respectively, $p < 0.01$). Interlukin-4 levels showed no differences between groups (data not shown).

3.4. mTS-IMX immunization triggered a marked IFN-γ secretion by CD8⁺ T and CD4⁺ T lymphocytes

IFN-γ expression by CD4⁺ Th1 and CD8⁺ cytotoxic T lymphocytes are crucial in the immune response against *T. cruzi*. To determine if mTS-IMX formulation favors the priming and activation of mTS-specific CD4⁺ and CD8⁺ T cells, we analyzed whether mTS-IMX formulation could improve IFN-γ secretion by mTS-experienced CD4⁺ and CD8⁺ lymphocytes compared with mTS or PBS immunization alone. Splenocytes from immunized mice were obtained 15 days after the last immunization. The presence of IFN-γ-producing cells in the spleen cells of immunized mice was determined after ex vivo restimulation with TS. As seen in Fig. 4A-C, after restimulation, the frequency of CD8⁺ T lymphocytes expressing INF-γ was substantially higher in mTS-IMX inoculated mice than in mTS and PBS groups ($p < 0.05$), whereas a lower IFN-γ secretion was observed in CD8⁺ T cells from mTS than in those from PBS immunized mice (Fig. 4C). Similar behavior was observed in the frequency of CD4⁺ T lymphocytes (Fig. 4B).

3.5. mTS-IMX immunization induced a strong protection against in vivo parasite challenge

To evaluate protective immunity elicited against *T. cruzi*, immunized mice were infected with a lethal dose of parasite. mTS-IMX and mTS-CFA animals showed a marked decrease in the parasitemia, which was evidently higher in mTS, IMX and PBS groups 21 days post-infection (Fig. 5A-E, $p < 0.05$). Furthermore, mTS-IMX and mTS-CFA showed no apparent systemic effects, since they revealed no changes in weight as compared to PBS-non-infected mice. By contrast, mTS, IMX and PBS mice exhibited a marked weight loss after 21 days post-infection (data not shown). Survival studies revealed that mTS-IMX immunization increased mice survival from 40% to 100% as compared to PBS group after a *T. cruzi* challenge ($p < 0.05$), whereas mTS-CFA, mTS and IMX immunized mice did not show difference from the PBS group (Fig. 5F).

3.6. mTS-IMX immunization induced high levels of IFN-γ and IL-10 secretion in acutely infected mice and protection against tissue damage

Splenocytes from mTS-IMX immunized and infected mice increased the levels of IFN-γ and IL-10 after ex vivo TS stimulation with respect to others groups (Fig. 6, $p < 0.01$). However, when IFN-γ/IL-10 ratio were compared, the values of all groups were similar. Levels of IL-4 showed low values and no differences between groups (data not shown).

To analyze whether mTS-IMX immunization attenuated tissue damage caused by *T. cruzi*, parasite load and myocarditis severity were evaluated in hearts of mice at day 14 and 100

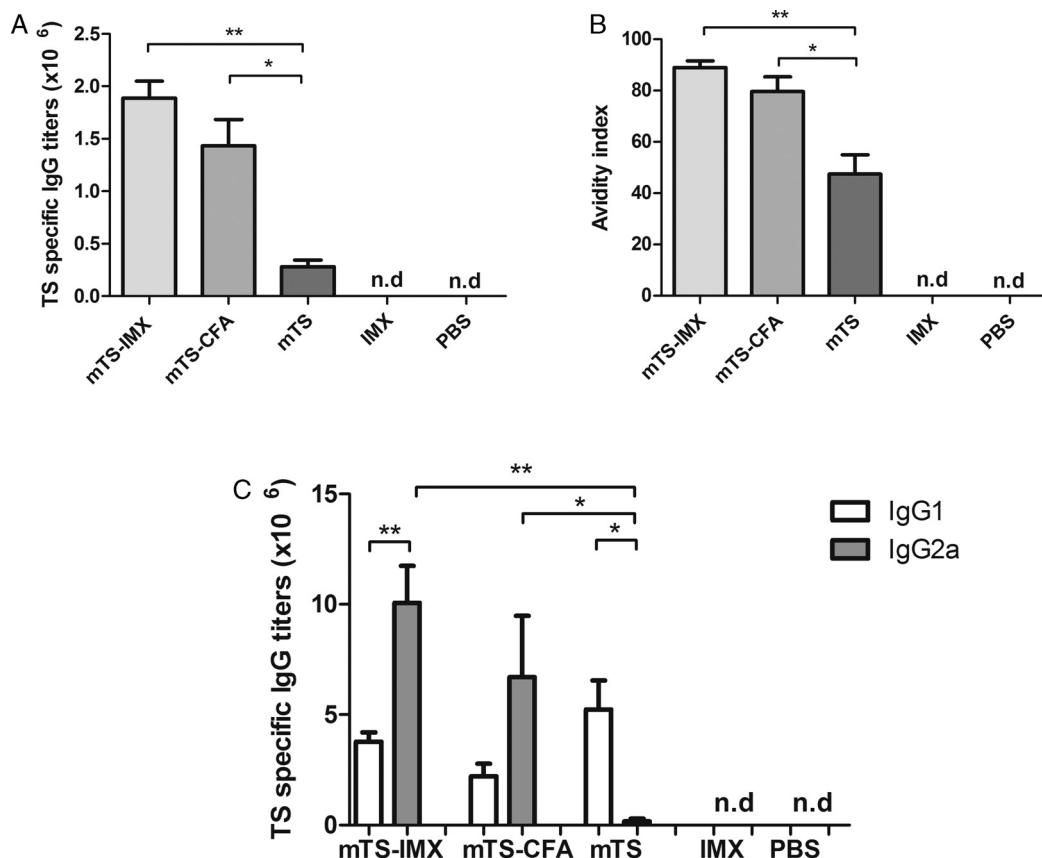


Fig. 2. Humoral immune response in vaccinated mice. Balb/c mice were immunized with mTS-IMX, mTS-CFA, mTS, IMX and PBS. Seven days after completion of immunization schedule, serum samples were analysed for TS-specific IgG, IgG1 and IgG2a antibodies titers and avidity by ELISA. (A) TS-specific IgG titers, (B) Avidity index of vaccine-induced IgGs (C) IgG1 and IgG2a subtypes titers. Results are expressed as mean \pm SD. Data shown represent three independent experiments ($n = 5$ /group). n.d. = not detected. * $p < 0.05$, ** $p < 0.01$.

days post infection. Even though the histological analysis revealed tissue infiltration and amastigote nests in all acutely *T. cruzi*-infected groups, mTS-IMX and mTS mice presented significantly fewer inflammatory foci than PBS infected controls (Fig. 7A and C, $p < 0.01$). In addition, only two parasite nests were typically observed in the slides from mTS-IMX acutely infected mice, whereas in mTS and PBS slides from acutely infected mice, six and four nests, respectively, were typically observed. Real time PCR showed a significant reduction (~4.7 times) of heart-parasite burden in mTS-IMX acutely infected mice compared to mTS and PBS infected controls (Fig. 7E, $p < 0.01$). In the chronic phase, mTS-IMX mice showed a drastic decrease of heart inflammatory infiltrates compared to PBS mice (~4 times, $p < 0.05$), which exhibited the persistence of diffused inflammatory foci (Fig. 7B, left and D). Real time PCR showed ~4.5 times of reduction of heart-parasite burden in

mTS-IMX chronically infected mice as compared to the PBS infected group (Fig. 7F, $p < 0.05$). Moreover, mTS and PBS infected mice showed an extensive fibrosis, whereas hearts of mTS-IMX infected mice only exhibited slight fibrosis when evaluated by picrosirus red staining (Fig. 7B, right).

4. Discussion

The present study evaluates the effectiveness of a new sub-unit vaccine candidate against *T. cruzi* infection. The antigen used was mTS, a TS without SAPA repeats whose enzymatic activity has been neutralized by an amino acid mutation to avoid previously described deleterious effects of the protein [19]. A higher production yield and purification degree of the mTS antigen was obtained than with the previous reported method [15], reaching

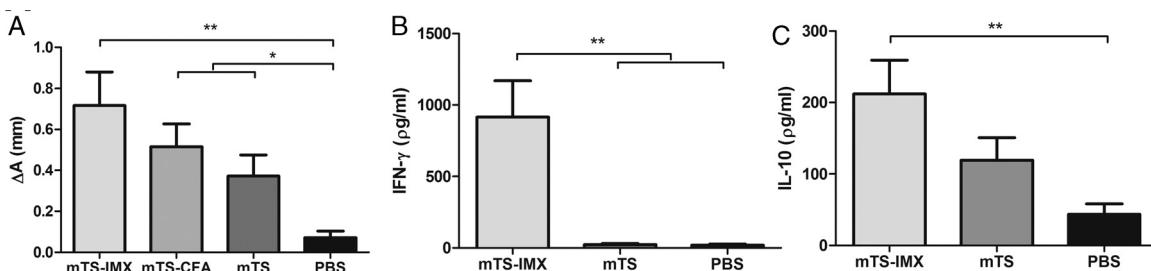


Fig. 3. Cellular immune response in immunized mice before the infection. (A) Delayed-type hypersensitivity test performed 12 days after the last immunization. (A) Footpad thickness was measured before and 48 h after inoculation of 5 μ g of mTS. Results are expressed as “delta A”: the difference between the values obtained after and before inoculation. (B and C) Splenocytes were cultured in triplicate for 72 h in the presence of TS and supernatants were assayed for: (B) IFN- γ and (C) IL-10. Cytokines were measured by capture ELISA. Data (mean \pm SD) are represent one of three independent experiments ($n = 5$ /group). * $p < 0.05$, ** $p < 0.01$.

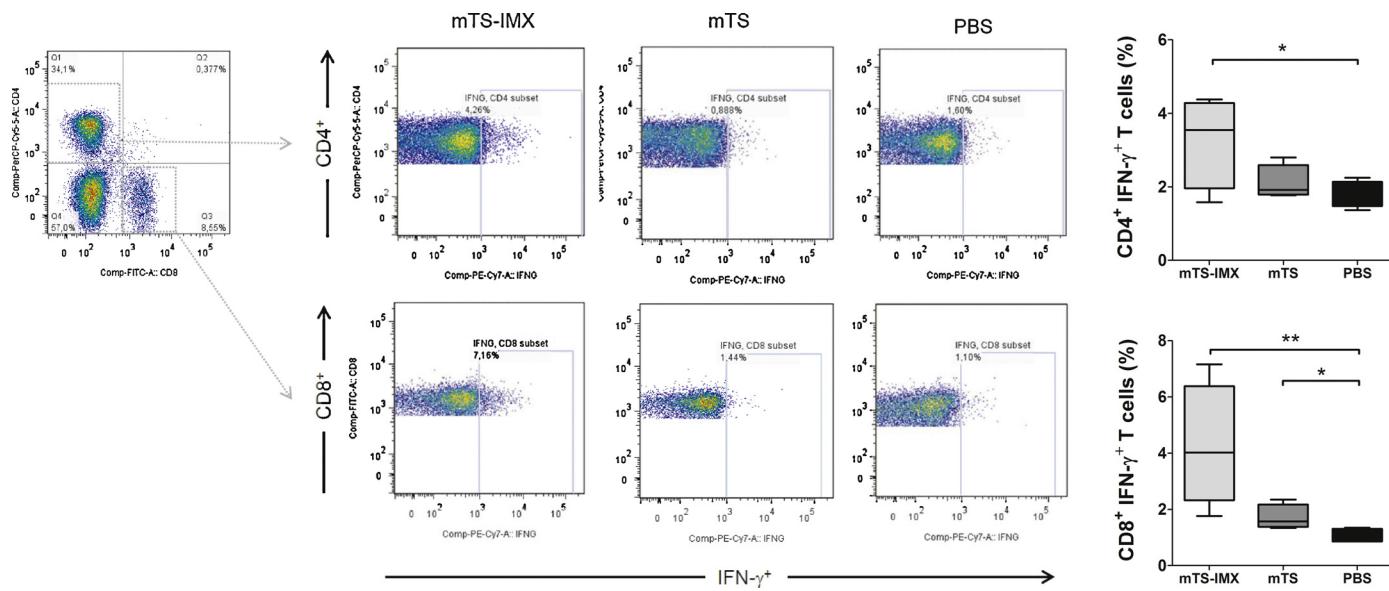


Fig. 4. mTS-IMX immunization triggered strongly IFN- γ secretion by mTS-specific CD8⁺ T and CD4⁺ T lymphocytes. Splenocytes were obtained 15 days after immunization and restimulated during 24 h ex vivo with TS to evaluate the IFN- γ secretion by mTS-primed lymphocytes. After that, to improve IFN- γ detection by T lymphocytes, splenocytes were briefly cultured with PMA plus ionomycin in the presence of brefeldin A, and afterwards, stained for CD4, CD8 and IFN- γ . (A) Representative dot plots illustrate IFN- γ intracellular staining profiles among both populations. (B) Frequency of IFN- γ production among splenic CD4⁺ T cells restimulated with TS ($n=4$ –5/group). * $p < 0.05$.

~15–20 fold increases; thus, the bottleneck of the production process is reduced, allowing further implementation in clinical testing for mTS antigen-based vaccines.

The adjuvant used was ISCOMATRIX, which has been widely assessed in other developed vaccines inducing a balanced cell-humoral response to fight against intracellular pathogens [16,25,26]. To our knowledge, this is the first assessment of IMX in the field of vaccine development against *T. cruzi* using a defined antigen although, in the early nineteen, extracted antigens of *T. cruzi* were assessed using ISCOM [8,27,28]. In the present study, we preferred the use of IMX instead of ISCOM because, although both particles have the same composition, the former allows us to formulate the antigen by direct absorption, avoiding antigen incorporation during its production, thereby facilitating a future production process [26]. IMX-based formulations can be efficiently taken up by APC due to the IMX particulate nature and saponin-mediated targeting of mannose receptor, DEC-205, which is present on the surface of DC, favoring endocytosis [29]. Recent reports described that IMX activates NOD receptors and MyD88 signaling, although the exact details of the signal pathways are still unknown [30,31].

In the first part of our immunological assessment, we evaluated humoral and cellular responses against TS in immunized mice. The quantitative humoral response was assessed by anti TS-IgG titers, being markedly improved in mTS-IMX with respect to mTS immunized mice. Even when comparing with mTS-CFA the response tends to be higher. The qualitative humoral response assessed by avidity index was also markedly improved in mTS-IMX with respect to mTS immunized mice, although it did not reach significant difference with respect to mTS-CFA. mTS-IMX also caused an increase in the IgG_{2a}/IgG₁ ratio, which is consistent with the development of a Th1 profile that is desirable to cope with *T. cruzi* infection [32]. In addition, DTH response was greatly increased in mTS-IMX immunized mice, indicating the development of specific cellular response to TS. TS-stimulated splenocytes from mTS-IMX mice released increased levels of IFN- γ , which is involved in key effector mechanisms against the parasite [33–35]. Interestingly, in this ex vivo assay, along with the IFN- γ increase, there was an

increase of IL-10. This result is in agreement with studies indicating that a regulatory response mediated by IL-10 is necessary to avoid detrimental effects caused by an exacerbated response during the acute stage of *T. cruzi* infection. [36,37]. Nevertheless, the high IFN- γ /IL-10 ratio exhibited by splenocytes from mTS-IMX immunized group clearly denote the induction of a skewed Th-1 profile.

In relation to CFA adjuvant performance, it is well known that this adjuvant ensures a good mixed humoral and cellular response that can hardly be improved by other adjuvants [38]. However it is not advised due to ethical concerns [38]. Consequently, it is particularly auspicious that mTS-IMX at least equaled mTS-CFA in terms of TS-specific antibody production, avidity, DTH response, Th1 profile and protection. On the other hand, in the absence of adjuvant, interestingly mTS induced an inadequate response, producing IgG avidities lower than 50% and mainly IgG₁ antibodies. Accordingly, a Th2-skewed profile was obtained when splenocytes were stimulated ex vivo with TS, which agrees with data indicating a detrimental immunological effect caused by TS on the host [12,13,39–41]. Although mTS lacks enzymatic activity, singular characteristics of the sequence that were not previously described may trigger this kind of response. Interestingly, our results indicate that IMX may switch mTS-driven detrimental response to a favorable one. The use of IMX to reduce the detrimental effect of the antigen has been previously described but due to another feature of this adjuvant: IMX required 1/100 lower doses of antigen, preventing associated toxic effects but maintaining the formulation immune efficacy [42,43]. In addition to a more balanced humoral response, mTS-IMX formulation improved the activation of primed CD4⁺ T Th1 and CD8⁺ T lymphocytes in terms of IFN- γ production. Both CD4⁺ and CD8⁺ T subpopulations are known to play a pivotal role in controlling *T. cruzi* infection [44–48]. Accordingly, mTS-driven IFN- γ production by CD4⁺ T cells might favor elimination of parasites by activated macrophages and help IgG_{2a} antibody production, coinciding with our observations (Fig. 1 and Fig. 4). Moreover, IFN- γ production by mTS-experienced CD4⁺ T cells is required for optimal activation and IFN- γ production by CD8⁺ T effector cells

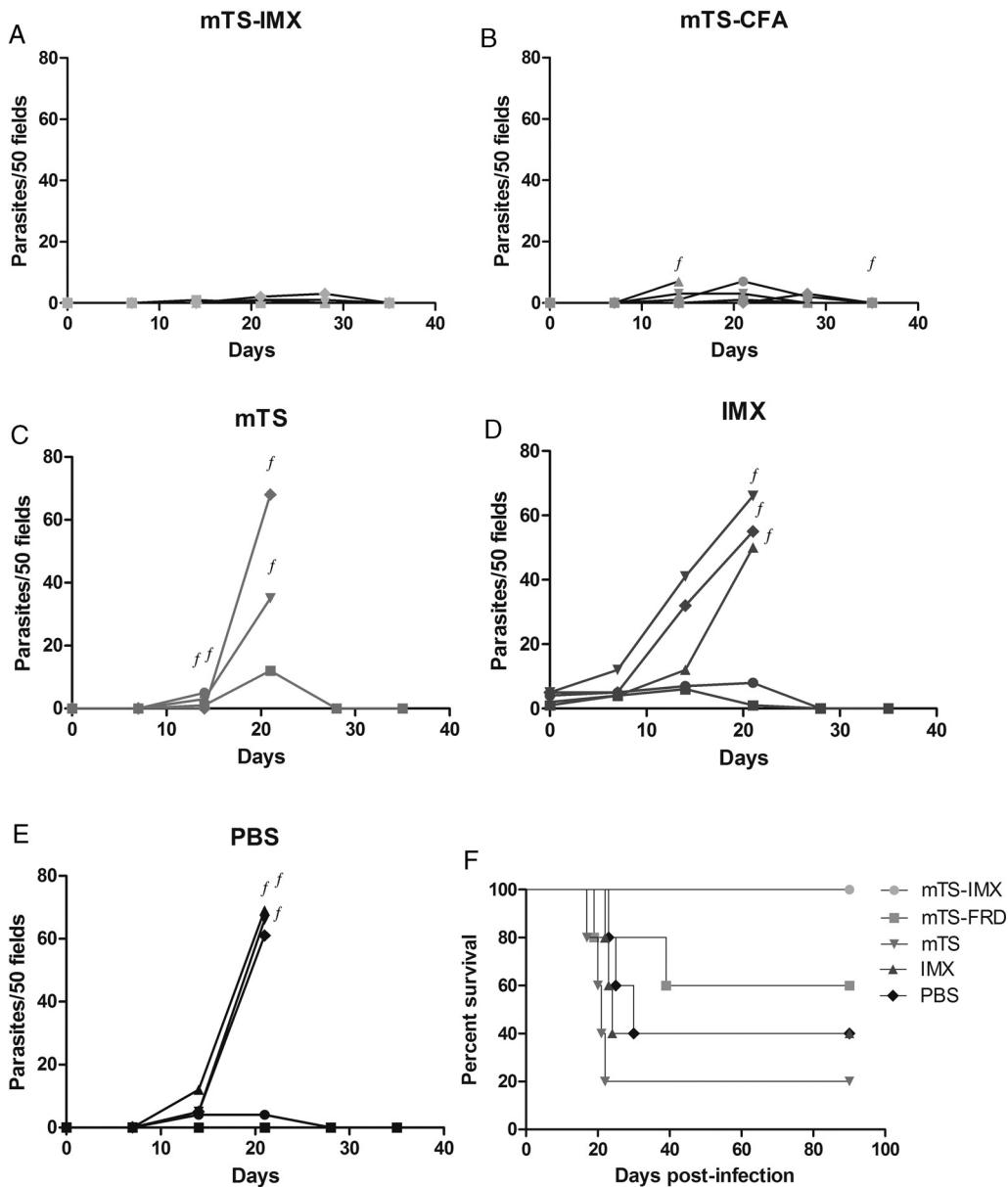


Fig. 5. Parasitemia and survival rates in immunized mice after *T. cruzi* challenge. Mice immunized with mTS-IMX, mTS-CFA, mTS, IMX and PBS ($n = 5$ /group) were challenged with 1000 trypanosomes of the Tulahuen strain 14 days after the last immunization. Individual parasitemias from mice immunized with (A) mTS-IMX, (B) mTS-CFA, (C) mTS, (D) IMX, (E) PBS. (F) Survival rates are shown. f indicates animal death. The results represent one from three independent experiments.

[46,49]. Interestingly, the frequency of INF- γ producing CD8 $^{+}$ T cells from mTS-IMX mice reached 8.5% after restimulation with TS, whereas mTS formulation slightly stimulated IFN- γ production compared to PBS, suggesting that IMX favors the priming

and activation of mTS-experienced CD8 $^{+}$ T lymphocytes. Similarly, Guptha and col [4], found similar levels of IFN- γ producing CD4 $^{+}$ and CD8 $^{+}$ T lymphocytes when a highly protective DNA-prime/MVA-boost vaccine based on TcG2 and TcG4 antigens was

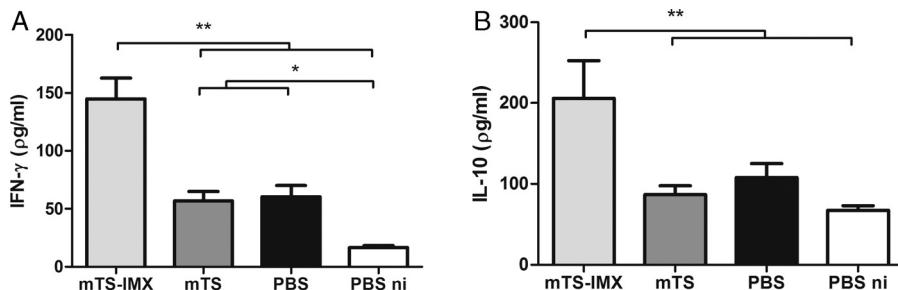


Fig. 6. Cellular immune response in immunized and infected mice. Splenocytes were cultured in triplicate for 72 h in the presence of mTS. (A) Levels of IFN- γ . (B) Levels of IL-10. Supernatant collected was measured by capture ELISA. Data (mean \pm SD) presented represent one from three independent experiments ($n = 5$ /group). * $p < 0.05$, ** $p < 0.01$.

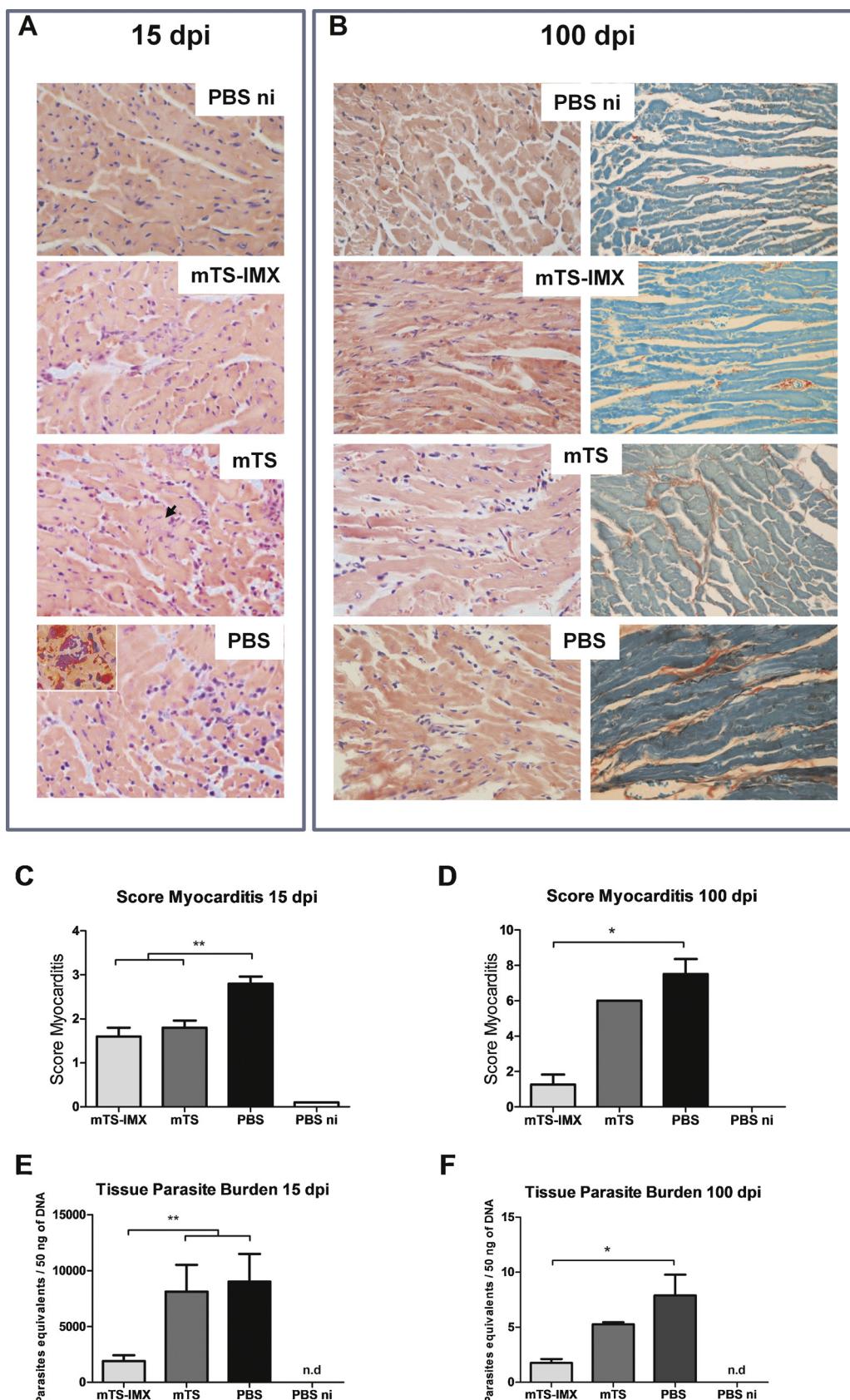


Fig. 7. Tissue pathology and parasite burden. (A and B left side) H&E staining (blue: nuclear staining, pink staining: muscle cytoplasm) of heart-tissue sections from immunized and control mice, harvested at 15 days post-infection (A) and 100 dpi (B left side, magnification: 200×). (B right panel) Picosirius red staining of hearts after 100 dpi (green: muscle/cytoplasm, red: collagen fibers). (C and D) score myocarditis at 15 dpi (C) and 100 dpi (D). (E and F) Real time PCR amplification of 195-bp satellite DNA *T. cruzi* sequence, normalized to TNF- α . Parasite nest showed in PBS group after 15 dpi are displayed in 400× magnification, ni = non-infected. Arrowhead shows destroyed myocardic fibers and parasite nest. n.d.=not detected. * $p<0.05$, ** $p<0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

tested, suggesting that mTS-IMX formulation can trigger a cellular response comparable to gene immunization approaches [4,50].

To prove the level of protection conferred by mTS-IMX formulation during infection, we challenged immunized mice with a lethal dose of Tulahuen strain. The protection level was the highest in mTS-IMX immunized mice. Notably, mTS-IMX immunized animals reached 100% of survival and exhibited a stronger decrease of parasite load, even better than mTS-CFA mice, which exhibited lower levels of protection. We also showed that mTS-IMX immunization reduced the effects of infection at the chronic stage, since 100% of surviving mTS-IMX mice showed the lowest tissue lesions of all the infected groups. Furthermore, parasite load in heart and muscle determined by RT-PCR was ~5-fold lower in mTS-IMX immunized mice than in the infected control. These results are highly promising, taking into account that in human Chagas disease, the presence of inflammatory infiltrate in the heart was correlated with parasite persistence [51,52]. Additionally, TS-stimulated splenocytes from infected mTS-IMX mice produced the highest levels of IFN- γ and IL-10 of all groups, suggesting a highly inflammatory but controlled response. This type of response is currently considered a good predictor of success for a *T. cruzi* vaccine [4,53,54]. Indeed, a specific but regulated effector immune response seems to be beneficial in human Chagas disease, as suggested by the immune profile observed in asymptomatic patients with respect to symptomatic ones (revised in [32]). Consequently, it can be hypothesized that a similar pattern of response would be desirable in human vaccines.

Strikingly, IFN- γ production was ~5 times higher in spleen cells from immunized mice (Fig. 2) than in splenocytes from immunized and *T. cruzi* infected mice (Fig. 4). Although further studies are required to fully explain this result, it can be speculated that during immunization protocol the proportion of mTS-specific IFN- γ secreting cells are higher than in immunized and infected animals due to the splenocyte polyclonal activation that occurs during the infectious process, which explains these differences at least partially. Another possibility could be related to the fact that immunized and infected groups might exhibit different kinetics of IFN- γ production. IFN- γ response may occur earlier in mTS-IMX immunized mice than in the other groups, and it is also possible that 15 days post-infection mTS-specific splenocytes are exhausted, whereas in the same period, the synthesis of IFN- γ may be just beginning in the other groups. Previous results obtained support this idea, since serum IFN- γ became noticeable by day 14 post-infection in non-immunized and infected Balb/c [22]. Nevertheless, in our study the analysis of the cytokine profile 15 days post-infection provides crucial data, since at this time, non-vaccinated and infected mice are highly symptomatic but still alive in our model.

In agreement with IMX-vaccine designs for other pathogens [16], our results prove that mTS-IMX formulation induce both optimal humoral and cellular immune responses, conferring strong protection against *T. cruzi*. Remarkably, mTS-IMX immunization prevents the development of severe myocarditis at the chronic stage, the hallmark of Chagas disease. Recombinant subunits potentiated with already assessed and approved adjuvants require lower evaluation steps than other approaches to achieve vaccines suitable for human use; therefore, the current formulation is a highly promising *T. cruzi* vaccine candidate.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2015.01.044>.

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