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# Genetic Immunization Elicits Antigen-Specific Protective Immune Responses and Decreases Disease Severity in *Trypanosoma cruzi* Infection

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**Immunity to *Trypanosoma cruzi* requires elicitation of humoral and cell-mediated immune responses to extracellular trypomastigotes and intracellular amastigotes. In this study, the effectiveness of the *T. cruzi* trans-sialidase family (ts) genes *ASP-1*, *ASP-2*, and *TSA-1* as genetic vaccines was assessed. Immunization of mice with plasmids encoding *ASP-1*, *ASP-2*, or *TSA-1* elicited poor antigen-specific cytotoxic-T-lymphocyte (CTL) activity and *T. cruzi*-specific antibody responses. Codelivery of interleukin-12 and granulocyte-macrophage colony-stimulating factor plasmids with antigen-encoding plasmids resulted in a substantial increase in CTL activity and antibody production and in increased resistance to *T. cruzi* infection. In pooled results from two to four experiments, 30 to 60% of mice immunized with antigen-encoding plasmids and 60 to 80% of mice immunized with antigen-encoding plasmids plus cytokine adjuvants survived a lethal challenge with *T. cruzi*. In comparison, 90% of control mice injected with empty plasmid DNA died during the acute phase of infection. However, the pool of three ts genes provided no greater protection than the most effective single gene (*ASP-2*) either with or without coadministration of cytokine plasmids. Importantly, the extent of tissue parasitism, inflammation, and associated tissue damage in skeletal muscles during the chronic phase of *T. cruzi* infection in mice immunized with antigen-encoding plasmids plus cytokine adjuvants was remarkably reduced compared to mice immunized with only cytokine adjuvants or empty plasmid DNA. These results identify new vaccine candidates and establish some of the methodologies that might be needed to develop effective vaccine-mediated control of *T. cruzi* infection. In addition, this work provides the first evidence that prophylactic genetic immunization can prevent the development of Chagas' disease.**

*Trypanosoma cruzi*, the causative agent of Chagas' disease in humans, is a major health problem in South and Central America. Infection with *T. cruzi* results in a generally mild acute phase, followed by a relatively long but asymptomatic "indeterminate" phase. Disease becomes clinically evident decades after the initial infection, effecting 30 to 40% of the infected individuals in the form of chronic chagasic cardiomyopathy and mega-syndrome (23, 34, 49).

In mammalian hosts, *T. cruzi* cycles between extracellular, nonreplicative trypomastigotes that circulate in the blood and intracellular replicative amastigotes. In murine *T. cruzi* infection, it is clear that the induction of a spectrum of host immune effector mechanisms is necessary to control *T. cruzi* infection (3, 39, 42). $CD4^+$ T cells assist in the control of *T. cruzi* through secretion of Th1 cytokines, resulting in amplification of the phagocytic activity of macrophages, the stimulation of B-cell proliferation and antibody production, and the enhancement of the $CD8^+$-T-cell response (3). $CD8^+$ T cells recognize processed parasite antigens presented in association with major histocompatibility complex (MHC) class I molecules on the surface of infected host cells and contribute to the control of *T. cruzi*, either by cytolysis of parasite-infected cells or by the secretion of cytokines that may induce trypanocidal activity (reviewed in references 39 and 42).

Effective vaccines for induction of protection against *T. cruzi* will likely need to elicit strong humoral and cellular immune responses. For this reason, genetic immunization is a particularly attractive vaccination strategy in *T. cruzi* infection, since it has been shown to elicit antibodies, Th1 cytokines, and $CD8^+$-T-cell immune responses (discussed in reference 6). Genetic immunization strategies have been explored for the induction of protective immune responses against a variety of infectious agents, including influenza virus, bovine herpes virus type I, human hepatitis B virus, and human immunodeficiency virus type I, as well as against the parasitic protozoans *Leishmania* spp., *Plasmodium* spp., and *T. cruzi* (4, 6, 14, 26, 36, 44, 46).

We have recently identified three glycosylphosphatidylinositol (GPI)-anchored proteins from *T. cruzi*—ASP-1, ASP-2, and TSA-1—that are the targets of $CD8^+$ cytotoxic T lymphocytes (CTLs) and that induce strong antibody responses in infected mice and humans (19, 20, 33, 47, 48, 50). The *T. cruzi* trans-sialidase family of genes (ts genes) is large, totaling perhaps a 1,000 or more distinct members dispersed in the *T. cruzi* genome. The family includes both bona fide trans-sialidases and trans-sialidase-like proteins that lack enzymatic activity (10). ts proteins are of particular interest as vaccine candidates because they are one of the two sets of proteins that are highly expressed on the parasite surface and because the enzymatically active members appear to have important roles in parasite survival (10).

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Genetic immunization with one of these trans-sialidase family members, TSA-1, provided substantial protection from *T. cruzi* infection in mice (46, 47). In the present study, we extended our investigation of vaccine candidates to ASP-1 and ASP-2 and addressed three specific questions. (i) Can vaccination with multiple trans-silidase family genes provide better protection than TSA-1 alone? (ii) Does coadministration of cytokine adjuvants boost the protective capacity of parasite genes? (iii) Can prophylactic genetic immunization have long-term benefits by decreasing the severity of chronic disease in mice infected with *T. cruzi*?

## MATERIALS AND METHODS

**Mice and parasites.** Six- to eight-week-old female C57BL/6J and C3H/HeSnJ mice (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 $10^3$ blood-form trypomastigotes (BFT) in C3H/HeSnJ mice (29) and by continuous in vitro passages of tissue culture-derived trypomastigotes in monolayers of Vero cells (28).

**Cell lines and culture reagents.** Vero (African green monkey kidney cells, ATCC CCL 81; American Type Culture Collection, Rockville, Md.) and RMA-S cells (an immunoselected variant of the RBL-5 lymphoma that is deficient in the expression of class I MHC molecules due to a mutation in the TAP-2 peptide transporter; a gift from M. B. Oldstone, The Scripps Research Institute, La Jolla, Calif.) were maintained in complete RPMI 1640 medium (Mediatech, Herndon, Va.) containing 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, Utah), 20 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 μg of gentamicin/ml (all from Gibco-BRL, Gaithersburg, Md.). COS7 cells (simian virus 40-transformed African green monkey kidney cells; ATCC CRL 1651) were grown in similarly supplemented Dulbecco modified Eagle medium (Mediatech). T-cell medium was prepared by supplementing RPMI–10% FBS with 50 μM 2-mercaptoethanol and 0.1 mM nonessential amino acids (Gibco-BRL).

**Peptides.** Peptides were synthesized by using Fmoc (9-fluorenylmethoxy carbonyl)-based, solid-phase chemistry on an ACT MPS 350-peptide synthesizer (Advanced Chem. Tech, Louisville, Ky.) by the Molecular Genetics Instrumentation Facility at the University of Georgia. The synthetic peptides pep77.2 ($TSA\text{-}1_{515\text{-}522}$) (47), PA8 ($ASP\text{-}2_{552\text{-}559}$), and PA14 ($ASP\text{-}1_{509\text{-}516}$) (19) represent *H-2K*$^b$-restricted CTL epitopes from *T. cruzi* proteins TSA-1, ASP-2, and ASP-1, respectively. The *H-2K*$^b$-restricted chicken ovalbumin CTL epitope $OVA_{257\text{-}264}$ (SIINFEKL) was used as a control peptide (31). Lyophilized peptides were dissolved at a 5 mM concentration in sterile phosphate-buffered saline (PBS; 10 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$, 137 mM NaCl, 8 mM KCl [pH 7.4]) and stored at −20°C.

**Plasmid construction.** The cDNA fragment of TSA-1, ASP-1, and ASP-2 genes (9, 33, 48) encoding amino acid residues 78 to 652, 27 to 641, and 61 to 705, respectively (excluding the N-terminal endoplasmic reticulum targeting signal sequence and the C-terminal GPI-anchor cleavage and/or attachment site and hydrophobic tail) were amplified by PCR. The recombinant pBluescript II SK(+) plasmids containing TSA-1 (GenBank accession no. AF085686; a gift from David Fouts, University of California, Irvine, Calif.), ASP-1 (GenBank accession no. U74494), and ASP-2 (GenBank accession no. U77951) were used as a templates for PCRs. Forward and reverse oligonucleotides for amplification of TSA-1, ASP-1, and ASP-2 cDNA were designed to incorporate, respectively, *Bam*HI and *Sma*I, *Bgl*II and *Xho*I, and *Bgl*II and *Sma*I restriction sites (underlined in the sequences below) for directional cloning. Oligonucleotides were constructed on an Applied Biosystems 394 DNA/RNA synthesizer (Foster City, Calif.) at the Molecular Genetics Instrumentation Facility. The forward and reverse oligonucleotides used for PCR amplification were 5′-AGGATCCATG ATTGCATTTGTCGAAGGC-3′ and 5′-ACCCGGGCATAGTTCACCGACA CTCAGTGG-3′ for TSA-1, 5′-AAGATCTTGTGGAAAGGAATTTGAGG-3′ and 5′-ACTCGAGTCACAGTGGGCGGTTGTACAG-3′ for ASP-1, and 5′-A AGATCTCTGTGAGGCTGCAGACGCTG-3′ and 5′-ACCCGGGTTATTGG TCGCCACCGTTTCC-3′ for ASP-2. The amplification products containing the A overhangs generated by *Taq* DNA polymerase during the PCR were cloned in pUC19(T) plasmid.

For expression in mammalian cells, the inserts from recombinant pUC19(T) plasmids were excised and cloned in the pCMVI.UBF3/2 plasmid (provided by Kathryn Sykes and Stephen A. Johnston, University of Texas Southwestern Medical Center, Dallas, Tex.) (Fig. 1). To construct pCMVI.UBF3/2.TSA-1, pUC19(T)TSA-1 was digested with *Bam*HI and *Sma*I, and the 1.7-kb TSA-1 fragment was cloned in pCMVI.UBF3/2 at similar restriction sites. To construct pCMVI.UBF3/2.ASP-1, pUC19(T)ASP-1 was digested with *Bgl*II and *Xho*I, and the 1.8-kb ASP-1 fragment was cloned in pCMVI.UBF3/2 at the *Bgl*II and *Sal*I sites. pCMVI.UBF3/2.ASP-2 was constructed by cloning at the *Bgl*II and *Sma*I sites the 1.8-kb ASP-2 fragment derived from pUC19(T)ASP-2 after digestion with similar restriction enzymes. The eukaryotic expression plasmids encoding murine cytokine interleukin-12 (IL-12; pcDNA3.msp35 and pcDNA3.msp40) and murine granulocyte-macrophage colony-stimulating factor (GM-CSF; pCMVI.GM-CSF) were provided by S. A. Johnston. Recombinant plasmids were transformed into *E. coli* DH5α-competent cells, grown in L broth containing 100 μg of ampicillin/ml, and purified by anion-exchange chromatography with the Qiagen Maxiprep kit (Qiagen, Chatsworth, Calif.) according to the manufacturer's specifications.

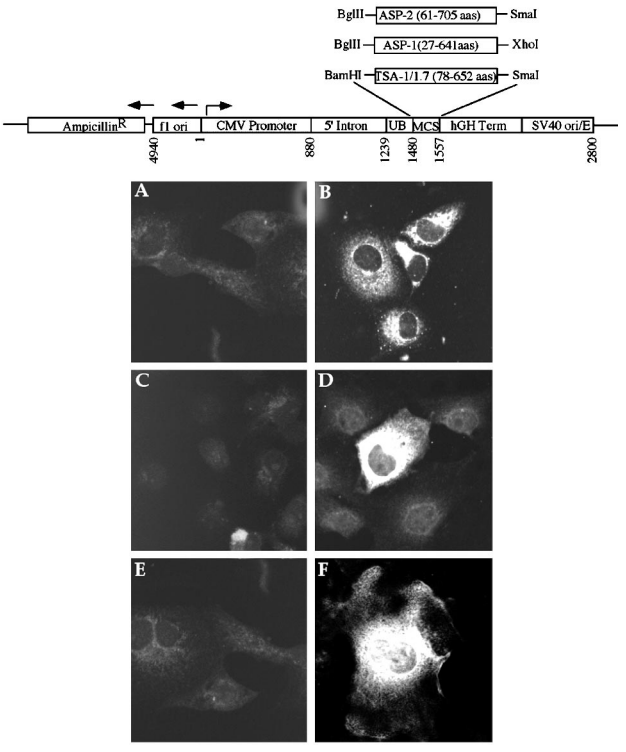


FIG. 1. Transient expression of *T. cruzi* proteins in COS7 cells. cDNAs encoding the *T. cruzi* proteins ASP-1 (27 to 641 amino acids), ASP-2 (61 to 705 amino acids), or TSA-1 (78 to 652 amino acids) were cloned in eukaryotic expression plasmid CMVI.UBF3/2 at the indicated restriction sites. COS7 cells were transfected with 5 μg of CMVI.UBF3/2 containing TSA-1 (E and F), ASP-1 (A and B), or ASP-2 (C and D) cDNA by using Lipofectin. Cells were incubated for 48 h, fixed and permeabilized with ice-cold methanol, and incubated with serum from mice in the acute (F) or chronic (B) phase of *T. cruzi* infection or with rabbit anti-ASP-2 polyclonal antiserum (D). Fluorescein isothiocyanate-labeled goat anti-mouse or anti-rabbit IgG was used as a secondary antibody, and cells were visualized by confocal microscopy. Cells incubated with normal mouse (A and E) or rabbit (C) serum as primary antibody were used as negative controls. Magnification, ×100.

**Gene expression.** Expression of the ASP-1, ASP-2, and TSA-1 was assessed by transient transfection of COS7 cells with pCMVI.UBF3/2.ASP-1, pCMVI.UBF3/2.ASP-2, and pCMVI.UBF3/2.TSA-1, respectively, as described previously (46). Briefly, COS7 cells seeded in six-well plates ($10^5$ cells/well) were transfected with 5 μg of each plasmid DNA by using Lipofectin (Gibco-BRL). After 48 h of incubation, cells were transferred to eight-well Lab Tek chamber slides (Nunc, Inc., Naperville, Ill.) at $10^4$ cells/well and incubated for an additional 24 h. Cells were fixed with ice-cold methanol and blocked with 1% bovine serum albumin (BSA) in PBS. After the blocking step, COS7 cells transfected with pCMVI-.UBF3/2.TSA-1 or pCMVI.UBF3/2.ASP-1 were incubated with polyclonal anti-*T. cruzi* serum (1:200 dilution in PBS–1% BSA) obtained from acutely or chronically infected mice, respectively. Cells transfected with pCMVI.UBF3/

2.ASP-2 were incubated with rabbit anti-ASP-2 polyclonal antiserum (1:200 dilution in PBS–1% BSA). Cells incubated with normal mouse or rabbit serum were used as negative controls. Cells were then stained with fluorescein isothiocyanate-labeled $F(ab')_2$ goat anti-mouse or anti-rabbit immunoglobulin G (IgG; 1:50 dilution in PBS–1% BSA, Southern Biotechnology, Birmingham, Ala.) (46). Slides were mounted in 10% glycerol–0.1 M sodium bicarbonate (pH 9)–2.5% 1,4-diazobicyclo-(2,2,2) octane and visualized by laser scanning confocal microscopy (MRC-600; Bio-Rad Laboratories, Hercules, Calif.).

**DNA immunization and infection.** Female C57BL/6 mice (six animals per group) were injected in the quadriceps muscles either with individual plasmid (pCMVI.UBF3/2.ASP-1, pCMVI.UBF3/2.ASP-2, or pCMVI.UBF3/2.TSA-1 at 100 μg of DNA/mouse) or with a mixture of ASP-1-, ASP-2-, and TSA-1-encoding plasmids (33 μg of each plasmid/mouse). In some experiments, an additional 100 μg of cytokine-encoding DNA (pcDNA3.msp35, pcDNA3.msp40 [IL-12], and pCMVI.GM-CSF, 33 μg of each) was injected along with the antigen-encoding DNA. Mice were boosted 6 weeks after the primary immunization with an identical dose of plasmid DNA. At 2 weeks after the second immunization, mice were infected by intraperitoneal injection of a lethal dose of *T. cruzi* BFT ($10^5$/mouse, five mice per group). Parasitemias were monitored by using hemacytometer counts of 10 μl of tail vein blood. Survival was recorded daily.

**Measurement of antibody responses.** Cell lysate obtained from culture-derived *T. cruzi* (70% amastigotes and 30% trypomastigotes, $1.0 \times 10^9$ parasites/ml) was used as a source of *T. cruzi*-soluble antigens for capturing serum antibodies (33). Pooled serum samples from immunized mice collected 2 weeks after the first and second immunizations were stored at −20°C until they were assayed for anti-*T. cruzi* antibodies by enzyme-linked immunosorbent assay as described previously (46). Briefly, flexible U-bottom (96-well) polyvinyl chloride plates (Becton Dickinson, Oxnard, Calif.) were coated overnight at 4°C with 100 μl of *T. cruzi*-soluble antigen/well (i.e., $5 \times 10^5$ parasite equivalents/well). Plates were blocked for 2 h at 37°C with 200 μl/well of 1% nonfat dry milk in PBS. After being washed with PBS–0.05%Tween 20 (PBST) and PBS, plates were incubated for 2 h with test sera (100 μl/well) added in twofold dilutions in triplicate. Plates were then incubated at room temperature for 30 min with 100 μl of horseradish peroxidase-labeled goat anti-mouse immunoglobulin (IgG+M; 1:2,000 dilution in PBST–1% nonfat dry milk; Cappel, West Chester, Pa.)/well. Color was developed with 100 μl of ABTS [2,2′azinobis(3-ethylbenzthiazolinesulfonic acid)]/well, and the optical density was read at 405 nm by using an automated enzyme-linked immunosorbent assay microplate reader (Bio-Tek Instruments, Winooski, Vt.).

**CTL activity.** The CTL activity of effector T cells obtained from plasmid DNA-immunized C57BL/6 mice was measured 2 weeks after the second immunization. Effector cells were generated by incubating $5 \times 10^6$ immune spleen cells ($2.5 \times 10^6$ cells/ml of T-cell medium, 2 ml/well in 24-well plates) with 1 μM antigenic peptide. After 2 days of culture at 37°C in 6% $CO_2$, the culture medium was supplemented with 5% Rat T-STIM without concanavalin A (Collaborative Biomedical Products, Bedford, Mass.) and incubated for 4 additional days. For targets, RMA-S (*H-2K*$^b$) cells preincubated for 24 h at 29°C, 6% $CO_2$ were seeded into 24-well plates (Costar, Cambridge, Mass.) at $10^6$ cells/well in 2 ml of RPMI–10% FBS. Cells were incubated overnight at 37°C in the presence of 100 μCi of $Na_2{}^{51}CrO_4$ (Amersham, Arlington Heights, Ill.) and 1 μM PA14 (ASP-$1_{509\text{-}516}$), PA8 (ASP-$2_{552\text{-}559}$), or pep77.2 (TSA-$1_{515\text{-}522}$) peptide or the OVA$_{257\text{-}264}$-negative control peptide. Cytolytic activity of effectors against targets was measured by the $^{51}$Cr release assay, as previously described (19, 48).

**Histology.** Some mice were sacrificed during the acute (30 to 45 days postinfection) or chronic (85 to 240 days postinfection) phase of *T. cruzi* infection for histologic examination of heart and skeletal muscle tissue. Heart and skeletal muscle tissue was removed and fixed in 10% buffered formalin for 24 h, dehydrated in absolute ethanol, cleared in xylene, and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin and evaluated by light microscopy. The tissue parasite burden was quantitated, based upon the number of parasitic pseudocysts present in sections of heart and skeletal muscles obtained from immunized and/or infected mice. Tissue sections were screened in >50 microscopic fields (mf) to assess the parasite load. Tissues were also scored according to extent of inflammation as previously described (38, 41).

## RESULTS

**Expression of *T. cruzi* genes in COS cells.** *T. cruzi* genes encoding ASP-1 (amino acids 27 to 641), ASP-2 (amino acids 61 to 705), and TSA-1 (amino acids 78 to 652) were cloned in CMVI.UBF3/2 mammalian expression plasmid containing the cytomegalovirus (CMV) immediate-early gene promoter, a synthetic intron, and a modified 3′ untranslated region from the human growth hormone (Fig. 1). The cloned genes were fused to a ubiquitin-encoding gene at the 5′ end (Fig. 1) to promote targeting of the expressed protein to the proteosome and entry into the MHC class I pathway of antigen presentation. The expression of ASP-1, ASP-2, and TSA-1 was determined by antibody staining of COS7 cells transiently transfected with CMVI.UBF3/2.ASP-1, CMVI.UBF3/2.ASP-2, or CMVI.UBF3/2.TSA-1, respectively (Fig. 1). The intense immunofluorescent staining with a polyclonal anti-*T. cruzi* serum of COS7 cells transiently transfected with CMVI.UBF3/2.ASP-1 (Fig. 1B) or CMVI.UBF3/2.TSA-1 (Fig. 1F) confirmed the cytoplasmic expression of ASP-1 and TSA-1, respectively. The cytoplasmic expression of ASP-2 in CMVI.UBF3/2-ASP-2 transfected COS7 cells was confirmed by immunofluorescent staining with a rabbit anti-ASP-2 polyclonal antiserum (Fig. 1D). In contrast, no fluorescence was detected when ASP-1-, ASP-2-, or TSA-1-transfected COS7 cells were stained with normal mouse or rabbit serum (Fig. 1A, C, and E), nor was it detected with either normal or chronically infected mouse serum in cells transfected with the empty plasmid DNA (data not shown).

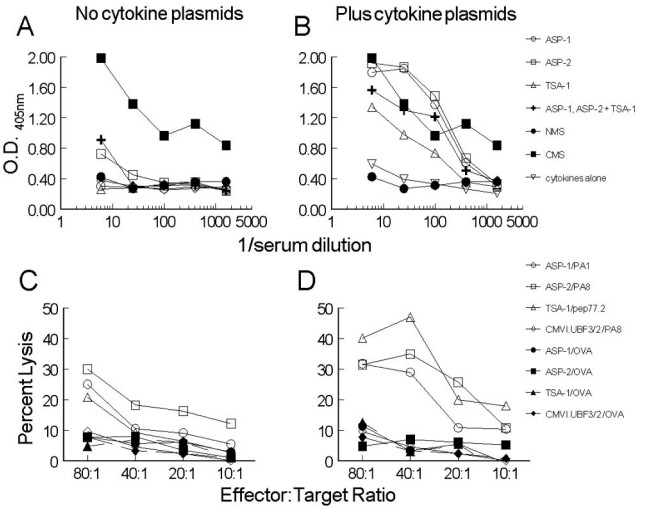


FIG. 2. Antibody production and cytolytic responses induced by intramuscular immunization with *T. cruzi* antigen-encoding plasmids C57BL/6 female mice were immunized with CMVI.UBF3/2 empty plasmid or CMVI.UBF3/2 containing ASP-1, ASP-2, and/or TSA-1 cDNA, with (B and D) or without (A and C) cytokine-encoding plasmids, twice at an interval of 6 weeks. The presence of parasite-specific antibodies in sera (A and B) and peptide-specific CTL responses (C and D) was assessed 2 weeks after the second immunization. Sera from normal mice (NMS) and mice chronically infected with *T. cruzi* (CMS) were used as negative and positive controls, respectively (A and B). For cytolytic assays (C and D), splenocytes from one mouse in each group were stimulated in vitro with ASP-1-, ASP-2-, or TSA-1-derived *H-2K*$^b$-restricted CTL epitope peptides (PA14, PA8, and pep77.2, respectively, at 1 μM). Cytolytic activity was measured in a 5-h $^{51}$Cr release assay against RMA-S target cells (*H-2K*$^b$) sensitized with specific (PA14, PA8, or pep77.2; open symbols) or nonspecific (SIINFEKL, OVA$_{257\text{-}264}$, solid symbols) peptides. The background lytic activity of splenocytes obtained from CMVI.UBF3/2-immunized mice and stimulated in vitro with ASP-2-specific peptide (PA8) against targets pulsed with the homologous peptide or heterologous peptide (SIINFEKL; solid symbols) is shown.

**Induction of *T. cruzi*-specific humoral and cellular immune**

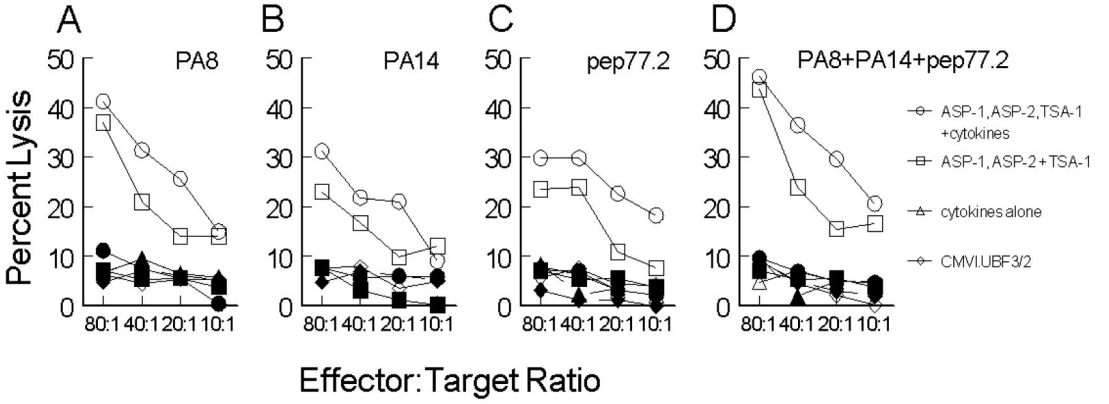


FIG. 3. Elicitation of cytolytic responses by a multicomponent nucleic acid vaccine can be augmented by cytokines. C57BL/6 mice were immunized with ASP-1-, ASP-2-, and TSA-1-encoding plasmids with or without cytokine-expressing plasmids. Mice injected with empty CMVI-.UBF3/2 plasmid or cytokine-encoding plasmids only were used as controls. At 2 weeks after the second immunization, splenocytes from immunized mice were stimulated in vitro with peptides PA8 (A), PA14 (B), pep77.2 (C), or a PA8-PA14-pep77.2 mixture (D). Effectors generated from these splenocytes were then tested in a 5-h $^{51}$Cr release assay against RMA-S target cells sensitized with either the homologous peptide (PA14, PA8, or pep77.2; open symbols) or pulsed with the control peptide (SIINFEKL, $OVA_{257-264}$; filled symbols).

**responses by genetic immunization.** To test the ability of the *T. cruzi* ts gene constructs to elicit humoral and cellular immune responses, mice were vaccinated as described in Materials and Methods and then assayed after the first and second immunizations for the presence of anti-parasite antibodies (i.e., by using a amastigote-trypomastigote lysate as the target) and peptide-specific CTL responses (Fig. 2). *T. cruzi*-specific antibodies were not detectable in sera collected after the first immunization (data not shown). However, 2 weeks after the second immunization, moderate levels of *T. cruzi*-specific antibodies were detected in the sera of mice immunized with CMVI.UBF3/2.ASP-2 alone or with the TSA-1, ASP-1, or ASP-2 constructs when codelivered with adjuvant plasmids but not in sera from mice immunized with CMVI.UBF3/2.ASP-1 or CMVI.UBF3/2.TSA-1 alone (Fig. 2A and B).

ASP-1, ASP-2, and TSA-1 all contain *H-2K*$^b$-restricted epitopes that are recognized by CTLs induced in *T. cruzi*-infected C57BL/6 mice (19, 47). To determine whether CTLs of this specificity are induced in mice after immunization with the ASP-1-, ASP-2-, or TSA-1-encoding plasmids, spleen cells were collected 2 weeks after the second immunization and stimulated in vitro in the presence of individual peptide(s). The effectors derived from these cultures were then tested for their ability to lyse $^{51}$Cr-labeled RMA-S cells pulsed with homologous peptide(s) or the irrelevant peptide $OVA_{257-264}$. As with the antibody response, the lytic activity to all three ts molecules was substantially boosted by coadministration of GM-CSF and IL-12 plasmids (Fig. 2C and D). Combined delivery of all three ts genes also elicited measurable CTL responses to each peptide that were enhanced by inclusion of the adjuvant plasmids (Fig. 3). Thus, the TSA-1, ASP-1, and ASP-2 constructs delivered either singly or in combination elicit both humoral and cellular immune responses that are boosted by the coadministration of IL-12- and GM-CSF-containing plasmids.

**DNA vaccine-induced protection from lethal *T. cruzi* infection.** We next sought to determine whether the immune responses elicited in mice after immunization with ASP-1, ASP-2, or TSA-1 constructs were protective against *T. cruzi* infection. For these studies, a high-dose challenge with the Brazil strain of *T. cruzi* was used. In contrast to some strains of *T. cruzi* that are lethal in mice within a week to 10 days, infections with the Brazil strain are more similar to the normal course of infections in humans and other species and are characterized by an acute phase which peaks in intensity at ca. 30 days postinfection (17). Of the ts constructs, ASP-2 consistently provided the best protection (Fig. 4). In pooled results from four experiments, 40% (4/10) of the mice immunized with CMVI.UBF3/2.ASP-1, 66% (10 of 15) of the mice immunized with CMVI.UBF3/2.ASP-2, and 30% (3 of 10) of the mice immunized with CMVI.UBF3/2.TSA-1 survived *T. cruzi* infection, whereas 90% (18 of 20) of the mice immunized with CMVI.UBF3/2 succumbed to infection. Codelivery of GM-CSF and IL-12 cytokine plasmids modestly enhanced the level of protection with all ts constructs: parasitemias in these mice became undetectable by 35 to 49 days postinfection (Fig. 4C), and all mice survived at least 70 days postinfection (Fig. 4D). Immunization with the pool of three ts constructs did not provide greater protection than ASP-2 alone, whether or not this pool was delivered with or without cytokine plasmids (Fig. 5). In pooled results from two experiments, 58% (7 of 12) of mice immunized with the three antigen-encoding plasmids and 83% (10 of 12) of mice immunized with antigen-encoding plasmids plus cytokine expression plasmids survived challenge infection with *T. cruzi*. It is also noteworthy that the cytokine plasmids alone, without ts genes, provided a significant degree of protection (Fig. 5). Altogether, these experiments demonstrate that (i) immunization with a single ts gene performs as well as a small pool of ts genes in providing protection from lethal acute infection with *T. cruzi*, (ii) immune responses and protection induced by ts genes can be enhanced by simultaneous delivery of cytokine adjuvant plasmids, and (iii) GM-CSF and IL-12 alone also enhance resistance to acute infection.

**Vaccine-induced protection from chronic disease.** The studies described above, as well as previously published results (1, 4, 11, 21, 27, 30, 36), demonstrate that genetic immunization

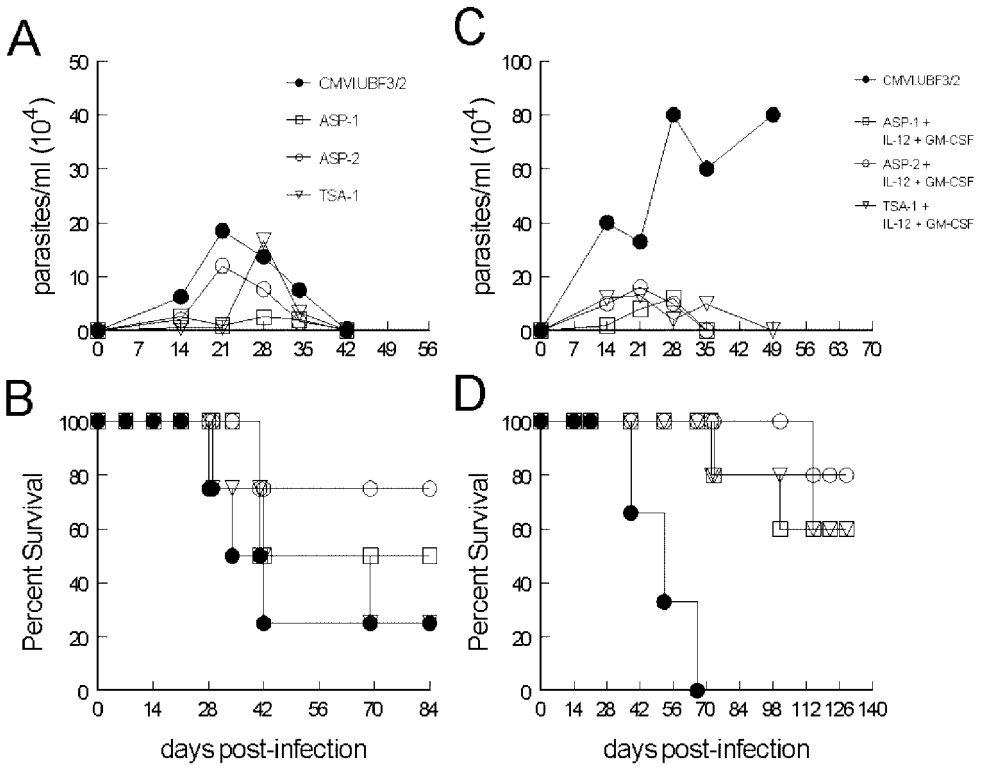


FIG. 4. Parasitemia and mortality in genetically immunized mice. C57BL/6 mice were injected with CMVI.UBF3/2 encoding ASP-1, ASP-2, or TSA-1, with (C and D) or without (A and B) cytokine adjuvants, twice at an interval of 6 weeks. Mice injected with the empty CMVI.UBF3/2 plasmid were used as controls. Two weeks after the second immunization, mice were challenged with a lethal dose of *T. cruzi* ($10^5$ BFT/mouse). Blood parasite levels (A and C) were monitored at weekly intervals, and survival (B and D) was recorded daily.

can enhance the survival of mice acutely infected with *T. cruzi*. However, none of these previous studies addressed the question of whether or not such vaccinations alter the course of disease development in the chronic phase of infection. For this analysis, sections from heart and skeletal muscles from mice immunized and challenged with $10^5$ BFT were assessed at various time intervals for tissue parasite burden and inflammation. Parasite persistence and hence disease severity in this particular mouse model of *T. cruzi* infection is highest in the skeletal muscle (51), and so this tissue was the primary focus of attention. Irrespective of the immunization conditions, mice in all groups exhibited moderate to high inflammatory responses in the skeletal muscles and heart during the acute phase of infection (45 days postinfection) (Fig. 6 and Table 1). However, mice immunized with a mixture of antigen-encoding plasmids plus cytokine-expression constructs exhibited the lowest level of tissue parasite burden (zero to two parasite pseudocysts/mf; Fig. 6D and Table 1). In comparison, mice immunized with ASP-1-, ASP-2-, or TSA-1-encoding plasmid (individually) plus cytokine expression plasmids (1 to 5 parasite nests/mf) or mice that received the antigen-encoding plasmid(s) (individually or in combination), cytokine plasmids only, or empty plasmid alone (2 to 10 parasite nests/mf) exhibited moderate-to-high levels of tissue parasites (Fig. 6A to C and Table 1)

The extent of inflammation and associated tissue damage in heart tissue and skeletal muscle during the chronic phase of infection was remarkably reduced in mice immunized with a mixture of *T. cruzi* antigen-encoding plasmids and cytokine-expression constructs (Fig. 7D). Although a majority of the animals in the control group (>90%) died due to high blood and tissue parasite burden during the acute phase of *T. cruzi* infection, a few animals that did survive to the chronic phase of infection showed extensive skeletal muscle inflammation and tissue necrosis (Fig. 7A and Table 1), the hallmarks of Chagas' disease development. In sharp contrast, all mice surviving infection after immunization with cytokine genes alone, with antigen-encoding plasmids (individually or in combination), or with ASP-1-, ASP-2-, or TSA-1-encoding plasmids (individually) plus cytokine expression plasmids exhibited a moderate inflammatory response up to day 240 postinfection (the time point after which experiments were terminated) (Fig. 7B and C and Table 1). These results demonstrate that the DNA vaccines used in the present study are effective in controlling the tissue parasite burden and thus the associated symptoms of chronic Chagas' disease.

## DISCUSSION

Genetic immunization is a promising approach for inducing protective immune responses against a variety of infectious agents. The effectiveness of *T. cruzi* genes as genetic vaccines for the elicitation of parasite-specific immune responses has recently been shown in a number of laboratories with several different *T. cruzi* genes (1, 4, 11, 21, 27, 30, 36). We have demonstrated the utility of TSA-1-encoding plasmids as genetic vaccines for the elicitation of *T. cruzi*-specific immune

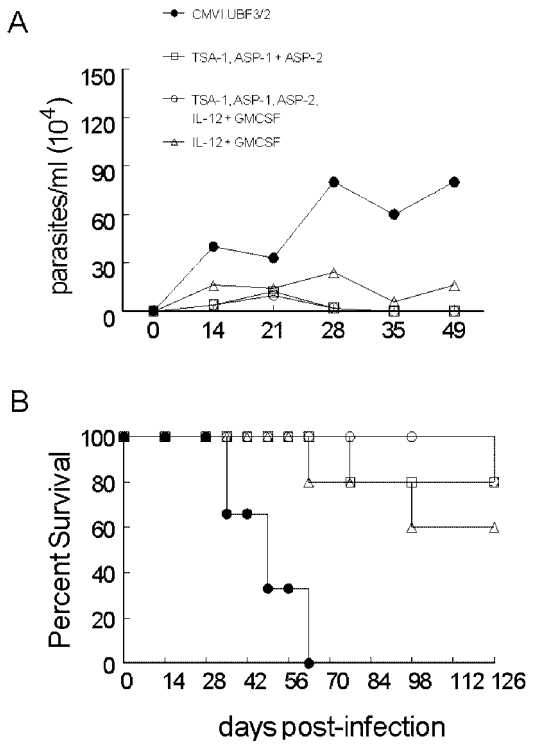


FIG. 5. Parasitemia and mortality in mice immunized with a multicompent genetic vaccine. C57BL/6 mice were injected with plasmids encoding ASP-1, ASP-2, and TSA-1, with or without cytokine adjuvants, twice at an interval of 6 weeks. Mice injected with the empty CMVI.UBF3/2 plasmid or cytokine genes alone were used as controls. Immunization of mice (as in Fig. 4) was followed 2 weeks later by intraperitoneal infection with *T. cruzi* ($10^5$ BFT/mouse). Blood parasitemia (A) and survival (B) were observed and determined as described in the legend to Fig. 4.

responses that provided a degree of protection from challenge infection in a murine model of *T. cruzi* infection (46). To further our understanding of the potential for genetic vaccines in *T. cruzi* infection, we addressed three questions in the present study. (i) Can pools of closely related genes enhance the protection provided by a single gene? (ii) Can protection be additionally enhanced with the use of cytokine genes as adjuvants? (iii) Does prophylactic vaccination have long-term benefits by altering the course and severity of chronic-phase disease?

To date, most attempts at genetic immunization in *T. cruzi* infection have used single parasite genes as the immunogen, and the majority of these genes have been members of the trans-sialidase-like gene family (4, 11, 30, 36, 46). The ts family is large, composed of up to 1,000 members that can be grouped into a number of subclasses (10). ts genes are appealing targets for vaccination because they are highly expressed as both surface-anchored and secreted proteins in trypomastigotes and amastigotes of *T. cruzi* and because they are among the most well-characterized targets of host immune responses in *T. cruzi*-infected hosts. The apparently enzymatically inactive TSA-1 gene (46), the catalytic region of the enzyme active trans-sialidase (11), and a complement regulatory protein member of this superfamily (36) have all been demonstrated to provide significant protection when delivered as genetic vaccines in mice. We chose to test the mixture of TSA-1, ASP-1, and ASP-2 in this study because we had evidence that all were targets of cellular and humoral immune responses in mice and/or humans infected with *T. cruzi* (19, 24, 33, 47, 48, 50).

Immunization with this mixture of ts-encoding plasmids elicited moderate parasite-specific antibody responses and substantial CTL activity and subsequently provided significant resistance to *T. cruzi* infection. However, the mixture of three trans-sialidase genes failed to provide a higher level of protection than single gene vaccination. This result may be a reflection of the fact that we used three relatively closely related members of the ts gene family. Although the proteins encoded by these three ts members are diverse—showing 51 to 55% sequence identity—they represent only a very small sample of one subfamily of the more than 1,000 ts superfamily of genes. Importantly, however, we also did not observe an inhibitory or competitive effect on the elicitation of antigen-specific immune responses when mice were immunized with the mixture of plasmids encoding *T. cruzi* genes. These results indicate that multicomponent genetic vaccines may be useful in expanding the diversity of vaccine-induced immune responses. However, a larger variety of ts genes may be needed to sufficiently broaden the gene diversity and the resulting effectiveness of a multicomponent genetic vaccine for *T. cruzi*.

In order to maximize the protective capacity of DNA vaccines for *T. cruzi* infection, we coadministered cytokine genes which can act as adjuvants to enhance the response to the antigen-encoding plasmid(s). IL-12 and GM-CSF were selected for coimmunization because they have shown utility as genetic adjuvants for enhancement and regulation of immune responses and because they were expected to enhance a subset of responses that are known to be important in the control of *T. cruzi* infection. Among many other activities, GM-CSF enhances the antigen-presenting capability of dendritic cells and facilitates B- and T-cell-mediated immunity (discussed in references 18 and 45), and IL-12 is a key cytokine involved in $CD8^+$-T-cell activation and proliferation, and in promoting type 1 cytokine production (discussed in references 5, 18, and 25). Augmentation of CTL activity, antibody production, and modulation of type I cytokine responses by codelivery of IL-12 and GM-CSF-encoding plasmids with DNA vaccines encoding a variety of viral, bacterial, or parasitic antigens has been documented (2, 8, 12, 13, 15, 16, 37, 43).

Coadministration of cytokine genes with *T. cruzi* antigen-encoding plasmids resulted in an increase in the level of antigen-specific CTL activity and humoral immune responses and provided substantially better protection from *T. cruzi* infection in comparison to mice immunized with antigen-encoding plasmids (individually or in combination) only. However, mice immunized with IL-12 and GM-CSF plasmids alone also had greater survival, lower parasite load, and less inflammatory disease compared to mice receiving the empty CMVI.UBF3/2 plasmid. It is likely that residual cytokines present at the time of infection (2 weeks after the second immunization) augmented the parasite-specific responses induced by the challenge infection. Support for this conclusion comes from the demonstration that administration of an IL-12 expression plasmid enhances the resistance of mice infected with *T. cruzi* (32).

Our most striking observation was the demonstration that the prior induction of systemic immunity by DNA vaccines substantially reduced the severity of disease during the chronic

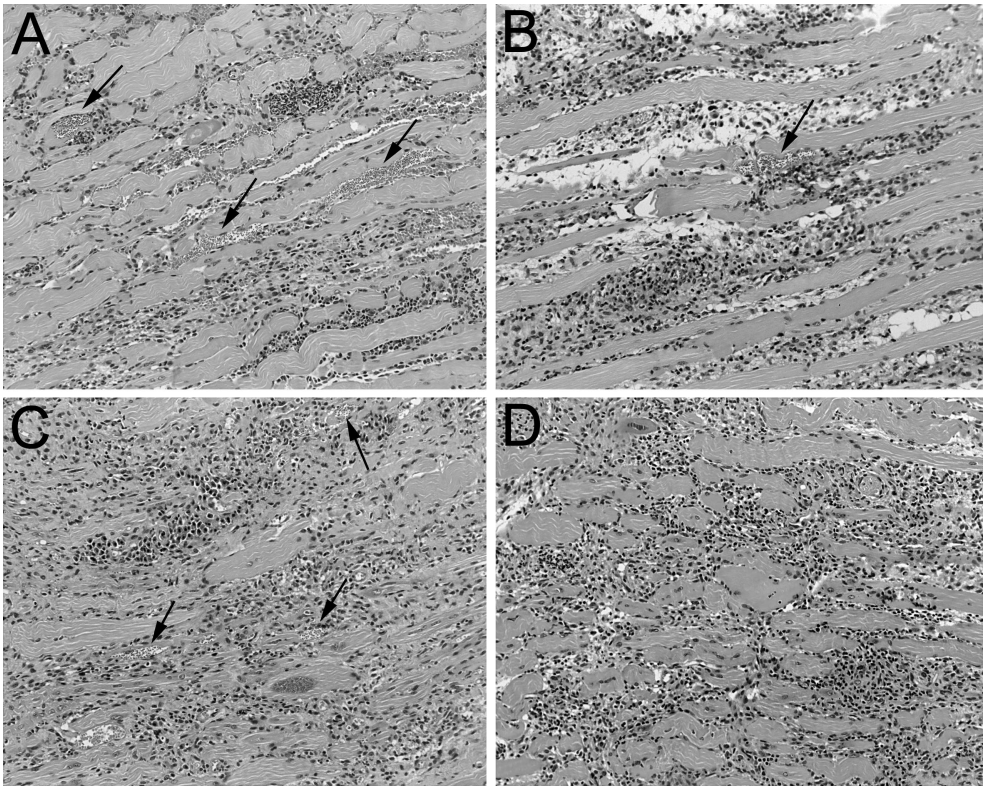


FIG. 6. Inflammation and tissue parasitemia in immunized and challenged mice. C57BL/6 mice were immunized with empty CMVI.UBF3/2 (A), with cytokine plasmids (B), with ASP-1-, ASP-2-, TSA-1-encoding plasmids (C), or with a mixture of antigen-encoding plasmids plus cytokine-expressing plasmids (D) twice at 6-week intervals. Mice were infected 2 weeks after their second immunization with a lethal dose of *T. cruzi* BFT ($10^5$/mouse). Skeletal muscle sections for histologic analysis were obtained at 45 days postinfection. Parasite-infected cells are indicated by arrows. Magnification, ×20.

phase of *T. cruzi* infection. The infection experiments described here were designed so that the efficacy of DNA vaccine(s) was determined based upon survival after a lethal challenge infection. Therefore, mice were infected with a high dose of *T. cruzi* ($10^5$ BFT) that was sufficient to kill most of the control animals during the acute phase of infection, long before the development of chronic chagasic symptoms. Under such conditions, mice immunized with antigen-encoding plasmid(s) and cytokine expression plasmids not only survived the challenge *T. cruzi* infection but also controlled the blood and tissue parasite burden and exhibited a dramatic reduction in skeletal and heart muscle inflammation and necrosis during the chronic phase of the infection. These results strongly suggest that effective immunological control of parasite load during the acute and chronic phases of infection results in reduced tissue parasite load and associated decreases in disease intensity. This result supports the hypothesis that the severity of disease in chronic *T. cruzi* infection is tightly linked to the

TABLE 1. Histopathologic analysis of the skeletal muscle from DNA-immunized mice upon infection with *T. cruzl*

| DNA immunization | IL-12 + GM-CSF[a] | Inflammation[b] | | Mean no. of pseudocysts/mf[e] ± SD |
|---|---|---|---|---|
| | | Acute phase[c] | Chronic phase[d] | |
| CMVI.UBF3/2 | − | ++++ | +++ | 9.37 ± 1.65 |
| CMVI.UBF3/2 | + | ++++ | +++ | 5.25 ± 1.56 |
| ASP-1 | − | ++ | +++ | 8.5 ± 1.41 |
| ASP-1 | + | +++ | ++ | 4.25 ± 1.19 |
| ASP-2 | − | +++ | ++ | 5.5 ± 1.93 |
| ASP-2 | + | ++ | + | 2.5 ± 1.11 |
| TSA-1 | − | ++/+++ | ++ | 4.88 ± 2.36 |
| TSA-1 | + | +++/++++ | +/++ | 3.38 ± 1.21 |
| ASP-1 + ASP-2 + TSA-1 | − | ++++ | + | 6.38 ± 1.57 |
| ASP-1 + ASP-2 + TSA-1 | + | ++++ | +/− | 1.12 ± 1.05 |

[a] When indicated, IL-12- and GM-CSF-encoding plasmids (33 μg of each/mouse) were administered with 100 μg of antigen-encoding DNA.
[b] Scores for degree of inflammation were determined as described in Materials and Methods.
[c] Tissues were collected for histopathologic analysis and quantitation of the parasitic pseudocysts on day 45 postinfection.
[d] Tissues were collected for histopathologic analysis during the chronic phase of infection (80 to 240 days postinfection).
[e] Results were derived from mean values of five different mf/section, two sections/tissue, and four to nine mice/determination.

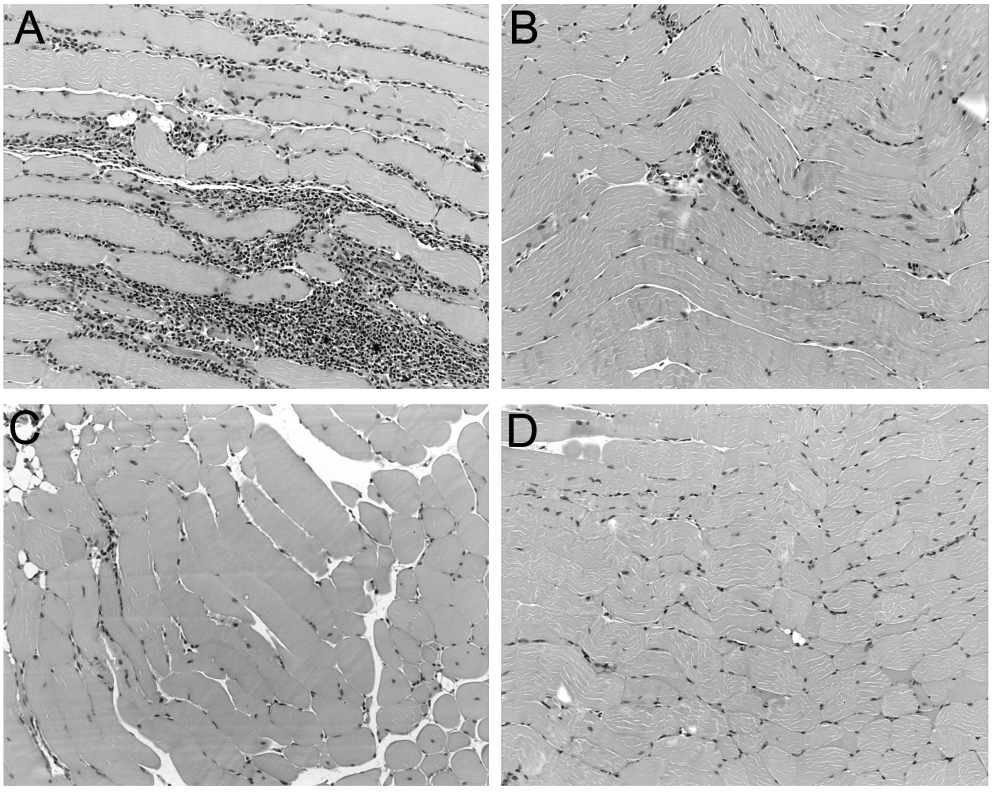


FIG. 7. Control of tissue inflammation and parasite burden by DNA immunization. Histologic analysis of skeletal muscles of mice immunized with empty CMVI.UBF3/2 plasmid alone (A), with cytokine-expressing plasmids (B), with ASP-1-, ASP-2-, and TSA-1-encoding plasmids (C), or with a mixture of antigen-encoding plasmids plus cytokine-expressing plasmids (D). Mice were infected 2 weeks after the second immunization with *T. cruzi* BFT ($10^5$/mouse), and tissues were collected at 150 days postinfection.

relative success in limiting parasite levels (40). Most importantly, these results demonstrate clearly that enhancing immune responses to parasite antigens via vaccination does not increase the severity of disease. This is the first evidence that DNA vaccination is a viable approach to reducing the severity of chronic *T. cruzi* infection.

In conclusion, we have demonstrated that (i) genetic vaccines composed of ASP-1, ASP-2, and TSA-1 provide partial protection from lethal *T. cruzi* infection and, more importantly, significantly modulate the severity of chronic Chagas' disease; (ii) individual or multiple genes can be codelivered to elicit antigen-specific immune responses; and (iii) the quality and quantity of immune responses elicited by *T. cruzi* DNA vaccine(s) can be enhanced by codelivery of cytokine expression plasmid. However, it is significant that, despite the control of *T. cruzi* infection and Chagas' disease, the three genes tested in the present study failed to inhibit infection or eliminate parasites from infected animals and also failed to prevent death from infection in 100% of vaccinated animals. Delivery of these genes under different experimental conditions or in different infection models (e.g., with different mouse or parasite strain or at a lower infection dose) might yield better protection (46). However, it is more than likely that the immune responses elicited by this protocol lack the diversity or magnitude to prevent establishment of *T. cruzi*. Efforts are needed to identify other genes that might constitute a multicomponent DNA vaccine capable of enhancing the protective immunity induced by trans-sialidase family members and cytokines. In addition, alternative mechanisms to boost the level of immune response to the components of a multigene vaccine, such as prime-boost regimens, (7, 22, 35) should be investigated.

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