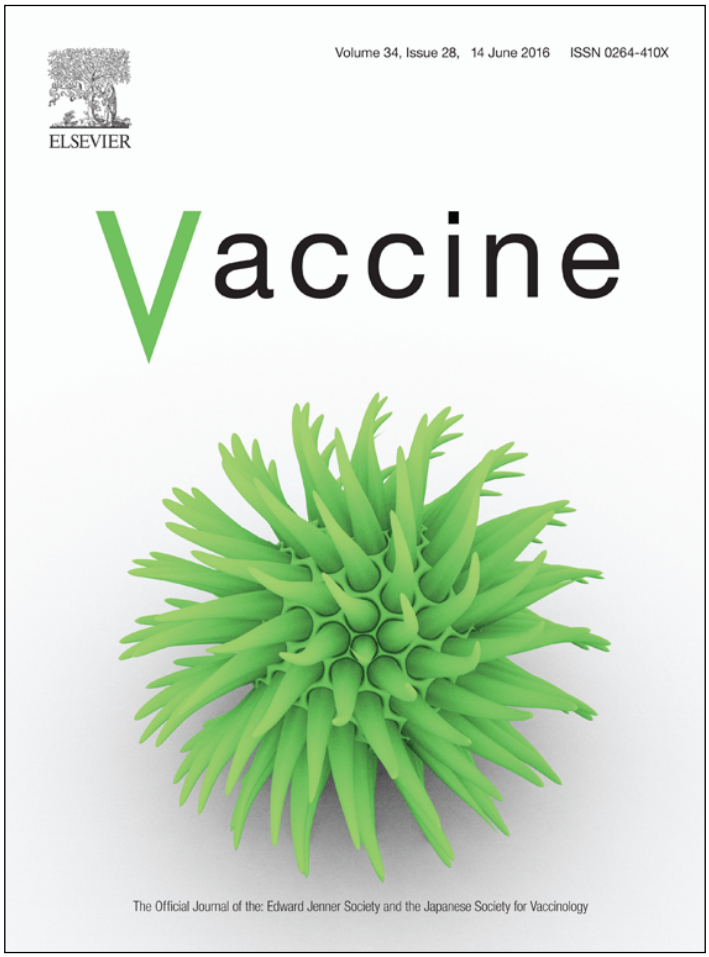

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A prime-boost immunization with Tc52 N-terminal domain DNA and the recombinant protein expressed in *Pichia pastoris* protects against *Trypanosoma cruzi* infection

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ABSTRACT

We have previously reported that the N-terminal domain of the antigen Tc52 (NTc52) is the section of the protein that confers the strongest protection against *Trypanosoma cruzi* infection. To improve vaccine efficacy, we conducted here a prime-boost strategy (NTc52PB) by inoculating two doses of pcDNA3.1 encoding the NTc52 DNA carried by attenuated *Salmonella* (SNTc52), followed by two doses of recombinant NTc52 expressed in *Picchia pastoris* plus ODN-CpG as adjuvant. This strategy was comparatively analyzed with the following protocols: (1) two doses of NTc52 + ODN-CpG by intranasal route followed by two doses of NTc52 + ODN-CpG by intradermal route (NTc52CpG); (2) four doses of SNTc52; and (3) a control group with four doses of *Salmonella* carrying the empty plasmid. All immunized groups developed a predominant Th1 cellular immune response but with important differences in antibody development and protection against infection. Thus, immunization with just SNTc52 induces a strong specific cellular response, a specific systemic antibody response that is weak yet functional (considering lysis of trypomastigotes and inhibition of cell invasion), and IgA mucosal immunity, protecting in both the acute and chronic stages of infection. The group that received only recombinant protein (NTc52CpG) developed a strong antibody immune response but weaker cellular immunity than the other groups, and the protection against infection was clear in the acute phase of infection but not in chronicity. The prime-boost strategy, which combines DNA and protein vaccine and both mucosal and systemic immunizations routes, was the best assayed protocol, inducing strong cellular and humoral responses as well as specific mucosal IgA, thus conferring better protection in the acute and chronic stages of infection.

1. Introduction

Trypanosoma cruzi is a protozoan intracellular parasite and the etiological agent of Chagas disease. The transmission modes for this parasite are diverse, namely vectorial, through blood transfusion and organ transplantation, vertically from mother to infant, and by oral route. The infection has an initial acute stage followed by a chronic stage, which can be symptomatic or not. Up to 30% of chronically infected individuals develop cardiac alterations whereas 10% of them develop digestive, neurological or mixed alterations [1]. Treatment is based on one of two drugs: nifurtimox and benznidazole. Both are effective in the acute stage of infection, losing effectiveness in the advanced phase. Furthermore, severe side effects are associated with the treatment. Currently, the number of worldwide infected individuals is estimated in 8-10 million. The disease affects not only people from endemic areas in South and Central America, but also from many countries of Western Europe and North America [1,2]. Efforts are focused not only in transmission control, but also in the development of more efficient and less toxic drugs as well as prophylactic and therapeutic vaccines.

In the field of vaccine development against *Trypanosoma cruzi*, several antigens such as cruzipain (Cz), amastigote surface protein 2 (ASP-2), trans-sialidase (TS), gp82 and paraflagellar rod proteins

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(PFR) have been tested among others, [reviewed in 3,4]. Recent strategies included DNA delivery systems comprising attenuated viruses and bacteria [5–8]. This kind of immunization induces a significant cellular immune response and the cytotoxic T cell activation required to control infection [9,10]. However, it has been shown that even when the cellular immune response is crucial for protecting against infection, antibodies are also required [11–13].

Tc52 is a *T. cruzi* protein having glutathione transferase activity [14], whose sequence is highly conserved among strains [15]. It is crucial for parasite survival since the knockout of both alleles is lethal [16]. Tc52 has many immunomodulatory properties: (1) it inhibits splenocyte proliferation induced by mitogen [17,18]; (2) it binds to dendritic cells (DC) and macrophage surface [19]; (3) it increases the expression of inducible nitric oxide synthase (iNOS) and nitric oxide production by macrophages in the presence of IFN-γ [20], among other properties. All these characteristics make Tc52 a promising vaccine candidate.

Tc52 has two domains: the N-terminal domain (NTc52) that has a molecular weight of 26 kDa and contains the enzyme active site, and a 25 kDa C-terminal (CTc52) domain, whose function is still unknown but could be responsible for some immunomodulatory activities [18]. The ability of recombinant Tc52 or its naked DNA to protect against *T. cruzi* infection yielded promising results [19,21]. More recently, we evaluated the ability of a *Salmonella* DNA delivery system encoding Tc52 and its N-and C-terminal domains to protect against *T. cruzi*. NTc52 conferred greater protection than CTc52 or full length Tc52 in the acute and chronic stages of infection [22]. The main goal of the present work was to improve the immune protection elicited by *Salmonella* as DNA delivery system of NTc52 using a prime-boost strategy.

Pichia pastoris is a eukaryotic organism that is easy to grow, allowing the production of recombinant proteins in large quantities. In addition, using suitable vectors, the protein of interest could be addressed either to the intracellular compartment or the secretion system. In the last few years, many recombinant proteins, including *T. cruzi* proteins, have been produced using *P. pastoris* as expression system [23,24]. We have previously expressed NTc52 as a recombinant protein in *E. coli* [22]. In this work we have chosen *P. pastoris* as expression system to improve soluble protein production.

2. Materials and methods

2.1. Parasites

T. cruzi epimastigotes (RA strain) were grown in LIT medium [22]. *T. cruzi* bloodstream RA trypomastigotes and the recombinant Tulahuen strain expressing β-galactosidase (Tul-β-Gal) [25] were isolated from infected mice.

2.2. Tc52 and NTc52 cloning and expression in Pichia pastoris

Tc52 and its N-terminal domain (residues 1–223) were cloned into pPICzα-A plasmids. For that purpose, genomic DNA was extracted from RA epimastigotes. The sequences were amplified by PCR using the following sets of primers: for full-length Tc52, the forward primer 5′-CGACTGGAATTCATGAAGGCTTTGAAACTTTTTAAAGA-3′ containing an *Eco*RI restriction site (underlined) and the reverse primer 5′-ACTAGCGCGGCCGCTCAGTGATGGTGATGGTGATGAGACGATGGAC-GCAA-3′ with a *Not*I restriction site and a sequence encoding a His_6 tag (both underlined). For the N-term domain, the Tc52 forward primer and the reverse primer 5′-ACTAGCGCGGCCGCTCAGTGATGGTGATGGTGATGCAATGACCAT-GTGACGTGC-3′ also with a *Not*I restriction site and a His_6 tag. The PCR products of 1,382 (Tc52) and 722 bp (NTc52) were digested with *Eco*RI and *Not*I and cloned in vector pPICzα-A (Invitrogen). Cloning was performed in *E. coli* DH5α, selecting positive clones by Zeocin resistance. Both pPICzα-Tc52 and pPICzα-NTc52 constructions were purified from the selected clones and sequenced.

Plasmids pPICzα-Tc52 and pPICzα-NTc52 were linearized with *Sac*I restriction enzyme, purified and used to transform 10^{10} electrocompetent *P. pastoris* KM71 or GS115 cells: pulsed 1.5 kV, 25 μF, 200 Ω, and then incubated for 2 h in cold 1 M sorbitol [26]. Recombinant yeast selection was performed in YPDS-Zeocin agar plates. Different Zeocin concentrations in the range 100-1000 mg/ml were tested, and clones with resistance to higher antibiotic concentrations were selected. The insertion of the DNA fragment was checked by colony PCR in selected Zeocin-resistant colonies [27]. PCR positive clones were cultured in minimal methanol histidine plates. Cultures were conducted at 28 °C for 4 days, daily adding methanol. Colony blotting was then assayed as described [26] using a mouse-specific anti-Tc52 or anti-NTc52 [22]. The yeasts colonies with higher expression levels were amplified as recommended (Invitrogen). Cultures (100 ml) were grown until $DO_{600\,nm}$ = 0.6–0.8. For protein expression, cells were centrifuged for 10 min at 2000 × *g* and suspended in 150 ml of BMMH and cultured for 5 days Methanol was added daily to 0.5% final concentration. Based on previous reports [26,28], other methanol concentrations (1%, 1.5%, 2% and 2.5%) were evaluated to enhance yield.

Recombinant Tc52 and NTc52 were purified under native conditions. *P. pastoris* induced-cultures were centrifuged at 4 °C, 20,000 × *g* for 30 min. Supernatants were concentrated 10 times by centrifugal ultrafiltration (Amicon, Millipore). Prior to purification, concentrated supernatants were dialyzed against PBS supplemented with 10 mM imidazole. Purifications of both proteins were performed in a nitrilotriacetic acid (NTA) column, at 4 °C, with purification buffer by imidazole gradient: 25 mM, pH 8.0, for washes, and 250 mM, pH 7.5, for elution. Purified proteins were then dialyzed against phosphate-buffered saline (PBS) and analyzed by SDS-PAGE and immunoblotting to verify purity and identity [22].

2.3. Cloning and expression of the N-terminal domain in a eukaryotic expression system

Cloning and expression of NTc52 in pcDNA3.1 eukaryotic plasmids and transformation of attenuated *Salmonella enterica* serovar Typhimurium *aroA* SL7207 were previously described [22].

2.4. Immunizations and challenge

Four groups (10 animals/group) of 6–8-week-old inbred female C3H/HeN mice were immunized four times every 10 days as follows: GI (control group): 10^9 CFU of *Salmonella* carrying the pcDNA3.1 vector (Sempty) by oral route; GII: 10^9 CFU of *Salmonella* harboring construct pcDNA3.1-NTc52 (SNTc52); GIII: a prime-boost vaccination protocol (PB) with 2 oral doses of SNTc52 and 2 doses of rNTc52 + ODN-CpG 1826 as adjuvant, by intradermal route (NTc52PB); GIV: rNTc52 + ODN-CpG 1826: two doses by intranasal route, and two by intradermal route (NTc52CpG). Intradermal and intranasal immunizations were performed with 10 μg of rNTc52 and 20 μg of ODN-CpG. For oral immunizations, attenuated *Salmonella* carrying the constructions (empty pcDNA3.1 or pcDNA3.1-NTc52) were grown in Brain Heart Infusion (BHI) medium supplemented with 100 μg/ml ampicillin, at 37 °C 70 rpm. Cultures reaching OD_{600} = 0.6 were centrifuged and suspended in 2.65% $NaHCO_3$ buffer, supplemented with 1.65% ascorbic acid and 0.2% lactose. Mice were deprived of water 2 h before immunization with 20 μl of the suspension (10^9 CFU). The number of CFU

was determined prior to immunization and verified in every immunization by plating serial dilutions of the cultures. One half of the animals of each group were randomly selected to study the immune response. The other half was infected by intraperitoneal route, 15 days after the last immunization with 10^3 *T. cruzi* bloodstream trypomastigotes of the highly virulent RA strain. We also performed a chronic model in immunized mice challenged with 200 RA trypomastigotes. *In vivo* experiments were approved by the Review Board of Ethics of the Faculty of Pharmacy and Biochemistry, UBA and conducted in accordance with the guidelines established by the National Research Council (CONICET) [29].

2.5. Antibody determination

Serum and intestinal lavage were collected 15 days after the last immunization for the determination of antigen-specific IgG, IgG1, IgG2a and IgA by ELISA as described [22]. Small intestines from mice were dissected and washed with cold PBS supplemented with 2 mM EDTA, 1 mM PMSF, 5 μM TLCK, 5 μM pepstatin, 5 μM leupeptin. Lavages were centrifuged at 1300 × *g* for 30 min and the supernatant was stored at −80 °C until IgA measurement. For the ELISA assays, plates were coated with 2 μg/ml of recombinant full-length Tc52 or NTc52. For the measurement of total IgA in intestinal lavages samples, plates were coated with anti-IgA polyclonal antibody (2 μg/ml). IgA specific titers were normalized to 10 μg of total IgA/well.

2.6. Trypomastigote lysis

Bloodstream trypomastigotes (RA strain) were incubated in duplicate with diluted (1/50) decomplemented sera from immunized mice. Incubation for 30 min at 37 °C with 5% CO_2 was performed in the presence of normal donor fresh serum. Then, the number of living parasites was determined by counting in a Neubauer chamber. The number of parasites in wells incubated with serum samples of nonimmunized mice was used as 100% lysis. Controls were carried out in the absence of mice sera and of complement source.

2.7. Cell invasion assay

Vero cells were infected with transfected blood trypomastigotes expressing β-galactosidase [30] at a parasite-to-cell ratio of 10:1 for 24 h at 37 °C. Trypomastigotes were previously incubated with diluted sera from immunized mice (1/10). Cell invasion by parasites was quantified using chlorophenol red-β-D-galactopyranoside (CPRG), measuring the absorbance at 595 nm. Uninfected cells were used as blanks and cells coincubated with parasites in the absence of mouse serum were used as 100% invasion. Cells infected with parasites pretreated with normal nonimmunized mice sera (in a 1/10 dilution) were used as controls.

2.8. DTH reaction

A delayed-type hypersensitivity (DTH) assay was performed 15 days after the last immunization by intradermal challenge with 5 μg of each recombinant protein (Tc52 and NTc52) in the footpads of the animals. Footpad thickness was measured before and 48 h after antigen inoculation.

2.9. Proliferation assays

Proliferation of spleen cells from immunized mice was done as previously described [22], 17 days after the last immunization. Two hundred thousand spleen cells were cultured for 5 days in the presence of 10 μg/ml Tc52 or NTc52, or 100 μg/ml of fraction F105 (soluble fraction of epimastigote lysate). For the last 18 h, cells were pulsed with 0.5 μCi/well of [^{3}H] thymidine. The results were expressed as proliferation index (PI), defined as the cpm (counts per minute) in the presence/cpm in the absence of the antigen.

2.10. Cytokine quantification

Four million spleen cells from each mouse were cultured in 24-well plates in the presence or absence of 10 μg/ml of recombinant Tc52. Supernatants were taken after 48 h of culture, and the concentrations of IFN-γ and IL-10 were measured by capture ELISA (BD).

2.11. Flow cytometric analysis of CD4 and CD8 IFN-γ^+ T cells

Spleen cells (4×10^6) of NTc52PB and Sempty immunized mice were incubated in 24 well plates for 24 h in the presence or absence of 10 μg/ml of Tc52. Brefeldin A in a final concentration 5 nM was added to the cultures in the last 5 h. Cells were stained with FITC rat anti-mouse CD4 (BD Pharmingen, 553729) and Alexa Fluor 647 anti-mouse CD8a (BD Pharmingen, 557682), fixed, permeabilized and then the intracellular staining was done with PE rat anti-mouse IFN-γ (BD Pharmingen, 554412). Stained cells were analyzed by flow cytometry.

2.12. Blood parasite levels and weight monitoring

Parasitemia was monitored by counting peripheral parasites every 2 days in 5 μl of blood diluted 1/5 in lysis buffer (0.75% NH4Cl, 0.2% Tris, pH 7.2) by direct microscopy examination in a Neubauer chamber.

As an indirect parameter of the protection conferred by vaccination, we evaluated weight as a function of time during the acute phase of infection. Results show the difference between the weight on each day and the weight registered on the day of the infection (day 0), expressed as a percentage. All comparisons were referred to the control group (Sempty).

2.13. Muscle damage determination

Mice sera was taken in the chronic stage of infection (100 days post-infection, dpi), and the activity of muscular enzymes was determined as markers of muscular damage. Aspartate transaminase (AST) and lactate dehydrogenase (LDH) enzyme activities were measured in a spectrophotometric assay in accordance with the manufacturer's protocol (Wiener Lab).

2.14. Histopathological analysis

Heart and skeletal (quadriceps) muscles from vaccinated and infected mice were analyzed at 100 dpi [22]. Inflammation was determined semiquantitatively according to distribution (focal, confluent, or diffuse) [31] and extent of inflammatory cells as follows: (1) single inflammatory focus; (2) multiple, non-confluent foci of inflammatory infiltrate; (3) confluent inflammation; and (4) diffuse inflammation extended throughout the section [32].

2.15. Statistical analysis

Statistical analyses were carried out using Prism software version 5.0 (GraphPad, San Diego, CA), and R [33] using a non-parametric Kruskal–Wallis test and Dunn's post-test. The survival curves were analyzed with a log rank Mantel-Cox test. All the comparisons were done with reference to the control group (Sempty),

except when indicated. p values of less than 0.05 were considered significant.

3. Results

3.1. Expression of recombinant Tc52 and its N-terminal domain in P. pastoris

DNA codifying for Tc52 and its N-terminal domain (NTc52) were amplified from the genomic DNA of *T. cruzi* RA strain epimastigotes and cloned into a pPICzα-A expression vector. The constructions showed 100% identity with a reported gene (GenBank accession number KM273041.1). The presence of the inserts in the pPICzα-A construction was verified by digestion with restriction enzymes (Fig. 1A) and by PCR with specific primers. *P. pastoris* strains GS115 and KM71H were transfected with the constructions linearized with *Sac*I. After selection by antibiotic resistance, the colony PCR using AOX primers verified the proper insertion of DNA fragments in the yeast genome.

In *P. pastoris*, recombinant protein expression levels can be affected by several factors, such as the number of copies of the foreign gene inserted into the genome and the host strain. Therefore, a screening of higher expressers was conducted. Colony blot was carried out after induction with methanol in 20 selected clones for each protein (Tc52 and NTc52) and for each *P. pastoris* strain (GS115 and KM71H). Clones showing higher expression levels were selected to use in further experiments. Methanol concentration was optimized, with great yields at 1.5% and 2%. Tc52 and NTc52 were properly expressed and secreted into culture medium with a molecular weight of 60 and 35 kDa, respectively (Fig. 1B). These molecular weights were slightly higher than those previously reported [22] and predicted based on amino acid sequences (52 and 26 kDa respectively), due to the glycosylation that *P. pastoris* adds to the expressed protein. Properly folded proteins purified under native conditions from culture medium showed yields of 18 ± 3 mg/l culture for Tc52, and 22 ± 4 mg/l culture for NTc52. These yields were higher than those obtained in *E. coli*: average yields of 0.8 and 2.7 mg purified refolded protein/l culture [22].

3.2. The prime-boost strategy elicits systemic and mucosal antibody responses.

The specific antibody response was analyzed by ELISA on plates coated with either recombinant full length Tc52, to evaluate whether the elicited antibodies could recognize their cognate epitope in the native Tc52 exposed by parasites, NTc52 or F105 [34] with similar results. All immunized groups developed specific IgG against Tc52, which was significantly different from that in the control group (Sempty) (Fig. 2A). Nevertheless, mice that received the recombinant protein by systemic route (NTc52PB and NTc52CpG) developed the highest titer. Moreover, the antibody response was significantly different between SNTc52 and NTc52PB groups, showing that the boost with recombinant protein was enough to give a strong antibody response, independently of the prime.

Tc52-specific IgG2a and IgG1 titers were measured to estimate the elicited T cell profile (Fig. 2B and C). Al immunized groups developed anti-Tc52 IgG2a titers, which were significantly higher than those in the Sempty group. In accordance with total IgG response, the NTc52PB group showed higher and significantly different IgG2a titers than SNTc52. Furthermore, all immunized groups exhibited significant differences between IgG2a and IgG1, indicating a Th1-driven immune response.

The ability of specific antibodies to target infective parasites was analyzed by performing two different assays: the capacity of elicited antibodies to lysate bloodstream trypomastigotes in the presence of complement (Fig. 2D), as well as their ability to inhibit parasite cell invasion (Fig. 2E). All immunized mice sera were able to activate complement and lysate bloodstream trypomastigotes (RA strain), showing lysis percentages greater than 20. Sera from mice of the SNTc52 group did not exhibit significant differences with respect to the Sempty control group. All immunized mice sera showed inhibitory activity against cell invasion, with significant differences with respect to control. It is noteworthy that sera from mice in the NTc52PB and NTc52CpG groups showed the highest levels of trypomastigote lysis and inhibition of cell invasion activities. Nevertheless, it must be highlighted that the significantly higher antibody titer in the groups that received at least two doses of recombinant protein were not reflected in the functional assays, especially in the inhibition of *T. cruzi* cell invasion.

We also analyzed the mucosal immune response elicited by the different strategies. Titers of Tc52-specific IgA were evaluated by ELISA in intestinal lavages (Fig. 2F). All immunized groups developed higher titers than in the control; however, the difference was significant only for the groups that received the DNA coding for NTc52 carried by attenuated *Salmonella* (SNTc52 and NTc52PB), highlighting that only the orally immunized mice developed mucosal IgA antibodies.

3.3. The prime-boost strategy induces a strong cellular response in immunized mice

Tc52-specific cellular immune response was analyzed *in vivo* by a delayed-type hypersensitivity (DTH) assay, after the inoculation of rTc52 in the footpads of immunized mice 15 days after the last immunization. All immunized mice showed an increase in footpad thickness 48 h after rTc52 inoculation, although the difference with the control group was only significant for SNTc52 and NTc52PB-vaccinated mice (Fig. 3A).

The cellular immune response was also evaluated *ex vivo*. Spleen cells from mice were removed 17 days after the last immunization, and restimulated *in vitro* with rTc52 (Fig. 3B). All immunized groups showed a higher proliferative response with respect to the control group with no significant differences among them.

We also analyzed cytokine secretion by spleen cell stimulation with rTc52 (Fig. 3C–E). The regulatory IL-10 cytokine was significantly higher than in the control mice (Sempty) for all immunized animals (Fig. 3D). In contrast, only spleen mice cells that received at least two doses of *Salmonella* as DNA delivery system for NTc52 released significant levels of IFN-γ, a Th1-related cytokine, (Fig. 3C). Ratios IFN-γ/IL-10 were 2.77, 2.12 and 1.69 for SNTc52, NTc52PB and NTc52CpG, respectively (Fig. 3E).

In addition, we analyzed by flow cytometry the cells responsible for the IFN-γ increase in the NTc52PB group. In concordance with results of cytokine quantification, when spleen cells were stimulated with Tc52 we found that NTc52PB group increased significantly the number of both $CD4^+$ and $CD8^+$ IFN-γ^+ cells compared with control group (Sempty), and that $CD4^+$ T cell had ~3 times more intracellular INF-γ than $CD8^+$ T cells (Fig. 3F).

3.4. Mice immunized in a prime-boost strategy were protected against a T. cruzi lethal challenge

Protection during the acute stage of infection was evaluated in mice challenged with a deadly dose of bloodstream trypomastigotes. As an indirect parameter of the protection conferred by vaccination, we evaluated weight as a function of time during the acute phase of the infection (Fig. 4A). Mice were weighed (day 0) and then infected. Results show the percentage difference between

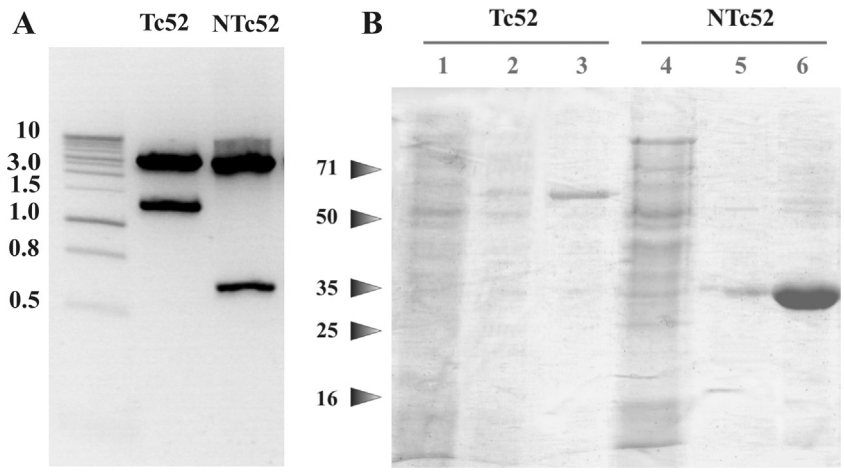

Fig. 1. Cloning, expression and purification of Tc52 and NTc52 in *P. pastoris*. (A) Enzymatic digestion of constructions pPICz-Tc52 (lane 2) and pPICz-NTc52 (lane 3), with *EcoR*I and *Not*I, showing the release of the corresponding inserts. Agarose gel (1.2%) stained with Gel-green is shown. The first lane shows the DNA marker (kbp) with selected band length indicated on the left. (B) Purification of recombinant proteins analyzed by SDS-PAGE. Lanes 1 and 4: Complete *P. pastoris* lysate after induction; lanes 2 and 5: *P. pastoris* culture supernatant after induction; lanes 3 and 6: purified proteins.

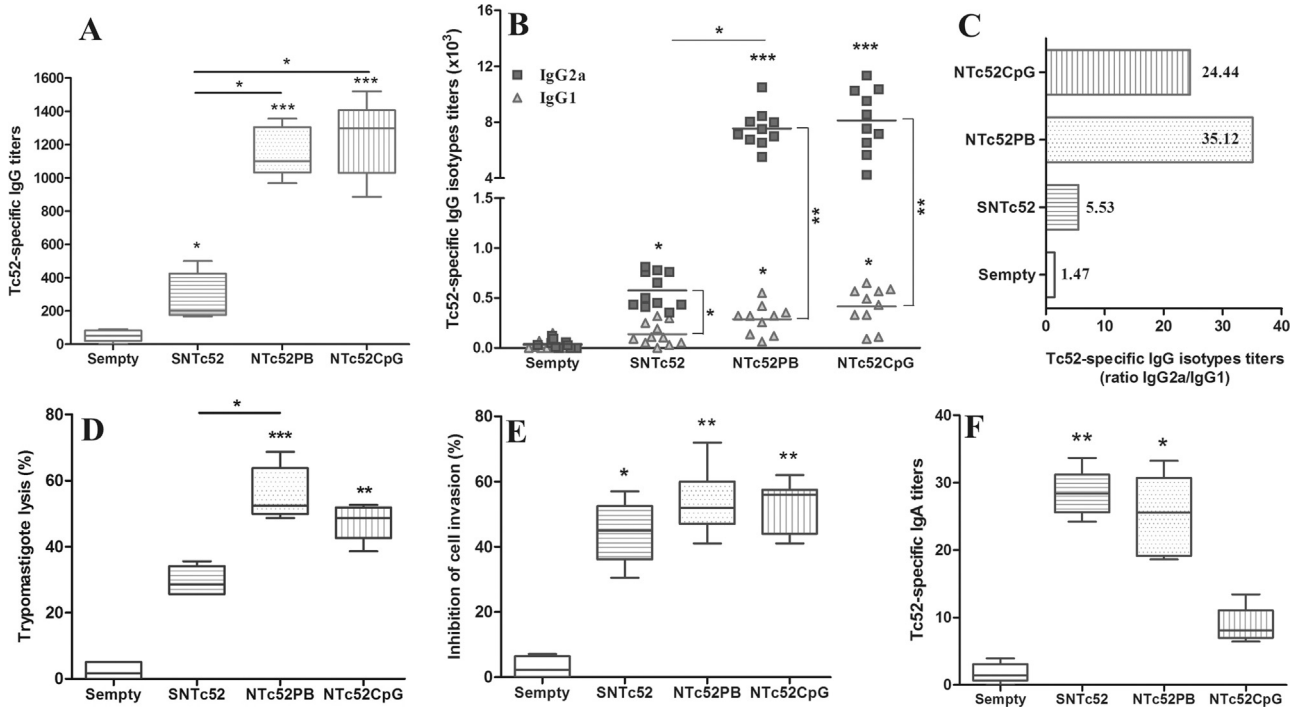

Fig. 2. Antibody response against Tc52 in immunized mice. Animals were vaccinated as: Sempty: attenuated *Salmonella* carrying the empty pcDNA3.1 vector by oral route; SNTc52: *Salmonella* delivering the construct pcDNA-NTc52, orally; NTc52PB: a prime-boost protocol consisting of 2 doses of SNTc52 orally and 2 doses of recombinant NTc52 + ODN-CpG by intradermal route; and NTc52CpG: recombinant NTc52 + ODN-CpG, two intranasal doses followed by two doses by intradermic route. Sera were taken 15 days after the last immunization. (A) Tc52-specific IgG titers in sera, measured by ELISA using immobilized full length Tc52 (2 μg/ml). (B) Tc52-specific IgG1 and IgG2a titers. (C) Titer ratios of Tc52-specific IgG2a/IgG1. (D) Trypomastigote lysis in the presence of immunized mice sera and complement, as previously described [22]. (E) Inhibition of mammalian cell invasion of Tulahuen beta-galactosidase bloodstream trypomastigotes in the presence of immunized mice sera [22]. (F) Tc52-specific IgA in intestinal lavage samples determined by ELISA. Total IgA in each sample was measured by the antibody capture ELISA, and the titer for each sample was then normalized to the total amount of IgA. Results are shown in box plots, the lines correspond to median, boxes to 25th and 75th percentiles, and whiskers to minimum and maximum values. Results are representative of at least three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

the weights on each day post infection and the weight at day 0, indicated by the grey straight line at 0%. As it can be appreciated in Fig. 4A, the uninfected mice gained weight, whereas the infected mice initially gained weight much faster, perhaps due to water retention resulting from the inflammation process, but then they lost weight. At the end of acute infection (21 dpi), only NTc52PB and SNTc52 immunized and infected mice showed body weight loss that was significantly different from that in the Sempty control group ($p < 0.05$).

Parasitemia was measured in each mouse every 2 days (Fig. 4B), and the area under the parasitemia concentration-time curve (AUC) was calculated for each group. The areas up to 21 dpi were 6.26, 5.17 and 3.74 times lower than in the control group for NTc52PB, SNTc52 and NTc52CpG, respectively.

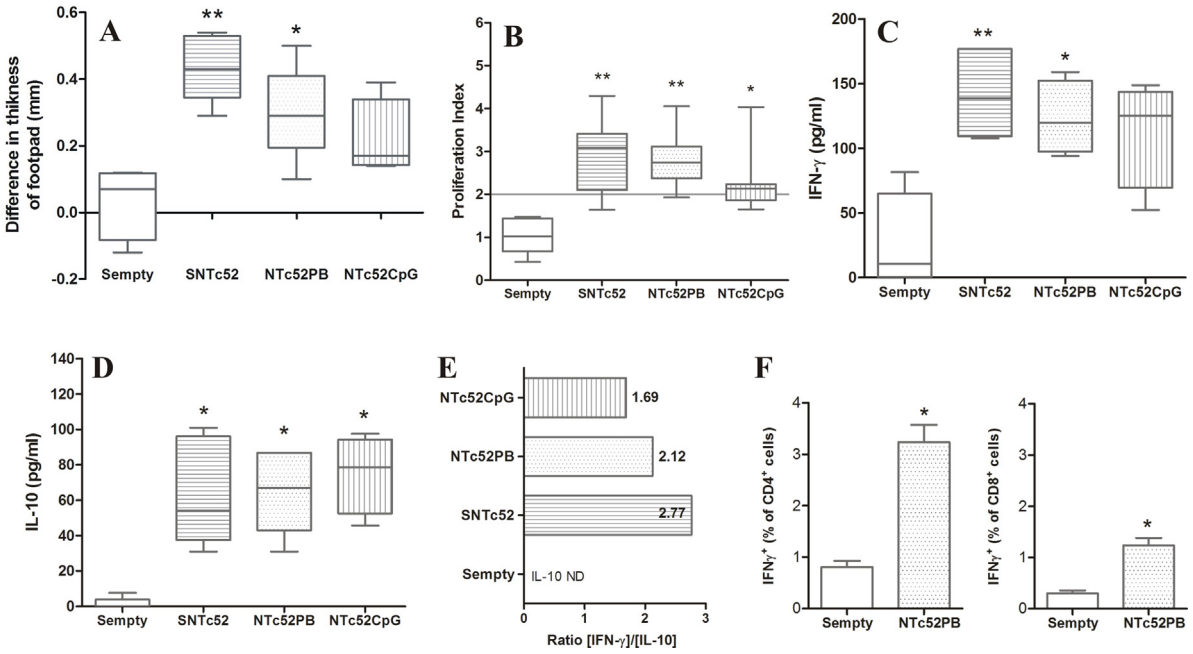

Fig. 3. Mice cellular immune response against Tc52 15 days after the last immunization. (A) Delayed-type hypersensitivity (DTH) test. Footpad thickness in vaccinated mice was measured before and 48 h after inoculation of 5 μg of recombinant Tc52. Results are expressed as the difference in footpad thickness before and after inoculation. Groups were as in Fig. 2. (B) Spleen cell proliferative responses expressed as proliferation index (PI): a ratio of [^{3}H] thymidine incorporation (cpm) among cells stimulated with Tc52 and non-stimulated. The assay was done as previously described [22], 200,000 spleen cells were cultured and 10 μg/ml Tc52 was used for stimulation (C–E). Concentration of cytokines IL-10 (C) and IFN-γ (D) in the supernatant of restimulated splenocytes measured by ELISA. (E) IFN-γ and IL-10 ratio. Results are shown in box plots; lines correspond to median, boxes to 25th and 75th percentiles, and whiskers to minimum and maximum values. (F) Intracellular IFN-γ in CD4$^+$ and CD8$^+$ T cells from spleen of NTc52PB and Sempty groups. Results are representative of two or three independent experiments. ND, non-detectable. $^*p < 0.05$; $^{**}p < 0.01$.

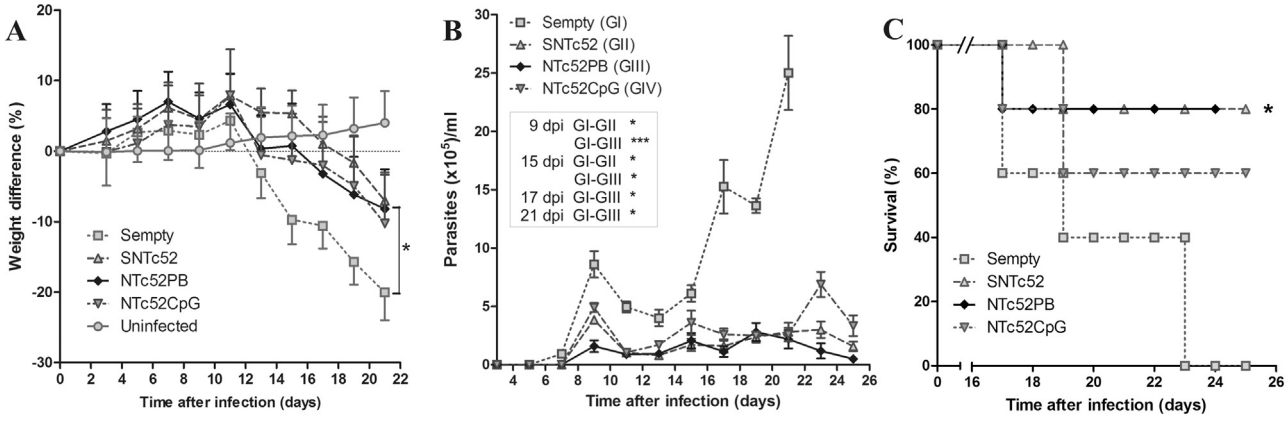

Fig. 4. Protection against a lethal *T. cruzi* challenge. Fifteen days after the last immunization, mice were lethally challenged with 1000 RA strain bloodstream trypomastigotes. (A) Mouse weight was determined every 2 days after infection and the weight difference was recorded for each group. Mice weight in uninfected mice is also shown. The change in weight was expressed as a percentage (W%) and calculated as follows: W% = (Wi − Wo) × 100%/Wo, where Wo is the weight of each mouse immediately before infection, and Wi is their weight on day 1 post infection. (B) Parasitemia after infection was monitored in 5 μl of blood taken every 2 days. Significance on several days post infection is indicated when applicable. (C) Survival was monitored daily. Results are representative of three independent experiments. $^*p < 0.05$; $^{***}p < 0.001$.

Survival was monitored daily after infection. At 25 dpi, all the control group mice (Sempty) had died, whereas the survival of the other groups was 80% for both SNTc52 and NTc52PB, and 60% for NTc52CpG immunized mice (Fig. 4C). Survival was significantly different with respect to the control for the SNTc52 and NTc52PB groups ($p < 0.05$).

3.5. *Vaccination with the NTc52 domain in a prime-boost protocol protects in the chronic stage of* T. cruzi *infection*

The ability of immunizations to limit tissue injury during the chronic stage of infection was evaluated after challenging immunized mice with a sublethal dose (200 parasites) of RA trypomastigotes. At 100 dpi, serum activity of aspartate transaminase (AST) and lactate dehydrogenase (LDH) was measured. Serum activity of these enzymes related to muscular tissue damage in SNTc52 and NTc52PB-immunized mice was significantly lower than that observed in the control group ($p < 0.05$), and most importantly, was similar to the values obtained in sera from non-infected mice (Fig. 5A). Moreover, although the differences were not statistically significant, serum activity in mice immunized following NTc52PB was slightly lower than that in SNTc52 vaccinated animals. Neither alteration nor parasites were observed in the histological sections of cardiac muscles in any of the groups. By contrast, the control group evidenced strong inflammatory infiltrates in skeletal muscle, with parasite debris in some cases,

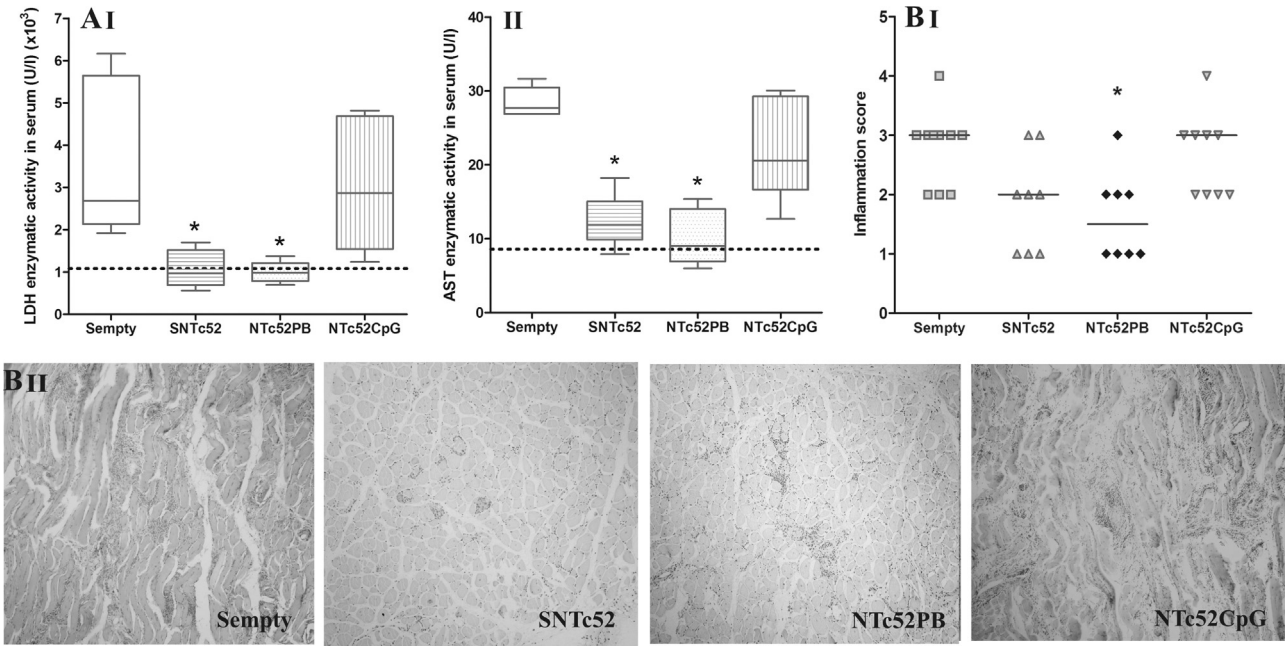

Fig. 5. Protection during chronic stage of *T. cruzi* infection. (A) Serum activity of muscular damage-associated enzymes in sera of immunized mice at 100 dpi. Enzyme levels in normal mice were 1087 ± 10.4, and 8.6 ± 4.6 IU/l for lactate dehydrogenase (LDH) and aspartate transaminase (AST), respectively; means are indicated in the figure with a dotted line. Results are representative of three independent experiments (*$p < 0.05$). (B) Micrographs of skeletal muscle of immunized and infected mice at 100 dpi, stained with hematoxylin and eosin. Inflammation scores are shown in panel BI. Each point represents an individual mouse in two experiments; the median of each group is indicated by a horizontal line. (BII) Representative histological sections (×400 magnifications) from Sempty, SNTc52, NTc52PB, and NTc52CpG immunized and infected mice.

which was not observed in any of the immunized groups (Fig. 5B). Median of the inflammatory score (IS) was 2 for SNTc52 and 1.5 for NTc52PB, both lower than in the Sempty control group (median of IS = 3); however, the difference with the control group was significant only for NTc52PB ($p < 0.05$).

4. Discussion

Tc52 and its N-terminal domain were cloned and expressed in the yeast *Pichia pastoris*, an easy to cultivate eukaryotic microorganism that allows the heterologous expression of recombinant proteins. With the adequate vectors, the protein of interest could be expressed in the intracellular compartment or routed to the secretion system. We previously cloned and expressed these proteins in *E. coli* as inclusion bodies that required refolding protocols to have a useful antigen as vaccine [22]. In this work, we set the conditions for *P. pastoris* to express both proteins in the extracellular medium as soluble and folded molecules. The yields were 22 mg/l culture for NTc52 and 18 mg/l culture for Tc52, similarly to other proteins [26,35,36].

In a previous work, pcDNA3.1 encoding NTc52 carried by attenuated *Salmonella* and administrated by oral route, showed to be an excellent vaccine candidate, conferring protection against *T. cruzi* infection [22]. To improve the efficacy of this vaccine, the NTc52 antigen was tested here using different immunization strategies to look for an improved cellular and antibody response, along with specific mucosal immunity. Even when the same antigen was used in all immunization protocols, the immune response was quantitatively and qualitatively different. While SNTc52 induces low specific IgG titers, NTc52PB and NTc52CpG groups, receiving two doses of the recombinant protein by intradermal route, showed a potent antibody response, which was predominantly of the IgG2a isotype. This was expected according to reports in which vaccination is carried out by this route with protein plus ODN-CpG [7,37,38]. However, the important differences in antibody titers among the groups that only received NTc52 DNA transported by *Salmonella* compared with the two groups that received recombinant protein were not proportionally reflected in the functional assays. Thus, SNTc52 showed trypomastigote lysis percentages over 20% but without significant differences with respect to the Sempty control group, in accordance with the slight antibody response developed. However, the slight antibody immune response developed by immunization with SNTc52 was more efficient in cell invasion inhibition (over 40%) than in parasite lysis (Fig. 2). Although further studies are needed, these results could be affected by several factors including: (1) sera dilution used was tested and selected independently to ensure the best performance of each assay, therefore, we used a 1/50 dilution for the lysis assay, and 1/10 for cell invasion inhibition [22]; (2) *T. cruzi* strains were different since RA was used for the lysis assay, and recombinant Tulahuen for the cell invasion inhibition assay; and (3) The profile of antibody isotypes is not the same in all immunization protocols. We just measured specific IgG1 and IgG2a; however other isotypes could also be involved and perhaps different proportions of complement-fixing antibodies could be developed in each protocol. There were no important differences in titers and functional antibody assays between groups NTc52PB and NTc52CpG, showing that at least 2 doses with the recombinant protein plus ODN-CpG are enough to develop strong specific IgG antibodies able to mediate trypomastigote lysis and cell invasion inhibition.

Transmission of *T. cruzi* infection could take place in different manners, and in the last years the importance of the oral route has increased [39]. By this route the parasite infects through the oral, esophageal, gastric and intestinal mucosa [40]. We are focused on the development of a vaccine conferring mucosal and systemic immunity. The challenge was conducted intraperitoneally as a heterologous route for a more rigorous test of vaccine efficacy. Both groups that received at least two doses of DNA coding for NTc52 carried by attenuated *Salmonella* (SNTc52 and NTc52PB) developed high specific IgA titers. By contrast, mice that received NTc52 in two doses by intranasal route, and two by intradermal

route (NTc52CpG) to avoid protein degradation by oral immunization, did not significantly increase anti-Tc52 IgA with respect to the control. In accordance with other reports [7,8,22,41], these results emphasize the potential of attenuated *Salmonella* as DNA delivery system for a mucosal vaccine [42].

All immunized groups developed a Tc52-specific cellular immune response; however, some differences were observed among them. Both SNTc52 and NTc52PB groups showed a strong cellular immune response, with a predominant Th1 profile including high IFN-γ and a regulatory component of IL-10. However, NTc52PB IgG2a/IgG1 ratio was much higher than in the SNTc52 group. In contrast, group NTc52CpG developed a weak cellular response, in terms of DTH, spleen cell proliferation and IFN-γ. Although the NTc52CpG group exhibited a high IgG2a/IgG1 ratio, suggesting a Th1-driven immune response, the IFN-γ was weak and the IFN-γ/IL-10 ratio was lower than in the other immunized groups. These somehow contradictory results could be due to the different cell types having ability for IL-10 secretion [43,44], and also to several factors influencing the stimulation of the IgG isotype [45,46]. The importance of a balanced immune response, including both effectors (such as specific Th1 cells) and regulatory components (such as IL-10 and Foxp3^{+} CD25^{high} CD4^{+} regulatory T cells), in the prevention of severe *T. cruzi*-induced disease was already established [47,48]. Cultures from PBMC from patients with different stages/degrees of cardiomyopathy, associated with Chagas disease, have shown that a reduced production of IL-10 and IL-17 in association with high levels of IFN-γ and TNF-α, correlates with the severity of Chagas disease cardiomyopathy [49,50]. In addition, it was also demonstrated in the vaccine development field that the induction of a balanced immune response with both inflammatory and regulatory cytokines protects against *T. cruzi* infection [36,51].

After *T. cruzi* challenge, all immunization prototypes conferred protection during the acute stage of the infection, in terms of parasitemia, weight loss control, and survival. Nevertheless, the NTc52CpG-vaccinated animals showed less protection than other immunization strategies as reflected by the death of mice during the acute phase of infection, parasitemia levels, as well as by the high serum levels of AST and LDH at the chronic phase of *T. cruzi* infection (Figs. 4 and 5).

A boost of NTc52 plus ODN-CpG in mice who have previously received *Salmonella* as DNA delivery system of NTc52 allowed better control of parasite infection with respect to SNTc52, as seen in parasitemia. This effect can be due, at least in part, to the strong antibody response elicited after the boost with the recombinant protein (combined with a strong, specific and balanced cellular immune response that we have observed in animals receiving at least 2 doses of SNTc52). This protection seems to be long lasting, since NTc52PB-immunized animals exhibited less tissue damage during chronic infection (Fig. 5).

The strength of prime-boost strategies combining DNA and protein vaccines was demonstrated in this case and also in other infections. Nevertheless, in some cases these strategies were effective [38,52–54] and not in others [7,55], depending on the antigen, the adjuvant and the targeted pathogen. Our prime-boost strategy, combining SNTc52 prime and a boost with NTc52 plus ODN-CpG, was the vaccine protocol that induced cellular and humoral immune responses, conferring the highest protection both in the acute and chronic stages of the infection.

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