Trans-Sialidase Recombinant Protein Mixed with CpG Motif-Containing Oligodeoxynucleotide Induces Protective Mucosal and Systemic Trypanosoma cruzi Immunity Involving CD8 T Cell-Mediated Cross-Priming

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**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**

Daniel F. Hoft, Christopher S. Eickhoff, Olivia K. Giddings, José R. C. Vasconcelos, and Maurício M. Rodrigues

The Trypanosoma cruzi trans-sialidase (TS) is a unique enzyme with neuraminidase and sialic acid transfer activities important for parasite infectivity. The T. cruzi genome contains a large family of TS homologous genes, and it has been suggested that TS homologues provide a mechanism of immune escape important for chronic infection. We have investigated whether the consensus TS enzymatic domain could induce immunity protective against acute and chronic, as well as mucosal and systemic, T. cruzi infection. We have shown that: 1) TS-specific immunity can protect against acute T. cruzi infection; 2) effective TS-specific immunity is maintained during chronic T. cruzi infection despite the expression of numerous related TS superfamily genes encoding altered peptide ligands that in theory could promote immune tolerization; and 3) the practical intranasal delivery of recombinant TS protein combined with a ssDNA oligodeoxynucleotide (ODN) adjuvant containing unmethylated CpG motifs can induce both mucosal and systemic protective immunity. We have further demonstrated that the intranasal delivery of soluble TS recombinant Ag combined with CpG ODN induces both TS-specific CD4⁺ and CD8⁺ T cells associated with vaccine-induced protective immunity. In addition, optimal protection induced by intranasal TS Ag combined with CpG ODN requires B cells, which, after treatment with CpG ODN, have the ability to induce TS-specific CD8⁺ T cell cross-priming. Our results support the development of TS vaccines for human use, suggest surrogate markers for use in future human vaccine trials, and mechanistically identify B cells as important APC targets for vaccines designed to induce CD8⁺ CTL responses. *The Journal of Immunology*, 2007, 179: 6889–6900.

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**Trypanosoma cruzi** infection; 2) effective TS-specific immunity is maintained during chronic T. cruzi infection despite the expression of numerous related TS superfamily genes encoding altered peptide ligands that in theory could promote immune tolerization; and 3) the practical intranasal delivery of recombinant TS protein combined with a ssDNA oligodeoxynucleotide (ODN) adjuvant containing unmethylated CpG motifs can induce both mucosal and systemic protective immunity. We have further demonstrated that the intranasal delivery of soluble TS recombinant Ag combined with CpG ODN induces both TS-specific CD4⁺ and CD8⁺ T cells associated with vaccine-induced protective immunity. In addition, optimal protection induced by intranasal TS Ag combined with CpG ODN requires B cells, which, after treatment with CpG ODN, have the ability to induce TS-specific CD8⁺ T cell cross-priming. Our results support the development of TS vaccines for human use, suggest surrogate markers for use in future human vaccine trials, and mechanistically identify B cells as important APC targets for vaccines designed to induce CD8⁺ CTL responses. *The Journal of Immunology*, 2007, 179: 6889–6900.

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3 Abbreviations used in this paper: IMT, insect-derived metacyclic trypanmastigotes (IMT) can infect through breaks in the skin (e.g., through the reduviid bite site) or via mucosal routes (e.g., conjunctival exposure and ingestion). Therefore, the induction of both mucosal and systemic immune responses is an important goal for T. cruzi vaccine development.

Serious chagasic cardiac and gastrointestinal pathologies occur in 30–50% of infected persons, and usually only after prolonged periods of chronic T. cruzi infection. For several decades it was commonly believed that chagasic pathology was caused by autoimmune triggers leading to parasitic infection (reviewed in Refs. 1 and 2). However, recent studies have demonstrated that human chagasic disease correlates with levels of parasites present in cardiac tissue (3–5) and that chagasic disease progression can be significantly reduced by specific chemotherapy that, although it may not be curative, effectively reduces parasite burden (6–8). These latter results strongly indicate that disease progression is predominantly caused by parasite-specific immunopathology, supporting the rationale for the development of both prophylactic and immunotherapeutic vaccines. In addition, murine studies have demonstrated that protective T. cruzi-specific vaccine immunity is not associated with enhanced inflammation or immunopathology before or after T. cruzi infection (9, 10).

Combined mucosal and systemic immunity could maximize protection against many mucosally invasive, intracellular pathogens (e.g., T. cruzi, *Mycobacterium tuberculosis*, and HIV). However, no vaccines available for human use are known to induce both optimal mucosal and systemic protection concurrently. There are theoretical reasons to hypothesize that mucosal and systemic protection could require different immune responses. T cells producing IL-4, IL-5, and IL-10 (type 2 phenotype) or high levels of...
TGF-β (type 3 phenotype) induce secretory IgA responses protective against mucosal infection (11, 12). In contrast, T cells producing IFN-γ, TNF-α, and IL-2 (type 1 phenotype) are clearly protective against systemic intracellular replication of many human pathogens (13, 14). Type 1 and type 2/3 responses have reciprocal inhibitory activities, presenting a significant obstacle for the development of vaccines designed to induce differential T cell responses in mucosal and systemic immune compartments (15–19). Therefore, it is of critical importance for the field of vaccine immunobiology to define the specific mucosal and systemic responses that are protective against mucosally invasive intracellular pathogens and to learn how to induce the appropriate responses in mucosal and systemic tissues by vaccination. In the T. cruzi murine model, despite the theoretical considerations described above we have shown that type 1 immunity provides optimal mucosal and systemic protection (20–23). Therefore, vaccines designed to protect against Chagas disease should induce a global type 1 immune response.

The T. cruzi genome contains multiple large gene families of uncertain function (24, 25). The largest family includes 1,430 genes containing at least 30–40% homology with the unique trans-sialidase (TS) enzyme sequence. Twelve of the 1,430 TS family members identified share ≥90% identity with a predicted 638-aa sequence encoding the TS consensus enzymatic domain. The importance of TS enzymatic activity for T. cruzi virulence (26, 27) and the discovery of such a large number of TS homologues suggest that this gene family may provide some mechanism of immune escape. However, recombinant vaccines inducing immunity against both the consensus TS enzymatic sequence and other TS-like family members protect BALB/c mice against virulent T. cruzi challenges (28–30). In the current work we have further investigated the potential of TS vaccines to protect against both acute and chronic T. cruzi infection, developed a practical strategy for TS vaccination capable of inducing both mucosal and systemic protection, and investigated mechanisms of protection induced by this TS vaccination strategy.

Materials and Methods

Mice and parasites

Six- to eight-week-old BALB/c background mice were used in these experiments. Wild-type mice were obtained from Harlan Sprague Dawley. CDA- and CD8− mice were provided courtesy of T. W. Mak (Artemis Institute, Toronto, Canada). B cell-deficient (μMT) mice were provided by L. Morrison (Saint Louis University, St. Louis, MO). Mice were bred and housed under pathogen-free conditions in an American Association for the Accreditation of Laboratory Animal Care accredited facility. The Tulahuen strain of T. cruzi was used throughout these studies. The T. cruzi life cycle was maintained by circulation through D. petdalogaster maximus reduvid bugs and BALB/c mice as described previously (22, 23). Blood-form trypanosomes (BFT) and IMT were obtained from heparinized mouse blood collected and transferred into SCID mice. After high numbers of parasites were detected in blood smears, TSIR BFT were collected in heparinized blood and frozen with 7.5% DMSO until needed.

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Cloning and purification of trans-sialidase

The TS gene used in the current work for TS protein expression and purification was the previously reported pTS-cat7 gene (obtained from Y strain TS gene 154; GenBank accession no. D50685) (31). This TS gene shares >90% identity along the predicted 638-aa consensus TS enzymatic domain sequence present within all previously published TS genes shown to encode enzymatically active TS proteins, and the protein product expressed from the pTS-cat7 gene has been shown to exhibit TS enzymatic activity (31). The pTS-cat7 gene was subcloned into the pET-14b Escherichia coli expression plasmid, which was then used to transform BL21(DE3) pLysE E. coli. Large scale cultures (2 liters) were grown in Luria-Bertani medium at 37°C and 250 rpm until an OD600 of 0.4–0.6 was attained and then induced overnight at 30°C and 150 rpm with 0.5 mM isopropyl β-D-thiogalactoside. Induced bacterial cultures were centrifuged and resuspended in 65 ml of BugBuster lysis buffer and 65 μl Benzonase reagent (Novagen). Lysates were mixed at room temperature for 25 min after the addition of PMSF (1 mM), leupeptin (10 μg/ml), antipain (1 μg/ml), pepstatin (1 μg/ml), and aprotinin (10 μg/ml). After centrifugation at 25,000 × g for 30 min at 4°C, the supernatant was collected and the recombinant TS protein (rTS) was purified using Ni-NTA agarose and mono-Q chromatography as previously described (31). Endotoxin levels of <10 endotoxin units/ml were detected in final rTS preparations using the Limulus amoebocyte lysate assay (Associates of Cape Cod). Bacteriophage 10 recombinant protein was purified in a similar manner and used as a negative control protein as described previously (21).

Negative control pcDNA3 and pTS154/13 (encoding the same active TS enzyme domain as pTS-cat7) (31) plasmid DNA were prepared for immunization using Qiagen Endo-free Giga-Prep kits using the manufacturer’s instructions. For the current studies we also amplified a TS gene from genomic Tulahuen strain DNA that shares 97% identity with the TS154 gene by using the primers described by Campetella et al. (32). This Tulahuen strain TS gene was subcloned into the pTARGET mammalian expression plasmid (Promega). Despite the 97% identity with the TS154 gene, our new Tulahuen strain TS gene encodes a mutation within the codon for amino acid position 342 that is critical for trans-sialidase enzymatic activity (33), resulting in the predicted expression of a histidine rather than a tyrosine at this position in the expressed protein. Therefore, the new Tulahuen strain TS gene was engineered to express an enzymatically inactive protein. We have named this pTARGET vector containing the Tulahuen strain TS gene pTARGET-ITS.

Immunizations

DNA-vaccinated mice were immunized twice, 2 wk apart, with 100 μg of negative control pcDNA3, pTS154/13, or pTARGET-ITS plasmid DNA (50 μg in each tibialis anterior muscle). Protein immunized mice were vaccinated with CpG-containing oligodeoxynucleotide (ODN) 1826 (TC CGATCAGCTTCTGCAAGT), with CpG underlined; courtesy of A. Krieg, Coley Pharmaceutical Group) mixed with purified negative control bacteriophage 10 protein, trans-sialidase, or T. cruzi lysate. In some experiments, the negative control 1982 ODN devoid of CpG motifs (TCCAG GTCCTTCTCAGGT; also provided by A. Krieg, Coley Pharmaceutical Group) was injected with the trans-sialidase Ag. Intramuscular and intranasal TS protein vaccinations were given twice, 2 wk apart, with 10–100 μg of TS154 and 20–50 μg of recombinant TS protein. Mice were immunized with 60 μg/kg of ketamine and 5 mg/kg xylazine before all immunizations. Mice were immunized intranasally with 2–10 μl Ag plus adjuvant per nostril during anesthesia using a P20 Pipetman.

In vitro studies of vaccine-induced Ab, lymphoproliferative, and secreted IFN-γ responses

Serum and spleen cells from immunized mice were studied preimmunization, 3 days after systemic infection, and 11–14 days after oral challenge (see figure legends for details). TS-specific serum IgG and falcot extract IgA ELISAs were performed using a previously described method, substituting 5.0 μg/ml rTS for recombinant cruzipain or T. cruzi lysate (21, 22). Lymphoproliferative responses were assayed by stimulating 2 × 106 spleen cells with 0.4–2.0 μg/ml rTS in 96-well round-bottom plates for 3 days at 37°C. After the collection of supernatants for cytokine secretion studies, stimulated cells were pulsed for 4–6 h with 0.5 μCi/well [3H]thymidine. Samples were harvested onto filter mats by using a Tomtec Mach-IHM automated harvester, dried, placed in sample bags with 4.0 ml of Ultima Gold F scintillation fluid (Packard Bioscience), and counted using a Wallac MicroBeta TriLux 1450 liquid scintillation counter. Secreted IFN-γ responses were quantitated in culture supernatants by ELISA as previously described (21).

IFN-γ ELISPOT assays

ELISPOT plates (Millipore) were coated overnight at 4°C with 10 μg/ml R46A2 (BD Pharmingen), washed four times with PBS, and then blocked with 10% FCS in RPMI 1640 for 2 h at room temperature. Spleen cells (5 × 105) were stimulated in these plates overnight with 1 × 105/ml control Ag A20J cells or 2 × 105/ml recombinant TS in 200 μl of medium providing early CMV promoter-driven expression of the TS154 protein) (29). A20J cells pulsed with 2.5 μM concentrations of a control peptide or the previously identified TS-specific and H-2Kb-restricted CD8 peptide epitope
Spleen cells from naive BALB/c mice were labeled with 0.25 or 2.5 μM CFSE (carboxyfluorescein diacetate, succinimidyl ester; Molecular Probes) for 8 min at 37°C, washed with RPMI 1640 plus 10% FBS, and then pulsed with 2.5 μM negative control or TS peptide (IYNVGQVSI) for 90 min at 37°C. Washed cells were then mixed 1:1 and 2 × 10^6 cells were injected into naive, vaccinated, or chronically infected recipient mice. After 16 h, spleen cells were harvested and populations were analyzed by flow cytometry. The percentage of specific lysis of TS peptide (IYNVGQVSI)-pulsed target cells was calculated as follows: 100 × [(percentage of naive CFSE<sup>high</sup> (percentage of sample CFSE<sup>high</sup>)/(percentage of sample CFSE<sup>high</sup>)].

FIGURE 1.

In vivo CTL assays

CTL assays were performed using a modified version of a previously reported method (35). Effector cell populations were prepared by culturing 3 × 10<sup>6</sup> spleen cells with 1 × 10<sup>6</sup> irradiated (6,000 rad) A20J-TS cells in upright T25 flasks in a total volume of 10 ml. After 2 days of culture, 0.5 ml of rat T-STAT without Con A (Collaborative Biomedical Products) was added and cultures were incubated for another 4 days. P815 targets were prepared from the excreta collected from T. cruzi-infected reduviid insects and enumerated by direct hemocytometer count. Orally challenged mice were fasted for 4 h, given 0.5 ml of 1.5% sodium bicarbonate in Hanks’ buffer intragastrically to neutralize stomach acid, and challenged mice were fasted for 4 h, given 0.5 ml of 1.5% sodium bicarbonate in Hanks’ buffer. CTL assays were performed using a modified version of a previously reported method (35). Effector cell populations were prepared by culturing 3 × 10<sup>6</sup> spleen cells with 1 × 10<sup>6</sup> irradiated (6,000 rad) A20J-TS cells in upright T25 flasks in a total volume of 10 ml. After 2 days of culture, 0.5 ml of rat T-STAT without Con A (Collaborative Biomedical Products) was added and cultures were incubated for another 4 days. P815 targets were prepared from the excreta collected from T. cruzi-infected reduviid insects and enumerated by direct hemocytometer count. Orally challenged mice were fasted for 4 h, given 0.5 ml of 1.5% sodium bicarbonate in Hanks’ buffer intragastrically to neutralize stomach acid, and challenged mice were fasted for 4 h, given 0.5 ml of 1.5% sodium bicarbonate in Hanks’ buffer. CTL assays were performed using a modified version of a previously reported method (35). Effector cell populations were prepared by culturing 3 × 10<sup>6</sup> spleen cells with 1 × 10<sup>6</sup> irradiated (6,000 rad) A20J-TS cells in upright T25 flasks in a total volume of 10 ml. After 2 days of culture, 0.5 ml of rat T-STAT without Con A (Collaborative Biomedical Products) was added and cultures were incubated for another 4 days. P815 targets were prepared from the excreta collected from T. cruzi-infected reduviid insects and enumerated by direct hemocytometer count. Orally challenged mice were fasted for 4 h, given 0.5 ml of 1.5% sodium bicarbonate in Hanks’ buffer intragastrically to neutralize stomach acid, and challenged mice were fasted for 4 h, given 0.5 ml of 1.5% sodium bicarbonate in Hanks’ buffer. CTL assays were performed using a modified version of a previously reported method (35). Effector cell populations were prepared by culturing 3 × 10<sup>6</sup> spleen cells with 1 × 10<sup>6</sup> irradiated (6,000 rad) A20J-TS cells in upright T25 flasks in a total volume of 10 ml. After 2 days of culture, 0.5 ml of rat T-STAT without Con A (Collaborative Biomedical Products) was added and cultures were incubated for another 4 days. P815 targets were prepared from the excreta collected from T. cruzi-infected reduviid insects and enumerated by direct hemocytometer count. Orally challenged mice were fasted for 4 h, given 0.5 ml of 1.5% sodium bicarbonate in Hanks’ buffer intragastrically to neutralize stomach acid, and challenged mice were fasted for 4 h, given 0.5 ml of 1.5% sodium bicarbonate in Hanks’ buffer.
either PE-labeled anti-MHC class I (H-2D\(^d\)) or anti-CD80 (BD Pharminogen). Levels of MHC class I and CD80 surface expression on gated CD19/H11001 B cells were measured using a BD FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

CpG-induced, B cell-mediated cross-priming of naive TS-specific CD8\(^+\) T cells

B cells were isolated from naive BALB/c spleen cells using Miltenyi anti-CD19 microbeads as directed by the manufacturer. Highly pure (>90% CD19\(^+\)) B cells were incubated in the presence of 100 μM ODN (control no.1982 or CpG no.1826) with and without 100 μg/ml rTS. One day later, naive CD8\(^+\) T cells were purified using a negative selection CD8 isolation kit (Miltenyi Biotec). Treated B cells were washed and cocultured with naive CD8\(^+\) T cells (>95% CD8\(^+\)) for 7 days in the presence of 10 U/ml rmIL-2. Cells were washed and added to ELISPot wells (1 x 10⁵ with 1 x 10⁴ A20J cells pulsed or not pulsed with 2.5 μM concentrations of the TS CD8 peptide (IYNVGQVSI) and incubated for 18 h. Plates were developed and spots were enumerated as described above.

Results

TS vaccines encoding the consensus T. cruzi trans-sialidase enzymatic domain induce protective immunity against acute parasitic infection

The combination of the importance of the TS enzyme as a T. cruzi virulence factor (26, 27) and the discovery of such a large number of TS homologous gene sequences (24, 25) has suggested that proteins encoded by this gene family may serve to provide some mechanism of immune escape. Secondary TCR encounters with homologous but nonidentical TS-encoded epitopes during progressive infection could lead to only partial TCR signaling and T cell anergy. However, previous immunizations with DNA encoding the consensus TS enzymatic sequence or recombinant TS protein have induced immunity in BALB/c mice that is protective against virulent challenges with the Y strain of T. cruzi (28, 29). We have confirmed these results here, demonstrating that immunizations with DNA encoding the consensus TS enzymatic domain can protect BALB/c mice against BFT challenges with Tulahuen strain parasites, a virulent strain of T. cruzi distinct from Y strain parasites. Furthermore, we found that TS enzymatic domains not encoding the tyrosine at position 342 required for trans-sialidase enzymatic activity (33) also were protective. Twenty five of 25 TS154 DNA-immunized mice and nine of 11 pTarget-iTS DNA-immunized mice survived >2 mo after a virulent Tulahuen BFT challenge, compared with only four of 44 negative control DNA-immunized mice (p < 0.0001, comparing both TS DNA groups with the negative controls by Fisher’s exact test). Therefore, these data confirm that vaccines expressing enzymatically active or inactive TS consensus domains can induce protective T. cruzi immunity.
The consensus T. cruzi trans-sialidase Ag induces immunity that persists during chronic parasitic infection

The TS immunization and challenge experiments performed to date evaluate protection against an acute parasite challenge, measuring survival after normally lethal challenges or levels of parasite replication after mucosal or less aggressive systemic challenges. Despite significant levels of protection measurable with these experimental endpoints, the vaccinated and protected mice still become chronically infected with T. cruzi. It is possible that during chronic infection different isoforms of TS gene family members could be expressed with altered T and B cell epitopes, allowing persistence of infection due to escape from the TS-specific immunity induced by vaccination. Alternatively, the expression of multiple homologous but nonidentical TS family member T cell epitopes could dampen optimal T cell responses after the control of acute T. cruzi infection via an altered peptide ligand phenomenon.

To further explore the possibility that TS-specific immunity could be involved in an immune escape mechanism during chronic T. cruzi infection, we have studied TS-specific immunity in mice chronically infected and repeatedly challenged with Tulahuen strain T. cruzi trypomastigotes. We and others have shown previously (20, 36) that although these mice become chronically infected, they develop highly protective immune responses against subsequent parasite challenges. To prove that these mice remain chronically infected with T. cruzi despite the hyperimmunization that results from repeated parasite challenges, we studied whether blood harvested from these mice could transfer T. cruzi infection to SCID mice. We found that seven of nine SCID mice developed patent T. cruzi parasitemia after being injected i.p. with 25–40 /H9262/ml of heparinized blood harvested from nine different BALB/c animals 3 mo after their fourth T. cruzi challenge. From these results we can conclude that although these repeatedly challenged mice develop potent immunity that limits parasitemia to levels as low as 20–40 parasites per milliliter of peripheral blood, they do remain chronically infected. It was important to demonstrate this to rule out the remote possibility that the mice repeatedly challenged with T. cruzi had developed such potent immunity that they had overcome any possible immune evasion strategy responsible for maintenance of persistent infection.

We next studied BALB/c mice that have been hyperimmunized by repeated parasite challenges to determine whether an altered peptide phenomenon had dampened TS-specific immunity during chronic infection. Spleen cells were harvested and stimulated in overnight IFN-γ ELISPOT assays with A20J cells transfected with a TS-expressing plasmid, A20J cells pulsed with the previously
identified H-2K<sup>d</sup>-restricted TS epitope (IYNVGQVSI) known to stimulate CD8<sup>+</sup> T cell responses, and unpulsed negative control A20J cells. The data shown in Fig. 1A clearly demonstrate that during chronic <i>T. cruzi</i> infection potent TS-specific, IFN-γ-producing T cells (including CD8<sup>+</sup> T cells) are present. In fact, the numbers of TS-specific, CD8<sup>+</sup> IFN-γ-producing T cells detectable in these chronically infected mice were higher than the levels detected in TS/DNA immunized mice preinfection (Fig. 1A). Therefore, during chronic <i>T. cruzi</i> infection the expression of many TS gene family homologues does not appear to result in the dampening of TS-specific immunity.

The previous results demonstrated that we could detect the persistence of TS-specific CD8<sup>+</sup> T cell responses in lymphocytes harvested from mice chronically infected with <i>T. cruzi</i> by using in vitro assays. Next, we investigated whether these TS-specific CD8<sup>+</sup> T cell responses were effective in vivo. We labeled naive BALB/c spleen cells with high and low concentrations of CFSE and pulsed them with the IYNVGQVSI TS-specific CD8<sup>+</sup> epitope or a control epitope, respectively. These preparations of CFSE-labeled and peptide-pulsed spleen cells were equally mixed and coadministered into naive, TS154 DNA immunized, and chronically infected BALB/c mice. The next day, spleen cells were harvested and studied by flow cytometry to determine the relative survival of spleen cells pulsed with the control and TS-specific peptides. Fig. 1B demonstrates that the highest level of TS-specific in vivo cytotoxicity was detected in mice chronically infected with <i>T. cruzi</i>. These results confirm that TS-specific T cell immunity remains effective in vivo during chronic <i>T. cruzi</i> infection.

We further explored the possibility that <i>T. cruzi</i> parasites may use different isoforms of the TS gene family during chronic infection, rendering the initial TS-specific immunity irrelevant for the clearance of persistent infection. We recovered persistent parasites from BALB/c mice that had been vaccinated with TS154 DNA and had survived 3 mo after virulent <i>T. cruzi</i> challenge. After passage through SCID mice to increase numbers of BFT, these parasites that survived TS-specific immunity were used to challenge naïve TS154 DNA immunized, and chronically infected BALB/c mice. The next day, spleen cells were harvested and studied by flow cytometry to determine the relative survival of spleen cells pulsed with the control and TS-specific peptides. Fig. 1B demonstrates that the highest level of TS-specific in vivo cytotoxicity was detected in mice chronically infected with <i>T. cruzi</i>. These results confirm that TS-specific T cell immunity remains effective in vivo during chronic <i>T. cruzi</i> infection.

FIGURE 4. Intranasal vaccination with TS protein plus CpG ODN induces robust protective <i>T. cruzi</i> mucosal immunity. Susceptible BALB/c mice were vaccinated intranasally with 50 μg of TS protein or irrelevant control Ag (NC) combined with 10 μg of CpG ODN. Mice were challenged perorally with 5,000 <i>T. cruzi</i> IMT 1 mo after the final vaccination. Draining gastric lymph nodes (GLN) (A) and gastric mucosa (B) from the initial site of mucosal invasion after oral <i>T. cruzi</i> challenge were studied by quantitative parasite culture and real-time PCR, respectively, for levels of replicating <i>T. cruzi</i> parasites 12 days postchallenge. TS-immunized mice were significantly protected against mucosal <i>T. cruzi</i> infection (∗,<i>p</i> < 0.05, by Mann-Whitney U test comparing the TS CpG immunized mice with both control groups).

Development of a subunit TS protein vaccine that induces levels of protection similar to that of TS DNA vaccines

DNA vaccines have been successful in animal studies, especially with small rodents, but have been less impressive in human trials to date. Therefore, we next devoted our attention to the development of a soluble TS protein vaccine that could be used in humans as a prophylactically vaccine and/or immunotherapy. Unmethylated CpG motifs within phosphorothioate-modified ODN sequences have been shown to bind to TLR-9 and trigger adjuvant properties including IL-12 production and enhanced Ag-presentation functions (37). Recombinant proteins are generally safe and, when mixed with CpG-containing ODN, have been successful in inducing type 1-related protective immunity in numerous mouse models of human intracellular infectious diseases, including <i>T. cruzi</i> (38–40). In addition, recombinant proteins mixed with CpG-containing ODN have been used successfully for the induction of both mucosal and systemic immune responses. For all of these reasons, we have pursued the strategy of CpG-adjuvanted TS protein vaccines.

First, we studied i.m. vaccinations in BALB/c mice with TS recombinant protein mixed with ODN containing a known murine TLR-9 stimulating CpG motif. Fig. 2 presents immunogenicity and efficacy data for these TS/CpG vaccinations. Intramuscular TS/CpG vaccination induced TS-specific serum IgG (Fig. 2A), lymphoproliferative (Fig. 2B), and IFN-γ responses (Fig. 2C). More importantly, TS/CpG vaccination induced complete protection against normally lethal <i>T. cruzi</i> BFT challenges (Fig. 2D; <i>n</i> = 5 per group; <i>p</i> < 0.01, comparing TS/CpG vaccinations with CpG vaccinations containing an irrelevant control protein by Fisher’s exact test). Moreover, TS/CpG vaccinations were more protective than CpG vaccinations containing the total Ags present in whole <i>T. cruzi</i> lysate.

We next studied whether intranasal vaccinations with TS/CpG vaccines could induce mucosal and/or systemic immunity (Fig. 3). Intranasal TS/CpG vaccination induced both TS-specific serum
IgG (Fig. 3A) and secretory IgA (Fig. 3B) detectable in fecal extracts. Robust numbers of splenic T cells from mice intranasally vaccinated with TS/CpG produced IFN-γ responses after restimulation in vitro with the TS-specific, H2-Kd-restricted CD8 epitope IYNVGQVSI and studied in IFN-γ ELISPOT assays (A). Mice immunized with either TS protein combined with CpG adjuvant intranasally or TS DNA i.m. developed similar IYNVGQVSI-specific responses. In addition, TS-specific CD8+ T cells induced by either TS protein combined with CpG adjuvant intranasally or TS DNA i.m. could lyse target cells pulsed with the IYNVGQVSI peptide epitope (B). To prove that the IYNVGQVSI peptide epitope does not include a CD4 T cell epitope that could explain the responses detected in A and B, we completed additional IFN-γ ELISPOT assays with immunized spleen cells immunomagnetically depleted of CD8+ T cells. CD8 depletion was highly efficient as shown in C (open and shaded histograms correspond to total and CD8 depleted spleen cells, respectively). Ten percent of total spleen cells were CD8+ T cells compared with <0.2% of residual CD8+ T cells in the depleted populations. In D, it is demonstrated that the depletion of CD8+ T cells abolished the IYNVGQVSI-specific IFN-γ ELISPOT responses detectable in total TS plus CpG-immunized spleen cells. The results shown in panels A-D are representative of multiple experiments with pooled samples. NC, Control; SFC, spot-forming cells.

To determine whether intranasal TS/CpG vaccinations could induce mucosal protection, we challenged immunized and control mice orally with IMT and studied levels of mucosal parasite replication 10–14 days later. We have previously shown that after oral IMT challenge the initial point of mucosal invasion occurs within the proximal gastric epithelium, followed by local spread to lymph nodes that enlarge within the lesser curvature of the stomach (36). Viable parasites replicating within the draining gastric lymph node can be quantified by limiting dilution parasite culture techniques. Parasite replication in the gastric mucosa itself can be analyzed by determination of the molecular equivalents of T. cruzi genomes present by real-time PCR. Fig. 4 demonstrates that mice vaccinated intranasally with TS/CpG had significantly reduced viable parasites (Fig. 4A) in the draining gastric lymph nodes and molecular equivalents of T. cruzi (Fig. 4B) recoverable from gastric mucosal epithelia (n = 5 per group; p < 0.05, by Mann-Whitney U tests). Therefore, intranasal TS/CpG vaccinations can induce both protective mucosal and systemic T. cruzi immunity in the susceptible BALB/c mouse strain. Immunization with TS in the absence of CpG did not induce Ag-specific immunity (data not shown).

**Intranasal TS/CpG vaccinations induce cross-priming of TS-specific CD8+ CTL responses**

CD8+ CTL are important for protective T. cruzi immunity (29, 40–44) but generally are not induced by soluble protein vaccines.
However, a mechanism known as cross-priming has been described whereby certain professional APC can induce CD8$^{+}$ T cell responses after the uptake of exogenous Ag (45–47). Cross-priming of CD8$^{+}$ CTL with exogenous Ags has been shown to occur with Ags taken up from apoptotic cells and Ags linked to ligands for receptor-mediated uptake. One report demonstrated that an OVA peptide linked to a CpG-containing synthetic ODN could induce cross-priming (48). Therefore, we hypothesized that our intranasal TS/CpG vaccinations were inducing cross-priming of TS-specific CD8$^{+}$ CTL. The results shown in Fig. 5 confirm this hypothesis. Using the IYNVGQVSI TS-specific CD8$^{+}$ CTL epitope, similar numbers of IFN-$\gamma$-producing T cells could be detected in ELISPOT assays containing spleen cells from mice hyperimmunized with either intranasal TS/CpG or TS/DNA vaccinations (Fig. 5A). DNA vaccinations in general are known to result in the encoded Ags being synthesized endogenously within APC, and TS DNA vaccinations have been shown previously to induce CD8$^{+}$ CTL specific for the IYNVGQVSI epitope (29). This is the first demonstration that TS/CpG vaccinations can induce CD8$^{+}$ T cell responses. Fig. 5B shows that both TS-specific CD8$^{+}$ CTL induced by TS/DNA and TS/CpG vaccinations can have lytic activity for targets sensitized with the IYNVGQVSI epitope. To further prove that these responses were due to CD8$^{+}$ T cells, we performed additional IYNVGQVSI-stimulated IFN-$\gamma$ ELISPOT assays with TS/CpG-immune spleen cells depleted of CD8$^{+}$ T cells. Fig. 5C demonstrates the efficacy of our CD8 depletion technique (>95%). Fig. 5D confirms that, after depleting the CD8$^{+}$ T cells, TS/CpG-immune spleen cells no longer respond to the IYNVGQVSI epitope.

Immunity induced by intranasal TS/CpG requires CD4$^{+}$ and CD8$^{+}$ T cells, as well as B cells

To determine which subsets of TS-specific immune responses were critical for the protection induced by intranasal TS/CpG, we studied the ability of intranasal TS/CpG vaccinations to induce protective immunity in CD4, CD8, and B cell (µMT) knockout mice. Wild-type and CD8 knockout mice developed similarly high-titered TS-specific serum Ab responses, whereas CD4 and B cell knockout mice had poor Ab responses (Fig. 6A). In addition, only wild-type and CD8 knockout mice developed detectable TS-specific lymphoproliferative (Fig. 6B) and IFN-$\gamma$ (Fig. 6C) responses. However, Fig. 6D presents the results of T. cruzi systemic protection studies. None of the intranasal TS/CpG-immunized knockout mice could survive after normally lethal s.c. BFT challenge (n = 5/group; p < 0.01, by Fisher’s exact test comparing wild-type intranasal TS/CpG-immunized mice with all other groups).

Protective immunity induced by intranasal TS/CpG vaccinations requires B cell APC functions

CD8 knockout mice produced TS-specific Ab responses similar to those of wild-type mice after intranasal TS/CpG vaccination (Fig. 6A). However, these immunized CD8 knockout mice were not protected against systemic T. cruzi challenge (Fig. 6D). These
combined results suggested that B cells may not be important for the Ab responses they produced after immunization with the intranasal TS/CpG vaccine, but rather that the APC functions of B cells may be important for the induction of optimal vaccine-induced responses. Consistent with this hypothesis, B cell knockout mice did not develop TS-specific lymphoproliferative (Fig. 6B) and IFN-γ (Fig. 6C) responses detectable after in vitro stimulation with a soluble recombinant TS protein. These latter results suggested that B cells may be important for the cross-priming of CD8+ T cell responses when immune spleen cells were stimulated with the IYNVGQVSI epitope (Fig. 7B).

These latter results suggested that B cells may be important for the cross-priming of CD8+ T cell responses when immune spleen cells were stimulated with the IYNVGQVSI epitope (Fig. 7B).
encode trans-sialidase enzymatic activity. The function of this large TS gene family, besides providing the important TS enzymatic activity critical for parasite virulence, is unknown. However, it has been hypothesized that the large number of TS Ags potentially expressed by this gene family may contain homologous but nonidentical T cell epitopes and that the concurrent expression of these homologous epitopes could serve in some mechanism of immune escape, perhaps via an altered peptide ligand phenomenon that could prevent optimal immune induction or even cause immunological tolerance to key protective TS epitopes (49, 50).

However, we and others have found that certain TS Ags can induce protective immunity in mice against T. cruzi infection. This provides important preliminary data for the development of a feasible vaccination strategy feasible for use in humans, the intranasal delivery of TS protein mixed with a CpG motif-containing ODN adjuvant, can induce mucosal and systemic protection against T. cruzi infection. We have also investigated the mechanisms by which intranasal delivery of TS protein plus CpG adjuvant induces protective T. cruzi immunity. Our previous work (20, 29) and the work of other groups (42, 55) have shown that both CD4+ and CD8+ T cells are important for protection against T. cruzi challenges. Although CD4+ Th1 cells produce IFN-γ, which can activate intracellular killing mechanisms effective against replicating T. cruzi parasites within macrophages, these Th1 cells appear in vivo to be most important as helper cells for the development of CD8+ CTL effector cells (20). This makes sense if one understands the biology of T. cruzi infection. T. cruzi can infect most nucleated mammalian cells, not simply macrophages, and replicates within the cytoplasm of the infected cell after escape from the phagolysosome. Thus, during both acute and chronic T. cruzi infection the majority of cells supporting the replication of the parasite are nonhematopoietic host cells that express only MHC class I and not MHC class II molecules, and only CD8+ CTL and not CD4+ Th1 cells can recognize and respond to these infected cells. The cytoplasmic location of parasite replication further biases toward the importance of CD8+ CTL as protective effector cells, because this facilitates access by the endosomal pathway of Ag presentation to parasite-specific, MHC class I-restricted T cell epitopes. For all of these reasons, we hypothesized that our intranasal delivery of TS protein plus CpG adjuvant must induce both CD4+ Th1 cells and CD8+ CTL. The results shown in Figs. 1–5 of the current study clearly demonstrate that this indeed is the case. We could detect TS-specific CD8+ CTL by both peptide-specific IFN-γ ELISPOT assays and by classical CTL chromium release assays in mice previously vaccinated with TS plus CpG adjuvant. The additional experiments conducted in CD4- and CD8- knockout mice (Fig. 6) further suggest the importance of both CD4+ Th1 cells and CD8+ CTL for the protective immunity induced by TS/CpG vaccination. These results are important, because they demonstrate that a protein subunit vaccine can induce both MHC class I- and II-restricted T cell responses associated with protective T. cruzi immunity. Generally, protein subunit vaccines do not induce CD8+ CTL responses because the Ags are taken up by pinocytosis, remain contained within the endosomal compartments of the APC, and the T cell peptide epitopes derived from these Ags are only accessible for MHC class II-restricted presentation. However, more recently a mechanism termed cross-presentation or cross-priming has been shown to occur where exogenous Ags can be taken up and processed in an unconventional manner, leading to MHC class I-restricted epitope presentation (45–47). Most of the published data have suggested that cross-priming is a normal but unique function of activated, mature dendritic cells. Furthermore, CpG motif-containing ODN have been shown to promote dendritic cell-mediated cross-priming (37) and, in at least one report (48), CpG motif-containing ODN covalently linked to an antigenic protein have induced cross-priming of naive CD8+ T cells by B cells. Because of these reports, we hypothesized that the importance of B cells for protective T. cruzi immunity induced by intranasal TS/CpG vaccinations as demonstrated in our B cell knockout experiments (Fig. 6) was related to the ability of B cells to cross-prime CD8+ T cell responses. Consistent with this hypothesis, we first investigated the effects of the absence of B cells on the induction of TS-specific CD8+ T cells and found that B cell knockout mice immunized with intranasal TS protein plus CpG adjuvant failed to develop
these responses. This was unique to the TS protein plus CpG vaccination, as TS DNA-vaccinated B cell knockout mice were shown to develop normal TS-specific CD8⁺ T cell responses (Fig. 7A) and were also protected against virulent T. cruzi challenges (Fig. 7B). Next, we conducted experiments proving that highly purified B cells pulsed with TS protein and CpG adjuvant up-regulated the surface expression of MHC class I and costimulatory molecules (CD80 and CD86) and induced naïve TS-specific CD8⁺ T cells to expand and produce enhanced Ag-specific IFN-γ responses (Fig. 8). These results strongly indicate that soluble TS Ag combined with CpG adjuvant can induce cross-priming of TS-specific CD8⁺ T cells by B cells. To our knowledge, these are the first results to demonstrate that immunization with a soluble protein not covalently linked to CpG-containing ODN can induce the cross-priming of CD8⁺ T cells by B cells. B cells also seemed to provide an important Ag presentation function for the induction of CD4⁺ T cell responses by CpG ODN plus TS (Figs. 6, B and C), consistent with other recent reports (56–58). Whether unique features of the TS Ag itself are at least partially responsible for these effects remain to be elucidated.

In summary, we have shown that: 1) TS-specific immunity can protect against acute T. cruzi infection; 2) TS-specific CD8⁺ T cell responses are maintained during chronic T. cruzi infection despite the expression of numerous related TS superfamily genes encoding altered peptide ligands that theoretically could diminish or tolerate TS-specific immune cells; 3) the practical intranasal delivery of recombinant TS protein combined with CpG adjuvant can induce both mucosal and systemic immunity; and 4) the protective immunity induced by intranasal TS protein plus CpG adjuvant is associated with the induction of CD4⁺ and CD8⁺ T cells as well as the potential for B cell-induced cross-priming. All of these results support the development of TS vaccines for human use, suggest surrogate markers for use in future human vaccine trials, and mechanistically identify B cells as important APC targets for future vaccines designed to induce CD8⁺ CTL responses. In addition, CpG adjuvanted mucosal immunization with T. cruzi recombinant Ags as described here can be a practical means for testing a number of potential vaccine candidates in preclinical models and subsequently in clinical trials.

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