

Research Paper

A DNA Vaccine Encoding for *TcSSP4* Induces Protection against Acute and Chronic Infection in Experimental Chagas Disease

Minerva Arce-Fonseca^{1,2,*}, Angel Ramos-Ligonio^{1,3,*}, Aracely López-Monteón^{1,3}, Berenice Salgado-Jiménez¹, Patricia Talamás-Rohana¹, and José Luis Rosales-Encina^{1,✉}

1. Departamento de Infectómica y Patogenesis Molecular, Centro de Investigación y de Estudios Avanzados del I.P.N., México D.F. 07360, México
2. Departamento de Biología Molecular, Instituto Nacional de Cardiología "Ignacio Chávez", México D.F.14080, México.
3. LADISER Inmunología y Biología Molecular, Facultad de Ciencias Químicas, Universidad Veracruzana, Orizaba, Veracruz, 94340, México.

* These authors contributed equally to this work.

✉ Corresponding author: José Luis Rosales-Encina. Departamento de Infectómica y Patogénesis Molecular, Centro de Investigación y de Estudios Avanzados del I.P.N., Avenida Instituto Politécnico Nacional No. 2508, Col. San Pedro Zacatenco, Delegación Gustavo A. Madero, México D.F., México. CP 07360. Telephone: (52) 55-5747-3349, Fax (52) 55-5747-3377. E-mail: rosales@cinvestav.mx.

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Abstract

Immunization of mice with plasmids containing genes of *Trypanosoma cruzi* induces protective immunity in the murine model of Chagas disease. A cDNA clone that codes for an amastigote-specific surface protein (*TcSSP4*) was used as a candidate to develop a DNA vaccine. Mice were immunized with the recombinant protein *rTcSSP4* and with cDNA for *TcSSP4*, and challenged with bloodstream trypomastigotes. Immunization with *rTcSSP4* protein makes mice more susceptible to trypomastigote infection, with high mortality rates, whereas mice immunized with a eukaryotic expression plasmid containing the *TcSSP4* cDNA were able to control the acute phase of infection. Heart tissue of gene-vaccinated animals did not show myocarditis and tissue damage at 365 days following infection, as compared with control animals. INF- γ was detected in sera of DNA vaccinated mice shortly after immunization, suggesting the development of a Th1 response. The *TcSSP4* gene is a promising candidate for the development of an anti-*T. cruzi* DNA vaccine.

Key words: DNA immunization; *Trypanosoma cruzi*; *TcSSP4*

Introduction

Chagas disease caused by *Trypanosoma cruzi* continues as a major health problem in South and Central America, although the estimated number of infected people has fallen from approximately 20 million in 1981 to around 10 million in 2009 due to the implementation of measures for vector control and safer blood transfusions. The risk of transmission of the disease is high because the infection has been detected in non-endemic areas of the Americas, the

Western Pacific Region, and the European Region [1-4].

The development of anti-*T. cruzi* vaccines could contribute significantly to controlling transmission of Chagas disease [5]. It has been shown that immunization with plasmids containing *T. cruzi* genes induces immune responses able to protect against lethal infection with *T. cruzi* [5-11]. The immune responses induced by DNA immunization are mediated by anti-

bodies, CD4⁺, and CD8⁺ T cells. Th1 cytokines secreted by CD4⁺ T cells stimulate macrophages' phagocytic activity, B-cell proliferation and antibody production. Additionally, increased cytolysis of parasite-infected cells by CD8⁺ T cells and secretion of cytokines (INF- γ) that may induce trypanocidal activity have been described [12-14]. The crucial role of CD8⁺ T cells to control *T. cruzi* infection has been demonstrated from studies showing the inability of mice lacking CD8⁺ T cell to survive infection (15-17), and the ability to increase the level of resistance to infection by boosting CD8⁺ T cell responses with strong immunodominant epitopes from the trans-sialidase family [18-21].

To date, DNA immunization protocols against *T. cruzi* infection have used genes associated with trypomastigotes in experimental models. However, the study and characterization of DNA vaccines from genes expressed by amastigotes, the form that multiplies inside the host cells, have been recently initiated [10,11]. We have identified a cDNA clone that codes for an amastigote-specific surface protein (*TcSSP4*). The recombinant protein *MBP::SSP4* was able to induce macrophage nitric oxide production. This recombinant protein was also shown to induce the expression of IL-1 α , IL-6, IL-12, IFN- γ , and TNF- α in macrophages from normal mice, and IL-10 in macrophages from immunized mice, suggesting that *TcSSP4* protein could participate in the immunoregulatory process during Chagas' disease [22]. In this work we first analyzed the effect of a single dose of the recombinant protein *rTcSSP4* on the course of parasitemia and survival of trypanomastigote infected mice, and then we analyzed the potential of the gene *TcSSP4* as a DNA vaccine.

Materials and Methods

Parasites

The H8 strain of *T. cruzi* [A gift from Dr. Jorge E. Zavala Castro, Centro de Investigaciones Regionales "Dr. Hideyo Noguchi", Universidad Autónoma de Yucatán, Mérida, Yucatán, México] was maintained *in vivo* by serial passages of blood form trypanomastigotes in BALB/c mice and by weekly *in vitro* passages of epimastigotes in Liver Infusion Tryptose (LIT) with 10 % of fetal calf serum (Gibco, Invitrogen), as described [22].

Plasmid construction and recombinant protein expression and purification

DNA encoding for the *TcSSP4* protein (GeneBank™ database accession number AF480943) was obtained from the plasmid pMAL-*TcSSP4* [22] as a 2.2

Eco RI fragment and ligated in the Eco RI site of plasmids pBk-CMV (Stratagene, La Jolla, CA) and pTWIN1 (New England BioLabs) for construction of DNA vectors pBCSSP4 and pTWSSP4, respectively. The plasmid pTWIN1 was used in order to obtain the recombinant protein *TcSSP4* (*rTcSSP4*) without the MBP tag. Purification of the recombinant protein *rTcSSP4* was achieved by transforming the plasmid pTWSSP4 into *E. coli* BL21(DE3). Cells were grown at 37 °C to OD₅₉₅ = 0.5 and induced with 0.3 mM IPTG for 6 h, then harvested by centrifugation and lysed by sonication in the presence of 10 μ g/ml lysozyme. The expressed *rTcSSP4* protein was purified from the lysate by using a chitin-affinity column, followed by intein cleavage by temperature shift from 4 °C to 25 °C at pH 7.0, according to the manufacturer's protocol. Analysis of protein purification was carried out by 10% SDS-PAGE and Coomassie blue staining.

Immunization and trypanomastigote infection of mice

Female 6-8-week old BALB/c mice were maintained according to the recommendations of our Institutional Animal Care and Use Committee. For protein immunization, each mouse was immunized i.p. with 10 μ g of *rTcSSP4* protein emulsified with Freund's complete adjuvant (Sigma, St Louis, MO). DNA immunizations were done twice at 2 week intervals in the *tibialis anterioris* muscle with the plasmids pBCSSP4 or pBk-CMV resuspended in PBS. Mice immunized with the *rTcSSP4* protein were challenged i.p. with 1x10⁴ or 3x10⁴ bloodstream trypanomastigotes (BT) 3 or 7 days post-immunization. Mice immunized with DNA were challenged 2 weeks after the last immunization with 1x10⁴ BT. Parasitemia was monitored every 3 days. All mice were randomly assigned into control or vaccinated groups of four mice each in two independent experiments (n=8).

Histopathology

Hearts from infected mice were removed, rinsed with PBS, and fixed for 24 h in 4% paraformaldehyde in PBS (pH 7.4). Fixed hearts were embedded in paraffin, sectioned (6 μ m), stained with hematoxylin-eosin, and examined by light microscopy (Nikon Eclipse E600, Nikon, Japan).

Serum cytokine analysis

Sera of mice immunized with DNA or *rTcSSP4* protein were collected at 3 and 12 h after the last immunization. Pools of sera from each group of immunized mice were analyzed for the presence of IL-2, IL-4, IL-6, IL-10, TNF- α , and IFN- γ with commercially available murine cytokine ELISA kits (OptEIA™ Sets

Mouse, PharMingen, San Diego, CA). Microwell plates (96-wells, Nunc Maxisorp) were coated with capture antibody diluted (1:250) in coating buffer (0.2 M sodium phosphate, pH 6.5 or 0.1 M sodium carbonate, pH 9.5), and incubated overnight at 4 °C. Wells were washed with PBS containing 0.05% Tween 20. Plates were blocked with 10% fetal bovine serum in PBS, incubated at room temperature for 1 h, and washed with PBS. Sera (1:8) and recombinant standards were added, and plates were incubated for 2 h at room temperature. After washing, detection antibody plus avidin-HRP were added to each well. Plates were incubated for 1 h at room temperature, washed, and developed with ABTS (Zymed Laboratories, South San Francisco, CA) to record the OD at 405 nm.

Statistical analysis

Results were expressed as means \pm 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

Immunization with rSSP4 increases parasitemia and mortality in the murine model.

Proliferation of spleen cells, from immunized or normal mice, stimulated *in vitro* with ConA or with the homologous antigen, was suppressed by the fusion protein MBP::SSP4. This suppression was inhibited by L-NAME [22], which indicates that nitric oxide is involved in suppressing spleen cells proliferation as it has been shown [23, 24]. On the other hand, mice peritoneal macrophages (pMΦs) obtained 3 and 7 days after immunization with a single dose of MBP::SSP4 showed high and null nitric oxide production respectively compared with pMΦs from normal mice [22]. Therefore we investigated parasitemia and mortality in mice immunized with a single dose of the recombinant protein rTcSSP4 and then challenged with bloodstream trypomastigotes at 3 and 7 days post-immunization.

The recombinant protein was purified from the intein-TcSSP4 fusion protein bound to chitin beads by temperature shift. Figure 1, lane 2 shows the Coomassie blue staining of the 60 kDa purified protein that was used to immunize the mice. To analyze whether immunization induced immunosuppression, mice were challenged with *T. cruzi* trypomastigotes. Both parasitemia and death rate were evaluated 3 and 7 days after a single immunization with 10 μ g of rTcSSP4. In mice challenged at both times after im-

munization with 3×10^4 trypomastigotes, (Fig. 2A, ●, ○), the parasitemia starts earlier than in normal animals (Fig. 2A, ▼). Although a peak of parasitemia was observed at day 26 in normal animals, all immunized mice died before a peak was reached. Death occurred at day 14 in those mice infected 7 days post-immunization (Fig. 2B, ○) and at day 17 in those infected 3 days after immunization (Fig. 2B, ●). Despite the peak of parasitemia shown by normal animals, they began dying at day 24 after challenge (Fig. 2B, ▼).

When the challenge dose of parasites was reduced to 1×10^4 (Fig. 2C and D), the beginning of parasitemia was similar in all animals and they presented the parasitemia peak at the same time. However, in immunized mice the peak was higher than in normal animals (Fig. 2C, ●, ○). In spite of a higher mortality than in normal animals, the survival rate was higher ($p < 0.05$) when compared to mice infected with 3×10^4 trypomastigotes (Fig. 2 B and D), suggesting that the lower number of parasites allowed them to better control the infection process.

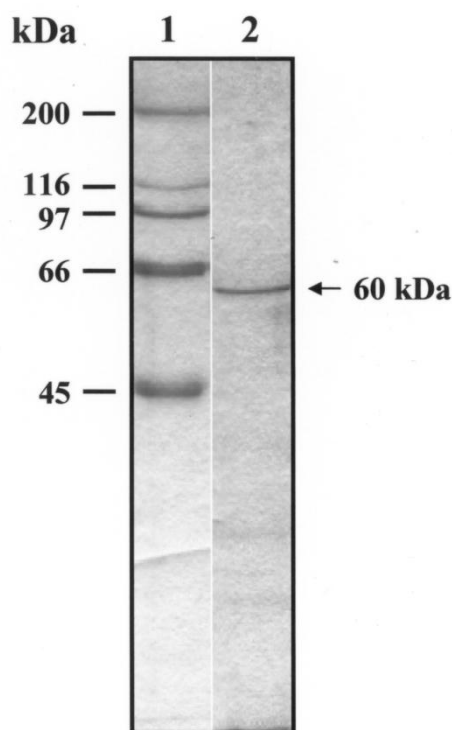


Figure 1. Purification of the rTcSSP4 protein. rTcSSP4 protein was purified from the Intein-TcSSP4 fusion protein bound to chitin beads by temperature shift from 4 °C to 25 °C at pH 7.0. (1) Molecular weight markers and (2) purified rTcSSP4.

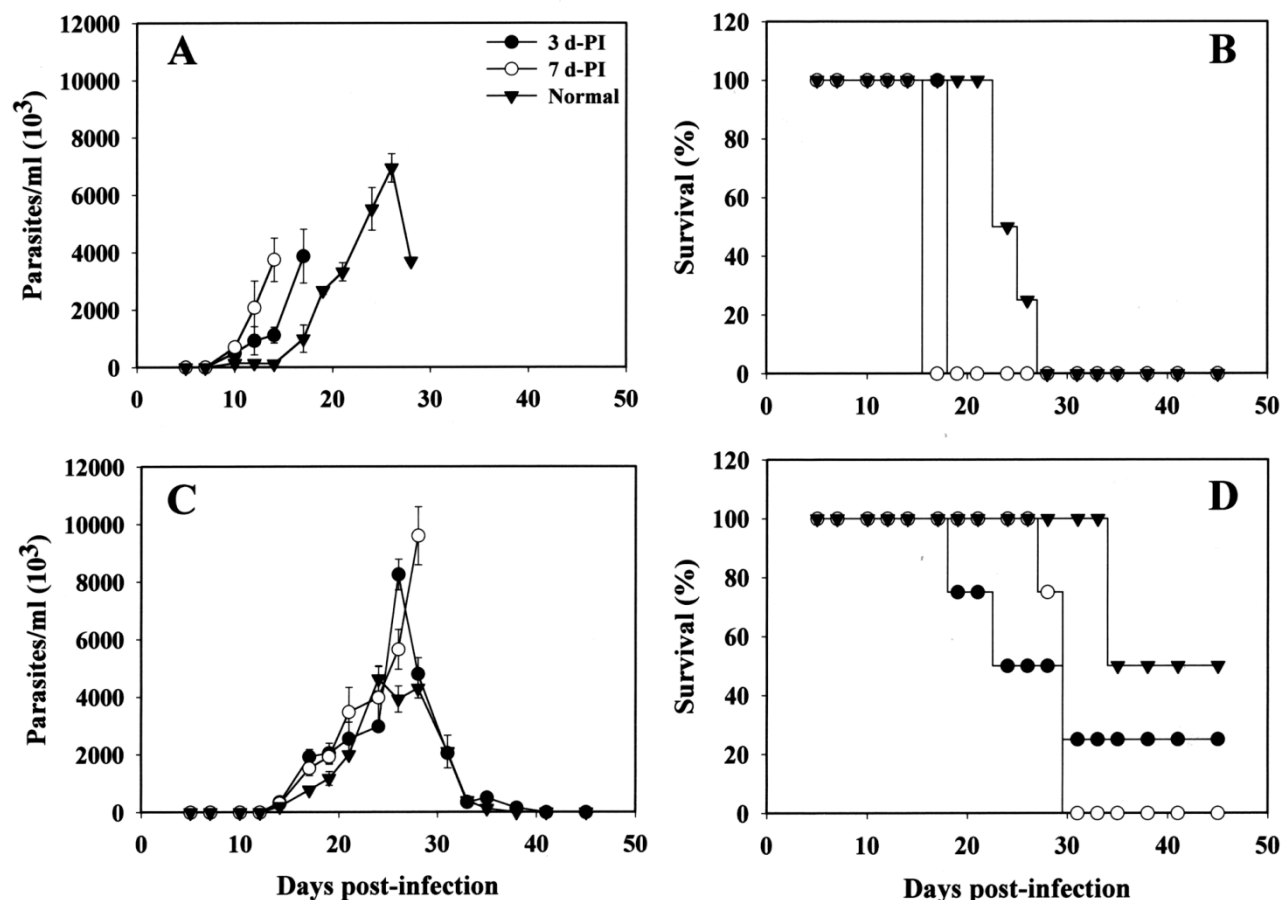


Figure 2. Parasitemia and mortality of *rTcSSP4* immunized mice challenged with BT. Mice were immunized with a single dose of *rTcSSP4* (10 μ g), and at 3 (\bullet) or 7 (\circ) days post-immunization (PI) animals were infected with 3×10^4 (A and B) or 1×10^4 (C and D) BT. Parasitemia (A and C) and mortality (B and D) rates were recorded up to 45 days. Normal animals received only Freund's complete adjuvant (\blacktriangledown). Parasitemia values represent mean \pm standard errors of the means for surviving mice.

Intramuscular immunization with anti-gen-expressing DNA plasmid induces protection against *T. cruzi* infection.

DNA immunization is a widely used procedure. DNA codified proteins are internally processed and presented by MHC class I and II antigens, and are therefore able to induce a Th1 type immune response in which CD8⁺ T cells are activated [25]. In mice vaccinated with the plasmid pBCSSP4 and later challenged with *T. cruzi* parasites, the parasitemia peak was observed at day 25 with the lowest number of parasites (Fig. 3A, \bullet), in comparison with the parasitemia peak of mice immunized with control plasmid pBk-CMV (29 days) (Fig. 3A, \circ), or animals injected with PBS (25 days) (Fig. 3 A, \blacktriangledown). Statistically significant differences ($p < 0.01$) were found in the parasitemia peak between vaccinated and control mice. In

terms of survival, the group immunized with pBCSSP4 showed the highest survival rate ($\sim 75\%$) even at 1 year after infection (Fig. 3B, \bullet) ($p < 0.05$).

Decreased tissue damage in mice immunized with pBCSSP4.

Heart tissue damage was analyzed after long term infection of immunized mice. One year after initial infection, surviving animals were sacrificed. Hearts were dissected and analyzed for the presence of myocarditis and tissue damage. No damage was found in hearts from mice immunized with the plasmid pBCSSP4 (Fig. 4A). In contrast, severe myocarditis and tissue damage were observed in hearts from mice immunized with the control plasmid pBKCMV (Fig. 4B) or from mice injected with PBS (Fig. 4D). Non-immunized, non-infected mice were used as control (Fig. 4C).

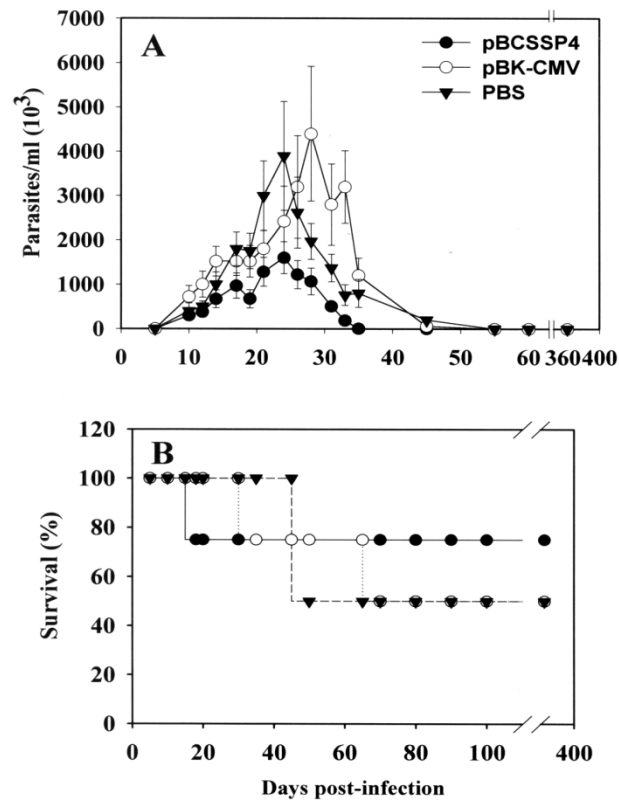


Figure 3. Parasitemia and mortality of pBCSSP4 immunized mice challenged with BT. Mice were immunized twice 15 days apart with 100 $\mu\text{g}/\text{dose}$ of the plasmids pBCSSP4 (●) and pBk-CMV (○). Two weeks after the last immunization they were infected with 10^4 BT. Control animals received only PBS (▼). Parasitemia (A) and mortality (B) rates were recorded up to 365 days. Parasitemia values represent mean \pm standard errors of the means for surviving mice.

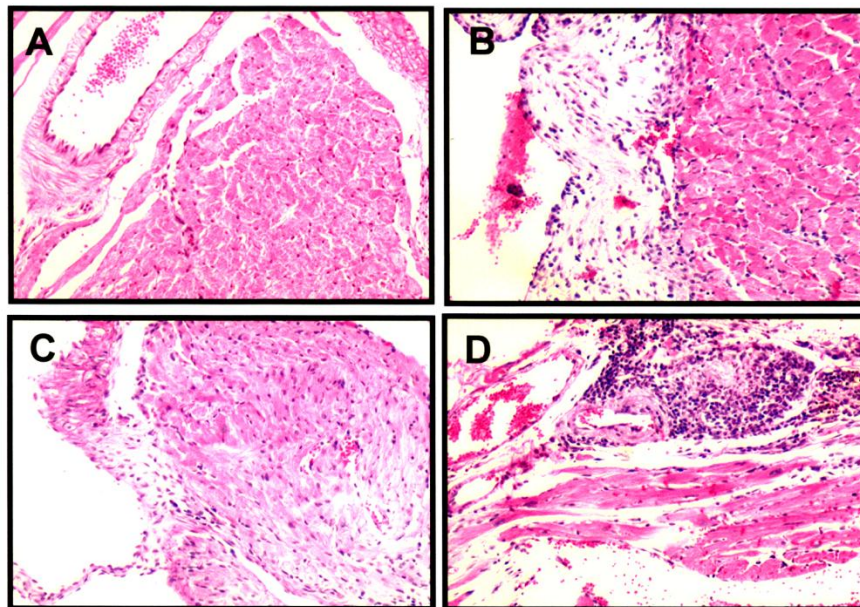


Figure 4. BALB/c mice immunized with plasmid pBCSSP4 and infected with *T. cruzi* did not develop myocarditis. BALB/c mice immunized with the plasmids pBCSSP4 (A), pBk-CMV (B), or injected with PBS (D) were infected with 10^4 BT. Control mice were non-immunized and non-infected (C). At 365 days post-infection, cardiac sections from corresponding mice were stained with hematoxylin and eosin. 40X magnification.

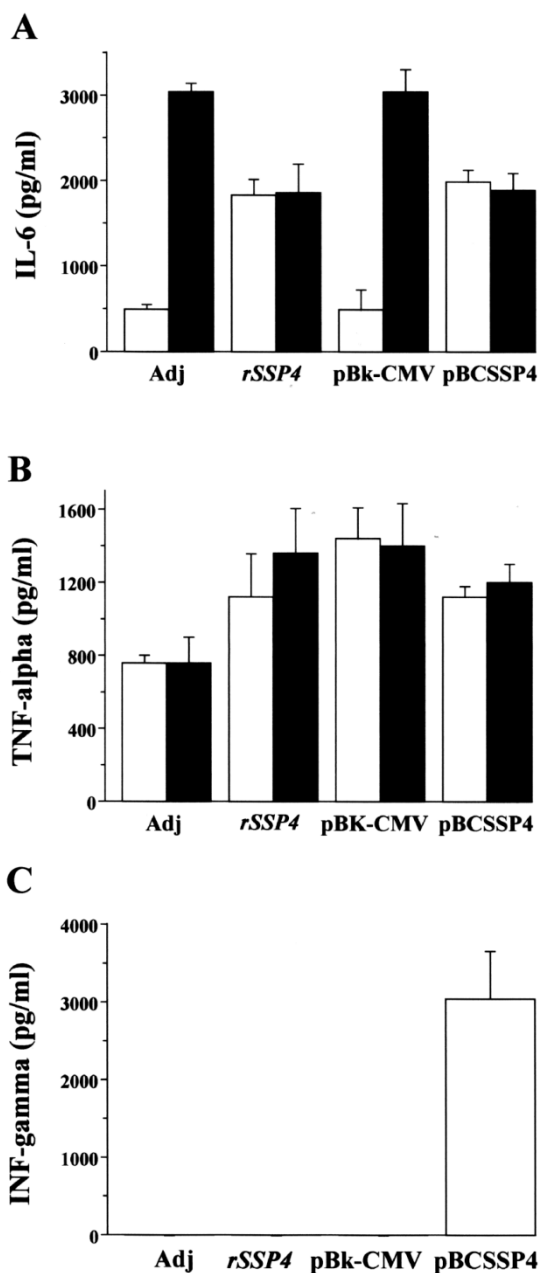


Figure 5. Serum cytokine levels in immunized mice. Pools of serum collected 3 (□) and 12 (■) h after final immunization and ELISA assays were performed for the presence of IL-6 (A), TNF- α (B) and IFN- γ (C). Sera were from mice immunized twice 15 days apart, with adjuvant (Adj), *rSSP4* protein (10 μ g), plasmid pBk-CMV (100 μ g), and plasmid pBCSSP4 (100 μ g). Values represent the mean \pm SD of a representative triplicate assay.

Serum cytokine levels in mice immunized with *rTcSSP4* and pBCSSP4.

As a first approach to analyze why protein or DNA vaccinated mice died or survived respectively, the serum cytokine profile of immunized mice was analyzed. Sera from immunized and control mice were collected 3 (□) and 12 (■) h after the last immunization and cytokine levels were measured by ELISA. When compared with controls, statistically significant differences were found in IL-6 levels in mice immunized with either *rTcSSP4* protein or pBCSSP4 plasmid. The IL-6 levels were higher at 3h ($p < 0.01$) and lower at 12 h ($p < 0.01$) (Fig. 5A). For TNF- α , statistically significant differences were found in mice immunized with *rTcSSP4* protein at 3 h ($p < 0.01$) and 12 h ($p < 0.01$) when compared with control mice, and no significant differences were found in mice immunized with pBCSSP4 plasmid (Fig. 5B). However, a clear difference was found regarding IFN- γ production; only 3 h sera from animals immunized with the pBCSSP4 plasmid showed significant amounts of this cytokine. In 12 h sera this cytokine was not detected. No detectable amounts of IFN- γ were found in the other conditions (Fig. 5C). Cytokines IL-1, IL-4, and IL-10 were not detected at these times of serum collection (data not shown).

Discussion

Mice immunized with the *rTcSSP4* protein and then challenged with a lethal dose of BT showed earlier parasitemia and death compared with non-immunized mice. However, when immunized animals were challenged with third of the lethal dose of parasites, parasitemia started at the same time as in non-immunized animals, but peaks of parasitemia and death rates were higher in immunized mice. These results could be explained in terms of immunosuppression and parasite load. We have previously shown that the fusion protein *MBP::SSP4* was able to induce nitric oxide (NO) production by spleen and peritoneal macrophages (pM ϕ s). Also we observed that this protein suppresses spleen cell proliferation. pM ϕ s from normal mice as well as those that recovered 3 and 5 days after one immunization with *MBP::SSP4* were able to synthesize NO, whereas pM ϕ s recovered at 7 days after one immunization and 3, 5 and 7 days after two immunizations were not able to produce NO. This recombinant protein was also shown to induce the expression of IL-1 α , IL-6, IL-12, IFN- γ , and TNF- α in pM ϕ s from normal mice, IL-6, TNF- α , and IL-10 in pM ϕ s recovered 3 days after one immunization, and IL-6 and IL-10 in pM ϕ s recovered 5 days after one immunization [22].

Earlier parasitemia and death of mice challenged 3 days after one immunization could be due mainly to NO production induced by *rTcSSP4*. It has been shown that NO production contributes to the immunosuppression observed during infection with *Toxoplasma gondii* [26], *Paracoccidioides brasiliensis* [27], *Echinococcus multilocularis* [28], *Trypanosoma brucei* [29], and *Trypanosoma cruzi* [30]. Although NO can kill intracellular parasites, overproduction can be detrimental, even lethal, to the host [26, 31]. In addition, earlier parasitemia and death of mice challenged 7 days after one immunization could be due to macrophage inactivation by IL-10 [32], and to the beginning of a Th2 response induced by *rTcSSP4*. Suppression of immune responses during *T. cruzi* infection results in a significantly increased parasite burden [14]. Thus, immunized mice that were able to control the initial phase of parasitemia (with a lower dose of BT) showed a parasitemia peak higher than that observed in non-immunized animals. Experiments are required to follow the kinetics of the immune response development and determine the type of T cells that are induced (Th1 or Th2) when a full immune response has been established.

In DNA based immunization, the antigen encoded by plasmid DNA is processed in the cytoplasm by transfected cells and presented to elicit an immune response [33]. Mice immunized with the plasmid pBCSSP4, and then challenged with BT showed a reduction in parasitemia and 75% survival, as compared with control animals. Similar results have been reported for DNA immunizations with *T. cruzi* genes, showing a reduction of parasitemia and mortality (Table 1). Besides using different genes, the variability in parasitemia and survival reported in these studies could be due to DNA doses, number of doses, interval between doses and time of challenge after the last dose, as well as parasite strain and number of parasites used to challenge. Therefore, it is necessary to develop and standardize a protocol for DNA immunization in experimental Chagas disease.

Immunization with pBCSSP4 altered the course of disease development in the chronic phase of infection in our experimental model. Immunized animals not only survived the trypomastigote infection but also showed low levels of parasitemia, and displayed undetectable levels of heart muscle inflammation and necrosis in the chronic phase of infection. Similarly, reduction in skeletal and heart muscle inflammation and necrosis in mice immunized with a mixture of plasmids containing the ASP-1, ASP-2 and TSA-1 genes and with antigen-encoding plasmids plus interleukin-12, and granulocyte-macrophage colony-stimulating factor plasmids has been reported [10].

These results support the hypothesis that severity of the disease in chronic *T. cruzi* infection is tightly linked to the relative success in limiting parasite levels [14, 34].

Table 1. DNA vaccines with plasmids containing *Trypanosoma cruzi* genes

Gene	Survival (%)	Reference
TSA-1	55-90	5
CPR	100	7
KMP11-HSP70	50	8
TS	100	9
ASP-1, ASP-2, TSA-1 Plus IL-12, GM-SF	30-60 60-80	10
Clone 9 (89.9% identity with ASP-2)	100	11
CRP	100	41
PFR/PFR3-HSP70	100	42
LYT1 Plus IL-12	75	43
TS family members	75	43
Mucin family members	25	43
SSP4	75	This work

It has been shown in the murine model that a Th1 response is able to control *T. cruzi* infection and to reduce the severity of the disease [35-40]. Although inflammatory cytokines IL-6 and TNF- α were detected at 3 h and 12 h post-immunization in sera of mice immunized with adjuvant, *rTcSSP4* protein, pBk-CMV, and pBCSSP4, INF- γ was detected only at 3 h post-immunization in sera of mice immunized with pBCSSP4. These results suggest that mice immunized with the gene coding for the TcSSP4 protein are primed to develop a Th1 response. Experiments are being conducted, varying the number of doses of plasmid DNA and parasites, to determine the type of response just before parasite challenge.

In summary, we have shown that immunization with rTCSSP4 protein makes mice more susceptible to trypomastigote infection, and a genetic vaccine based on TcSSP4 cDNA provides partial protection against lethal infection with *T. cruzi*. Further investigations are needed to determine whether different DNA immunization protocols (e.g. total DNA dose, number and interval between doses, time of challenge, cytokine adjuvants) might yield better protection.

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Conflict of Interests

The authors have declared that no conflict of interest exists.

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