Chagas’ disease, caused by the intracellular protozoan parasite Trypanosoma cruzi, is a lifelong health problem in Central and South America, where an estimated 18 million people are infected with this parasite and 90 million are at risk of infection (35, 65). Following a short-lived acute-phase illness characterized by fever and a patent parasitemia, infected individuals enter a nearly parasitemic asymptomatic chronic phase, where most remain for the remainder of their lifetime. However, at 10 to 20 years postinfection nearly 30% of infected individuals develop severe cardiomyopathy, which is responsible for more than 50,000 deaths caused by Chagas’ disease each year (45). Although redundant vector control and blood bank screening measures have had a major impact in reducing transmission of T. cruzi (65), the operational costs to maintain such control programs, behavioral differences among vector species, existence of animal reservoirs, persistence of parasites in chronically infected patients, and lack of adequate chemotherapies to treat the infection will likely prevent these control measures alone from completely eradicating T. cruzi. An additional approach that could contribute significantly to control the transmission of Chagas’ disease is the development of anti-T. cruzi vaccines. To date, however, vaccine production for T. cruzi has been a low priority despite the current knowledge about the protective roles that antibodies, type 1 cytokines, and CD8 T cells play in resistance to experimental T. cruzi infections (53).

During T. cruzi infection, both chagasic patients and experimental animals produce strong immune responses to molecules from the infective nonreplicative trypomastigote stage and the replicative amastigote forms (3, 4, 14, 29). Among these, trypomastigote surface antigen 1 (TSA-1) (15, 38), a major trypomastigote surface antigen and the first identified member of the trans-sialidase gene superfamily (48), is a target of protective immune responses in mice (61, 66). Immunization with an amino-proximal fragment of TSA-1 induces a strong antibody response and protects mice against an otherwise lethal challenge with T. cruzi (66). Our studies have recently identified TSA-1 as the first bona fide target of CD8+ cytotoxic T lymphocytes (CTL) in T. cruzi-infected mice and demonstrated that the adoptive transfer of TSA-1-specific gamma interferon (IFN-γ)- and tumor necrosis factor alpha-producing CTL lines protects naive animals against lethal T. cruzi infection (61). Moreover, we have recently determined that TSA-1 and amastigote surface protein-1 and -2 (33, 44), which are also recognized by murine CTL (32), represent three target molecules of T. cruzi-specific human CD8+ CTL (62). These studies demonstrated the validity of the mouse model to identify target antigens of protective anti-T. cruzi immune responses and provide a strong incentive for the development of vaccines as a potential control measure against Chagas’ disease. For this purpose, and given the success of plasmid DNA vaccination in specifically stimulating a broad spectrum of immune responses to the vector-encoded target antigen (12), we have chosen to investigate DNA-based immunization as a system to generate vaccine-induced resistance against T. cruzi and have used TSA-1 as a model antigen for its initial evaluation. In this report we document that intramuscular injection of BALB/c and C57BL/6J mice with TSA-1-encoding plasmid DNA induces antibodies, CTL, and significant protection against lethal challenge with T. cruzi.

**MATERIALS AND METHODS**

**Mice and parasites.** Six- to 8-week-old female C57BL/6J (B6) and BALB/cByJ (BALB/c) mice (breeding pairs obtained from The Jackson Laboratory, Bar Harbor, Maine) were used in all experiments. The Brazil strain of T. cruzi was maintained in vivo by serial biweekly passage of 10^5 blood-form trypomastigotes (BFT) in C3H/HeSnJ mice (30) and by continuous in vitro passages of tissue culture-derived trypomastigotes (TCT) in monolayers of Vero cells (18). B6 mice

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**Vaccination with Trypomastigote Surface Antigen 1-Encoding Plasmid DNA Confers Protection against Lethal Trypanosoma cruzi Infection**

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DNA vaccination was evaluated with the experimental murine model of Trypanosoma cruzi infection as a means to induce antiparasite protective immunity, and the trypomastigote surface antigen 1 (TSA-1), a target of anti-T. cruzi antibody and major histocompatibility complex (MHC) class I-restricted CD8+ cytotoxic T-lymphocyte (CTL) responses, was used as the model antigen. Following the intramuscular immunization of H-2^b and H-2^d mice with a plasmid DNA encoding an N-terminally truncated TSA-1 lacking or containing the C-terminal nonapeptide tandem repeats, the antibody level, CTL response, and protection against challenge with T. cruzi were assessed. In H-2^b mice, antiparasite antibodies were induced only by immunization with the DNA construct encoding TSA-1 containing the C-terminal repeats. However, both DNA constructs were efficient in eliciting long-lasting CTL responses against the protective H-2K^d-restricted TSA-1^515–522 epitope. In H-2^d mice, inoculation with either of the two TSA-1-expressing vectors effectively generated antiparasite antibodies and primed CTLs that lysed T. cruzi-infected cells in an antigen-specific, MHC class I-restricted, and CD8^+ T-cell-dependent manner. When TSA-1 DNA-vaccinated animals were challenged with T. cruzi, 14 of 22 (64%) H-2^b and 16 of 18 (89%) H-2^d mice survived the infection. The ability to induce significant murine anti-T. cruzi protective immunity by immunization with plasmid DNA expressing TSA-1 provides the basis for the application of this technology in the design of optimal DNA multicomponent anti-T. cruzi vaccines which may ultimately be used for the prevention or treatment of Chagas’ disease.
were infected intraperitoneally with 10^7 BFT and challenged 3 months later with 10^6 TCT by subcutaneous injection at the base of the tail.

**Cell lines and culture reagents**. P815 cells (H-2d: mastocytoma cells; ATCC TIB 64), 3774 cells (H-2d: macrophages; ATCC TIB 67), 3T3 cells (H-2b, fibroblasts; ATCC, Manassas, Va.), NRK and Vero cells (ATCC CCL 81) (all from the American Type Culture Collection, Rockville, Md.). RMA-S cells (peptide TAP transporter-deficient, low H-2d expression mutant of the RBL-5 Rauscher virus-induced T-cell lymphoma; provided by S. Jameison, University of Minnesota, Minneapolis) were maintained in complete RPMI 1640 (Mediatech, Herndon, Va.) medium (CR) containing, respectively, 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids and 50 μg of gentamicin per ml (all from Gibco BRL, Gaithersburg, Md.). COS-7 cells (simian-transformed African Green monkey kidney cells; ATCC CRL 1651) were grown in 24-well plates at 20°C until assayed for IL-4 production. Cells were incubated overnight with 4 μg/ml of complete CR and incubated for an additional hour with 100 μl of a horseradish peroxidase-labeled goat anti-mouse immunoglobulin G (1:50 dilution in PBS) (Cappel, Organon Teknika Corp., West Chester, Pa.). Washed cells were developed with 100 μl of substrate solution (5 mg/ml 4-chloro-1-naphthol, 1 mg/ml hydrogen peroxide, 0.05% w/v H_{2}O_{2}) and incubated for 1 hour at room temperature. Absorbance was read at 405 nm with a automated ELISA microplate reader (Bio-Tek Instruments, Winooski, Vt.).

**Generation of effector cells**. Unless otherwise indicated, spleens from DNA-immunized BALB/c mice were harvested after the last dose and immune spleen cell (SC) suspensions were prepared in CR. In the case of B6 mice, SCs were cultured in 24-well plates at 5 × 10^5 cells/well. TSA1.7, VR1012 TSA2.1, or control VR1012 suspended in 50 μl of PBS by using a 27-gauge needle. Mice were boosted 4 weeks later with an identical dose of plasmid (100 μg total) given by the same bilateral intramuscular injection. Tail blood samples were collected 3 and 2 weeks after the first and second doses, respectively, and sera were collected 6 weeks after the second dose, animals were infected by intraperitoneal injection of 10^7 (B6) or 10^8 (BALB/c) T. cruzi BFT. Parasitemia were monitored periodical by hemocytometer counts of 10 μl of tail vein blood in an ammonium chloride solution. Mortality was recorded daily.

**Determination of serum antibody levels**. Antibody responses induced by the immunization of mice with plasmid DNA were evaluated by a solid-phase enzyme-linked immunosorbent assay (ELISA). In brief, capture antigen was prepared by sonication of 5 × 10^5 P815 cells (Costar) in PBS and then incubated at 4°C for 1 hour with 50 μl of 50 mM carbonate-bicarbonate buffer (pH 9.6). Sonicated material was spun for 1 hour at 100,000 × g at 4°C. Wells of flexible polystyrene 96-well plates (Falcon, Becton Dickinson & Co., Oxford, N.J.) were coated overnight at 4°C with 1 μg/ml of the soluble antigen. Washed wells were blocked with 1% BSA in PBS-0.05% Tween 20 (PBS) for 1 hour at 37°C. After blocking, 100 μl of pooled mouse sera (1:10 dilution in PBS) was added to the plates and incubated at 4°C for 1 hour. Plates were washed six times with PBS and incubated for an additional hour with 100 μl of a horseradish peroxidase-labeled goat anti-mouse immunoglobulin G (A, G, M) (1:10,000 dilution in PBS) (Cappel, Organon Teknika Corp., West Chester, Pa.). Washed wells were developed with 100 μl of substrate solution (5 mg/ml 4-chloro-1-naphthol, 1 mg/ml hydrogen peroxide, 0.05% w/v H_{2}O_{2}) and incubated for 1 hour at room temperature. Absorbance was read at 405 nm with a automated ELISA microplate reader (Bio-Tek Instruments, Winooski, Vt.).

**Preparation of peptide-pulsed target cells**. Peptide-pulsed targets were used to measure CTL activity of peptide-stimulated effector cells generated from plasmid DNA-immunized B6 mice. RMA-S (H-2d) cells preincubated for 24 h at 26°C and 6% CO2 were seeded into 24-well plates (Costar) at 10^6 cells/well in 2 ml of CR and incubated overnight under the same conditions in the presence of 0.05 μM TSA1.7,222 peptide or OVA257–264 negative control peptide and 100 μg of a sterile Na_{2}CO_{3} solution (0.1%) (Amersh颜m Life Science Corporation, Rockford, Ill.). Two hours after the final washing step, the target cells were infected at 37°C with trypomastigotes, 20% amastigotes) in 50 mM carbonate-bicarbonate buffer (pH 9.6). Sonicated material was spun for 1 hour at 100,000 × g at 4°C. Wells of flexible polystyrene 96-well plates (Falcon, Becton Dickinson & Co., Oxford, N.J.) were coated overnight at 4°C with 1 μg/ml of the soluble antigen. Washed wells were blocked with 1% BSA in PBS-0.05% Tween 20 (PBS) for 1 hour at 37°C. After blocking, 100 μl of pooled mouse sera (1:10 dilution in PBS) was added to the plates and incubated at 4°C for 1 hour. Plates were washed six times with PBS and incubated for an additional hour with 100 μl of a horseradish peroxidase-labeled goat anti-mouse immunoglobulin G (A, G, M) (1:10,000 dilution in PBS) (Cappel, Organon Teknika Corp., West Chester, Pa.). Washed wells were developed with 100 μl of substrate solution (5 mg/ml 4-chloro-1-naphthol, 1 mg/ml hydrogen peroxide, 0.05% w/v H_{2}O_{2}) and incubated for 1 hour at room temperature. Absorbance was read at 405 nm with a automated ELISA microplate reader (Bio-Tek Instruments, Winooski, Vt.).

**4.3.1.4. Detection of T. cruzi-infected stimulator and target cells.** T. cruzi-infected cells were used to generate and measure the CTL activity of peptide-stimulated effector cells from plasmid DNA-immunized BALB/c mice. Monolayers of J774 cells (60% confluent) prepared in upright 25-cm2 tissue culture flasks (Corning, Corning, N.Y.) at 10^5 cells/ml, washed, and then infected overnight with 50 μl of complete CR containing 10^6 T. cruzi cells (Costar) at 10^6 cells/well in 2 ml of CR and incubated overnight under the same conditions in the presence of 0.05 μM TSA1.7,222 peptide or OVA257–264 negative control peptide and 100 μg of a sterile Na_{2}CO_{3} solution (0.1%) (Amersh颜m Life Science Corporation, Rockford, Ill.). Two hours after the final washing step, the target cells were infected at 37°C with trypomastigotes, 20% amastigotes) in 50 mM carbonate-bicarbonate buffer (pH 9.6). Sonicated material was spun for 1 hour at 100,000 × g at 4°C. Wells of flexible polystyrene 96-well plates (Falcon, Becton Dickinson & Co., Oxford, N.J.) were coated overnight at 4°C with 1 μg/ml of the soluble antigen. Washed wells were blocked with 1% BSA in PBS-0.05% Tween 20 (PBS) for 1 hour at 37°C. After blocking, 100 μl of pooled mouse sera (1:10 dilution in PBS) was added to the plates and incubated at 4°C for 1 hour. Plates were washed six times with PBS and incubated for an additional hour with 100 μl of a horseradish peroxidase-labeled goat anti-mouse immunoglobulin G (A, G, M) (1:10,000 dilution in PBS) (Cappel, Organon Teknika Corp., West Chester, Pa.). Washed wells were developed with 100 μl of substrate solution (5 mg/ml 4-chloro-1-naphthol, 1 mg/ml hydrogen peroxide, 0.05% w/v H_{2}O_{2}) and incubated for 1 hour at room temperature. Absorbance was read at 405 nm with a automated ELISA microplate reader (Bio-Tek Instruments, Winooski, Vt.).

**General immunizations and challenges.** Groups of B6 and BALB/c mice were injected intramuscularly into each tibialis anterior muscle with 50 μg of VR1012

**TSA1.7, VR1012 TSA2.1, or control VR1012 suspended in 50 μl of PBS by using a 27-gauge needle. Mice were boosted 4 weeks later with an identical dose of plasmid (100 μg total) given by the same bilateral intramuscular injection. Tail blood samples were collected 3 and 2 weeks after the first and second doses, respectively, and sera were collected 6 weeks after the second dose, animals were infected by intraperitoneal injection of 10^7 (B6) or 10^8 (BALB/c) T. cruzi BFT. Parasitemia were monitored periodical by hemocytometer counts of 10 μl of tail vein blood in an ammonium chloride solution. Mortality was recorded daily.

**CTL assay.** Cytolytic activity was measured by the ^51Cr release assay, as previously described (65). In brief, ^51Cr-labeled target cells were washed three times with CR and resuspended in CR to 1 × 10^6 cells/ml. 1 × 10^5 target cells were added to effector cells (100 μl) at various effector cell-to-target cell (E/T) ratios in 96-well round-bottom plates (Corning). After a 5-h incubation at 37°C and 6% CO2, supernatants were harvested with the SCS System (Skatron, Sterling, Va.), and percent specific lysis was calculated.
from the mean of triplicates as 100 × [(experimental release – spontaneous release)/(maximum release – spontaneous release)]. Maximum and spontaneous releases were determined in wells containing no effectors in the presence or absence of 2% Triton X-100, respectively. In experiments where CTL activity of CD8\(^+\) and CD4\(^+\) T cells was tested, effector cells were depleted by incubation on ice for 30 min with predetermined dilutions of culture supernatants from hybridomas 3.155 (anti-CD8) (ATCC TIB 211) (46) and RL172 (anti-CD4) (8), followed by 30 min at 37°C in the presence of 1.6-diluted rabbit complement (Pel-Freez, Brown Deer, Wis.). Spontaneous release did not exceed 20% of the maximum release. The standard error ranged between 0.02 to 6.1% of the mean.

**RESULTS**

Expression of TSA-1 in transiently transfected cells. To study the effectiveness of genetic immunization against *T. cruzi*, the TSA-1 gene was subcloned into the VR1012 mammalian expression vector (19), containing the cytomegalovirus promoter and the bovine growth hormone polyadenylation sequences. The constructs VR1012 TSA1.7 and VR1012 TSA2.1 were generated to drive the expression of two N-terminally truncated TSA-1 gene products lacking and bearing, respectively, the five nonapeptide repeats located near the C-terminal end of the TSA-1 protein. Both plasmid constructs expressed the inserted TSA-1 gene fragment upon transient transfection of COS-7 cells. The cytoplasmic expression of TSA-1 in VR1012 TSA1.7- and VR1012 TSA2.1-transfected cells was intense as detected by immunofluorescent staining with a polyclonal anti-*T. cruzi* serum (Fig. 1D and F). In contrast, similarly transfected cells stained with normal mouse serum showed no evidence of immunofluorescence (Fig. 1C and E). No expression was detected in cells transfected with the unmodified VR1012 vector and stained with either serum (Fig. 1A and B).

Immunization with TSA-1 plasmid DNA elicits a parasite-specific antibody response. A strong humoral immune response has been widely implicated as a major effector mechanism that participates in the immune control of *T. cruzi* (28, 29, 71), and immunization of mice with a recombinant N-proximal portion of TSA-1 induces an antibody response which correlates with survival after a lethal challenge infection (66). To ascertain whether a *T. cruzi*-specific antibody response could be elicited by the expression of the TSA-1 protein fragments following intramuscular DNA immunization, BALB/c and B6 mice were injected twice with 100 μg of VR1012 TSA1.7, VR1012 TSA2.1, or control plasmid VR1012. The presence of parasite-specific antibodies in pooled sera prepared from each group of mice was assessed by ELISA (Fig. 2). Three weeks following the first dose, sera from BALB/c mice immunized with either VR1012 TSA1.7 or VR1012 TSA2.1 showed comparable antibody responses against the sonicated parasite material used as capture antigen. Two weeks after the second dose, while a boosting of the parasite-specific antibody level was detected in the sera from the VR1012 TSA1.7-immunized group, the level of antibodies in the sera from the VR1012 TSA2.1-immunized animals remained essentially unchanged. When a similar analysis was conducted for the pooled sera from similarly immunized B6 mice, the antibody levels after the first dose did not exceed the level found in normal mouse serum. However, after the second dose, only the VR1012 TSA2.1-immunized group showed a parasite-specific antibody response. In all cases, the antibody levels detected in the sera from groups of mice immunized with unmodified VR1012 vector were no different than the level measured in normal mouse serum.

Induction of a long-lasting TSA-1-specific CTL response in TSA-1 plasmid DNA-immunized B6 mice. TSA-1\(_{1515-522}\) is a target of H-2K\(^b\)-restricted protective CTL responses induced in B6 mice infected with *T. cruzi* (61). We therefore wanted to determine whether immunization of this strain of mice with the TSA-1-encoding DNA vectors could induce a TSA-1\(_{1515-522}\)-specific CTL response. Two weeks after the second intramuscular injection of either VR1012 TSA1.7 or VR1012 TSA2.1, immune SCs were stimulated with TSA-1\(_{1515-522}\) and 6 days later, the lytic activity of effectors was tested against peptide-sensitized target cells. CTL activity was antigen specific, MHC class I restricted, and dependent on CD8\(^+\) T lymphocytes (Fig. 3A). The H-2\(^d\) effector cells lysed matched RMA-S cells (H-2\(^d\)) sensitized with TSA-1\(_{1515-522}\) but were unable to lyse the same cells pulsed with control peptide OVA\(_{277-284}\) or MHC mismatched P815 cells (H-2\(^e\)) pulsed with TSA-1\(_{1515-522}\). Detected lytic activity was abrogated by CD8\(^+\)-T-cell depletion but not by depletion of CD4\(^+\) effectors. In no case did TSA-1\(_{1515-522}\)-stimulated SCs from mice immunized with unmodified VR1012 vector display CTL activity against peptide-sensitized target cells. Similar TSA-1\(_{1515-522}\)-specific CTL activity was detected in the peptide-stimulated SC cultures established 7 months after mice had received the second 100-μg dose of the TSA-1-encoding DNA vectors (Fig. 3B). The magnitude of such recall CTL responses was comparable to the CTL activity detected for TSA-1\(_{1515-522}\)-stimulated effectors from *T. cruzi*-infected mice. Hence, immunization of B6 mice with both TSA-1-encoding DNA constructs generates a long-lasting TSA-1\(_{1515-522}\)-specific CTL response which closely resembles the recall response induced in *T. cruzi*-infected animals.

The CTL response induced in BALB/c mice by TSA-1 plasmid DNA immunization is parasite specific, MHC class I restricted, and CD8\(^+\) T cell dependent. Despite the fact that the target antigens recognized by CTL from *T. cruzi*-infected BALB/c mice (H-2\(^d\)) have not been identified, SCs from these animals display genetically restricted CTL activity against *T. cruzi*-infected target cells (36). Thus, we used this system to determine whether parasite-specific CTL could be induced in BALB/c mice following immunization with the TSA-1-encoding plasmid DNA constructs. First, spleens were collected 2 weeks after the second dose of DNA, and on the same day the CTL activity of SCs against infected and uninfected target cells was measured (Fig. 4A). Infection of J774 cells (H-2\(^d\)) with *T. cruzi* efficiently targeted these macrophages for lysis by the H-2\(^d\) effector cells harvested from either VR1012 TSA1.7- or VR1012 TSA2.1-immunized mice. In contrast, minimal or no lysis against uninfected J774 cells and against mismatched *T. cruzi*-infected 5A.Kb.e3 fibroblasts (H-2\(^d\); H-2\(^K\)) was detected. None of the target cells tested was recognized by effector cells obtained from control VR1012-immunized animals. Next, CTL activity of immune SCs that had been stimulated for 6 days with *T. cruzi*-infected J774 macrophages against uninfected and *T. cruzi*-infected fibroblasts was assessed (Fig. 4B). Again, the specificity and MHC class I-restricted nature of the recall CTL response was demonstrated by the ability of effector cells derived from VR1012 TSA1.7- and VR1012 TSA2.1-immunized mice to lyse infected but not uninfected 3T3 cells (H-2\(^d\)) and by their inability to recognize infected 5A.Kb.e3 cells (H-2\(^d\); H-2\(^K\)). When the phenotype of the VR1012 TSA1.7-derived effectors was tested, it was found that they were CD8\(^+\) CD4\(^-\), because the lytic activity of these cells was significantly reduced by the depletion of CD8\(^+\) T cells and was minimally affected by the depletion of CD4\(^+\) T cells. Similarly stimulated VR1012 immune SCs failed to lyse all the target cells tested. Together, these data indicated that immunization of BALB/c mice with TSA-1-encoding DNA plasmids efficiently primed parasite-specific CD8\(^+\) CTL precursors and that these in vivo-expanded cells were in sufficient numbers to allow the detection of their genetically restricted lytic activity without in vitro restimulation.
A TSA-1 plasmid DNA-based vaccine significantly protects mice from T. cruzi-induced mortality. Having established that B6 and BALB/c mice generated T. cruzi-specific immune responses upon immunization with either of the TSA-1-expressing constructs, we next determined whether DNA vaccination could provide these animals with any degree of protection against challenge with T. cruzi. Two weeks after the second immunizing dose, groups of B6 and BALB/c mice were challenged with $10^5$ or $10^3$ T. cruzi BFT, respectively. The difference in the challenging dose was to compensate for the ob-

![FIG. 1. Expression of TSA-1 in transiently transfected cells. COS-7 cells were transfected with 10 µg of unmodified VR1012 plasmid (A and B), TSA-1-encoding VR1012 TSA1.7 (C and D), or VR1012 TSA2.1 (E and F) by using Lipofection. After 72 h, cells were fixed in ice-cold methanol and stained by immunofluorescence with a polyclonal anti-T. cruzi serum obtained from acutely infected mice (B, D, and F) or with a control normal mouse serum (A, C, and E) followed by a fluorescein isothiocyanate-conjugated secondary antibody. Photomicrographs (magnification, ×100) were taken by confocal microscopy. Note the cytoplasmic localization of the transgene-expressed TSA-1 products (D and F).](http://iai.asm.org/)
served differences in susceptibility of the two strains of mice. Both strains of mice showed a significant degree of protection against T. cruzi-induced mortality. As illustrated in one of the three conducted experiments, B6 mice vaccinated with either of the TSA-1-encoding vectors showed a 7-day delay in the onset of parasitemia and a consistently reduced level of parasites compared to control animals immunized with the unmodified VR1012 vector (Fig. 5A). Moreover, all control animals died before 45 days postinfection, whereas 50% of mice in each of the test groups survived the infection (Fig. 5B). In the case of BALB/c mice, however, the steady increase in parasitemia noted in TSA-1 DNA-vaccinated animals was strikingly similar to the kinetics of infection observed for mice immunized with the unmodified plasmid DNA (Fig. 6A). Despite similar levels of circulating parasites in test and control animals, none of the mice vaccinated with either of the TSA-1-encoding vectors succumbed to T. cruzi infection, whereas 75% of control mice developed fatal infections within 27 days postinfection (Fig. 6B). Overall, protection against an otherwise lethal inoculum of trypanosomes was observed in 73 and 55% of VR1012 TSA1.7- and VR1012 TSA2.1-vaccinated B6 mice, respectively, and in 91 and 86% of similarly vaccinated BALB/c mice, respectively (Table 1). In contrast, control VR1012-vaccinated mice remained highly susceptible to T. cruzi-induced lethality, as only 9% overall survival was observed for both strains (Table 1).

**DISCUSSION**

Several observations on T. cruzi-infected hosts regarding the mechanisms involved in disease development and protective immunity provide strong support for the development of vaccines as a means to prevent or lessen the severity of Chagas' disease (20–22, 41, 53, 56). Thus far, the exploration of vaccines as a means to prevent a disease that many still consider to have an autoimmune etiology (26). However, a growing body of evidence indicates that it is the persistence of T. cruzi in the diseased tissue and not the parasite-induced immune responses to self molecules which correlates best with the induction and maintenance of the inflammatory disease process (5, 7, 25, 31, 56).

![T. cruzi-specific serum antibody response in TSA-1 DNA-vaccinated mice.](Image)

**FIG. 2.** T. cruzi-specific serum antibody response in TSA-1 DNA-vaccinated mice. BALB/c and B6 mice were injected with 50 μg of VR1012 TSA1.7, VR1012 TSA2.1, or unmodified VR1012 plasmid in each tibialis anterior muscle. Mice were boosted after 4 weeks with the same dose of plasmid. The presence of parasite-specific antibodies was assessed by ELISA with a 1:100 dilution of sera pooled from individual tail blood samples (four or five mice per group) and collected 3 and 2 weeks after the first (bars 1) and second (bars 2) doses. Negative and positive controls were sera from normal mice (NMS) and from mice acutely infected with T. cruzi (TcIS). OD450, optical density at 450 nm.

This link between parasite load and severity of disease is further supported by the critical role that CD8+ T cells play in parasite control and survival after infection. CD8+ T cells constitute the major component in inflammatory foci of T. cruzi-infected tissues (22, 40, 47, 51), and in their absence (52, 54, 55), infected mice have increased mortality rates and tissue parasite loads with a decreased or absent inflammatory response. The recent demonstration of CD8+ CTL in T. cruzi-infected mice and humans with a specificity for defined trypanomastigote and amastigote surface molecules (32, 61, 62) and of the immunoprotective phenotype that these cells express (61) prompted us to initiate the development of immunization strategies to further characterize the vaccine potential of parasite components known to be targets of protective anti-T. cruzi immune responses.

DNA-based immunization has been shown in animal models to easily, safely, and effectively elicit and modulate the spectrum of immune responses necessary for the prevention of
infectious diseases (17, 34, 50, 59, 69, 70) and for the treatment of neoplastic (10, 24, 49), allergic (23, 39), and autoimmune (60) disorders. Thus, we chose this vaccination method to induce T. cruzi-specific antibody and class I-restricted CD8+ CTL responses in two inbred mouse strains and to assess its protective efficacy against parasite challenge. Our recent demonstration of TSA-1 as a target of protective CTL (61) made this parasite molecule a prime model antigen to evaluate this immunization method, inasmuch as (i) the N-proximal portion of TSA-1 had already been shown to induce antibody responses which correlate with survival after lethal T. cruzi infection (66) and (ii) TSA-1 is a member of the large 85-kDa family of trypomastigote surface proteins which are recognized by human sera and rodent-derived protective antibodies (2, 37).

Plasmid DNA vaccines VR1012 TSA1.7 and VR1012 TSA2.1 were constructed to drive the expression of products TSA-178–652 and TSA-178–790, which are truncated at the N terminus by 77 residues and at the C terminus by 183 and 45 amino acids, respectively. The main reasons for such a design were twofold: first, because removal of the N-terminal endoplasmic reticulum translocation signal sequence would ensure the cytoplasmic retention of de novo-synthesized TSA-1 protein, its subsequent cytosolic degradation, and an efficient priming of CTL responses; second, because conventional TSA-1 protein-based immunization of BALB/c mice has shown that the C-proximal portion encompassing residues 618 to 835 contains epitopes which interfere with the generation of antibodies to the protective determinants within residues 78 to 619 of the N-proximal portion (66).

Both VR1012 TSA-1 constructs directed the in vitro expression of cytoplasmically retained proteins with immunoreactivity to sera from T. cruzi-infected mice, and in BALB/c mice, both TSA-1-encoding vectors, with and without the repeat sequence, elicited parasite-specific antibody responses. Such responses were detected after the priming dose, and a modest boosting was achieved after the second dose with the VR1012 TSA2.1 vector. By contrast, in B6 mice, parasite-specific antibodies were detected only after the second dose of the VR1012 TSA2.1 vector alone. Similar strain-dependent variability in the induction of antibody responses following plasmid DNA immunization has been reported for the Plasmodium yoelii
circumsporozoite and hepatocyte-erythrocyte proteins (13). This discrepancy, however, might be a reflection of the well-established genetic control of immune responses to *T. cruzi* (58, 67). We are currently seeking to improve the antibody responses to levels comparable to or higher than those generated by *T. cruzi* infection through the immunization with *T. cruzi*-encoding DNA vaccines that co-deliver cytokine genes that had been reported to enhance both humoral and cellular immune responses (9, 16, 24, 27, 68).

The fact that immunization with *T. cruzi*-expressing plasmid DNA vaccines efficiently elicited MHC class I-restricted CTL responses in B6 (*H-2 b*) and BALB/c (*H-2 d*) mice is notable, inasmuch as prior to these studies, *T. cruzi*-specific CD8<sup>+</sup> CTL had been primed only by parasite infection (32, 36, 61) and TSA-1 had been identified only as a CTL target molecule of B6 mice (61). The demonstration with B6 mice that *T. cruzi* DNA vaccination and *T. cruzi* infection were able to prime CD8<sup>+</sup> CTL populations with specificity for the same protective H-2K<sup>b</sup>-restricted TSA-1<sub>515–522</sub> epitope indicated that similar immunogenic peptides are generated when a cell is transiently transfected in vivo or when it is expressed by an infected cell. In agreement with other studies where DNA immunization has been found to elicit long-lasting CTL responses (6, 57), TSA-

### Table 1. Protection against lethal *T. cruzi* challenge conferred by DNA vaccination

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<td></td>
<td>no. challenged</td>
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<td>no. challenged</td>
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<tr>
<td>VR1012</td>
<td>0/3 0/4 1/4</td>
<td>0/3 1/4 0/4</td>
<td>0/4 0/4 1/4</td>
<td>0/4 1/4 0/4</td>
</tr>
<tr>
<td>VR1012 TSA2.1</td>
<td>2/3 2/4 2/4</td>
<td>2/3 2/4 2/4</td>
<td>Total 6/11 55 2/4 55</td>
<td>6/7 86 2/4 86</td>
</tr>
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</table>

* Mice were primed intramuscularly with 100 μg of VR1012 TSA1.7, VR1012 TSA2.1, or control VR1012 plasmid and boosted 4 weeks later with a similar dose of the respective construct. After 2 weeks, immunized animals were infected by intraperitoneal injection with 10<sup>3</sup> *T. cruzi* (Brazil strain) BFT. Percent survival was assessed at day 100 postinfection.

* ND, not determined.

...
and BALB/c mice significant levels of protection against lethal *T. cruzi* challenge infection. Overall survival rates of B6 mice vaccinated with VR1012 TSA1.7 or VR1012 TSA2.1 were 73 and 55%, respectively. The same constructs furnished BALB/c mice with nearly complete protection, as 91 and 86% of vaccinated animals, respectively, survived *T. cruzi* infection. These results are in sharp contrast to the 9% survival observed for animals immunized with the unmodified VR1012 plasmid for both strains of mice. It should be noted, though, that immunization with the TSA-1-encoding vectors did not prevent recipient mice from getting infected, and DNA-vaccinated mice from both strains developed parasitemias, albeit at different levels. In B6 mice, the number of circulating parasites in test animals was lower than that observed for recipients of the control DNA vaccine, whereas in BALB/c mice, parasitemias were frequently similar in both groups of animals.

The results presented here lay the foundation for DNA immunization as a strategy for the design of anti-*T. cruzi* vaccines. Using TSA-1 as the model antigen, we demonstrated that this type of antigen delivery was efficient in the induction of parasite-specific antibody and CTL responses as well as in providing significant protection in two inbred strains of mice against *T. cruzi*-induced lethality. Work is now in progress to determine whether the simultaneous delivery of plasmids encoding additional parasite antigens (33, 44) and immunomodulatory cytokines (9, 16, 24, 27, 68) can improve protection and induce efficacious immune responses in genetically diverse strains of mice. Such information may provide strong support for the development of DNA-based vaccines that not only might protect humans at risk of infection with *T. cruzi* but also may alleviate or prevent the pathogenic responses characteristic of chronic Chagas’ disease by reducing or perhaps eliminating tissue parasites from infected patients.

**ACKNOWLEDGMENTS**

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