Infection and Immunity

Tc52 Amino-Terminal-Domain DNA Carried by Attenuated Salmonella enterica Serovar Typhimurium Induces Protection against a Trypanosoma cruzi Lethal Challenge

Marina N. Matos, Silvia I. Cazorla, Augusto E. Bivona, Celina Morales, Carlos A. Guzmán and Emilio L. Malchiodi

Infect. Immun. **2014, 82(10):4265. DOI: 10.1128/IAI.02190-14. Published Ahead of Print 28 July 2014.**

IAI Journals.ASM.org

Tc52 Amino-Terminal-Domain DNA Carried by Attenuated *Salmonella enterica* Serovar Typhimurium Induces Protection against a *Trypanosoma cruzi* Lethal Challenge

Marina N. Matos,[a,b] Silvia I. Cazorla,[a,b] Augusto E. Bivona,[a,b] Celina Morales,[c] Carlos A. Guzmán,[d] Emilio L. Malchiodi[a,b]

Cátedra de Inmunología and Instituto de Estudios de la Inmunidad Humoral, UBA-CONICET, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina[a]; Instituto de Microbiología y Parasitología Médica, UBA-CONICET, and Departamento de Microbiología, Parasitología e Inmunología, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina[b]; Instituto de Fisiopatología Cardiovascular, Departamento de Patología, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina[c]; Department of Vaccinology and Applied Microbiology, Helmholtz Centre for Infection Research, Braunschweig, Germany[d]

In this work we immunized mice with DNA encoding full-length Tc52 or its amino- or carboxy-terminal (N- and C-term, respectively) domain carried by attenuated *Salmonella* as a DNA delivery system. As expected, *Salmonella*-mediated DNA delivery resulted in low antibody titers and a predominantly Th1 response, as shown by the ratio of IgG2a/IgG1-specific antibodies. Despite modest expression of Tc52 in trypomastigotes, the antibodies elicited by vaccination were able to mediate lysis of the trypomastigotes in the presence of complement and inhibit their invasion of mammal cells *in vitro*. The strongest functional activity was observed with sera from mice immunized with *Salmonella* carrying the N-term domain (SN-term), followed by Tc52 (STc52), and the C-term domain (SC-term). All immunized groups developed strong cellular responses, with predominant activation of Th1 cells. However, mice immunized with SN-term showed higher levels of interleukin-10 (IL-10), counterbalancing the inflammatory reaction, and also strong activation of Tc52-specific gamma interferon-positive (IFN-γ^+) CD8$^+$ T cells. In agreement with this, although all prototypes conferred protection against infection, immunization with SN-term promoted greater protection than that with SC-term for all parameters tested and slightly better protection than that with STc52, especially in the acute stage of infection. We conclude that the N-terminal domain of Tc52 is the section of the protein that confers maximal protection against infection and propose it as a promising candidate for vaccine development.

Trypanosoma cruzi is an intracellular protozoan parasite and the etiological agent of Chagas disease. The parasite is transmitted to humans by infected feces of triatomine insects that feed on blood. At present, approximately 7 to 10 million people are infected with *T. cruzi* in areas of endemicity in Latin America, and there is an incidence of 56,000 new cases per year considering all forms of transmission; Chagas disease causes 12,000 deaths annually, and 100 million people are at risk of infection (1). Up to 30% of chronically infected people develop cardiac alterations, and up to 10% develop digestive, neurological, or mixed alterations, for which specific treatment may become necessary (2). The number of cases of infection in areas where the parasite is not endemic is increasing because of the migration of people from areas of endemicity and the absence of adequate control in blood banks, which promotes transfusion transmission. Furthermore, in countries where the parasite is not endemic, the majority of people infected by the parasite that causes Chagas disease ignore that they are infected. The CDC estimates that in the United States more than 300,000 people are infected with *T. cruzi* (3). Although there are triatomine bugs in the United States, only rare vector-borne cases of Chagas disease have been documented. The WHO estimates that the number of infected people in Europe exceeds 80,000, with more than 3,900 laboratory-confirmed cases during the past 10 years in Belgium, France, Italy, Spain, Switzerland, and the United Kingdom (4).

The pharmacological treatment with benznidazole or nifurtimox is efficient only in the acute phase of the infection and is highly toxic due to the extension of the treatment, with important associated side effects. Thus, not only the development of new efficient treatments and better vectorial control but also the development of efficient prophylactic and therapeutic vaccines is important. Several attempts have been made to confer protection against *T. cruzi* experimental infection using recombinant proteins including cruzipain (Cz), amastigote surface protein 2 (ASP-2), paraflagellar rod proteins (PFR), Tc24, and trans-sialidase (TS), among others (reviewed in references 5 to 7). Viruses (8–11) and bacteria (12) have been used as delivery systems for DNA vaccines, and new adjuvants have also been tested (13–15).

Tc52 is a *T. cruzi* protein with glutathione transferase activity (16) and immunomodulatory properties (17, 18). Tc52 has two domains: the amino-terminal (N-term) domain of 26 kDa that carries the enzyme active site and a carboxy-terminal (C-term) domain of 25 kDa whose function is not understood. By alignment of the amino acid sequences, the domains have 27% identity and an additional 27% homology (18, 19). Tc52 is crucial for the survival of the parasite as the knockout of both alleles is lethal (20). Tc52 is also highly conserved, with the presence of single nucleotide polymorphisms (SNPs) between some strains, and its expres-

Received 10 June 2014 Returned for modification 11 July 2014
Accepted 22 July 2014
Published ahead of print 28 July 2014
Editor: J. A. Appleton
Address correspondence to Emilio L. Malchiodi, emalchio@ffyb.uba.ar.

doi:10.1128/IAI.02190-14

sion was demonstrated in different strains of *T. cruzi* (21–24). All of these characteristics make Tc52 a promising vaccine candidate. Early immunization attempts making use of native Tc52 purified from the parasite formulated with *Bordetella pertussis* and alum hydroxide as an adjuvant resulted in partial protection against infection (17). It was also shown that therapeutic vaccination using naked DNA coding for Tc52 and $AlPO_4$ as an adjuvant promotes parasite clearance (25). However, the use of an antigen purified from parasites and the fact that DNA is rapidly degraded in living organisms represent serious limitations to a vaccine candidate.

Attenuated bacteria have been shown to be good delivery systems for DNA coding for different proteins in the development of vaccines against different infectious diseases (26–30; reviewed in references 31 and 32). We previously studied the protection generated by the DNA encoding cruzipain, the major cysteine proteinase of *T. cruzi*, carried by attenuated *Salmonella enterica* against infection with *Trypanosoma cruzi* (12). Here, we analyzed the immune response generated by attenuated *Salmonella* carrying a plasmid encoding full-length Tc52 or its N-term and C-term domains and the protection elicited by them against *T. cruzi* infection.

MATERIALS AND METHODS

Parasite. *T. cruzi* epimastigotes (RA strain) were grown in LIT medium (5 g/liter liver infusion, 5 g/liter tryptose, 2 g/liter glucose, 68 mM NaCl, 5.4 mM KCl, 22 mM HPO_4Na_2), with 20 mg/liter hemin and 10% (vol/vol) fetal calf serum (FCS). The *T. cruzi* bloodstream trypomastigotes (RA strain) and the recombinant Tulahuen strain expressing β-galactosidase (Tul-β-Gal) (33) were isolated from infected mice.

Tc52 cloning, expression, and purification. Tc52 and its N-term (residues 1 to 223) and C-term (residues 224 to 445) domains were cloned in pET23a plasmids. For that purpose, genomic DNA was extracted from epimastigotes (RA strain) using a genomic DNA extraction kit (Qiagen, CA, USA). The sequences coding for Tc52 and its N-term and C-term domains were amplified by PCR using the following sets of primers: for full-length Tc52, the forward primer 5′-CGACTGCATATGAAGGCTTTGAAACTTTTTAAAGA-3′ containing an NdeI restriction site (underlined) and the reverse primer 5′-ACTAGCAAGCTTAGACGATGGACGCAAAAACG-3′ with an HindIII restriction site; for the N-term domain, the forward primer of Tc52 and the reverse primer 5′-ACTAGCAAGCTTTCAGTGATGGTGATGGTGATGCAATGACCATGTGACGTGC-3′ with an HindIII site and a sequence encoding a His_6 tag (both underlined); and for the C-term domain, the forward primer 5′-CGACTGCATATGGCTCCTGGCTATGTACTTTTTGTT-3′ (NdeI restriction site) and the reverse primer 5′-ACTAGCAAGCTTTCAGTGATGGTGATGGTGATGAGACGATGGACGCAAAAACG-3′ with an HindIII restriction site and a His_6 tag. The PCR products of 1,377, 717, and 708 bp, respectively, were digested with NdeI and HindIII and cloned in the prokaryotic expression vector pET23a. The three constructions were sequenced and showed more than 99% identity with the Y strain (AAG08957.1) and identity of 100% for each domain with the full-length sequence, indicating that no mutations were generated during the PCR amplification.

Escherichia coli BL21(DE3) cells were chemically transformed with the constructions and cultured to an optical density (OD) of 0.7, and expression was induced for 4.5 h with 1 mM isopropyl-β-D-thiogalactopyranoside. Full-length Tc52 and its N-term and C-term domains were expressed as inclusion bodies (IBs). After induction, the cells were lysed with 2.5% Triton X-100 in phosphate-buffered saline (PBS) and subjected to sonication. The IBs were washed twice with 1% Triton X-100 in PBS and once with PBS. Then, the IBs were solubilized in denaturation buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea), pH 8, sampled in a Ni-nitrilotriacetic acid (NTA) column, and purified under denaturing conditions by pH gradient. The proteins were then dialyzed against PBS with decreasing concentrations of urea. Endotoxin in the recombinant proteins was measured by the *Limulus* amebocyte lysate (LAL) assay; the levels were <10 endotoxin units (EU)/mg protein.

Purified proteins were analyzed by SDS-PAGE and immunoblotting to verify purity. Briefly, 10 μg of each purified protein and 100 μg of F105 (soluble fraction of epimastigote lysate) were separated by 12.5% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. As primary antibodies, sera from mice immunized with the recombinant protein Tc52, the N-term domain, or the C-term domain or sera from chronically infected mice were used. As secondary antibody, rabbit anti-mouse Fc conjugated with peroxidase was used; the reaction was reveled using 4-chloro-1-naftol and H_2O_2.

Cloning and expression of Tc52 and the N-term and C-term domains in a eukaryotic expression system. The sequences coding for Tc52 and the N-term and C-term domains were amplified by PCR using the following sets of primers: for full-length Tc52, the forward primer 5′-CGACTGAAGCTTGCGATGAAGGCTTTGAAACTTTTTAAAGA-3′ (HindIII and Kozak sequence underlined), and the reverse primer 5′-ACTAGCGAATTCTCAAGACGATGGACGCAAAAA-3′ (EcoRI); for Tc52 N-term domain, the forward primer used for Tc52 and the reverse primer 5′-ACTAGCGAATTCTCACAATGACCATGTGACGTGC-3′ (EcoRI); for the Tc52 C-term domain, forward primer 5′-CGACTGAAGCTTGCGATGGCTCCTGGCTATGTACTTTTTGTT-3′ (HindIII and Kozak sequence) and the reverse primer used for Tc52. The PCR products of 1,365, 702, and 696 bp, respectively, were digested with EcoRI and HindIII and cloned in the pcDNA3.1(+) eukaryotic expression vector, thereby generating pcDNA-Tc52, pcDNA–N-term, and pcDNA–C-term, respectively. The three constructions were sequenced and showed more than 99% identity to the Y strain, and identity of 100% for each domain compared with the full-length sequence, indicating that no mutations were generated during the PCR amplification.

COS-7 cells in 90% confluence were transfected in the presence of Lipofectamine (Invitrogen, CA, USA) with the constructions pcDNA-Tc52, pcDNA–N-term, pcDNA–C-term, and pcDNA3.1 without the insert as a control and incubated for 48 h at 37°C with 5% CO_2. Expression was detected by immunofluorescence assay using specific serum from BALB/c mice immunized with the purified proteins and Freund's adjuvant against the three proteins.

Attenuated *Salmonella enterica* serovar Typhimurium *aroA* SL7207 bacteria were transformed by electroporation (34) with the pcDNA-Tc52, pcDNA–N-term, or pcDNA–C-term construction or with the pcDNA3.1(+)vector without insert.

Immunizations and challenge. Four groups (10 animals/group) of inbred female 6- to 8-week-old C3H/HeN (H-2K haplotype) mice were immunized four times every 10 days with 10^9 CFU of *Salmonella* SL7207 carrying the following: group I (GI), the pcDNA3.1 vector (S-empty); GII, the construct pcDNA3.1-Tc52 (STc52); GIII, the construct pcDNA3.1-N-term domain (SN-term); and GIV, the construct pcDNA3.1-C-term domain (SC-term). The oral immunization was carried out by feeding each mouse 20 μl of PBS carrying 10^9 CFU of the corresponding *Salmonella* construct. One half of the animals of each group were selected randomly and used to study the immune response, and the other half were infected 15 days after the last immunization with 10^3 *T. cruzi* bloodstream trypomastigotes of the highly virulent RA strain. Another set of mice was used for chronic infection experiments, receiving 200 RA strain trypomastigotes. Experiments that used animals were approved by the Review Board of Ethics of the IDEHU and conducted in accordance with the guidelines established by the National Research Council (CONICET).

Antibody determination. Serum samples and intestinal lavage samples were collected 15 days after the last immunization for the measurement of antigen-specific IgG, IgG1, IgG2a, and IgA. Polyvinylchloride 96-well plates (Nunc/Thermo Scientific) were coated with recombinant Tc52 at a concentration of 2 μg/ml in PBS and incubated overnight at 4°C. The plates were then blocked with 3% bovine serum albumin (BSA) in PBS, washed, and incubated with the following antibodies: anti-IgG-biotin (B6398; Sigma), anti-IgG1-biotin (02232D; Pharmingen, Becton

Dickinson), anti-IgG2a-biotin (Pharmingen, Becton Dickinson), and anti-IgA-horseradish peroxidase (HRP; Sigma). For the IgG, IgG1, and IgG2a measurements, after several washes the plates were incubated for 30 min at 37°C with streptavidin-HRP (BD Biosciences). The reaction was developed by the addition of *o*-phenylenediamine dihydrochloride (OPD; 1 mg/ml) (ICN Biomedicals, Inc.) and 0.045% H_2O_2 in citrate buffer (0.1 M citric acid, 0.1 M PO_4HNa_2, pH 5.0). The absorbance was measured at 492 nm in an enzyme-linked immunosorbent assay (ELISA) reader (Labsystems Multiscan EX). Endpoint titers were defined as the dilution that resulted in an OD value greater than a cutoff, which was calculated as the median plus 2 standard deviations (SD) of negative-control serum samples.

Total IgA in intestinal lavage samples was measured by ELISA in plates coated with anti-IgA polyclonal antibody (M8769; Sigma) at a concentration of 2 μg/ml in PBS and incubated with the samples in different dilutions or with an IgA standard (M1421; Sigma). Plates were washed and then incubated with anti-IgA-peroxidase (A4789; Sigma). The reaction was developed with OPD and H_2O_2. Anti-Tc52 IgA titers were normalized to the amount of total IgA in each sample.

Trypomastigote lysis assay. Bloodstream trypomastigotes (RA strain) were purified from the blood of infected mice. A total of 500,000 parasites were coincubated (30 min, 37°C) with the serum at a final 1/50 dilution and fresh serum as a source of complement in Mayer buffer (142 mM NaCl, 5 mM sodium veronal, 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, pH 7.5). Then, live parasites were counted using a Neubauer chamber. The number of parasites from serum samples of normal nonimmunized mice was used as 0% lysis. A control without fresh serum was also included.

Cell invasion assay. Purified *T. cruzi* bloodstream trypomastigotes expressing β-galactosidase (Tul-β-Gal) were pretreated for 30 min at 37°C with the serum (in a 1/10 dilution) and then coincubated (37°C, 5% CO_2, 18 h) with Vero cells at a ratio of 10:1 in RPMI medium–5% fetal bovine serum in 96-well plates. Then, cells were washed with PBS to remove extracellular trypomastigotes and incubated for 5 days with RPMI medium without phenol red and 5% FBS. Nonidet P-40 and chlorophenol red-β-D-galactopyranoside (CPRG) were added to final concentrations of 1% (vol/vol) and 0.1 mM, respectively, and incubated for 4 h. Absorbance was measured 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 mouse serum (in a 1/10 dilution) were used as controls.

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 (full-length Tc52 and its N-term or C-term domain) in the footpads of the animals. The thickness of the footpads was measured before and 48 h after the antigen inoculation.

Proliferation assays. Mice were sacrificed 17 days after the last immunization, the spleens were taken aseptically, and cell suspensions were prepared. A total of 200,000 spleen cells were cultured in the presence or absence of 10 μg/ml of recombinant Tc52 in RPMI medium in 96-well plates. The cells were pulsed 3 days later with [^{3}H]thymidine (0.5 μCi/well). After 18 h the cells were harvested, and the incorporated radioactivity was measured in a liquid scintillation counter (35). The results were expressed as proliferation index (PI), defined as the cpm (counts per minute) in the presence of the antigen/cpm in the absence of the antigen.

Cytokine quantification. Four million spleen cells of each mouse were cultured in 24-well plates in the presence or absence of 10 μg/ml of recombinant Tc52. After 48 h of culture, the supernatant was taken. The concentrations of interleukin-2 (IL-2), gamma interferon (IFN-γ), and IL-10 were measured by capture ELISA (R&D System, Minneapolis, MN).

Flow cytometric analysis of $CD8^+$ IFN-γ^+ cells. For the determination of $CD8^+$ T cells activated in the presence of Tc52, 4×10^6 spleen cells of each group 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 of 5 nM was added to the cultures in the last 5 h. Cells were stained with fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD4 (553729; BD Pharmingen) and Alexa Fluor 647-conjugated anti-mouse CD8a (557682; BD Pharmingen), fixed, and permeabilized, and then intracellular staining was done with phycoerythrin (PE)-conjugated rat anti-mouse IFN-γ (554412; BD Pharmingen). Stained cells were analyzed by flow cytometry in a PAS-III flow cytometer (Partec, Görlitz, Germany).

Peptide-specific $CD8^+$ T cell cytotoxicity assay. Based on the Tc52 sequence, we designed and synthetized peptides that would be presented in the context of major histocompatibility complex (MHC) class I H2-K. For that purpose, we analyzed the sequence of the whole protein with four databases, SYFPEITHI (http://www.syfpeithi.de/), Rankpep (http://bio.dfci.harvard.edu/RANKPEP/), BIMAS (http://www-bimas.cit.nih.gov/molbio/hla_bind/), and MAPPP (http://www.mpiib-berlin.mpg.de/MAPPP/binding.html), and selected those that showed high scores at least in two databases: peptide 1 (pep1), RETVPTLQV (residues 50 to 58); pep2, HKTAYIEGI (127 to 135); pep3, AEVMVVPFL (154 to 161); pep4, EEYIIGFK (205 to 212); pep5, KDADGLYHV (310 to 318); and pep6, SEAGKKVF (420 to 427). The ranking of the peptides chosen, from higher to lower score, is pep4, pep6, pep1, pep3, pep2, and pep5. An unrelated peptide (AEEAFRLSV) was used as a control.

Spleen cells from immunized mice were incubated with 10 μg/ml of recombinant Tc52 for 5 days at 37°C with 5% CO_2. The $CD4^+$ cells were lysed by incubation with GK1.5 hybridoma in the presence of fresh serum for 30 min at 4°C, washed twice with RPMI medium, and used as effector cells. Peritoneal cells (target) taken from C3H/HeN (H-2K haplotype) mice were incubated (1 h at 4°C) with 1 μM each peptide, washed with PBS, and stained for 30 min at 4°C with 1 μM carboxyfluorescein succinimidyl ester (CFSE). The stain was stopped by the addition of fetal bovine serum (FBS) to a final concentration of 15%. Effector cells were coincubated with target cells at a ratio of 20:1 for 4 h at 37°C in 5% CO_2. Propidium iodide (PI) was added, and immediately afterwards stained cells were analyzed by flow cytometry in a PAS-III flow cytometer (Partec, Görlitz, Germany). The results were expressed as ratios of the percentages of CFSE-positive ($CFSE^+$) and PI-positive (PI^+) cells as follows: (% $CFSE^+$ PI^+ cells in the presence of peptide/% $CFSE^+$ cells in the presence of peptide)/(% $CFSE^+$ PI^+ cells in the absence of peptide/% $CFSE^+$ cells in the absence of peptide).

Blood parasite levels and weight monitoring. Mice were infected 15 days after the last immunization with 10^3 *T. cruzi* bloodstream trypomastigotes of the highly virulent RA strain. Every 2 or 3 days after the challenge, the weight and parasitemia were measured. Five microliters of blood was taken from the tail of each mouse; the number of parasites was determined using a Neubauer chamber. The change in weight was expressed as a percentage (Δ*W*%) and calculated as follows: $\Delta W\% = (W_i - W_o) \times 100\%/W_o$, where W_o is the weight of each mouse immediately before infection, and W_i is its weight at day *i* postinfection.

Muscle damage determination. The activity of muscular enzymes was determined in sera of mice in the chronic stage of the infection as markers of muscle injury. Creatinine kinase (CK), aspartate transaminase (AST), and lactate dehydrogenase (LDH) enzymes were measured at 100 days after the challenge. The enzyme activity was measured in a spectrophotometric assay according to the description of the manufacturer (Wiener Lab).

Histopathologic analysis. The histological features of heart and skeletal (quadriceps) muscles from vaccinated and infected mice at 100 days postinfection (dpi) were also investigated. The removed organs were rinsed with PBS, fixed for 24 h in 10% buffered formalin, embedded in paraffin, sectioned, stained with hematoxylin-eosin, and examined by light microscopy. A blind histological test was done, analyzing 10 microscopic fields in 10 sections of each organ. Inflammation was evaluated semiquantitatively according to distribution (focal, confluent, or diffuse) (36) and extent of inflammatory cells (1+ for a single inflammatory focus; 2+ for multiple, nonconfluent foci of inflammatory infiltrate; 3+ for confluent inflammation; and 4+ for diffuse inflammation extended throughout the section) (37, 38).

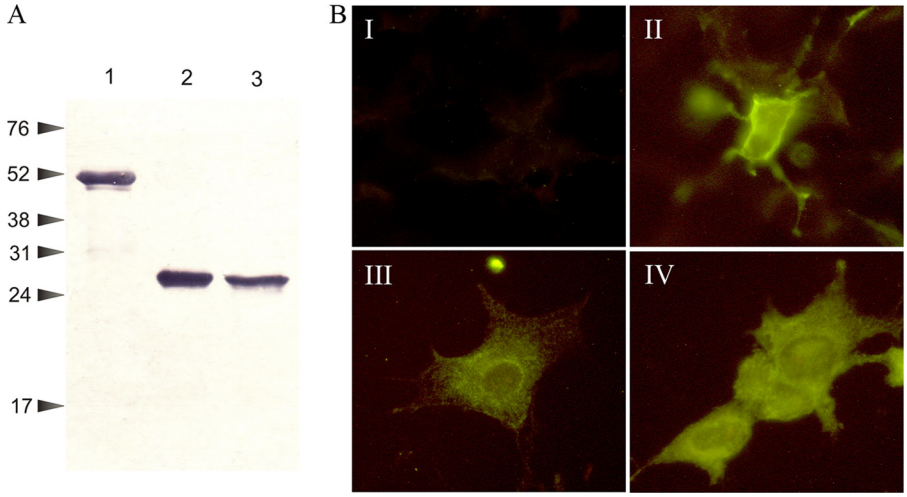

FIG 1 Expression of recombinant Tc52 and its N-term and C-term domains. (A) Immunoblotting of the recombinant proteins expressed in *E. coli* using sera from *T. cruzi* chronically infected mice. Lane 1, rTc52; lane 2, N-term domain; lane 3, C-term domain. At left, the molecular size markers (in kDa) are shown. (B) Immunofluorescence assay of COS-7 cells transfected with pcDNA3.1 (frame I; empty), pcDNA-Tc52 (II), pcDNA-N-term (III), and pcDNA-C-term (IV). The first antibodies were mouse polyclonal antibodies against the recombinant proteins Tc52 (frames I and II), N-term domain (III), and C-term domain (IV). The second antibody was FITC-labeled rabbit anti-mouse IgG.

Statistical analysis. Statistical analyses were carried out with Prism, version 5.0, software (GraphPad, San Diego, CA), using a nonparametric Kruskal-Wallis test and Dunn's posttest. The survival curves were analyzed with a log rank Mantel-Cox test. All the comparisons were in reference to the control group (immunized with *Salmonella* carrying the empty vector; GI), except when indicated. *P* values of <0.05 were considered significant.

Nucleotide sequence accession number. The sequence data for Tc52 have been deposited in GenBank under accession no. KM273041.

RESULTS

Expression and characterization of the recombinant proteins. The DNA coding for full-length Tc52 and its N-term and C-term domains were amplified from *T. cruzi* RA strain genomic DNA and cloned in the prokaryotic expression vector pET23a. Recombinant Tc52 (rTc52) and the N-term and C-term domains were expressed in *E. coli* BL21(DE3) as inclusion bodies, purified, and refolded. The proper folding of the proteins was assessed by circular dichroism (data not shown). The recombinant proteins were recognized by sera of chronically infected mice in ELISAs (data not shown) and in Western blotting, showing the expected molecular sizes (Fig. 1A). Polyclonal serum obtained from immunizing mice with either rTc52 or the N-term or C-term domain recognized native Tc52 present in the soluble fraction of lysed epimastigotes (F105) (35) of the RA strain (data not shown). These results suggested that the recombinant proteins would be able to generate an immune response able to recognize the parasite in the context of an infection.

In addition, the DNA coding for Tc52 or the N-term or C-term domain was cloned in the eukaryotic expression vector pcDNA3.1. The ability of mammalian eukaryotic cells to express the proteins was verified by transfecting COS-7 cells with the constructions. The transient expression in transfected cells was verified by immunofluorescence assays using the polyclonal serum against each one of the recombinant proteins (Fig. 1B).

The *Salmonella* Tc52 DNA delivery system elicited a specific antibody response. We analyzed the specific immune response against full-length Tc52 by ELISA in groups of mice immunized four times orally with attenuated *Salmonella* bacteria harboring the pcDNA3.1 vector carrying Tc52 (STc52) or the N-term (SN-term) or C-term (SC-term) domain. The reason for using full-length Tc52 to test humoral responses instead of the immunizing antigen used for each group was that the elicited antibodies would be effective only if they could recognize their cognate epitope in the full-length Tc52. Control mice were inoculated with *Salmonella* transformed with the empty pcDNA3.1 (S-empty) vector. Immunization with the recombinant proteins elicited a slight but detectable specific humoral immune response 15 days after the last immunization (Fig. 2A). To estimate the developed T cell profile, we analyzed the titers of the IgG1 and IgG2a isotypes specific against Tc52 and its domains. As expected, all of the immunized groups (STc52, SN-term, and SC-term) developed higher titers of IgG2a than IgG1 antibodies, suggesting that the immune response was predominantly driven by Th1 cells (Fig. 2B and C).

To analyze the ability of the specific antibodies to target infective parasites, we carried out two different assays. To evaluate the capacity of the elicited antibodies to activate complement and lysate parasites, bloodstream trypomastigotes (RA strain) were incubated with sera from the immunized mice and a fresh source of complement (Fig. 2D). To study whether the antibodies are able to inhibit parasite cell invasion, bloodstream trypomastigotes of recombinant *T. cruzi* (Tulahuen strain) expressing β-galactosidase (33) were incubated with specific serum previous to infection in nonphagocytic Vero cells (Fig. 2E). We found that even when the antibody titers in serum were low (Fig. 2A), the antibodies developed in the immunized groups showed the capacity to lyse infecting parasites in the presence of complement and to neutralize parasite invasion of mammal cells. In both assays the patterns were similar, showing that the group receiving immunization with SN-term developed the highest antibody activity and that the SC-term group produced antibodies with lower activity.

To test the ability of the immunization protocol using *Salmonella* as a DNA delivery system to generate IgA antibodies in mu-

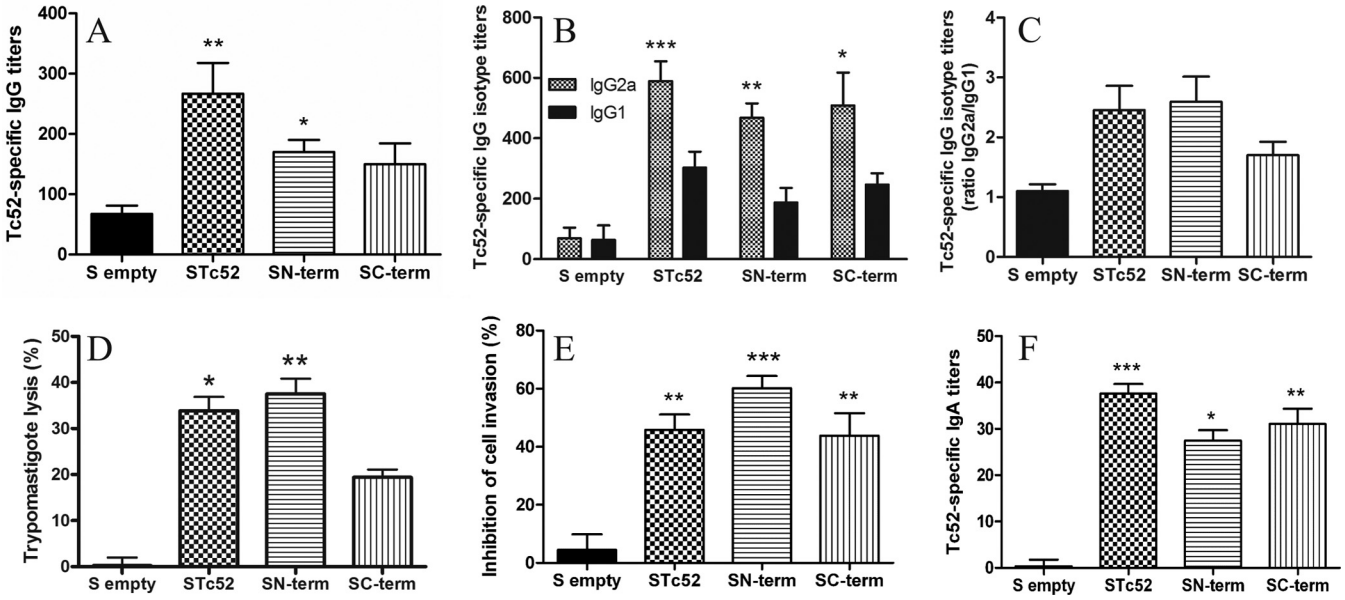

FIG 2 Antibody response against Tc52 in immunized mice. Groups of mice were immunized with STc52, SN-term, SC-term, and S-empty (control group), and sera were taken 15 days after the last immunization. Tc52-specific IgG (A) and IgG1 and IgG2a (B) titers in sera were measured by ELISA. (C) Titer ratios of Tc52-specific IgG2a/IgG1. (D) A trypomastigote lysis assay in the presence of antibodies and fresh complement was conducted using RA strain bloodstream trypomastigotes and the specific antisera. (E) Inhibition of cell invasion assay using Tulahuen beta-galactosidase strain bloodstream trypomastigotes, Vero cells, and the sera studied. (F) Tc52-specific IgA in intestinal lavage samples determined by ELISA. Samples were taken 15 days after the last immunization of mice in the STc52, SN-term, SC-term, and control (S-empty) groups. Total IgA in each sample was also measured by ELISA, and the titer for each sample was then normalized to the total amount of IgA. Results are representative of at least three independent experiments.*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

cosal tissues, 15 days after the last immunization, intestinal lavage fluids were obtained from all of the groups, and Tc52-specific and total IgA were measured. The titer of Tc52-specific IgA was normalized to the total amount of IgA in each sample. As shown in Fig. 2F, all of the immunized groups were able to develop antigen-specific mucosal immune responses compared to the control group without significant differences among them.

***Salmonella* as an antigen DNA delivery system induces a strong cellular immune response.** The Tc52-specific cellular immune response was assessed by *in vivo* and *in vitro* experiments. Fifteen days after the last immunization, a DTH test was conducted by injecting the hind footpad of the mice with 5 μg of Tc52 or N-term or C-term domain recombinant antigen. The thicknesses of the footpads were measured before and 48 h after the inoculation. Figure 3A shows that a significant DTH was induced in all the immunized groups compared to the control group (S-empty). The group immunized with STc52 showed a cellular immune response against rTc52 (Fig. 3A, panel I) and also when challenged with the N-term (Fig. 3A, panel II) and the C-term (Fig. 3A, panel III) domains. Similarly, the groups immunized with SN-term and SC-term reacted against the immunizing domains (Fig. 3A, panels II and III) and also against the full-length rTc52 (Fig. 3A, panel I). The DTH reaction was stronger in mice challenged with the antigen homolog to the immunizing antigen for STc52 (Fig. 3A, panel I) and SN-term (Fig. 3A, panel II). In contrast, when the challenge was done with the C-term domain (Fig. 3A, panel III), the group immunized with STc52 was the one that developed the highest DTH. As shown in Fig. 3A, panels II and III, SN-term- and SC-term-immunized groups showed cross-reactivity with the heterologous domain.

The ability of STc52, SN-term, and SC-term to induce a cellular immune response was also evaluated in *ex vivo* assays. Spleen cells taken from mice 15 days after the last immunization were stimulated with rTc52, and the activation of specific T cells was measured in a proliferation assay as [^{3}H]thymidine incorporation. Splenocytes of groups immunized with STc52, SN-term, and SC-term proliferated in response to rTc52, with proliferation indexes higher than 2, which is significantly different with respect to the control group value (Fig. 3B). In addition, cytokine concentration was also measured by ELISA in the supernatant of rTc52-stimulated spleen cells. The level of secreted IL-2 correlated with the proliferation index, showing in all groups significant differences with respect to the control values, with the highest level in the STc52-immunized group (homolog antigen) (Fig. 3C, panel I). Immunized groups also showed high levels of IFN-γ compared to the control group (Fig. 3C, panel II). Again, the STc52-immunized mice showed the highest level of this cytokine. In contrast, the secreted IL-10 level was low in all groups. Despite the fact that the immunized mice had higher levels than controls, only animals from SN-term-immunized group showed significant differences with respect to the S-empty-immunized group (Fig. 3C, panel III). No significant differences among immunized groups were detected in these assays. Analysis of the IFN-γ/IL-10 ratios shows that the strongest inflammatory reaction without an anti-inflammatory counterpart to protect tissues was developed in the full-length protein- and SC-term-immunized groups (Fig. 3, panel IV).

All immunized groups induce CD8$^+$ T cells that are able to produce IFN-γ in response to rTc52. Since IFN-γ-secreting CD8$^+$ T cells are very important in controlling *T. cruzi* infection (10, 39), we analyzed the intracellular expression of IFN-γ in spleen CD8$^+$ T cells by flow cytometry. Figure 4A shows that immunized groups had a higher number of IFN-γ$^+$ CD8$^+$ T cells

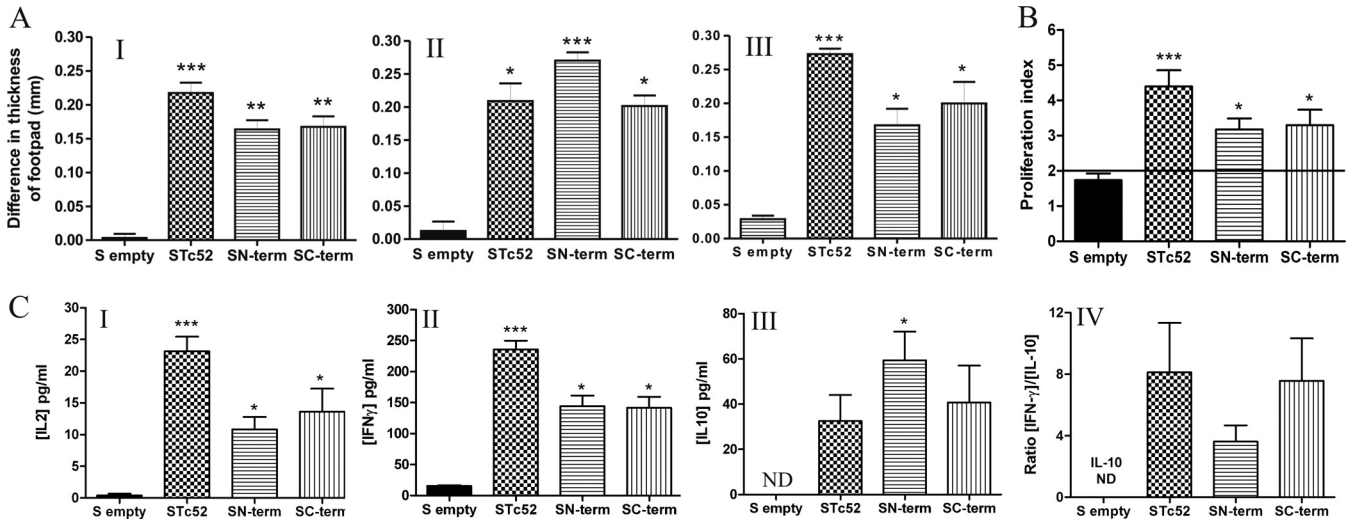

FIG 3 Cellular immune response against Tc52, N-term domain, and C-term domain in immunized mice. (A) DTH test 15 days after the last immunization. The figure shows the difference between the thickness of the footpads in mice of the groups immunized with STc52, SN-term, SC-term, and S-empty, before and 48 h after inoculation with 5 μg of antigen, as follows: I, Tc52; II, N-term domain; III, C-term domain. (B) Proliferative response of spleen cells from immunized mice 15 days after the last immunization. The proliferation index is expressed as a ratio of [^{3}H]thymidine incorporation (cpm) between cells with and without rTc52. (C) Concentration of the cytokines IL-2 (I), IFN-γ (II), and IL-10 (III) and ratio of IFN-γ/IL-10 (IV) in culture supernatant. Spleen cells were cocultivated with or without the antigen Tc52. The concentration of each cytokine in culture supernatant was measured by ELISA. For each sample, the absorbance in the absence of antigen was subtracted. Results are representative of two or three independent experiments. ND, nondetectable. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

than the control group in response to rTc52. The SN-term group showed the strongest responses, with approximately three times more IFN-γ$^+$ CD8$^+$ T cells than the control group.

In order to explore the specificity of the CD8$^+$ T cells activated during the immunization, we used bioinformatics tools to search for peptides able to bind MHC class I molecules of the H-2K haplotype in the Tc52 sequence. We selected the six peptides showing the highest scores and performed a cytotoxicity assay. Spleen cells from C3H/HeN (H-2K haplotype) mice immunized with STc52 were depleted of CD4$^+$ T cells and coincubated with mouse peritoneal cells loaded with the peptides. CD8$^+$ T cells from mice immunized with Tc52 showed cytotoxic activity to target cells carrying four of the six Tc52-specific peptides tested, but the difference compared to cells loaded with a control unrelated peptide was significant only for pep1 and pep4, which belong to the N-terminal domain, and pep6 of the C-terminal domain of Tc52 (Fig. 4B).

All immunized groups were protected against a lethal challenge. To analyze the protection conferred by immunization, the

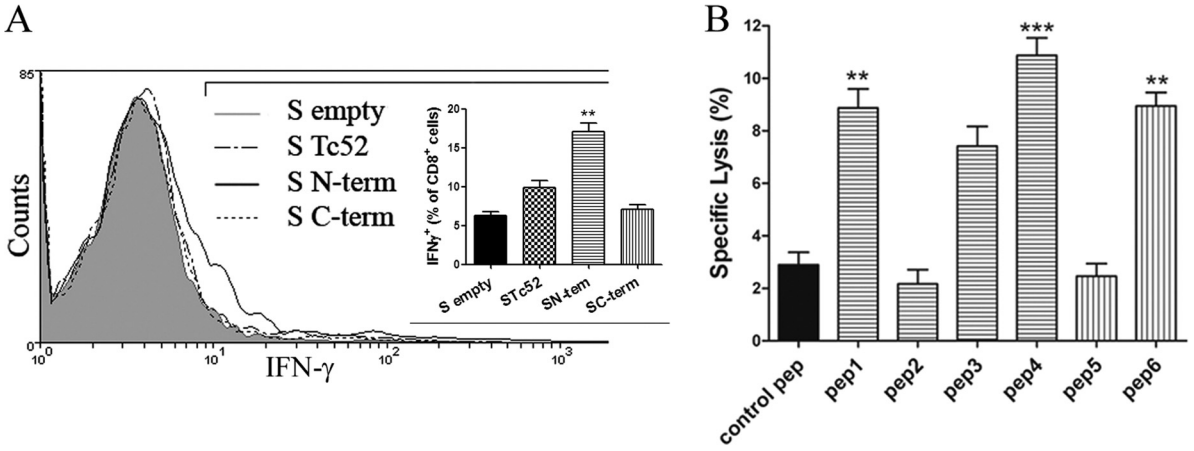

FIG 4 Immune response of CD8$^+$ cells from immunized mice against Tc52. (A) Expression of IFN-γ in CD8$^+$ cells stimulated with Tc52. Fifteen days after the last immunization with S-empty, STc52, SN-term, and SC-term, spleen cells were stimulated for 24 h with Tc52 and then stained with anti-CD4, anti-CD8, and anti-IFN-γ. CD8$^+$ CD4$^-$ cells were gated. The figure shows the expression of IFN-γ in this population of Tc52-stimulated cells from mice immunized with S-empty, STc52, SN-term, and SC-term. The percentages of IFN-γ$^+$ cells (in the CD8$^+$ population) are also shown in the figure. (B) Lysis of cells carrying specific MHC class I peptides mediated by CD8$^+$ cells. Mouse peritoneal cells were loaded with Tc52-specific peptides and also a control peptide. Peptides 1 to 4 belong to the N-term domain, whereas peptides 5 and 6 belong to the C-term domain. Spleen cells from mice immunized with STc52, depleted in CD4$^+$ cells, were coincubated with the peritoneal cells stained with CFSE and loaded with the peptides. Lysis was measured, and results are expressed as percentages. Results are representative of at least two independent experiments. **, $P < 0.01$; ***, $P < 0.001$.

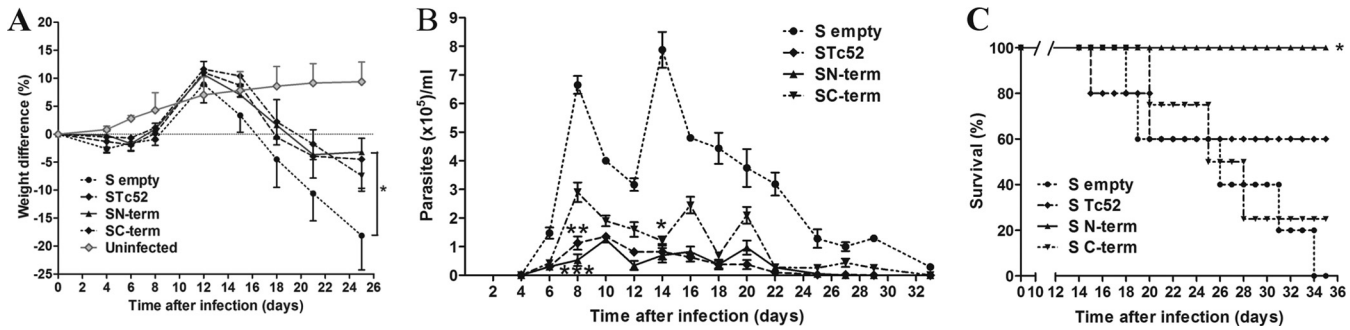

FIG 5 Protection against a lethal challenge. Fifteen days after the last immunization, mice were lethally challenged with 1,000 RA strain bloodstream trypomastigotes. (A) Mouse weight was determined every 2 to 4 days after infection, and the weight difference was recorded for every group. The weight difference in uninfected mice is also shown. (B) Level of parasites in blood after infection was monitored in 5 μl of blood taken from the tail every 2 to 3 days and recorded. (C) The number of dead mice was recorded daily. Results are representative of three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

mice were challenged with a deadly amount of bloodstream trypomastigotes. The mouse weight was measured after infection as a parameter of physiological status. The protection in the acute stage of infection was assessed by measuring the levels of parasite in the blood and monitoring survival. Thus, 15 days after the last immunization, mice were challenged with 1,000 bloodstream trypomastigotes of the highly virulent RA strain. All immunized groups had less weight loss than the nonimmunized and challenged control mice, but the difference was significant only for mice immunized with SN-term at 25 dpi (Fig. 5A). Furthermore, at the first peak of parasitemia (8 dpi) (Fig. 5B), immunizations with STc52 and SN-term resulted in a significant reduction in the number of circulating parasites ($P < 0.01$ and $P < 0.001$, respectively). At the second peak (14 dpi), all immunized groups showed differences from the control ($P < 0.05$). We also calculated the area under the parasitemia concentration-time curve (AUC) to assess the ability of the vaccination to reduce the total parasite load. The calculated AUCs were 7.2, 7.0, and 3.0 times lower than the control for groups immunized with SN-term, STc52, and SC-term, respectively (Fig. 5B). The survival of the mice was checked daily. All the mice from the control group died by 34 dpi, whereas the survival of the other groups was 100% for SN-term, 60% for STc52, and 25% for SC-term (Fig. 5C). Survival was monitored further, and all the mice of the SN-term-immunized group survived until sacrifice at 100 dpi (data not shown), with significant differences compared to the control group ($P < 0.05$).

Vaccination also confers protection in the chronic stage of infection. To investigate whether immunizations were effective in limiting tissue injury in the chronic stage of infection, we challenged immunized mice with a sublethal amount (200 parasites) of bloodstream trypomastigotes (RA strain). At 100 days after infection, we quantified the serum activity of enzymes related to muscular tissue damage: CK, AST, and LDH. Two mice from the S-empty-immunized and SC-term-immunized groups died before samples were taken at 100 dpi. The experiment was repeated three times, and in all cases one or more mice from the control group died before samples were taken. The groups immunized with STc52 and SN-term showed significantly lower levels of the three enzymes than the control (Fig. 6A). In addition, the levels of the enzymes in the STc52 and SN-term groups were similar to those of the uninfected mice (28.7 ± 4.6, 8.6 ± 4.6, 1,087 ± 10.4 IU/liter for CK, AST, and LDH, respectively). No differences were detected among the immunized groups. In addition, at 100 dpi we took samples of heart and skeletal muscles of mice of each group. Histological sections of cardiac muscles did not present alterations (even in the control group). Nevertheless, in skeletal muscle, the control group showed strong inflammatory infiltrates and necrosis, whereas these were moderate in the other groups. The median of the inflammation score was 3 for S-empty, 2 for SC-term, and 1 for STc52 and SN-term. Moreover, the control group was the only one that showed amastigote debris in the tissue (Fig. 6B).

DISCUSSION

We cloned and expressed in a bacterial system the *T. cruzi* protein Tc52 and its N- and C-terminal domains. In addition, we cloned the Tc52 gene and the sequences of its N- and C-terminal domains in plasmids for eukaryotic expression, which were tested *in vitro* for expression of the recombinant proteins by COS-7 cells. These plasmids were used to transfect attenuated *S. enterica* serovar Typhimurium as a DNA delivery system. A DNA delivery system has the advantage, over traditional naked DNA vaccination, that bacteria protect the DNA vaccine from the environment until it reaches the macrophages, where the *Salmonella* is disrupted, releasing its intracellular plasmid cargo and enabling expression of the recombinant protein by the eukaryotic cell machinery. It was previously shown that oral immunization with this system is able to induce specific antibody and cellular responses, with Th1 and $CD8^+$ cell activation, as well as specific mucosal immunity (40–42).

There are several routes of transmission of *T. cruzi*, including vectorial, transfusional, transplacental, and oral. As a consequence of active control in dwellings and their surroundings and the serological screening in blood banks, the transmission of the infection by vectorial and transfusional routes fell sharply in the last years (43). In this scenario, oral transmission appears as a route of emerging relevance (44–47). Potential sources of food contamination are triatomine bugs or triatomine feces, raw meat from infected animals, and gland secretions of infected opossums (*Didephis marsupalis*) (44). When the infection occurs by the oral route, metacyclic trypomastigotes invade the epithelium of the oral, esophageal, gastric, and intestinal mucosae and rapidly become systemic (48). In this work we focus on the development of an oral vaccine that could confer mucosal and systemic immunity, and the challenge was conducted intraperitoneally as a heterologous route for a more rigorous test of vaccine efficacy.

Groups of mice were fed with attenuated *Salmonella* bacteria carrying the empty vector and the pcDNA-Tc52, pcDNA-N-term,

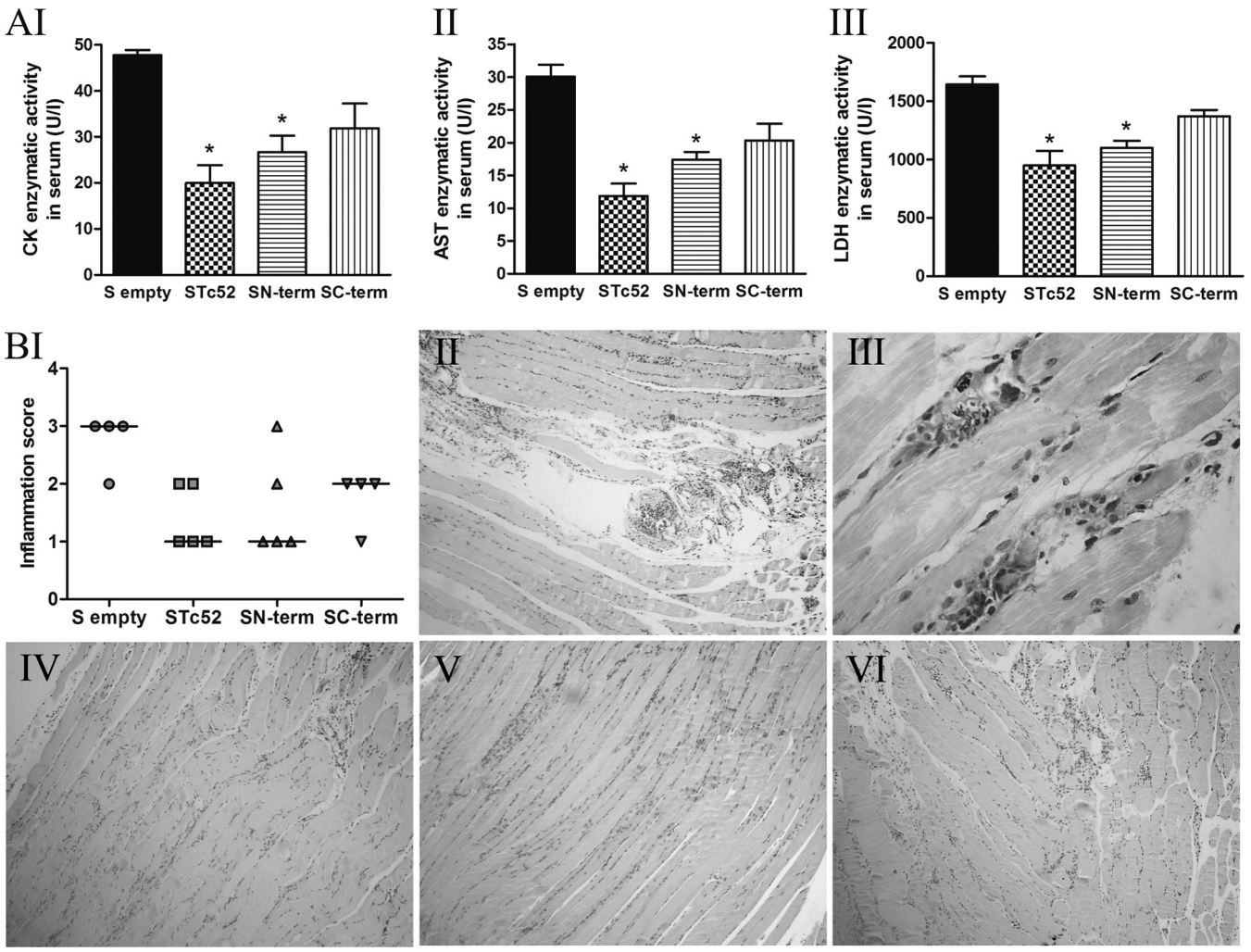

FIG 6 Protection in chronic stage of infection. (A) Activity of muscular enzymes in sera of immunized mice at 100 dpi, as indicated. Results are representative of three independent experiments (*, $P < 0.05$). Levels detected in normal mice were 28.7 ± 4.6, 8.6 ± 4.6, and 1,087 ± 10.4 IU/liter for CK, AST, and LDH, respectively. (B) Micrographs of skeletal muscle of immunized and infected mice at 100 dpi, stained with hematoxylin and eosin. Inflammation scores are shown in panel I. Each point represents an individual mouse; the median of each group is indicated by a horizontal line. Representative histological sections from one mouse of each group are shown, as follows: S-empty (II and III), STc52 (IV), SN-term (V), and SC-term (VI). Histological sections are shown at magnifications of ×400 (II, IV, V, and VI) and ×1,000 (III). Images show amastigotes and amastigote debris, which were found only in micrographs of skeletal muscle from mice of the S-empty-immunized group.

and pcDNA-C-term constructions. The delivery of the plasmid vector was efficient, leading to expression, processing, and presentation of the recombinant proteins since Tc52-specific antibodies were elicited in all vaccination groups. The antibody titers were low, as expected in a DNA immunization, but the antibodies efficiently mediated trypomastigote lysis in the presence of complement. This is particularly important considering that the expression of Tc52 in this parasite stage is poor (21). Furthermore, the antibodies inhibited trypomastigote invasion of mammal cells in an *in vitro* assay. In these two functional assays, the patterns were similar: sera from all immunization groups showed activity, but the strongest response was observed in serum from the SN-term-immunized group, followed by responses from mice vaccinated with STc52. The differential pattern between titers and activities of the sera of the three groups could be attributed to the position of the protein in the parasite membrane, which is unfortunately still not known, and therefore the availability of each one of the epitopes to the antibodies is not known. In this regard, Tc52 amino acid sequence analysis was unable to detect a glycosylphosphatidylinositol (GPI) anchor to the membrane using big-PI Predictor (http://mendel.imp.ac.at) (49) or the α-helix transmembrane region using the TMHMM server, version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). In contrast, using the dense alignment surface method (DAS [http://www.sbc.su.se/%7Emiklos/DAS/maindas.html]), we identified a short region between residues 157 and 164 belonging to the N-term domain that could have transmembrane localization. More studies should be conducted in order to define Tc52 localization on the parasite. However, our results showing that antibodies against full-length Tc52 and its domains are able to lyse trypomastigotes in the presence of complement further support a membrane location of Tc52, as previously shown (21). These results also suggest a potential role of Tc52 in cell invasion, which was previously investigated with phagocytic, but not with nonphagocytic, cells (20).

The profile of elicited antibodies in all vaccination groups indicated higher IgG2a than IgG1 antibodies, suggesting that the immune response was predominantly oriented by the Th1 subset, which is desirable in the design of vaccines against intracellular pathogens. In most cases, when *Salmonella* is used as a delivery system for foreign DNA, the immune response is predominantly Th1 (12, 50). There are cases, though, where the immune response depends on the antigen, and eventually a Th2-oriented immune response is developed (51). In addition, the *Salmonella*-based DNA delivery system was able to induce a mucosal Tc52-specific immunity, as indicated by the secretory IgA found in intestinal lavage samples.

All vaccination groups developed a strong cellular immune response against Tc52. In the DTH assay, as well as in the *ex vivo* spleen cell proliferation assay, the group immunized with STc52 showed a stronger response against rTc52 than the other groups; nevertheless, no statistically significant differences were detected between STc52, SN-term, and SC-term groups. The STc52-immunized group showed a higher proliferation index and higher levels of IL-2 and IFN-γ than the SN-term and SC-term groups. This can be explained because the STc52 group developed an immune response for T epitopes encompassed within the full-length of Tc52, whereas the SN-term and SC-term groups developed an immune response only for T epitopes present in each of the domains. In addition, splenocytes were stimulated with full-length Tc52 for all groups. Only the splenocytes from the group immunized with SN-term showed levels of secreted IL-10 significantly different from the control level. Even when the absolute levels of this cytokine were low, the results suggest that SN-term immunization results in a predominantly Th1 cellular response, which is counterbalanced in its proinflammatory potential by IL-10 produced by other cell subset(s). In this regard, the role of regulatory T (Treg) cells ($CD4^+$ $CD25^+$ $Foxp3^+$) in the persistence of *T. cruzi* infection is controversial. While Treg cells could restrict tissue damage, they inhibit IFN-γ secretion by $CD4^+$ and $CD8^+$ T cells and downregulate the cytotoxic activity of $CD8^+$ T cells, thereby suppressing an effective immune response against infection. Depletion of $CD4^+$ $CD25^+$ T cells did not correlate with an increase in $CD8^+$ T cells in tissue or even a change in parasitemia in the acute stage of the infection (52). In contrast to this, studies conducted with patients showed that high numbers of $CD4^+$ $CD25^+$ $Foxp3^+$ T cells secreting IL-10 and IL-17 correlated with the absence of cardiac pathology, whereas in patients with chronic cardiac disease the dominant cytokines were IL-6, IFN-γ, and tumor necrosis factor alpha (TNF-α), thereby suggesting a protective role for Treg cells in cardiac pathology (53). Further studies are necessary to identify the cells producing IL-10 after vaccination.

Due to the importance of cytotoxic T cells in parasite clearance (10, 39), we studied $CD8^+$ T cells activated in the presence of Tc52 (IFN-γ^+ $CD8^+$ T cells). We found that immunization with SN-term was the most efficient in the generation of IFN-γ-secreting $CD8^+$ T cells specific for Tc52. In order to know which part of Tc52 is the target of the specific cytotoxic cellular response, we selected peptides of the Tc52 sequence that were predicted to be presented in the context of MHC class I. We found that the peptides with a high predictive index belong to the N-term domain. When we analyzed the cytotoxic activity of $CD8^+$ T cells from the spleens of immunized mice, we found that out of the six selected peptides, three were targets for cytotoxic activity, with two belonging to the N-term domain. This could explain, at least in part, why SN-term promoted stronger activation of Tc52-specific $CD8^+$ T cells than SC-term.

In the acute stage of infection, we evaluated weight loss, parasitemia, and survival. The three vaccines conferred protection in terms of all of three of these parameters compared to the control group. However, the groups immunized with STc52 and SN-term showed better protection in terms of parasitemia than the one immunized with SC-term. Additionally, the SN-term-immunized group was the only one that, at 25 dpi, presented a significant difference in weight with respect to the controls. It was also the only group with 100% survival until 100 dpi (data not shown). In contrast, mice immunized with STc52 and with SC-term and the control group showed 60%, 25%, and 0% survival, respectively, at that time.

In the chronic stage of the infection, no tissue inflammation, damage, or amastigote presence was detected in cardiac muscle in any group (data not shown). In skeletal muscle of the quadriceps, a certain degree of inflammation was seen in all groups. However, the three immunized groups showed lower inflammation scores than the control group, with the STc52 and SN-term groups displaying the lowest ones. Moreover, amastigotes (as parasite debris) could be found only in the control group. This correlates with the serum activity of enzymes related to muscular tissue damage, in which case only the STc52 and SN-term groups showed significant differences from the control group. These results are similar to the ones observed in the acute stage of infection, indicating that control of parasitemia and parasite dissemination in all immunized mice, particularly those receiving STc52 and SN-term, prevents chronic tissue inflammation.

Previously, we demonstrated that the N-term domain of cruzipain (Cz), the major cysteine protease of *T. cruzi*, is the enzymatic and protective domain of the enzyme (54). The C-term domain of Cz is immunodominant during natural infection, eliciting a high titer of specific antibodies. Similarly, immunization with full-length Cz triggers an immune response mostly directed against the C-term domain, which is poorly protective. In contrast, immunization with the N-term domain of Cz redirects the immune response to the protective domain, thereby enhancing protection (54). In the case of Tc52, immunization with DNA vectors delivered by attenuated *Salmonella* encoding full-length Tc52 or its N-term or C-term domain did not result in marked differences in protection, as for Cz. Nevertheless, there are considerable differences in the responses triggered by these three vaccine prototypes. In particular, immunization with SN-term seems to promote more efficient responses. Vaccination with SN-term resulted in efficient mucosal and systemic humoral responses with antibodies able to both mediate trypomastigote lyses and prevent parasite invasion of eukaryotic cells. It also promoted a cellular immune response characterized by stimulation of Th1 cells and activation of Tc52-specific $CD8^+$ T cells. This prototype was also the more efficient in terms of conferring protection against acute and chronic infection. We conclude that, despite the protective capacity exhibited by full-length Tc52, the superior responses stimulated by the N-term domain and the need to reduce the antigen used due to autoimmunity concerns (55, 56) make the N-term domain a more promising candidate for inclusion in vaccine formulations against Chagas disease.

ACKNOWLEDGMENTS

Financial support was received from the University of Buenos Aires, the National Research Council of Argentina (CONICET), and Agencia Nacional de Promoción Científica y Técnica (PICT-2006-608 and PICT-2010-657 to E.L.M.), Argentina. E.L.M. is also supported by the Fogarty International Center (TW007972) and International Centre for Genetic Engineering and Biotechnology (CRP/ARG09-02).

We are grateful to Mariana Mundo and Jose Maria Delfino for the circular dichroism analysis, Gerardo Mirkin for helping with immunofluorescence assays, and Blair Prochnow for critical reading of the manuscript.

We declare that we have no competing financial interests.

REFERENCES

1. **Pan American Health Organization.** 2012. Chagas Disease (American typanosomiasis). Pan American Health Organization, Washington, DC. http://new.paho.org/hq/index.php?option=com_content&view=category&layout=blog&id=3591&Itemid=3921.
2. **World Health Organization.** 2013. Chagas disease (American trypanosomiasis) fact sheet. World Health Organization, Geneva, Switzerland. http://www.who.int/mediacentre/factsheets/fs340/en/.
3. **Centers for Disease Control and Prevention.** 2013. Parasites: American trypanosomiasis (also known as Chagas disease). Epidemiology and risk factors. Centers for Disease Control and Prevention, Atlanta, GA. http://www.cdc.gov/parasites/chagas/epi.html.
4. **World Health Organization.** 2010. Neglected tropical diseases. Statement: Chagas disease in Europe. Recommendations of an informal consultation meeting on Chagas disease control and prevention in Europe, WHO headquarters, Geneva, Switzerland, 17–18 December 2009. World Health Organization, Geneva, Switzerland. http://www.who.int/neglected_diseases/integrated_media_chagas_statement/en/index.html.
5. **Quijano-Hernandez I, Dumonteil E.** 2011. Advances and challenges towards a vaccine against Chagas disease. Hum. Vaccin. **7:**1184–1191. http://dx.doi.org/10.4161/hv.7.11.17016.
6. **Vázquez-Chagoyán JC, Gupta S, Garg NJ.** 2011. Vaccine development against *Trypanosoma cruzi* and Chagas disease. Adv. Parasitol. **75:**121–146. http://dx.doi.org/10.1016/B978-0-12-385863-4.00006-X.
7. **Cazorla SI, Frank FM, Malchiodi EL.** 2009. Vaccination approaches against *Trypanosoma cruzi* infection. Expert Rev. Vaccines **8:**921–935. http://dx.doi.org/10.1586/erv.09.45.
8. **de Alencar BC, Persechini PM, Haolla FA, de Oliveira G, Silverio JC, Lannes-Vieira J, Machado AV, Gazzinelli RT, Bruna-Romero O, Rodrigues MM.** 2009. Perforin and gamma interferon expression are required for $CD4^+$ and $CD8^+$ T-cell-dependent protective immunity against a human parasite, Trypanosoma cruzi, elicited by heterologous plasmid DNA prime-recombinant adenovirus 5 boost vaccination. Infect. Immun. **77:**4383–4395. http://dx.doi.org/10.1128/IAI.01459-08.
9. **Machado AV, Cardoso JE, Claser C, Rodrigues MM, Gazzinelli RT, Bruna-Romero O.** 2006. Long-term protective immunity induced against *Trypanosoma cruzi* infection after vaccination with recombinant adenoviruses encoding amastigote surface protein-2 and trans-sialidase. Hum. Gene Ther. **17:**898–908. http://dx.doi.org/10.1089/hum.2006.17.898.
10. **Rigato PO, de Alencar BC, de Vasconcelos JR, Dominguez MR, Araújo AF, Machado AV, Gazzinelli RT, Bruna-Romero O, Rodrigues MM.** 2011. Heterologous plasmid DNA prime-recombinant human adenovirus 5 boost vaccination generates a stable pool of protective long-lived $CD8^+$ T effector memory cells specific for a human parasite, *Trypanosoma cruzi*. Infect. Immun. **79:**2120–2130. http://dx.doi.org/10.1128/IAI.01190-10.
11. **Gupta S, Garg NJ.** 2013. TcVac3 induced control of *Trypanosoma cruzi* infection and chronic myocarditis in mice. PLoS One **8:**e59434. http://dx.doi.org/10.1371/journal.pone.0059434.
12. **Cazorla SI, Becker PD, Frank FM, Ebensen T, Sartori MJ, Corral RS, Malchiodi EL, Guzmán CA.** 2008. Oral vaccination with *Salmonella enterica* as a cruzipain-DNA delivery system confers protective immunity against *Trypanosoma cruzi*. Infect. Immun. **76:**324–333. http://dx.doi.org/10.1128/IAI.01163-07.
13. **Araújo AF, de Alencar BC, Vasconcelos JR, Hiyane MI, Marinho CR, Penido ML, Boscardin SB, Hoft DF, Gazzinelli RT, Rodrigues MM.** 2005. $CD8^+$-T-cell-dependent control of *Trypanosoma cruzi* infection in a highly susceptible mouse strain after immunization with recombinant proteins based on amastigote surface protein 2. Infect. Immun. **73:**6017–6025. http://dx.doi.org/10.1128/IAI.73.9.6017-6025.2005.
14. **Hoft DF, Eickhoff CS, Giddings OK, Vasconcelos JR, Rodrigues MM.** 2007. Trans-sialidase recombinant protein mixed with CpG motif-containing oligodeoxynucleotide induces protective mucosal and systemic *Trypanosoma cruzi* immunity involving $CD8^+$ CTL and B cell-mediated cross-priming. J. Immunol. **179:**6889–6900. http://dx.doi.org/10.4049/jimmunol.179.10.6889.
15. **Cazorla SI, Frank FM, Becker PD, Corral RS, Guzmán CA, Malchiodi EL.** 2008. Prime-boost immunization with cruzipain co-administered with MALP-2 triggers a protective immune response able to decrease parasite burden and tissue injury in an experimental *Trypanosoma cruzi* infection model. Vaccine **26:**1999–2009. http://dx.doi.org/10.1016/j.vaccine.2008.02.011.
16. **Moutiez M, Quéméneur E, Sergheraert C, Lucas V, Tartar A, Davioud-Charvet E.** 1997. Glutathione-dependent activities of *Trypanosoma cruzi* p52 makes it a new member of the thiol:disulphide oxidoreductase family. Biochem. J. **322:**43–48.
17. **Ouaissi A, Guilvard E, Delneste Y, Caron G, Magistrelli G, Herbault N, Thieblemont N, Jeannin P.** 2002. The *Trypanosoma cruzi* Tc52-released protein induces human dendritic cell maturation, signals via Toll-like receptor 2, and confers protection against lethal infection. J. Immunol. **168:**6366–6374. http://dx.doi.org/10.4049/jimmunol.168.12.6366.
18. **Borges M, Da Silva AC, Sereno D, Ouaissi A.** 2003. Peptide-based analysis of the amino acid sequence important to the immunoregulatory function of *Trypanosoma cruzi* Tc52 virulence factor. Immunology **109:**147–155. http://dx.doi.org/10.1046/j.1365-2567.2003.01637.x.
19. **Schöneck R, Plumas-Marty B, Taibi A, Billaut-Mulot O, Loyens M, Gras-Masse H, Capron A, Ouaissi A.** 1994. *Trypanosoma cruzi* cDNA encodes a tandemly repeated domain structure characteristic of small stress proteins and glutathione S-transferases. Biol. Cell **80:**1–10. http://dx.doi.org/10.1016/0248-4900(94)90011-6.
20. **Allaoui A, François C, Zemzoumi K, Guilvard E, Ouaissi A.** 1999. Intracellular growth and metacyclogenesis defects in *Trypanosoma cruzi* carrying a targeted deletion of a Tc52 protein-encoding allele. Mol. Microbiol. **32:**1273–1286. http://dx.doi.org/10.1046/j.1365-2958.1999.01440.x.
21. **Ouaissi MA, Dubremetz JF, Schöneck R, Fernandez-Gomez R, Gomez-Corvera R, Billaut-Mulot O, Taibi A, Loyens M, Tartar A, Sergheraert C, Kusnierz JP.** 1995. *Trypanosoma cruzi*: a 52-kDa protein sharing sequence homology with glutathione S-transferase is localized in parasite organelles morphologically resembling reservosomes. Exp. Parasitol. **81:**453–461. http://dx.doi.org/10.1006/expr.1995.1138.
22. **Oury B, Tarrieu F, Monte-Alegre A, Ouaissi A.** 2005. *Trypanosoma cruzi*: Sequence polymorphism of the gene encoding the Tc52 immunoregulatory released factor in relation to the phylogenetic diversity of the species. Exp. Parasitol. **111:**198–206. http://dx.doi.org/10.1016/j.exppara.2005.07.001.
23. **Mathieu-Daudé F, Bosseno M, Garzon E, Lelièvre J, Sereno D, Ouaissi A, Brenière SF.** 2007. Sequence diversity and differential expression of Tc52 immuno-regulatory protein in *Trypanosoma cruzi*: potential implications in the biological variability of strains. Parasitol. Res. **101:**1355–1363. http://dx.doi.org/10.1007/s00436-007-0651-3.
24. **Roellig DM, Savage MY, Fujita AW, Barnabé C, Tibayrenc M, Steurer FJ, Yabsley MJ.** 2013. Genetic variation and exchange in *Trypanosoma cruzi* isolates from the United States. PLoS One **8:**e56198. http://dx.doi.org/10.1371/journal.pone.0056198.
25. **Sanchez-Burgos G, Mezquita-Vega RG, Escobedo-Ortegon J, Ramirez-Sierra MJ, Arjona-Torres A, Ouaissi A, Rodrigues MM, Dumonteil E.** 2007. Comparative evaluation of therapeutic DNA vaccines against *Trypanosoma cruzi* in mice. FEMS Immunol. Med. Microbiol. **50:**333–341. http://dx.doi.org/10.1111/j.1574-695X.2007.00251.x.
26. **Pompa-Mera EN, Yépez-Mulia L, Ocaña-Mondragón A, García-Zepeda EA, Ortega-Pierres G, González-Bonilla CR.** 2011. *Trichinella spiralis*: intranasal immunization with attenuated Salmonella enterica carrying a gp43 antigen-derived 30 mer epitope elicits protection in BALB/c mice. Exp. Parasitol. **129:**393–401. http://dx.doi.org/10.1016/j.exppara.2011.08.013.
27. **Chen G, Dai Y, Chen J, Wang X, Tang B, Zhu Y, Hua Z.** 2011. Oral delivery of the Sj23LHD-GST antigen by *Salmonella typhimurium* type III secretion system protects against *Schistosoma japonicum* infection in mice. PLoS Negl. Trop. Dis. **5:**e1313. http://dx.doi.org/10.1371/journal.pntd.0001313.
28. **Schroeder J, Brown N, Kaye P, Aebischer T.** 2011. Single dose novel

Salmonella vaccine enhances resistance against visceralizing *L. major* and *L. donovani* infection in susceptible BALB/c mice. PLoS Negl. Trop. Dis. **5:**e1406. http://dx.doi.org/10.1371/journal.pntd.0001406.
29. **Jazayeri SD, Ideris A, Zakaria Z, Yeap SK, Omar AR.** 2012. Improved immune responses against avian influenza virus following oral vaccination of chickens with HA DNA vaccine using attenuated *Salmonella typhimurium* as carrier. Comp. Immunol. Microbiol. Infect. Dis. **35:**417–427. http://dx.doi.org/10.1016/j.cimid.2012.03.007.
30. **Ding J, Zheng Y, Wang Y, Dou Y, Chen X, Zhu X, Wang S, Zhang S, Liu Z, Hou J, Zhai J, Yan H, Luo X, Cai X.** 2013. Immune responses to a recombinant attenuated *Salmonella typhimurium* strain expressing a *Taenia solium* oncosphere antigen TSOL18. Comp. Immunol. Microbiol. Infect. Dis. **36:**17–23. http://dx.doi.org/10.1016/j.cimid.2012.09.006.
31. **Becker PD, Noerder M, Guzmán CA.** 2008. Genetic immunization: bacteria as DNA vaccine delivery vehicles. Hum. Vaccin. **4:**189–202. http://dx.doi.org/10.4161/hv.4.3.6314.
32. **Hegazy WAH, Hensel M.** 2012. *Salmonella enterica* as a vaccine carrier. Future Microbiol. **7:**111–127. http://dx.doi.org/10.2217/fmb.11.144.
33. **Buckner FS, Verlinde CL, La Flamme AC, Van Voorhis WC.** 1996. Efficient technique for screening drugs for activity against *Trypanosoma cruzi* using parasites expressing beta-galactosidase. Antimicrob. Agents Chemother. **40:**2592–2597.
34. **Basso H, Rohde M, Guzmán CA.** 2000. Vectors to achieve selective expression of vaccine antigens within eukaryotic cells using *Salmonella* spp. as carrier strains. FEMS Microbiol. Lett. **182:**219–223. http://dx.doi.org/10.1111/j.1574-6968.2000.tb08898.x.
35. **Frank FM, Petray PB, Cazorla SI, Muñoz MC, Corral RS, Malchiodi EL.** 2003. Use of a purified *Trypanosoma cruzi* antigen and CpG oligodeoxynucleotides for immunoprotection against a lethal challenge with trypomastigotes. Vaccine **22:**77–86. http://dx.doi.org/10.1016/S0264-410X(03)00541-3.
36. **Aretz HT, Billingham ME, Edwards WD, Factor SM, Fallon JT, Fenoglio JJ, Jr, Olsen EG, Schoen FJ.** 1987. Myocarditis. A histopathologic definition and classification. Am. J. Cardiovasc. Pathol. **1:**3–14.
37. **Postan M, Bailey JJ, Dvorak JA, McDaniel JP, Pottala EW.** 1987. Studies of *Trypanosoma cruzi* clones in inbred mice. III. Histopathological and electrocardiographical responses to chronic infection. Am. J. Trop. Med. Hyg. **37:**541–549.
38. **Martin DL, Postan M, Lucas P, Gress R, Tarleton RL.** 2007. TGF-beta regulates pathology but not tissue $CD8^+$ T cell dysfunction during experimental *Trypanosoma cruzi* infection. Eur. J. Immunol. **37:**2764–2771. http://dx.doi.org/10.1002/eji.200737033.
39. **Padilla A, Xu D, Martin D, Tarleton R.** 2007. Limited role for $CD4^+$ T-cell help in the initial priming of *Trypanosoma cruzi*-specific $CD8^+$ T cells. Infect. Immun. **75:**231–235. http://dx.doi.org/10.1128/IAI.01245-06.
40. **Hopkins SA, Niedergang F, Corthesy-Theulaz IE, Kraehenbuhl JP.** 2000. A recombinant *Salmonella typhimurium* vaccine strain is taken up and survives within murine Peyer's patch dendritic cells. Cell. Microbiol. **2:**59–68. http://dx.doi.org/10.1046/j.1462-5822.2000.00035.x.
41. **Darji A, Zur Lage S, Garbe AI, Chakraborty T, Weiss S.** 2000. Oral delivery of DNA vaccines using attenuated *Salmonella typhimurium* as carrier. FEMS Immunol. Med. Microbiol. **27:**341–349. http://dx.doi.org/10.1111/j.1574-695X.2000.tb01448.x.
42. **Berger E, Soldati R, Huebener N, Hohn O, Stermann A, Durmus T, Lobitz S, Zenclussen AC, Christiansen H, Lode HN, Fest S.** 2013. *Salmonella* SL7207 application is the most effective DNA vaccine delivery method for successful tumor eradication in a murine model for neuroblastoma. Cancer Lett. **331:**167–173. http://dx.doi.org/10.1016/j.canlet.2012.12.026.
43. **Moncayo A, Silveira AC.** 2009. Current epidemiological trends for Chagas disease in Latin America and future challenges in epidemiology, surveillance and health policy. Mem. Inst. Oswaldo Cruz **104**(Suppl 1)**:**17–30. http://dx.doi.org/10.1590/S0074-02762009000900005.
44. **Coura JR, Junqueira AC, Fernandes O, Valente SA, Miles MA.** 2002. Emerging Chagas disease in Amazonian Brazil. Trends Parasitol. **18:**171–176. http://dx.doi.org/10.1016/S1471-4922(01)02200-0.
45. **Igreja RP.** 2009. Chagas disease 100 years after its discovery. Lancet **373:**1340. http://dx.doi.org/10.1016/S0140-6736(09)60775-3.
46. **Muñoz-Calderón A, Díaz-Bello Z, Valladares B, Noya O, López MC, Alarcón de Noya B, Thomas MC.** 2013. Oral transmission of Chagas disease: typing of *Trypanosoma cruzi* from five outbreaks occurred in Venezuela shows multiclonal and common infections in patients, vectors and reservoirs. Infect. Genet. Evol. **17:**113–122. http://dx.doi.org/10.1016/j.meegid.2013.03.036.
47. **Sánchez LV, Ramírez JD.** 2013. Congenital and oral transmission of American trypanosomiasis: an overview of physiopathogenic aspects. Parasitology **140:**147–159. http://dx.doi.org/10.1017/S0031182012001394.
48. **Collins MH, Craft JM, Bustamante JM, Tarleton RL.** 2011. Oral exposure to *Trypanosoma cruzi* elicits a systemic $CD8^+$ T cell response and protection against heterotopic challenge. Infect. Immun. **79:**3397–3406. http://dx.doi.org/10.1128/IAI.01080-10.
49. **Sunyaev SR, Eisenhaber F, Rodchenkov IV, Eisenhaber B, Tumanyan VG, Kuznetsov EN.** 1999. Prediction of potential GPI-modification sites in proprotein sequences. Protein Eng. **12:**387–394. http://dx.doi.org/10.1093/protein/12.5.387.
50. **Koesling J, Lucas B, Develioglou L, Aebischer T, Meyer TF.** 2001. Vaccination of mice with live recombinant *Salmonella typhimurium aroA* against *H. pylori*: parameters associated with prophylactic and therapeutic vaccine efficacy. Vaccine **20:**413–420. http://dx.doi.org/10.1016/S0264-410X(01)00355-3.
51. **Eo SK, Yoon HA, Aleyas AG, Park SO, Han YW, Chae JS, Lee JH, Song HJ, Cho JG.** 2006. Systemic and mucosal immunity induced by oral somatic transgene vaccination against glycoprotein B of pseudorabies virus using live attenuated *Salmonella typhimurium*. FEMS Immunol. Med. Microbiol. **47:**451–461. http://dx.doi.org/10.1111/j.1574-695X.2006.00117.x.
52. **Kotner J, Tarleton R.** 2007. Endogenous $CD4^+$ $CD25^+$ regulatory T cells have a limited role in the control of *Trypanosoma cruzi* infection in mice. Infect. Immun. **75:**861–869. http://dx.doi.org/10.1128/IAI.01500-06.
53. **de Araújo FF, Corrêa-Oliveira R, Rocha MO, Chaves AT, Fiuza JA, Fares RC, Ferreira KS, Nunes MC, Keesen TS, Damasio MP, Teixeira-Carvalho A, Gomes JA.** 2012. $Foxp3^+$ $CD25^{high}$ $CD4^+$ regulatory T cells from indeterminate patients with Chagas disease can suppress the effector cells and cytokines and reveal altered correlations with disease severity. Immunobiology **217:**768–777. http://dx.doi.org/10.1016/j.imbio.2012.04.008.
54. **Cazorla SI, Frank FM, Becker PD, Arnaiz M, Mirkin GA, Corral RS, Guzmán CA, Malchiodi EL.** 2010. Redirection of the immune response to the functional catalytic domain of the cystein proteinase cruzipain improves protective immunity against *Trypanosoma cruzi* infection. J. Infect. Dis. **202:**136–144. http://dx.doi.org/10.1086/652872.
55. **Cunha-Neto E, Teixeira PC, Nogueira LG, Kalil J.** 2011. Autoimmunity. Adv. Parasitol. **76:**129–152. http://dx.doi.org/10.1016/B978-0-12-385895-5.00006-2.
56. **Gutierrez FR, Guedes PM, Gazzinelli RT, Silva JS.** 2009. The role of parasite persistence in pathogenesis of Chagas heart disease. Parasite Immunol. **31:**673–685. http://dx.doi.org/10.1111/j.1365-3024.2009.01108.x.